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Embryonic Origins of Central Chemosensitivity in the Avian Hindbrain: role of chloride in neurodevelopmental “switches” in the pH response and recovery of neural activity during pH stress

by

Jessica Whitaker-Fornek

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biological Sciences

Idaho State University

Fall 2020
Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of JESSICA WHITAKER-FORNEK find it satisfactory and recommend that it be accepted.

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December 10, 2019

Jason Q. Pilarski, PhD
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RE: Full-board review regarding study number 784: Breeding, Husbandry, and Experimental Protocols for Investigations into the Development of Breathing Circuitry in Birds (Zebra Finch)

Dear Dr. Pilarski:

Your request for approval of the new study listed above was reviewed at the December 9th, 2019 meeting of the Idaho State University IACUC. This is to confirm that your protocol was approved. Your study is approved through 12/9/22.

You are granted permission to conduct your study as submitted effective immediately. The date for annual review is 12/9/20, unless the study closes before that date.

Please note that any further changes to the study must be promptly reported and approved. Contact Tom Bailey (animalcare@isu.edu; 208-282-2179) if you have any questions or require further information.

Sincerely,

Erin Rasmussen, PhD
IACUC Chair
Acknowledgements

I am extremely thankful for the tremendous support I have received over the last four years as a PhD student. There are so many people that have helped me during this journey and I’d like to try to give them the credit that they deserve. But, I don’t think that my words in this brief section will come close to expressing my endless gratitude to my advisor, lab team, committee, the biology department, animal care facility, and my friends and family.

First, I would like to thank my advisor, Dr. Jason Q. Pilarski, for being exactly the kind of mentor that I’ve needed in this stage of my science journey. From my very first visit to Pocatello, I have been able to ask Jason for help with anything—science-related issues or if he could help me fix up my bike. His number one priority has always been making sure that I would be able to successfully complete my PhD program and learn as much as I can during this time. He has shown dedication to this task by ensuring that I have been funded for all four years and during the summer semesters. Also, he has enthusiastically supported me in gaining teaching experience by giving me opportunities to lead his embryology lab and do a pedagogical study one of his lecture classes. One last aspect of Jason’s mentorship that I’d like to mention is that he has supported me in taking time for myself to do things outside of the laboratory. I am so happy that I had Jason as my advisor and I am looking forward to continuing to work together as colleagues since I plan to stay in the respiratory neurobiology field.

The research outlined in this document would not have been possible without the undergraduate student members of Team Bird Brain. From the beginning, I learned pro-tips for microsurgery and extracellular recordings from senior undergraduates in the lab,
Kaci Pickett and Kaitlyn Whitesitt. Casetin Lybbert performed at least 30 of the experiments included in this document and he built my favorite electrophysiology rig in the lab. I am also thankful for the chance to mentor undergraduate students, Erik Nakken, Kory Andersen, Camdon Kay and Layton Elledge, and a high school student, Janet Leon, during my time in Team Bird Brain. I learned so much from all of these students as they asked me questions that I didn’t know the answers to, so we got to figure things out together. I’d like to especially thank recently fledged Team Bird Brain members, Jayden Burton and Jennie Nelson, for finishing up data collection while I worked from home this past spring and summer. Jayden performed 21 experiments that are included in my dissertation research. I’d like to emphasize that I have been so fortunate to work alongside Jennie for the last 3 years. Jennie’s commitment to objectivity and sound experimental design has helped me control for confounding variables to the best of my ability. In the words of Jennie, “It’s all about the baseline.” Jennie worked approximately 3,860 hours over the 3 years she spent in Team Bird Brain and she performed 86 experiments that are included in my dissertation research.

My research outlined in this document has benefitted from the guidance of my committee members including Dr. Michele Brumley, Dr. James Groome, Dr. Michael Thomas, Shawn Bearden and Jared Barrott. I am so appreciative to have been able to depend on all of you for critical feedback on my research and progress as a scientist.

One of my favorite things about my experience as a PhD student has been being surrounded by faculty, graduate students, and staff who are positive and caring. I would like to especially thank the Biology Graduate Student Association for welcoming me when I first arrived on campus. I have benefitted from experience in leadership roles
and travel grant funding through BGSA. But most importantly, it has been so much fun to make a homecoming parade float, spend time with my fellow graduate students, and do so many other activities. Another big aspect of the biology department I want to highlight is the staff. Thank you to Jackie Coffin and Joyell Bedwell for always being available to help me with tasks that were critical to completing my degree program and coordinating BGSA related activities like guest speaker lunches. I also am thankful for help from Greg Stowell and Cindy Russell. Last but not least, when I was working in the lab in the evening, I always looked forward to interacting with the custodial staff, including Tyson Anderson and Rocky Haddon, and sharing a little about what was going on in the lab.

My research would not have been possible without the excellent animal care facility managed by Mia Benkenstein. The staff have really spoiled our zebra finch colony, even painting a mural on the wall of the large free flight aviary. I am thankful for their efforts in keeping the birds happy so the colony would produce plenty of eggs for my experiments.

Finally, I am extremely thankful for being supported by my friends and family while pursuing a career in science. My husband, Jon Fornek, has waited patiently for me in the lab on many Saturdays while I collected eggs or checked on the incubator. He has listened to countless practice presentations and, most importantly, has encouraged me to go after my dreams. Similarly, my family has always been excited to hear what I’m studying in the lab. I credit my family with nurturing my interests in the natural world from a young age. I am thankful for their support in me moving all the way from Southern Illinois to Idaho to follow my dreams.
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**List of Abbreviations**

5HT; 5-hydroxytryptamine or serotonin
aCSF; artificial cerebrospinal fluid
AE3; anion exchanger 3
CN; cranial nerve
CNS; central nervous system
DIDS; 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid
E#; embryonic day #
\(E_{\text{ion}}\); equilibrium potential for a given ion
GABA; gamma aminobutyric acid
HCVR; hypercapnic ventilatory responses
[ion]; concentration of a given ion
[ion]_; intracellular concentration of a given ion
[ion]_; extracellular concentration of a given ion
IF; instantaneous frequency
IPC; intrapulmonary chemoreceptors
KCC2; K⁺ Cl⁻ co-transporter 2
NKCC1; Na⁺ K⁺ 2Cl⁻ cotransporter 1
\(P(\text{gas})\); partial pressure of a given gas
PTX; picrotoxin
rSNA; rhythmic spontaneous neural activity
\(\dot{V}E\); total ventilation
Embryonic Origins of Central Chemosensitivity in the Avian Hindbrain: role of chloride in neurodevelopmental “switches” in the pH response and recovery of neural activity during pH stress

Dissertation Abstract—Idaho State University (2020)

Although the mechanistic details of central respiratory chemosensitivity have been studied for a long time, the emergence of central chemosensitivity in embryos remains largely out of reach. In this dissertation research project, I used the altricial zebra finch embryo to study the neurogenesis of breathing-related motor activity spanning the time period when the microcircuits are not yet required for respiration in ovo, through the establishment of continuous air-breathing.

The present research encompasses three aims. The first aim was to describe the sensitivity of avian embryo hindbrain motor rhythms to metabolic and respiratory acidic/alkaline conditions. Using this approach, I discovered a neurodevelopmental switch in pH chemosensitivity on rhythmic frequency bookmarked near embryonic (E) day 11. Previous work in the Pilarski Laboratory shows that ~E11 defines a shift in the role of chloride (Cl\(^{-}\)) neurotransmission from excitatory to the more typical adult role of inhibition (Pickett et al., 2018). Accordingly, the second aim was to test whether Cl\(^{-}\) played a role in the age-dependent pH response. By lowering pH in combination with antagonists for a Cl\(^{-}\) ion channel and a Cl\(^{-}\) transporter, as well as using Cl\(^{-}\) free artificial cerebrospinal fluid (aCSF), results showed that the age-dependent effects of pH on rhythm frequency were reversible. These findings suggest the Cl\(^{-}\) concentration gradient is a critical factor determining the pH response of rhythm frequency. The final aim was to assess whether Cl\(^{-}\) contributed to motor rhythm recovery often observed during low
pH exposure and determine whether this effect was age-dependent. Results showed that rebound or recovery was blocked by disrupting Cl⁻ transport and removing extracellular Cl⁻.

Understanding the rules of developing central circuits that support homeostatic control of the electrochemical environment could help elucidate how early experiences shape functional outcomes. Newborns are prone to ventilatory control issues that impair breathing and threaten life (Carroll and Agarwal, 2010) and avian embryos in the wild are subject to fluctuating temperature and pH conditions as incubating parents periodically leave the nest (MacDonald et al., 2014). My dissertation research could provide some insights that help inform future investigations in these clinical and ecological contexts.

Key Words: development, motor rhythm, pH, neural activity, zebra finch, avian
1. Background

1.1 The structure and function of the avian respiratory system

The respiratory system of adult birds is unique among air-breathing vertebrates. Unlike mammals, bird lungs do not expand and shrink with the passage of air. Rather, air flows through the rigid lungs by the active expansion and constriction of air sacs (Powell, 1986). Air enters the respiratory system through the nares and oral cavity. The opening of the glottis during inspiration allows air to pass through the larynx and trachea (Ole Næsbye Larsen and Goller, 2002). Before entering the primary bronchi, air flows through the syrinx. The syrinx is a structure that birds use to produce vocalizations. Beyond the syrinx, air flows through the primary bronchi and can enter air sacs via the intrapulmonary bronchus and secondary bronchi. At the level of the secondary bronchi, branches separate to serve the cranial, caudal, and ventral regions of the lung. In each of these areas, tertiary bronchi form parallel connections between the secondary bronchi. The tertiary bronchi, also known as parabronchii, contain air capillaries that are ~2-10 micrometers in diameter. Pulmonary blood capillaries are closely associated with the air capillaries; creating the air-blood interface needed for the diffusion of respiratory gases.

The arrangement of the conducting airways, air sacs, and the site of gas exchange (i.e., parabronchii in the lungs) results in fresh air ventilating the lungs during inspiration and expiration. Birds accomplish this feat by receiving fresh air in the caudal air sacs during inspiration. At the same time, stale air moves out of the lung and into the cranial air sacs. During expiration, fresh air moves from the caudal air sacs into the lung while stale air is exhaled from the cranial air sacs. Air always moves in the same
direction through the parabronchi in the lung due to aerodynamic valving which involves the morphology of branching between secondary and primary bronchi (Scheid and Piiper, 1989).

1.2 Transport of respiratory gases in avian blood

Supplying tissues with oxygen (O₂) and eliminating carbon dioxide (CO₂) generated by cellular respiration depends on the ease of diffusion. At the air-blood interface in the bird lung, gases and blood encounter each other in a cross-current arrangement (i.e., blood vessels run perpendicular to the parabronchi) (Scheid and Piiper, 1989). O₂ is carried in the blood mainly bound to hemoglobin while CO₂ is found dissolved in blood and associated with proteins. Within the red blood cell and in plasma, CO₂ undergoes its hydration reaction and forms carbonic acid (Equation 1.1). This reaction is facilitated by the enzyme carbonic anhydrase. Carbonic acid dissociates into hydrogen carbonate (HCO₃⁻) and hydrogen ions (H⁺). Thus, HCO₃⁻ is the major form of CO₂ carried in the blood. This reaction is important for acid-base homeostasis since the proportions of HCO₃⁻, H⁺, and dissolved CO₂ greatly inform blood pH.

\[ CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \]

Equation 1.1 The carbon dioxide hydration reaction. Carbon dioxide and water form carbonic acid. This step is catalyzed by the enzyme carbonic anhydrase. Carbonic acid disassociates into hydrogen ions and hydrogen carbonate.
One mathematical model that relates CO₂, HCO₃⁻, and H⁺ concentrations to blood pH is the Henderson-Hasselbalch equation (Equation 1.2). This model shows that a change in the ratio of the buffer, HCO₃⁻, and the acid, CO₂, changes pH. The levels of HCO₃⁻ ([HCO₃⁻]), pH, and PCO₂ can be used to identify respiratory acidosis/alkalosis or metabolic acidosis/alkalosis. Respiratory acidosis results from an increased PCO₂ due to hypoventilation, ventilation-perfusion inequality, or breathing obstruction. Respiratory alkalosis can occur when PCO₂ drops during hyperventilation. Metabolic acidosis can occur due to a decrease in HCO₃⁻ brought on by acids building up in the blood (e.g., uncontrolled diabetes mellitus). Metabolic alkalosis results when HCO₃⁻ is above normal levels which occurs in conditions where acids are lost via vomiting or excess bases are consumed (e.g., taking too many antacids).

Prolonged acidosis and alkalosis can impair protein function since proteins’ structure can be influenced by pH. Thus, respiratory and metabolic pH imbalances are corrected in the short-term by adjustments in ventilation or in the long-term by changes in absorption or excretion of HCO₃⁻ in the kidneys.

$$pH = pka + \log \frac{[HCO3^-]}{PCO2}$$

Equation 1.2 The Henderson-Hasselbalch Equation. The key variables in this equation is the proportion of the base (hydrogen carbonate, HCO₃⁻) and acid (partial pressure of carbon dioxide, PCO₂). Changes to this ratio modify pH.

1.3 Peripheral chemoreceptors in adult birds

The first step in adjusting pH to maintain acid-base homeostasis is sensing and responding to unusual pH, blood gas, and HCO₃⁻ levels. Birds have some of the same
mechanisms for monitoring blood gases and pH as mammals (i.e., carotid bodies, stretch receptors). However, they also possess intrapulmonary chemoreceptors within the parabronchial mantle of the lung that monitor phasic and tonic CO2.

Just like mammals, one of the main sites for peripheral blood PO2 monitoring are the carotid bodies. The carotid bodies sample blood gases at the bifurcation of the internal and external carotid arteries. The carotid bodies are innervated by the vagus nerve and signal the central respiratory circuit to increase ventilation when low PO2 is sensed, and to a lesser extent, high PCO2. Thus, carotid bodies can monitor blood pH and contribute to some of birds’ drive to breathe under acidotic conditions.

A unique feature of avian respiratory control are the intrapulmonary chemoreceptors (IPC). The morphology of these structures is still mysterious. However, their function has been studied for a long time (Peterson and Fedde, 1968). IPC firing rate increases during periods of low CO2 such as the drop in PCO2 accompanying during inspiration. This inverse relationship between CO2 and firing rate may help to shut off inspiration when CO2 phasically drops within the lung parenchyma. The IPC are vagally innervated similarly to the carotid bodies.

The bird’s respiratory system presents an ideal system for studying peripheral breathing control. Experiments employing the unidirectionally ventilated duckling, have revealed that IPC are sensitive to shifting CO2 levels at least 12 hours before hatching (Pilarski and Hempleman, 2007). Also, the cellular mechanisms by which changes in PCO2 is transduced to changes in IPC firing is becoming more clear (Hempleman and Posner, 2004). Data show that IPC primarily respond to changes in intracellular pH that result from the flux of CO2 and the corresponding hydration and dehydration reactions.
1.4 Central chemoreceptors in adult birds

Compared with peripheral CO₂ chemosensation, much less is known about the sensitivity of central breathing circuits to local PCO₂ and pH fluctuations. Milsom and colleagues (1981) altered the perfusion of central chemoreceptors in unanesthetized ducks to study chemoreceptor contribution to the hypercapnic ventilatory response. They observed a ~150% increase in total ventilation during exposure to raised PCO₂ in vasculature serving the head. Despite acknowledging the functional presence of avian central chemoreceptors in adult ducks, cellular mechanisms of chemosensation and the anatomical location of chemoreceptors in the hindbrain have remained elusive. Further, the overarching aim of the work presented in this dissertation is to characterize the embryonic origins of central chemosensitivity in the avian embryo. To begin to understand the birth of ventilatory chemosensitivity, it is necessary to review the initial gas exchange modes that lead up to pulmonary ventilation.

1.5 Gas exchange and ventilation during the incubation period

In this section, I provide an overview of the egg environment including gas exchange structures employed by the embryo and the changing respiratory gas levels in the egg compartment. For a great review about gas exchange within the bird egg, please see How bird eggs breathe (Rahn et al., 1979).

Figure 1.1 diagrams key milestones of avian embryogenesis. As soon as the egg is laid, respiratory gases and water vapor diffuse through microscopic pores within the eggshell. CO₂, which accumulated in the egg as it traveled along the female bird’s
oviduct, leaves the egg through pores in the eggshell. This results in somewhat alkaline environment within the eggshell at this stage. Once incubation begins, the developing embryo produces CO₂ as a byproduct of cellular respiration and O₂ consumption. The eggshell presents a barrier to the movement of O₂ as well as CO₂, so the embryo becomes relatively hypoxic and acidotic over time.

To increase the surface area for the diffusion of respiratory gases as the demands for gas exchange intensify, the embryo employs vascularized membranes at the egg-air interface. First, the area vasculosa forms at around E4. This membrane serves as a major site of diffusive gas exchange until around E8 when the chorioallantoic membrane assumes this role. The chorioallantoic membrane increases in size for the next several days of incubation until it fully encloses the egg contents. The increased surface area for gas exchange provided by the chorioallantoic membrane allows for an increase in PO₂. However, PCO₂ continues to gently rise despite increased diffusion of gases across the chorioallantoic membrane and increasing levels of HCO₃⁻. Thus, pH tends to decrease over incubation. Meanwhile, water is lost at a constant rate over the course of incubation. This water loss results in the formation of an air-filled space at the blunt end of the egg called the air cell. This space is important toward hatching when the embryo’s bill tears through the overlying chorioallantoic and inner shell membranes (i.e., internal pipping) and begins to breathe air before hatching.

As the bird rebreathes air within the air cell of the egg, PCO₂ increases and lungs become the primary site of gas exchange. During the hatching process, the paranatal bird cracks the eggshell and breathes the ambient air. Breathing fresh air alleviates the high PCO₂, allowing blood pH to raise. This period is known as external pipping.
Over incubation, gas exchange transitions from mainly diffusive to mainly convective which influences the physiochemical environment of the egg. A central question in this context is the role of *in ovo* conditions for signaling the normal development of motor rhythms for breathing. The following section outlines how acute and chronic exposure to heightened CO$_2$ levels changes motor activity in early embryonic and paranatal birds, which suggests that developing neural circuits could be sensitive to altered pH experience.

1.6 Ventilatory chemosensation in bird embryos and hatchlings

1.6.1 Effects of acute CO$_2$ exposure

Exposing an egg to high CO$_2$ (hypercapnia) has visible effects on the freely behaving bird embryo behavior *in ovo*. Data show that bird embryos around embryonic days (E) 13-20 display increased embryonic motility when CO$_2$ levels are raised (Windle and Barcroft, 1938). Rather than evoking a respiratory-specific response to adjust acid-base balance, these first signs of CO$_2$ influencing behavior could be related to overall changes in the activity of the nervous system.

By the time bird embryos breathe air following internal pipping, increases in CO$_2$ results in increased total ventilation, which is usually accomplished by elevated tidal volumes or, occasionally, more rapid tidal frequency (for a review see Whitaker-Fornek et al., 2019). Hatchling chickens, red-winged blackbirds, ducks, and wedge-tailed shearwaters show increased tidal volume upon breathing hypercapnic gas mixtures (Dzialowski et al., 2016; Mortola and Toro-Velasquez, 2014; Pettit and Whittow, 1982;
Sirsat and Dzialowski, 2016). Heightened ventilation is the typical acute response to a respiratory acidosis shown by air-breathing vertebrates (Powell, 1986). Increased tidal volume and/or tidal frequency is effective at eliminating excess CO\textsubscript{2} and compensating for an acidosis.

1.6.2 Effects of chronic CO\textsubscript{2} exposure

To date, laboratory studies examining the effects of chronic hypercapnia on chemoreflex development in freely behaving birds has been examined in chicken, quail, and zebra finch (Bavis and Kilgore, 2001; Ferner and Mortola, 2009a; Szdzuy and Mortola, 2008; Williams and Kilgore, 1992). In chickens, hatchlings with a history of chronic hypercapnia during incubation showed decreased tidal volume and total ventilation when their hypercapnic ventilatory responses (HCVR) were tested compared with control hatchlings. Surprisingly, quail do not consistently show blunted HCVR responses following hypercapnic exposure during incubation, at least not as an adult. Rather, sex and other inherited factors seem to play a role since only females and quail from specific hatcheries show reduced total ventilation (\(\dot{V}E\)) when their HCVR was quantified (Bavis and Kilgore, 2001; Bavis and Simons, 2008). For zebra finch, the HCVR was measured in adults after they had been exposed to increased CO\textsubscript{2} during one of three time periods during incubation (Williams and Kilgore, 1992). In adult zebra finch, as in quail, the hypercapnic experience during the embryonic, post-hatch, or both embryonic and post-hatch periods resulted in significantly blunted HCVRs.

In nature, bird embryos can be exposed to elevated CO\textsubscript{2} if they are raised in burrows and this experience can also lead to blunted HCVRs. The eggs of bank
swallows naturally experience hypoxic (~17% O\textsubscript{2}) and hypercapnic (~3% CO\textsubscript{2}) conditions within their nest chambers (Birchard and Kilgore, 1980). Similar to the open-nesting bird species described above, ~2-week-old bank swallow nestlings showed an intact HCVR when evoked with acute respiratory gas disturbances. Colby et al. (1987) reported these ventilatory changes in juveniles as “blunted” compared with adults. However, this comparison is fundamentally different than those in the experimental studies listed above since it is assumed that the adult bank swallows experienced the same hypoxic and hypercapnic conditions during their own “chickhood” and may also have relatively blunted respiratory chemoreflexes.

Since experiencing elevated CO\textsubscript{2} during incubation results in changes in ventilatory chemoreflexes, it is reasonable to propose that the breathing control network is malleable to pH influences during the embryonic period. However, only whole-animal breathing parameters have been assessed after these experiences. There is a need for studies that test whether these changes in behavior can be partially explained by differences in the structure and function of central breathing circuits. The following subsection presents the current evidence for the maturation of chemoreceptors in avian embryos which provide feedback to the breathing control network.

1.6.3 Peripheral chemosensors and mechanosensors

Overall, birds possess functional ventilatory chemoreflexes from at least the beginning of air-breathing through hatching and these reflexes can be shaped by embryonic experience. However, relatively little is resolved about when peripheral chemosensors and mechanosensors first appear developmentally. Here, I provide a
brief overview of the maturation of peripheral chemosensors including intrapulmonary chemoreceptors (IPC), carotid bodies, and mechanoreceptors.

To examine the maturation of IPC, a series of experiments were performed in which the inspired CO\textsubscript{2} was changed in unidirectionally ventilated ducklings at prenatal, paranatal, and hatchling periods. These data suggest that IPC are able to sense abrupt changes in CO\textsubscript{2} within the airway by about 12 hours before hatching (Pilarski and Hempleman, 2007). Similar studies have not yet been performed in other bird species, therefore it is unclear whether the chronology of IPC development is a general property of birds. However, the responses of paranatal birds to the acute hypercapnia hints that IPC could be similarly functional in other bird species since all of them show changes in ventilation during acute CO\textsubscript{2} exposure where O\textsubscript{2} levels remained at a constant, normal level (Dzialowski et al., 2016; Menna and Mortola, 2003; Mortola and Toro-Velasquez, 2014; Pettit and Whittow, 1982; Sirsat and Dzialowski, 2016; Szdzuy and Mortola, 2008).

Changes in O\textsubscript{2} concentration are primarily relayed to the central nervous system (CNS) by the carotid body. Avian carotid body glomus cells detect hypoxia and respond by increasing the rate of action potential firing which is conveyed to respiratory control areas via the vagus nerve (Milsom and Burleson, 2007). Hypercapnia can also be detected by carotid bodies and triggers an increase in vagal afferent firing rate (Hempleman et al., 1992; Nye and Powell, 1984). The embryological development of carotid body has been followed using electron microscopy and histochemical labeling techniques (Fontaine, 1973; Hempleman and Warburton, 2013; Kameda, 1994). After migrating to their mature position, carotid bodies start to function just before hatching,
which is inferred from ventilatory changes following acute perturbations in O₂ during internal pipping, external pipping, and hatchling phases (see Table 1 and Mortola and Toro-Velasquez, 2014; Pettit and Whittow, 1982; Szdzuy and Mortola, 2007b). Carotid bodies may play a role in the changes to ventilatory chemoreflexes following chronic hypoxic exposure during incubation. For example, exposure to hypoxia only resulted in blunted chemoreflexes when chick embryos were exposed during either the full 3-week incubation or the last week, during which carotid bodies become functional (Ferner and Mortola, 2009b).

Ventilatory mechanoreceptors in birds are found in the air sac system (Kubke et al., 2004). Air sac mechanoreceptors detect the expansion and compression of these spaces during the phases of ventilation (Leitner and Roumy, 1974; Molony, 1974). Mechanoreceptors from avian air sacs provide vagal afferent input to ventilatory control centers via the nucleus of the solitary tract, but relatively little detail is known about this system in birds (but see Arends et al., 1988). Even less is known about the development and maturation of stretch sensitive air sac mechanoreceptors. We speculate that airway afferents are likely to remain nonfunctional until air-breathing begins at internal pipping and fluid is removed from the airway and air sac system. In mammals, including humans, vagal afferents, such as pulmonary stretch receptors, are somewhat functional, although not necessarily mature, at birth and can even evoke inspiratory and expiratory reflexes in the newborn (Fisher and Sant'Ambrogio, 1982; Frappell and MacFarlane, 2005; Hannam et al., 2001).
1.6.4 Central chemosensors

Recent work in the zebra finch embryo has yielded evidence for central chemosensation in the avian embryo hindbrain which will be described in Chapter 2 and has been published in part (Whitaker-Fornek et al., 2019). Further, the ventilatory responses to acute hypercapnia in young birds outlined above could be partly due to stimulation of central chemoreceptors. The main goal of this dissertation research is to describe the central chemosensitivity of the zebra finch hindbrain. To do this, it is necessary to study the motor rhythms generated by hindbrain neural circuits. The following section introduces general motor rhythms produced by vertebrate embryos.

1.7 Origin of motor behaviors in the vertebrate embryo nervous system

The normal development of the nervous system depends on genetic factors and the activity of newborn neurons in response to environmental conditions. First, neurons and glial cells differentiate from their ectodermal and neural crest progenitors. Genes and transcription factors broadly guide the transformation of neural progenitor cells into distinct cell types, but as soon as axons stretch toward their synaptic partners, neurons begin to generate spontaneous electrical activity. The electrical activity is thought to be a signal itself that ultimately guides axon pathfinding, synapse formation, and neurotransmitter phenotype (Moody and Bosma, 2005). Within specific segments of the nervous system, large populations of neurons begin to spontaneously synchronize and discharge their membrane potentials with action potentials (Fortin et al., 1995; Momose-Sato and Sato, 2013). Waves of these episodes oscillate with quiescent periods, which
are characterized by low levels of activity. Fortuitously, these rhythmic oscillations can be sampled and recorded using electrodes (Chub and O'Donovan, 1998) and voltage-sensitive dyes (Sato and Momose-Sato, 2017). This bioelectric activity is referred to as rhythmic spontaneous neural or network activity (rSNA). For the remainder of this document, I will refer to the synchronous activation events that characterize SNA as “bursts” but the reader should keep in mind that these events may be referred to as “episodes” comprised of multiple “bursts” or “burstlets” in the literature.

The study of rSNA is not new and has a long history beginning with observations and questions about the nature of early vertebrate embryos that regularly and rhythmically move their bodies and limbs within the uterine or egg environment (Momose-Sato and Sato, 2016; Preyer et al., 1937). Embryonic motility has important implications for normal development. In humans, abnormal embryonic movement behavior in the womb can be associated with arthrogryposis or anencephaly in severe cases (De Vries and Fong, 2007). In vivo and in vitro studies of rSNA in avian embryos show that embryonic “exercise” or “practice” is a byproduct of rSNA in spinal motor circuits (Bekoff, 1976; O'Donovan, 1999). These primordial movements have been further linked with normal locomotor development through experimental manipulations of the frequency of movement episodes in living chicken embryos (Oppenheim et al., 1978).
1.8 Breathing-related motor behavior in the embryo hindbrain

Given the connection between rSNA and an embryo's spontaneous movement behavior, it is reasonable to suggest that rSNA drives other emerging motor behaviors such as fetal breathing movements. In humans, fetal breathing movements have been detected as early as 10-12 weeks of gestation (Darnall, 2010; Pilarski et al., 2019). In lambs, the absence of fetal breathing movements results in poor lung development (Harding et al., 1993). Therefore, it is imperative to understand the role of rSNA in the maturation of breathing behavior. To do this, neural activity has been recorded in isolated preparations of the avian and mammalian embryo hindbrain. Much like studying rSNA in the lumbar spinal cord to determine how it corresponds with chicken embryo leg movements, it is necessary to first describe the age-dependent patterns of neural activity in the hindbrain prior to exploring its particular roles in neurodevelopment.

Regardless of the axial segment of the nervous system, data show that the location, timing and shape of the motor bursts that make up rSNA change as vertebrate embryos age (Fortin et al., 1994; Vincen-Brown et al., 2016b). Figure 1.2 shows examples of SNA recorded from either the hindbrain (chicken, zebra finch, mouse, frog) or cervical spinal cord (rat, opossum) of six different vertebrate embryos. Chicken and zebra finch embryos provide the earliest glimpses of rSNA during the first third of gestation. In these birds, the initial flickers of neural activity consist of short-duration bursts that regularly occur every few minutes (Fortin et al., 1994; Vincen-Brown et al., 2016b). As these avian embryos age, burst duration and the quiescent time between bursts (i.e., the interburst interval) substantially increase. In the days leading up to hatching, the zebra finch embryo's rSNA undergoes a second major transformation to a
much faster rhythm composed of short-duration bursts. No records of hindbrain spontaneous electrical activity are available for the paranatal chicken embryo (i.e., once continuous air-breathing begins during internal pipping). Since chicken embryos hatch at a relatively more mature state than the zebra finch, the health of the chicken embryo nervous system may be more difficult to maintain in the in vitro conditions necessary for direct recordings of neural activity. The absence of data on hindbrain neural activity may not indicate that the nervous system stops producing rSNA in the late-stage chicken embryo. Rather, rSNA is likely difficult to detect via extracellular suction recordings of cranial nerve motor output due to the physical constraints of the tissue in vitro (e.g., thickness of tissue determines ease of diffusion of respiratory gases). Nonetheless, this matter remains unresolved.

The rodent embryo’s pattern and SNA rhythms mirror that of the avian embryo but over a condensed time scale closer to birth (Figure 1.2). Mouse and rat embryos initially display arrhythmic bursts of neural excitation that vary in pattern (Abadie et al., 2000; Ren and Greer, 2003). By the 12th-14th days of gestation, rodent embryos exhibit rSNA that appears similar to the embryonic day (E) 4.5 chicken. During the final third of gestation, the rodent embryos display two dramatic changes in the shape and rhythm of SNA. First, burst duration increases for both species and rat embryos exhibit a much slower rate of bursting. The second major change occurs at about 4 days before birth; mouse and rat embryos’ rSNA become faster and burst duration drops. This fast-paced rSNA showed by older mouse and rat embryos is akin to rSNA recorded from the cervical spinal cords of opossum neonates (Eugenín and Nicholls, 1997) and the hindbrains of paranatal zebra finch embryos (Vincen-Brown et al., 2016b).
The common patterns and rates of endogenous neural activity in late-gestation birds and mammals may be linked to the onset of air-breathing in which the rapidly increasing metabolic rate must be matched to ventilation. Birth, even the “birth” of a bird is a time when respiratory physiology changes abruptly, i.e., a rapid transition from aqueous to aerial breathing. Further evidence for the necessity of increased ventilatory motor output across taxa is exemplified by the conversion to air-breathing behaviors in amphibian preparations. Frogs also show similar alterations in ventilatory pattern and timing when advancing from pre- to post-metamorphic time points (Hedrick, 2005). The frog hindbrain rSNA increases in the rate of bursting as it metamorphoses from a gill-ventilating tadpole to a primarily air-breathing froglet.

Despite much observational knowledge about the developmental chronology of hindbrain rSNA patterns, how the changing hindbrain signals relates to the specific details of breathing motor function remains largely unclear. In other areas of the nervous system, rSNA contributes to proper sensory and motor system development. As rSNA propagates across sensory epithelia such as the retina and cochlea, sensory maps are built and refined (Blankenship and Feller, 2010). In motor networks, rSNA assists motor neurons in finding their way to target muscle fibers (Kastanenka and Landmesser, 2010) and honing nerve-muscle synapses (Ding et al., 1983). Thus, the age-dependent differentiation of hindbrain SNA may reflect changes in the structure and functional connectivity of neuronal circuits therein.

However, much less attention has been paid to the development of breathing circuits in general (but see Eugenín et al., 2006; Greer et al., 2006; Mellen and Thoby-Brisson, 2012), and little is known about these circuits in birds. Birds are a great model
to study respiratory rhythmogenesis in embryos since their young grow outside the mother in hard shelled eggs. Also, birds’ unique respiratory system provides an opportunity to compare what is known about central respiratory control circuits in mammals. As described in a previous section, birds engage in active inspiration and expiration at rest which is different from mammals. These comparative aspects may reveal unique or fundamental aspects of central breathing circuit morphology and function.

Recently, the zebra finch embryo has provided some of the details of early maturation of hindbrain motor circuitry via measurements of rSNA made simultaneously from pairs of cranial nerves (Figure 1.3). When rhythmic motor output begins, synchronous motor outflow can be recorded from pairs of cranial nerves which normally innervate breathing muscles in life. Bursts recorded from the glossopharyngeal nerve occur in-phase with bursts recorded from the vagus nerve (Figure 1.3B). Similar synchronization occurs across the trigeminal, facial, glossopharyngeal, vagus, and hypoglossal nerves in the E4.5 chicken embryo hindbrain as well (Fortin et al., 1994). The coincident timing and pattern of motor output, among different populations, suggests that hindbrain circuitry is relatively undifferentiated at this time and shares circuit connectivity.

In contrast, by E13, Figure 1.3C shows distinct phases of rSNA travel along cranial nerves of the air-breathing paranatal zebra finch, suggesting developmental specialization during the incubation period (Whitaker-Fornek et al., 2019). When the glossopharyngeal nerve is active, motor output in the vagus nerve decrements (Figure 2D). Figure 1.3D also shows that the vagus nerve becomes increasingly active
immediately following the glossopharyngeal episode. By this stage of incubation, the glossopharyngeal nerve innervates the glottis musculature, which opens during inspiration in Aves (Ole Naesbye Larsen and Goller, 2002). The vagus nerve may innervate upper respiratory tract muscles involved in post-inspiratory braking (Richter and Smith, 2014).

When the embryo’s mandible and tongue can be observed in situ it is possible to simultaneously record systems level rhythmic motor output from multiple sources while observing tongue movements and glottis opening. Figure 1.4 shows recordings from both the glossopharyngeal (glottal opener) and spinal accessory nerves (inspiratory muscles) in a semi-intact preparation of the hindbrain with the lower bill attached. Bursts of activity occurred during protrusion of the tongue and opening of the glottis. In mammals, data show that the movement of the tongue forward in the oral cavity helps expand the upper airway during inspiration while the glottis opens the trachea during each inhalation (Miller, 2002; Sawczuk and Mosier, 2001). Hatchling zebra finch also show visible tongue movements when breathing with the bill open (personal observation). The vagus nerve was most active when the tongue returned to its original position. This vagal activity persisted until the tongue protruded again. The timing of vagal activity is consistent with post-inspiratory activity or expiratory activity in other animals including humans (Richter and Smith, 2014). A schematic drawing of these phasic activation patterns was modeled using audio recordings of the motor output in combination with a video recording of tongue movements (Figure 1.4). Future studies using this semi-intact preparation will seek to record from the glossopharyngeal and vagus nerves simultaneously.
Other recent studies have described the early maturational changes in the context of neurotransmitters responsible for rSNA patterns. For example, the dominant neurotransmitters that drive hindbrain rSNA during the first third of development are acetylcholine, glycine, and \( \gamma \)-aminobutyric acid (GABA) (Pickett et al., 2018; Vincen-Brown et al., 2016a). Over time, glutamate becomes a more important excitatory rhythmogenic component than GABA and glycine. Close to hatching, GABA becomes an important inhibitory component for shaping cranial nerve motor output from the central pattern generating network, and may play a role in the link between phasic ventilation and central chemosensitivity. It is this pattern in neurotransmitter profile over embryogenesis that forms the basis for my dissertation project.

The dynamic and rapidly changing nature of chloride neurotransmission is attributed to developmental changes in transmembrane ion transport (Schulte et al., 2018). Briefly, a sodium-potassium-chloride cotransporter (NKCC1) predominates during early embryogenesis. NKCC1 mediates chloride influx, leading to an increased concentration of intracellular chloride relative to the extracellular fluid. This greater intracellular chloride load results in a more depolarized equilibrium potential for GABA (\( E_{\text{GABA}} \)) compared with resting membrane potential (\( V_{\text{rest}} \)). Therefore, when GABA binds to its receptor, chloride efflux occurs which drives \( V_{\text{rest}} \) closer to the threshold for firing action potentials. Later during prenatal or early postnatal development, a potassium-chloride cotransporter (KCC2) becomes more dominantly expressed and facilitates chloride efflux. The action of KCC2 lowers the amount of intracellular chloride relative to extracellular chloride. With this shift in chloride concentration gradient, \( E_{\text{GABA}} \) becomes more hyperpolarized compared with \( V_{\text{rest}} \). Therefore, when GABA binds to its receptor,
chloride entry will occur and hyperpolarize membrane potential making it less likely for neuronal excitation. These developmental shifts in excitatory and inhibitory neurotransmission may be important for regulating the maturation of function within hindbrain breathing circuits.

1.9 Overview of the dissertation research

I hypothesize that CO$_2$ and pH contribute to the neurogenesis of rSNA and breathing-related cranial nerve motor output throughout the entire window of embryonic development, but in unique ways depending on age. To test this hypothesis, I formed three thematically connected research aims. **Aim 1** was to examine the role of central pH and CO$_2$ in the generation and maintenance of rSNA during prenatal development. It is known that internally pipped birds, which are continuously breathing air, are sensitive to pH/CO$_2$ stimuli (Szdzuy and Mortola, 2007a). High CO$_2$/low pH stimulates breathing and low CO$_2$/high pH attenuates breathing. It is also known that high CO$_2$/low pH inhibits non-respiratory neurons and low CO$_2$/high pH excites them (Sinning and Hübner, 2013). However, it is completely unknown how pH and CO$_2$ chemosensitivity develops in the avian hindbrain as neurons transition from non-respiratory to specialized respiratory neurons during the prenatal and perinatal period. **Aim 2** was to determine whether chloride is involved with the development of central pH/CO$_2$ chemosensitivity. It is known that GABAergic neurotransmission achieves its adult phenotype (i.e., inhibitory neurotransmission) around internal pipping (E12) and the establishment of air-breathing (Pickett et al., 2018). Thus, I tested the degree to which chloride could explain the similar time course of pH/CO$_2$ chemotransduction. My rationale was that CO$_2$ hydration,
and alterations in pH and [HCO₃⁻], are linked to chloride-mediated neurotransmission and chloride transport. **Aim 3** was to test whether chloride contributed to the homeostatic recovery of rhythmic SNA after low pH had depressed motor rhythm in younger zebra finch embryos. It is known that rSNA overcomes multiple “stressors” that tend to decrease or eliminate neural spiking behavior by increasing neural activity (Turrigiano and Nelson, 2004). This phenomenon occurs most strongly during early embryonic development, prior to internal pipping, at a time when the embryo is trapped within the aqueous environment of the eggshell and respiratory-related circuits in the brainstem are generic and not yet specialized for aerial breathing behavior (Vincen-Brown et al., 2016a; Wenner, 2011).

The following chapters of this dissertation are presented in manuscript format. Chapter 2 of this dissertation entitled “*Effects of central metabolic and respiratory disturbances on avian hindbrain motor rhythms before and after breathing begins*” describes the day-by-day effects of metabolic acidosis/alkalosis and respiratory acidosis/alkalosis on hindbrain rhythms in the altricial zebra finch. Part of the data in Chapter 2 have been published (Whitaker-Fornek et al., 2019). Chapter 3, entitled “*Role of chloride in the age-dependent effects of pH on rhythmic neural activity from early embryogenesis through hatching,*” provides insights into the mechanisms of pH sensitivity over the course of zebra finch embryogenesis. Specifically, I describe three experimental series that test the hypothesis that chloride is involved in the changes I observed in rSNA and breathing-related activity during low pH exposure. Chapter 4, entitled “*Role of chloride in the recovery of avian respiratory motor rhythms during acidosis,*” examines how rSNA can be robust to prolonged pH changes during specific
periods of incubation. Here, I examine how chloride removal, blocking chloride transport, and disrupting GABA<sub>A</sub> receptor function affect “recovery” or homeostatic plasticity of early rhythms under stress. Chapter 5, entitled “Conclusions,” contains an overall summary of this dissertation and proposes future studies that build on the findings described here.

Figure 1.1 A developmental time-line for avian embryos in the context of respiration. From the early prenatal period through hatching, the physiochemical environment changes, resulting in embryos transitioning from purely diffusive gas exchange to convective pulmonary ventilation. Due to embryonic metabolism, the blood of vitelline and chorioallantoic arteries become acidic (dotted line). This acidification is somewhat blunted by [HCO<sub>3</sub>⁻] (solid line) that compensates for the ramifications of rising PCO<sub>2</sub> (dashed and dotted line). The in ovo environment shifts during the transition to air breathing as PO<sub>2</sub> rises (dashed line) with the onset of internal and external pipping (dark gray and light gray shading, respectively). Rhythmic spontaneous neural activity (SNA) can be recorded from cranial nerves of the hindbrain that innervate accessory respiratory muscles in both bird embryos on E4. Accordingly, the rhythmic
SNA measured via cranial nerve XI shows changing pattern and complexity over chronological time (schematic drawing above time-lines, | = single burstlet, █ = multiple burstlets). SNA transitions to an air-breathing motor outflow near E11-12 in the zebra finch and likely at E18-19 in the chicken. Also, changes in the neurotransmitter profile responsible for SNA shift from acetylcholine to excitatory chloride during the early to middle days of incubation. Just before air-breathing begins, excitatory glutamatergic inputs drive SNA and GABA/glycine take on inhibitory roles once breathing is underway. References: 1. (Ackerman and Rahn, 1981), 2. (Wangensteen and Rahn, 1970), 3. (Chiba et al., 2002), 4. (Tazawa et al., 1971), 5. (Baumann and Meuer, 1992), 6. (Meuer et al., 1989), 7. (Meuer and Tietke, 1990), 8. (Vincen-Brown et al., 2016b), 9. (Fortin et al., 1994), 10. (Pickett et al., 2018), 11. (Chatonnet et al., 2002).
Figure 1.2 Early rhythmic spontaneous neural activity produced by vertebrate embryos of different ages. During the prenatal period, the nervous system actively produces episodes of excitation known as rhythmic spontaneous neural activity (SNA). Since vertebrate embryos exhibit different total lengths of gestation (e.g., chicken = 21 days until hatching vs zebra finch = 14 days until hatching), examples of rSNA are binned into three windows of time representing thirds of the embryonic period. Chicken, zebra finch, mouse, and frog examples of hindbrain rSNA recorded from cranial nerves are shown. Electrical recordings for the rat embryos and opossum neonates represent cervical spinal cord rSNA recorded from en bloc brainstem spinal cord preparations. For birds, rSNA begins in the first third of incubation. By the second third of gestation, SNA is detected in mice, rats, and frog tadpoles. The gray short-tailed opossum displays rSNA at birth. Despite different onset times of rSNA, both avian and rodent embryos show longer duration rSNA episodes during the second third of the prenatal period and all three groups show increased episode duration and frequency during the final third of prenatal life.

Figure 1.3 Zebra finch embryo hindbrain anatomy and motor outflow over time. 

A. Drawing of the embryonic day (E) 6 zebra finch hindbrain with cranial nerves labeled. B. On E6, cranial nerve (CN) IX and CN X show synchronous activation when both nerves are recorded from simultaneously. C. Drawing of the E14 zebra finch hindbrain with CNs labeled. D. A dual recording of CN IX and CN X on E13 shows alternation of activation periods across the two nerves. The alternating activity patterns may correspond with inspiration and expiration phases of the breathing cycle. Specific phase relationships among motor nerves suggests the presence of more specialized neural circuits for breathing control in the air-breathing E13 bird.
Figure 1.4 Model of zebra finch embryo tongue movements and rhythm. Extracellular recordings of cranial nerve (CN) XI and X were performed on an embryonic day (E) 14 zebra finch embryo with the lower jaw and tongue intact. Using a video recording of the preparation and audio recording, the relative timing of tongue movements were aligned with traces of CN activity. CN XI (depicted above) and CN IX (not depicted above) motor neurons discharged when the tongue moved forward in the mouth. The forward movement of the tongue may occur during inspiration in birds similar to mammals. CN X was active as the tongue moved back into the mouth cavity which is consistent with vagal activation during post-inspiration in mammals.
2. Effects of central metabolic and respiratory disturbances on avian hindbrain motor rhythms before and after breathing begins

2.1 Abstract

Similar to mammals, birds adjust their ventilation to compensate for acid-base imbalances. Typically, low pH is sensed within the brainstem chemosensitive neurons that become excited by acidification. The opposite would occur in response to high pH. These increases and decreases in neural activity control ventilatory muscles such that ventilation corrects deviations from the proper electrochemical environment. Unlike mammals, very little is known about central respiratory-related chemosensitivity in the avian hindbrain. Further, it is unknown how central chemosensitivity emerges embryonically such that hatchling birds can produce appropriate ventilatory responses when breathing begins. Therefore, I used the in vitro zebra finch hindbrain preparation to assess the sensitivity of hindbrain breathing circuits to pH from embryonic day (E) 4 through hatching (E14). I found that a neurodevelopmental “switch” occurred in the response of motor rhythms to pH. Lowering pH resulted in slower rates of motor rhythms in E7-11 birds (66 ± 11%; n = 17; P = 0.048) while the same stimulus evoked faster rhythmic activity in E12-14 birds (151 ± 19%; n = 11; P = 0.009). Raising pH increased the frequency of rhythm in E7-11 birds (219 ± 23%; n = 15; P < 0.0001) while this perturbation tended to decrease the frequency of E12-14 rhythm (58 ± 9%; n = 8; P = 0.5). Exposing hindbrain preparations to higher or lower CO₂ levels resulted in a similar pattern of responses as observed when manipulating sodium bicarbonate (NaHCO₃) levels in the artificial cerebrospinal fluid. However, the gaseous stimuli did not evoke statistically significant changes in rhythmic activity. This finding was likely due
to the fact that altering CO$_2$ gas concentration resulted in pH changes of smaller magnitudes compared with the aCSF containing lower or higher NaHCO$_3$\textsuperscript{−}. These results show that the avian embryo hindbrain possesses central chemosensitivity approximately 1 week prior to hatching and that the influence of pH on motor rhythm frequency switches from inhibitory to excitatory as air-breathing begins.

\section*{2.2 Introduction}

Despite recognizing the function of central chemosensitive areas in the adult bird brain (Milsom et al., 1981), it is still largely unknown how these sensitive regions form during embryogenesis in time to be functional during a bird’s first breath. Birds are ideal for studying how central chemoreceptive brain areas emerge during the embryonic period. Avian embryos are conveniently located in hard shelled eggs outside of the mother and birds begin pulmonary ventilation over an extended period of time leading up to hatching. The extended hatching sequence is amenable to observation and experimentation. There is a long tradition of testing the effects of acute and chronic alterations in respiratory gases on whole animal breathing parameters in hatchling birds (Bavis and Simons, 2008; Colby et al., 1987; Szdzuy and Mortola, 2007b). However, little is known about the embryonic origins of central chemosensitive regions in the hindbrain. Further, it can be technically challenging to access or manipulate central chemoreceptors in intact bird embryos. To address this gap, I employed the \textit{en bloc} hindbrain preparation of the altricial zebra finch embryo (Vincen-Brown et al., 2016b). Using this preparation, it is possible to track, day-by-day, the rhythmic spontaneous neural activity (rSNA) carried by cranial nerves that will eventually supply respiratory
muscles with motor commands for breathing. In addition, this preparation allows for the fine control of the hindbrain’s extracellular environment by altering properties of the artificial cerebrospinal fluid (aCSF) superfusing the neural tissue. This experimental platform is well-suited for testing the effects of metabolic or respiratory acid-base disturbances on the production of the first sparks of rSNA through the more mature motor output driving air breathing.

From the beginning of incubation, a bird embryo is faced with acid-base balance challenges that could impact the function of the nervous system including developmental signals from rSNA. Therefore, I hypothesized that manipulating the acidity and alkalinity of the extracellular fluid bathing the hindbrain could reveal different roles for these stimuli depending on embryonic age. Earlier in development, prior to air-breathing, the hindbrain may adjust neural activity in a manner reminiscent of non-respiratory neurons experiencing pH shifts (i.e., acidity dampens neural excitation while alkalinity enhances activity), thus preserving neural function (Sinning and Hübner, 2013). Alternatively, I hypothesized that once the embryo initiates pulmonary ventilation, acidification or alkalinization of the hindbrain may alter breathing-related motor output to respiratory muscles in a way consistent with the whole animal hypercapnic or hypocapnic ventilatory responses (e.g., hypercapnic acidosis would result in increased ventilatory effort or greater frequency, duration, or amplitude of motor output which would help blow off excess CO₂ in an intact bird).

I specifically tested whether rSNA and breathing-related motor rhythms were sensitive to conditions modeled after respiratory acidosis/alkalosis or metabolic acidosis/alkalosis. To my knowledge, this is the first study to examine the maturation of
central CO$_2$ and pH chemosensitivity continuously through prenatal and early paranatal development. Some of the data in this chapter were previously published (Whitaker-Fornek et al., 2019).

2.3 Methods

2.3.1 Experimental animals

All procedures were approved by the Idaho State University Institutional Animal Care and Use Committee. I collected zebra finch eggs from an in-house breeding colony, candelled eggs to estimate developmental age, and placed eggs into a temperature-controlled (38°C), humidified (60-70%), forced-draft incubator (GQF 1502 Sportsman). A total number of 125 embryos were used for the experiments. Embryos aged E4-E14 were used for all experimental protocols. Ages were grouped into three categories: early (E4-E6), middle (E7-E10), and late (E12-E14) unless otherwise specified. These age groups display similar patterns of baseline rhythm frequencies and durations of rSNA episodes (Pickett et al., 2018). I estimated the number of animals needed for each type of experiment using a power analysis calculator, G*Power software (version 3.0.10). Using conservative values for effect size (0.25) and power (0.8), I determined that a minimum of 5 animals per age group for each experiment would be sufficient to detect a significant treatment effect (P < 0.05) using a two-way ANOVA with repeated measures. However, to better assess the responses of individual ages day-by-day, I collected data from two to three individual embryos at each day of development which resulted in larger numbers of animals per age group.
2.3.2 Preparation of control and experimental cerebrospinal fluid

Control artificial cerebrospinal fluid (aCSF) was prepared using millipore-filtered deionized water (NanoPure, Mt. Pleasant, SC) and contained the following components (in mM): 120 NaCl, 26 NaHCO₃, 30 D-Glucose, 1 MgCl₂, 3 KCl, 1.25 NaH₂PO₄, 1 CaCl₂. Control aCSF was equilibrated with 95% O₂ and 5% CO₂ gas (Airgas, Pocatello, ID) until a pH of 7.45 was reached (Accumet model 10 pH meter, Waltham, MA). In order to determine the roles of [H⁺] and CO₂ on rSNA and respiratory related neural activity, four experimental aCSF treatments were prepared that modeled acid-base disturbances, 1.) metabolic acidosis: 13 mM NaHCO₃ and the solution was bubbled with 95% O₂ and 5% CO₂ until a pH of 7.21 was reached; 2.) metabolic alkalosis: 52 mM NaHCO₃ and the solution was bubbled with 95% O₂ and 5% CO₂ until a pH of 7.8 was reached; 3.) respiratory acidosis: same recipe as control aCSF only bubbled with 90% O₂, 8% CO₂, and balance N₂ until a pH of 7.2 was reached; 4.) respiratory alkalosis: same recipe as control aCSF only bubbled with 90% O₂, 2% CO₂, and 8% N₂ until a pH of 7.8 was reached. Table 2.1 lists the constituents for each experimental aCSF. I selected the pH values and associated [NaHCO₃⁻] concentrations or CO₂ levels according to experiments investigating central pH chemosensitivity in neonatal rodent brainstem tissue in vitro (Eugenín et al., 2006; Kawai et al., 2006; Voipo and Ballanyi, 1997). Additionally, I performed “dose-response” experiments with step changes in [NaHCO₃⁻] to settle on the values for metabolic acidosis and alkalosis used in this study.
### Table 2.1 Artificial cerebrospinal fluid constituents by treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>CO₂ (%)</th>
<th>NaHCO₃ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic Acidosis</td>
<td>7.21</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Respiratory Acidosis</td>
<td>7.21</td>
<td>8</td>
<td>26</td>
</tr>
<tr>
<td>Metabolic Alkalosis</td>
<td>7.8</td>
<td>5</td>
<td>52</td>
</tr>
<tr>
<td>Respiratory Alkalosis</td>
<td>7.8</td>
<td>2</td>
<td>26</td>
</tr>
</tbody>
</table>

#### 2.3.3 Surgical isolation of brainstem preparation

Embryos were anaesthetized using ice and quickly decerebrated to ensure the exclusion of somatic or visceral pain sensations during surgery. Embryos were placed in room temperature, control aCSF bubbled with control gas (90% or 95% O₂ and 5% CO₂; pH 7.45). Developmental stage was determined using external morphological features such as limbs, eyes, feathers, and the beak (Murray et al., 2013). Once rhythmic neural activity was recorded, burst characteristics and baseline frequency were used to more finely determine age (see Appendix for details). All experiments were checked for adherence to rhythm-based age guidelines with the help of Jennie Nelson (undergraduate Team Bird Brain lab member). Subsequently, tissues surrounding the neuraxis were carefully cut away. After exposing the brainstem and spinal cord, the neuraxis was isolated into a segment with the rostral border between the pons and mesencephalon and caudal border at the first segment of the spinal cord. On the dorsal aspect of the isolated segment, the cerebellum was removed. Finally, the spinal accessory nerve, henceforth referred to as cranial nerve (CN) XI, was identified and gently cleared of overlying meninges. CN XI innervates the cucullaris muscle (Tada and
Kuratani, 2015). This is a neck muscle that we speculate is involved in the maintaining the integrity of the upper airway during inspiration.

2.3.4 Extracellular recording and data collection

During the extracellular recording procedure, the brainstem preparation was carefully pinned to the bottom of a sylgard-lined chamber and superfused with room temperature, control aCSF at a flow-rate of 3 ml/min (Figure 2.1, panel A). The temperature of the aCSF was slowly raised using an in-line heater and chamber heater to 26.5 ± 1 °C (Warner Dual Automatic Temperature Controller, model TC-344B, Holliston, MA). A pulled glass micropipette tip was broken and fire-polished in order to snugly accommodate the entire transected CN XI and/or rootlets connecting CN XI to the medulla. The nerve was aspirated into the tip of the glass micropipette using gentle suction. Differential amplification was used to detect neural signals by comparing signals recorded by an active Ag/AgCl electrode in contact with the CN XI to a reference electrode in the bath (HI-Z differential pre-amplifier, Grass Instruments, Quincy, MA). Signals from the pre-amplifier were further amplified (20 to 20000) and band pass filtered (0.03-3 kHz) using an amplifier (Grass Instruments). Signals were further processed using an analog to digital converter (Power1401, Cambridge Electronic Design, Cambridge, UK) and recorded directly to a PC for storage and analysis using Spike2 software (Version 8).
2.3.5 General experiment protocol

Figure 2.1 (panel B) shows a schematic diagram of the general treatment protocol. Before beginning experimental treatments, stable baseline CN XI activity was recorded for 30 minutes while the preparation was superfused with the appropriate control aCSF. Next, the control aCSF was replaced by a single experimental aCSF by switching to a reservoir containing an experimental aCSF for 1 hour. I superfused each hindbrain with only one experimental aCSF solution. This approach provided an assessment of the effects of metabolic or respiratory acidosis/alkalosis on CN XI activity without any chance of earlier treatments influencing later activity. Each treatment was applied for 1 hour because it allowed enough time for 2-3 episodes of SNA to occur at developmental stages where rhythmic activity is slowest. Following the treatment, control aCSF bathed the preparation for 30 min or until rhythmic neural activity resembled the original, baseline rhythm. The pH of superfusate was regularly checked during experiments using a small pH electrode placed in the bath (Orion PerpHecT ROSS Combination pH Micro Electrode, Thermo Scientific, Waltham, MA).

2.3.6 Ensuring independence, summarizing data, and analyzing data

The zebra finch brainstem preparation was the unit of replication for all experiments. Zebra finch brainstems were selected randomly without specific knowledge of their exact age, sex, or parentage. To further minimize the effect of confounding variables, I did not consecutively perform all of the experiments for a single experiment series and then move to another experiment series (i.e., perform all of the low pH and then all of the high pH experiments). Instead, I selected treatments on a
rotating basis to prevent confounding variables that could arise from performing one set of experiments in the spring and another set of experiments in the winter.

I collected summary data (i.e., frequency, amplitude, duration, area) from Spike 2 software data files recorded during each experiment. I used a previously written in-house Spike2 script to measure frequency, duration, and amplitude of cranial nerve motor rhythms (see Appendix). The average frequency, duration, and amplitude was calculated for the entire baseline and treatment periods for each experiment. I divided the average frequency during treatment by the average frequency during the control period to normalize data to the percentage of control period frequency. The same procedure was done to duration, amplitude, and area data. This adjusted for slight differences in baseline rhythms between animals. These summary data were tabulated in Excel and I calculated means ± SEM by age and age group. Means of response variables ± SEM were normalized to percentage of control for a given variable. Normalized, averaged data were plotted by treatment type to determine relationships between dependent and independent variables. To test for age and treatment effects, data were imported into JMP Pro (version 14). I used a two-way ANOVA to test for significant effects of treatment, age, and treatment*age interaction (P<0.05). Tukey’s post hoc test was used to compare treatment effects between age groups.

2.4 Results

2.4.1 Effects of metabolic acidosis and alkalosis on CN XI motor rhythms

Treatment of the hindbrain with solutions mimicking metabolic acidosis (pH 7.2) and metabolic alkalosis (pH 7.8) influenced CN XI rhythm in an age-dependent manner.
Figure 2.2 shows representative traces of rectified and integrated CN XI motor output recorded over the course of low pH (panel A) and high pH exposures (panel B) for specific days of development within the early, middle, and late age groups. The age-dependent differences in response to either acidosis or alkalosis can be seen by looking down each column of traces.

Figure 2.3 shows relative changes in the frequency of rhythmic CN XI activity, day-by-day during superfusion of low pH (panel A) or high pH (panel B) aCSF. The same age-dependent switch in the effects of pH on rhythm frequency can be seen across the day-by-day data. After binning the frequency data into early, middle, and late age groups, statistical analyses revealed significant overall effects for treatment, age group, and treatment*age group interaction (P = 0.0007, P < 0.0001, and P < 0.0001, respectively) (Figure 2.3). Embryos within the early age group showed a non-significant decrease in rSNA frequency during acidosis compared with the frequency during control aCSF (78 ± 7%; n = 8; P = 0.79). Middle age embryos (E7-11) showed decreased rSNA frequency relative to control conditions during the metabolic acidotic treatment (66 ± 11%; n = 17; P = 0.05). E12-14 (late) embryos exhibited increased frequency of CN XI motor rhythms during low pH treatment compared with control periods (152 ± 19%; n = 11; P = 0.009) which differed from the early and middle age groups (P < 0.0001, P = 0.0002, respectively).

Metabolic alkalotic aCSF resulted in an opposite pattern of changes to the frequency of rSNA and breathing-related activity compared with metabolic acidotic treatment (Figure 2.2, panel B; Figure 2.3, panels B and D). E4-6 embryos displayed little change in rSNA frequency during high pH exposure relative to control conditions.
(118 ± 15%; n = 8; P = 0.97). In middle age embryos, rSNA frequency increased relative to control periods (219 ± 23%; n = 15; P < 0.0001) which was different compared with all other age groups (P < 0.0001). Late embryos tended to decrease the frequency of CN XI motor rhythms compared to frequency of rhythm during control aCSF, although this was not statistically significant (57 ± 9%; n = 8; P = 0.5).

Metabolic acidic aCSF had no influence on burst duration or burst amplitude. Metabolic alkaline aCSF increased the burst amplitude of E7-11 rhythmic activity (136 ± 14%, n = 15, P = 0.02) compared with control activity. Figure 2.4 shows summary data grouped by early, middle, and late age groups for low pH (panels A and C) and high pH (panels B and D). High pH did not influence burst duration for any age group.

### 2.4.2 Effects of respiratory acidosis and alkalosis on CN XI motor rhythms

Respiratory acidosis (8% CO₂) and alkalosis (2% CO₂) tended to influence the frequency of rhythmic CN XI activity similarly to metabolic pH disturbances. Figure 2.5 shows representative neurograms of CN XI activity during respiratory acidosis (panel A) and respiratory alkalosis (panel B). Figure 2.6 shows relative changes in the frequency of CN XI activity day-by-day (panels A and B) and by age group (panes C and D). No significant treatment effects were observed for respiratory acidosis in any age group. However, a subtle pattern can be seen where early and middle age groups show dampened frequency during high CO₂ treatment and E11 and E12 show slightly increased frequency. Mean relative frequency for early-, middle-, and late-age groups when exposed to high CO₂ (8%) were E4-6: 87 ± 5%; n = 7; P = 0.85, E7-11: 93 ± 8%; n
= 17; \( P = 0.91 \); E12-14: 89 ± 15\%; n = 8; \( P = 1.0 \)), respectively. For respiratory alkalosis, data show a significant increase in rhythmic SNA frequency for the middle age group (E7-11: 150 ± 7\%; n = 12; \( P = 0.0001 \)). However, neither the early nor late age group exhibited significant changes in the frequency of rhythmic SNA during low CO2 (E4-6: 122 ± 12\%; n = 7; \( P = 0.19 \), E12-14: 88 ± 7\%; n = 8; \( P = 0.66 \)).

Respiratory acidosis and alkalosis had little influence over burst duration and amplitude (Figure 2.7). High CO2 treatment resulted in greater burst duration in E7-11 birds (117 ± 4\%; n = 17; \( P = 0.0053 \)), although no other age group showed significant effects of high or low CO2 on burst duration or amplitude. Respiratory alkalosis tended to increase burst duration in the middle age group but this effect was not significant (148 ± 33\%; n = 12; \( P = 0.49 \)). The middle age group also showed increased burst amplitude during low CO2 treatment relative to control conditions but this was not significant (121 ± 8\%; n = 12; \( P = 0.16 \)). The late age group showed a slight tendency for burst amplitude to increase during low CO2, although this was also not significant (126 ± 22\%; n = 4; \( P = 0.52 \)).

2.5 Discussion

When a mammal is born or when a bird hatches, their respiratory control circuitry must be ready to sense acid-base status and tune breathing to maintain balance. Although whole-animal ventilatory chemoreflexes are functional in paranatal birds and hatchlings, little is known about the embryonic origins of central pH and CO2 chemosensitivity. Therefore, in the current study, I evaluated the effects of metabolic
and respiratory acid-base disturbances on the production of biorhythms in the zebra finch hindbrain from the time when rhythmic activity is first observed through hatching.

2.5.1 pH alters the frequency of rSNA depending on embryonic age

The results show that pH effects depend on embryonic age, with early and middle embryos exhibiting opposite changes in the frequency of CN XI motor output compared with late embryos. These results support the hypothesis that earlier embryos display a more generalized rhythmic response to pH changes (e.g., low pH lowers excitability) while more mature embryos exhibit rhythmic responses consistent with compensating for acid-base imbalance (e.g., low pH increases ventilatory drive).

Besides the present work in the zebra finch embryo, no studies have reported an age-dependent switch in the frequency response of hindbrain motor rhythms to pH. Tests of central pH and CO₂ chemosensitivity in tadpoles (Reed et al., 2019), opossums (Eugenín and Nicholls, 1997), mice (Eugenín et al., 2006), and rats (Kawai et al., 2006) have shown that hindbrain motor rhythms are primarily augmented by acidification. This difference in the zebra finch embryo hindbrain could be related to the relative period of gestation when central chemosensitivity tests were made. In other words, perhaps the developmental switch in the pH response could be detected in other vertebrates if they were tested at earlier stages of the prenatal period.

It is difficult to compare stages of embryonic development across vertebrate groups due to differences in relative maturity at birth, species-specific life history traits such as metamorphosis or pouch-rearing, and varying lengths of gestation. One coarse
method for comparing the age of embryos across taxa is to calculate the relative percentage of gestation represented by the embryos of various species. Using this metric, the E7-11 period (i.e., the earliest time point when pH altered hindbrain rhythmic activity) roughly represents 50-78% of gestation in the zebra finch. Tests of central chemosensitivity in the mouse hindbrain have been performed as early as E13 which is about 65% of gestation (Eugenín et al., 2006). E13 mouse hindbrain rhythm increased in frequency during low pH exposure. The age of mouse embryos tested for central pH chemosensitivity is within the same relative window of time when a dampening of neural activity was observed in the zebra finch during metabolic acidotic treatment. Thus, it appears that the age-dependent switch in response to central pH stimuli shown by the zebra finch is unique compared to the mouse embryo and may reflect phylogenetic differences.

In contrast with the frequency of rSNA, other burst characteristics such as duration and amplitude were largely unaffected by metabolic and respiratory acidosis or alkalosis. The only significant changes were observed in the burst duration of the middle age group embryos during respiratory acidosis and in burst amplitude of the middle age group during metabolic alkalosis. During high CO₂, E7-10 birds exhibited longer bursts during exposure to high CO₂. Hypercapnia may result in a more rapid and broad decline in intracellular pH since neuronal cell membranes are permeable to CO₂. Perhaps diffusion of CO₂ into large swathes of the hindbrain in the middle age group altered recruitment of motor neurons in the CN XI motor pool which resulted in longer bursts. The same age group showed increased burst amplitude during high pH which may be related to the overall increased excitability of hindbrain rhythms during alkalinization.
The increased amplitude may be related to the recruitment of additional motor neurons during high pH as well.

### 2.5.2 Working models of pH sensitivity in the embryo hindbrain

To begin to understand how identical pH stimuli could have opposing effects on the frequency of CN XI motor rhythms depending on age, it is helpful to consider co-occurring changes in the hindbrain breathing network. The change in the polarity of responses to pH occurs sometime between E11 and E12. During the same time period, it has been shown that GABAergic and glycinergic neurotransmission shift from mediating excitatory activity to providing inhibitory inputs within the breathing network (Pickett et al., 2018). The mechanisms underlying this excitatory to inhibitory switch may involve a maturational “changing of the guard” in cation-chloride-co-transporters (Ben-Ari et al., 2007). Initially the Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporter 1 (NKCC1) is abundantly expressed in the embryonic central nervous system (Ben-Ari et al., 2007). NKCC1 mediates Na\(^{+}\), K\(^{+}\) and Cl\(^{-}\) influx which raises the intracellular Cl\(^{-}\) \([\text{Cl}^{-}]_i\) relative to extracellular Cl\(^{-}\) \([\text{Cl}^{-}]_e\) (Schulte et al., 2018). This results in the equilibrium potential for Cl\(^{-}\) \((E_{\text{Cl}^{-}})\) becoming more depolarized than the resting membrane potential. Later in development, the K\(^{+}\) Cl\(^{-}\) co-transporter 2 (KCC2), which mediates K\(^{+}\) and Cl\(^{-}\) efflux, becomes dominantly expressed. Accordingly, \(E_{\text{Cl}^{-}}\) becomes hyperpolarized compared with resting membrane potential and GABA and glycine switch to their typical inhibitory roles. It is important to note that \([\text{Cl}^{-}]_i\) and extracellular \([\text{Cl}^{-}]_e\) do not completely flip in concentration as a result of changing Cl\(^{-}\) transporter expression (e.g., \([\text{Cl}^{-}]_j\) does not fall to zero). Rather, the relative Cl\(^{-}\) levels of the intra- and extracellular spaces become
closer to each other. Currently, no data exist for immunohistological labeling of Cl− transporters in the zebra finch hindbrain. Therefore, it is only possible to speculate that Cl− ionic gradients change at around E11 due to the outcomes of pharmacologic agonists or antagonists of Cl− neurotransmission on rSNA (Pickett et al., 2018).

In the zebra finch hindbrain, the emergence of Cl−-mediated inhibition co-occurs with the switch in response to metabolic acidosis and alkalosis. This raises the question: Is an ontogenetic change in Cl− ion currents or Cl− ion homeostasis involved in the age-dependent pH sensitivity of hindbrain rhythms? Broadly, I hypothesize that the maturational switch in the rhythmic consequences of metabolic acidosis and alkalosis is a result of two age-related factors: 1. changes in the direction of Cl− ion flux, and 2. changes in the specialization and connectivity of hindbrain breathing control circuits which involve inhibitory inputs.

First, pH may have opposing influences on the frequency of CN XI motor output due to the direction of Cl− movement exhibited during the earlier part of the prenatal period. I hypothesize that rSNA frequency may decrease during metabolic acidosis due to the effects of pH on two chloride pathways involved in rSNA production. In cultured mammalian neurons, GABA A receptor activation is enhanced by low pH (Krishek et al., 1996). Thus, GABA A receptors recruited during spontaneous activity may allow greater Cl− efflux during low pH conditions. In the chicken embryo spinal cord, the restoration of [Cl−] stores during the quiescent period between bursts involves the activity of NKCC1 (Gonzalez-Islas et al., 2009). Protonation of NKCC1 has been found to inhibit the function of this cation-Cl− cotransporter in duck red blood cells (Hegde and Palfrey, 1992). Accordingly, low pH exposure may strain the ability of NKCC1 to reaccumulate
[Cl\textsuperscript{-}] stores leading to a gradual slowing of CN XI motor output frequency. Figure 2.8 models this speculative mechanism of Cl\textsuperscript{-} mediated low pH sensitivity in the immature zebra finch hindbrain. Once air-breathing begins at around E12, the influence of low pH on rhythmic frequency may occur independently of high [Cl\textsubscript{i}], since NKCC1 expression is thought to decrease over time (Ben-Ari et al., 2007). Also, the response of speeding up CN XI motor output cannot be explained by acidification-induced enhancement of endogenous GABA\textsubscript{A} receptor activity which could result in a slowing of rhythm as is seen during GABA\textsubscript{A} agonist application (Pickett et al., 2018). Figure 2.9 summarizes the previous ideas showing how the effects of low pH may act independently of Cl\textsuperscript{-} pathways to speed up CN XI motor output in older zebra finch embryos.

Second, age-related growth and specialization in the morphology of hindbrain breathing control circuits may explain the divergence in rhythmic outcomes to pH alterations. I hypothesize that specialized brain regions for detecting altered pH and relaying this information to breathing control neurons may not become functional until the onset of air-breathing (Figure 2.10). Prior to the formation of specialized chemosensitive regions, acidification may lead to a general dampening of neural activity (Sinning and Hübner, 2013). Once air-breathing begins, the need to adjust ventilation according to acid-base status becomes paramount for the health of the developing embryo. Therefore, the emergence or new connectivity of chemosensing regions may result in a switch in the response of systems level output (CN XI motor output) to acidosis. For example, in the raphe region of the rodent hindbrain, a population of GABAergic neurons were identified that are inhibited by high CO\textsubscript{2} or low pH (Iceman et al., 2014). In this scenario, acidity releases serotonergic raphe neurons from inhibition.
so they can provide excitatory inputs to the respiratory rhythm and pattern generating regions. I hypothesize that the establishment of connections between the raphe and breathing rhythmogenic neurons could contribute to the increased CN XI motor output observed in >E11 zebra finch hindbrains.

2.5.3 Considerations and limitations of the current study

The results showed that pre-breathing cranial nerve motor output as well as putative breathing-related rhythms in the zebra finch embryo hindbrain were more sensitive to metabolic (low HCO$_3^-$) pH disturbances than respiratory (high CO$_2$) pH disturbances. Data suggest that the prenatal and paranatal zebra finch hindbrain is relatively insensitive to respiratory acidotic (high CO$_2$) aCSF treatment and minimally sensitive to respiratory alkalotic (low CO$_2$) treatment. These findings were surprising since the rodent brainstem-spinal cord preparation shows the opposite pattern with high CO$_2$ more strongly enhancing the frequency of fictive ventilation \textit{in vitro} compared with metabolic acidosis (Kawai et al., 2006). I assessed the pH in the recording chamber to determine if differences in the pH level achieved by the metabolic vs respiratory stimuli could be responsible for these findings. Figure 2.11 shows in-bath measurements for pH during the course of metabolic and respiratory acidotic treatment (panel A) and metabolic and respiratory alkalotic treatment (panel B). The blunted effects of respiratory acidosis likely occurred because decreasing [HCO$_3^-$] resulted in more acidic bath pH values compared with raising the percentage of CO$_2$ gas. Interestingly, bath pH levels were more similar during metabolic and respiratory alkalosis (Figure 2.8B). This observation for alkalosis corresponds well with the significant increases in rhythm
frequency observed for E7-11 embryos exposed to both metabolic and respiratory stimuli. Future experiments will employ a gas mixture with a higher CO$_2$ percentage to distinguish whether the avian hindbrain is less sensitive to respiratory acidosis or if the blunted response was due to weaker acidification during high CO$_2$ treatment.

Also during the present study, the aCSF that modeled metabolic or respiratory acidosis or alkalosis was bath applied to the entire hindbrain. Therefore, there is little spatial resolution as to what areas of the hindbrain house central pH or CO$_2$ chemoreceptors. In the tadpole hindbrain, locations of central chemoreceptive regions have been identified using focal acidification as well as silencing via cooling or protease injections (Reed et al., 2019). In the opossum hindbrain, different regions of the hindbrain have been exposed to treatment aCSF by partitioning the bath into different compartments (Eugenín and Nicholls, 1997). Similar approaches could be used in future experiments in the zebra finch hindbrain to identify where pH stimuli are sensed and transduced into a change in rhythmic CN XI motor output.

Overall, the key finding of this study is that the zebra finch embryo hindbrain alters its rSNA or breathing-related motor activity in response to pH in an age dependent manner. The direction of changes in rhythm frequency are largely determined by embryonic age with a switch occurring at around the time air-breathing begins. In Chapter 3, I will present experiments that test the hypotheses proposed by the working models shared here.
Figure 2.1 Overview of experimental methods. A. Diagram of the superfusion system showing artificial cerebrospinal fluid (aCSF) entering the bath via gravity and exiting through an outlet attached to an aquarium pump. In the bath chamber, the isolated hindbrain can be seen submerged in temperature-controlled fluid. Spontaneous motor rhythms are recorded from the spinal accessory nerve (CN XI) using a suction electrode illustrated by the micropipette pictured here. B. General experiment protocol includes 30 min of baseline or control activity recorded from CN XI, followed by 60 min of treatment aCSF, and concluding with recording at least 30 min of activity during superfusion with control aCSF.
Figure 2.2 Representative examples of cranial nerve (CN) XI motor output during either metabolic acidosis (low pH) or metabolic alkalosis (high pH) treatments. A. Embryos from both the early (E4) and middle (E8) age groups show slower rhythmic SNA frequency during low pH treatment. The embryo from the late age group (E12-14) shows a greater frequency of rhythmic SNA during the identical low pH treatment. B. Rhythmic SNA increases in frequency during high pH treatment in embryos from early (E4) and middle (E8) age groups. The embryo representing the late age group (E13) shows a reduced frequency of SNA under the same alkaline conditions.
Figure 2.3 Summary data for the effects of bath application of metabolic acidotic and alkalotic aCSF on CN XI activity in the zebra finch embryo hindbrain. **A.** Mean frequency of rhythmic CN XI activity as a percent of control frequency during 1 hour of acidic aCSF treatment. **B.** Mean frequency of rhythmic CN XI motor output as a percent of control frequency during 1 hour of alkaline aCSF treatment. **C.** Mean frequency of rhythmic CN XI activity as a percent of control frequency for developmental ages E4-6, E7-11, and E12-14 during low pH treatment. **D.** Mean frequency of CN XI activity as a percent of control frequency for developmental ages E4-6, E7-11, and E12-14 during high pH treatment. Error bars represent standard error of the mean (± SEM). Dashed line represents rhythmic SNA frequency under baseline conditions (i.e., 100% of control frequency). Lower case letters (a, b) indicate significant differences between age groups (P ≤ 0.05) and * indicates differences from control SNA frequency (P ≤ 0.05).
Figure 2.4 Summary data for the effects of bath application of metabolic acidic and alkaline aCSF on burst characteristics. **A.** Mean duration of rhythmic CN XI activity as a percent of control duration after 1 hour of acidic aCSF treatment. **B.** Mean duration of rhythmic CN XI motor output as a percent of control duration after 1 hour of alkaline aCSF treatment. **C.** Mean amplitude of rhythmic CN XI activity as a percent of control amplitude during low pH treatment. **D.** Mean amplitude of CN XI activity as a percent of control amplitude during high pH treatment. Error bars represent standard error of the mean (± SEM). Dashed line represents rhythmic SNA frequency under baseline conditions (i.e., 100% of control duration or amplitude). Sample sizes were smaller for duration and amplitude due to the exclusion of data due to nerves slipping out of suction electrodes. * indicates differences from control SNA frequency (P ≤ 0.05).
Figure 2.5 Representative examples of CN XI activity during respiratory acidic and respiratory alkalotic treatment. A. Early and middle age groups show subtle slowing of rhythm during high CO$_2$ treatment while little change is visible in the late age group. B. Low CO$_2$ results in increased rhythmic SNA frequency in early and middle age groups. In the late age group, low CO$_2$ decreases the rate of rhythm.
Figure 2.6 Summary data for the effects of bath application of respiratory acidotic and alkalotic aCSF on CN XI activity in the zebra finch embryo hindbrain. A. Mean frequency of rhythmic CN XI activity as a percent of control frequency during 1 hour of high CO$_2$ aCSF treatment. B. Mean frequency of rhythmic CN XI motor output as a percent of control frequency during 1 hour of low CO$_2$ aCSF treatment. C. Mean frequency of rhythmic CN XI activity as a percent of control frequency for developmental ages E4-6, E7-11, and E12-14 during high CO$_2$ treatment. D. Mean frequency of CN XI activity as a percent of control frequency for developmental ages E4-6, E7-11, and E12-14 during low CO$_2$ treatment. Error bars represent standard error of the mean (± SEM). Dashed line represents rhythmic SNA frequency under baseline conditions (i.e., 100% of control frequency). * indicates differences from control (P ≤ 0.05).
Figure 2.7 Summary data for the effects of bath application of respiratory acidic and alkaline aCSF on burst characteristics. A. Mean duration of rhythmic CN XI activity as a percent of control duration during 1 hour of high CO₂ aCSF treatment. B. Mean duration of rhythmic CN XI motor output as a percent of control duration during 1 hour of low CO₂ aCSF treatment. C. Mean amplitude of rhythmic CN XI activity as a percent of control amplitude during high CO₂ treatment. D. Mean amplitude of CN XI activity as a percent of control amplitude during low CO₂ treatment. Error bars represent standard error of the mean (± SEM). Dashed line represents rhythmic SNA frequency under baseline conditions (i.e., 100% of control duration or amplitude). Sample sizes were smaller for duration and amplitude due to the exclusion of data due to nerves slipping out of suction electrodes. The gradual escape of nerves from the recording electrode precludes the measurement of amplitude or duration. * indicates differences from control (P ≤ 0.05).
Figure 2.8 Working model for the role of Cl⁻ in the pH sensitivity of early hindbrain rhythms. During early-mid incubation, the activation of Cl⁻ mediated neurotransmitter systems tends to depolarize neurons due to Cl⁻ efflux. When the pH of the aCSF is lowered, Cl⁻ currents through GABA_A receptors may be potentiated. This action depletes intracellular Cl⁻ stores which are typically restored by the action of the cation-Cl⁻ cotransporter NKCC1. In low pH conditions, NKCC1 function may be dampened. Increased Cl⁻ release during rhythmic episodes of CN XI motor output coupled with poor Cl⁻ reuptake by NKCC1 could result in longer quiescent periods in between bursts. Thus, CN XI motor output frequency decreases.
Figure 2.9 Working model for the relationship between pH sensitivity and Cl⁻ in more mature hindbrains. During the onset of air-breathing, Cl⁻ mediated neurotransmission may no longer be as important for the pH sensitivity of hindbrain rhythms. Potentiated GABA<sub>A</sub> receptor function by low pH would tend to modulate burst shape, change phase relations of respiratory motor pools, and perhaps slow CN XI motor output frequency. However, the main influence of low pH at ages >E11 is to speed up systems level output. In addition, the Cl⁻ cotransporter NKCC1 is no longer a major contributor to Cl⁻ ion homeostasis. Therefore, protonation of NKCC1 likely would not be involved in the pH effect at this age.
Figure 2.10 Working model of age-related changes in breathing circuitry and influences on pH sensitivity. Age-related reconfiguration or specialization of populations of neurons could explain divergent rhythmic outcomes of the same stimulus. Prior to air-breathing, the hindbrain may be largely undifferentiated and neurons are generally inhibited by acidification. Later, once pulmonary ventilation initiates, chemosensitive regions could emerge that convey signals to the breathing rhythm and pattern generating centers. For example, the raphe is a region in mammals where low pH disinhibits serotonergic neurons that provide excitatory drive to the breathing central pattern generator.
Figure 2.11 Summary data for the pH measured in the bath over the course of experiments. A. Average pH in the recording chamber during metabolic acidosis (closed circles) or respiratory acidosis (open circles, solid line). Error bars are mean +/- SEM. n = 3 per treatment. B. Average pH recorded in the bath chamber during metabolic alkalosis (closed circles, solid line) or respiratory alkalosis (open circles, dashed line). n = 3 per treatment.
3. Role of chloride in the age-dependent effects of pH on rhythmic neural activity from early embryogenesis through hatching

3.1 Abstract

Newly born and hatched terrestrial vertebrates benefit from some ability to adjust their breathing to suit their current activity level or environment. Accordingly, young mammals and birds exhibit ventilatory chemoreflexes when exposed to high CO$_2$ (hypercapnia) or low O$_2$ (hypoxia). Although whole-animal breathing parameters and peripheral chemosensors have been studied in young birds exposed to altered respiratory gases, the mechanisms of central chemosensitive reflexes for breathing control are largely unknown in birds. Recently, the onset and development of pH sensitivity in the hindbrain breathing circuits of zebra finch were documented across the embryonic period (Whitaker-Fornek et al., 2019). The major finding was that the effect of pH on rhythmic neural activity was age-dependent. The goal of the present work was to determine the mechanisms of pH sensitivity that could explain the diametrical effects of pH on rhythm frequency over embryonic age. Since the transition in pH effect co-occurred with the maturation of Cl$^-$ mediated inhibitory neurotransmission, I tested whether Cl$^-$ could be involved in pH sensitivity in the hindbrain breathing network. I found that blocking GABA$_A$ receptor activity blocked the slowing effect of low pH on E7-11 but did not change the effects of acidosis in early or late stage embryos. NKCC1 function was not involved in the pH sensitivity of E12-14 hindbrain pH sensitivity. Similarly, Cl$^-$ removal did not change the influence of low pH on rhythm frequency in early or middle-stage embryos. However, blocking NKCC1 transporter activity and removing Cl$^-$ reversed the effects of pH on rhythm frequency in middle and late age.
groups of zebra finch, respectively. These results suggest that the Cl\textsuperscript{−} concentration gradient may mediate the influence of pH on hindbrain motor rhythms for breathing.

3.2 Introduction

In animals, such as rodents and the zebra finch, data show that hindbrain motor rhythms for breathing are sensitive to pH before air-breathing begins (Eugenín et al., 2006; Whitaker-Fornek et al., 2019). Exposure to acidic pH increases the frequency of hindbrain rhythms in the mouse embryo beginning on embryonic day (E) 13.5 (Eugenín et al., 2006). This excitatory pH effect aligns with the whole-animal acute ventilatory response during hypercapnia in rat pups \textit{i.e.}, increased breathing rates) (Bavis et al., 2006). Using the zebra finch embryo, data also reveal a neurodevelopmental “switch” in the central pH response centered around E11-12 (Whitaker-Fornek et al., 2019).

Therefore, the ramifications of central pH sensitivity may change over the course of embryogenesis as much needed specialized neural circuits for breathing behaviors emerge. Understanding the mechanisms of central pH sensitivity in the bird embryo could uncover fundamental steps for constructing proper breathing circuitry \textit{in ovo}.

In the zebra finch hindbrain, one question is whether the maturation of chloride (Cl\textsuperscript{−})-mediated inhibition underlies the observed switch in the pH response (see Chapter 2 Discussion and Pickett et al., 2018). During earlier embryonic stages, the Na\textsuperscript{+} K\textsuperscript{+} 2Cl\textsuperscript{−} transporter (NKCC1) facilitates Cl\textsuperscript{−} entry into immature neurons and sets up a Cl\textsuperscript{−} concentration gradient favoring Cl\textsuperscript{−} efflux upon stimulation of Cl\textsuperscript{−} ion channels (Schulte et al., 2018). Later in the paranatal period, increased expression of the Cl\textsuperscript{−} exporter, KCC2,
facilitates a switch in the Cl\textsuperscript{−} concentration gradient that favors Cl\textsuperscript{−} entry through Cl\textsuperscript{−} ion channels. Data show that the excitatory to inhibitory Cl\textsuperscript{−} switch occurs functionally by E11-12 in the zebra finch embryo—the same time as the switch in the pH response was observed (Pickett et al., 2018).

In Chapter 2, I proposed models for the role of Cl\textsuperscript{−} in the pH sensitivity of zebra finch hindbrains during early and late embryonic stages (Figures 2.8, 2.9, and 2.10). Two central hypotheses emerged from the models: 1. metabolic acidosis decreases hindbrain rhythm frequency by increasing the activity of GABA\textsubscript{A} receptors and decreasing NKCC1 activity in zebra finch embryos younger than E11, and 2. increased rhythm frequency during metabolic acidosis in >E11 embryos is independent of NKCC1 and involves GABA\textsubscript{A} receptors differently than in younger embryos. The rationale for the first hypothesis is based on data that show GABA\textsubscript{A} receptor currents can be enhanced by acidosis and protonation inhibits NKCC1 in duck red blood cells (Hegde and Palfrey, 1992; Krishek et al., 1996). Accordingly, slower rhythm during acidosis occurs because of excess Cl\textsuperscript{−} efflux and limited Cl\textsuperscript{−} accumulation. For older embryos, NKCC1 expression is thought to be outweighed by KCC2 expression such that protonation of the transporter no longer has any bearing on the systems level motor output. Further, the excitatory effect of low pH on rhythm in more mature embryos does not correspond with acid-induced enhancement of GABA\textsubscript{A} receptor activity. These two strands of reasoning provide the basis for the second hypothesis mentioned above.

In this chapter, the first goal was to test whether GABA\textsubscript{A} receptors were involved in the effects of pH on motor rhythms for breathing in the zebra finch embryo from both the prenatal and paranatal embryo. To do this, I pharmacologically blocked GABA\textsubscript{A}
receptors while lowering the pH of the fluid bathing the hindbrain preparation. The second goal was to test whether Cl⁻ transport through NKCC1 was involved in the effects of pH on zebra finch embryo hindbrain motor rhythms. To assess the role of NKCC1 in central pH chemosensation, I blocked NKCC1 activity with bumetanide while lowering pH of the superfusate bathing zebra finch hindbrain preparations. The third goal was to determine if Cl⁻ was involved in the mechanisms of pH sensitivity more broadly. To do this, low pH experiments were carried out in Cl⁻ free conditions.

Results show that the effects of pH are independent of GABA_A receptor activity and that the pH effect on frequency is reversible by blocking NKCC1 activity in E8-11 zebra finch or removing Cl⁻ in E12-14 zebra finch. However, the effects of acidosis on rhythm in older zebra finch embryos were not affected by blocking NKCC1 activity and Cl⁻ removal did not change the effect of low pH on younger embryo rhythms. The ability to alter the pH effect on frequency using different Cl⁻ manipulations in younger and older zebra finch embryos suggests that the Cl⁻ concentration gradient may be a key factor in avian central pH sensitivity.

3.3 Methods

3.3.1 Experimental animals

All procedures were approved by the Idaho State University Institutional Animal Care and Use Committee. I collected zebra finch eggs from an in-house breeding colony, candled eggs to estimate developmental age, and placed eggs into a
temperature-controlled (38°C), humidified (60-70%), forced-draft incubator (GQF 1502 Sportsman). A total number of 138 embryos were used for the experiments.

3.3.2 Preparation of control and experimental cerebrospinal fluid

Control artificial cerebrospinal fluid (aCSF) was prepared using millipore-filtered deionized water (Nanopure) and contained the following components (in mM): 120 NaCl, 26 NaHCO₃, 30 D-Glucose, 1 MgCl₂, 3 KCl, 1.25 NaH₂PO₄, 1 CaCl₂. Control aCSF was equilibrated with 95% O₂ and 5% CO₂ gas (Airgas) until a pH of 7.45 was reached (Accumet model 10 pH meter). Low pH aCSF was prepared following the previous recipe with the only alteration being a reduction in NaHCO₃ to 13 mM. Low pH aCSF was bubbled with 95% O₂ and 5% CO₂ gas until a pH of 7.2 was reached. To test whether chloride was involved in the acid-induced response of hindbrain breathing rhythms, chloride-free aCSF solution was prepared in which gluconate replaced chloride. Chloride-free aCSF was prepared using millipore-filtered and deionized water (Nanopure) and contained the following components (in mM): 120 Sodium gluconate, 26 NaHCO₃-, 30 D-Glucose, 1 Mg D-gluconate hydrate, 3 Potassium gluconate, 1.25 Sodium phosphate monobasic monohydrate, 1 Calcium gluconate. Chloride-free solution was bubbled with 95% O₂ and 5% CO₂ until a pH of 7.45 was reached. Low pH chloride-free solution contained the identical ingredients as the previous recipe with the exception of 13 mM NaHCO₃. The chloride-free low pH solution was bubbled with 95% O₂, 5% CO₂ until a pH of 7.2 was reached.
3.3.3 Pharmacologic agents

Drugs were purchased in powder form and reconstituted in appropriate solvents to make stock solutions (Tocris Biosciences). We used bumetanide to interfere with NKCC1 function and picrotoxin (PTX) to disrupt GABA_A receptor activation. Drug concentrations were based on dosage response experiments performed on embryos of varying ages. Drugs were added to aCSF to reach the following concentrations: bumetanide 3-4 \( \mu \text{M} \), picrotoxin 0.01 \( \mu \text{M} \) for E4-11 and 0.1 \( \mu \text{M} \) for E12-14. Since I was interested in comparing the effects of low pH alone to low pH in combination with the drug, it was not essential to keep drug concentrations constant for all ages. Therefore, higher concentrations of PTX were used in E12-14 embryos than the younger age groups. Also, bumetanide concentrations were kept below 10 \( \mu \text{M} \) to prevent cross-reactivity with other Cl\(^-\) transporters (Payne et al., 2003).

3.3.4 Surgical isolation of the brainstem preparation

Same as described in Chapter 2 Methods.

3.3.5 Extracellular recording and data collection

Same as described in Chapter 2 Methods.

3.3.6 General experiment protocol

Figure 3.1, panel A shows a schematic diagram of the general experimental protocol. Before beginning experimental treatments, stable baseline CN XI activity was
recorded for 30 minutes while the preparation was superfused with the appropriate control aCSF. Next, the control aCSF was replaced by aCSF containing either PTX, bumetanide, or Cl⁻ free aCSF by switching reservoirs for 30 minutes. I referred to this period as the “drug baseline” or “Cl⁻ free baseline.” Next, low pH aCSF containing drug or Cl⁻ free aCSF was added to a clean reservoir for 1 hour. This period was called the “treatment period.” Finally, control aCSF bathed the preparation for 30 min or until rhythmic neural activity resembled the original, baseline rhythm. The pH of superfusate was regularly checked during experiments using a small pH electrode placed in the bath (Orion PerpHecT ROSS Combination pH Micro Electrode, 8220BNWP).

3.3.7 Summarizing data and analyzing data

The frequency of CN XI motor rhythm was calculated for the drug/Cl⁻ free baseline period and treatment period for each experiment. To isolate the effect of pH alone on rhythm, the frequency during the treatment period was divided by the frequency during the drug/Cl⁻ free baseline period and multiplied by 100. The resulting value represented the relative change in rhythm frequency as a result of low pH under each condition. The same procedure was used to acquire relative changes in burst duration, amplitude, and area for the treatment period. Data were grouped into three age bins: E4-6, E7-11, and E12-14 as previously described in Chapter 2. JMP statistical software was used to test for differences between the effects of low pH alone and low pH in combination with drugs or Cl⁻ free solution. I used one-way ANOVA to test for significant effects of treatment within each age group (P<0.05). I did not include age, or age*treatment as factors because I was asking questions about the effect of acidosis.
alone vs acidosis under different conditions within each age group. Also, different dosages of drugs were used in some age groups which prevented comparing treatment effects between age groups.

The coefficient of variation was calculated to investigate whether manipulating Cl- during acidosis influenced the variability of rhythmic CN XI motor output. First, the average coefficient of variation for rhythm was determined by dividing the standard deviation of interburst interval by the mean interburst interval for the control low pH treatment period. Coefficient of variation values for rhythm during acidotic exposure were averaged by age. Second, the coefficient of variation was calculated for the experimental treatment period (e.g., PTX + low pH) and similarly averaged by age.

3.4 Results

3.4.1 Effects of blocking GABA<sub>A</sub> receptors during low pH treatment

As previously reported, pH alters the frequency of zebra finch embryo hindbrain breathing rhythms in an age-dependent manner (Whitaker-Fornek et al., 2019). Prior to the onset of aerial ventilation (~E12), acidosis tends to decrease the frequency of rhythmic neural activity. In E12-14 zebra finch that have begun pulmonary ventilation, lowering aCSF pH increases rhythm frequency. Since the turning point for the effects of pH on rhythm co-occurred with the onset of inhibitory Cl- neurotransmission, I hypothesized that Cl- mediated neurotransmission contributes to the development of central pH sensitivity. To test this, GABA<sub>A</sub> receptors, ionotropic receptors that conduct Cl-, were blocked using the drug picrotoxin (PTX) while lowering the pH of the
superfusate. Figure 3.2 shows electroneurograms of cranial rhythms during PTX + low pH treatment.

To quantitatively assess the combined effects of PTX and low pH compared with acidosis alone, Figure 3.3 shows day-by-day summary data for frequency, duration, amplitude, and area for each condition (PTX + low pH: solid orange bars and low pH alone: solid black bars). To test whether broad age classes with higher sample sizes exhibited statistically different patterns of rhythmic activity during PTX + low pH vs control, data were binned by age according to baseline rhythm features (Figure 3.4). On average, E4-6 zebra finch showed no change in the effects of pH on rhythm frequency when PTX was added compared with low pH alone (64 ± 4%, n = 7, P = 0.13). The addition of PTX blocked the low pH-induced slowing effect typically seen in E7-11 hindbrains (115 ± 19%, n = 20, P = 0.04). This result was due to E10 and E11 embryos that showed increases in motor output frequency during PTX + low pH aCSF (177 ± 29%, n = 9, P = 0.035). This increase in frequency can be observed in the E10 whole nerve recording shown in Figure 3.2. Lastly, the excitatory effect of low pH on E12-14 rhythm frequency was non-significantly blunted in the presence of PTX (124 ± 14%, n = 10, P = 0.28). Burst characteristics such as duration, amplitude and area did not deviate from levels observed under low pH control for any age group when PTX + low pH was bath-applied.
3.4.2 Effects of blocking NKCC1 transporters during low pH treatment

Figure 3.5 shows suction electrode recordings of CN XI during bumetanide + low pH treatment. NKCC1 blockade did not change the effects of lowering pH on rhythm in the early or late age group. However, zebra finch within the middle age group exhibited increased frequency of rhythm that included shorter duration bursts as is depicted in the E9 example (Figure 3.5 middle trace). This excitatory effect was observed in 2 out of 5 E8 experiments, 2 out of 5 E9 experiments and in 5 out of 5 E10 experiments.

For a comprehensive overview of the combined effects of bumetanide + low pH vs acidosis alone, Figure 3.6 shows summary data for the frequency of rhythm and other burst characteristics by embryonic age. Summary data by age group are consistent with the qualitative observations in the electroneurograms and daily averages (Figure 3.7). In the early age group, rhythm frequency was no different from control when NKCC1 was blocked during acidosis (84 ± 12%, n = 8, P = 0.48). Bumetanide + low pH aCSF significantly increased the frequency of CN XI motor output in E7-11 zebra finch compared with low pH alone (239 ± 54%, n = 20, P = 0.006). E12-14 zebra finch showed no change in CN XI motor output frequency during acidosis and NKCC1 blockade compared with low pH alone (122 ± 9%, n = 7, P = 0.27). Also, burst duration and area were decreased in all three age groups compared with control, but only E12-14 animals showed significant decreases in amplitude (77 ± 7%, n = 18, P = 0.006). The decrease in amplitude was a significant concern for us while performing experiments and treatments were only performed if the signal to noise ratio was adequate.
3.4.3 Effects of chloride free solution during low pH treatment

Cl⁻ free aCSF was used to broadly investigate the Cl⁻ dependence of pH sensitivity in the zebra finch embryo hindbrain. Bursting was abolished in E4-5 zebra finch hindbrains when Cl⁻ free solution was bath-applied. In E6 and older zebra finch, Cl⁻ free aCSF increased tonic excitatory activity which can be seen in the large increase in baseline amplitude of the traces in Figure 3.8. In E12-14 embryos, Cl⁻ free solution dramatically changed the pattern and rhythm of inspiratory activity. In control aCSF conditions, E12-14 rhythms consist of short-duration bursts every 5-20 seconds. With Cl⁻ removal, the rhythm became much slower (i.e., average inter-burst interval of 458 ± 47 seconds) and consisted of long-duration oscillations (i.e., average burst duration of 52 ± 11 seconds). Two animals in this age range showed low amplitude oscillatory activity where individual bursts were unidentifiable (data not shown). For the sake of clarity, only E12-14 experiments with distinguishable bursts were included in summary data.

Eliminating extracellular Cl⁻ altered the effects of low pH in E12-14 zebra finch while E4-6 and E7-10 zebra finch showed largely unchanged responses to acidosis. Figure 3.9 shows daily summary data for CN XI rhythm frequency and burst characteristics during acidosis alone and in the absence of Cl⁻. When these data were grouped into three windows of embryonic development, data show that Cl⁻ free + low pH treatment significantly reduced the frequency of inspiratory activity in E12-14 birds compared to low pH alone (41 ± 17%, n = 7, P < 0.0001) (Figure 3.10). E4-6 and E7-11 zebra finch showed decreased rhythm frequency when pH was lowered in Cl⁻ free superfusate no different than control (low pH alone) (84 ± 16%, n = 5, P = 0.69 and 76 ±
9%, n = 17, P = 0.99, respectively). E12-14 burst amplitude decreased during Cl⁻ free + low pH (70 ± 2%, n = 4, P = 0.006). E7-11 burst duration and area increased during Cl⁻ free + low pH (164 ± 22%, n = 17, p = 0.01; 154 ± 19%, n = 17, p = 0.008). Finally, Cl⁻ free + low pH aCSF resulted in lower mean coefficient of variation for CN XI motor rhythms in E11 zebra finch embryos (Cl⁻ free + low pH: 0.13 ± 0.02, n = 2; low pH alone: 1.14 ± 0.1, n = 3).

3.5 Discussion

Since motor rhythms produced by the embryonic zebra finch hindbrain showed age-dependent responses to pH, I hypothesized that the age-dependent shift in Cl⁻ neurotransmission from excitatory to inhibitory could be involved in the mechanisms of pH sensitivity. Specifically, low pH may attenuate the rate of bursting in <E11 birds by enhancing Cl⁻ efflux through GABAₐ receptors and limiting Cl⁻ reuptake by inhibiting NKCC1 (see Figure 2.8 in Chapter 2 discussion). In >E11 birds, lowering pH could evoke an adult-like increase in inspiratory activity without requiring NKCC1 function and by involving GABAₐ receptors in specific chemosensing regions such as the raphe (see Figure 2.9 and 2.10 in Chapter 2 discussion). To test these hypotheses, I lowered the pH of aCSF bathing the in vitro hindbrain preparation while bath-applying a GABAₐ receptor antagonist, a NKCC1 antagonist, and Cl⁻ free solution. Results show that manipulating Cl⁻ transporter activity and removing Cl⁻ reverse the effects of acidosis on rhythmic motor outflow in younger (<E11) and older (>E11) zebra finch hindbrains, respectively.
3.5.1 Blocking GABA\(_A\) receptors reversed the pH effect in E7-11 embryos

I hypothesized that the slowing effect of low pH on <E11 motor rhythms was partly due to enhanced Cl\(^-\) currents via GABA\(_A\) receptors exposed to low pH (Krishek et al., 1996). My prediction was that if GABA\(_A\) receptor activation was blocked during low pH, then the slowing effect of low pH would be weakened. However, only E7-11 zebra finch showed a different response to acidosis when PTX was bath-applied.

In E4-6 embryos, GABA\(_A\) receptors may not be a part of the pH sensing machinery due to the role of GABAergic neurotransmission in supplying part of the fundamental drive for rhythm production (Pickett et al., 2018). Perhaps, PTX inhibition of GABA\(_A\) receptors simply slowed the production of baseline rhythm but did not interfere with the mechanisms by which low pH slowed rSNA frequency. Acidity can broadly influence protein structure and cellular processes which often results in an overall dampening of excitation in neurons that are not involved in respiratory control (Sinning and Hübner, 2013). Utilizing voltage sensitive dyes during metabolic acidotic exposures to the zebra finch embryo hindbrain could determine whether overall excitation decreases as a function of reduced pH (Momose-Sato et al., 2009).

E7-11 embryos showed significant reversal of the low pH effect during GABA\(_A\) receptor antagonism. These results support the hypothesis that GABA\(_A\) receptor activity may be enhanced as a result of acidosis, leading to excess Cl\(^-\) efflux and slower rSNA frequency. However, I did not anticipate that blocking the Cl\(^-\) ion channel would result in faster rSNA frequency during acidosis for the middle age group. An alternate explanation for the increase in rSNA frequency could be that the excitatory effect of blocking GABA\(_A\) receptors with PTX masked the slowing effect of low pH alone. In fact,
bath application of PTX alone for 60 minutes resulted in increased frequency of rSNA in E10-11 embryos that was similar in magnitude to the response evoked by low pH + PTX aCSF (personal observation). It is possible that PTX excited the developing breathing network of embryos that had already shifted to inhibitory Cl⁻ neurotransmission. Thus, blocking GABA_A receptor activity would be functionally excitatory for these embryos.

In embryos older than E11, I hypothesized that GABA_A receptors may be involved in pH sensitivity in a different way compared with younger embryos to result in the increase in CN XI motor output frequency. By the time air-breathing begins, data show there are specific populations of GABAergic neurons in the rodent hindbrain, such as within the raphe, that become inhibited by low pH (Iceman et al., 2014). I predicted that pharmacologically blocking GABA_A receptors could strengthen the excitatory influence of acidosis on breathing-related motor output. However, low pH + PTX did not influence the low pH response compared to low pH alone. The broad GABA_A receptor antagonism across the whole hindbrain generally increased excitation which prevented disentangling the role of GABA_A receptors in central pH ventilatory chemoreflexes. In future experiments, direct application of PTX to the medullary raphe would be a better approach to test whether GABA_A receptors on serotonergic raphe neurons are involved in the increase in CN XI rhythm frequency observed under low pH conditions. Further, additional Cl⁻ channels may be involved in pH sensitivity which could be assessed using agonists or antagonists for glycinergic neurotransmission, for example.
3.5.2 Blocking NKCC1 function reverses the pH effect in E7-11 embryos

I hypothesized that the slowing effect of low pH on <E11 motor rhythms was partly due to the inhibition of NKCC1 activity by acidosis (Hegde and Palfrey, 1992). I predicted that blocking NKCC1 function in <E11 zebra finch hindbrains would strengthen the decrementing effect of acidosis on rSNA frequency. Results showed no change and the opposite effect on motor rhythm frequency in E4-6 and E7-11 embryos, respectively. NKCC1 expression levels and its relative importance in establishing [Cl\(^-\)]\(_i\) changes with increasing embryonic age (Schulte et al., 2018). Therefore, data suggest that NKCC1 expression is highest between E7-11 in the zebra finch embryo hindbrain since the greatest effect of bumetanide application was observed at this time. Histological approaches examining NKCC1 expression will be necessary to confirm this pharmacological evidence.

The stimulating effects of bumetanide + low pH aCSF on rSNA was unexpected in E7-11 zebra finch hindbrains. Rather, I predicted that blocking Cl\(^-\) entry during acidosis would attenuate the frequency of CN XI motor output even more than low pH alone (Figure 2.8). NKCC1 antagonism has been shown to prematurely hyperpolarize the Cl\(^-\) equilibrium potential relative to resting membrane potential (Gonzalez-Islas et al., 2009). The combination of bumetanide and low pH may have hampered NKCC1 function to such a degree that the Cl\(^-\) concentration gradient switched to favoring Cl\(^-\) influx through pH-regulating exchangers such as anion exchanger 3 (AE3). AE3 allows Cl\(^-\) to enter cells in exchange for HCO\(_3^-\) efflux (Ruffin et al., 2014). Higher extracellular HCO\(_3^-\) levels may have buffered the low pH stimulus and alkalinized pH which could serve to excite immature, non-respiratory neurons (Sinning and Hübner, 2013). A
follow-up experiment would be to apply bumetanide in combination with a GABA or glycine agonist to test whether rhythmic activity frequency is increased or decreased. This would determine whether blocking NKCC1 activity reduces intracellular Cl\(^-\) to an extent that Cl\(^-\) mediated neurotransmission prematurely takes on an inhibitory role. Other approaches to assess intracellular Cl\(^-\) concentrations could use a fluorescent Cl\(^-\) indicator (Lindsly et al., 2014) or estimate intracellular Cl\(^-\) by measuring GABA minipostsynaptic currents while clamping membrane voltage to determine the GABAergic reversal potential (\(E_{GABA}\)) (Gonzalez-Islas et al., 2009).

In more mature zebra finch embryos (>E11), I hypothesized that NKCC1 would not be involved in central pH sensitivity of hindbrain breathing circuits. Data demonstrated decreased amplitude as the only difference in the effects of bumetanide + low pH compared with acidosis alone for this age group. It was common to observe decreased amplitude during bumetanide treatment but it the mechanisms of this effect are unknown.

Finally, results from control experiments (i.e., bumetanide was bath-applied for 60 min while holding pH constant) show that blocking NKCC1 activity directly influenced the effects of acidosis on rhythm frequency and burst amplitude. These findings were not due to bumetanide’s individual influence.
3.5.3 Removing extracellular chloride reversed the effects of pH on hindbrain rhythms for breathing

The overarching hypothesis for the present work was that the age-dependent switch in the pH response depended on Cl\(^-\). I predicted that eliminating extracellular Cl\(^-\) would alter the effects of pH on rhythm frequency. Results show that the actions of low pH were unaffected in <E11 embryos and completely switched in E12-14 embryos when Cl\(^-\) was reduced to 0 mM in aCSF.

On E4-5, rSNA production ceased under Cl\(^-\) free conditions, which shows that rSNA depends on Cl\(^-\) at this age. Bath-application of Cl\(^-\) free solution has been found to block rhythmic gill bursting in isolated frog tadpole brainstems while lung bursts in tadpoles and adults were modulated but not abolished (Broch et al., 2002; Galante et al., 1996). Similarly, CN XI motor bursts were possible in Cl\(^-\) free conditions for E6 and older embryos. Control experiments where Cl\(^-\) was removed for 60 minutes while holding pH constant revealed that burst duration and area increased as a result of Cl\(^-\) free solution alone (personal observation). This result suggests increased excitability and, perhaps, increased recruitment of motor neurons in these conditions. During Cl\(^-\) free + low pH exposure, rSNA frequency decreased similar to the level observed during low pH alone for E4-6 and E7-11 embryos. These data show that the central pH sensitivity of early and middle age embryos is intact under low extracellular Cl\(^-\) conditions. I speculate that Cl\(^-\) free aCSF did not change the response to low pH at these ages because <E11 zebra finch are thought to already possess a Cl\(^-\) concentration gradient favoring Cl\(^-\) efflux at this time (Pickett et al., 2018). Therefore,
higher [Cl⁻] may be more important for determining the effects of acidosis in younger zebra finch embryos (see Figure 3.12 for more details about this hypothesis).

Data show that E11 rhythmic CN XI activity became more regular during Cl⁻ free aCSF exposure. Baseline E11 rhythm is associated with high variability, in general, therefore it was noteworthy when the combination of Cl⁻ free + low pH superfusate resulted in a more regular rhythm. This qualitative observation was consistent with the coefficient of variation data. Eliminating Cl⁻ may stabilize CN XI motor rhythm frequency because E11 is a transitional age where the Cl⁻ equilibrium potential is becoming more hyperpolarized than resting membrane potential. Perhaps shifting to a depolarized Cl⁻ equilibrium potential by reducing extracellular Cl⁻ to zero promoted slow, steady rates of bursting in E11 embryos. These findings suggest that the irregularity of E11 rhythm may reflect maturational changes in Cl⁻ ion homeostasis.

In E12-14 embryos, Cl⁻ free solution excited baseline activity and transformed rhythmic activity into long-duration, low frequency bursts. The majority of embryos demonstrated large-amplitude oscillations that were identifiable as bursts. Also, bursting still occurred during 90-minute long Cl⁻ free treatments where pH was held constant although burst amplitude significantly decreased. In the adult amphibian hindbrain preparation, Cl⁻ removal has been found to decrease the frequency of lung bursts (Broch et al., 2002). In mammalian neurons, total Cl⁻ removal evokes seizure like activity (Dick et al., 2018). Paton and colleagues report that blocking Cl⁻ mediated inhibition changes the shape of bursts and a lack of inhibition could also disrupt the coordination of phases relationships among the activation of respiratory control motor pools (Paton and Richter, 1995). Perhaps the overexcitation of neurons across the
hindbrain may have contributed to the marked changes in activity pattern recorded for E12-14 zebra finch embryos during Cl⁻ free solution exposure.

In addition to the contrasts between the baseline rhythm and Cl⁻ free rhythm exhibited by E12-14 zebra finch, the effect of acidosis was reversed in the absence of Cl⁻. In other words, the combination of Cl⁻ free superfusate and lowering pH decreased the frequency of inspiratory activity in the internally pipped, air-breathing embryos. I conjecture that low pH may slow the breathing-related rhythmic activity during Cl⁻ free treatment because reduced extracellular Cl⁻ forces the hindbrain breathing circuit into an ionic situation more similar to a younger embryo. With the Cl⁻ concentration gradient favoring Cl⁻ efflux, pH regulating transporters such as the Na⁺ dependent Cl⁻ HCO₃⁻ exchanger and AE3 may alter the direction of HCO₃⁻ and Cl⁻ shuttling. This could result in net HCO₃⁻ entry which could alkalinize intracellular pH and lower the overall excitability of respiratory neurons. In life, increases in pH can be remedied by hypoventilation, which would appear as slower rates of inspiratory activity produced by central breathing networks (Javaheri et al., 1982; Kawai et al., 2006). Further, removal of Cl⁻ in the aCSF bathing medullary slices has been found to alkalinize intracellular pH in neurons from breathing control areas in the rodent hindbrain (Ritucci et al., 1998). To test whether AE3 is involved in central pH sensitivity during Cl⁻ free aCSF superfusion, I could block AE3 using 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS). DIDS has been used to block AE3 in chicken embryo motor neurons and to study chemosensation in duck intrapulmonary chemoreceptors (Gonzalez-Islas et al., 2009; Shoemaker and Hempleman, 2001). I predict that AE3 antagonism would prevent intracellular pH increases and keep bursting frequency from slowing down in E12-14
hindbrains. Another approach to test this hypothesis would be to measure intracellular pH using a fluorescent indicator during the treatment period to assess whether intracellular pH increases (Ritucci et al., 1996).

3.5.4 Speculative models for the role of Cl\(^-\) in the pH sensitivity of avian embryo breathing rhythms

Overall, results from the present research suggest that the Cl\(^-\) concentration gradient across neuronal membranes is a key factor in the embryonic development of avian central pH sensitivity. Three lines of evidence provide the rationale for this claim. First, the bath-application of GABA\(_A\) receptor antagonist reversed the slowing effect of low pH in middle stage embryos. Second, blocking NKCC1 function reversed the effect of metabolic acidosis on CN XI motor rhythm frequency in E7-11 hindbrains. Third, removing extracellular Cl\(^-\) reversed the effect of acidosis on the frequency of breathing-related motor rhythms in E12-14 zebra finch.

In light of these results, I propose revised models for Cl\(^-\) mechanisms mediating the rhythmic consequences of lowering extracellular pH (Figure 3.12). These models are not exhaustive and focus specifically on ways that Cl\(^-\) may be involved in pH sensation and changes in neuronal excitability. For these models, my goal was to outline the current understanding of Cl\(^-\) in the pH sensitivity of the zebra finch embryo breathing network.

In the zebra finch embryo hindbrain, the main effect of metabolic acidosis on rhythm is to alter the frequency of CN XI motor rhythm, but the polarity of this effect
depends on age. Results show that disrupting GABA_A receptor activity blunts the slowing effect of acidosis in middle stage zebra finch but does not alter the effects of pH on rhythm in early or late stage zebra finch. Therefore, in the early (<E11) neuron model the Cl^- ion channel is shown in light shading since it may be involved in pH sensitivity at this time (Figure 3.11, star). In the late (>E11) neuron model, this Cl^- ion channel is faded out since results show its activity is not required for pH sensitivity. I speculate that metabolic acidosis may potentiate Cl^- currents through GABA_A receptors and deplete intracellular Cl^- which could slow rSNA production. NKCC1 function is necessary to maintain the normal Cl^- concentration gradient in early zebra finch embryo neurons (star, Figure 3.11). When pH drops in the extracellular environment, pH regulating exchangers such as AE3 may buffer intracellular pH changes by allowing HCO_3^- entry in exchange for Cl^- . However, AE3 activity may not fully prevent reductions in intracellular pH which would lead to dampened excitability, typical of non-respiratory neurons (Sinning and Hübner, 2013). In late stage embryos (>E11), KCC2, the Cl^- extruder, is needed to maintain the normal Cl^- concentration gradient which is necessary for the excitatory effect of acidosis on breathing-related motor output (star, Figure 3.11). When the extracellular fluid becomes acidic, the intracellular pH of respiratory neurons decreases and triggers pH-sensitive ion channels, molecules, and other transmembrane proteins (Putnam, 2001; Ruffin et al., 2014). The acidification of intracellular pH can be accomplished by AE3 which mediates HCO_3^- efflux in exchange for Cl^- . As a result of the lasting acidification of intracellular pH, some respiratory neurons become excited and increase rhythmic motor output to drive ventilation and alleviate acidosis (Huckstepp and Dale, 2011).
These speculative models draw attention to opportunities for future study of the mechanisms of low pH sensing across embryonic development in the zebra finch hindbrain. The work described up to this point focused on roles of Cl\(^-\) but did not address all means of intercellular Cl\(^-\) movement. For example, the use of an antagonist for AE3, such as DIDS, could be used to test whether this transporter is involved in pH sensitivity as proposed in the models. Finally, the age-dependent effects of acidosis on hindbrain rhythms could be explained by the maturation of other pH detection and regulating proteins such as acid-sensing cation channels, tandem pore acid sensing potassium channels, and gap junctions (Huckstepp and Dale, 2011).

![Figure 3.1 Overview of experiment protocol.](image)

A. Once CN XI motor output was detected, I recorded activity for at least 30 min in control artificial cerebrospinal fluid (aCSF). Next, aCSF containing either picrotoxin, bumetanide, or chloride (Cl\(^-\)) free aCSF was superfused over the hindbrain for 30 min. This period is referred to as the “drug/Cl\(^-\) free baseline.” Then, low pH aCSF with the same drug or Cl\(^-\) free conditions is bath-applied for 60 min for the “treatment period.” Finally, the hindbrain was washed with control aCSF for 30 minutes.
Figure 3.2. Representative cranial nerve (CN) XI activity during GABA$_A$ receptor blockade and metabolic acidosis. Rectified and integrated whole-nerve discharge was recorded from CN XI of hindbrain preparations from early (E5), middle (E10), and late (E13) stage zebra finch. The GABA$_A$ receptor antagonist picrotoxin (PTX) was bath applied for 30 min (orange single bar) prior to a 60-min period of constant PTX and metabolic acidotic aCSF (orange and black double bar). The low pH-induced changes in rhythm frequency are mostly unchanged in the presence of PTX with early embryos showing decreased rhythm frequency and late embryos showing increased rhythm frequency. On E10, frequency increased during PTX + low pH exposure which differed from the slowing effect of acidosis alone.
Figure 3.3 Daily summary data for the effects of GABA_A receptor antagonism and metabolic acidosis. A. Mean frequency of rhythmic CN XI motor output as percent of control frequency after 1 h of either metabolic acidotic aCSF (black bars, low pH control) or picrotoxin (PTX) and low pH (orange bars). PTX did not influence the effects of acidosis on rhythm frequency except for on E10 and 11 where frequency increased. B-D. Same as in A except shows relative changes in duration, amplitude, and area for each condition. No differences were found between the two conditions for any of these variables. Error bars represent standard error of the mean.
Figure 3.4 Summary data for the effects of GABA_A receptor antagonism and metabolic acidosis by age group. A. Average relative change in frequency during PTX + low pH (solid orange bars) or low pH exposure (control; solid black bars). GABA_A receptor antagonist exposure significantly altered the effects of acidosis on frequency for the middle age group. B-D. Same as in A for burst duration, amplitude, and area. Error bars represent standard error of the mean. * indicates differences from control (low pH alone).
Figure 3.5 Representative cranial nerve (CN) XI activity during NKCC1 blockade and metabolic acidosis. The NKCC1 antagonist bumetanide was bath applied for 30 min (gray single bar) prior to a 60-min period of constant bumetanide and metabolic acidotic aCSF (gray and black double bar). Blocking NKCC1 activity reversed the effect of acidosis in zebra finch embryos in the middle age group (E7-10). When bumetanide was added to the low pH superfusate, the frequency of CN XI motor output is seen to increase in the representative E9 (center). Blocking NKCC1 activity does not change the response to metabolic acidosis in early (E4-6) or late stage (E12-14) zebra finch embryos.
Figure 3.6 Daily summary data for the effects of NKCC1 antagonism and metabolic acidosis. A. Blocking NKCC1 transporter function during low pH reversed the effects of low pH on frequency between E8-11. Lowering pH resulted in similar effects on rhythm frequency in the presence of bumetanide (BMT) for E4-7 and E12-14 animals. B-D. Burst duration, amplitude, and area were similar when low pH was bath-applied alone or in combination with NKCC1 antagonism. Error bars represent standard error of the mean.
Figure 3.7 Summary data for the effects of NKCC1 antagonism and metabolic acidosis by age group. A. Blocking NKCC1 transporter activity during acidosis resulted in significantly increased frequency of rhythm in E7-11 embryos. B-D. On average, bumetanide in combination with low pH tended to decrease burst duration and area in all age groups, although this was not significant. Burst amplitude was significantly decreased as a result of blocking NKCC1 transport for the late age group. Error bars represent standard error of the mean. * indicates differences from control (low pH alone).
Figure 3.8 Representative cranial nerve (CN) XI activity during Cl⁻ free solution and metabolic acidosis. Cl⁻ free aCSF was bath applied for 30 min (blue single bar) prior to a 60-min period of constant Cl⁻ free and metabolic acidotic aCSF (blue and black double bar). Removal of Cl⁻ from the aCSF increased tonic activity as evidenced by thicker baseline signal widths. Bursts can be seen superimposed on this tonic activity. The frequency of rhythm in <E11 zebra finch was slightly slowed by Cl⁻ removal. In E12-14 zebra finch, Cl⁻ free solution greatly slowed rhythm and increased the duration of bursts. The E12 suction electrode recording shows an example of clearly identifiable, large amplitude oscillations that were classified as bursts. Only experiments where bursts were similarly identifiable were included in summary data. During acidosis + Cl⁻ free (blue and black double bar), rhythm frequency slowed down in early and middle embryos similar to control. Cl⁻ free and low pH aCSF decreased the frequency of rhythm for late stage embryos in contrast to low pH alone. During wash, rhythm returned that resembled the original activity prior to Cl⁻ free treatment. Often, wash periods greater than 30 minutes were necessary to observe rhythm recovery. Therefore, additional wash period data are shown to capture rhythm onset in control aCSF.
Figure 3.9 Daily summary data for the effects of Cl⁻ free solution and metabolic acidosis. Removing Cl⁻ blocked rhythm in E4-5 zebra finch, therefore data for those ages were excluded. A. The combination of acidosis and Cl⁻ free aCSF did not differ from the effects of low pH alone on frequency. B-D. Patterns of other burst characteristics were similar in low pH control and Cl⁻ free + low pH except duration and area which tended to be higher in Cl⁻ free conditions for E7-11 animals. Error bars represent standard error of the mean.
Figure 3.10 Summary data for the effects of Cl⁻ free solution and metabolic acidosis by age group. A. The removal of Cl⁻ reversed the effect of low pH on E12-14 rhythm frequency. The attenuating influence of low pH on rhythm frequency was non-significantly weakened by Cl⁻ removal in middle and early age groups. B-D. Cl⁻ free solution significantly increased burst duration and area for the middle age group compared to low pH alone. The Cl⁻ free aCSF and acidosis significantly dampened burst amplitude for the late age group compared to control (low pH alone). Error bars represent standard error of the mean. * indicates differences from control (low pH alone).
Figure 3.11 Speculative models for the roles of Cl⁻ in the effects of acidosis on cranial rhythms in the zebra finch embryo. Two models are proposed for how Cl⁻ underlies the systems level motor output observed during acidosis in both early (<E11) and late (>E11) zebra finch. First, the activity of GABA<A receptors was involved in the effects of low pH on rhythm in the middle age group but no other age group. Therefore, the ion channel is shown in light purple shading in the early neuron model and faded out in the late neuron model. Second, NKCC1 and KCC2 activity are needed to establish the age-appropriate Cl⁻ concentration gradients for early and late embryos, respectively. I hypothesize that the activity of the Cl⁻/HCO₃⁻ exchanger AE3 is involved in the pH sensitivity of early and late embryos. In early embryos, AE3 may buffer intracellular pH changes incompletely (small downward arrow) with reductions in pH leading to general decreases in excitability typical of non-respiratory neurons. In late embryos, AE3 activity may help rapidly acidify intracellular pH (large downward arrow) during an extracellular acidosis to trigger pH sensing transmembrane proteins that depolarize respiratory neurons. Stars indicate important factors for each age category. Question marks indicate candidate proteins that have yet to be experimentally tested.
4. Role of chloride in the recovery of avian respiratory motor rhythms during acidosis

4.1 Abstract

Throughout the central nervous system, rhythmic spontaneous neural activity (rSNA) supports the normal development of motor networks during embryogenesis. However, we know little about how normal and irregular disturbances in the embryonic environment, and thus, the extracellular milieu of the embryo, alters potential neurodevelopmental signals orchestrated by rSNA. In the early- to mid-incubation zebra finch embryo hindbrain, acidity is a stressor that impedes the normal production of motor rhythms, at least in vitro. The goal of the present work was to describe and test the response of early motor rhythms to the depressant effects of pH. In the chicken embryo spinal cord, chloride (Cl\(^-\)) contributes to the recovery of rSNA when pharmacologic agents directly disrupt neurotransmission. Therefore, we studied the recovery of rSNA during acidosis in the zebra finch embryo hindbrain, the site for controlling future ventilatory behaviors customized for breathing control, while manipulating Cl\(^-\) neurotransmission, Cl\(^-\) transport, and extracellular Cl\(^-\). The results of these experiments show that Cl\(^-\) transport and the concentration of extracellular Cl\(^-\) are key players in the process of recovery during metabolic acidosis. In addition, blocking gamma aminobutyric acid A (GABA\(_A\)) receptor activity did not change recovery of rSNA during acidosis at any age.
4.2 Introduction

Maintaining a constant internal homeostatic range for temperature, pH, electrolyte concentrations, and other physiological variables helps ensure an organism’s survival. Often, homeostasis emerges from feedback systems that monitor physiological variables and trigger adjustments when these variables drift from a set range of values (Cannon, 1929). Balance is maintained at the level of individual organ systems as well, such as the central nervous system (CNS) as well (Turrigiano and Nelson, 2004).

In the CNS, homeostatic processes stabilize neuronal activity in part by performing “housekeeping” roles in electrolyte and acid-base balance. When the firing rates of some neurons rise or fall from a given set point, modifications to intrinsic neuronal properties and synaptic interactions can help restore the firing rate back toward the set point; this process is known as homeostatic plasticity (Turrigiano and Nelson, 2004). A simplified overview of this process is shown in Figure 4.1. Data show that homeostatic plasticity helps maintain developing systems-level motor output. The rate of bursts depends on the level of synaptic drive within the motor circuit. When the burst rate falls below the target range (blue shading) due to a perturbation that slows activity, mechanisms of homeostatic plasticity (black arrow to the left) are engaged to increase excitability. For example, in some systems, additional receptors are inserted into the postsynaptic membrane or additional neurotransmitter vesicles could are recruited to the active zone of presynaptic neurons (Malinow and Malenka, 2002; Prange and Murphy, 1999). In cases where the bursting rate is increased relative to the target level, mechanisms are engaged to decrease excitability and lower bursting rate.
back into the target range, often by opposite mechanisms to those described above (Malinow and Malenka, 2002).

Homeostatic plasticity has been studied in *en bloc, in vitro* preparations including the avian embryonic nervous system destined to contain motor circuits that generate rhythmic motor behaviors (Chub and O’Donovan, 1998; Vincen-Brown et al., 2016; for a review see Wenner, 2014). The developing CNS is notable for far-reaching depolarizations of large populations of neurons that regularly excite the brain and spinal cord (Blankenship and Feller, 2010; Hughes et al., 2009; Momose-Sato and Sato, 2013). This rhythmic spontaneous neural activity (rSNA) has been found to be resilient to change under conditions that either increases or decreases its frequency. For example, rSNA recorded from the E8 chicken embryo spinal cord recovered to control levels after 1-2 hours of excitatory blockade (*i.e.*, glutamate receptor antagonists) (Wilhelm and Wenner, 2008). Similarly, spontaneous cranial nerve (CN) IX motor rhythms in the E5 chicken embryo hindbrain began to recover after 5-10 minutes of low extracellular potassium or 6 hours of chronic nicotine exposure (Vincen-Brown et al., 2016a). When employing more intact preparations, it can be difficult to resolve the details of cellular mechanisms at the same level that can be reached studying smaller populations of neurons in cell culture. However, experiments that employ pharmacologic agents to disrupt neurotransmission in the avian hindbrain and spinal cord have revealed that gamma aminobutyric acid A (GABA\textsubscript{A}) receptors may play a role in the homeostatic plasticity of rSNA during conditions that block motor outflow, at least when rSNA is still generic and undifferentiated (Vincen-Brown et al., 2016a; Wenner, 2011). This opens the possibility that GABA is an important signal for homeostatic corrections.
in rSNA, which may have consequences for the rules of circuit formation alter when specific motor behaviors must be functional at hatching.

Before air-breathing begins, excitatory GABAergic transmission drives rSNA production in chicken embryos and zebra finch embryos to varying degrees as a function of age (Gonzalez-Islas et al., 2010; Pickett et al., 2018). Similar to other young vertebrates, GABA receptor activation tends to depolarize membrane potential due to the relatively higher intracellular chloride, [Cl\(^-\)]. The greater [Cl\(^-\)] is accomplished by a relative abundance of the cation-chloride cotransporter (NKCC1) which mediates the import of 2Cl\(^-\) along with K\(^+\) and Na\(^+\) (Ben-Ari et al., 2007). Later on, a cation-chloride cotransporter that extrudes Cl\(^-\) (KCC2) becomes more prevalent which leads to relatively lower [Cl\(^-\)] and an inhibitory effect of GABA\(_A\) receptor activation.

The contribution of GABA\(_A\) receptor activation to synaptic drive could be a key player in the ability of rSNA to persist in the presence of glutamatergic antagonists (Chub and O’Donovan, 1998; Wilhelm and Wenner, 2008) or when bursting stops due to over-excitation of respiratory circuits by nicotine (Vincen-Brown et al., 2016a). During perturbations that slow rSNA production, a net increase in [Cl\(^-\)] can be observed using a genetically encoded chloride indicator (Lindsly et al., 2014). Cl\(^-\) accumulation is thought to occur during the absence of rSNA because of background activity of NKCC1 and a Cl\(^-\) bicarbonate (HCO\(_3^\)\) transporter, anion exchanger 3 (AE3), which both facilitate neuronal Cl\(^-\) entry (Gonzalez-Islas et al., 2009). Higher [Cl\(^-\)] relative to extracellular Cl\(^-\) depolarizes resting membrane potential making it easier for neurons to reach the threshold for the opening of voltage gated Na\(^+\) channels despite stressors that slow or block neural activity.
To date, studies examining homeostatic plasticity mechanisms have taken place in the context of pharmacologically induced perturbations to rSNA that directly impede normal neurotransmission via pharmacologic agents (Turrigiano and Nelson, 2004). There is a need to explore homeostatic mechanisms during exposure to natural cues, such as pH changes, that could alters neural activity in developing vertebrate embryos. Santin and colleagues demonstrated that increases in synaptic strength occur in the adult bullfrog respiratory circuit during the absence of pulmonary ventilation as the frogs spend colder months of the year hibernating underwater (Santin et al., 2017). Maintaining the excitability of the respiratory circuit during hibernation is thought to prepare frogs for air-breathing once they leave the water in the spring. Similar to the bullfrog’s capacity for sustaining respiratory motor rhythms through winter, the zebra finch embryo hindbrain motor rhythms have been observed to persist during acidification that initially slows rhythmic bursting. This phenomenon presents an opportunity to study mechanisms of homeostatic plasticity in response to a more physiologically relevant stressor to the bird embryo.

The role of Cl⁻ mediated neurotransmission and Cl⁻ transport in the recovery of chicken embryo rSNA during chronic neurotransmission blockade led us to hypothesize that Cl⁻ is also involved in the recovery of rSNA during acidosis, especially considering the role of Cl⁻ in pH regulation (Vincen-Brown et al., 2016a; Wenner, 2014). To test this hypothesis, recovery of rSNA during pH exposure was examined under experimental conditions manipulating chloride-mediated neurotransmission (i.e., GABA₄ receptor antagonist present), chloride transport (i.e., NKCC1 antagonist present), or disrupting all Cl⁻ dependent processes by bath applying Cl⁻ free aCSF.
Results show that Cl⁻ is responsible for the recovery of rSNA during acidosis and the rebound-overshoot of rSNA during pH washout in a context dependent manner. For example, while rSNA recovery persists in zebra finch when GABAₐ receptor activity is inhibited, antagonism of NKCC1 transporters eliminates recovery and rebound upon pH washout in E4-6 embryos. By E7-10, bath application of the NKCC1 blocker bumetanide prevented recovery in some animals while evoking faster rhythms in others. Finally, removal of Cl⁻ blocked rSNA recovery in nearly all embryos while rebound-overshoot upon pH washout was present but delayed.

4.3 Methods

4.3.1 Experimental animals

All procedures were approved by the Idaho State University Institutional Animal Care and Use Committee. I collected zebra finch eggs from an in-house breeding colony, candled eggs to estimate developmental age, and placed eggs into a temperature-controlled (38°C), humidified (60-70%), forced-draft incubator (GQF 1502 Sportsman). 70 embryos were used for the experiments.

4.3.2 Preparation of control and experimental cerebrospinal fluid

Same as described in Chapter 3 methods except for the following. In three experiments, pH was lowered to 7.03 by reducing NaHCO₃ to ~6.5 mM while keep the other components of the aCSF the same as control fluid. This “extra low pH” was used to more routinely evoke recovery in E4-7 zebra finch.
4.3.3 Pharmacologic agents

Same as described in Chapter 3 Methods.

4.3.4 Surgical isolation of the brainstem preparation

Same as described in Chapter 2 Methods.

4.3.5 Extracellular recording and data collection

Same as described in Chapter 2 Methods.

4.3.6 General experiment protocol

Before beginning experimental treatments, stable baseline CN XI activity was recorded for at least 30 minutes while the preparation was superfused with the appropriate control aCSF. Next, the control aCSF was replaced by aCSF containing either PTX, bumetanide, or Cl⁻ free aCSF by switching reservoirs for 30 minutes. I referred to this period as the drug or Cl⁻ free baseline. Next, low pH aCSF containing a drug or Cl⁻ free aCSF was added to a clean reservoir for 1 hour. Finally, control aCSF bathed the preparation for 30 min or until rhythmic neural activity resembled the original, baseline rhythm. Figure 4.2 shows a diagram of the experiment protocol. The pH of superfusate was checked during experiments using a small pH electrode placed in the bath (Orion PerpHecT ROSS Combination pH Micro Electrode, 8220BNWP).
4.3.7 Experimental protocol for overnight experiments

To gain more insight into the time course of recovery, overnight experiments were performed where low pH solution was applied to the hindbrain preparation overnight (8.5-14 hours, n = 3, age = E8 and E10). A peristaltic pump (Ismatec Reglo ISM834, Wertheim, Germany) was used to recirculate low pH aCSF that was continuously oxygenated with a 95% O₂/5% CO₂ gas mixture (Airgas). An in-line and bath chamber temperature controller maintained the bath temperature at 27°C (Warner Dual Automatic Temperature Controller, model TC-344B, Holliston, MA). Spike2 software (version 8) continuously recorded cranial nerve motor output for the entire duration of the experiment until the recording was stopped or the nerve became dislodged from the suction electrode.

4.3.8 Summarizing and analyzing data

The zebra finch brainstem preparation was the unit of replication for all experiments. Zebra finch brainstems were selected randomly without specific knowledge of their exact age, sex, or parentage. To further minimize the effect of confounding variables, the experimental treatment given to any zebra finch brainstem was haphazardly selected. For recovery studies, data were pooled into two age groups: E4-6 and E7-10. These groupings were selected since they both showed recovery during low pH treatment. Data analysis involved quantitative and qualitative methods described in the following sections.
4.3.9 Identification of recovery of CN XI motor output during acidosis

After the discovery that CN XI activity only temporarily changed during hour-long pH treatments in some ages, the next step was to create an objective definition for this recovery or plasticity. First, experiments were included for recovery analysis where a solid nerve-micropipette-electrode connection lasted the entire duration of the treatment period (i.e., “slipping” did not occur). This was a conservative measure that ensured a plastic response was being captured rather than simply losing the nerve and then regaining it after increasing negative backpressure. Second, to determine if recovery occurred, a clear treatment effect (e.g., either decreased or increased frequency) followed by a reversal of the treatment effect (e.g., a change in rhythm opposite to the treatment effect) must have been present. If the reversal did not persist for the remainder of the treatment period, recovery was deemed absent. Finally, if the baseline rhythm showed an inconsistent, irregular rhythm or poor noise to signal ratio, it was not evaluated for recovery. This rigorous set of criteria allowed for a careful and conservative analysis of recovery.

4.3.10 Quantification and statistical analysis of recovery

Although recovery was detectable by eye, two different analysis methods were used to measure it. Jennie Nelson, an undergraduate student and Team Bird Brain lab member, developed the following methods as part of her summer INBRE research project completed in the Pilarski Laboratory.
First, Spike2 was used to calculate the instantaneous frequency for the control, treatment, and wash periods. This was accomplished using built-in analysis features in Spike2 and the script DumpInstFreq (http://ced.co.uk/downloads/scriptspkport). Since embryos of different ages displayed varying burst frequency, instantaneous frequency was collected for 100-second time windows for the duration of the entire experiment. Each value specifically represented the instantaneous frequency at the beginning of each 100 second time interval. This method excluded some bursts in embryos with faster rhythms (i.e., interburst intervals < 100 sec) and counted bursts multiple times in embryos with slower rhythms (i.e., interburst intervals > 100 sec). The binned instantaneous frequency values were plotted as a function of the experimental time course. To assess recovery, the slope of the instantaneous frequency was examined during the last ~8 min of the treatment period.

Linear regression was used to determine whether the slope of the instantaneous frequency (IF) during the recovery period differed from zero (JMP). A significant deviation in slope from zero was used to identify recovery (P < 0.05).

The second approach evaluated when recovery began during the hour-long acidotic exposure. First, the onset time of the first burst in a series of bursts that exhibited a reversal in the treatment effect was recorded. This metric provided the best estimate of the timing of recovery initiation. In some cases, a long gap would occur following bursts that were showing a reversal in treatment effect. If the gap was less than the maximum interburst interval induced by pH, recovery onset time was still counted. If the gap was longer, recovery onset time was counted as the time of the first burst in the subsequent series of bursts showing a reversal in treatment effect. Next, the
relative time during the 3600 second treatment period was calculated that corresponded with recovery onset (recovery time - treatment start time). Then, resulting time (seconds) was divided by 60 to arrive at “minutes of the treatment period.” These data were summarized by calculating the mean recovery onset time for the E4-6 and E7-10 age groups.

4.3.11 Quantification of rebound

Rebound was assessed by plotting the average instantaneous frequency (IF) values for the 100-second time windows for the 30-minute wash period. Next, the average IF for the total baseline period was plotted on the graph to provide a visual reference for the baseline relative to the wash period IF. A positive shift of the wash period IF above the baseline IF was identified as rebound.

4.4 Results

4.4.1 Characteristics of recovery during hour-long acidosis

Overall, E4-6 and E7-11 hindbrain motor rhythms decrease in frequency during acidosis (see Chapter 2). However, the dampening effect of acidic pH was found to be transient in some embryos with the slowed rhythm reversing and rSNA frequency increasing despite a constant low pH stimulus. This reversal of rSNA rhythm frequency was termed homeostatic recovery or simply “recovery.” The majority of E4-6 embryos (6 out of 7 experiments) and more than half of E7-10 embryos (9 out of 12 experiments)
showed recovery during the 1-hour acidotic treatment period. Figure 4.3 shows
examples of recovery during metabolic acidosis treatment for embryos representing the
E4-6 (early) and E7-10 (middle) age groups. Note that homeostatic recovery did not
occur in embryonic hindbrains older than E11. Recovery began on average near or just
after the halfway point of treatment (E4-6: 31 ± 4 min; E7-10: 40 ± 4 min).

To measure recovery over the low pH treatment period, instantaneous frequency
of CN XI motor rhythms was first plotted over time. This method allowed for a
standardized assessment of recovery by examining the slope of instantaneous
frequency (IF) values during the last 8 minutes of the treatment period when recovery
was underway. Figure 4.4 shows an example plot of IF data for 100-sec time bins over
the entire experimental period shown in the accompanying trace. Figure 4.5 shows the
same type of plots for E4-6 and E7-10 embryos exposed to low pH treatment. On
average, IF for E4-6 embryos showed recovery which was indicated by the slope of the
regression line through IF values being unequal to zero (slope = 1 * 10^-6, n = 7, P =
0.02). Similarly, E7-10 embryos showed recovery as evidenced by non-zero slope of the
regression line through IF values for the recovery period (slope = 5 * 10^-7, n = 12, P =
0.01).

4.4.2 Role of GABA\textsubscript{A} receptor activity in recovery

Blocking GABAergic neurotransmission using picrotoxin (PTX) during acidosis
did not block recovery in E4-6 or E7-10 zebra finch. All of the E4-6 embryos (6 out of 6)
and all of the E7-10 embryos (10 out of 10) exhibited recovery when PTX was bath
applied in combination with low pH. Figure 4.6 shows representative electroneurograms during low pH + PTX for embryos representing both age groups. Similar to low pH alone, recovery onset was identified near the middle of the low pH + PTX treatment (E4-6: 23 ± 1 min; E7-10: 36 ± 5 min). Figure 4.7 shows the average IF for both age groups while exposed to the GABA\textsubscript{A} receptor antagonist and acidosis. During the recovery periods, the slopes of the regression lines through the average IF were not different than zero (E4-6: slope = 1 * 10\textsuperscript{-7}, n = 6, P = 0.53; E7-10: slope = 5 *10\textsuperscript{-6}, n = 10, P = 0.47). This result indicates that recovery was not occurring during the last portion of the treatment period. Rather, recovery had already occurred earlier as can be seen in the suction electrode recordings.

4.4.3 Role of NKCC1 activity in recovery

Disrupting the activity of the Cl\textsuperscript{-} cation co-transporter NKCC1 attenuated rSNA recovery in some zebra finch. Recovery occurred in only half of E4-6 embryos (4 out of 8) and nearly half of E7-10 embryos (8 out of 19). Similar to low pH alone, the onset of recovery was identified at the midpoint of treatment for the E4-6 (31 ± 4 min) and E7-10 (32 ± 4 min) age groups. Representative whole nerve recordings show blockage of recovery in embryos from both age groups and an example of the increased frequency observed in some E7-10 experiments (Figure 4.8).

Using the average IF slope analysis, recovery was found to be blocked in E4-6 and E7-10 embryos in the presence of the NKCC1 antagonist bumetanide. On average for E4-6, the slope of the average IF during the recovery period was no different than
zero (slope = -3 *10^{-8}; n = 8, P = 0.93) (Figure 4.9). Within the E7-10 age group, recovery was absent during the recovery period as evidenced by slope of the regression line no different than zero (slope = 2*10^{-7}, n = 19, P = 0.87).

4.4.4 Role of extracellular Cl⁻ in recovery

Removing Cl⁻ from the superfusate completely blocked recovery in E4-6 embryos and in all but one embryo in the E7-10 age group, showing that pH homeostasis is not possible without Cl⁻ in the extracellular environment (Figure 4.10). Figure 4.11 shows plots of the average IF values by age group for the entire experimental period and expanded views of the recovery periods. The slope of the average IF for the early age groups was found to be no different from zero (E4-6: slope = -7*10^{-21}, n = 7; P = 0.99). The slope of the average IF during the recovery period for middle age embryos was significantly different from zero (slope = -1 *10^{-7}, n = 11; P = 0.009). However, this finding likely reflects slower rates of bursting resulting in a non-zero slope of the IF values during the recovery period which would indicate persistent slowing rather than recovery.

4.4.5 Rebound of CN XI motor output

In addition to observing recovery during hour-long low pH treatments, rSNA was observed to overshoot baseline burst rates or “rebound” during the wash period, which suggested that compensatory mechanisms were engaged during the acidic pH exposure and subsequently revealed during wash. After pH-induced slowing of CN XI
motor frequency, wash often resulted in faster rhythms. To assess rebound in rSNA frequency during wash compared to baseline, the average IF for 100 sec time bins was plotted for the entire wash period with reference to the average IF for the entire baseline rhythm. E4-6 zebra finch showed greater average IF during the wash period compared with baseline beginning after ~5 minutes of wash and reaching a stable plateau just above baseline average IF (Figure 4.12, panel A). E7-10 embryos displayed rebound in rhythm frequency after ~15 minutes of wash that continued increasing through the end of wash (Figure 4.12, panel B).

Manipulating Cl- mediated neurotransmission, Cl- transport, and removing extracellular Cl- influenced rebound in an age-dependent. Bath applying a GABA\textsubscript{A} receptor antagonist during acidosis blocked rebound in E4-6 embryos while rebound remained intact in E7-10 embryos (Figure 4.13). Bath application of the NKCC1 antagonist, bumetanide, primarily dampened rebound in E4-6 zebra finch with the exception of some variable increases mid-wash compared to the rebound observed after washout of low pH alone (Figure 4.14, panel A). For E7-10, rebound appeared inconsistently during the wash period interspersed with baseline or sub-baseline IF (Figure 4.14, panel B). Finally, rebound occurred following the washout of Cl- free aCSF in E4-6 and E7-10 embryos (Figure 4.15). However, rebound occurred after 20 minutes of wash in the E7-10 embryos compared with only 10 minutes of wash after low pH alone. In E4-6 embryos, rebound occurred only slightly later than control.
4.4.6 Recovery during chronic acidosis

A full recovery to baseline rhythm was not observed within the 60 minute treatment period for E7-10 embryos. Therefore, an E8 zebra finch was exposed to acidosis overnight to assess recovery that may occur over longer time scales. Figure 4.16 shows the extracellular recording during the baseline, 1-hour acidosis, 5-hour acidosis, and 10-hour acidosis time points. During the first hour of low pH, rhythm decreased in frequency. However, rhythm gradually increased in frequency over the next several hours until by 10-hours of acidosis, bursting occurred at a greater rate than the original baseline. Further, rhythm became more regular after 10 hours of acidosis. Overnight experiments were performed on two E10 embryos as well. However, the rhythm characteristics of E10s, including short duration bursts interspersed with long duration bursts, complicated the assessment of recovery (data not shown).

4.5 Discussion

The first goal of this study was to describe the homeostatic recovery of rSNA during a pH challenge. E4-6 and E7-10 zebra finch embryo hindbrain rhythms were resilient to the dampening effects of acidosis. In other words, the central breathing network was capable of maintaining rhythmic output in the face of a pH stressor that initially weakened motor output frequency. In addition to recovery, an increase in rhythm frequency above baseline frequency was often observed during washout of the pH treatment. This phenomenon was termed “rebound”. Rebound was observed in embryos of early and middle age groups during low pH washout. Rebound in respiratory
motor output has been observed during washout of serotonin (5-HT) in the turtle brainstem spinal cord preparation (Johnson et al., 2001). Similar to low pH exposure to the zebra finch embryo hindbrain, 5-HT has a depressant effect on the frequency of CN XII motor rhythm in the turtle brainstem. Rebound in frequency lasted for ~2 hours during wash in the turtle. The duration of rebound during low pH washout in the zebra finch hindbrain is not yet known. In the future, assessing the duration of rebound could provide insights into the timescale of recovery mechanisms in the developing avian nervous system. In addition, it would be interesting to assess whether a second low pH exposure yielded more rapid recovery after being “primed” by the first exposure.

The second goal of the present work was to test whether a Cl⁻ ion channel, Cl⁻ transporter, or extracellular Cl⁻ contributes to the recovery of CN XI motor rhythms during acidosis. To do this, I assessed recovery under three conditions where 1.) GABAₐ receptors were blocked using picrotoxin (PTX), 2.) Cl⁻ transport was blocked with bumetanide, or 3.) Cl⁻ was removed from the superfusate. In addition, I examined the effects of the previously mentioned manipulations on rebound during wash since rebound could reflect an unmasking of compensatory mechanisms in play that support rhythm generation under challenging conditions. The results of these experiments show that Cl⁻ is involved in both the recovery and rebound seen in cranial motor rhythms of the zebra finch embryo during acidosis. Three lines of evidence suggest that Cl⁻ participates in the compensatory mechanisms responsible for the recovery of CN XI motor rhythms during pH perturbations.

First, data show that recovery was still possible in both E4-6 and E7-10 embryos during GABAergic antagonism and acidosis. But, blocking GABAₐ receptor activity
eliminated overshoot rebound in E4-6 zebra finch. A lack of rebound during washout of experimental aCSF in E4-6 embryos suggests that GABAergic activity may contribute a non-essential part of the compensatory mechanisms during low pH exposure. GABA receptors are known to contribute to the recovery of rSNA in the chicken embryo spinal cord and hindbrain (Chub and O'Donovan, 1998; Vincen-Brown et al., 2016a; Wilhelm and Wenner, 2008). GABA ion channels have been proposed to serve as sensors for activity that in turn engage plasticity mechanisms (Gonzalez-Islas et al., 2010). I speculate that recovery occurring in response to acidosis may incorporate multiple pathways in addition to GABAergic neurotransmission.

Second, disrupting the activity of the Cl⁻ cation co-transporter NKCC1 blocked recovery in E4-6 and E7-10 embryos. These results suggest that intracellular Cl⁻ accumulation could be involved in promoting bursting during low pH. In the chicken embryo spinal cord, NKCC1 mediates Cl⁻ uptake that leads to higher intracellular Cl⁻, depolarized GABA equilibrium potential, and depolarizing GABAergic neurotransmission (Gonzalez-Islas et al., 2009). However, blocking NKCC1 activity in E7-10 zebra finch did not block recovery in as straightforward of a fashion compared with E4-6 animals. Five embryos between the ages of E9-10 displayed increased rhythm frequency without recovery during bumetanide + low pH. Since these experiments were included in the analysis of IF during the recovery period, IF data were quite variable over the course of the treatment period. It is possible that the slope of the IF during the recovery period was found to be equivalent with zero because bumetanide tended to increase rhythm frequency in some animals and IF values reached a plateau. The occurrence of variable
effects of blocking NKCC1 may have been due to the proximity to the natural transition of Cl⁻ neurotransmission from excitatory to inhibitory (Pickett et al., 2018).

Third, eliminating Cl⁻ from the superfusate blocked recovery in all E4-6 and almost all E7-10 embryos. Removal of extracellular Cl⁻ likely interrupted the normal function of Cl⁻ transport proteins such as NKCC1 and Cl⁻/HCO₃⁻ exchangers such as anion exchanger 3 (AE3). In addition, reducing Cl⁻ to zero likely depolarized the equilibrium potential for Cl⁻. By enhancing the driving force for Cl⁻ efflux and preventing intracellular Cl⁻ restoration for a time, I speculate that recovery could only occur once a normal Cl⁻ concentration gradient was restored. Indeed, rebound occurred during washout of Cl⁻ free + low pH treatment in several E4-6 and E7-10 zebra finch. One way to experimentally test this hypothesis could be to expose zebra finch hindbrains to different levels of Cl⁻ depletion during acidosis, washout with control aCSF, and assess the timing of rebound relative to the degree of extracellular Cl⁻ depletion. Further, washout of Cl⁻ free low pH solution could keep the bumetanide concentration constant which could hinder the reaccumulation of Cl⁻ via NKCC1.

4.5.1 Considerations and limitations of the current study

I am planning to refine the analysis method for studying recovery that involves comparing the slope of the IF during different experimental conditions. The current problem with this analysis method is exemplified by the fact that recovery was observed during GABAergic neurotransmission blockade and acidosis but the analysis of the IF data suggested that recovery was not occurring. I believe that these contradictory
results occurred because the last 8 minutes of treatment does not capture recovery in all experiments. I am planning to examine the individual experiments again to identify a better time frame for studying the slope of IF to compare recovery in experimental and control (metabolic acidosis) treatments.

Also, my experiments tested whether Cl\(^-\) was involved in the homeostatic recovery of rSNA but there are other ions and molecules that may also contribute to the recovery described here. For example, activity-dependent flux in intracellular calcium (Ca\(^{2+}\)) or neurotrophic factors such as brain derived neurotrophic factor may act as a signal for initiating homeostatic plasticity mechanisms (Turrigiano and Nelson, 2004). To assess possible contributions of Ca\(^{2+}\) to recovery in the zebra finch hindbrain, Ca\(^{2+}\) imaging during the recovery process could be employed (Russell, 2011).

Recently, a fast-acting depolarization in resting membrane potential was identified in the early stages of recovery in the chicken embryo spinal cord (Gonzalez-Islas et al., 2020). The depolarization is thought to be due to either changes in K\(^+\) leak channel currents or adjustments in Na-K\(^+\) ATPase activity. To address this alternate hypothesis, additional studies in a more reduced preparation such as a rhythmic slice could be ideal. For example, whole cell current clamp experiments could be used to measure membrane potential of CN XI motor neurons during early recovery (i.e., during 1-hour long low pH exposure) or after overnight exposure. Finally, a whole cell voltage clamp approach would also be useful for assessing whether gradual Cl\(^-\) accumulation and a depolarization of the Cl\(^-\) equilibrium potential is contributing to recovery in the present work.
Another limitation of the current research is that it is unclear if the recovery of CN XI motor output is due to homeostatic regulation of intracellular pH. Vertebrate embryos including the common snapping turtle have the capacity to maintain a set intracellular pH (pHᵢ) level when exposed to acute acidosis that lowers extracellular pH (pHₑ) (Shartau et al., 2016). This concept has been put forth as the “preferential pHᵢ hypothesis.” Turtle embryos have been found to protect pHᵢ so changes in pHₑ have little influence on the pH of the cellular compartment. It is unknown whether avian embryos display this cellular behavior. Given the ability to regulate and maintain pHᵢ, it is possible that the resurgence of rSNA during acidosis could reflect the restoration of physiological pHᵢ in the zebra finch hindbrain. To test this hypothesis, pHᵢ could be measured using a biochemical approach or fluorescent pHᵢ indicator during control conditions and during acidosis at timepoints that capture initial slowing of rhythm and recovery (Gdovin et al., 2009; Portner et al., 1990).

The alternate hypotheses raised here provide exciting new directions for future study. The main finding of the current research was that the embryonic zebra finch hindbrain mounts a compensatory response to acidosis using Cl⁻ mediated mechanisms. However, additional processes are likely involved in recovery since the sustained production of rSNA is important for proper neurodevelopment.
Figure 4.1 Overview of homeostatic plasticity in the nervous system. Homeostatic mechanisms (black arrows) help maintain neural activity within a target range (blue shaded region). During conditions that lower synaptic drive, such as low pH in the zebra finch embryo hindbrain, homeostatic mechanisms that increase excitability promote increased synaptic drive and greater rates of motor output (bottom arrow below the target rate). In contrast, if bursting exceeds the target rate due to exposure to alkaline pH stimuli then plasticity mechanisms decrease excitability and limit bursting rate through lower synaptic drive (top arrow). This figure was adapted with permission from Figure 2 in Turrigiano & Nelson 2004 Nature Reviews Neuroscience.

Figure 4.2 Protocol for recovery experiments. First, stable baseline CN XI motor activity is recorded for 30 min. Drugs or Cl⁻ free fluid superfuses the hindbrain for the next 30 min (dotted fill). Next, low pH fluid (gray shading with dots) containing drugs or without Cl⁻ is applied for 60 minutes. Treatment is washed out for 30 min. The last 8 minutes of the treatment period are used for the analysis of recovery using the instantaneous frequency slope method (see Figure 4.4 for a description of this analysis approach).
Figure 4.3 Representative CN XI nerve recordings showing recovery during metabolic acidosis. During bath-application of acidic aCSF, rhythmic CN XI motor bursts decrease in frequency. In early embryos (E4-6), this decrement in frequency is short-lived with rhythmic output gradually increasing in frequency during sustained acidosis. In middle age embryos (E7-10), the slowing effect of low pH is greater and longer-lasting. However, just before wash begins, bursting resumes. The reversals of the acid-induced slowing seen in these recordings were referred to as “recovery.” The first burst denoting the time of recovery onset is marked with an asterisk (*). The E9 recording exhibits rebound-overshoot during wash where the frequency and amplitude of bursting overshoots the baseline activity level.
Figure 4.4 Example of quantifying recovery using instantaneous frequency. Top shows a rectified and integrated suction electrode recording of a full-length low pH experiment including baseline, treatment, and wash periods. Below the trace is a plot of the instantaneous frequency (IF) of 100 second time windows of the above trace. During the metabolic acidosis treatment (yellow shading), IF data show a constant IF corresponding with the last IF value from the baseline period since no bursts occurred during more than half of the low pH treatment period. The last ~8 minutes of treatment is the period when recovery was analyzed.
Figure 4.5 Mean instantaneous frequency (IF) during full metabolic acidosis experiments. 

A. Plot of average IF for E4-6 zebra finch over low pH experiments. Yellow shading shows the duration of low pH exposure. Average IF initially declines during acidosis and is seen to gradually increase over the last 8 minutes of the treatment period. Below, the average IF for the recovery period is shown with a linear regression line with slope that was significantly different from zero. This finding shows recovery occurred. 

B. Plot of average IF for E7-10 zebra finch during low pH experiments. Average IF decreases over the course of acidic aCSF exposure but increases during the recovery period. The slope of the regression line through the average values during the last 8 minutes of treatment was not equal to zero, showing that recovery occurred.
Figure 4.6 Recovery of CN XI motor rhythms persists in the presence of a GABA$_A$ receptor antagonist and metabolic acidotic aCSF. Cranial nerve bursting slows and recovers prior to washout of picrotoxin + low pH aCSF in early (E6, top trace) and middle age zebra finch embryos (E7, bottom trace). Asterisks (*) denote the burst representing the onset of recovery.
Figure 4.7 Average instantaneous frequency during bath-application of picrotoxin and acidotic aCSF. A. When picrotoxin (PTX) was applied in combination with low pH aCSF, the slope of the regression line that was not different from zero for E4-6 zebra finch during the recovery period (slope = 1 \times 10^{-7}, n = 6, P = 0.53). B. Similarly, the slope of the regression line through IF values during the recovery period was no different from zero for E7-10 zebra finch (slope = 5 \times 10^{-6}, n = 10, P = 0.47). It is noteworthy that recovery was observed in all E4-6 and E7-10 zebra finch, but recovery occurred before the last 8 minutes of treatment which was not captured by this analysis method.
Figure 4.8 Representative traces of CN XI motor output during bumetanide and low pH experiments. Rhythmic motor output continues to slow down throughout the duration of the low pH + bumetanide treatment in the E5 zebra finch. The E9 electroneurogram shows the persistent slowing effect of bumetanide + low pH aCSF on rhythm frequency.
Figure 4.9 Average instantaneous frequency during bumetanide and metabolic acidosis experiments. Plots of average IF over experimental time during bumetanide + low pH experiments for E4-6 (A.) and E7-10 (B.) embryos. On E4-6, average IF is lower during NKCC1 antagonist exposure and recovery does not happen as indicated by the slope of recovery period equivalent with zero (slope = -3 *10^{-8}; n = 8, P = 0.93). For E7-10, during the recovery period, average IF slope was not different from zero which suggests recovery did not occur (slope = 2*10^{-7}, n = 19, P = 0.87). However, the increased and variable frequency during bumetanide exposure may have enhanced recovery earlier in the treatment period such that recovery was not detected during the last 8 minutes of treatment.
Figure 4.10 Removing Cl⁻ prevents recovery in all ages. Representative electroneurograms of CN XI activity during Cl⁻ free + low pH experiments show persistent slowing of rhythm without recovery. Two examples of E6 experiments show a range of responses for early embryos. Some exhibited no bursting during the Cl⁻ free + low pH aCSF superfusion while bursting was still possible in others. E7-10 embryos showed persistent slowing of rSNA without recovery as in the E9 shown in the bottom trace. During washout, rhythm returns and can be seen to rebound in amplitude and frequency.
Figure 4.11 Average instantaneous frequency during removal of Cl\(^{-}\) and acidosis. A. Average IF over the Cl\(^{-}\) free + low pH treatment period for E4-6 zebra finch shows the absence of recovery as evidenced by slope of the regression line through average IF values equivalent to zero (E4-6: slope = \(-7\times10^{-21}\), n = 7; P = 0.99). B. E7-10 zebra finch show slope of the IF values during the recovery period that are significantly different from zero (slope = \(-1\times10^{-7}\), n = 11; P = 0.009). However, recovery is not seen to occur in the suction electrode recordings. Thus, the analysis of the average IF during the recovery period likely reflects a persistent slowing in rhythm frequency rather than recovery occurring.
Figure 4.12 Rebound in the frequency of CN XI motor rhythms after metabolic acidosis exposure. **A.** Average instantaneous frequency (IF) for E4-6 zebra finch (n = 7) over the wash period following low pH treatment with reference to the average IF for the total baseline period (horizontal black line). Rebound is present as an increase in IF above baseline that reaches a plateau. **B.** Same as in A for E7-10 zebra finch (n = 12). Rebound begins after ~10 minutes of wash as IF gradually increases.

Figure 4.13 Rebound in rSNA frequency after GABAergic antagonism and metabolic acidosis exposure. **A.** Average IF over washout of PTX + low pH for E4-6 zebra finch (n = 6) with reference to the average IF for the baseline period (orange line). Rebound is absent during washout of PTX + low pH. **B.** Same as in A for E7-10 zebra finch (n = 10). Rebound is variable during wash.
Figure 4.14 Rebound in rSNA frequency after NKCC1 antagonism and metabolic acidosis exposure. A. Average IF over washout of Bumetanide + low pH for E4-6 zebra finch (n = 8) with reference to the average IF for the baseline period (gray line). Rebound is depressed with variable increases in IF during washout. B. Same as in A for all experiments on E7-10 zebra finch (n = 19). Rebound is present and variable during wash.

Figure 4.15 Rebound in rSNA frequency after washout of Cl⁻ free + low pH aCSF. A. Average IF over the wash period for E4-6 embryos during washout of Cl⁻ free + low pH aCSF (n = 7). Rebound can be seen to occur after ~8 minutes of wash. B. For E7-10 embryos (n = 11), rebound in average IF is seen as average IF positively overshoots baseline after ~18 minutes of wash.
Figure 4.16 Recovery of CN XI motor output during overnight exposure to metabolic acidosis. During the first hour of low pH treatment, a profound decrease in rhythm frequency is observed which is similar to what is seen in the majority of E7-10 low pH experiments previously described. After 5 hours of low pH treatment, rhythm can be seen to recover. After 10 hours of sustained low pH, rhythmic activity appears faster than baseline.
5. Conclusions

5.1 Summary Statement

The first takeaway from my dissertation research is that newly formed hindbrain circuits change their motor output when acid/base imbalance occurs. In the zebra finch embryo, the pH sensitivity of hindbrain circuits changes over incubation. During the part of the embryonic period when the embryo satisfies gas exchange via diffusion, pH disturbances tend to lower excitability or raise excitability similar to the response of non-respiratory neurons during acidosis or alkalosis, respectively (Sinning and Hübner, 2013). Once the embryo begins pulmonary ventilation, hindbrain motor rhythms behave in the exact opposite manner during acidosis and alkalosis. This neurodevelopmental switch may reflect the formation of a specialized feedback system capable of adjusting whole-animal behavior to compensate for a metabolic acidosis.

Second, results show that maturational changes in chloride (Cl⁻) ion homeostasis partly contribute to the switch observed in the rhythmic consequences of acid/base stimuli. In younger (<E11) zebra finch embryos, bath applying a Cl⁻ cation transporter antagonist and blocking GABAergic neurotransmission resulted in an increased frequency rhythm during acidosis. In older (>E11) zebra finch embryos, superfusing the hindbrain with Cl⁻ free aCSF resulted in decreased frequency rhythm during acidosis. But, the effect of acidosis on breathing-related motor output was not affected by bath-application of antagonists for GABA_A receptors or the Cl⁻ transporter NKCC1. Together these data suggest that interfering with Cl⁻ concentration gradients is sufficient to reverse the effects of acidosis on systems level motor output compared to the natural, age-dependent responses. Thus, the maturation of inhibitory Cl⁻ neurotransmission may also contribute to the birth central pH chemosensation.

Third, homeostatic recovery of hindbrain motor rhythms in early zebra finch embryos depends on Cl⁻ as well. The cranial rhythms of E4-10 zebra finch initially slowed during acidosis
but rhythm began to recovery before the acidic fluid was washed out. Recovery was impossible in either the presence of antagonists for the Cl\(^{-}\) cation transporter, NKCC1, or Cl\(^{-}\) free solution. These results suggest that gradual restoration of intracellular Cl\(^{-}\) contributes to the recovery of rhythmic bursting in the zebra finch embryo hindbrain. Importantly, recovery was absent in embryos older than E11 which suggests that the low pH signal may be an important cue for increasing ventilatory behavior in older embryos. I speculate that the increased frequency of breathing-related hindbrain rhythm persists for the full duration of acidosis until pH changes as a result of respiratory compensation for the metabolic acidosis.

### 5.2 Future Directions

The findings of my dissertation research provide a foundation for additional studies on the embryonic emergence of central pH sensitivity and maturation of central feedback systems for regulating whole animal ventilation in birds. Future studies could address the following questions: 1. What is the anatomical organization of central pH sensing regions in the zebra finch embryo hindbrain? 2. Do developmental changes in the expression of specific pH-sensing ion channels contribute to the switch in pH response? 3. Is intracellular Cl\(^{-}\) concentration changing in younger and older zebra finch embryos as a result of blocking NKCC1 function or removing extracellular Cl\(^{-}\), respectively? 4. What are the mechanisms of the age-dependent switch in response to alkaline pH? 5. How could chronic exposure to pH imbalance via incubation in hypercapnic gas influence the development of respiratory circuits in the avian hindbrain?

All of the experiments performed in this project used bath-application of pH stimuli and pharmacologic agents to the whole hindbrain. Therefore, spatial information is missing about where pH is sensed in the avian hindbrain. The mammalian hindbrain possesses several nuclei
noted for their role in pH and carbon dioxide sensing including the retrotrapezoid, locus coeruleus, raphe, and others (Huckstepp and Dale, 2011). A straightforward series of experiments applying focal acid stimuli to different locations in the zebra finch hindbrain could performed to answer these questions. In addition, it is unknown if respiratory-specific chemosensing nuclei arise early on in incubation or only become functional when air-breathing begins. The focal acidosis studies could be performed on hindbrain preparations from a range of embryonic ages to test for changes in the location of pH sensing regions. I tentatively hypothesize that during early embryogenesis, cranial rhythms could respond to acidosis applied to any region of the hindbrain while in air-breathing embryos, rhythmic motor output could be altered by stimulating individual nuclei.

Studies of pH sensitivity in the mammalian hindbrain provide a cast of characters of pH sensing ion channels that could also play a role in the switch in the effects of pH on zebra finch embryo cranial rhythms (Huckstepp and Dale, 2011). Pharmacologic assays could address whether blocking the activity of acid sensing ion channels or tandem pore acid sensing potassium channels affects the pH sensitivity of avian hindbrain motor rhythms. Further, these experiments could be performed at different embryonic time points which could reveal when specialized ion channels contribute to central respiratory chemosensation. In addition, immunohistochemical approaches could be used to support functional data by assessing the developmental expression profile of these ion channels over incubation.

In Chapters 3 and 4, results suggested that Cl⁻ ionic gradients were determining factors for how acidosis modulated systems level motor output in the bird brain. To experimentally test this idea, it is necessary to estimate intracellular Cl⁻ levels using either fluorescent indicators or intracellular recordings of Cl⁻ currents while holding membrane potential constant. Since these techniques require specialized equipment, a more accessible experiment could be performed
using GABA agonists or antagonists applied following the perturbations thought to shift the Cl\textsuperscript{-} concentration gradient to a more mature or immature state.

Since increased acidity over incubation is a natural occurrence in bird eggs, I focused on understanding the neurodevelopmental switch in the response to low pH rather than high pH. However, data in Chapter 1 showed a parallel switch in the effects of alkalosis on rhythmic motor output. Given what I have learned about the age-dependence of the response to acidity, I hypothesize that the Cl\textsuperscript{-} concentration gradient could also be a determining factor for the switch in the effects of alkalosis. To test this hypothesis, high pH experiments could be performed on E7-11 zebra finch embryos in the presence of bumetanide and on E12-14 zebra finch embryos under Cl\textsuperscript{-} free conditions.

Finally, it is known that incubation in high CO\textsubscript{2} gas influences whole-animal ventilatory chemoreflexes in hatchling and adult birds (Bavis and Kilgore, 2001; Menna and Mortola, 2003; Williams and Kilgore, 1992). However, it is unknown if these whole-animal behavioral changes reflect alterations to the hindbrain breathing control network. In the future, I would really enjoy testing whether altered gaseous experience during incubation influences the central chemosensitivity of the hindbrain.
6. References


Cannon, W.B., 1929. ORGANIZATION FOR PHYSIOLOGICAL HOMEOSTASIS. Physiol. Rev. 9, 399–431. https://doi.org/10.1152/physrev.1929.9.3.399


Figure S1. Burst identification in whole nerve recordings. The integrated and rectified CN XI recording above shows a graphical representation of how I distinguished bursts of motor output from noise. An amplitude threshold (2w; gray dashed line) was created for each recording by multiplying the amplitude of the baseline (w) by 2. Bursts were identified as increases in activity that crossed the amplitude threshold (B). Events were classified as noise (n) if the spike did not cross the threshold or if the spike peaked in a square wave pattern (shown in inset circle).
I analyzed whole nerve recordings from each experiment using a custom script written by Dr. Ann Revill from Midwestern University in Glendale, Arizona. The script employs a user-assigned amplitude threshold to calculate the burst duration, amplitude, and area for bursts within a user-defined time period within an experiment.

'Spike2 Scripts

I analyzed whole nerve recordings from each experiment using a custom script written by Dr. Ann Revill from Midwestern University in Glendale, Arizona. The script employs a user-assigned amplitude threshold to calculate the burst duration, amplitude, and area for bursts within a user-defined time period within an experiment.

'current quantification

'Filename --> EMG script for ampl-duration-area

'Jason Pilarski

'last update --> June 5, 2013

Var st, l%, et, test, EMG, base, sdbase, fsave$, burst, nbursts, stb, etb, loop%, FiltEMG%, tc, id1%, thr, stcurr, bs[1000], n%;
Var ii%, id%, id11%, cond$, age, name$, datafile$, dur, ampl, area, duri, bnum[1000], a, j%, m%, limit%, test2, count2, goodburst$, name2$, gbnum;
datafile$:=filename$(3)+filename$(4); 'gets the filename of current file
message(" Set pathway for data analysis output");
Filepathset(fsave$);
fsave$:=filepath$();
name$:=Input$("enter name of output data file for frequency:", "Bursting_freq.xls");
name2$:=Input$("enter name of output data file for burst characteristics:", "Burst_characteristics.xls");
cond$:=input$("condition in utero (unex/sal/nic)", "unex", 4, "unxnisal");
age:=input("enter age of animal (days)", 1);
EMG:=Input("enter in channel number for EMG", 1);
'FiltEMG%:=ChanDuplicate(EMG);
'Chanshow(FiltEMG%);
'optimise(FiltEMG%,0, maxtime());
'ii%:=ChanProcessAdd(FiltEMG%,2); 'Add DC Remove to channel 3
'id%:=ChanProcessAdd(FiltEMG%,0); 'Add Rectify to channel 3
'id1%:=ChanProcessAdd(FiltEMG%,1, 0.02); 'Add Smooth to channel 3
'id11%:=ChanProcessAdd(FiltEMG%,3,3,1,0.02); 'Set Smooth Time constant (s)
CursorSet(2);
CursorLabel(2);
HCursorDelete(-1); ' get rid of any horizontal cursors
'Repeat
  'ChanProcessClear(FiltEMG%,id11%+1); ' clear out any low pass filtering
  'tc:=Input("enter in time constant (s) for filtering current",0.02); 'variable to allow user to change filtering level, default = 0.02s
  'ChanProcessAdd(FiltEMG%, 1, tc); ' low pass filters, time constant = tc
  'interact("view results of filtering",724); 'allows user to examine results of filtering
  'optimise(FiltEMG%,0, maxtime());
  'Draw (0,maxtime());
  'Until Query ("Is this ok for filtering?");
'Draw (0,maxtime()); ' show whole trial
'optimise(EMG,0, maxtime());
Interact ("set cursors to enclose all bursts to analyze",256);
st:=Cursor(1);
et:=Cursor(2);
Hcursornew(EMG,0); 'place a horizontal cursor on the filtered channel at a level of zero
Interact("set horizontal cursor at threshold for detecting onset of current events",256); ' let the user set threshold
thr:=Hcursor(1); 'save the value of the horizontal cursor, i.e. the threshold
CursorActive(1, 7, EMG,"Cursor(1)","Cursor(2)", "Cursor(1)", thr, 0, 0); ' make cursor 1 'active', to search for positive threshold crossings of thr
bs[1]:=CursorSearch(1); 'find first threshold crossing, save time in first element of array bs
CursorActive(1, 7, EMG,"Cursor(1)+ 5","Cursor(2)", "Cursor(1)", thr, 0, 0); ' make cursor 1 'active', to search for positive threshold crossings of thr
'repeat
  ' goodburst$ := input$("Do you want to analyze this burst?", "y",1,"yn"); '
  ' if goodburst$ = "y" then
    '    gbnum := 1
  ' else
    '    gbnum := 0
' endif
' docase
' case goodburst$ = "y" then
' case goodburst$ = "n" then
'   bs[1]:=CursorSearch(1); 'find first threshold crossing, save time in first element of array bs
'   endcase
'until gbnum = 1;
'CursorActive(1, 7, EMG,"Cursor(1)+ 5","Cursor(2)", "Cursor(1)", thr, 0, 0);' make cursor 1 'active', to search for positive threshold crossings of thr
n%:=1;' set counter
bnum[0]:=1; 'bnum is vector that counts the number of bursts per episode
repeat   'loop that looks for all events that cross threshold
   st:=CursorSearch(1);
   If st> 0 then
      goodburst$ := input$("Do you want to analyze this burst?", "y",1,"yn");   ' docase
         case goodburst$ = "y" then
            n%:=n%+1;
            'printlog(n%);
            bs[n%]:=st;
         case goodburst$ = "n" then
            Endif;
      Endif;
   Until st < 0;
'printlog(bs);
a := 1;
for j%:=2 to n% do   'loop that determines which bursts are part of the same episode
   duri := bs[j%]-bs[j%-1]; 'calculates the duration between bursts
   'printlog(duri);
   if duri<.25 then   'if duration is less than 5 s, these two bursts are part of the same episode
      bnum[j%-1] := a; 'burst number for this burst stays the same
else                     'if duration is greater than 20 s, these two bursts are from different episodes
     a := a+1;            'increment a, since this is part of a new burst
     bnum[j-1] := a;
endif
next
 'printlog(bnum);
Message("number of burst detected = %6.0f", a);
filepathset(fsave$);
fileopen(name$, 8, 3);
print("%6.2f \n %6.2f \n", bs[1:999], bnum);
fileclose();
Draw(0, maxtime());
optimise(EMG, maxtime ());
Hcursornew(EMG,0);
Cursornew(1,3);'activate cursor 3
Cursornew(1,4);'activate cursor 4
cursorlabel(4,3,"S");
cursorlabel(4,4,"E");
'For the first burst episode
I%:=1;  'counter used to demarcate each bursting episode in the analyzed region
test2 := 1; 'counter used to step through each burst, regardless of which episode it is part of
count2 :=0; 'counter used to determine how many bursts are in an episode
Draw (bs[test2]-3,30); 'show a 30 s window, beginning 3 seconds before burst onset
'goodburst$ := input$("Do you want to analyze this burst?", "y",1,"yn");
'docase
   case goodburst$ = "y" then    'If this is a good burst, then analyze the parameters
      base:=ChanMeasure(EMG, 2, bs[I%]-0.7,bs[I%]-0.2);'get mean value in an 0.5 s window, 0.7 - 0.2 s before burst onset
      'sdbase:=ChanMeasure(FiltEMG%,12, bs[I%]-0.7,bs[I%]-0.2);'get SD in an 0.5 s window, 0.7 - 0.2 s before burst onset
      Hcursor(2, base); 'put horizontal cursor at baseline
      for m% := 0 to n% do    'find all bursts that are part of the first episode
if bnum[m%] = 1% then
  count2 := count2 + 1;
  'printlog(count2);
endif
next;
if count2 = 1 then 'if there is only one burst in the episode, use what is below to calculate
  test2 := test2;
  stb:=bs[test2];'set time stb to the threshold crossing time
else 'if there are multiple bursts in the episode, use what is below to calculate
  test2 := test2+count2-1;
  stb:=bs[test2-count2+1];'set time stb to the threshold crossing time
  count2 := count2+1;
endif
  'message("count = %6.2f", count2, "test2 = %6.2f", test2);
  'message("test2 = %6.2f",test2);
Repeat 'loop to find onset of bursting episode, i.e. where burst crosses baseline (determined by base)
  test:=ChanValue(EMG,stb);'get value of current at time stb
  if test >= base then 'if have not yet crossed threshold, then
    stb:=stb-0.001; 'decrement time count by 1 ms
  endif
  until test < base; 'keep cycling until test value exceeds threshold
  cursor(3, stb); 'place Cursor 3 at start of burst
  etb:=bs[test2]; 'set time etb to the threshold crossing time
  'printlog(limit%);
  Repeat 'loop to find end of bursting episode
  test:=ChanValue(EMG,etb);'get value of current at time etb
  if test >= base then 'if have not yet crossed threshold, then
    etb:=etb+0.001; 'INCREMENT time count by 1 ms
  endif
  until test < base;'keep cycling until test value exceeds threshold
  cursor(4, etb); 'place Cursor 3 at start of burst
Interact("change cursor locations if you wish", 256);

stb:=cursor(3);
etb:=cursor(4);
dur:=etb-stb; ' duration of burst
ampl:=ChanMeasure(EMG, 14, stb, etb); ' ampl of burst
area:=ChanMeasure(EMG, 1, stb, etb); ' area of burst
message("duration = %6.2f ", dur, ", amplitude = %6.2f ", ampl, ", area = %6.2f ", area);
test2 := test2 + 1;

' For all subsequent bursts
for I%:=2 to a do
    count2 :=0; ' counter used to determine how many bursts are in an episode
    Draw (bs[test2]-3,30); ' show a 40 s window, beginning 3 seconds before burst onset
    ' Do you want to include this burst in the analysis?
    ' goodburst$ := input$("Do you want to analyze this burst?", "y", 1, "yn");
    ' docase
    case goodburst$ = "y" then
        base:=ChanMeasure(EMG, 2, bs[I%]-0.7, bs[I%]-0.2); ' get mean value in an 0.5 s window, 0.7 - 0.2 s before burst onset
        ' sdbase:=ChanMeasure(FiltEMG%,12, bs[I%]-0.7, bs[I%]-0.2); ' get SD in an 0.5 s window, 0.7 - 0.2 s before burst onset
        Hcursor(2, base);
        for m% := 1 to n% do
            if bnum[m%] = I% then
                count2 := count2 + 1;
# if count2 = 1 then
# test2 := test2;
# stb := bs[test2]; 'set time stb to the threshold crossing time
# 'printlog(I%, " ");
# 'printlog("one ");
# 'printlog(test2);
# else
# test2 := test2 + count2 - 1;
# stb := bs[test2 - count2 + 1]; 'set time stb to the threshold crossing time
# 'printlog("two ");
# 'printlog(test2);
# endif
# 'message("count = %6.2f", count2);
# 'message("test2 = %6.2f", test2);

Repeat
  test := ChanValue(EMG, stb); 'get value of current at time stb
  if test >= base then 'if have not yet crossed threshold, then
    stb := stb - 0.001; 'decrement time count by 1 ms
  endif
until test < base; 'keep cycling until test value exceeds threshold

cursor(3, stb); 'place Cursor 3 at start of burst
etb := bs[test2]; 'set time etb to the threshold crossing time
'printlog(limit%);

Repeat
  test := ChanValue(EMG, etb); 'get value of current at time etb
  if test >= base then 'if have not yet crossed threshold, then
    etb := etb + 0.001; 'INCREMENT time count by 1 ms
  endif
until test < base;'keep cycling until test value exceeds threshold
cursor(4, etb); 'place Cursor 3 at start of burst
Interact("change cursor locations if you wish",256);
stb:=cursor(3);
etb:=cursor(4);
    dur:=etb-stb;'duration of burst
ampl:=ChanMeasure(EMG, 14, stb, etb); 'ampl of burst
area:=ChanMeasure(EMG, 1, stb, etb); 'area of burst
message("duration = %6.2f", dur, ", amplitude = ", ampl, ", area = ", area);
test2 := test2 + 1;
'printlog(dur);
filepathset(fsave$);
fileopen(name2$, 8, 3);
    print("%s %s %s %6.2f %6.2f %6.2f %6.2f \n", datafile$, age, cond$, dur, ampl, area, count2);
    fileclose();
'case goodburst$ = "n" then
    ' test2 := test2 + 1;
    'endcase
next;
halt;
I collected instantaneous frequency data from whole nerve recordings using a script from Cambridge Electronic Design Limited. The script entitled DumpInstFreq is provided below.

'DumpInstFreq|Script to write instantaneous frequency values as text to the Log window

'The script is a 'work in progress' and is offered without guarantees.
'You must test it to see whether it is suitable for your application.
'CED 19/07/10

var evChan%;
var numEvents%;
var data%;
var sTime, eTime;
var a%;
var num%;

if ViewKind() = 0 then              'Check we have a data file open
data%:= View();
FrontView(data%);
else
    data%:= FileOpen("",0);        'If not prompt user to select one
    WindowVisible(1);
endif;
if data% < 0 then
    Message("Could not open a data file!");     'Quit if no file opened
    halt;endif;
View(LogHandle());
Window(0,0,50,100);
WIndowVisible(0);

150
EditSelectAll();
EditClear();
View(data%);
DlgCreate("Spike channel selection");
DlgChan(1,"Select spike channel",30);
DlgShow(evChan%);
CursorSet(2);
Interact("Position cursors around area to extract intervals",1023);
CursorRenumber();
sTime := Cursor(1);
eTime := Cursor(2);
num% := Count(evChan%,sTime,eTime);
if num% > 0 then
    DumpFrequencies(num%+1);
endif;
Proc DumpFrequencies(numEv%)
    var evTimes[numEv%];
    var inst[numEv%];
    numEvents% := ChanData(evChan%,evTimes[1:],sTime,eTime); 'Put event times into array
    if Count(evChan%,0,sTime) > 0 then 'Fill first array
evTimes[0] := LastTime(evChan%,sTime); 'If first event, put zero in first element
    else
        evTimes[0] := 0;
    endif;
    ArrConst(inst[],evTimes[]);
    ArrDiff(inst[]); 'Convert time array to intervals
    ArrDivR(inst[],1); 'Convert to instantaneous frequencies
PrintLog("Event times and instantaneous frequencies between \%g s and \%g s on channel \%d\n\n", sTime, eTime, evChan%);
PrintLog("Time\n\n\nInst freq\n\n");
for a% := 1 to numEv% -1 do
    PrintLog("\%g\t\%g\n", evTimes[a%], inst[a%]);
next;
FrontView(LogHandle());
end;
Table S1. Guide for determining embryonic age using whole nerve recordings

<table>
<thead>
<tr>
<th>Embryonic Age (days)</th>
<th>Average Interburst Interval (sec ± SEM)</th>
<th>Distinctive Burst Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>209 ± 30</td>
<td>singlets; burst duration of ~1.25-2 sec</td>
</tr>
<tr>
<td>5</td>
<td>212 ± 21</td>
<td>singlets; burst duration of ~2.5-4 sec</td>
</tr>
<tr>
<td>6</td>
<td>246 ± 18</td>
<td>doublets (i.e., large amplitude burstlet followed by a shorter amplitude burstlet)</td>
</tr>
<tr>
<td>7</td>
<td>399 ± 27</td>
<td>more than two burstlets per episode; burst duration of ~20-30 sec</td>
</tr>
<tr>
<td>8</td>
<td>470 ± 29</td>
<td>many burstlets per episode; burst duration of ~30-50 sec</td>
</tr>
<tr>
<td>9</td>
<td>715 ± 64</td>
<td>many burstlets per episode; burst duration of ~60-70 sec</td>
</tr>
<tr>
<td>10</td>
<td>676 ± 103</td>
<td>both short-duration singlets and long-duration episodes containing multiple burstlets</td>
</tr>
<tr>
<td>11</td>
<td>231 ± 31</td>
<td>same as E10</td>
</tr>
<tr>
<td>12</td>
<td>80 ± 17</td>
<td>primarily singlet bursts; occasionally doublets with an initial short-duration burst followed by a slightly longer-duration burst</td>
</tr>
<tr>
<td>13</td>
<td>16 ± 2</td>
<td>same as E12</td>
</tr>
<tr>
<td>14</td>
<td>18 ± 3</td>
<td>same as E12</td>
</tr>
</tbody>
</table>