The role of ephrin-B3 on midbrain topography and auditory function

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The Role of Ephrin-B3 on Midbrain Topography and Auditory Function

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In

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Abstract

Eph-ephrins are a family of molecular guidance proteins that provide cell-cell interactions necessary for topographic mapping and pattern formation in the developing nervous system. Studies in our laboratory have shown in mouse the transient expression of certain Eph-ephrin members in the developing inferior colliculus (IC) prior to hearing onset. Ephrin-B3 expression, while absent in the central nucleus (CNIC), is highly expressed in extramodular domains of the lateral cortex (LCIC) as well as the mesencephalic midline. We utilize multiple-labeling approaches in control and ephrin-B3 mutants to explore the development of converging CNIC and LCIC afferent patterns. Tract-tracing studies describe the relative distribution patterns of local and commissural CNIC connections together with an ascending input arising from the auditory brainstem. Additionally, we performed auditory brainstem responses (ABRs) as a physiological assessment of the auditory circuitry for each of our experimental groups. While tract-tracing experiments reveal no gross anatomical abnormalities between wild-type and ephrin-B3 mutants, ABRs show effects on auditory threshold, peak amplitude, and waveform fidelity in ephrin-B3 mutant mice. Taken together, these findings suggest that while ephrin-B3 may not influence the construction of topographic maps for the examined IC afferents, it is indeed necessary for ensuring fully functional auditory circuits prior to experience.
INTRODUCTION

I. Significance

Clinical Relevance

Tinnitus is a common disorder characterized by a perceived ringing in one’s ears while no stimulus is actually present in the surrounding environment. A current theory suggests tinnitus results from circuit plasticity or rearrangements in the mature auditory system (Engineer et al., 2011). Such changes in auditory cortex in tinnitus sufferers are somewhat analogous to cortical changes associated with phantom limb pain (Mühlnickel et al., 1998).

Implant devices are an increasingly common treatment strategy for deafness and hearing loss. Cochlear implants involve the surgical implantation of electrodes into the auditory nerve fibers of the patient, while auditory brainstem implants bypass the nerve and directly stimulate central structures. Despite many successes involving implants and improved hearing, significant drawbacks still exist. The most advanced cochlear implants still lack the ability to completely process the full frequency spectrum. Furthermore, noisy environments with complex and cluttered sounds can also be problematic for users. Another consideration is that regions of the auditory system are bypassed to artificially stimulate higher auditory centers by relatively crude means. Little research has been done to explore the possible effects cochlear implants may have on forcing plasticity on an established auditory system.

For both of these conditions, the understanding of the anatomy of the auditory system, as well as the mechanisms that help guide its formation, is of great importance.
and may provide insights concerning the root causes of these auditory pathologies as well as more rational ways to treat them.

The goal of my thesis is two-fold. The first goal is to describe the patterns and mapping of specific connections of the auditory midbrain during neural development, as well as what happens to these circuits when ephrin-B3, a protein associated with development, is functionally compromised or removed. The second goal is to describe the role of ephrin-B3 in auditory function by physiologically assessing ephrin-B3 mutant mice.

**Neural Development and Circuit Formation**

The nervous system involves an interplay of elaborate circuits that allows individual organisms to perceive their surrounding environment and to interact with it. The brain is a highly organized structure such that different regions encode information for different modalities. For example, certain regions are dedicated to the processing and perception of visual stimuli, whereas other areas are dedicated to the planning and execution of motor commands. Within these areas, functional neuronal populations are grouped as deep nuclei, or as cortical layers that superficially line a structure. Processing within and between these areas enables us to organize, analyze, and interact with our environment.

During development of the nervous system, neuronal populations and their connections become organized in highly specific ways. These precise arrangements are based on neural or topographic maps that preserve information in a given pathway. The characteristic mapping feature of neurons in an auditory structure is their spatial organization based on frequency. Other systems, like olfaction, have maps that reflect
circuits segregated by “type” rather than spatial position (Semple and Aitkin, 1979; Sanes et al., 2006; Luo and Flanagan, 2007). During development, these projections often traverse significant distances to identify a target nucleus. Once in the target structure, pioneer axons systematically make connections, preserving the information processed downstream.

This necessity for highly organized circuits is the impetus for studies that explore the mechanisms that guide such connections. The chemoaffinity theory provides one explanation, postulating that neurochemical gradients provide positional information for the systematic organization of neuronal populations and their connections (Sperry, 1963). In such cases, growing axons must express signaling proteins that detect and respond to corresponding expressed chemicals. Expression patterns guide axons to specific coordinate regions of the target nucleus to ensure proper neuronal connections. These chemical signals can have attractive, repulsive, and even hindrance properties to push and pull axons along a pathway towards its termination point (Cowan and Henkemeyer, 2002).

**Topographic Mapping**

Two types of neural topographic maps have been identified: continuous maps and discrete maps. The type of map that characterizes a nucleus depends on the type of information processed and the specific needs of the involved system. During development, the expression pattern of chemical guidance molecules within a nucleus is often a helpful clue in categorizing it as either a continuously or discretely mapped structure (Luo and Flanagan, 2007).
Continuous Maps

Continuous maps allow for connections that preserve a spatial order established in the periphery or in a source nucleus. The visual system uses continuous mapping to preserve the encoding of the visual world based on retinal position. The spatial pattern of light impinging on the retina is preserved throughout the visual system, such that nasal and temporal portions of the retina map to distinct regions of their central target.

The creation of continuous topographic maps is typically aided by the expression of multiple guidance molecules in gradients within a given nucleus. The patterns of guidance molecules are often not co-expressed, but rather are complementary to each other (Figure 1). This arrangement allows for even more specificity in regards to providing positional information for developing axonal connections along a given axis. In order for neurons and axons to be spatially arranged in multiple dimensions, gradients along differing axes are usually necessary (Luo and Flanagan, 2007). These gradients run orthogonal to one another and create a coordinate system to allow for axonal mapping along additional axes of the target structure.

Discrete Maps

Discrete topographic maps are not organized based on a continuum of stimuli or spatial position. Instead, discrete maps segregate inputs to a target structure based on the “type” of stimuli they encode (Figure 2). Aspects of the olfactory system exemplify discrete mapping, where connections from distinct receptors in the nasal epithelium are segregated centrally into discrete zones based on the odorant type that elicits a response (Luo and Flanagan, 2007). Another example of discrete mapping is found in the gustatory
system, where taste receptors on the tongue are already segregated in discrete regions and remain segregated in higher centers of the brain (Luo and Flanagan, 2007).

In contrast to continuous maps, expression patterns of guidance molecules in discrete maps do not exist as gradients. Rather, their expression is discontinuous and only present in specific entities of the target nucleus. Furthermore, other guidance molecules may be expressed around these entities to provide additional information to help with the segregation of multiple inputs within a target.
Figure 1. Diagram of a continuous map. Neighboring neurons in the source nucleus connect with neighboring neurons in a target nucleus, preserving spatial order. Neurochemical countergradients serve as guidance signals for growing axons through attractive and/or repulsive mechanisms. Examples of continuous maps are found in various levels of the visual system as well as in the auditory brainstem of the chick (Cramer, 2005). Modified reconstruction of Figure 1 in Luo and Flanagan, 2007.
Neurons in the source nucleus segregate in the target nucleus based on discrete guidance mechanisms. Expression patterns exemplify how types of inputs are segregated and organize to confined areas (red and blue), or to regions surrounding such domains (purple). Examples of sensory systems that have discrete neural maps include the olfactory system and the gustatory system. Modified reconstruction of Figure 1 of Luo and Flanagan, 2007.
II. Auditory Circuit Formation Prior to Hearing Onset

The potential for both continuous and discrete maps in the auditory system exists as sound is not only processed for its tonal properties (i.e. continuous maps), but also integrates inputs from other systems, such as motor pathways for reflexive responsiveness to auditory stimuli (i.e. discrete maps; Parham and Willott, 1990). With very few exceptions, the auditory system and its connections exhibit a strict adherence to a continuum of frequencies established in the cochlea. This frequency-specific mapping is referred to as tonotopy. In the cochlea, high frequency waves stimulate receptor cells at its base, while lower frequency waves activate receptors closer to the apex. This mechanical sorting in the cochlea is preserved at higher centers, where one end of an auditory nucleus is tuned to high frequencies, while the other encodes lower frequencies. While there is considerable evidence in the adult system for continuous frequency mapping, to date there is little evidence elucidating discrete maps and their functional importance for auditory processing.

Interestingly, the strict adherence to frequency-specific circuits emerges early in development, prior to any experience of hearing. This suggests intrinsic mechanisms that drive the organization and patterning of developing auditory connections. My research investigates the mechanisms that aid in the shaping of neuronal maps in the auditory midbrain prior to the onset of hearing.

Inferior Colliculus

The inferior colliculus (IC) is a midbrain structure that serves as an integrative relay hub in the ascending auditory pathway. The IC is organized into three subdivisions: the central nucleus (CNIC), the lateral cortex (LCIC), and the dorsal cortex (DCIC;
Herbert et al., 1991; Loftus et al., 2008). The most highly studied region of the IC is the CNIC. The CNIC is dedicated purely to the processing of auditory stimuli. Its neurons receive a vast array of afferent inputs that influence its narrowly-tuned and low threshold response properties (Aitkin et al., 1975; Ota et al., 2004). This region exhibits the characteristic tonotopic arrangement, where high frequency neurons are located in the ventromedial portions, and lower frequency neurons reside in more dorsolateral aspects. Within this tonotopic organization, a finer layered arrangement exists. Fibers of afferent projections parallel dendritic arbors of resident disc-shaped neurons and comprise the well-described fibrodendritic laminar architecture of the CNIC (Schreiner and Langner, 1997; Gabriele et al., 2000; Fathke and Gabriele, 2009).

The LCIC is situated just superficial and lateral to the central nucleus. Neurons in the LCIC are more broadly tuned and respond to spectrally complex auditory stimuli such as vocalizations (Ota et al., 2004; Zhou and Shore, 2006). The LCIC is divided into three distinct layers, layer 1 being the most superficial and layer 3 being the deepest (Faye-Lund and Osen, 1985; Herbert et al., 1991). The LCIC is a multimodal region, receiving inputs from both auditory nuclei as well as somatosensory structures (Zhou and Shore, 2006). It has also been shown to be associated with the startle reflex in mice and coordinating motor control with perceived auditory stimuli (Parham and Willott, 1990). While not described in detail to date, the neural topography of the LCIC appears more in keeping with a discretely mapped structure, as inputs to the LCIC exhibit patchy or extramodular projection distributions in guinea pig, rat, and the developing mouse (Saldaña and Merchán, 1992; Zhou and Shore, 2006; Wallace et al., 2013).

Immunostaining and histochemistry for glutamic acid decarboxylase, parvalbumin,
acetylcholinesterase, and cytochrome oxidase shows a modular patterning within the LCIC, which suggests the presence of a discretely mapped structure (Chernock et al., 2004).

The DCIC is the third subdivision and is located superficial and dorsal to the CNIC. The DCIC shares characteristics with the LCIC in that it is also subdivided into three layers, with cell response properties again more broadly tuned than the CNIC. However, the DCIC differs from the LCIC in that inputs into the DCIC are mostly descending connections from auditory cortex and receives no ascending inputs from somatosensory source nuclei. Furthermore, no patchy or modular inputs have been described in the DCIC, and a laminar arrangement has been observed in keeping with a topography more similar to the CNIC (Coleman and Clerici, 1987).

As mentioned previously, the inferior colliculus is an important part of the auditory system as it is a key integration hub of all downstream auditory nuclei. Before any auditory information is processed to higher centers of auditory thalamus and auditory cortex, nearly all axonal projections first synapse in the IC. Major downstream inputs arise from the cochlear nuclei, the lateral superior olive (LSO), the superior paraolivary nucleus, and the lateral lemniscal nuclei (Cant, 1982; Oliver, 1987; Schneiderman and Henkel, 1987; Schofield and Cant, 1996; Gabriele et al., 2000; Henkel et al., 2007; Saldaña et al., 2009).

Inputs from the LSO have received much attention, as it is a prominent nucleus in the binaural processing of sound. The LSO receives information from both ears, and subsequently sends bilateral projections to the IC (Wallace et al., 2013). These inputs terminate in the CNIC in a frequency-specific layered arrangement consistent with
continuous mapping (Figure 3). Interestingly, axons seemingly arising from the LSO also project to ipsilateral LCIC modular fields, which is in keeping with discrete mapping (Wallace et al., 2013).

In addition to receiving converging ascending inputs, the IC also makes local connections between subdivisions as well as commissural connections, allowing for IC crosstalk (Figure 3). In adult rat, there is evidence for a projection from the CNIC that targets extramodular LCIC domains (Saldaña and Merchán, 1992; Malmierca et al., 1996). The IC also sends commissural projections across the midline that sparsely terminate in contralateral LCIC extramodular regions as well as in layers within frequency-matched regions of the CNIC (Saldaña and Merchán, 1992). To date, none of these local or commissural IC connections have been described in the adult or developing mouse.

The current project aims to further our understanding of the development of patterned inputs to the CNIC and LCIC, to test the role of an individual signaling protein, and to assess its functional importance. It is our working hypothesis that the IC includes both continuously (CNIC) and discretely (LCIC) mapped inputs, and that patterning of both of these types emerge prior to experience. Furthermore, we anticipate based on previous expression data that ephrin-B3, a signaling protein, is involved in shaping discrete inputs to the LCIC, but not continuous CNIC inputs. To test this, fluorescent tract-tracing experiments labeling LSO-IC projections as well as local and commissural IC projections were made in wild-type (WT) and ephrin-B3 mutants.
Figure 3. Axonal projections from an auditory brainstem nucleus (red) and the IC itself (green). LSO axons bilaterally project to IC and terminate in the CNIC in a frequency-specific, layered arrangement (only ipsilateral projection shown). Closed contours surrounding the LSO depict other major auditory nuclei of the superior olivary complex and its associated periolivary nuclei. Intrinsic and commissural IC axonal projections form layered bands in the contralateral CNIC as well as bilateral extramodular terminal fields in the LCIC (Saldaña and Merchán, 1992).
**Eph-Ephrins**

The nervous system and its processing requires highly organized circuits. Some of these connections require the preservation of spatial attributes (continuously mapped), while others require the functional segregation of types of input (discretely mapped). One established means of affecting guidance behavior is via a family of receptor tyrosine kinase signaling proteins known as Eph-ephrins. This family is known to have roles in various aspects of development, including axon guidance, segmentation, cell migration, and angiogenesis (Davy and Soriano, 2005).

The Eph-ephrin family has 14 known receptors (Ephs), and 8 known ligands (ephrins). These Eph-ephrins are categorized into A- and B-subgroups, based on structural characteristics. All Eph receptors feature an extracellular cysteine rich domain linked to a transmembrane domain for signal transduction. Unlike Eph receptors, ephrin ligands can be split into two distinct structural morphologies. All ephrin ligands have an extracellular cysteine core and are also bound to the cell membrane, but differ in their attachment. Ephrin-A ligands are linked by way of a glycosyl phosphatidylinositol membrane anchor, while ephrin-B ligands have a dedicated transmembrane domain. In general, ephrin-A ligands bind to EphA receptors, and ephrin-B ligands bind to EphB receptors. However there are some exceptions, namely EphA4’s high binding affinity for both ephrin-B2 and ephrin-B3 (Flanagan and Vanderhaeghen, 1998).

Eph-ephrin interactions are characterized by their ability to signal bidirectionally, allowing for not only ephrin-to-Eph signaling (forward), but also for Eph-to-ephrin signaling (reverse; Cowan and Henkemeyer, 2002). The functional importance of reverse signaling is underscored by studies that show when an ephrin’s ability to reverse signal is
compromised, so too are processes such as long term potentiation, axon pathfinding, cell migration, and target selection (Cowan et al., 2004; Davy and Soriano, 2005; Armstrong et al., 2006; Wallace et al., 2013)

The molecular guidance properties of the Eph-ephrin family have been described in a variety of systems: EphB2 has been shown to aid in the migration of progenitor cells of the dentate gyrus of the hippocampus, which is associated with learning and memory (Catchpole and Henkemeyer, 2011); EphA/ephrin-A signaling has been implicated in the visual system helping to guide the formation of retinotopic maps in the superior colliculus (Cang et al., 2008); and EphA4 and ephrin-A5 have been shown to aid in target selection in the somatosensory systems (Dufour et al., 2003). Disruption or manipulation of Eph-ephrin signaling also appears to impact nearly all levels of the auditory system, affecting tonotopy and topographic mapping in the auditory brainstem and midbrain, as well as frequency tuning in the auditory cortex (Huffman and Cramer, 2007; Intskirveli et al., 2011; Wallace et al., 2013)

Expression gradients within the IC and auditory brainstem nuclei prior to hearing onset have been described previously for EphA4 and ephrin-B2 (Gabriele et al., 2011; Wallace et al., 2013). My project looks at the anatomical role of ephrin-B3 in the circuit formation of the IC and the formation of topographic maps in its distinct subdivisions. Ephrin-B3 has been implicated in various aspects of the nervous system, though little has been done to investigate ephrin-B3’s role in the development of the auditory system. Clues to what role ephrin-B3 might have on the auditory system come from preliminary research in our lab, as well as research others have done on ephrin-B3’s effect on other systems. Such studies have suggested that ephrin-B3 has a role as a repulsive barrier,
aiding in the prevention of recrossing behaviors of descending corticospinal axons that have already navigated the midline (Kullander et al., 2001; Yokoyama et al., 2001).

In our lab, preliminary ephrin-B3 expression studies show protein concentration in areas surrounding the CNIC, as well as conspicuous pockets of no expression in the LCIC (Figure 5A). Furthermore, ephrin-B3 is highly expressed at the midline (Figure 5B, arrowheads), which is in keeping with the notion of ephrin-B3’s involvement in midline decision making. In the auditory brainstem, there is no expression of ephrin-B3 in the LSO (Figure 5C), but ephrin-B3 is present in the surrounding periolivary regions. All ephrin-B3 expression in these regions has been identified as being transient, with ephrin-B3 expression significantly downregulated upon hearing onset. These expression patterns of ephrin-B3 show no obvious gradients, which is the basis for our hypothesis that it may play a role in discrete mapping of the LCIC but not in establishing the continuous CNIC map.
Figure 4. The inferior colliculus and downstream auditory brainstem nuclei with known patterns of EphA4 and ephrin-B2 expression. Countergradients of expression indicated by red and blue triangles, where the base of each triangle represents high expression. Tonotopic axis of each nuclei indicated by dual-sided arrow (HF = high frequency, LF = low frequency). Question marks indicate unidentified gradients of expression. CN = cochlear nucleus; DLPO = dorsolateral periolivary nucleus; DNLL = dorsal nucleus of the lateral lemniscus; DPO = dorsoperiolivary nucleus; LNTB = lateral nucleus of the trapezoid body; MNTB = medial nucleus of the trapezoid body; MSO = medial superior olive, SPON = superior paraolivary nucleus; VNTB = ventral nucleus of the trapezoid body.
Figure 5. Expression patterns of ephrin-B3 in the auditory midbrain and the auditory brainstem prior to hearing onset. X-gal staining uses the lacZ gene inserted in ephrin-B3lacZ mutants as a reporter for the localization of ephrin-B3 protein. A. Ephrin-B3 is highly expressed in regions surrounding the CNIC and in extramodular domains of the LCIC (dashed closed contours). B. Midline expression of ephrin-B3 is very high at both the dorsal midbrain commissure and ventrally at the brainstem (arrowheads). C. Expression patterns of auditory brainstem nuclei, showing an absence of ephrin-B3 in the LSO with considerable expression in surrounding areas.
III. Auditory Brainstem Responses

In addition to investigating the potential effect of ephrin-B3 on the establishment of IC connections, the current project also assesses the influence ephrin-B3 has on auditory function. Auditory brainstem response testing (ABRs) was performed to determine if the ability of ephrin-B3 mutant mice to detect and process auditory stimuli is compromised in any way. ABRs record brainwaves of activity in response to presented stimuli. In response to auditory stimuli, ABRs exhibit five prominent peaks which correspond to activity at various levels of the auditory system. The first peak in an ABR waveform is indicative of auditory nerve, or cochlear nerve, activity. The second peak corresponds to activity in the cochlear nucleus, or the central target of the cochlear nerve. Peaks I and II are the most prominent waveforms in mice. Subsequent activation in the superior olivary complex corresponds to Peak III. From here, activity in the lateral lemniscus and ultimately in the IC correspond to Peaks IV and V, respectively. Though Peak III is still identifiable in many ABR waveforms, it is not as pronounced as Peaks I and II. Peaks IV and V are harder still to distinguish and are sometimes indistinguishable from the background noise of the waveform.

ABRs use pure tones or broadband click stimuli at varying levels of intensity to determine audible frequency range, as well as response thresholds and latencies. Comparisons between WT mice and ephrin-B3 mutant mice were made in the current studies to determine the role ephrin-B3 has on auditory function. Previous ABR studies implicate other members of the Eph-ephrin family in auditory function, as ephrin-B2 and EphA4 mutant mice exhibit increased thresholds and latencies when compared to WT mice (Miko et al., 2008). Interestingly, ABRs on ephrin-A2 and ephrin-A5 mutant mice
show increased sensitivity to stimuli compared to WT mice, suggesting that the modification or removal of Eph-ephrin proteins can lead to a broad spectrum of changes in auditory function. (Yates et al., 2014).

IV. Specific Aims

**Specific Aim 1:** To determine the topography of LSO-IC and intrinsic and commissural IC projections in the developing mouse prior to experience.

**Specific Aim 2:** To identify the role of ephrin-B3 in instructing hypothesized LCIC discrete maps and to determine its influence, if any, on the construction of continuous maps in the CNIC.

**Specific Aim 3:** To assess the functional role of ephrin-B3 by comparing ABR waveforms in WT and mutant mice.
METHODS

I. Experimental Groups and Genotyping

Two different strains of ephrin-B3 mutant mice were used as experimental groups to determine the role of ephrin-B3 on the organization of axonal projections in the IC as well as its role in functional hearing. Ephrin-B3\textsuperscript{null} mice are a mutant strain that have been genetically manipulated to completely knock out ephrin-B3 expression in the homozygous animal (ephrin-B3\textsuperscript{−/−}). Ephrin-B3\textsuperscript{lacZ} mice are a slightly different mutant in that a lacZ insertion allows for localization of ephrin-B3 via β-galactosidase staining. Furthermore, while these mutants retain the ability to forward signal, their ability to reverse signal is compromised and completely lacking in homozygous mutants (ephrin-B3\textsuperscript{lacZ/lacZ}). This shutdown of reverse signaling is due to the deletion of a required cytoplasmic domain of the ephrin-B3 protein necessary for signal transduction.

C57BL/6J mice (Jackson Labs) and WT genotypes of the ephrin-B3 mutant groups were used as controls for all experiments.

Tail samples for genotyping were digested, isolated, and precipitated with an Easy-DNA kit (Invitrogen, Carlsbad, CA). Primer sequences used for PCR amplification were as follows: ephrin-B3-forward 5’-GACGGCGGGCAAGCCTTCGGAGAG-3’, ephrin-B3-reverse 5’-ATAGCCAGGAGGAGCCAAAGAG-3’ and lacZ 5’-AGGCGATTAAGTTGGGTAAACG-3’. Gel electrophoresis visualized ephrin-B3 WT bands (401-bp) and/or mutant (142-bp) allele bands. All experimental procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and received prior approval from the Institutional Animal Care and Use Committee (Protocol No. A17-12).
II. Fluorescent Tract-Tracing of Developing IC Circuits

Mice

Experiments were performed on developing mice at postnatal stages from birth (P0) to onset of hearing, postnatal day 12 (P12). Stages of particular interest were P4, as this time point coincides with axonal organization within the target IC, and P8, when developing inputs exhibit a mature projection arrangement.

NeuroVue Tract-Tracing of Axonal Projections

Tract-tracing experiments were performed similarly to the protocol previously described by the Gabriele lab (Wallace et al., 2013). In short, mice are given an overdose of a ketamine/xylazine cocktail (10 mg/kg: 1mg/kg ketamine-to-xylazine) and perfused transcardially with physiological rinse followed by 4% paraformaldehyde fixative solution. Brains were carefully harvested from the skull and stored at 4°C in paraformaldehyde solution. Brains were blocked in the coronal plane and embedded in a gelatin/egg yolk mixture with a slight tilt to facilitate a sectioning plane that incorporated both the LSO and slightly more rostral IC. Embedded brain tissue was then once again stored overnight at 4°C in paraformaldehyde fixative solution. Within a week of embedding, tissue blocks were cut on a Vibratome from caudal-to-rostral in 75µm slices until caudal aspects of both the IC and LSO were visible under darkfield microscopy.

In most cases, a combination of two fluorescent lipophilic dyes were used to observe the convergence of commissural IC axonal projections and ipsilateral LSO projections into IC. A small sliver of lipophilic NeuroVue Maroon dye-soaked filter paper was cut using Vannas scissors and positioned in the CNIC contralateral to the target IC with the aid of a dissecting microscope. A second fluorescent dye-soaked filter
paper, NeuroVue Red, was similarly cut and placed in the LSO ipsilateral to the target IC. Slivers of fluorescent dye-soaked filter paper were kept as consistent as possible between cases and multiple placements were made for each experimental group to account for any variability in the placement size and spread. In the case of NeuroVue Maroon labeling of intrinsic and commissural IC projections, enough small and large placements of dye were made in each experimental group to be able to discuss the results of both frequency-matching and pattern formation.

Following dye placement, remaining tissue blocks were incubated at 37°C in the dark for one month in 4% paraformaldehyde solution to allow for adequate diffusion. At this time, the tissue was once again cut on the Vibratome in 75µm sections and counterstained with bis-benzimide (Hoechst 33258; Invitrogen, Carlsbad, CA) for 5 minutes to visualize the surrounding cytoarchitectural boundaries of the LSO and IC. Following counterstaining, sections were rinsed in phosphate buffered saline solution, mounted and coverslipped with Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA).

**Wide-Field Fluorescence Microscopy**

Tissue sections were viewed with a Nikon TE 2000 microscope using a monochrome, cooled CCD CoolSnap HQ digital camera (Roper Scientific). Excitation/emission spectra for the bis-benzimide and both NeuroVue dyes are such that they are easily distinguishable with the aid of appropriate filter sets (NeuroVue Maroon maximum excitation/emission = 647nm/667nm; NeuroVue Red = 567nm/588nm; bis-benzimide = 352nm/461nm).
All image capturing was performed using Nikon Elements Advance Research software version 4.13.04. A separate monochrome channel was created for each fluorophore, pseudocolored, and then merged (green: NeuroVue Maroon, red: NeuroVue Red, blue: bis-benzimide counterstain). 4X (numerical aperture [N.A.] 0.2; working distance [W.D.] 20 mm), 10X (N.A. 0.45; W.D. 4.0 mm), and 20X (N.A. 0.75; W.D. 1.0 mm) images were taken throughout the rostral-to-caudal extent of the LSO and IC. Images were auto-exposed using Nikon elements software, and finer refinements were made manually based on the histogram adjustment tool. Three-dimensional Z-series were performed at 10X and 20X magnifications to allow for the capture of all labeling throughout the 75µm section. Z-series images were merged to a single image using a maximum intensity projection and a deconvolution algorithm (Create Focused Image function in Nikon Elements). All images were saved in the Nikon Elements native file format of JPEG2000 to preserve raw image profiles and avoid compression. In some cases, Adobe Photoshop was used to make slight adjustments to brightness or contrast for illustrative purposes or to accentuate cytoarchitectural boundaries.

III. Auditory Brainstem Responses

Experimental Overview

Monaural auditory brainstem responses were recorded from 40 adult mice up to postnatal day 90. Auditory circuitry is fully matured by this stage, and the mice are old enough to have been weaned and are completely independent of their mother. Mice older than three months were excluded from testing to avoid the effects of reported age-related hearing loss common in C57BL/6J mice (Parham, 1997).
Analysis of the ABRs compared the average peak latencies, amplitudes, and response thresholds between groups. Waveforms were recorded in each mouse for brainstem responses to three separate auditory stimuli: 8 kHz pure tone pips, 12 kHz pure tone pips, and broadband clicks. Each auditory stimulus was presented separately to each mouse in a randomized order. In order to gather information on the auditory response thresholds of the experimental groups, a series of waveforms was also recorded for each stimulus at decreasing nominal (uncalibrated) intensities in the following succession: 90 dB, 70 dB, 60 dB, 55 dB, 50 dB, 45 dB, 40 dB, 35 dB, 30 dB, and back up to 90 dB a second time to corroborate data recorded at the beginning of the test to ensure stability of the animal and recording equipment. Actual values of intensity varied slightly for each stimulus such that 12 kHz and 8 kHz pure-tones had approximate starting output intensities of 92 dB and 103 dB respectively when calibrated. Peak energy for the click stimulus was at 2.6 kHz and had an average band power of 90 dB (200-20,000 Hz).

Data Collection

Testing was performed in a double-walled, double floored Industrial Acoustics Corp sound attenuating booth. Anesthesia was initiated with 5% isofluorane and maintained with ketamine/xylazine (150 mg/kg ketamine and 30 mg/kg xylazine; IM route). Dosage of injections were calculated by weight, with additional dosing used as needed to ensure sufficient anesthesia. Mice were placed on a Harvard Animal Blanket Control Unit to maintain body temperature throughout experimentation. Speakers within the sound proof chamber were connected by cables that ran through a sound proof opening. A computer and auditory hardware rig controlled the experiment from the outside of the chamber.
Tucker-Davis Technology (TDT; Achula, FL) hardware and software were used to perform ABR experiments. The parameters for the generation of auditory stimuli were programmed using the TDT SigGen software application. 8 kHz and 12 kHz pure tones were generated to last 5 ms in duration, with .5 ms Blackman windowed rise and decay times. Clicks were 0.1 ms in duration with instantaneous electrical onset and offset. The rate of stimulation for each treatment was 39.1 stimuli/sec. Presentation of stimuli at manipulated intensities and the recording of auditory brainstem response waveforms were performed using the TDT BioSig application. Digital signal from the computer software was sent to a TDT RP2.1 Enhanced Real-Time Processor to convert the digital signal to analog auditory signal. Signal was then sent through a TDT PA5 Programmable Attenuator to allow the BioSig software to be able to adjust the intensity of the auditory stimuli during the experiment. Stimuli was then sent through the output channel of the attenuator into the input channel of a TDT ED1 Electrostatic Speaker Driver to adjust initial gain level and prepare the signal for the TDT EC-1 high-frequency electrostatic closed-tube speaker within the soundproof booth. Auditory stimulus was distributed through a closed Y-tube at the ear of the test mouse to allow for the presentation of stimulation at one end of the Y-tube, reception of stimuli by the test mouse’s ear, and real-time calibration of auditory stimuli with an Etymotic Research ER-7C probe microphone on the third arm of the Y-tube. The probe microphone was attached to an Agilent 35670A Dynamic Signal Analyzer to allow for the recording of peak frequency, amplitude and bandwidth of stimulus at the nominal 90 dB intensity level.

Evoked potentials were recorded from subdermal needle electrodes: non-inverting input at the vertex of the brain, inverting input at the mastoid just ventral to the test ear of
the mouse, and a ground placed on the back of the mouse towards the tail. Electrodes were connected to a TDT RA4PA 4-channel Medusa Pre-amp and this impedance was checked to ensure that values were 1kΩ or less. Electrodes were adjusted if impedance values were higher. Auditory brainstem response signals were transferred to a TDT RA16 Medusa Base Station before being recorded and assimilated as raw waveforms in the TDT BioSig software application.

Four different traces, each the average of 100 sweeps made below the limit of artifact rejection were recorded at each intensity of each stimulus. The four traces alternated polarities such that one trace of 100 rarefaction clicks was followed by 100 condensation clicks, and then this process was repeated once more before resuming at a decreased intensity, described above.

**Calibration**

The Agilent 35670A Dynamic Signal Analyzer was first calibrated to confirm that the machine was recording stimuli at the proper intensity and frequency. A ¼ inch Brüel & Kjær (B&K) 4939 high frequency microphone was attached to a B&K 4230 Sound Level Calibrator via a ¼ adapter. The sound level calibrator is engineered to consistently transmit an acoustic signal at a frequency of 1 kHz at an intensity of 94 dB. The output of the ¼ high frequency microphone was connected to a pre-amplifier, which was then connected to the dynamic signal analyzer. When the signal was transmitted, the correct intensity and frequency of the calibrator’s acoustic stimulus was observed on the analyzer.

Calibrations of the experimental stimuli took place using the same setup as used to test mice with the substitution of the ¼ inch high frequency microphone in the place of
the test mouse. The microphone was affixed to the Y-tube system using an adapter that simulates the volume of the average adult mouse ear. The microphone output was connected to a microphone pre-amplifier before the input of the Agilent Dynamic Signal Analyzer as well as an Agilent 541D Digital Oscilloscope. One other notable difference between the calibration setup and the experimental setup was that the stimuli were adjusted in length to allow for accurate recording of frequency, intensity and bandwidth. 8 kHz and 12 kHz pure-tones were lengthened to be 1-s in duration using the TDT SigGen software, while calibration of clicks were initially simulated as continuous white-noise to record the spectrum of frequencies used in the click stimuli. Secondary calibration used a continuous series of clicks at the experimental duration to measure the peak-to-peak voltage of the clicks.

**Quantitative Analysis**

Waveforms were transferred to the MATLAB (V2013a Mathworks Inc. Natick, MA) software application and a program was created to allow for the determination of thresholds and identification of peaks for every trial (coding by Dr. Lincoln Gray). In order to reduce bias and ensure replicability, thresholds and peaks were identified separately by two observers. To confirm validity of acquired ABRs, mean z latency-intensity functions were performed. Under normal conditions, latencies should decrease with increased intensity of the delivered stimulus. Subsequently, thresholds were defined as an intensity level between the last of the ABR series in which an auditory response was obtained and the first level in which no response was observed. Peaks were carefully selected for every waveform in which clear rarefaction and condensation components were present. Peaks I and II were easily identified in every case, while Peaks III-V were
increasingly difficult to observe, particularly in mutant cases. Peak III was evident in the majority of cases, with Peaks IV and V most difficult to distinguish. Peak-to-trough amplitudes were also measured for Peaks I and II. Upon identification of all available peaks for each stimulus at each intensity, the program exported threshold information and coordinates of every peak to an excel file for further data analysis. Inter-observer reliability was tested to ensure that peaks were unbiased and replicable. Marginal means of thresholds, peak latencies, and amplitudes were obtained to determine significance on the effect of mutations by way of a repeated-measures ANOVA using SPSS (V21 IBM Inc. Chicago, IL).
RESULTS

I. Fluorescent Tract-Tracing of Developing IC Circuits

Intrinsic Connections of the Inferior Colliculus

Following placement of fluorescent dye in the CNIC, a V-shaped isofrequency axonal pattern was observed locally in the IC for all experimental groups (Figure 6A-E). A band of labeling extended into the CNIC and DCIC corresponding to the placement of fluorescent dye in the CNIC. The CNIC-DCIC bands merged ventrally with labeled projections into layer 3 of the LCIC, creating the characteristic V-shaped pattern as previously described in the adult rat (Saldaña and Merchán, 1992; Malmierca et al., 1996).

Projections from the CNIC to the ipsilateral LCIC terminated in a clear extramodular pattern. These data are the first to describe this projection pattern in any species. The extramodular arrangement in mouse emerged as early as P4 and was present regardless of strain or genotype (Figure 6A-E). Cross-bridges of axonal labeling through layer 2 of the LCIC, presumably between neighboring modules, connected the projection distribution in layer 3 to additional label in layer 1. This pattern was most obvious in the rostral third of the IC.

Commissural Connections of the Inferior Colliculus

Commissural connections were also observed from the CNIC to contralateral IC. Labeled projections crossed the midline and terminated as a dense band extending from the DCIC into the CNIC. This resultant laminar plexus matched the position of the dye placement in the opposite CNIC. This frequency-specific axonal targeting of the commissural CNIC projection was observed in all experimental groups (Figure 6F-J).
Figure 6. Intrinsic and commissural connections of the IC prior to onset of hearing. A-E. Intrinsic connections originating from the CNIC exhibit a characteristic V-shaped axonal plexus in the CNIC and extending into deep aspects of the LCIC. CNIC-LCIC projections exhibited a consistent extramodular arrangement ipsilaterally in WT and all ephrin-B3 mutants (green label surrounding dashed modular domains). F-J. Commissural connections originating in the CNIC projecting across the midline (arrowheads) and terminating in contralateral IC show connectivity in frequency-matched aspects of the DCIC and dorsal aspects of the CNIC. Panels B,C,G, and H represent ephrin-B3lacZ mutant mice, and D,E,I, and J represent ephrin-B3null mice. D = dorsal, V = ventral, M = medial, and L = lateral. Scale bar in A = 500 μm, applies for all panels.
Laminar arrangements of labeled commissural axons that target the contralateral IC are shown for ephrin-B3 mutants in Figure 7. A low magnification montage illustrates an axonal plexus in frequency-matched aspects of the IC contralateral to the dye placement (Figure 7A). Cases with small, localized placements of dye in the source CNIC yielded a single axonal layer in comparable areas of the contralateral target CNIC (Figure 7B-C). In cases with larger dye placements, multiple layers of axons were observed in appropriate regions of the contralateral IC (Figure 7D-E).

Extramodular labeling was observed in the contralateral LCIC following a CNIC dye placement, but was considerably more sparse than that observed for the described intrinsic CNIC-LCIC projection (Figure 6). Commissural fiber fascicles to the contralateral LCIC split shortly after the midline into two characteristic trajectories, either streaming dorsally or passing through the DCIC and CNIC before invading the LCIC ventrally (data not shown).

**Layered LSO-CNIC Projection Patterns**

Ascending projections from the LSO exhibit a clear laminar organization in the ipsilateral CNIC prior to hearing onset (Figure 8). LSO axons enter the IC ventrally and occupy characteristic layers in the CNIC, terminating just short of the CNIC-DCIC boundary. LSO layers were observed consistently regardless of strain and genotype. Modular inputs into layer 2 of the LCIC were also observed, but only on occasion. As many of the dye placements encompassed much of the LSO, it was difficult to determine any effect on the tonotopicity of this projection.
Figure 7. Tonotopic organization and pattern formation of commissural IC projections in ephrin-B3 mutant mice. Images shown are from heterozygous mice (A-E), although similar observations were seen in WT and homozygous mutants. A. Composite image of commissural connections exhibiting laminar plexes in regions corresponding to the placement of fluorescent dye in the contralateral IC. B. A single labeled axonal layer was observed in the contralateral IC following a small dye placement (higher magnification of inset box shown in C). D. Large fluorescent dye placements in the IC label multiple contralateral laminae, similar to that observed in WT mice (higher magnification of inset box shown in E). Scale bars in A, B, D = 500 μm; C, E = 100 μm.
Figure 8. Characteristic LSO layers in the CNIC in ephrin-B3 mutant mice. Large placements of NeuroVue Red dye in the LSO (closed contour in upper right) yielded a pattern of labeled axonal plexes in the target CNIC consistent with its underlying fibrodendritic architecture. Shown is an ephrin-B3+ / lacZ mouse, yet all experimental groups showed distinctly layered LSO-IC projection patterns. Higher magnification of inset in (A) shown in (B). Arrowheads highlight 3 axonal layers. Scale bars in A = 200 μm, B = 100 μm.
Patterns of Convergence of LSO and Commissural Inputs to the IC

Convergence patterns were observed in cases with prominent labeling of both ascending ipsilateral LSO and commissural IC axons. Convergence of axons was most obvious in the CNIC, where laminar projections from both LSO and contralateral IC were complementary to one another with very little overlap (Figure 9). A noticeable boundary was also observed between the CNIC and DCIC, where very few LSO axons crossed. Along that same boundary, dense contralateral IC projections into DCIC gave way to laminar arrangements in the CNIC (Figure 9).

Convergence within the LCIC was seen infrequently, but when present, ascending and commissural projections were segregated into a modular-extramodular arrangement with no overlap (Figure 9C-F). Commissural connections were sparse in the LCIC, but terminated in the extramodular regions surrounding modular projections arising from lower centers.
Figure 9. Convergence patterns of commissural midbrain projections and ascending brainstem projections in developing mice. Similar complementary patterns were seen in all experimental groups. A-D highlight two ephrin-B3^{+/+lacZ} cases; E, F are from an ephrin-B3^{lacZ/lacZ} mouse. A-B. Labeling of both ascending LSO axons (red) and commissural IC axons (green) terminate at the boundary of the CNIC and DCIC, with LSO axons labeled in the CNIC and commissural axons primarily in the DCIC. Little to no overlap was observed between projections. C-D. Labeling in the LCIC shows a modular-extramodular arrangement of axons, with commissural connections forming sparsely labeled extramodular rings around patchy ascending projections originating downstream from the IC. E-F. Qualitatively, convergence patterns in both the CNIC and LCIC of homozygous mutants were similar to that observed in WT and heterozygous mutants. Layers of the LCIC are numbered from superficial (1) to deep (3). Scale bars in A, C, E = 500 μm; B, D = 100μm; F = 200μm.
II. Auditory Brainstem Responses

ABR waveforms were traced from broadband clicks, 8 kHz, and 12 kHz pure tone experiments performed on WT and ephrin-B3 mutant mice (Figure 10). Peaks I-III were easily observable in all cases, while Peaks IV-V were only recorded occasionally. Peak I was intact in most ephrin-B3\textsuperscript{lacZ} cases (Figure 10A-C, \textit{blue} traces), in contrast to ephrin-B3\textsuperscript{null} mutant mice (Figure 10D-F, \textit{black} traces) that showed temporal splitting of Peak I, most notably in heterozygous mutants (Figure 10D-F). Latency-intensity functions confirmed successful performance of ABRs by supporting that waveforms were real neural responses that were under direct influence of stimulus intensity as evidenced by the progressive decrease in Peak I latency (Figure 11).

**Inter-Observer Reliability**

Seven points (Peaks I-V, as well as troughs following Peaks I and II) were picked on all waveform traces by two separate observers to reduce bias and ensure replicability. Following a coordinated, careful method of peak picking, inter-observer reliabilities were calculated and found to be highly correlated: $r^2=.586$ for Peak I latency, .815 for amplitude I, and .549 for threshold (Figure 12, threshold data not shown). As traces were similar between observers, the highly correlated values were averaged for further analysis.

**Effects of Stimulus on Neural Activity**

ABR testing showed a clear effect of stimulus on neural activity. Broadband click stimulus triggered lower thresholds of hearing (Figure 13), faster neural response or decreased latencies (Figure 14), and increased waveform maxima amplitudes (Figure 15) in all mice as compared with 8 or 12 kHz pure tone pip presentations.
Figure 10. Averaged ABR waveforms in WT and ephrin-B3 mutants. Traces are shown for decreasing intensities from broadband clicks experiments. Similar waveforms were recorded for the 8 kHz and 12 kHz pure tone experiments. Stimulus presentation begins at 0 ms. A-C. ephrin-B3^{+/+} (n=8), ephrin-B3^{+/lacZ} (n=7), and ephrin-B3^{lacZ/lacZ} (n=3) mice. D-F. ephrin-B3^{+/+} (n=2), ephrin-B3^{+/+} (n=11), and ephrin-B3^- mice (n=6). Ephrin-B3^- mice qualitatively show reduced waveform fidelity, with Peak I splintered temporally into two distinct peaks.
Figure 11. Averaged latency-intensity function of all experimental groups normalized for genotype and stimulus type. As intensity (dB) increases, Peak I latency (ms) decreases as expected.
Figure 12. Peak I latency and amplitude reliability between two observers. Inter-observer reliability values between observers were highly similar. A. Peak I latency ($r^2 = 0.586$) B. Peak I amplitude ($r^2 = 0.815$)
Auditory Thresholds

The ABR thresholds of each experimental group were analyzed via repeated-measures ANOVA and marginal means were compared (Figure 13). Average thresholds for ephrin-B3\textsuperscript{lacZ} mice increase from WT to mutant genotypes, with homozygous mutants experiencing the most elevated thresholds. Marginal means of WT ephrin-B3\textsuperscript{lacZ} mice ranged from 49.2-56.0 dB, depending on the stimulus. Threshold ranges of marginal means for heterozygous and homozygous mutations were 57.2-60.5 dB and 58.5-75.5 dB, respectively. Interestingly, thresholds for ephrin-B3\textsuperscript{null} mice are highly elevated in the heterozygous genotype (56.2-74.3 dB compared to 48.1-53.0 dB in WT, depending on stimulus), while less so in homozygous animals (ranging from 51.0-61.0 dB).

Peak Latencies

The timing of Peaks I and II was recorded for each experimental group and analyzed to determine if mutation of ephrin-B3 had any effect on latencies (Figure 14). No discernible effect of mutation on the latencies of Peaks I or II was observed. Latency patterns varied by stimulus in all experiment groups, with no consistent pattern observed across stimuli. Latencies of broadband click stimulus ABRs were much lower than those of 8 and 12 kHz pure tones as expected.
Figure 13. ABR thresholds in WT and ephrin-B3 mutants. A. Elevated thresholds are observed in both heterozygous and homozygous ephrin-B3<sup>lacZ</sup> mutants. B. In ephrin-B3<sup>null</sup> mice, threshold elevation for each stimulus is most pronounced in the heterozygous mutations. C. Marginal means from a repeated-measures ANOVA of thresholds showing significant effects of ephrin-B3 between strains and genotypes (p < 0.05, p = 0.047).
Figure 14. Marginal means of ABR Peak I and II latencies. No significant effects on latency were observed for either ephrin-B3 mutant strains. A, B. Peak I latencies for ephrin-B3$^{lacZ}$ (A) and ephrin-B3$^{null}$ (B) mutants. C, D. Peak II latencies for ephrin-B3$^{lacZ}$ (C) and ephrin-B3$^{null}$ (D) mice.
Peak Amplitudes

Peak I and II amplitudes were analyzed via repeated-measures ANOVA to determine if ephrin-B3 mutation influenced the magnitude of waveform maxima (Figure 15). Marginal means of Peak I show significant decreases in amplitude in ephrin-B3<sup>lacZ</sup> mutant mice, with the most pronounced reduction observed in homozygous animals (Figure 15A). Ephrin-B3<sup>null</sup> waveforms showed decreased Peak I amplitudes for heterozygous mice exposed to broadband click stimuli and 12 kHz pure tones. Interestingly, amplitudes increase for all stimuli in homozygous ephrin-B3<sup>null</sup> animals (Figure 15B). Peak II amplitudes also decrease in ephrin-B3<sup>lacZ</sup> mutant mice, but the difference in amplitude between heterozygous and homozygous mutations varies based on stimulus. Broadband click stimuli and 8 kHz pure tone pips displayed lower amplitudes in heterozygous ephrin-B3<sup>lacZ</sup> animals as compared to the homozygous mutant. This was not the case however for the 12 kHz presentation, where Peak II amplitudes continued to decrease across genotypes (Figure 15C). No significant effects on Peak II amplitude was observed in ephrin-B3<sup>null</sup> mice (Figure 15D).
Figure 15. Marginal means of ABR Peak I and II amplitudes (µV). A. Peak I amplitudes for ephrin-B3\textsuperscript{lacZ} mice significantly decrease with mutations for all stimuli tested. (p < 0.05; p = 0.038) B. Decreases in Peak I amplitudes for ephrin-B3\textsuperscript{null} mice are most pronounced in heterozygous mutants, particularly when tested for click or 12 kHz pure tone presentations. C. Ephrin-B3\textsuperscript{lacZ} mutant waveforms have noticeably lower Peak II amplitudes compared to WT. D. Peak II amplitudes were unaffected by mutation in ephrin-B3\textsuperscript{null} mice.
DISCUSSION

These present experiments have significantly advanced our understanding of the development and topographic mapping of the IC, as well as the impact of ephrin-B3 on emerging connectivity prior to experience. The results provide the first description of extramodular projection patterns for CNIC-LCIC connections in any species. These findings confirm in the developing mouse previous findings in the adult rat concerning intrinsic and commissural IC projections (Saldaña and Merchán, 1992; Malmierca et al., 1996). Furthermore, this work provides the first insights concerning the mapping and convergence of multiple inputs to the LCIC. While still preliminary, these findings suggest frequency-specific mapping of the CNIC, and modular-extramodular mapping of the LCIC that is in keeping with our model of continuously and discretely mapped subdivisions of the IC. While not anticipated, ephrin-B3 was not required for the development of any of the examined projections. Despite seemingly normal midbrain connections in ephrin-B3 mutants, ABR results suggest a pivotal role for ephrin-B3 in the development of auditory circuitry and its function.

I. Fluorescent Tract-Tracing of Developing IC Circuits

The IC makes local connections between its three subdivisions. As mentioned previously, evidence exists in rat for a projection from the CNIC that targets extramodular LCIC domains (Saldaña and Merchán, 1992). None of these local IC connections have been described in the adult or developing mouse. Our study documents that not only are these terminal fields present in mice as well, but established as early as P4, one week prior to hearing onset. Extramodular terminal domains were consistently observed for the ipsilateral CNIC-LCIC projection independent of experimental group.
Thus, ephrin-B3 is not required for the establishment of this particular extramodular topography. However, due to the nature of the extramodular expression of ephrin-B3, there may be a repulsive role for ephrin-B3 in boundary formation of modular inputs to the LCIC. Future studies exploring other inputs to the LCIC modular/extramodular fields and their response to ephrin-B3 will prove important in determining its exact role.

Crossing fibers arising from the CNIC navigated the midline with no obvious obstruction, regardless of genotype. This suggests ephrin-B3 is unlikely to provide an attractive signal that guides projections across the midline. It is unclear, however, whether ephrin-B3 has a role in preventing already-crossed axons from recrossing the midline, as has been described in other systems (Kullander et al., 2001; Yokoyama et al., 2001). Data from our lab suggest axons are able to cross the midline during the period of ephrin-B3 expression. Future experiments aimed at comparing the initial expression of ephrin-B3 at the midline with the time projections cross may help provide more insight in the role of ephrin-B3 on midline decisions.

Eph-ephrins influence the organization of certain inputs to the CNIC, as evidenced by previous studies in our lab showing involvement in the development of the tonotopic LSO-IC projection (Wallace et al., 2013). While ephrin-B2 was required for accurate frequency-specific mapping, it was not necessary for layer formation. In the current study, projection patterns in the CNIC appeared to be unchanged with the modification or removal of ephrin-B3 expression. Labeled projections in the CNIC and DCIC matched to the location of dye placement in contralateral IC. Furthermore, the laminar arrangement of CNIC afferents remained intact in regards to incoming projections from ipsilateral LSO and contralateral IC. In regards to topography mapping,
our hypothesis that the organization of the CNIC is influenced by a continuous map paradigm is consistent with the result that ephrin-B3, a guidance protein not found in the CNIC, had no effect on either frequency-specificity or pattern formation.

Preliminary data in our lab clearly show that ephrin-B3 is expressed extramodularly in the LCIC surrounding patches of no expression in layer 2. This is indicative of a discrete map, where axons of different “type” would segregate into different regions as dictated by guidance proteins. Indeed, projection patterns in the LCIC displayed a modular-extramodular arrangement of axons, with commissural IC axons occupying the extramodal regions. Axons labeled from a placement of fluorescent dye in the LSO were seen, on occasion, to occupy the modular regions of the LCIC. This lack of consistent labeling may be due to the unintentional labeling of neighboring fibers from the spinal trigeminal tract (Sp5), in addition to the LSO, which are known to target LCIC modules (Zhou and Shore, 2006). These somatosensory projections travel across the auditory brainstem just rostral to the LSO, and it is possible that some LSO dye placements clipped these somatosensory fibers. Nonetheless, discrete mapping is an appropriate scheme for regions such as the LCIC, which is multimodal in nature and may segregate and incorporate inputs from various systems, including the somatosensory system.

II. Auditory Brainstem Responses

Although changes in anatomy within the IC were not observed, auditory function was compromised in experimental mice as evidenced by ABR testing. ABR data show significant changes in thresholds and peak amplitudes in mice according to genotype. The dramatic increase in thresholds in lacZ mutant mice demonstrate the importance of
reverse signaling on proper auditory function, as auditory impairment was most obvious in the homozygous ephrin-B3\textsuperscript{lacZ} mutant mice. The impairment of function in ephrin-B3\textsuperscript{lacZ} mutants underscores the importance of bi-directional signaling, as ligands not only send information upon binding to corresponding receptors (forward signaling), but also receive signals from the Eph-expressing cell (reverse signaling).

Increases in ABR thresholds are seen most prominently in heterozygous ephrin-B3\textsuperscript{null} mice, while thresholds are only slightly elevated in the homozygous mutation. This result is not easily explained, but may involve the known redundancy of function shared between numerous Eph-ephrin guidance members. It is plausible that the effect of partially expressed ephrin-B3 is more dramatic because when ephrin-B3 is completely lacking, other proteins that share similar binding affinities may assume some of ephrin-B3’s guidance responsibilities.

Despite anatomical evidence showing no effect of ephrin-B3 on the developing projections of interest, ABR data suggest that changes to ephrin-B3 do in fact affect the auditory system and its processing. Amplitudes for Peak I and II were affected by ephrin-B3 mutation, particularly in ephrin-B3\textsuperscript{lacZ} mice. This highlights a reverse-signaling ephrin-B3 role in proper auditory function. The reduction of Peak I and II amplitudes not only indicate that ephrin-B3 has an effect on the magnitude of ABR responses, it also gives us potential clues regarding where anatomical differences may be occurring. As mentioned previously, ABR Peaks I-III are most easily identified and correlate with activity in the auditory nerve, cochlear nucleus, and superior olivary complex, respectively. These data suggest it might be worthwhile to explore the possibility that crude anatomical differences exist in these more peripheral structures downstream of the
midbrain IC. That said, it remains possible that anatomical changes in IC connectivity are in fact present in ephrin-B3 mutants. For one, the fidelity of Peak V that correlates with IC activity is poor in ABRs, precluding any direct physiological midbrain assessments in these mice. Furthermore, the IC receives an impressive array of inputs beyond the three examined in the present studies, and certainly some of these may be guided in part by ephrin-B3 signaling. Finally, it is possible that despite clear differences in ABRs, no obvious anatomical aberrations exist at any level. In other words, the general scheme for major projections patterns may be unaffected by changes in ephrin-B3, while finer-scale synaptic rearrangements on individual neurons may be the root cause of the observed physiological differences.

The present physiological evidence that suggests that ephrin-B3 is important for the establishment of proper auditory function, coupled with previous studies suggesting more concrete roles for ephrin-B3 in other systems, should guide future experiments to pinpoint ephrin-B3’s exact role in the auditory system. Ephrin-B3 has been implicated in preventing contralaterally projecting axons from recrossing the midline of the spinal cord, allowing for unilateral control of individual limbs (Kullander et al., 2001; Yokoyama et al., 2001). Indeed, in mice with mutations to ephrin-B3, unilateral control of limbs is compromised, resulting in a phenotypic hopping gait due to simultaneous movement of both the left and right limbs. Ephrin-B3, being heavily expressed at the midline of the auditory brainstem and auditory midbrain, could be responsible for similar midline interactions in the auditory system. This may impact the ability of contralaterally projecting nuclei to make proper connections, and may have implications on auditory processes such as sound localization that involve comparisons of interaural differences in
timing and intensity of stimuli. Pre-pulse inhibition experiments ongoing in the lab should allow us to test this hypothesis by helping to determine if affected mice can detect and localize stimuli in space.

III. Summary

Neural topography is important for proper function of the nervous system. Organization of neural connections is driven by molecular forces (Eph-ephrins and other guidance proteins) and further refined by both activity-independent (spontaneous firing of hair cells) and activity-dependent (onset of hearing) mechanisms. Our study investigated the influence a sole member of the Eph-ephrin family has on establishing proper connections within the developing IC. We also investigated the establishment of continuous and discrete maps in the IC to determine if both map types exist in the subdivisions of the IC and whether ephrin-B3 is required for the construction of these maps. Our data support the notion that discrete and continuous mapping paradigms do exist in the IC, with continuous mapping properties present in the CNIC and discrete properties exhibited in the LCIC. Furthermore, we were able to conclude that manipulating or removing ephrin-B3 had no qualitative effect on the establishment of proper connections in the IC. However, subtle changes may still exist where we were unable to make conclusions, such as ephrin-B3’s influence on the size and shape of extramodular LCIC labeling. Nevertheless, testing for auditory function through the use of ABRs demonstrated that though no obvious changes were observed in the studied anatomy, ephrin-B3 mutations significantly affect auditory function. Many of the physiological observations are consistent with changes in nuclei downstream of the IC, so anatomical investigations of peripheral and auditory brainstem connections in ephrin-B3
mutants should be considered. There were also considerable differences in auditory function between our two experimental strains of mice, ephrin-B3\textsuperscript{lacZ} and ephrin-B3\textsuperscript{null}. Given the differences in the mutant strains, the results from ABR testing highlight the importance of both the presence of the guidance protein as a whole, as well as reverse signaling mechanisms in axonal guidance.

Members of the Eph-ephrin family have been previously implicated in the development of topographic maps in other systems. In regards to continuous maps, the role of Eph-ephrins in guiding connections that preserve nearest neighbor relationships through the use of countergradients has been well established in the visual system (Luo and Flanagan, 2007). As ephrin-B3 expression is not only not graded but lacking entirely in CNIC, we hypothesized and our data support the notion that it is not involved in establishing a continuous CNIC map. Our extramodular LCIC ephrin-B3 expression data however, suggest a guidance role in the formation of discrete LCIC maps. These data are reminiscent of patch-matrix Eph-ephrin expression maps in the developing striatum (Janis et al., 1999), as well as similar discontinuous patterns in the developing olfactory and somatosensory systems. A center critical to the coordination of sensorimotor and psychomotor behavior (Janis et al., 1999), the striatum perhaps provides the best correlate for our proposed LCIC model. EphA4 and EphA7 receptors are expressed in mosaic-like domains in the striatum that are comparable to the modular-extramodular regions of the LCIC. EphA4 expression correlates directly with ephrin-A4 binding patterns here, while ephrin-A2 and –A5 binding patterns match with EphA7 expression. Similar expression patterns of Eph-ephrins with known binding affinities may exist in the LCIC, and future
experiments are needed to determine the precise interactions that instruct its modular-extramodular arrangement in the absence of experience.

Elucidating the mechanisms that help guide the organization of the nervous system is important for our understanding of several brain conditions. Investigations of the development and plasticity of auditory connections may help to provide more rational treatments and possibly even cures to conditions such as tinnitus and hearing loss. We hope that our investigation of the Eph-ephrin family of guidance proteins and the development of neural maps will help contribute to the growing base of knowledge of the auditory system and the fields of development, plasticity, and functional regeneration.
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