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Research paper

Graded and discontinuous EphA–ephrinB expression patterns in the developing auditory brainstem

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ABSTRACT

Eph–ephrin interactions guide topographic mapping and pattern formation in a variety of systems. In contrast to other sensory pathways, their precise role in the assembly of central auditory circuits remains poorly understood. The auditory midbrain, or inferior colliculus (IC) is an intriguing structure for exploring guidance of patterned projections as adjacent subdivisions exhibit distinct organizational features. The central nucleus of the IC (CNIC) and deep aspects of its neighboring lateral cortex (LCIC, Layer 3) are tonotopically-organized and receive layered inputs from primarily downstream auditory sources. While less is known about more superficial aspects of the LCIC, its inputs are multimodal, lack a clear tonotopic order, and appear discontinuous, terminating in modular, patch/matrix-like distributions. Here we utilize X-Gal staining approaches in *lacZ* mutant mice (ephrin-B2, -B3, and EphA4) to reveal EphA–ephrinB expression patterns in the nascent IC during the period of projection shaping that precedes hearing onset. We also report early postnatal protein expression in the cochlear nuclei, the superior olivary complex, the nuclei of the lateral lemniscus, and relevant midline structures. Continuous ephrin-B2 and EphA4 expression gradients exist along frequency axes of the CNIC and LCIC Layer 3. In contrast, more superficial LCIC localization is not graded, but confined to a series of discrete ephrin-B2 and EphA4-positive Layer 2 modules. While heavily expressed in the midline, much of the auditory brainstem is devoid of ephrin-B3, including the CNIC, LCIC Layer 2 modular fields, the dorsal nucleus of the lateral lemniscus (DNLL), as well as much of the superior olivary complex and cochlear nuclei. Ephrin-B3 LCIC expression appears complementary to that of ephrin-B2 and EphA4, with protein most concentrated in presumptive extramodular zones. Described tonotopic gradients and seemingly complementary modular/extramodular patterns suggest Eph–ephrin guidance in establishing juxtaposed continuous and discrete neural maps in the developing IC prior to experience.

1. Introduction

Centrally located in the mesencephalon, the inferior colliculus (IC) receives a complex combination of extrinsic inputs (Winer and Schreiner, 2005) amongst a dense network of intrinsic connections (Sturm et al., 2014). Projections from a host of auditory nuclei converge on its central nucleus (CNIC), terminating in a tonotopic manner as afferent layers that extend along its laminar continuum and into deep aspects of the neighboring lateral cortex (LCIC, Layer 3). In contrast to these primarily auditory areas, more superficial LCIC regions are multimodal and lack a clear frequency order (Aitkin et al., 1981; Gruters and Groh, 2012). In lieu of layers or fibrodendritic flaminae, a series of neurochemically-distinct modules define the multimodal LCIC (Mugnaini and Oertel, 1985; Chernock et al., 2004; Lesicko and Llano, 2015) with afferents preferentially targeting Layer 2 modular fields or surrounding extramodular domains. While not fully characterized, patterned inputs to these areas include auditory projections from the dorsal cochlear nucleus (Shore and Zhou, 2006; Zhou and Shore, 2006), the CNIC (Saldaña and Merchán, 1992; Noftz et al., 2014), and auditory cortex (Saldaña et al., 1996; Torii et al., 2013; Stebbings et al., 2014; Barnstedt et al., 2015), as well as a diverse array arising from nonauditory sources (Olazábal and Moore, 1989; Shammah-Lagnado et al., 1996), including somatosensory projections from the spinal trigeminal (Sp5, Shore and Zhou, 2006;

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Zhou and Shore, 2006) and dorsal column nuclei (Li and Mizuno, 1997).

Previously, we demonstrated that topographic layered inputs to the CNIC emerge prior to experience in a variety of species (Gabriele et al., 2000a,b; Henkel et al., 2005; Gabriele et al., 2007; Fathke and Gabriele, 2009; Gabriele et al., 2011; Wallace et al., 2013). Distinct axonal plexuses are evident by postnatal day 4 (P4) and exhibit highly refined adult-like projection patterns by hearing onset (P12, in rat and mouse). The early spatial alignment of alternating and partially overlapping layered inputs provides evidence that highly precise CNIC maps form in the absence of experience (Fathke and Gabriele, 2009). While less is known about LCIC circuit assembly, preliminary findings from our laboratory suggest a similar accuracy in initial targeting of multimodal discontinuous modular/extramodular fields (Noftz et al., 2014; Balsamo and Gabriele, 2015).

The Eph–ephrin signaling family fulfills all criteria for topographic guidance molecules (McLaughlin and O'Leary, 2005) and is well-documented for its instructive role in the establishment of two kinds of neural maps, *continuous* and *discrete* (Luo and Flanagan, 2007). Eph–ephrin gradients provide positional information necessary for continuous maps that preserve nearest neighbor relationships from source to target (e.g. retinotectal multiaxes mapping: Triplett and Feldheim, 2012). Discontinuous or segregated Eph–ephrin expression, on the other hand, is consistent with discrete mapping, whereby connections are arranged according to input type as opposed to spatial position (e.g. striosome/matrix map: Gerfen, 1992; Janis et al., 1999, olfactory glomerular map; Strotmann and Breer, 2006). Typically, auditory maps are categorized as continuous with their emphasis on tonotopic order and preservation of cochlear place code. Tonotopic gradients of Eph family proteins at various levels suggest their involvement in constructing orderly frequency-specific circuits (Person et al., 2004; Miko et al., 2007; Gabriele et al., 2011). Manipulations of certain Eph–ephrin members indeed affect *in vivo* mapping of tonotopic circuits at the level of the auditory brainstem (Cramer, 2005; Huffman and Cramer, 2007; Nakamura et al., 2012; Wallace et al., 2013) and even shape aspects of cortical auditory response properties (Intskirveli et al., 2011). Even with this recent progress, considerable work remains in identifying the full spectrum of signaling members, expression gradients, and mechanisms that drive continuous auditory map formation. Multimodal targets like LCIC modular/extramodular fields exhibit unique organizational features, and thus, appear more in keeping with discrete neural maps (Cramer and Gabriele, 2014). The presence of both continuous and discrete maps within a single system would not be unprecedented, given the somatosensory system's respective elements of body surface representation and whisker-specific barreloids/barrel fields.

Functional roles for ephrin-B2, -B3 and EphA4 in aspects of the developing auditory system downstream of the IC have been previously reported in the literature (Bianchi and Gale, 1998; Pickles et al., 2002; Brors et al., 2003; Cramer, 2005; Miko et al., 2007; Defourny et al., 2013), although less is known about their expression and involvement in midbrain mapping (Gabriele et al., 2011; Wallace et al., 2013; Cramer and Gabriele, 2014). Transgenic lines with *lacZ* reporter gene manipulations in coding regions of Eph–ephrin genes of interest provide the means for mapping endogenous gene expression in developing auditory structures of animals carrying a mutant allele (Bianchi et al., 2002; Miko et al., 2007, 2008). The present expression study provides the first step in exploring the notion that continuous and discrete guidance maps exist juxtaposed in neighboring IC subdivisions. Quantification of X-Gal staining in *lacZ* mutants reveals clear EphA4 and ephrin-B2 gradients in the tonotopic CNIC and LCIC Layer 3. Within the multimodal LCIC, periodic modular (ephrin-B2, EphA4) and extramodular (ephrin-B3) expression patterns appear complementary. Despite belonging to different Eph–ephrin subfamilies, EphA4 has strong binding affinities for both ephrin-A and ephrin-B ligands (Gale et al., 1996; Pasquale, 1997; Bergemann et al., 1998). Expression patterns are also noted for other major auditory brainstem nuclei and relevant midline structures. The potential instructive role of the described graded and modular expression patterns in establishing continuous tonotopic and discrete multimodal neural maps within the CNIC and LCIC are discussed.

2. Materials and methods

2.1. Animal subjects

Early postnatal mice (P0, P4, P8, P12) were studied leading up to hearing onset (P12). Timepoints were chosen to directly correlate with stages from previous studies documenting the development of multiple topographic and patterned inputs to the IC (Gabriele et al., 2000a, 2000b, 2007, 2011; Henkel et al., 2005; Fathke and Gabriele, 2009; Wallace et al., 2013). Results include data from 36 heterozygous mice of three different Eph–ephrin *lacZ* mutations (ephrin-B2, CD1/129 background, n = 19; EphA4, C57BL/6J background, n = 8; ephrin-B3, CD1 background n = 9). Breeding pairs for ephrin-B2 and ephrin-B3 colonies were provided by Dr. Mark Henkemeyer; EphA4 colony was generated from breeding pairs acquired from the Mutant Mouse Regional Resource Center (MMRRC, NCRR-NIH; donated by Marc Tessier-Lavigne). *LacZ* insertions afford β-galactosidase or X-Gal histochemical staining (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; X-Gal, Sigma–Aldrich, St. Louis, MO), faithfully reporting gene expression for the proteins of interest. Positive X-Gal staining results in a blue reaction product for visualization with brightfield microscopy.

Visualized β-galactosidase in our mutant strains results from *lacZ* reporter gene manipulations in coding regions for the genes of interest. In the ephrin-B2 and -B3 strains, mutant alleles encode for membrane-bound ephrin-B2 (or -B3) – β-galactosidase fusion proteins in which the cytoplasmic domain has been deleted and replaced with β-gal (Dravis et al., 2004). Affixing β-gal to truncated ephrin-B2 or ephrin-B3 enables precise spatial and subcellular visualization of these proteins (Yokoyama et al., 2001). In contrast, the mutant allele for the EphA4 gene trap strain produces no EphA4 protein and expresses cytoplasmic β-galactosidase (Leighton et al., 2001).

2.2. Genotyping procedures

Ephrin-B2 mice were genotyped as previously described (Gabriele et al., 2011; Wallace et al., 2013). Similar methods were employed for EphA4 and ephrin-B3 genotyping. In short, EphA4 and ephrin-B3 tail samples were digested, isolated, and precipitated with an Easy-DNA kit (Invitrogen, Carlsbad, CA). EphA4 (EphA4-forward 5′-GTTTCCGCTCTGAGCTTATACTGC-3′, EphA4-reverse 5′-ACAGTGAGTGGACAAAGAGACAGG-3′, lacZ 5′-CGCTCTTACCAAAGGGCAAACC-3′) and ephrin-B3 primers (EB3-forward 5′-GACGGCGGGCCAAGCCTTCGGAGAG-3′, EB3-reverse 5′-ATAGCCAGGAGGAGCCAAAGAG-3′, lacZ 5′-AGGCGATTAAGTTGGGTAACG-3′) were used for PCR amplification (Dravis et al., 2004; Miko et al., 2007). Visualization of PCR product via gel electrophoresis results in EphA4 wild-type (WT; 639-bp) and/or mutant (800-bp) allele bands, and ephrin-B3 WT (401-bp) and/or mutant (142-bp) allele bands. All experimental procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and received prior approval from the Institutional