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Studying respiratory rhythm generation in a developing bird: hatching a new experimental model using the classic *in vitro* brainstem-spinal cord preparatio

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Abstract

It has been more than thirty years since the *in vitro* brainstem-spinal cord preparation was first presented as a method to study automatic breathing behaviors in the neonatal rat. This straightforward preparation has led to an incredible burst of information about the location and coordination of several spontaneously active microcircuits that form the ventrolateral respiratory network of the brainstem. Despite these advances, our knowledge of the mechanisms that regulate central breathing behaviors is still incomplete. Investigations into the nature of spontaneous breathing rhythmicity have almost exclusively focused on mammals, and there is a need for comparative experimental models to evaluate several unresolved issues from a different perspective. With this in mind, we sought to develop a new avian *in vitro* model with the long term goal to better understand questions associated with the ontogeny of respiratory rhythm generation, neuroplasticity, and whether multiple, independent oscillators drive the major phases of breathing. The fact that birds develop *in ovo* provides unparalleled access to central neuronal networks throughout the prenatal period—from embryo to hatchling—that are free from confounding interactions with mother. Previous studies using *in vitro* avian models have been strictly limited to the early embryonic period. Consequently, the details and even the presence of brainstem derived breathing-related rhythmogenesis in birds have never been described. In the present study, we used the altricial zebra finch (*Taeniopygia guttata*) and show robust spontaneous motor outflow through cranial motor nerve IX, which is first detectable on embryonic day four and continues through prenatal and early postnatal development without interruption. We also show that brainstem oscillations change dramatically over the course of prenatal development, sometimes within hours, which suggests rapid maturational modifications in growth and

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Author contributions: Michael Vincen-Brown performed most of the experiments, wrote and edited portions of the manuscript, and analyzed and helped interpret the data. MVB was integral to all phases of these experiments and preparation of the manuscript. Kaitlyn C. Whitesitt analyzed and contributed to the interpretation of the data, and edited the manuscript. Forrest G. Quick performed some of the experiments, edited the manuscript, and contributed to the interpretation of the data. Jason Q. Pilarski performed some of the experiments, interpreted the findings, and wrote the majority of the manuscript.

connectivity. We propose that this experimental preparation will be useful for a variety of studies aimed at testing the biophysical and synaptic properties of neurons that participate in the unique spatiotemporal patterns of avian breathing behaviors, especially in the context of early development.

Keywords

Development and maturation; breathing; *in vitro* brainstem-spinal cord preparation; zebra finch; birds; central pattern generation

1. Introduction

Apart from mammals, birds are the only other class of vertebrates that can be described as homeothermic endotherms. Birds, like mammals, live in extremely diverse environments, exhibit high basal metabolism, possess a four-chambered heart, and are capable of very high sustained peak metabolic rates. Accordingly, birds have been used as an important comparative model of respiratory physiology, including studies of pulmonary gas exchange and circulation (Hempleman et al. 1979; Powell & Hempleman 1988; for reviews see West 2011; West 2013), and several aspects of the peripheral (afferent) control of ventilation (Burger et al. 1974; Kubke et al. 2004; Pilarski & Hempleman 2007; for a review see Hempleman & Posner 2004).

Interestingly, despite the considerable contributions of birds to understanding vertebrate respiration, the central brainstem networks that produce, organize, and maintain breathing periodicity are largely unexplored (but see Richards 1970; Davey & Seller 1984; Schmidt et al. 2012; Sturdy et al. 2003; Wild et al. 2009; McLean et al. 2013). Perhaps a common assumption is that neural mechanisms of respiratory rhythmogenesis in birds are similar to mammals as a result of anatomically comparable nuclei in the ventrolateral medulla (Reinke and Wild, 1998, 1997). However, evidence also suggests that birds are likely to exhibit a number of distinct differences in their spatiotemporal pattern of respiratory rhythmogenesis due to the unique structure of their respiratory system (Scheid 1979; Maina et al., 2010). For example, unlike mammals, birds lack a phrenic nerve and thoracic diaphragm and their mechanical production of tidal flows employs air sacs that necessitates active muscle contractions for both inspiration and expiration, even at rest (Bouverot, 1978).

We therefore aim to introduce a new comparative model, the altricial zebra finch (*Taeniopygia guttata*), to examine the details of breathing-related rhythmogenesis generated by the avian brainstem. We begin this endeavor using the *in vitro* brainstem spinal cord preparation pioneered by Suzue (1984), and we examine how spontaneously occurring brainstem neural activity transforms during prenatal development into a functional breathing-related rhythm and pattern generator. While several laboratory groups have studied the respiratory-related brainstem of birds, these investigations have been experimentally restricted to early developmental stages in the precocial chicken (*Gallus gallus domesticus*) when large-scale depolarization waves in the spinal cord (for reviews see Hanson et al. 2008; O'Donovan et al. 1998) and brainstem (Fortin et al. 1995; Mochida et al. 2009; Hughes et al. 2009; Abadie et al. 2000; and for a review see Momose-Sato & Sato

2013) are primordial and undifferentiated. We hypothesize that the altricial development of the zebra finch, which is analogous to the developmental strategy of many mammalian rodents (*i.e.*, Rodentia and Muridae) and humans, will permit the first comprehensive description of breathing-related rhythm generation in the isolated avian brainstem.

Our results show that bioelectric rhythms in the zebra finch brainstem can be measured day-by-day from cranial nerve IX as it changes from a generic and widespread depolarization wave to a derived, spatially restricted breathing-related motor output. We also show that the development of breathing oscillations in the embryonic zebra finch *in vitro* produced two distinct waveforms, short duration and long duration episodes (hereafter referred to as SD and LD episodes), whose temporal and neurochemical characteristics evolve over the course of prenatal development. These data have been previously presented in abstract form (Vincen-Brown et al., 2015).

2. Methods

2.1. Research animals

All procedures were approved in advance by the Animal Care and Use Committee at the Idaho State University. For all experiments, we used zebra finch embryos (*Taeniopygia guttata*) from embryonic day 3 (E3) through embryonic day 14 (external pipping or hatching, E14), according to morphological staging parameters developed by Murray et al. (2013). We studied a total of 97 embryos of either sex. Embryos were acquired as fertilized eggs from closed breeding colonies maintained in a dedicated on-site animal facility at Idaho State University in the Department of Biological Sciences. A total of 14 breeding birds (6 males and 8 females) were provided water and feed (Mazuri Small Bird Breeder Diet #56A7) *ad libitum*. Each week eggs were collected from nest boxes which were connected to a large aviary (diameter = 5 ft). The eggs were examined via candling with an optic light source, separated into approximate ages, and then obliged to develop in a forced-draft cabinet incubator maintained at 38°C, 60–70% humidity and turned automatically every hour until their use at specific embryological stages. Chronological age of the embryos was assessed by candling from the blunt end of the egg. If there was no clear blastodisc and/or vascularization of the yolk surface, the embryo was deemed embryonic day zero (E0). The age of each embryo was then confirmed by morphological staging before each experiment (Murray et al., 2013). Individual embryos that had been previously incubated in the nest (> E0) were staged immediately prior to each experiment.

2.2. Experimental preparation

We used an isolated brainstem spinal cord preparation *en bloc* for these experiments, which is a widely established experimental model to study brainstem neural circuits during development in both birds and mammals (Suzue 1984; Smith & Feldman 1987; Ballanyi et al. 1999; Sholomenko & O'Donovan 1995). Embryos that were younger than E11 were anesthetized using hypothermia until limb withdrawal reflexes were abolished. Individuals that were older than E11 were anesthetized with isoflurane in a closed chamber until the limb withdrawal reflexes were eliminated. The embryo was then transferred to a Sylgard-covered dish and decerebrated. The brainstems were removed using a ventral approach that

first consisted of removing the skin, followed by targeted sections of the skull and several cervical vertebrae. The isolated neuraxis was then pinned to the Sylgard surface and the surrounding connective tissue was removed from the ventral aspect. Cranial nerves (CN) V–XII were dissected free as rootlets (Fig. 1A). The target rootlet was associated with cranial nerve IX or the glossopharyngeal nerve. Cranial nerve IX contains both sensory and motor pathways and therefore was transected close to the brainstem surface adjacent the nerve root using microscissors. Cranial nerve IX was targeted because it carries respiratory-related motor outflow to the larynx and glottis in continuously breathing birds (Larsen and Goller, 2002).

The rostral and caudal boundaries of the isolated brainstem tissue consisted of the isthmus (mesencephalic-rhombomeric junction) and a small section of the cervical spinal cord (C1–2), respectively. During the surgical isolation and subsequent electrophysiological recordings, we used a custom artificial cerebrospinal fluid (aCSF). The aCSF included (in mM) 120 NaCl, 12 D-Glucose, 1.15 MgCl₂, 1.26 CaCl₂, 5–8 KCl, 26 NaHCO₃, 0.58 NaH₂PO₄ bubbled with a 95% O₂, 5% CO₂ gas mixture producing a final pH = 7.4 ± 0.1, as described previously (Fortin et al., 1999, 1995, 1994; Thoby-Brisson et al., 2005). The aCSF was maintained at 2–4 °C during the surgical isolation and the temperature of the aCSF during suction electrode recordings was raised and maintained at 25–27 °C. All brainstems were allowed to recover at room temperature (21–23 °C) for 30 min to 1h prior to beginning the experimental protocols.

2.3. Electrophysiological recordings

Isolated brainstems were superfused with aCSF using a flow rate of 3 ml/min. To record spontaneous cranial nerve IX activity, glass suction electrodes were positioned near the transected nerve and gentle back pressure was applied to the glass micropipette that was previously filled with aCSF (Fig. 1B). Raw signals were referenced to an indifferent electrode contained in the bath chamber 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 activity was processed by an analog to digital converter (Power1401, Cambridge Electronic Design, Cambridge, UK) at 5 kHz and integrated at a time constant of 200 ms with a moving average using Spike II software (Cambridge Electronic Design, Cambridge, UK). Files were stored for subsequent offline data analysis.

2.4. Pharmacology

Stock solutions of drugs were prepared as concentrates by dilution in aCSF. Drugs were added to the superfusate by changing to reservoirs containing the new solution. We used: 2–20 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block non-NMDA receptors and 50 µM 2-amino-5-phosphonovaleric acid (AP5) to block NMDA receptors. We also used a mixture of metabotropic glutamate receptor antagonists including: 50 µM 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) to block mGlu₁ receptors, 100 µM 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP) to block mGlu₅ receptors, and 100 µM RS-1-amino-5-phosphonindan-1-carboxylic acid

(RS)-APICA to block mGlu_{2/3} receptors. All drugs were purchased from Tocris Bioscience (Ellisville, MO).

2.5. Experimental protocols

Following the surgical recovery period, stable spontaneous rhythmic activity was recorded from the embryonic glossopharyngeal (IX) nerve at stages E4 through E14 (hatching). In all experiments, cranial nerve IX activity was recorded for an initial period of 15–30 minutes, which served as the reference or baseline activity for that age. Pharmacological agents were bath applied for 15–30 min to the superfusate which was followed by a washout period. If drug clearance was not apparent, *i.e.*, activity did not return to near baseline levels, the record was not analyzed. At a flow rate of 3 ml/min, we determined that at least 2 min was required for the drug-loaded superfusate to reach the recording chamber.

2.6. Data analysis and statistics

For all protocols, the average frequency and duration of cranial nerve IX motor output was quantified off-line during stable 5–15 min windows for control (reference) activity, drug infusion, and washout periods. Windows of data were rectified and integrated and signals were identified relative to baseline. Periodic signals that deviated from the stable baseline according to a pre-determined threshold were categorized as an episode of either short duration SD or LD. SD episodes were defined as episodes < 25 s and LD episodes were defined as episodes ≥ 40 s in which the rectified integrated signal did not return to baseline. We also refer to the cranial nerve motor activity as ‘rhythmic spontaneous neural activity’ during the embryonic period prior to the onset of breathing behavior (< E12) and as ‘breathin-grelated rhythmic activity’ after continuous breathing begins (≥ E12). Previous data indicate that embryonic breathing movements start just prior to 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 at E13, and external pipping or hatching occurs at E14. To quantify differences between control and drug treatment groups, means of cranial IX activity were analyzed with a paired Student’s t-test. When appropriate we also used a one-way ANOVA (main effect: time) for statistical inference and corrections for multiple comparisons. Post-hoc contrasts were performed if a significant model effect was detected using Tukey’s procedure. A probability value < 0.05 was considered significant. All values are expressed as means ± standard error of the mean (SEM) unless stated otherwise. All statistical analyses were performed with JMP-IN software (SAS, Cary, SC).

3. Results

3.1. Ontogeny of breathing-related rhythm generation in the avian brainstem

We recorded motor outflow through cranial nerve IX and determined the age-dependent temporal pattern of spontaneously active circuits as they differentiate from widespread nonspecific ensembles to a spatially restricted respiratory-related neural network (Fig. 2A–K). Rhythmic spontaneous neural activity first appeared on E4 (stage 22) as regular single SD episodes with a mean frequency of 0.7 episodes/min and duration of 2.18 s (n=8; Figs. 2 and 3), which is similar to studies using precocial chicken embryos (Fortin et al. 1994, 1995,

1999). This SD pattern was observed in 100% of the preparations at E4 (8 of 8) and 100% of the preparations at E5 (12 of 12; Fig. 4). It is important to note that cranial nerve IX activity was not detected in eight brainstems younger than E4.

As development and maturation continued, the overall SD episode frequency increased (ANOVA, $P < 0.001$; see Fig. 3A). SD episodes also increased in duration from E4 to E9 (ANOVA, $P < 0.003$) but then remained stable as hatching approached (Fig. 3B). At E6.75, SD episode lengthening was a result of rhythmic trains of burstlets (see panel Cc. Fig. 2). Thus, late E6 and early E7 marked the first appearance of LD episodes (Fig. 2C–D, 3A and B and Fig. 4), which were defined as rectified and integrated activity lasting ≥ 40 s. By late E7 and E8, SD episodes were completely absent, obscured, or transformed into the LD phenotype. Figure 4 shows that zero out of 13 preparations during this time contained SD episodes (≥ 20 s in duration). Figures 2D–J and Figures 3 and 4 show that LD episodes, once initiated, persisted from E6 until near hatching and exhibited a decline in baseline frequency beginning at E6 until E8 (ANOVA, $P < 0.0001$), which then stabilized through E14 (Fig. 3A). Similarly, LD episode duration increased from E6 until E9 (ANOVA, $P = 0.0004$) then remained stable (Fig. 3B). Figures 2 (panels F–K) and Figures 3 and 4 show that, beginning near E9, short duration episodes returned to produce a composite SD-LD pattern. Once breathing was initiated (E12–14), the occurrence of LD episodes abruptly decreased (Fig. 4). At E13–14 only 2 of 15 preparations contained LD episodes. Figure 2 (panel K) shows a record of uninterrupted SD episodes at E14. The average frequency of SD episodes during this period was 4.08 episodes/min with a range of 0.66–9.80 episodes/min with a mean duration of 4.02 s ($n = 15$).

These data suggest that network drive not only increased throughout development but was partitioned among two distinct waveforms that at times coexisted and at other times existed independently. There appeared to be two significant developmental transitions in cranial nerve IX activity. The first occurred between E6–E9, where SD episodes temporarily vanished and LD episode appeared, and the second occurred near hatching when LD episodes weakened and SD episodes further strengthened.

3.2. Maturation of chemical neurotransmission

In addition to quantifying the age-dependent temporal pattern of cranial IX motor outflow, we bath applied receptor agonists to determine the contribution of excitatory neurotransmission from E4–E14. Our main focus in the second set of experiments was to assess the role of ionotropic glutamate neurotransmission in the development and maturation of breathing-related SD and LD motor patterns.

During early embryonic development, bath application of CNQX (2–20 μM) and AP5 (50 μM) to block AMPA/kainate receptors and NMDA receptors, respectively, reduced the frequency of SD episodes at each day between E4–E6 compared to baseline conditions by an average of 75.3% (ANOVA, $P < 0.0001$, $n = 10$; Fig. 5A) but had no effect on the duration at the same chronological age (ANOVA, $P = 0.063$; Fig. 5B). These data suggest that other excitatory neurotransmitters besides glutamate participate in rhythm generation when spontaneous neural activity first appears.

Therefore, we also bath applied several nicotinic antagonists separately and in combination, including dihydro- β -erythroidine hydrobromide (20 μ M), α -bugarotoxin (20 μ M), hexamethonium (20 μ M), mecamylamine (20 μ M), and chlorisodamine (20 μ M), to address the role of endogenous acetylcholine in producing SD episodes. On E4, we found that nicotinic antagonists slowed the SD episode frequency by 52% (Student's t-test, $P < 0.008$, $n = 8$; data not shown) and decreased SD duration by 38% (Student's t-test, $P < 0.05$, $n = 8$, data not shown). In contrast, rhythmic SD activity beginning at E9 through hatching at E14 was completely abolished by CNQX (2–20 μ M) and AP5 (50 μ M) (Fig. 5A and B).

Figures 6A and B show that between E4 and E12 there were no significant effects of ionotropic glutamate blockage on LD episodes compared to baseline frequency and duration. This is despite a complete block of SD in the same preparations (see Fig. 7A for an example at E11.5 with both SD and LD present). These data demonstrate that from E4 until E12, before breathing starts *in ovo*, SD and LD episodes are independent waveforms and can be separated pharmacologically at these ages where both SD and LD are present. As an additional example, Figure 7B shows an experiment in which a cocktail of metabotropic glutamate receptor blockers were used to eliminate LD while preserving SD. Interestingly, once breathing was initiated at E13–14, antagonism of ionotropic glutamate neurotransmission eliminates both the SD and LD episodes, as shown in Figures 6A and B.

4. Discussion

In the present study, we used the *in vitro* brainstem-spinal cord preparation and tracked the development and maturation of spontaneous respiratory-related cranial nerve IX activity throughout the prenatal and early postnatal period. The avian cranial nerve IX houses axons from motor neurons that provide innervation to the dilator muscles of the avian glottis *in vivo* and transmits rhythmic activity during the breathing cycle (Larsen and Goller 2002; White & Chubb 1967). We found that the embryonic zebra finch brainstem produces a strong spontaneously activated rhythm from embryonic day 4 (E4) thru hatching (E14). We also found that the overall pattern of rhythmic activity incorporates two types of episodes, *i.e.*, short duration (SD) episode and a long duration (LD) episode, which occurred both independently and together at various chronological ages.

Early SD episodes were partially driven by ionotropic glutamate neurotransmission and also by nicotinic cholinergic neurotransmission when they first appeared at E4, similar to the chicken embryo (Mochida et al. 2009; Fortin et al. 1995; Hughes et al. 2009; Wang et al. 2009). By E7, early SD episodes were lost or perhaps obscured by LD episodes. The emergence of LD episodes is reminiscent of breathing patterns in frogs and other intermittent breathers in which lung-related motor outflow occurs in clusters separated by silent periods of variable lengths (Vasilakos et al. 2006; Kinkead & Milsom 1997; Douse & Mitchell 1992; Straus et al. 2000 and for a review see Milsom 1991). Importantly, the loss of early SD episodes and appearance of LD episodes coincided with the increased dominance of glutamatergic neurotransmission and possibly a much decreased cholinergic input to cranial nerve IX motor neurons. When SD episodes re-appeared near E8, rhythmogenesis is critically dependent on ionotropic glutamate receptors through the hatching period. The importance of glutamate is a consistent finding from rhythmic respiratory networks among

many diverse taxa, including mammals (Ireland et al., 2008; Pace et al., 2007; Smith et al., 1991), turtles (Johnson and Mitchell, 1998), and frogs (Chen and Hedrick, 2008).

The second embryonic waveform, LD episodes, initially appeared during late E6 and was then joined by SD episodes at E9, producing a mixed SD-LD phenotype that was observed almost until hatching. Over time, the SD episodes became more numerous as LD episodes waned near E14. LD episodes were not blocked by ionotropic glutamate receptor antagonism between E7 and E12, even when SD episodes were abolished by CNQX and AP5 at the same age (see Fig. 7A). Moreover, at least at E12, metabotropic glutamate receptor antagonism could separate SD and LD episodes by eliminating the LD component without effects on the SD component (see Fig. 7B). More experiments are required to understand the synaptic details of the LD episode and how it may interact with the SD episode when they are both present. By E13, when continuous breathing begins in the zebra finch, respiratory-related rhythmic motor outflow was regular and predominately composed of glutamatergic-dependent SD episodes, similar to the motor outflow described in neonatal rats and mice (Ballanyi et al. 1999; Smith & Feldman 1987).

4.1. Critique of methods

The *in vitro* embryonic spinal cord and brainstem from the chicken has been used extensively to better understand the nature of early neural network formation, circuit behavior and the propensity for plasticity (for reviews see O'Donovan et al. 1998; Hanson et al. 2008; Moody & Bosma 2005; Momose-Sato & Sato 2013). However, there were previously no available reports in which the *in vitro* primordial rhythms in birds had been studied continuously throughout the post-rhombomeric prenatal period, as there are in mammals (Thoby-Brisson, 2005). In particular, there were also no available reports describing the rhythmic respiratory-related behavior of the isolated brainstem from any avian species at or near external pipping (*i.e.*, hatching) when continuous breathing is well established. This large gap in our understanding of avian respiratory oscillations is due to the frequently reported loss of spontaneous neural activity following about E13 (Abadie et al., 2000). The disappearance or weakening of early large scale depolarization waves recorded in cranial nerves is presumed to result from the difficulty in recording a spatially restricted signal from neurons that are actively transforming into a specialized and functional microcircuit (for review see Momose-Sato & Sato, 2013). While a restricted developmental window for rhythmic spontaneous activity has support (Blankenship et al., 2009; Bosma, 2010; Momose-Sato and Sato, 2013), it has also led to some cross-taxa confusion, such as why spontaneous neural activity seems to disappear in birds but not consistently in mammals under similar experimental conditions (Abadie et al., 2000; Ren and Greer, 2003; Thoby-Brisson, 2005; Yvert et al., 2004).

Our data indicate that the ontogenetic development of spontaneous neural activity in the brainstem of mammals and birds are more similar than previously suggested. Both groups show similar temporal patterns of cranial nerve activity during the embryonic period when activity is measured day-by-day through birth. We show that the two types of cranial nerve rhythms observed in mammals are also present in birds under identical experimental conditions (Thoby-Brisson, 2005), and, at no time is rhythmic activity through cranial nerve

IX silent, despite significant alterations to the rhythm and pattern over time. Furthermore, the development of cranial nerve activity in birds and mammals is similarly dependent on cholinergic and ionic glutamatergic neurotransmission in the same chronological sequence.

It is our opinion that the previously reported differences between the ontogeny of rhythmic cranial nerve activity in *G. gallus* compared to mammals is a result of critical differences between the developmental strategies of mice and chickens and how their state of maturity at or near birth tolerates tissue isolation (Murray et al., 2013). Among mammals, including humans and rodents, most are altricial and thus are immature at birth. This immaturity has enabled the experimental isolation of relatively intact and functional nervous tissue without significant deleterious consequences from hypoxia, metabolic failure, and ion disturbances. In contrast, *G. gallus* is precocial and large at birth (approximate weights for comparison: hatchling chicken = 40–60 grams; neonatal rat = 7–9 grams; neonatal mouse = 1–2 grams; zebra finch = 1 gram). This makes tissue isolation and viability particularly problematic, similar to *in vitro* mammalian preparations older than about three weeks (Mitra and Brownstone, 2012; Paton et al., 1994). We conjecture that previously unsuccessful attempts to study cranial nerve activities in the embryonic chicken brainstem near hatching have led to the perhaps premature conclusion that spontaneous neural activity in birds is a specific developmental phenomenon that disappears and is largely unrelated to the future output of the functionally mature central rhythm and pattern generator(s).

Conclusions and future directions—Much like the early development of respiratory-rhythm generation in mammals and other vertebrates, the avian brainstem generates primordial biorhythms that are renovated and or replaced to produce one or more respiratory-related central pattern generators over the course of incubation. We report significant developmental alterations to both the pattern of cranial nerve activity and the phenotype of the neurotransmitters that are involved. These data provide a baseline and a convenient model for future investigations into the longstanding question of whether spontaneous neural activity is a primordial version of mature central pattern generators, a completely independent phenomenon, or a blend of these two positions. The avian pattern of SD episodes presented here suggests the latter. Moreover, the replacement of LD episodes with SD episodes near hatching infers a critical role for glutamate neurotransmission and perhaps an engagement of endogenously excitable glutamatergic interneurons that represents a topographical and ionic homolog to the pre-Bötzinger complex in birds. Future studies will need to address the location and ionic phenotype of the central rhythm and pattern generators in the zebra finch, as well as assess the similarities and difference between patterns of activity *in vitro* and respiratory-related cranial nerve activity *in vivo*.

It is also of interest to aim future investigations at the role of metabotropic glutamate receptors (mGluRs) in the maturation of avian respiratory rhythm generation. mGluRs are class-C G-protein coupled glutamate receptors that are linked to intracellular second messenger pathways and can modulate neuronal activity (Anwyl, 1999; El Manira et al., 2002). mGluRs have been shown to enhance excitability and rhythmicity in respiratory neurons of mammals (Nattie & Li 1995; Feldman et al. 2005; and for reviews see Reikling et al. 2000 and Nistri et al. 2006). Nonetheless, few studies have examined the potential for mGluRs to shape synaptic transmission and plasticity during normal embryonic

development of the respiratory-related brainstem. This is despite data that show mGluRs can orchestrate synapse elimination and apoptosis in several other brain regions (Catania et al., 2007; Ichise et al., 2000; Kano et al., 2008; Zirpel et al., 2000).

Taken together, there remains a shortage of investigations into the developmental, maturation, and plasticity of breathing control in embryos as mammalian models are experimentally cumbersome and expensive. At the same time, it has not been possible to study the mechanisms of central breathing rhythmogenesis in birds because of experimental limitations associated with the most available avian model system. The embryonic zebra finch model, in concert with various *in vitro* preparations, should expand our understanding of these topics as well as provide significant opportunities to explore mechanisms of rhythm generation from a new perspective.

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Highlights

- We studied central respiratory-related rhythm generation *in vitro* in the altricial zebra finch
- We measured continuous neural oscillations from cranial nerve IX beginning on embryonic day 4 through hatching
- Zebra finch exhibit two types of spontaneous episodes that are independent
- Respiratory-related rhythm generation in birds depends on ionotropic glutamate receptors

Figure 1.

Anatomy of the zebra finch brainstem showing the relative positions of spinal (C1 & 2) and cranial (V–XII) nerve roots. The electrophysiological recording site is marked by * in both panels. *A*: Ventral view of archetypal mature avian brainstem and cervical spinal cord from the pontomedullary border rostrally to the spinomedullary border caudally. These are the boundaries of the experiments described in the text. P=pons; M=medulla; V1=ophthalmic branch of V; V2=maxillary branch of V; V3=mandibular branch of V. Drawing modified from Bubien-Waluszewska, 1981. *B*: Ventral/ventrolateral view of the *in vitro* brainstem spinal cord *en bloc* plus recording chamber with suction electrode targeting cranial nerve IX shown with the broken white line outline. Cranial nerve XI is shown transected near spinomedullary border. Inset depicts a section of the rectified and integrated cranial nerve IX signal from this embryo. CN=cranial nerve.

Figure 2.

Developmental changes in spontaneous neural activity and respiratory-related activity in the zebra finch brainstem from embryonic day 4 (E4) through external pipping or hatching (E14). A–K: Representative examples of rectified and integrated suction electrode measurements positioned on the transected stump of cranial nerve IX *in vitro* for each day of incubation. Spontaneous neural activity was not detected prior to E4. Short duration episodes were defined as episodes ≤ 25 s (see panels A. and G.) and long duration episodes were defined as episodes ≥ 40 s (see panels D. and G.).

Figure 3.

Summary data for cranial nerve IX episode characteristics during embryonic development. *A*: Mean frequency of short duration (SD) and long duration (LD) episodes from E4–14. *B*: Mean duration for SD and LD episodes from E4–E14. Black bars=SD episodes and white bars=LD episode. Values are means $\pm$ standard error of the mean (SEM).

Figure 4. Distribution of *in vitro* brainstem spinal cord preparations that contained short duration (SD) and long duration (LD) episodes relative to total number preparations studied from each embryonic day (E4–14). Black bars=SD episodes and white bars=LD episodes.

Figure 5.

Effects of ionotropic glutamate antagonism on short duration cranial nerve IX episode characteristics during embryonic development. **A:** Mean frequency of short duration (SD) episodes as percent of control frequency following bath application of CNQX (2–20 μ M) and AP5 (50 μ M) from E4–14. **B:** Mean duration of short duration (SD) episodes as percent of control frequency following bath application of CNQX (2–20 μ M) and AP5 (50 μ M) from E4–14. Black bars=SD episodes. Values are means $\pm$ standard error of the mean (SEM). * indicates $P < 0.05$.

Figure 6.

Effects of ionotropic glutamate antagonism on long duration cranial nerve IX episode characteristics during embryonic development. **A:** Mean frequency of long duration (LD) episodes as percent of control frequency following bath application of CNQX (2–20 μ M) and AP5 (50 μ M) from E7–14. **B:** Mean duration of long duration (LD) episodes as percent of control frequency following bath application of CNQX (2–20 μ M) and AP5 (50 μ M) from E7–14. White bars=LD episodes. Values are means $\pm$ standard error of the mean (SEM). * indicates $P < 0.05$.

Figure 7.

Effects of ionotropic and metabotropic glutamate receptor antagonism on rhythmic cranial IX nerve activity at E11–12. *A*: Example rectified integrated suction electrode recording showing the effect of ionotropic glutamate block (CNQX, 10 μ M and AP5, 50 μ M) on composite SD-LD episodes in an E11.5 embryo. Note the inhibition of SD episodes but not LD episodes. *B*: Example rectified integrated suction electrode recording showing the effect of metabotropic glutamate block, including 50 μ M 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) to block mGlu₁ receptors, 100 μ M 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) to block mGlu₅ receptors, and 100 μ M RS)-1-amino-5-phosphonoindan-1-carboxylic acid (RS)-APICA to block mGlu_{2/3} receptors, on composite SD-LD episodes in an E12 embryo. Note the inhibition of LD episodes but not SD episodes.