Published in final edited form as:

Dev Neurobiol. 2018 November ; 78(11): 1081–1096. doi:10.1002/dneu.22632.

Maturation of breathing-related inhibitory neurotransmission in the medulla oblongata of the embryonic and perinatal zebra finch (*Taeniopygia guttata*)

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Abstract

The medullary portion of the embryonic zebra finch hindbrain was isolated and superfused with physiologically relevant artificial cerebral spinal fluid (aCSF). This *in vitro* preparation produced uninterrupted rhythmic episodes of neural activity via cranial nerve IX (glossopharyngeal) from embryonic day 4 (E4) through hatching on E14. Cranial nerve IX carries motor activity to the glottis during the inspiratory phase of breathing, and we focused on the role of synaptic inhibition during the embryonic and perinatal maturation of this branchiomotor outflow. We show that spontaneous neural activity (SNA) is first observed on E4 and temporally transforms as the embryo ages. To start, SNA is dependent on the excitatory actions of GABA_A and glycine. As the embryo continues to develop, GABAergic and glycinergic neurotransmission take on a modulatory role, albeit an excitatory one, through E10. After that, data show that GABAergic and glycinergic neurotransmission switches to a phenotype consistent with inhibition, coincident with the onset of functional breathing. We also report that the inhibitory action of GABAergic and glycinergic receptor gating is not necessary for the spontaneous generation of branchiomotor motor rhythms in these birds near hatching. This is the first report focusing on the development of central breathing-related inhibitory neurotransmission in birds during the entire period of embryogenesis.

Keywords

birds; development; breathing; GABA; glycine; inhibitory; hindbrain; embryo

Introduction

A fundamental goal of respiratory neurobiology is to understand how motor rhythms that generate air-breathing behaviors emerge and transform during the whole of embryonic development. Terrestrial amniote vertebrates develop within vascularized extraembryonic membranes that serve as the first respiratory organs of the embryo. At nearly the same chronological time in many animals, the hindbrain, as well as future respiratory-related motor nuclei, become rhythmically active. Studies show that these early spontaneous

bioelectric signals have the potential to regulate a suite of activity-dependent outcomes, including axon pathfinding, ion channel specification, and synaptogenesis, even while cells of the nervous system continue to migrate, differentiate and specialize to yield a functional breathing circuit at birth (Moody and Bosma 2005).

Nevertheless, studies investigating the ontogeny of respiratory motor output, especially those details that precede the appearance of aerial breathing behaviors, have been relatively uncommon and are limited by technical details associated with placentation and viviparity in mammals. Where data do exist studies have concentrated on the perinatal period in rats and mice (>60% of gestational term), because data show that the embryonic parafacial region and preBötzinger complex are established during this period (Pagliardini et al. 2003; Thoby-Brisson et al. 2005; Eugenin et al. 2006; Fortin and Thoby-Brisson 2009 and for a recent review see Beltrán-Castillo et al. 2017). Accordingly, we know little about the ontogeny of breathing motor rhythms, except for the final week of rodent gestation, which is analogous to the third trimester in the human fetus, a chronological period that is significantly later than the period in which the onset of breathing motor rhythms begin in other mammals.

In contrast to rodents, data from avian species show that primordial respiratory-like motor outflow is present during the early embryonic period (<30% of incubation term; Fortin, Champagnat and Lumsden, 1994) and throughout the entirety of incubation without interruption (Vincen-Brown et al. 2016). This type of phylogenetic diversity, perhaps an example of developmental heterochrony, is advantageous for studying the growth and organization of central breathing circuits (Abadie et al. 2000). Moreover, the extended period of respiratory-like rhythm generation within the embryonic avian hindbrain may be similar to other mammals, such as sheep, and even intact human fetuses *in utero* (for a review see Koos and Rajaei 2014). In fetal sheep (Barcroft and Barron 1936) and in the human fetus, the first sign of breathing movements also occurs near the conclusion of the first trimester, *i.e.*, week 10–12 or <30% of gestational term, much earlier than the arrival of the preBötzinger complex and fetal breathing movements in rodents (Greer, 2012).

Consequently, we used a comparative approach to examine the pattern of motor rhythm generation from the avian medulla oblongata spanning the period of widespread respiratory-like spontaneous neural activity (SNA) through the onset of respiratory-specific rhythmogenesis. The latter is defined simply as the period in which the functional breathing cycle begins. We used the oviparity of the zebra finch as a tool in the present study. Oviparity allows for experimental embryonic access at any time during incubation. Birds are an excellent developmental model for this reason. However, the central control of breathing in birds, at any age, has received little attention outside of vocal-respiratory interactions (Kubke et al. 2005; McLean et al. 2013), and the cellular and synaptic mechanisms associated with respiratory-specific rhythmogenesis are largely unknown (but see Fortin, Champagnat and Lumsden, 1994; Fortin et al., 1995; Kubke et al., 2005; Vincen-Brown et al., 2016). Understanding the temporal sequence of branchiomotor circuit development will allow us to test whether early rhythms represent a causal sequence for more specialized circuit formation. To start, we are particularly interested in the role of synaptic inhibition, day-by-day over the course of embryonic and perinatal development.

This investigation aims to: (1) augment current data about the ontogeny of central branchiomotor rhythmogenesis in general and in birds specifically by following the pattern of inspiratory-related motor rhythms *in vitro* from its onset at E4 through external pipping or hatching (E14); (2) test the role that GABAergic- and glycinergic-mediated chloride gradients play in the maturation of inspiratory rhythmicity; and (3) determine if spontaneous inspiratory rhythm endures during pharmacological block of GABAergic and glycinergic-mediated inhibition once continuous air breathing begins near E12. We hypothesize that central inspiratory rhythm is critically dependent on inhibition for the generation of rhythmic activity, especially near birth, because rhythm generation in birds is dependent on active muscular phases for both inspiration and expiration.

Methods

Research animals

Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Idaho State University and conducted in accordance with the principles required by the U.S. Department of Agriculture (USDA) and the Office of Laboratory Animal Welfare. We studied the medullary portion of the zebra finch hindbrain each day from embryonic day 4 (E4) through hatching (E14). Embryos were obtained from an on-site zebra finch breeding colony housed in a dedicated animal facility at Idaho State University in the Department of Biological Sciences. The breeding colony was composed of approximately 32 adult birds (16 males, 16 females) with access to water and feed (Mazuri Small Bird Breeder Diet #56A7) *ad libitum*, as well as perching and nesting areas within a large round aviary (diameter=1.525m; height=2.44m). Room conditions were maintained at a temperature of 24 ± 1 °C and 14:10 day-night cycling. Eggs were collected every 7–10 days from the nest boxes attached to the walls of the aviary. A total of seventy-seven embryos of either sex were used. Chronological examination of embryos *in ovo* was initially performed by candling from the blunt end of the egg to separate eggs into approximate ages. If there was no vascularization of the yolk sack or appearance of a clear blastodisc, the embryo was deemed embryonic day 0 (E0). Eggs were subsequently allowed to develop in a forced-draft cabinet incubator maintained at 35–38° C and 60–70% humidity until their use at specific embryological stages. The age of embryos at the time of the experiment was confirmed by morphological staging of zebra finch as described by Murray et al. (2013). The morphological staging precision for each day was equal to ~6 hours.

Experimental preparation

The *in vitro* isolated medulla oblongata preparation (called a medullary preparation going forward) was used for the majority of these experiments as it is a well-established model for studying respiratory-related developing neural circuits in mammals (Suzue 1984; Smith and Feldman 1987; Sholomenko and O'Donovan 1995; Ballanyi et al. 1999) and birds (Fortin et al. 1994; Vincen-Brown et al. 2016). Briefly, embryos that were still within the chorioallantoic membrane were anesthetized via hypothermia until limb withdrawal reflexes were abolished. Embryos that were internally and/or externally pipping and breathing air were anesthetized by either hypothermia (~2 min) or inhaled isoflurane (~30 sec) until limb withdrawal reflexes were abolished. To control for our use of different anesthesia techniques

within the older age group (E11–14), we compared cranial nerve IX activity from three isolated medullary preparations that experienced hypothermic anesthesia and three that experienced inhaled isofluorane anesthesia. Comparisons were made on E12 during control conditions and following bath application of bicuculline and strychnine, identical to experimental protocols described below. We observed no differences in branchiomotor episode characteristics as a function of anesthesia technique and therefore we pooled these groups ($P=0.491$ for the comparison of mean episode frequency following GABA_A and glycine receptor block). All embryos were subsequently transferred into a Sylgard-covered dish and decerebrated thus eliminating any chance of pain perception. The removal of the hindbrain was accomplished via a ventral approach removing skin, skeletal muscles, skull and several cervical vertebrae. The isolated hindbrain was then cleared of surrounding tissue from the ventral aspect while still pinned to the Sylgard surface. Cranial nerves (CN) V–XII were dissected free as rootlets. Cranial nerve IX was then transected close to the surface adjacent the nerve root using microscissors and mini-pins. The rostral and caudal boundaries of the isolated hindbrain consisted of the pontomedullary intersection using the anterior superior cerebellar artery (AICA) as a gross landmark and the spinomedullary border near the exit of the first cervical spinal nerves.

A custom artificial cerebrospinal fluid (aCSF) was used during all surgical isolations and subsequent electrophysiological recordings. The aCSF superfusate flowed from a reservoir at 3ml/min and included (in mM) 120 NaCl, 12 d-Glucose, 1.15 MgCl₂, 1.26 CaCl₂, 3 KCl, 26 NaHCO₃, 0.58 NaH₂PO₄ bubbled with a 95% O₂, 5% CO₂ gas mixture producing a final pH of 7.4. The aCSF was maintained at room temperature during the surgical isolation and the temperature of the aCSF during suction electrode recordings was raised and maintained at 25–27°C.

Electrophysiological recordings

To record cranial nerve IX activity, glass micropipette suction electrodes (A-M Systems Inc., Sequim, WA) with an open-air port were fixed in a micromanipulator (Narishige International USA). The suction electrodes were then positioned near the transected cranial nerve IX and gentle backpressure was applied to the micropipette, which was filled with aCSF. The zebra finch medulla can produce stable and consistent motor rhythms for more than 6 hours. Raw signals were referenced to an indifferent electrode positioned in the bath chamber or around the tip of the suction electrode and were amplified using a HI-Z differential pre-amplifier (Grass Instruments, Quincy, MA) connected to a 511 alternating current (AC) amplifier (Grass Instruments, Quincy, MA). The signal was amplified (gain = 1000–10000) and band pass filtered (0.1–1 kHz). The waveforms were further processed by an analog to digital converter (Power1401, Cambridge Electronic Design, Cambridge, UK) at 5 kHz, full-wave rectified and integrated at a time constant of 200 ms with a moving average using a personal computer (Dell, Inc. Round Rock, TX) loaded with Spike II software (Cambridge Electronic Design, Cambridge, UK). Files were stored for subsequent offline data analysis.

Pharmacology

Stock solutions of drugs were prepared in the selected concentrations as concentrates by dilution in aCSF. Drugs were added to the superfusate by switching to reservoirs containing the treatment solutions. The following drugs were used: 5–10 μ M bicuculline methiodide (GABA_A receptor antagonist), 0.4 μ M strychnine hydrochloride (glycine receptor antagonist), and 0.1–1.0 μ M 5-aminomethyl-3-hydroxyisoxazole, also known as muscimol (GABA_A receptor agonist). Earlier work using similar concentrations of these drugs on avian spinal cords *in vitro* have been described (Chub and O'Donovan 1998; Hughes et al. 2009; Vincen-Brown et al. 2016), and we use this information along with pilot trials of dose responses to determine effective concentrations. All drugs were purchased from Tocris Bioscience (Bristol, UK).

Experimental protocols

In every experiment presented here, spontaneous activity was recorded from the transected cranial nerve IX (glossopharyngeal). The avian cranial nerve IX innervates the glottis via its laryngopharyngeal branch and carries rhythmic inspiratory activity for abduction of the dilator muscle to open the airway during inspiration (Larsen and Goller 2002). At the beginning of each recording session, baseline cranial nerve IX activity was measured until regular stable oscillations in motor outflow were observed followed by a period of at least 15–30 minutes in which data were collected and saved. Once this baseline was obtained, the reservoirs were switched to the experimental superfusate for another 15–30 minutes. The range was dependent on the specific drug being used. The volume of the tissue chamber was 4 ml. At a flow rate of 3ml/min, we assessed that the bath application of the drug required about 2 minutes to reach the tissue in the recording chamber. The drug treatment period was then followed by a washout period with the control aCSF. If activity did not return to near control values, the file was not analyzed. In all cases, only one drug or drug combination was used for each preparation.

Medullary slice preparation

In several experiments (n=12), the isolated medullary preparation previously described was surgically “cut down” to a thick slice of approximately 500 microns above and below the exit root of cranial nerve IX. We reasoned that further isolation of the rhythm-generating region, and removal of potentially confounding synaptic interactions, such as disinhibition, would help clarify the details of the development of synaptic inhibition and the changing role of GABAergic/glycinergic neurotransmission around E10–11. We also aimed to further focus our attention on the possible anatomical region(s) that produces autonomous inspiratory-related rhythms in birds, which was completely unknown at the time of this study. The transections were made using microscissors after a baseline recording was obtained from the *en bloc* medullary preparation described above. The 1000-micron slice was bookended by the facial nucleus rostrally and the nucleus retroambiguus caudally. Once the cranial nerve IX rhythm recovered from the transection (15–30 min), the same protocol as described above was employed, *i.e.*, 15 minutes of stable baseline activity followed by 15 minutes of drug treatment and then drug washout. We used this thick medullary slice

preparation in embryos staged to E8-E10 and E12-E14, *i.e.*, younger and older chronologically than E11.

Data analysis

Embryos were categorized by age within approximately 6 hours according to morphological staging criteria (Murray, 2016) and were grouped together by day for the ten gestational days that cranial motor rhythms were present (E4 through E14). For example, E4 included embryos staged to E4, 4.25, E4.5, and E4.75. For every experiment, the mean frequency (episodes/min) and duration (sec) of cranial nerve IX motor output was quantified off-line during 5–15 minute windows for control (reference) activity and the drug infusion period, capturing a minimum of 3–5 episodes. Data windows were rectified and integrated and signals were identified relative to baseline using a custom script in Spike II software (Cambridge Electronic Design, Cambridge, UK). We refer to the cranial nerve motor activity as rhythmic ‘spontaneous neural activity’ during the early embryonic period (*i.e.*, SNA), prior to the onset of breathing behavior (E11), and as ‘breathing-related rhythmic activity’ after continuous breathing begins (E12). According to previous data, embryonic breathing movements arise just before internal pipping in birds (Dawes 1976; Mortola 2009). Internal pipping in zebra finches, in which the developing embryo tears the inner shell membrane and begins aerial ventilation via the air cell, occurs on E12, and external pipping or hatching occurs at E14. To quantify differences between and within control and treatment groups, we used a matched pairs two-tailed Student’s *t*-test for single comparisons and a one-way ANOVA (main effects: age, treatment, and age x treatment) with repeated measures when multiple comparisons were required. If the model effect was significant, we then employed a Tukey’s post hoc analysis. All summary data was analyzed using Microsoft Excel (Redmond, WA) and inferential statistics employed JMP software (Cary, NC). Data are presented as means $\pm$ SEM.

Results

Early maturation of avian branchiomotor rhythmogenesis in physiological K^+ and Ca^{2+}

We studied the development and maturation of medulla-derived motor rhythms in 53 embryonic zebra finch hindbrains from E4 through E14. Cranial nerve IX activity exiting the medulla first appeared on E4 (see Fig. 1), and exhibited a mean frequency of 0.44 ± 0.09 episodes/min and a duration of 1.81 ± 0.2 sec (Fig. 2A and B), similar to previous reports investigating the onset of respiratory-like rhythms using an aCSF with higher K^+ and Ca^{2+} concentrations (Vincen-Brown et al., 2016). Within the first 48 hours following the onset of rhythmogenesis (E4-E5), activity appeared as single episodes and tended to increase in frequency. As embryonic age advanced over the next four days, episode frequency gradually declined below the initial discharge frequency at E4, but this was not statistically significant (see Fig. 1 and 2A, E7-E10). Nonetheless, episodes during this time clearly displayed increased waveform complexity within a single episode (Fig. 1, E7–10). The increase in episode complexity, which resulted from trains of bursts within each episode, significantly increased episode duration from E7–10 (Fig. 2B). Finally, cranial nerve IX frequency displayed an abrupt increase at E13 (Fig. 2A) and duration was sharply reduced at E11 (Fig. 2B).

Unlike previous data using higher superfusate K^+ (5–8 mM) concentrations, cranial nerve IX motor outflow never showed a second independent waveform (compared Figs. 1 here and Fig. 2 in Vincen-Brown et al., 2016). Beyond E4, physiological K^+ concentrations produced a single respiratory-like motor waveform carried in cranial nerve IX (Fig. 1). Nonetheless, the generalized developmental pattern of respiratory rhythms in the embryonic zebra finch hindbrain in 3 mM K^+ compared to higher K^+ (5–8 mM) concentrations is similar (Vincen-Brown et al., 2016) and is characterized by two main transitions in waveform shape, frequency and duration. The first marked shift in pattern was near 30% of gestation (~E7), when cranial nerve IX rhythm slowed below its initial rate and the episode duration lengthened. This pattern remained until approximately 80–90% of gestation, until ~E11–12, when the respiratory rhythm began to increase its rate and the duration fell significantly. The latter pattern was associated with the beginning of functional breathing in the zebra finch embryo *in ovo* (i.e., internal pipping).

Using the results from the day-by-day measurements of cranial IX motor rhythm, as well as the statistical evaluation of the waveform transitions described above, we grouped the data into three incubation periods, *i.e.*, early (E4–6), middle (E7–10), and late (E11–14) for further analysis. As shown in Figure 2C and D, the mean discharge frequency for developmental periods encompassing E4–6, E7–10 and E11–14 was 0.61 ± 0.1 , 0.21 ± 0.02 and 1.09 ± 0.3 (episodes per min) and the mean duration was 4.3 ± 0.9 , 28.5 ± 3 and 3.8 ± 1 (seconds), respectively. The results of the one-way ANOVA (main effect: age) were consistent with the daily measurements and showed that motor rhythms in cranial nerve IX tended to decrease in frequency during the middle of incubation. However, episode frequency subsequently increased from E7–10 compared to E11–14 ($P=0.0013$). At the same time, cranial nerve IX episode duration markedly increased during the middle of incubation ($P<0.0001$), followed by a sharp decrease in duration from internal pipping through hatching (compared E7–10 to E11–14, $P<0.0001$).

Role of inhibitory neurotransmission in the medullary preparation during avian embryonic branchiomotor development

On each day, following stable baseline measurements, we applied two antagonists to chloride conductance, bicuculline and strychnine, which target the most common ionotropic inhibitory neurotransmitters in the CNS, GABA_A and glycine receptors, respectively. Fig. 3A and B show that cranial nerve IX activity ceased when bicuculline and strychnine were collectively added to the bath superfusate on E4. GABA_A and glycine blockage also significantly decreased discharge frequency at E6 and E7 (ANOVA; $P=0.0026$ and $P=0.0048$, respectively). Similarly, the duration of the individual episodes was shorter compared to control during this same period (see E6). From E8 through E10, there was also a trend toward lower oscillatory activity following drug treatment, although the only statistically significance blunting effect of bicuculline and strychnine was on E10 ($P=0.0083$). The polarity of chloride gradients appear to gradually change as hatching approaches. Figure 3A shows that blocking GABA_A and glycine receptors tended to increase cranial nerve IX discharge frequency after E10, suggesting the effect of chloride conductance may be changing or have switched from an outward to an inward current. We also observed that antagonism of GABA_A and glycine begins to lengthen the episode

duration beginning on E10 and was significantly longer on E13, before decreasing (shortening) again as hatching approached (Fig. 3B). Importantly, these data show that while the sensitivity of motor rhythms to bicuculline and strychnine are changing throughout development, GABAergic/glycinergic neurotransmission is not necessary for the generation of inspiratory-related oscillatory behavior once continuous breathing is established.

As shown in Figure 3C and D, the mean percent change in discharge frequency following bath application of bicuculline and strychnine for E4–6, E7–10 and E11–14 was 0.61 ± 0.1 , 0.21 ± 0.01 and 1.1 ± 0.3 episodes per min and the mean percent change in duration was 4.3 ± 0.9 , 28.5 ± 2.9 and 3.8 ± 0.9 seconds, respectively. The results of the one-way ANOVA (main effect: age) were consistent with the daily measurements and showed that the sensitivity of motor rhythms carried in cranial nerve IX to GABA_A and glycine receptor blockage changed from early to late incubation periods. As shown in Fig. 3C, the frequency of branchiomotor rhythms during early incubation was significantly depressed following the bath administration of bicuculline and strychnine (E4–6; $P < 0.0001$). Bath application of bicuculline and strychnine also depressed cranial nerve IX frequency during the middle incubation period (E7–10; $P = 0.0075$), although the decrease in rate of discharge was less pronounced at E7–10 than E4–6 ($P = 0.0044$). We further show that during late incubation (E11–14), the sensitivity of respiratory-related motor rhythms to GABA_A and glycine receptor blockage continued to change when compared to E7–10, but the frequency was no longer significantly depressed by the drug treatment (Fig. 3C; $P = 0.5372$). Although, in 12 out of 19 hindbrains during this time, cranial nerve IX frequency increased compared to controls. Fig. 3D shows that cranial nerve IX episode duration also changed as a function of developmental age in response to bath application of bicuculline and strychnine. During early and middle incubation periods, cranial nerve IX episode duration was unaffected by bicuculline and strychnine (E4–E10), but, following E10, duration was markedly increased overall ($P = 0.0001$). It is important to note that the grouped mean data obscures the previously mentioned abrupt decrease in episode duration when GABA_A and glycine receptors are blocked at hatching (E14). Fig. 4A, B, and C are example nerve recordings from experiments on E6, E8, and E13, respectively. The left panels are under control conditions and the right panels depict activity following bath application of 5 μ M bicuculline and 0.4 μ M strychnine.

In a second series of experiments, we again employed the medullary preparation *en bloc* and used the GABA_A receptor agonist muscimol, as well as glycine, to test the polarity of chloride gradients in zebra finch hindbrains during the early development of the breathing network. We focused on two incubation groups, early to middle (E6–10), *i.e.*, prior to internal pipping, and late (E12–14), immediately following internal pipping, which was based on the summary data presented above and in Figures 2 and 3. When we applied both muscimol and glycine together, or glycine alone, at effective concentrations, tonic branchiomotor activity increased and became hyper-excited. This result consistently prevented synchronous oscillation from occurring for more than one oscillatory cycle (data not shown). Thus, we applied muscimol alone to examine the effect of increasing chloride movement, because blocking experiments indicated that GABA_A receptors were the dominant chloride-mediated ionotropic receptor in the embryo, consistent with previous studies using chicken embryos (Hughes et al. 2009). Figure 5A, C and D shows that

muscimol had no effect on frequency or duration of rhythmic activity in younger hindbrains (range E6–10). In 3 out of 7 E6–10 hindbrains, muscimol increased cranial nerve IX frequency and in 4 out of 7 hindbrains muscimol decreased frequency. Fig. 5A shows an example recording where 0.3 μ M muscimol increased cranial nerve IX discharge frequency. However, on average, the low concentrations of muscimol necessary to prevent over stimulation of cranial nerve IX activity had no significant effect on discharge frequency or duration. This is in contrast to late stage hindbrains (E12–E14; see Fig. 5B, C, and D), at time when continuous air breathing had begun, where muscimol had a significant inhibitory effect on frequency ($P=0.0178$) with a non-significant and variable effect on mean duration ($P=0.489$). Figure 5B shows an example recording on E12 in which 0.3 μ M muscimol nearly abolished cranial nerve IX activity.

Role of inhibitory neurotransmission in a thick medullary slice preparation during avian embryological branchiomotor development

Figure 6A (inset) shows the boundaries of the tissue slice contained in the “cut down” thick medullary slice preparation. The slice we generated should contain medullary regions homologous to the neural networks for inspiratory rhythms described in the mammalian medulla. Initially, the experimental transection resulted in a cessation of rhythm. However, after approximately 15–30 minutes, the rhythmic discharges carried in cranial nerve IX returned and confirmed that the episodic rhythms carried in cranial nerve IX were generated in a 1000-micron region of the medulla similar to the mammalian hindbrain (Smith et al. 1991). Figure 6A shows an example branchiomotor (cranial nerve IX) recording from an E8 hindbrain and Figure 6B shows branchiomotor (cranial nerve IX) outflow from an E12 hindbrain. Fig. 6C shows that the post-transection episode frequency was significantly elevated in seven medullary slices examined from E6–E10 ($P=0.0195$), but the frequency was statistically unaffected in five slices E12–E14 when compared to the data from the medulla *en bloc*. Post-transection cranial nerve IX episode duration was statistically similar to control slices in both age groups (Fig. 6D), although the older medullary slices tended to respond with longer and more variable episode durations. We bath applied GABAergic/glycinergic antagonists to the medullary slice, as described in Methods for the *en bloc* preparations. Figures 7A and B show example cranial nerve IX recordings from isolated E8 and E12 hindbrains in which baseline activity was established and then bicuculline and strychnine were applied to the superfusate. Figure 7C shows that when bicuculline and strychnine were bath applied to the early stage slices (E6–E10) compared to late stage slices (E12–E14) cranial nerve IX frequency showed an opposing response, *i.e.*, a decrease in rhythmic frequency in E6–10 slices ($37\% \pm 11$ of control values; $P=0.0064$) and an increase in frequency in E12–14 slices ($305\% \pm 67$ of control; $P=0.0277$). These data suggest a reversal in the polarity of chloride gradients around E10–11 ($P=0.0185$). Similarly, mean data show that cranial nerve IX episode duration was only $46\% \pm 18$ of control values ($P=0.0501$) in early stage slices and $107\% \pm 27$ of control values ($P=0.739$) in late stage slices (Fig. 7D).

Discussion

Our primary aim was to delineate the embryonic maturation of chloride gradients from the onset of primordial respiratory-like biorhythms (*i.e.*, SNA) to the period of external hatching when breathing circuits must successfully manage functional ventilation. We chose to use GABA_A and glycine receptor pharmacology to test our hypotheses. Whereas GABA_A and glycine-mediated neurotransmission is well studied in neonatal and adult mammals, an understanding of their developmental role in the embryo is more limited, especially in the context of building a well-defined neuromuscular behavior like breathing. Altricial birds and their isolated and protected *in ovo* developmental strategy provide an excellent opportunity to examine the influence of receptor-mediated chloride gradients on the normal development of breathing-related rhythmicity. We hypothesized that the influence of GABA_A and glycine neurotransmission would evolve over time and reverse polarity, *i.e.*, from excitatory to inhibitory effects on spontaneous rhythms, near internal pipping on E12, because that is when continuous air-breathing behavior begins. We speculated that mutual inhibitory synaptic circuitry would be critically important to produce the timing and pattern of the inspiratory phase of breathing since, unlike mammals, the breathing phases produced in all birds require active neuromuscular contractions during both inspiration and expiration even at rest. The mechanically neutral position of avian ventilation is sandwiched between the tidal maxima of the inspiratory and expiratory phases.

Data from the present study indeed showed that the onset of GABA_A and glycine-mediated inhibitory synaptic transmission is associated with the beginning of continuous air breathing on E12, if not slightly earlier near embryonic day 11. However, except for the onset of SNA on E4, GABA_A and glycine receptor function was not required for rhythmogenesis. Instead, we discovered that blocking GABA_A and glycine receptors with bicuculline and strychnine using the *en bloc* medullary preparation, or the thick medullary slice preparation, as well as activating GABA_A receptors with muscimol, collectively demonstrated that inhibitory synaptic transmission has only a modulatory effect on the pattern of branchiomotor outflow. For most of the incubation period, the action of GABAergic/glycinergic neurotransmission is excitatory. We also suggest that inhibition is not critical for the inspiratory duty cycle when breathing begins. Blockade of GABA_A and glycine receptors beyond E11, when GABA_A and glycine receptor activation is inhibitory in its action, seemed to strengthen rhythmic activity overall, which is consistent with previous works in mammals (Feldman and Smith 1989; Shao and Feldman 1997). As far as we can determine, this is the first study to show that avian central rhythmogenesis perseveres despite the significant reduction of GABAergic and glycinergic inhibitory neurotransmission within the medulla.

If synaptic inhibition is not required for central breathing-related rhythm generation during the perinatal period in birds, then the avian hindbrain might employ rhythmogenic cells that have specialized pacemaker properties similar to mammalian models (Dunin-Barkowski et al. 2003; Del Negro et al. 2005; Purvis et al. 2006; Ramirez et al. 2011). However, data show that the pacemaker mechanisms that produce some types of spontaneous breathing rhythms in mammals are still disputed and regularly debated, especially the role of pacemaker-specific currents (Del Negro et al. 2002; Rubin et al. 2009; Montandon and Horner 2013). In rodents, the balance of evidence for the cellular machinery underlying breathing-related

rhythm generation seems to favor a group-pacemaker situation involving recurrent excitatory oscillations (Rekling and Feldman 1998; Feldman et al. 2013; Feldman and Kam 2015; Morgado-Valle and Beltran-Parrazal 2017). It is presently unknown if the avian hindbrain employs endogenously active pacemaker-driven neurons or a group-pacemaker mechanisms or some other rhythmogenic mechanism, and the present study does not elucidate this particular issue. Further experiments are needed to test a variety of ionic fluxes within and between regions of the avian hindbrain that may generate each breathing phase.

It is also equivocal as to how the onset of synaptic inhibition in the avian medulla is functionally associated with continuous air breathing. Perhaps, the timing of the onset of inhibition allows the rhythm generator to achieve the much higher tidal frequencies once aerial breathing starts. Higher ventilatory frequencies may be required to meet the demands of the increased metabolic rate associated with internal and external pipping (hatching) and eventual independence from the egg. Coincident with embryonic day (E11), an important respiratory transition occurs as the bird breaks into the air cell compartment and begins pulmonary breathing. Prior to the bird internally pipping, the chorioallantoic membrane completely sub-serves gas exchange and central respiratory drive is not yet providing functional ventilation. Following internal pipping a dual gas exchange system exists and ventilation of the lungs becomes critical to help meet metabolic demands. During this period, gas exchange involves both the chorioallantoic membranes and the lungs. As metabolic rate increases further and the needs of gas exchange also surges, the bird externally pips or hatches from the hard egg shell. This event is accompanied by an abrupt transition in which the lungs completely take over gas exchange, while the role of the chorioallantoic membrane is abruptly terminated (Tazawa et al. 1983).

Comparison with other similar studies

Previous work using domestic fowl embryonic hindbrains (*Gallus gallus domesticus*) have reported that early rhythmic SNA is observed during a limited embryonic period and subsequently disappears (Momose-Sato and Sato, 2013, 2017). Thus, the early rhythm generator is thought to be usurped and entirely replaced by an independent adult central pattern and rhythm generator. Although abundant amounts of novel and important information have come from these investigations (e.g., motor axon pathfinding decisions, maturation of neurotransmitter systems genetic underpinnings of rhombomeric organization), the chicken model system has also helped to disseminate the idea that early motor rhythms are singular, isolated developmental phenomenon instead of focusing on their potential contribution to the development of mature central pattern and rhythm networks. The data presented here, and in a previous report (Vincen-Brown et al., 2016), suggest an alternative developmental scenario for early biorhythms. Instead of a transient phenomenon, we propose that the previously reported cessation of spontaneous hindbrain rhythms in the *Gallus* embryo toward the end of incubation (> 50% gestation) is likely due to differences in relative maturity (i.e., chickens are precocial), as well as the large size of perinatal chickens when compared to the altricial zebra finch. The chicken embryo is approximately fifty times larger than the zebra finch at hatching and thus the isolated CNS preparations likely suffers from a suite of cellular insults (e.g., hypoxia, metabolic disturbances, etc.) that preclude the production of continuous bioelectric rhythms over most of incubation period. Perhaps due to

these details, fictive spinal and/or hindbrain central pattern and rhythm generating rhythms have never been reported in hatchling chickens or any other birds to our knowledge. This is despite reports of avian embryonic motility in the form of limb movements and even fetal breathing movements throughout prenatal and perinatal periods (Vince 1976; Landmesser and O'Donovan 1984; Chiba et al. 2002; Bradley et al. 2005)

We propose instead that the earliest rhythmic activity recorded from the avian hindbrain is a relevant primordial version of the central pattern and rhythm generators, albeit with a distinctive spatial, temporal and morphological architecture. Activity-dependent processes likely play a role in the proper development of functional sensory-motor networks for breathing behaviors. The mechanisms of rhythm generation during early, middle, and later incubation may be different, but they appear continuous and uninterrupted, and the nature of their origin, connectivity, and maturation is a critical step in understanding the rules of neural circuit formation. We therefore consider the zebra finch model and the data presented here to be an important tool for studying real-time central breathing-related circuit development.

We also show that branchiomotor rhythms observed between E4 and E14 are evident in physiological relevant aCSF using this altricial zebra finch model. Previously, Vincen-Brown et al. (2016) used elevated levels of K^+ to maximize drive and increase the likelihood of observing neural rhythms throughout the embryonic period in this reduced preparation. Using higher K^+ (and higher Ca^{2+}) concentrations led to the presence of multiple waveforms during middle and late incubation that varied in duration and frequency, consistent with comparable studies using nearly identical *in vitro* superfusate recipes (Fortin et al. 1994; Fortin et al. 1995; Fortin et al. 1999; Yvert et al. 2004; Toboy-Brisson et al. 2005). We show here that elevated K^+ is not required for the manifestation of rhythmic activity in the prenatal and perinatal zebra finch, even when using a 1000-micron medullary slice.

Future Directions

Given that little is known about avian breathing circuits, significant attention will need to be directed toward the following topics. First, while the present study examines the inspiratory phase of breathing and its independence from GABAergic and glycinergic synaptic transmission, the expiratory phase has never been studied. Unlike mammals the expiratory phase in an active muscular movement even during resting conditions. It will be valuable to understand how the inspiratory and expiratory phases are coupled and whether inhibition is necessary for the entirety of the cycle. Second, it is of interest to understand how early rhythms are controlled and maintained during embryonic development. For example, how are early breathing rhythms embryologically initiated, and when, has not been settled from a phylotypic point of view and has only been thoroughly examined in rodents (Eugenín et al. 2006; Darnall 2010; Beltrán-Castillo et al. 2017). Third, data suggest that chloride conductance plays an important role in maintaining early excitability of the large scale depolarization waves in the developing CNS, and this depolarizing current can be strengthened when activity is pharmacologically reduced, suggesting a form of developmental plasticity that may be critical for proper circuit construction. Future studies are underway to examine the

possible mechanisms that tie together SNA, fetal breathing movements, and signals that control these prenatal and perinatal behaviors.

Acknowledgments

The authors would like to thank Casetin Lybbert and Kaitlyn Whitesitt for their contributions to this manuscript. We also gratefully appreciate the funding and support from the NIH and the National Institutes of Neurological Disorders and Stroke to JQP (grant #1R15NS087521) and the Idaho IDeA Network of Biomedical Research Excellence (INBRE) program funded by the National Institute of General Medical Sciences via the NIH (grant # P20GM103408).

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Figure 1.

Rhythmic cranial nerve IX activity produced by the zebra finch medulla *in vitro* from embryonic day 4 (E4) through external pipping (E14). Each day of incubation includes a representative example of a rectified, integrated and averaged suction electrode measurement in the presence of 3 mM K^+ and 1.26 mM Ca^{2+} aCSF (left panel). The right panels shows a representative episode for each day between E4 and E14 with an expanded time base.

Figure 2.

Summary data of glossopharyngeal nerve (cranial nerve IX) activity during developmental days E4-E14. (A.) Mean episode frequency (min^{-1}); (B.) Mean episode duration (sec); (C.) Mean discharge frequency for developmental periods encompassing E4-6, E7-10 and E11-14; (D.) Mean duration for developmental periods encompassing E4-6, E7-10 and E11-14. Values are means $\pm$ SEM. Unique lowercase letters (a, b, c.) above the bars on individual embryonic days indicate statistical differences among ages with $P < 0.05$.

Figure 3.

Summary data of glossopharyngeal nerve (cranial nerve IX) activity following bath application of GABA/glycine antagonists from E4 through hatching (E14). (A.) Episode frequency as a percent of control; (B.) Episode duration as a percent of control; (C.) Episode frequency as a percent of control for developmental periods encompassing E4-6, E7-10 and E11-14; (D.) Episode duration as a percent of control for developmental periods encompassing E4-6, E7-10 and E11-14. Values are means $\pm$ SEM. * above bars in panels A. and B. indicates differences from control ($P < 0.05$). For grouped means in panels C. (frequency) and D. (duration), unique letters above bars indicate differences in age groups ($P < 0.05$) and + above bars indicates differences from control ($P < 0.05$).

Figure 4.

Example recordings of rectified and integrated cranial nerve IX activity before and during application of GABA_A and glycine antagonists. (A.) Shows rhythmic episodes generated at E6 in control (left panel) and following bath application of bicuculline (5 μ M) and strychnine (0.4 μ M) (right panel); (B.) Shows rhythmic episodes generated at E8 in control (left panel) and following bath application of bicuculline (5 μ M) and strychnine (0.4 μ M) (right panel); (C.) Shows rhythmic episodes generated at E8 in control (left panel) and following bath application of bicuculline (5 μ M) and strychnine (0.4 μ M) (right panel). Each panel (horizontally) in A., B., and C. represents the same animal and experiment.

Figure 5.

Summary data for the effects of GABA_A agonist muscimol on rhythmic cranial nerve IX activity in the zebra finch with data grouped into embryonic ages E6-E10, and E12-E14. (A.) Shows rhythmic episodes generated at E7 in control and following bath application of muscimol (0.3 μ M) (right panel); (B.) Shows rhythmic episodes generated at E12 in control and following bath application of muscimol (0.3 μ M). Each panel (horizontally) in A. and B. represents the same animal and experiment. (C.) Shows mean episode frequency as a percent of control in each age group; (D.) Shows mean episode duration as a percent of control in each age group; Values in C. and D. are means $\pm$ SEM. Unique letters (a, b) indicate difference between ages $P < 0.05$ and + indicates difference from control $P < 0.05$.

Figure 6.

Example recordings of rectified and integrated cranial nerve IX activity and summary data comparing rhythmic branchiomotor features from the zebra finch medullary and the thick medullary slice preparation. In all cases, bars represent mean cranial nerve IX activity with data grouped into embryonic ages E6-E10, and E12-E14. (A.) Inset depicts the approximate boundaries of the medullary slice: sn1=spinal nerve 1; cn12=cranial nerve 12; cn9/10=cranial nerve 9/10; cn7/8=cranial nerve 7/8; cn5=cranial nerve 5. The left panel (A.) shows rhythmic episodes generated at E8 in control conditions using the entire medulla and then following the transection of the medulla 500 μ M above and below cranial nerve IX (right panel); (B.) The left panel shows rhythmic episodes generated at E12 in control conditions using the entire medulla and then following the transection of the medulla 500 μ M above and below cranial nerve IX (right panel); (C.) Episode frequency as a percent of control (*i.e.*, entire medulla) following transections that produce a 1000-micron thick slice of the medulla; (D.) Episode duration as a percent of control (*i.e.*, entire medulla) following transections that produce a 1000-micron thick slice of the medulla. Values are means $\pm$ SEM. + indicates difference from control $P < 0.05$.

Figure. 7.

Example recordings of rectified and integrated cranial nerve IX activity and summary data examining the effects of bicuculline and strychnine antagonism on branchiomotor output using the thick medullary slice. (A.) Shows cranial nerve IX activity during baseline conditions at E8 and then following bath application of bicuculline (5 μ M) and strychnine (0.4 μ M), which was followed by a washout period. (B.) Shows cranial nerve IX activity during baseline conditions at E12 and then following bath application of bicuculline (5 μ M) and strychnine (0.4 μ M), which was followed by a washout period. Each panel (horizontally) represents the same animal and experiment. (C.) Shows mean episode frequency as a percent of control following bath application of GABA/glycine antagonists in the thick slice preparation for ages E6-10 and E12-14; (D.) Shows episode duration as a percent of control following bath application of GABA/glycine antagonists in the thick slice preparation for ages E6-10 and E12-14. Values are means $\pm$ SEM. Unique letters (a, b, c) indicate difference between ages $P < 0.05$ and $^+$ indicates difference from control $P < 0.05$.