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Testing the evolutionary conservation of vocal motoneurons in vertebrates

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ABSTRACT

Medullary motoneurons drive vocalization in many vertebrate lineages including fish, amphibians, birds, and mammals. The developmental history of vocal motoneuron populations in each of these lineages remains largely unknown. The highly conserved transcription factor Paired-like Homeobox 2b (Phox2b) is presumed to be expressed in all vertebrate hindbrain branchial motoneurons, including laryngeal motoneurons essential for vocalization in humans. We used immunohistochemistry and in situ hybridization to examine Phox2b protein and mRNA expression in caudal hindbrain and rostral spinal cord motoneuron populations in seven species across five chordate classes. Phox2b was present in motoneurons dedicated to sound production in mice and frogs (bullfrog, African clawed frog), but not those in bird (zebra finch) or bony fish (midshipman, channel catfish). Overall, the pattern of caudal medullary motoneuron Phox2b expression was conserved across vertebrates and similar to expression in sea lamprey. These observations suggest that motoneurons dedicated to sound production in vertebrates are not derived from a single developmentally or evolutionarily conserved progenitor pool.

1. Introduction

Many vertebrates use sound production to communicate. Comparative analysis of development led to the hypothesis that an evolutionarily conserved neural network in the caudal medulla generates vocalization (Bass, 2014; Bass and Baker, 1997; Bass et al., 2008). Output from this conserved vocal generator projects to hindbrain and cervical spinal cord premotor and motor neurons leading to species-specific vocalization behaviors (Bass, 2014). Whether these premotor and motor neurons are also evolutionarily conserved is unclear.

The variety of motor behaviors encompassing vocalization across vertebrates requires the use of widely different and often species-specific muscles, innervated by different caudal hindbrain and rostral spinal cord motoneuron pools (Bass et al., 2008; Kelley and Bass, 2010; Ladich and Bass, 1998; Onuki and Somiya, 2007; Vasilakos et al., 2005; Wake, 1993; Wild, 1997). Vocalization in mammals employs the larynx, tongue, diaphragm and intercostal muscles for control of air and phonation (Brudzynski, 2009). Vocal production in songbirds similarly uses respiratory airflow through the uniquely avian syrinx (Wild, 1997). The syrinx is innervated by motoneurons of the tracheosyringeal division of the hypoglossal motor nucleus (XIIts) that makes up the posterior two thirds of the XII nuclear group with the remainder innervating the tongue (Manogue and Paton, 1982; Nottebohm et al., 1976).

In contrast, a variety of species have evolved non-respiratory mechanisms for sound production (Bass and Baker, 1997). While terrestrial frogs use air movement to support vocalization, fully

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aquatic species such as African clawed frogs (*Xenopus laevis*) communicate while under water using rhythmic contractions of intrinsic laryngeal muscles to produce brief sound pulses when the laryngeal arytenoid disks move (Brahic and Kelley, 2003; Ryan and Guerra, 2014; Tobias and Kelley, 1987; Yager, 1992). In both terrestrial and fully aquatic anurans, the larynx and the glottis are innervated by vagal motoneurons in the caudal medulla (Simpson et al., 1986; Straka et al., 2006).

In some teleost fish, sound is generated by the contraction of muscles that vibrate the swim bladder (Bass et al., 2008). These sonic muscles are innervated by motoneurons that run in either lateral or medial columns beginning at caudal levels of the vagal motor pool and extending into the cervical spinal cord (Bass et al., 2008; Ladich and Bass, 1998). The swim bladder muscles and motoneurons of toadfishes are referred to as vocal, in part, because they innervate muscle dedicated to sound production, like the syringeal muscles of birds (Bass et al., 1994).

Channel and other catfish species can produce sound by moving pectoral spines across grooves in the pectoral girdle, although some catfish species also have a sonic swim bladder mechanism (Fine et al., 1996; Ladich and Bass, 1996). As discussed elsewhere (Bass and Chagnaud, 2012), pectoral-dependent mechanisms for sound production are best designated as sonic, a term inclusive of a broad range of sound producing mechanisms that include the use of the pectoral skeletal-muscular system in fishes and tetrapods (e.g. avian wings) to generate sounds. Hence, sonic mechanisms include vocal ones, but have other functions as well (e.g. locomotion). The pectoral fins are innervated by motoneurons either located or born within the caudal medulla at the same level as dedicated vocal motoneurons (Hale, 2014; Ladich and Bass, 1996; Ma et al., 2010). In sum, sound production across vertebrates uses motoneurons of the caudal medulla and rostral spinal cord.

Based upon relative location and axonal projection patterns, caudal medullary motoneuron populations across different vertebrates have been suggested, using mammals as the example, to be extensions of vagal, hypoglossal, spinal accessory, or spinal motor pools (Benninger and McNeil, 2011; Ma et al., 2010; Tada and Kuratani, 2015; Wake, 1993). It remains unclear, however, the extent to which these different motoneurons might have shared developmental and hence evolutionary origins between species (Bass, 2014; Bass and Chagnaud, 2012; Benninger and McNeil, 2011; Cambronero and Puelles, 2000; Gilland and Baker, 2005; Hale, 2014; Ma et al., 2010).

Evolutionarily conserved patterns of gene expression are being used to identify homologies for structures and neural populations between species, and even between vertebrates and invertebrates. For example, expression of the highly conserved Paired-like Homeobox 2 (Phox2) transcription factor (TF) gene family in branchial motoneurons in vertebrates and ingestion-related motoneurons in invertebrates has been used to argue for homology of these motoneuron populations (Dufour et al., 2006; Nomaksteinsky et al., 2013; Wilson et al., 2009). In mammals, laryngeal motoneurons express Phox2b during development, which persists in some neurons into adulthood. Hypoglossal (XII) motoneurons, however, do not express Phox2b (Bilodeau et al., 2001; Gray, 2013; Kang et al., 2007; Pattyn et al., 1999). The highly conserved nature of the Phox2b protein and advances in genome and transcriptome sequencing led us to wonder whether comparisons of Phox2b expression in sonic/vocal motoneurons across species might provide a clear test to determine whether these diverse neural populations were homologous between species, i.e. derived from an evolutionarily conserved precursor population. We found that while Phox2b was expressed in vagal motoneurons innervating vocal laryngeal muscles in mouse and amphibians, it was not expressed in motoneurons innervating vocal/sonic non-laryngeal muscles in birds (zebra finch) or bony fish (midshipman, channel catfish). This suggests the motoneurons driving sound production in different vertebrate species can be derived from motor pools with divergent developmental and likely divergent evolutionary origins.

2. Methods

2.1. Animals

African clawed frogs (*Xenopus laevis*): Staged tadpoles were acquired from Xenopus Express (Brooksville, FL). Bullfrog (*Lithobates catesbeiana*, formerly called *Rana catesbeiana*): premetamorphic tadpoles were acquired from the Sullivan Company (Nashville, TN). Channel Catfish (*Ictalurus punctatus*): Paraformaldehyde fixed brains were acquired from Mark Burleson (Department of Biology, University of North Texas). Brain tissue for mRNA isolation was acquired from discarded catfish heads from a local retailer (Seafood City, University City MO). Mouse (*Mus musculus*): Mice were bred on site with a mixed CD1/C57BL6 background (Jackson Laboratory, Bar Harbor ME). P0 indicates age at birth. Plainfin midshipman (*Porichthys notatus*): Paraformaldehyde fixed brains and tissue for mRNA isolation were acquired from field-collected specimens (see (Bass et al., 1994)). Sea lamprey (*Petromyzon marinus*): Paraformaldehyde fixed brains were acquired from field-collected and laboratory raised specimens. Zebra finch (*Taeniopygia guttata*): Paraformaldehyde fixed brains and tissues for mRNA isolation were acquired from adult male birds ranging in age from 120 to 500 days of age, obtained from Canary Bird Farm, NJ. All the experiments were performed in accordance with guidelines laid down by the NIH in the US and Canadian Council on Animal Care regarding the care and use of animals for experimental procedures, the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, and in compliance with protocols approved by the Animal Care Committee at the University of Calgary, the Institutional Animal Care and Use Committees at Cornell University, the Université de Montréal, the Université du Québec à Montréal, and the University of Pennsylvania, and the Animal Studies Committee at Washington University School of Medicine.

2.2. Tissue acquisition

Bullfrog, catfish, lamprey, and *Xenopus laevis* were anesthetized in deionized water containing tricaine methanesulfonate (50 mg/l). Midshipman fish were anesthetized in 0.025% benzocaine (ethyl p-amino benzoate; Sigma, St. Louis, Mo.). Neonate mice were anesthetized by hypothermia. Zebra finches were anesthetized with 0.04 ml euthasol (Virbac AH, Inc, Fort Worth, Texas). All species were either transcardially perfused with 4% PFA in 0.1 M phosphate buffered saline (PBS) for histology or their brains removed to sterile PBS for mRNA isolation. Fixed tissues were postfixed in PFA overnight at 4 °C, cryoprotected in 25-30% sucrose in PBS, blocked, frozen in Optimal Cutting Temperature compound (Sakura Finetek, Torrance CA), and stored at −75 °C. Brains were sectioned in sets of 4–6 on a Hacker (Winnsboro, SC) cryostat at 20 μm and sections thaw mounted onto Superfrost Plus (Fisher Scientific, Hampton, New Hampshire) slides and stored at −20° C until use.

2.3. In situ hybridization (ISH)

As previously described (Gray, 2013), slides were immersed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), permeabilized with radioimmunoprecipitation assay buffer, washed in 0.1 M triethanolamine-HCl with 0.25% acetic anhydride, blocked in hybridization buffer at 65 °C, then placed into slide mailers containing hybridization buffer with digoxigenin labeled antisense Phox2 or Phox2b cRNA at 1 μg/ml overnight at 65 °C.