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# NEUROSCIENCE FOREFRONT REVIEW

# AXON GUIDANCE IN THE AUDITORY SYSTEM: MULTIPLE FUNCTIONS OF EPH RECEPTORS

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**Abstract—The neural pathways of the auditory system underlie our ability to detect sounds and to transform amplitude and frequency information into rich and meaningful perception. While it shares some organizational features with other sensory systems, the auditory system has some unique functions that impose special demands on precision in circuit assembly. In particular, the cochlear epithelium creates a frequency map rather than a space map, and specialized pathways extract information on interaural time and intensity differences to permit sound source localization. The assembly of auditory circuitry requires the coordinated function of multiple molecular cues. Eph receptors and their ephrin ligands constitute a large family of axon guidance molecules with developmentally regulated expression throughout the auditory system. Functional studies of Eph/ephrin signaling have revealed important roles at multiple levels of the auditory pathway, from the cochlea to the auditory cortex. These proteins provide graded cues used in establishing tonotopically ordered connections between auditory areas, as well as discrete cues that enable axons to form connections with appropriate postsynaptic partners within a target area. Throughout the auditory system, Eph proteins help to establish patterning in neural pathways during early development. This early targeting, which is further refined with neuronal activity, establishes the precision needed for auditory perception.**


**Key words: cochlea, brainstem, inferior colliculus, auditory cortex, Eph receptor, ephrin.**

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*Abbreviations:* AVCN, anterior ventral cochlear nucleus; CNIC, central nucleus of the inferior colliculus; DCN, dorsal cochlear nucleus; DCIC, dorsal cortex of the inferior colliculus; DNLL, dorsal nucleus of the lateral lemniscus; ECIC, external cortex of the inferior colliculus; IC, inferior colliculus; ILD's, interaural level differences; ITD's, interaural time differences; LCIC, lateral cortex of the inferior colliculus; LGN, lateral geniculate nucleus; LSO, lateral superior olive; MGB, medial geniculate body; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; NL, *n. laminaris*; NM, *n. magnocellularis*; SGNs, spiral ganglion neurons; VCN, ventral cochlear nuclei.

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Contents



## INTRODUCTION

Mature neural circuitry in the auditory system reflects the culmination of multiple coordinated developmental processes. In early embryonic development, morphogenesis of the elaborate structures in the inner ear relies on multiple signaling pathways. Mechanisms for cell fate specification in the peripheral and central portions of the auditory system are just beginning to be understood. During late embryonic and early postnatal development, axons are guided to appropriate targets and form synapses, which in some parts of the auditory pathway exhibit highly specialized structures. The extent and mechanisms of neuronal migration remain elusive. Some of these processes likely share molecular mechanisms with other developing systems, whereas the differences are likely to shed light on the specializations needed for auditory function. In this review we focus on the mechanisms by which axons of cells in the auditory system are guided to their targets. At each level of the auditory system, axons project to appropriate target regions, which include specialized nuclei or cortical

areas. Here we consider specifically the guidance of auditory axons to appropriate regions or cell types within their target nucleus or cortical area, and we highlight the role of a family of axon guidance molecules, the Eph receptors and their ephrin ligands, in this targeting.

## EPH RECEPTORS AND EPHRINS

Eph proteins, including the Eph receptors and their ligands, the ephrins, are broadly expressed in the developing auditory system (for reviews see Bianchi et al., 2002; Pickles, 2003; Cramer, 2005; Gabriele et al., 2011). They provide multiple mechanisms for signaling and for generating precise connectivity. Eph/ephrin interactions generally regulate cellular movement and adhesion, and thus these proteins play significant roles not only in axon guidance, but also in cell migration, angiogenesis, cancer, synaptogenesis, and synaptic plasticity (Lai and Ip, 2009; Pasquale, 2010; Bush and Soriano, 2012). The basis for this broad array of functions lies in their complex interactions in a variety of biological contexts.

### Binding properties

Eph receptors, the largest family of receptor tyrosine kinases in vertebrates, were first identified from an erythropoietin-producing hepatocellular carcinoma (Hirai et al., 1987). Eph receptors and ephrins (Eph receptor Interacting proteins), are divided into the A and B classes on the basis of sequence homology and binding affinity (Gale et al., 1996). In mammals there are ten EphA receptors (EphA1–A10) and six EphB receptors (EphB1–B6). Ephrin-A proteins (A1–A6) bind to all of the EphA receptors and ephrin-B proteins (B1–B3) bind to all the EphB receptors. The broad binding properties within each class provide considerable redundancy, and several family members are often co-expressed in populations of cells. Exceptions to these binding restrictions provide some degree of crosstalk between the A and B families: EphA4 can bind to both ephrin-A and ephrin-B ligands, and EphB2 can bind to ephrin-A5 (Gale et al., 1996; Himanen et al., 2004; Himanen, 2012).

Ephrins are unusual as ligands for receptor tyrosine kinases in that they are attached to cell membranes, thereby facilitating cell–cell interactions. Ephrin-A proteins are tethered to the plasma membrane with a glycosyl phosphatidyl inositol (GPI) linkage, whereas ephrin-B ligands are integral membrane proteins with a transmembrane domain. Several facets of Eph/ephrin signaling result from this property of ephrins. First, as cells must come into contact with one another, the signaling occurs at small distances and thus tends to be important for axon targeting over limited areas. In the auditory pathways discussed here, Eph protein signaling generally regulates choice of location or postsynaptic partner within a designated target area. Second, the association of both Eph receptors and ephrins with cell membranes facilitates bidirectional signaling. In addition to traditional forward signaling, reverse signaling also occurs, whereby the activation of ephrins by Eph receptor binding activates cell signaling events in the cell expressing the ephrins (Davy et al., 1999; Huai and Drescher, 2001; Cowan and Henkemeyer, 2002; Kullander and Klein, 2002; Lim et al., 2008). It was initially thought that Eph/ephrin interactions elicited only chemorepulsion in growth cones through forward signaling; however, it has now been shown that both forward and reverse signaling are active in axon guidance, and that interactions may be attractive or repulsive (for review see Xu and Henkemeyer, 2012; Klein and Kania, 2014).

### Regulation of Eph/ephrin signaling

Unlike most receptor tyrosine kinases, which can be activated by a single molecule of ligand, Eph receptors are unusual in that their activation requires membrane attached or clustered ligands (Davis et al., 1994). Activated Eph receptors are arranged as a tetramer with two ephrin molecules and two Eph receptors. Moreover, this binding results in formation of large clusters of activated Eph receptors, which reside in plasma membrane microdomains known as lipid rafts (Marquardt et al., 2005; Janes et al., 2012). These clusters can contain multiple types of Eph receptors, and may serve as an additional mechanism for crosstalk between the Eph subclasses (Janes et al., 2011; Nikolov et al., 2013). Multiple Eph receptors and ephrins are often expressed within a single tissue or cell. Ephrins can bind to Eph receptors within the same cell in *cis*, thereby inhibiting protein interactions with molecules on other cells in *trans* (Arvanitis and Davy, 2008; Kao and Kania, 2011; Falivelli et al., 2013).

While ephrins are generally associated with cell membranes, the extracellular domain of the proteins can be cleaved by metalloproteases. This cleavage was originally observed for ephrin-A proteins and is seen as a mechanism to promote contact-mediated cell repulsion. Further, it has been shown that the cleaved, soluble extracellular domains of the Eph proteins can in some cases have signaling properties on their own (Ieguchi et al., 2013). To facilitate repulsion between cells expressing EphB receptors and ephrin-B ligands, it is thought that endocytosis of the bound complex is needed (Zimmer et al., 2003). However, cleavage of EphB proteins by matrix metalloproteases has also been reported (Lin et al., 2008).

### Axon guidance

Eph receptors and ephrins have a significant role in axon guidance in many areas of the developing nervous system. This function was first discovered in the retinotectal pathway of the chick embryo, in which retinal ganglion cells express a gradient of EphA3 along the nasal-temporal axis and the tectum expresses an opposing gradient of ephrin-A2 and ephrin-A5 in the recipient anterior–posterior axis (Cheng and Flanagan, 1994; Cheng et al., 1995; Drescher et al., 1995). A similar pattern was seen in mammals, with EphA5 expressed in a gradient in retinal ganglion cells. Mutations in these ephrin-A genes disrupt mapping along this axis in the superior colliculus and in the lateral geniculate nucleus (LGN). The effects are more pronounced when spontaneous activity is blocked, suggesting that topography requires both fine

axon guidance and subsequent activity-dependent refinement (Feldheim et al., 2004; Pfeiffenberger et al., 2006; Cang et al., 2008b; Triplett and Feldheim, 2012). Interestingly, mutations in ephrin-A proteins resulted in erroneous placement of eye-specific layers in the LGN (Pfeiffenberger et al., 2005), and overexpression of EphA5 in ferrets resulted in an alteration of eye-specific projections (Huberman et al., 2005). In addition, control of retinal ganglion cell growth at the optic chiasm is regulated by ephrin-B proteins at the midline (Petros et al., 2009; Chenaux and Henkemeyer, 2011). These studies in the visual system demonstrate roles for Eph proteins in both graded axon guidance as well as in guidance at discrete choice points.

## PERIPHERAL AUDITORY PATHWAYS

### Organization of peripheral auditory circuits

Neural processing of auditory stimuli in mammals begins in the cochlea, where sensory receptors known as hair cells in the organ of Corti encode changes in sound pressure that are transduced through movement of the basilar membrane. Deflections in hair cell stereocilia in response to this movement result in transmitter release onto distal processes of spiral ganglion neurons (SGNs). Because the basilar membrane varies systematically in its mechanical properties, there is a strong correlation between position in the organ of Corti and the frequency that elicits the largest displacement. This relationship forms the basis of tonotopy, whereby ordered representations of frequency are seen within auditory areas. Topographic connections convey this frequency map to SGNs. Central projections of spiral ganglion cells then propagate this map into the central auditory system.

The peripherally projecting axons of SGNs form radial bundles that innervate the hair cells (Rubel and Fritzsch, 2002). Mammals have two types of auditory hair cells. Along the organ of Corti, there is one row of inner hair cells and three rows of outer hair cells. These cells have distinct functions and innervation patterns (Kiang et al., 1982). Inner hair cells contact peripheral processes of numerous type I SGNs. In contrast, outer hair cells are more sparsely innervated, making contact with relatively few type II SGNs (Fig. 1). Genetic fate mapping studies suggest that these subtypes of SGN are determined at early developmental ages, and that projections are correlated with their neurogenesis (Koundakjian et al., 2007). Initial projections may undergo some refinement, however, as individual SGN axons have been shown to initially contact both inner and outer hair cells (Echteler, 1992).

### Eph/ephrin signaling and axon guidance in peripheral auditory axons

Several studies have demonstrated that Eph receptors and ephrins are expressed in the developing inner ear in mice (Henkemeyer et al., 1994; Bianchi and Gale, 1998; Bianchi et al., 2002; Pickles et al., 2002; Zhou et al., 2011; Coate et al., 2012; Defourny et al., 2013).

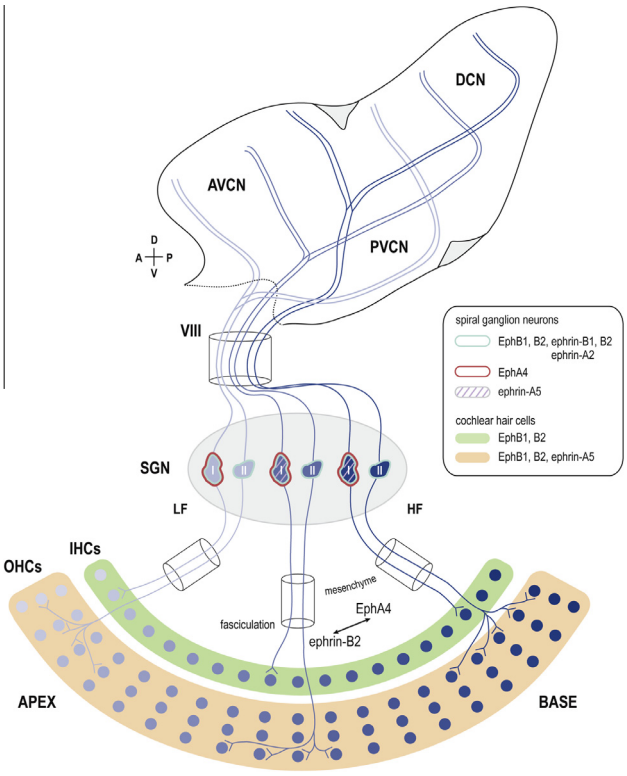


**Fig. 1.** Mapping of afferents from the auditory periphery to the cochlear nucleus complex. Precise topography (indicated by *shades of blue* in this and all subsequent figures) preserves centrally the frequency order established in the cochlea. The organ of Corti, SGNs, and their developing connections exhibit complex complementary and overlapping Eph/ephrin expression patterns. Guidance mechanisms involving both subfamilies are responsible for accurate circuit assembly (*inset key*). Inner radial bundle formation and proper fasciculation require ephrin-B2/EphA4 signaling between growing SGN peripheral processes and the surrounding otic mesenchyme. Ephrin-A5-expressing outer hair cells (OHCs) repel EphA4-positive type I SGNs that preferentially map to the inner hair cell (IHCs) rank. A subpopulation of type I SGNs are ephrin-A5-positive (*purple stripes*). LF = low frequency, HF = high frequency.

SGNs express ephrin-B1 and ephrin-B2, and growth of SGN processes is inhibited *in vitro* by EphB2 through reverse signaling (Bianchi and Gray, 2002; Zhou et al., 2011). Null mutations in EphB1–3 or ephrin-B1 lead to excessive growth of SGN axons beyond the third row of outer hair cells (Zhou et al., 2011). These studies suggest that EphB signaling delimits the region through which SGN axons grow. This view is supported by studies of SGN axon fasciculation. EphA4, which binds both ephrin-A and ephrin-B ligands, is needed for normal fasciculation of SGN axons in their peripheral trajectories (Coate et al., 2012). SGN peripheral axons in mice lacking EphA4 were more splayed and formed significantly smaller fascicles. Ephrin-B2 mutations resulted in a similar effect, suggesting that ephrin-B2/EphA4 signaling is needed for proper fasciculation.

During formation of peripheral projections, EphA4 is expressed in type I SGNs, with very limited expression in type II SGNs, and Ephrin-A5 is expressed in outer hair cells, but not inner hair cells (Defourny et al., 2013). In *ephrin-A5*$^{-/-}$ mice, outer hair cell innervation is more

extensive, largely due to type I SGN axons that extend past inner hair cells into the inappropriate region. The distinct expression pattern of ephrin-A5 suggests that this protein acts on growing SGN axons via forward signaling. This study provides further evidence that Eph signaling provides instructive signals that culminate in appropriate peripheral auditory connections. An interesting possibility is that Eph molecules participate in correction of initial errors in cell contacts (Echteler, 1992).

Central projections from the cochlea toward the brainstem travel within the VIIIth cranial nerve, which contains processes from auditory and vestibular ganglia. Expression of Eph proteins in central axons has been demonstrated in chick embryos (Siddiqui and Cramer, 2005). During the formation of auditory circuitry these fibers express several Eph receptors and ephrins from both classes. Expression of EphA4 is graded in a manner consistent with the future frequency axes, but the role of the protein in forming topographic maps has not been tested. Interestingly, EphB2 and EphA4 have complementary expression patterns in the VIIIth nerve (Siddiqui and Cramer, 2005), with greater EphA4 expression in putative auditory regions of the nerve and greater EphB2 expression in putative vestibular regions. Misexpression of these genes or treatment with inhibitory fusion proteins did not result in axon mistargeting to inappropriate auditory vs. vestibular regions (Allen-Sharpley et al., 2013). Instead, these manipulations resulted in a shifting of the boundaries demarcating the auditory and vestibular projection areas of the nerve within the brainstem. In mice, EphA5 shows significantly more expression in the spiral ganglion than in vestibular ganglia (Lu et al., 2011), but the contribution of this protein to modality-specific targeting is not known.

## AUDITORY BRAINSTEM

Topographic mapping from sensory epithelia into higher sensory areas is a feature shared with other systems, and hence tonotopy is similar to retinotopy or somatotopy. However, the auditory system is unique in that the stimulus frequency, and not the stimulus location, is mapped along the sensory receptive surface. Thus, while these systems may share common mechanisms to establish topographic projections, the central auditory system incorporates specific circuitry to obtain location information from binaural cues.

### Eph proteins shape connections in avian auditory brainstem

Neuroanatomical and electrophysiological studies in birds have advanced our understanding of the role of brainstem nuclei in sound localization (Carr and Konishi, 1990; Hyson, 2005). In chicks, the central projections of the cochlear ganglion bifurcate and terminate in *n. angularis* and *n. magnocellularis* (NM) (Rubel and Fritzsch, 2002). NM axons also bifurcate, sending one branch to ipsilateral *n. laminaris* (NL) and the other branch to contralateral NL. Ipsilateral NM axon branches terminate on dorsal dendrites and somata of NL neurons, whereas contralateral projections terminate on ventral NL dendrites and somata (Young and Rubel, 1983). The contralateral projection creates a delay line through which signals from NM require longer time to reach lateral NL cells than to reach medial NL cells. This circuitry, together with fine coincidence detection in NL neurons, allows for the computation of interaural time differences (ITD's) used to localize low-frequency sounds (Overholt et al., 1992; Hyson, 2005; Koppl and Carr, 2008).

The NM–NL projection thus contains a graded map alongside a binary separation of ipsilateral and contralateral projections. Eph protein signaling has a demonstrated role in both aspects of this projection. During the formation of these connections, which are seen by embryonic day 10 (E10), EphA4 is more heavily expressed on dorsal dendrites of NL (Cramer et al., 2000). In this dorsal region, a concentration gradient is seen so that high frequency regions display more expression than do low-frequency regions (Person et al., 2004). Disruption of EphA4 using *in ovo* plasmid electroporation resulted in errors in dorsal–ventral segregation of inputs, as well as broadening the topography of the NM–NL projection (Cramer et al., 2004; Huffman and Cramer, 2007).

While EphA4 has an asymmetric dorsoventral expression pattern, several other Eph proteins are expressed in both dorsal and ventral regions (Cramer et al., 2002). EphB2 is expressed similarly on both sides, and appears to work together with EphA4 in restricting NM inputs appropriately. When EphB2 signaling was blocked using either electroporation or treatment with inhibiting fusion proteins, axons made errors in dorsoventral targeting; when both EphB and EphA signaling were blocked, this effect was significantly greater (Allen-Sharpley and Cramer, 2012). Graded expression of ephrin-B2, a ligand for EphA4 and EphB2, was observed along the frequency axis in NL cell bodies, as well as in the glial cells alongside NL (Person et al., 2004). The contributions of ephrin-B2 to tonotopy and patterning in this pathway have not yet been tested. However, unlike the opposing gradients seen in central projections of the retina, the gradients seen in this pathway appear to be parallel.

Formation of brainstem pathways in the hindbrain in chick embryos requires an extended period of migration and morphogenesis (Rubel et al., 1976; Cramer and Rubel, 1998). At the earliest ages the hindbrain is divided into distinct rhombomeres. Movement across rhombomere boundaries is limited at early ages, at least in part by Eph protein-mediated repulsion at these boundaries (Xu et al., 1999). NM and NL neurons are born early in the hindbrain, from embryonic day 2 (E2) to E4 (Rubel et al., 1976). Precursors for NM and NL then coalesce to form a laterally oriented auditory anlage at about E7 (Book and Morest, 1990). The nuclei are then separate and move medially, and NL takes on its characteristic laminar appearance by E9–10, when the synapses from NM inputs form. Thus, the period of axon guidance and synaptogenesis coincides with the formation of the mature nuclei (Hendricks et al., 2006). Eph protein signaling appears to influence both processes. Disruption of EphA4 and EphB2 not only alters axon targeting, but also

results in abnormally shaped auditory nuclei (Cramer et al., 2004; Allen-Sharpley and Cramer, 2012). These studies suggest that Eph proteins may coordinate the position of cells together with their arriving inputs. This dual role of Eph signaling in migration and axon targeting has been seen in several regions of the developing mammalian cerebral cortex (North et al., 2013) and suggests a potential role for coordinating cues that influence cell movement and axon outgrowth.

### Sound localization circuits in mammals

In mammals, central SGN axons entering the brainstem branch and project tonotopically to three subdivisions of the cochlear nucleus, the anterior ventral cochlear nucleus (AVCN), the posterior ventral cochlear nucleus (PVCN), and the dorsal cochlear nucleus (DCN). Branches of axons from AVCN project to ipsilateral and contralateral medial superior olive (MSO), with inputs segregated onto lateral and medial dendrites, respectively, in a pathway that detects ITD's. In addition, the globular bushy cells of the ventral cochlear nuclei (VCN) project to the medial nucleus of the trapezoid body (MNTB). These axons terminate on principal cells of the contralateral MNTB in a large synapse, the calyx of Held, which encapsulates the postsynaptic neuron (Cant, 1992). MNTB neurons provide inhibitory input to a number of auditory brainstem nuclei, including the MSO. Another target is the ipsilateral lateral superior olive (LSO), where cells receive tonotopically matched excitatory input from the spherical bushy cells of the ipsilateral ventral cochlear nucleus. This pathway allows for detection of interaural level differences (ILD's), a cue used to determine locations of high frequency sounds.

### Eph proteins shape multiple aspects of brainstem pathways

Studies of mutant mouse lines have revealed several roles for Eph proteins in the circuitry of the auditory brainstem. Evidence for a role in establishing tonotopy comes from a study in which animals were exposed to pure tones and then histologically processed to examine expression of the immediate early gene *c-fos* (Miko et al., 2007). Mice with reduced levels of ephrin-B2 showed increased positional spread of c-fos-positive cells in DCN in response to pure tone stimulation. Additionally, mice lacking EphA4 showed a shift in the position of c-fos-positive cells in MNTB in response to pure tones. The results suggest that ephrin-B2 and EphA4 are needed to form appropriately restricted tonotopic maps in some of the auditory brainstem nuclei. Expression studies showed gradients during development in several areas of the developing brainstem, consistent with this result. The altered frequency bands may reflect a role for Eph proteins in topographic mapping to these nuclei.

Eph proteins have an additional role in establishing VCN–MNTB projections. While this projection is normally strictly contralateral, a significant number of ipsilateral terminations are seen in *ephrin-B2*$^{lacZ/+}$ mice and in *EphB2*$^{-/-}$; *EphB3*$^{-/-}$ mice (Hsieh et al., 2010; Nakamura and Cramer, 2011). These ipsilateral projections have calyceal terminations that form at the same time as normal contralateral connections. Unlike the aberrant growth of vestibular inner ear efferents seen in embryonic *EphB2*$^{-/-}$; *EphB3*$^{-/-}$ mice, this ipsilateral projection is not eliminated at later ages. The connections to ipsilateral MNTB in most cases arise from axon branches that project contralaterally, but in some cases ipsilateral calyces appear to emerge directly from VCN (Fig. 2). These latter projections could reflect pruning of the contralateral branch, but could also indicate a failure of VCN axons to reach and cross the midline. The axon guidance molecules netrin-1 and DCC are needed for VCN axon growth to the midline (Howell et al., 2007), and absence of Robo3 receptor in VCN cells results in an entirely ipsilateral projection to VCN (Renier et al., 2010). In EphB mutants it appears that the majority of

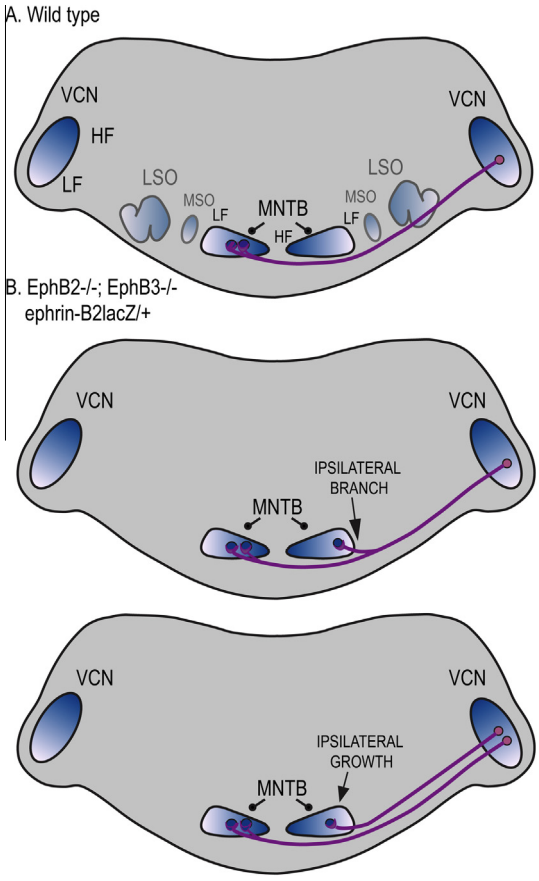


**Fig. 2.** Altered brainstem projections in mice with mutations that reduce EphB signaling. (**A**) Normal pathway from AVCN and PVCN (indicated as VCN) to MNTB in wild type mice. Nearly all of the projections are contralateral, terminating in a calyx of Held in the appropriate frequency region. Blue color gradients refer to frequency axis; dark blue represents high frequencies. LSO, lateral superior olive; MSO, medial superior olive. (**B**) Mutations that block reverse signaling through ephrin-B receptors result in a significant number of ipsilateral calyceal projections (Hsieh et al., 2010). These ipsilateral projections in most cases appear as branches from contralaterally projecting VCN axons. In some cases, ipsilateral projections grow directly to the MNTB in the absence of a contralaterally projecting branch. For clarity, MSO and LSO are omitted; these nuclei have not been studied in the context of Eph signaling.

axons reach the midline, and that the mutations result in a more favorable environment for ipsilateral axon branches that may be stabilized in ipsilateral MNTB. These results together highlight the role of Eph signaling in determining local targets; in this case, once the axons have been instructed by other signals to cross the midline and synapse in contralateral MNTB. Consistent with this view, these mutations also lead to an enhancement of induced ipsilateral connections after unilateral cochlear removal during early postnatal development (Nakamura et al., 2012; Nakamura and Cramer, 2013).

## AUDITORY MIDBRAIN

### Continuous and compartmentalized pathways in the inferior colliculus (IC)

The IC is a strategically located midbrain hub that integrates a multitude of converging inputs. Its position and rich multi-tiered innervation scheme emphasize its pivotal role coordinating both ascending and descending auditory circuits. In addition to its vast array of auditory afferents (Casseday et al., 2002), the IC also receives non-auditory inputs and is thereby thought to be involved in aspects of multisensory integration (Jain and Shore, 2006; Zhou and Shore, 2006). While purely auditory areas of the IC reliably preserve the tonotopic order established in the periphery (Merzenich and Reid, 1974; Schreiner and Langner, 1997; Malmierca et al., 2008), such a frequency continuum is less apparent in certain multimodal regions where topographically mapped inputs target discrete, or discontinuous terminal fields.

The IC is functionally organized into a central nucleus (CNIC), lateral or external cortex (LCIC, ECIC: Loftus et al., 2008), and dorsal cortex (DCIC). Besides each exhibiting a unique connectivity, substantial intrinsic and commissural projections functionally link these major subdivisions (Saldaña and Merchán, 1992; Malmierca et al., 1995). The CNIC and LCIC in particular provide an intriguing model for considering guidance mechanisms as each has tonotopically defined regions, yet differ significantly in other basic organizational features.

The CNIC is the largest subdivision and the most thoroughly studied to date, as it is the site of convergence for ascending streams that process in parallel complex spectral, temporal, and spatial signal attributes. It receives direct projections from the cochlear nuclear complex, as well as numerous pathways arising from various nuclei of the superior olivary complex (SOC) and lateral lemniscus (Winer and Schreiner, 2005). Despite its homogeneous appearance in routine cellular stains, the CNIC exhibits a layered arrangement consisting of a series of fibrodendritic or isofrequency laminae that run perpendicular to its tonotopic axis (Morest and Oliver, 1984; Oliver and Morest, 1984). Inputs identify precise sublayers of target isofrequency laminae and terminate in characteristic patterns of alternating axonal layers. Spatial alignment of converging layered afferents is well documented (Shneiderman and Henkel, 1987; Oliver et al., 1997; Loftus et al., 2004, 2010; Malmierca et al., 2005) and thought to define functional compartments or CNIC synaptic domains that receive various monaural and binaural input combinations (Oliver, 2005). Assembly of such complex circuitry requires considerable topographic precision and is necessary for the accurate assimilation of various stimulus features (Ehret and Merzenich, 1985; Schreiner and Langner, 1997) before being conveyed on to the thalamus and cortex.

Far less is known concerning the topographic mapping and functionality of the LCIC. In rodent, the LCIC has a layered structure (Faye-Lund and Osen, 1985). Its deepest aspect, Layer 3, exhibits a clear frequency order with refined axonal layers similar to those in the adjacent CNIC. Layer 3 inputs are also comparable to its neighbor, arising primarily from lemniscal, intrinsic, or commissural origins (Saldaña and Merchán, 1992; Malmierca et al., 1995; Saldaña et al., 2009). In contrast, LCIC Layers 1 and 2 lack any evidence of a tonotopic organization and receive a largely unique set of connections. Despite considerable connections from the CNIC itself and modest inputs from lateral lemniscal nuclei (Rockel and Jones, 1973; Coleman and Clerici, 1987; González-Hernández et al., 1996), the heaviest influences to these more superficial areas arise primarily from the auditory cortex (Herbert et al., 1991; Saldaña et al., 1996; Druga et al., 1997; Winer et al., 1998) and extramodal sources, including the dorsal column nuclei (Li and Mizuno, 1997), spinal trigeminal tract (Aitkin et al., 1981), and basal ganglia (Olazábal and Moore, 1989; Shammah-Lagnado et al., 1996). Interestingly, projection patterns to these multimodal areas are not layered, but rather appear to either preferentially target a periodic network of Layer 2 modules (Chernock et al., 2004; Zhou and Shore, 2006; Malmierca et al., 2011; Ouda and Syka, 2012), or spare these clusters of presumptive GABAergic cells and terminate in surrounding extramodular domains. Such patch-matrix-like distributions, while common in other sensory and motor systems (striatum: Gerfen and Engber, 1992, visual: Illing, 1996, somatosensory: Petersen, 2007, olfactory: Imai et al., 2010), are conspicuously absent from most auditory structures. Taken together, the CNIC and LCIC provide a promising model system for examining emergent topographic, laminar, and modular arrangements prior to experience.

### Eph signaling in IC circuit assembly

Since patterns of afferent projections define functional compartments that occur within and between IC subdivisions, it is essential to understand the mechanisms that shape its early topography. Innervation of the nascent IC and the subsequent emergence of its orderly connectivity occur prior to hearing onset (postnatal day 12 in rat and mouse). In short, pioneer fibers invade appropriate subdivisions of the embryonic IC and exhibit initially diffuse projection distributions. Shortly after birth, characteristic CNIC layers become apparent as axonal arbors refine themselves, coupling selective pruning with continued elaboration within appropriate postsynaptic sublayers (Kandler and Friauf, 1993; Gabriele et al., 2000a,b, 2007; Henkel et al., 2007; Fathke and Gabriele, 2009). Bilateral LSO patterns and those arising from the

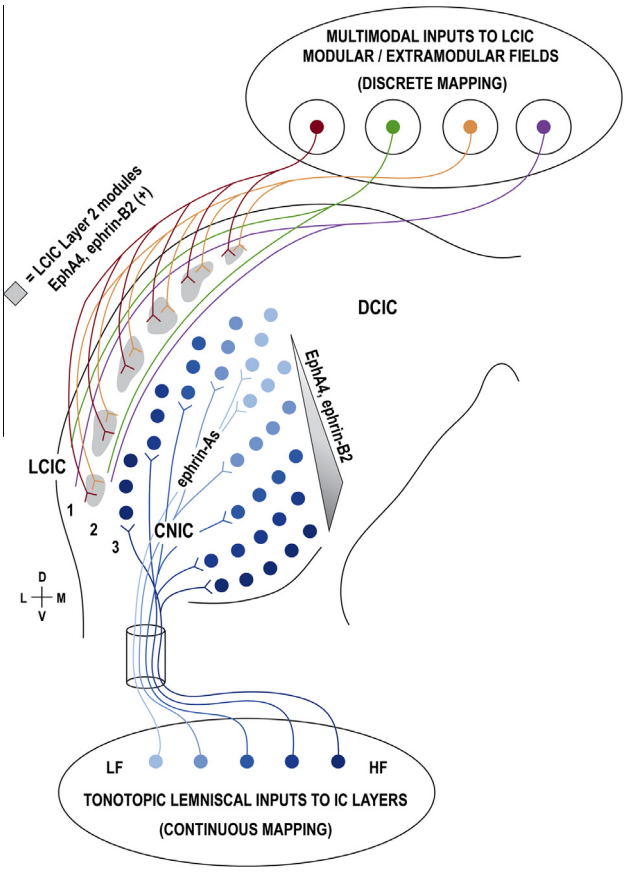


**Fig. 3.** Schematic of proposed continuous vs. discrete Eph/ephrin mapping of the auditory midbrain. The CNIC and deep LCIC exhibit characteristic frequency laminae (*shades of blue*) that receive tonotopic inputs from multiple lemniscal sources (including the cochlear nuclei, LSO, MSO, SPN, DPO, VNLL, and DNLL). Graded expressions of certain Eph/ephrins along the ventromedial-to-dorsolateral CNIC frequency continuum appear to influence axonal targeting prior to experience. Ephrin-As are also highly expressed in the nascent CNIC, and while not yet quantified, may provide positional information necessary for mapping afferents along additional IC axes. While less characterized, inputs to more superficial aspects of the LCIC are multimodal, lack a clear frequency order, and exhibit discontinuous modular or extramodular projection distributions. Descending inputs from the auditory cortex as well as intrinsic and commissural connections from the CNIC appear to spare Layer 2 modules (*green, purple*), while somatosensory inputs arising from the dorsal column and spinal trigeminal nuclei preferentially target these domains (*red, orange*). In lieu of continuous gradients, LCIC Eph/ephrin expression is conspicuously modular (*gray*), suggesting a role in instructing discrete maps that reflect "type" rather than "position/frequency" of inputs. SPN = superior paraolivary nucleus; DPO = dorsal periolivary group.

contralateral DCN and dorsal nucleus of the lateral lemniscus (DNLL) all exhibit remarkable projection specificity prior to experience, targeting defined synaptic domains of frequency-band laminae (Fathke and Gabriele, 2009). Much like their layered counterparts in the CNIC, modular (Wallace et al., 2013) and extramodular (Torii et al., 2013) LCIC arrangements emerge during early postnatal development.

The precision with which IC inputs adhere to the local cytoarchitectural framework in the absence of experience suggests involvement of close-contact signaling mechanisms. An important first step in assessing whether Eph/ephrins play an instructive role in auditory midbrain map formation is the identification of protein expression patterns that correlate with the early period of projection shaping (Fig. 3). Localization studies utilizing immunocytochemical and X-Gal staining approaches in *lacZ* mutants show a transient expression of EphA4 and ephrin-B2 in the IC leading up to the functional onset of hearing (Gabriele et al., 2011). In rat and mouse, each is expressed in a graded fashion across the tonotopic axis of the CNIC, with protein most concentrated in ventromedial, high-frequency regions. CNIC gradients are steep at birth through P4 (period of axonal sorting), before flattening to more homogeneous expression as experience ensues (Miko et al., 2007; Gabriele et al., 2011). In contrast to their continuous CNIC expression, both exhibit discrete, discontinuous patterns in the LCIC, with protein localized to presumptive modular fields that mimic those neurochemically described in the adult (Chernock et al., 2004; Malmierca et al., 2011; Ouda and Syka, 2012). This pattern remains prominent throughout the first postnatal week prior to being noticeably downregulated by P12. EphA4 and ephrin-B2-positive cells are also evident during this period in several auditory brainstem nuclei that send patterned inputs to the IC, namely the cochlear nuclei, LSO, DNLL, and ventral nucleus of the lateral lemniscus (VNLL).

To assess Eph/ephrin cues in establishing continuous CNIC and discrete LCIC maps, a recent study explored the specific involvement of ephrin-B2 in developing topographic projections from the LSO to IC (Wallace et al., 2013). In contrast to the strict tonotopy observed in wild-type mice, *ephrin-B2*$^{lacZ/+}$ mutants (compromised reverse signaling) lacked any clear projection topography. Whereas focal LSO dye placements in WTs resulted in one or two frequency-matched axonal layers in the CNIC, comparably sized placements consistently yielded unrefined projection distributions encompassing a larger frequency axis extent in heterozygous mutants. Interestingly, ephrin-B2 reverse signaling was not required for aspects of pattern formation as characteristic CNIC layers still form prior to experience.

If previous findings in the retinotectal and other analogous systems are any indication (Luo and Flanagan, 2007), accurate IC map construction likely involves additional Eph/ephrin members, as well as potentially complex interactions with other signaling families. In addition to *EphA4* and *ephrin-B2*, *ephrin-A2* and *ephrin*-A5 mRNA is present in the embryonic IC (Zhang et al., 1996). EphA7-Fc chimeric protein binding studies reveal the presence of ephrin-As in the P4 mouse IC (Torii et al., 2013). In this same study, EphA7 overexpression in the auditory cortex significantly alters targeting of corticocollicular projections to the DCIC and LCIC. Descending inputs that normally avoid Layer 2 modules exhibit a more even LCIC distribution pattern. More recently, comprehensive *in situ* hybridization studies implicate a host of other EphA/B proteins in the developing IC (www.brain-map.org; Allen Institute for Brain Science). Finally, preliminary studies suggest ephrin-B3 midbrain expression patterns are complementary to those for EphA4 and ephrin-B2 (Klotz et al., 2013). Ephrin-B3 is

also highly expressed in the mesencephalic midline (Noftz et al., 2014), yet it remains to be determined whether it is involved in crossing decisions for intercollicular and commissural auditory fibers. Taken together, the temporal and spatial correlation of afferent shaping and Eph/ephrin expression patterns suggest their involvement in the IC circuit construction. Future experiments employing a variety of approaches are necessary to determine the precise interactions that instruct IC topography.

## THALAMUS AND AUDITORY CORTEX

Neurons from IC project to the auditory thalamus, where they form tonotopic projections terminating in the medial geniculate body (MGB). Eph receptors and ephrins are expressed in the developing MGB as in other thalamic nuclei (Intskirveli et al., 2011; Lehigh et al., 2013; Torii et al., 2013), with graded expression of some family members. The distinct expression patterns of Eph proteins in the developing thalamus suggest a role in defining boundaries between distinct nuclei (Lehigh et al., 2013).

The role of Eph proteins in guidance of MGB axons to appropriate locations in the primary auditory cortex has not been tested. Nonetheless, Eph proteins have multiple roles in every stage of cortical development (North et al., 2013), and thus an understanding of their integrated function will be of value in understanding assembly of auditory pathways. Eph proteins regulate areal specification of the cortex and guidance of thalamocortical axons to the appropriate cortical areas (Dufour et al., 2003; Robichaux et al., 2014). They influence cortical cell migration (Steinecke et al., 2014), laminar specification (Mann et al., 2002), topography (Cang et al., 2008a), and columnar organization (Torii et al., 2009; Dimidschstein et al., 2013). In primary auditory cortex, null mutations in *EphB2* and *EphB3* result in degraded frequency selectivity (Intskirveli et al., 2011). This result could signify an effect on thalamocortical topographic mapping, but could also reflect divergent projections in lower auditory areas.

## CONCLUSIONS

Eph receptors and ephrins make multiple contributions to the organization of neural circuitry. Emerging evidence suggests that this protein family has a significant role in the development of auditory pathways, from the peripheral projections to the auditory cortex. Early in development, Eph signaling influences the movement of cells as nuclei form and at the same time regulates the growth of their axons to appropriate targets. Later in development, the function in axon guidance dominates, as Eph signaling seems to fine tune projections that have arrived in the correct target. Throughout this process, these molecules seem to work together with other cues, including other axon guidance signals as well as activity-dependent refinement known to occur in topographic mapping.

As a consequence of cell contact-mediated binding, Eph/ephrin signaling appears to be most important for selection of regions within a target, rather than for selecting a target region. These subregions include topographic locations that rely on graded expression patterns, resulting in tonotopic maps. They also include discrete locations, such as inner vs. outer hair cells, dorsal vs. ventral NL dendrites, ipsilateral vs. contralateral nuclei, or modular vs. extramodular domains. In many cases, Eph proteins serve as an inhibitory signal that delineates compartments permitting axon growth. Nearly every stage of auditory system development exhibits expression of several ligands and receptors. These proteins have overlapping binding properties and function in many stages of neural development, including proliferation, migration, axon guidance, and synaptogenesis. Their expression and signaling capabilities help to coordinate the formation of auditory nuclei, axon growth, and precise formation of synapses throughout the auditory system.

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