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Evidence for rhombomeric organization of multiple respiratory oscillators in the bullfrog brainstem

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ABSTRACT

The anuran brainstem is segmentally organized into rhombomeres (r) and this segmental organization is uniquely preserved throughout development. We hypothesized that rhombomeres associated with cranial nerves (CN) also contain oscillators that are capable of producing rhythmic motor output (bursts) in isolation. We used in vitro brainstem preparations from pre- and post-metamorphic bullfrogs (*Lithobates catesbeianus*) to determine if rhombomeric organization of oscillators is present throughout development. Brainstems were transected into segments containing one or more rhombomeres and motor output was measured with suction electrodes attached to CN V, X and XII. Rhythmic motor output was observed in 85% of tadpoles and 91% of frogs in an anterior segment (r0–r5), 27% of tadpoles and 18% of frogs in the middle segment (r6–r7), and 77% of tadpoles and 55% of frogs in the caudal segment (r8). There were significant reductions in burst frequency and whole nerve amplitude following transections. These data support the hypothesis that brainstem oscillators associated with specific groups of rhombomeres are present throughout development in anurans.

1. Introduction

During development, the vertebrate hindbrain neuroepithelium becomes transiently organized into distinct morphological segments termed rhombomeres (Lumsden and Keynes, 1989). In the chick, rhombomeres are present from stage 9 to stage 24 whereas in the mouse, rhombomeres appear between E8 and E12 (Chatonnet et al., 2003). Within these rhombomeres, distinct neuronal populations including motor, reticular and vestibular nuclei are arranged in clearly defined segmental patterns (Straka et al., 2002, 2006). In most taxa, this transient segmental pattern is generally distorted by extensive cell migration later in development (Chatonnet et al., 2003). However, the frog brainstem is unique in that the segmental pattern of rhombomeres is retained throughout development and following metamorphosis (Straka et al., 2002, 2006). Thus, the frog brainstem presents a unique opportunity to map physiologically identified neurons onto the developmentally persistent segmental brainstem pattern.

The rhombomeric organization of the brainstem has important implications for respiratory rhythm generation. There is evidence from transection studies in perinatal rats and embryonic chicks that respiratory oscillators are present in multiple rhombomeres during early stages of development, and that they become inactive or incorporated into the mature respiratory network as rhombomeres disappear and cell migration occurs (Champagnat and Fortin, 1997; Pagliardini et al., 2003). Transection of the frog brainstem at the approximate border of rhombomeres 5 and 6 allows independent bursts of fictive lung and buccal bursts, thus providing some evidence for the rhombomeric organization of multiple oscillators (Wilson et al., 2002). Because rhombomeres are retained throughout development and post-metamorphically in amphibians, it is likely that respiratory oscillators are also retained in multiple rhombomeric segments throughout development.

Transection studies have also provided insight as to the locations of respiratory oscillators in the amphibian brainstem over the course of development. In pre-metamorphic tadpoles, the smallest segment capable of generating lung burst activity was located in a caudal brainstem region containing cranial nerves (CN) X and XII; however, in post-metamorphic frogs, the smallest segment was located in a rostral brainstem region between CN VII and IX (Torgerson et al., 2001). The explanation for these results was that the primary lung rhythm-generating region of the brainstem underwent a caudal to rostral 'translocation' within the brainstem. Although this translocation hypothesis adequately explains the results observed in the study by Torgerson et al. (2001), the physical mechanism behind this hypothesis was not clear. Given that substantial cell migration does not occur in the developing amphibian brainstem (Straka et al., 2002, 2006), a likely explanation for the

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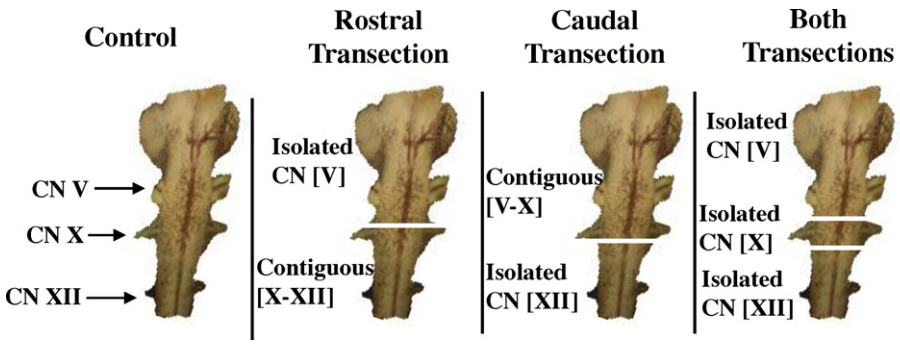

Fig. 1. Brainstem segments formed after transections outlined on a magnified (40×) image of a pre-metamorphic brainstem in a recording chamber. Following control recordings, a primary transection was made either rostral to CN IX (A) or caudal to CN XI (B). Brackets ([]) are used to designate the various slices after transections. This was followed by a secondary transection isolating CN [V], [X], and [XII] (C) into three segments. Isolated CN [V] contains r0–r5, isolated CN [X] contains r6 and r7, and the isolated CN [XII] segment contains r8.

translocation of the primary respiratory rhythm generator is the switching between distinct rhythm generating circuits located in different rhombomeres, each capable of independently producing burst activity. If different rhombomeric segments exerted primary control over brainstem neural circuits at different times in development to produce respiratory motor output, this would give the appearance of a translocated site for rhythmogenesis.

The goal of this study is to determine if rhombomeric segments in the amphibian brainstem contain respiratory oscillators capable of independently generating respiratory motor burst activity in isolation, and if rhombomeric organization of respiratory activity changes with development. We hypothesized that respiratory oscillators responsible for generating respiratory motor activity are present in multiple brainstem rhombomeres throughout development in amphibians.

2. Materials and methods

2.1. Animals

Experiments were performed on 13 pre-metamorphic and 11 post-metamorphic North American bullfrogs (*Lithobates catesbeianus*). Pre-metamorphic tadpoles were classified according to the staging criteria of Taylor and Köllros (1946) and ranged from T-K stages V–XIII (mean mass 4.3 g). Post-metamorphic frogs were T-K stage >25 (mean mass 4.8 g). Animals were acquired from a commercial supplier (Charles D. Sullivan Co., Inc., Nashville, TN, USA). Pre-metamorphic tadpoles were kept in plastic tank aquaria with oxygenated, dechlorinated tap water and were fed boiled spinach twice per week. Frogs were kept in plastic aquaria that provided dechlorinated water and a dry area. Frogs were fed small crickets twice per week. All animals were maintained at room temperature (20–22 °C). All experimental procedures were approved by the CSUEB Institutional Animal Care and Use Committee.

2.2. In vitro brainstem preparation

Animals were anesthetized prior to surgery with a dilute (0.5%) solution of ethyl-*m*-aminobenzoate (MS-222) buffered to pH 7.8 with sodium bicarbonate. Once the breathing movements ceased (2–5 min for pre-metamorphic tadpoles; and 5–10 min for frogs) and withdrawal and eye blink reflexes were abolished, the animals were removed from anesthetic. A small opening was made in the cranium using iris scissors for the transection of brainstem at rostral to the optic lobes to remove the forebrain. The brainstem was exposed and all nerves anterior to the brachial nerves were carefully cut at their exit from the skull. During decerebration and dissection, the brainstem was supplied with constant perfusion of cold (5–10 °C), oxygenated (98% O_2 and 2% CO_2) artificial cerebrospinal fluid (aCSF) with following composition (mmol l^{-1}): NaCl 104.0, KCl 4.0, $MgCl_2$ 1.4, $NaHCO_3$ 25.0, $CaCl_2$ 2.4, glucose 10.0. The entire dissection was completed in approximately 5–10 min.

Once the brainstem was removed, it was placed in a recording chamber (7 ml). The brainstem was pinned ventral side up and the dura gently removed. The recording chamber was continuously supplied with oxygenated (98% O_2 and 2% CO_2) aCSF equilibrated to room temperature (20–22 °C) from a reservoir at a rate of 5–10 ml min^{-1} at a pH of 7.8–7.9.

Nerve roots of cranial nerves (CN) V (trigeminal), X (vagus), and XII (hypoglossal) from the brainstem that normally innervate the glottis and branchial muscles were attached to suction electrodes fabricated from 1 mm diameter thin-walled capillary glass tubing (A-M Systems, Carlsborg, WA, USA). Previous studies with amphibian brainstem preparations have verified that the neural activities in CN V, VII, X and XII are correlated with breathing in intact animals (Gdovin et al., 1998; Sakakibara, 1984). In isolated brainstems, putative lung bursts were defined as large amplitude bursts with duration of approximately 1 s (Gdovin et al., 1998; Chen and Hedrick, 2008). Neural signals were amplified 10,000 times and filtered (low cutoff 10 Hz, high cutoff 1 kHz using a differential AC amplifier (A-M Systems model 1700, Carlsborg, WA). Raw nerve signals were then full-wave rectified and integrated (time constant 100 ms) with a moving time averager (CWE, Inc. Model MA-821, Ardmore, PA). Integrated signals were digitized at 200 Hz with a data acquisition system (Powerlab 8/S; AD Instruments, Colorado Springs, CO, USA) and saved to personal computer for off-line analysis with Chart software (AD Instruments).

2.3. Experimental protocol

Transection of the bullfrog brainstem into rhombomeric segments was based on previous studies showing that groups of rhombomeres can be delineated from the relative location of cranial nerve emergence from the brainstem (Straka et al., 2002, 2006). This allowed the creation of brainstem segments containing one or more rhombomeres and a CN from which respiratory-related activity could be recorded. After control respiratory activity was measured from CN V, X and XII for a period of 45 min, suction electrodes were removed and a primary transection was made with iris scissors at the rostral margin of CN IX ($N = 8$ pre-metamorphic, $N = 7$ post-metamorphic) or at the caudal margin of CN XI ($N = 5$ pre-metamorphic, $N = 4$ post-metamorphic) (Fig. 1). Cranial nerves IX, X and XI form a complex that approximates the caudal boundary of r5 and the rostral boundary of r7 in the anuran brainstem (see Straka et al., 2006). After the primary transection, the suction