Respiratory Physiology & Neurobiology 224 (2016) 62–70

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

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

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ARTICLE INFO

Article history:
Received 5 May 2015
Received in revised form 18 August 2015
Accepted 19 August 2015
Available online 24 August 2015

Keywords:
Development and maturation
Breathing
In vitro brainstem-spinal cord preparation
Zebra finch
Birds
Central pattern generation

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 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.

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 and Hempleman, 1988; for reviews see West, 2011, 2013), and several aspects of the peripheral (afferent) control of ventilation (Burger et al., 1974; Kubke et al., 2004; Pilarski and Hempleman, 2007; for a review see Hempleman and 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 and 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

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http://dx.doi.org/10.1016/j.resp.2015.08.007
1569-9048/

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., 2009a,b; Hughes et al., 2009; Abadie et al., 2000; and for a review see Momose-Sato and 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 (*T. 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 and Feldman, 1987; Ballanyi et al., 1999; Sholomenko and 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 inspiratory 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 $MgCl_2$, 1.26 $CaCl_2$, 5–8 KCl, 26 $NaHCO_3$, 0.58 NaH_2PO_4 bubbled with a 95% O_2, 5% CO_2 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 1 h 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.