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Development of inputs from the Lateral Superior Olivary Nucleus to the Inferior Colliculus in EphA4 wild type and mutant mice prior to hearing onset

Allyse Bailey Harris
James Madison University

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Development of Inputs from the Lateral Superior Olivary Nucleus to the Inferior Colliculus in EphA4 Wild-Type and Mutant Mice Prior to Hearing Onset

A Project Presented to

the Faculty of the Undergraduate

College of Science and Mathematics

James Madison University

in Partial Fulfillment of the Requirements

for the Degree of Bachelor of Science

by Allyse Bailey Harris

May 2014

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science.

FACULTY COMMITTEE:  

Project Advisor: Mark Gabriele, Ph.D., Associate Professor, Biology

Reader: Janet Daniel, Ph.D., Associate Professor, Biology

HONORS PROGRAM APPROVAL:

Barry Falk, Ph.D.,  
Director, Honors Program

Reader: Justin Brown, Ph.D.,  
Associate Professor, Biology
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Abstract

Processing of sophisticated auditory tasks requires complex circuitry in the brain. These pathways are highly organized, as central connections preserve and integrate stimulus attributes received from the periphery. The focus of our laboratory is centered upon understanding how central auditory connections develop prior to experience (postnatal day 12, P12 in mouse). The present study examines the establishment of projection maps from an auditory center in the brainstem, the lateral superior olivary nucleus (LSO), to the auditory midbrain or inferior colliculus (IC). Previous results from our lab show that LSO to IC projections are arranged tonotopically (i.e. frequency-mapped) before hearing onset, and subsequently these frequency-specific inputs segregate into characteristic axonal layers. Here we test the influence a signaling molecule, EphA4, exerts in establishing this early topography. The Eph family of receptor tyrosine kinases and the ephrin ligands are proteins that are involved in guiding the development of axonal targeting and topographic map formation in other systems. Comparatively less is known about their involvement in the development of the auditory system, particularly their role in the development of patterned inputs to the auditory midbrain. Recent studies reveal EphA4-positive LSO neurons as well as an EphA4 expression gradient along the IC tonotopic axis during the early developmental period when axonal layers emerge. Anterograde fluorescent tract-tracing approaches are used to compare the establishment of ordered LSO-IC projection patterns in wild-type and EphA4 mutants. We hypothesize that EphA4 signaling is necessary for accurate LSO point-to-point mapping and pattern formation in the IC prior to experience. Results indicate no qualitative errors in EphA4 mutants concerning the targeting ability of LSO projections to the IC and subsequent formation of characteristic axonal layers. These findings
provide necessary insights regarding the role Eph-ephrin signaling plays in constructing complex auditory circuits prior to experience.
Introduction

Hearing loss is a disability that affects many US citizens. Approximately thirty-six million Americans, roughly seventeen percent of the nation’s population, have reported some degree of hearing loss (NIDCD, 2010). Artificial hearing implants (i.e. cochlear implants) have assisted in restoring aspects of hearing in some patients. However, these technologies have not been sufficient for all patients suffering from hearing loss. Relatively little is known about the auditory system’s development in establishing connections necessary for hearing. The organization of the auditory circuitry is complex and requires a better foundational knowledge in order to continue to improve treatment strategies for hearing loss. Furthermore, tinnitus is a widespread problem in which patients perceive auditory stimuli (ringing) when none are actually present. Recent studies suggest that alterations in established topographic maps of the auditory system may contribute to tinnitus (Engineer, et al., 2011). Thus, it is important to better understand the manner in which these organized connections are established and ultimately maintained.

When a stimulus reaches the ear, the sound waves are first collected by the external ear, and then pass through the air-filled middle ear cavity. The sound waves then strike the tympanic membrane, causing the malleus, incus and stapes (auditory ossicles) to vibrate and transmit the waves to the fluid-filled cochlea in the inner ear. Ultimately the waves reach the receptor hair cells situated on the basilar membrane housed within the cochlea. Displacement of the basilar membrane causes the stereocilia in the hair cells to bend which subsequently initiates depolarization. Hair cell depolarization creates an action potential that is processed centrally via the auditory nerve. This information is organized based on frequency such that high frequency stimuli are processed at the base and low frequency stimuli are encoded more toward the apex of
the basilar membrane of the cochlea. This frequency-based organization (or tonotopy) established in the cochlea is maintained throughout the ascending auditory pathways. Once in the brain, the signal is sent from the cochlear nucleus (CN) to the superior olivary complex (SOC) (Figure 1). The SOC is a collection of auditory brainstem nuclei which includes the medial nucleus of trapezoid body (MNTB), the medial superior olivary nucleus (MSO), and the lateral superior olive (LSO). The LSO sends efferent projections from the auditory brainstem to the auditory midbrain, or inferior colliculus (IC). The projection from the LSO to the IC is bilateral in that projections are sent from the LSO to both the ipsilateral and contralateral IC (Figure 2).

Before the onset of hearing (postnatal day 12 in mouse), projections from the LSO terminating in the IC are arranged tonotopically (Wallace, et al., 2013). The LSO frequency axis is such that low frequency neurons are located laterally and high frequency neurons more medially (Kelly, et al., 1998). Similarly, the IC exhibits a strict tonotopy, with low frequency regions located dorsolaterally, and higher frequencies represented ventromedially (Schreiner and Langner, 1997). Inputs to the IC are not only frequency-specific, but also arrange into precise patterns of alternating axonal layers (Gabriele, et al., 2000). Tract-tracing experiments confirm adult findings that LSO frequency-specific axonal layers from the two sides terminate in an interdigitating pattern and emerge prior to hearing onset (Gabriele, et al., 2007; Gabriele, et al., 2011; Wallace, et al., 2013).

The Eph family of receptor tyrosine kinases and the ephrin ligands are signaling proteins that are involved in guiding the development of axonal targeting and topographic map formation in a variety of systems. The ephrin ligands are unique in that they are membrane bound, which necessitates cell-to-cell contact to phosphorylate specific tyrosine residues in order to initiate a signaling cascade in the corresponding neuron. This binding may lead to cytoskeletal
Figure 1. Hierarchy and organization of the ascending auditory system.
Schematic illustrating major pathways in the ascending auditory system. The vestibulocochlear nerve carries the signal from the periphery to the brainstem, synapsing in the cochlear nucleus (CN). Auditory information is then channeled bilaterally to various brainstem structures, before converging at the level of the midbrain. The information ascends through the system to levels of thalamus and cortex for processing of higher order auditory tasks. Solid symbols indicate excitatory connections, whereas open symbols indicate inhibitory connections. Colors correspond to ear of origin contributing to input for any given structure. Abbreviations: CN = cochlear nucleus, DNLL = dorsal nucleus of the lateral lemniscus, IC = inferior colliculus, MGN = medial geniculate nucleus, A1 = primary auditory cortex.
Figure 2. LSO to IC projections illustrating pattern formation in CNIC. Schematic depicting layered CNIC pattern. LSO sends bilateral layered projections to ipsilateral and contralateral CNIC.
remodeling by which growth cones exhibit either adhesive or repulsive behaviors (Klein, 2004). Another characteristic of the Eph-ephrin interactions is their capability for bidirectional signaling, both forward and reverse (Figure 3). In forward signaling, ephrins bind to the Eph receptors and trigger a response in the Eph receptor-expressing cell. In the opposite direction, reverse signaling activates the cytoplasmic domain of the ephrin ligand-expressing cell (Cowan and Henkemeyer, 2001; Wilkinson, 2001; Davy and Soriano, 2005; Egea and Klein, 2007).

The Eph-ephrins are divided into two families, the Eph-ephrin As and the Eph-ephrin Bs (Figure 4). Generally, EphAs bind to ephrin-As, and EphBs bind to ephrin-Bs. Cross family binding has been found to occur, however, such as EphA4 binds to ephrin-B2 and ephrin-B3 ligands (Figure 3) (Gale, et al., 1996; Himanen, et al., 2004). Eph-ephrin interactions have been found to have significant roles in other sensory systems. Ephrins and Eph receptors have been found to be expressed in the retina and tectum in gradients that instruct topographic mapping in the visual midbrain in both the anterior-posterior and dorsal-ventral axes (Flanagan and Vanderhaeghen, 1998).

Comparatively less data is available concerning Eph-ephrin involvement in the development of the auditory system, and little is known about their role in the development of axonal layers and topographic projections in the auditory midbrain. However, recent data from our laboratory suggests a role of these proteins in establishing orderly connections in the IC prior to experience. EphA4 and ephrin-B2 in particular, are expressed in the LSO and central nucleus of the inferior colliculus (CNIC) during the period of initial projection shaping (Figure 5) (Gabriele, et al., 2011). Further, their graded expression along the CNIC frequency axis implicates them in a possible role of instructing a tonotopic organization. Additional c-fos neural activation studies and auditory brainstem responses in EphA4 mutants further
Figure 3. Eph-ephrin directional signaling in wild-type and mutant EphA4 $\text{lacZ}$ mice.
Schematic depicting Eph-ephrin bidirectional signaling among specific family members thought to influence developing LSO-IC projections. EphA4 and ephrin-B2, -B3 are known to have strong cross-family binding affinities. LSO axons expressing EphA4 target neurons which express ephrin-B2 in the CNIC. The EphA4 $\text{lacZ}$ heterozygous mutant is capable of forward signaling, however, due to a deletion mutation in a cytoplasmic kinase domain, is incapable of reverse signaling.
Figure 4. Known members of Eph-ephrin signaling family.
The Eph receptor tyrosine kinases and their corresponding membrane-bound ephrin ligands. The Eph-ephrin family is subdivided into A and B subfamilies. Eph-ephrin interactions are involved in the development of various neural systems. Typically EphAs bind to ephrin-As, and EphBs bind to ephrin-Bs. However, there are exceptions where cross family binding also occurs, such as EphA4 binds to ephrin-B2 and ephrin-B3 ligands.
Figure 5. EphA4 expression pattern in early postnatal mouse.
(A) Immunohistochemistry demonstrates a graded expression pattern in CNIC along the
tonotopic axis (ventromedial-dorsolateral). (B) X-Gal staining in EphA4 lacZ mouse confirms
EphA4 graded CNIC expression. Dashed lines indicate ventromedial border of CNIC. Scale
bars=500 μm.
suggest a prominent role in establishing functional auditory circuits (Miko, et al., 2007; Miko, et al., 2008).

Dr. Gabriele’s lab research team has shown three members of the Eph-ephrin family to be expressed in the developing IC when LSO axons are being shaped (EphA4, ephrin-B2, and ephrin-B3) (Figure 5). This study focuses on EphA4-mediated signaling and its role in establishing ordered connections in the projection from the LSO to the IC prior to experience. This was accomplished by confirming established topographic and patterned projections from the LSO to the IC in wild-type (WT) mice at a series of postnatal days leading up to the onset of hearing. WT LSO-IC topographic projection pattern results were compared against EphA4 heterozygous mutants. A future study will explore differences in the homozygous mutation. Our working hypothesis is EphA4 is necessary for accurate mapping of LSO inputs to the IC prior to hearing onset. This data will be helpful in understanding the mechanisms that are responsible for the assembly of early auditory circuits, which could be beneficial in designing new treatments for certain pathologies that are thought to result from alterations in existing topographic maps (i.e. tinnitus).
Materials and Methods

All procedures were approved by the Institution’s Animal Care and Use Committee and conformed to NIH standards (Gabriele IACUC Protocol #A08-09).

Animal Perfusion and Tissue Preparation

EphA4 lacZ mutant mice (n=3 EphA4+/+ and n=7 EphA4lacZ+/+) were used in this study. The lacZ insertion facilitates protein localization studies using X-Gal staining approaches. Additionally, EphA4 mutants have a deletion mutation in a cytoplasmic kinase domain that precludes reverse signaling. Mice were perfused transcardially at early developmental periods, postnatal days P0, P4, and P12 (n=3 at P0, n=4 at P4, and n=3 at P12). An injection of ketamine (100mg/kg) and xylazine (12 mg/kg) was administered intramuscularly. Each pup was allowed sufficient time to be properly anesthetized, which was confirmed by checking for the absence of a toe pinch withdrawal reflex. Tail snip samples were collected from each pup to be used for genotyping procedures, and were stored in separate microcentrifuge tubes in a freezer kept at -80°C. After tail snip collection, a small incision was made just below the rib cage to gain access to the thoracic cavity and heart. A needle, which was attached to a mini-osmotic pump was inserted into the left ventricle, and the right atrium was then cut to allow proper circulation of rinse and fixative. The pump was then switched on in order to deliver a saline rinse (0.9% saline, 0.5% NaNO₂) to the heart, followed by a 4% paraformaldehyde fixative solution. After the appropriate amount of fixative was delivered and the pup was adequately perfused, the skull was removed and placed into a jar of fixative and kept at 4°C. The brains were then removed carefully from their skulls using a dissecting microscope, and placed back into the jar of fixative and stored in the 4°C refrigerator until the tissue was ready to be used.
Genotyping

In order to determine the genotypes for each case, a three day genotyping procedure was conducted. On the first day, tail snips underwent a procedure to digest them and ready them for DNA isolation using an Invitrogen Easy-DNA kit. A solution of 320μL of TE Buffer, 20μL of Solution A, 10μL of Solution B, and 5μL of Protein Degrader was added to each microcentrifuge tube containing a mouse tail. The tubes were then placed into a heat block set at 68°C, and were rocked overnight for a period of about 20 hours.

After the tails had been sufficiently digested, the second day of genotyping protocol started by adding 300μL of Solution A and 120μL of Solution B to each microcentrifuge tube. Each tube was then inverted several times and vortexed until the solution was uniform. Then, approximately 750μL of chloroform was added to each tube, and each tube was then vortexed until the solution in each tube appeared homogeneous. The tubes were then centrifuged for 10 minutes in a centrifuge set to 4°C. After centrifugation, the upper aqueous phases from each tube were transferred to new microcentrifuge tubes, labeled respectively, to start DNA precipitation. Each tube received approximately 1.0 mL of 100% ethanol, previously kept at -20°C, and was then vortexed and incubated on ice for 30 minutes. After the ice incubation, each tube was centrifuged for 15 minutes, and the ethanol in each was gently poured off. Another ethanol rinse was added of 500μL 80% ethanol, also previously kept at -20°C. The tubes were mixed by inversion, and then centrifuged again for 5 minutes. The ethanol was poured off gently from each tube, followed by a last centrifugation for 3 minutes. Any residual ethanol left in the tube was carefully drawn out using a 20μL pipet. To resuspend the DNA pellet now present in each tube, a mix of 49μL of TE buffer and 1μL of RNAse was added to each tube and vortexed. The tubes were then placed in a heat block set to 37°C for 30 minutes.
After the incubation in the heating block, the DNA was ready for PCR preparation. A solution of 23μL of dH2O and 0.5μL of 10μM primer working solutions was added to each illustraPuRe Taq PCR tube (GE Healthcare Piscataway, NJ). Then 1.5μL of the template DNA from each sample was added to its appropriate PCR tubes. The PCR tubes were vortexed and centrifuged in a minifuge for one minute. The tubes were then placed into a PCR machine, and the PCR program cycle was run. During each cycle, the machine would run these steps 31 times: 94°C for 30 seconds, 94°C for 30 seconds, 56°C for 30 seconds, and then 72°C for two minutes. Then after the 31 repetitions were complete, the machine would keep the tubes at 72°C for 10 minutes, and then kept at 4°C until ready to be run in the gels.

The PCR products were then ready to be loaded into the wells of Invitrogen pre-stained ethidium bromide gels. Before loading, 5μL of each PCR product was individually mixed with 2μL of agarose loading dye and 10μL of ultrapure dH2O. Then, the mixture containing the product, loading dye, and dH2O was loaded into individual wells. Gels were run for 30 minutes, and subsequently imaged by a Bio-Rad Gel Documentation System (Bio-Rad Hercules, CA) using Quantity One software. The wild-type bands ran out at 639 bp and the mutant bands ran out at 800 bp (Figure 6).

Tissue Labeling, Sectioning, and Mounting

In order to label the developing neuronal pathways from the LSO to the IC, the fixed brain tissue was first blocked rostral to the superior colliculus and caudal to the cerebellum. Blocked brains were then embedded in an egg yolk-gelatin mixture and allowed to sit in fixative in the 4°C refrigerator for 24 hours. Coronal sections beginning at the caudal end of the tissue block were then taken using a Vibratome until the LSO was reached, as confirmed with darkfield microscopy. With the aid of a dissecting microscope, a small incision was made in the
Figure 6. Gel electrophoresis of PCR products for identification of EphA4 lacZ genotypes.
PCR products from EphA4 tail snips were run on 2% agarose gel. Two lanes correspond to genotype for one animal, with different lanes corresponding to different primers for wild-type and mutant alleles. For EphA4 wild-type animals, only a 639-bp band was observed (wild-type EphA4 allele, lane 4; no product for the mutant allele, lane 5). Heterozygous EphA4 lacZ mutants revealed both a 639-bp band for the wild-type allele and an 800-bp band for the mutant allele (lanes 2, 3). 100-bp DNA ladder shown in lane 1.
Figure 7. Schematic depicting anterograde experiment using NeuroVue dye. Anterograde approach tract-tracing studies. Localized NeuroVue dye placements in the LSO yield resultant labeling of axons in the CNIC.
LSO. Then, a minute sliver of NeuroVue Red (Molecular Targeting Technologies, Inc., West Chester, PA) was inserted into the LSO (Figure 7). After the dye placement, the brains were then incubated at 37°C in formaldehyde fixative for at least 2-3 months to allow for proper dye diffusion.

After the incubation period, the remaining tissue block containing the LSO-IC projection was sectioned on the Vibratome at 75μm in 0.1M phosphate buffer. The sections were treated with a fluorescent bis-benzimide counterstain for five minutes, and then rinsed with 0.1M phosphate buffer three more times, for five minutes each rinse, in petri dishes. Then, sections were mounted and coverslipped onto charged glass slides using GelMount media. Finally, slides were sealed with Cytoseal a few hours after mounting and were ready for imaging.

**Microscopy and Imaging**

A Nikon TE 2000 microscope (Nikon Inc., Melville, NY) equipped with a monochrome cooled CCD CoolSnap HQ digital camera (Roper Scientific, Tucson, AZ) were used to image the fluorescent material. Specific filter sets were applied in order to visualize the NeuroVue Red Dye (R and B Phycoeythrin filter set) and bis-benzimide stain (DAPI filter set). In order to observe landmarks in the tissue, nuclear boundaries, and fluorescent labeling, monochrome channels were merged together and pseudocolored red and blue. Images were taken at different magnifications, including 4x and 10x, of the injection site, LSO, and ipsilateral and contralateral IC’s through the rostrocaudal extent of each case. For images taken at a magnification of 10x or higher, a series of images along the z-axis of the 75μm section using the three dimensional z-stack application (NIH Elements Software, Nikon Inc.) was captured in order to visualize all axonal layers within the section. The z-stack was then flattened into a two-dimensional image.
through the maximum projection application, and focused. Every image was saved as a JPEG2000 file.
Results

Anterograde tract-tracing experiments were conducted in order to assess the role that EphA4 plays in the developmental organization of afferent layers in the CNIC prior to experience in EphA4$^{+/+}$ and EphA4$^{lacZ+}$ mice. As anticipated, focal dye placements made in lateral aspects of LSO in a P12 EphA4 wild-type mouse yield clear laminae in ventral, high-frequency regions of CNIC (Figure 8). This data confirms previous findings that LSO projects to the IC and forms refined layers in the CNIC by the onset of hearing. After this confirmation, we then focused on earlier postnatal ages (P0, P4), as this is the time LSO pioneer axons invade the CNIC and afferent layers emerge. P0 cases demonstrate LSO projections have entered the IC by birth, and at P4 projection-shaping and clear laminar layers are evident.

At P0, dye in the lateral LSO yielded layered input to the lateral ipsilateral CNIC in an EphA4 wild-type mouse (Figure 9). Multiple laminae covering more of the ventromedial-dorsolateral axis were also observed in the contralateral CNIC in the same animal. The presence of this organized pattern formation indicates that projections entering the CNIC from the LSO begin to already order themselves into distinct isofrequency laminae at birth. The observed presence of this pattern in the contralateral CNIC also indicates that the projections are able to cross the midline beginning by birth.

In age-matched heterozygous mutants (EphA4$^{lacZ+}$), medial LSO labeling is similarly seen in the CNIC both ipsilaterally and contralaterally (Figure 10). The contralateral CNIC input is clearly organized into laminae in the more medial aspect of the nucleus (arrowheads). This indicates that these inputs in the heterozygous mutant are able to effectively navigate the midline, unlike contralateral projections in Eph-ephrin mutants in other systems (Petros, et al., 2008). Ipsilateral input is also observed at birth in the heterozygous mutant in frequency-
**Figure 8. LSO-IC projection and pattern formation in EphA4 wild-type at hearing onset.**

Anterograde tracing experiment of the projection from LSO to IC in a P12 wild-type mouse. As anticipated based on previous experiments, dye placements centered in lateral aspects of LSO (A) resulted in highly refined axonal layers (*arrows*) in lateral matched domain of target IC (B, C). Inset in (B) is shown at a higher magnification in (C). Scale bars in A, B=500 μm; C=200 μm.
Figure 9. LSO-IC projections and layered input organization in EphA4 wild-type at birth. NeuroVue dye placed in lateral LSO (A) to label inputs to the CNIC in an EphA4 wild-type mouse at birth, P0. Pioneer LSO axons have entered the ipsilateral (B) and contralateral (C) CNIC. The organization of invading axons appears to be patterned in a layered laminar formation as indicated by the arrowheads. Contralateral CNIC laminae cover more of the frequency axis (C), whereas input in the ipsilateral CNIC appears to be refined into a single visible isofrequency lamina (B). Dashed lines indicated ventromedial borders of CNIC. Scale bars in A=500μm; B, C=200μm.
Figure 10. LSO-IC layered projection distributions at birth in EphA4<sup>lacZ/+</sup> mouse. Medial LSO injection (A) yields resultant labeling in ipsilateral and contralateral CNIC in an EphA4 heterozygous mutant at birth, P0. Arrowhead in (A) indicates retrograde labeling of the MNTB cell bodies, confirming that the injection was made in LSO. Ipsilateral CNIC (B) labeling is present but not as prominent likely due to labeling of lateral aspects of the superior periolivary nucleus. The arrowhead in (C) identifies the distinct dense layer labeled in the corresponding ventromedial, high-frequency aspect of contralateral CNIC. Dashed lines represent the ventromedial borders of CNIC. Scale bars in A=500μm; B, C=200μm.
matched high-frequency aspects of the CNIC that correspond to a medial LSO injection. In Figure 10, the ipsilateral labeling does not appear as clearly organized into specific laminae as it does in the contralateral CNIC. This is likely due to unforeseen leaching of the dye into lateral aspects of the neighboring superior periolivary nucleus, which sends projections to the ipsilateral IC.

In P4 EphA4\textsuperscript{lacZ/+} mice, dye placements in mid-frequency regions of the LSO result in corresponding mid-frequency labeling in the ipsilateral CNIC (Figure 11). Contralateral CNIC labeling was also observed in the P4 mutant, forming several distinct bands centered in the mid-frequency region. This confirms that projections are able to cross the midline in this later developmental period in the mutant. These observations indicate that during the postnatal period when projection-shaping is at its development peak (P4) neurons are able to target the CNIC bilaterally from the LSO and form organized afferent layers in the EphA4 heterozygous mutant.

Another P4 EphA4\textsuperscript{lacZ/+} case confirms the previously described LSO-IC projection findings in a similar mid-frequency LSO injection (Figure 12). Several distinct layered bands are observed in the ipsilateral CNIC, found in the more central aspect of the nucleus corresponding to the mid-frequency LSO injection. In the contralateral CNIC, layers are also observed in the central aspect in an ordered laminar fashion. This contralateral labeling again confirms that during the P4 postnatal developmental period, projections are crossing the midline and targeting the contralateral CNIC.

Thus, in comparing the wild-type EphA4s and the heterozygous mutants, both genotypes demonstrate bilateral frequency-specific LSO projections to the CNIC. Additionally, these inputs exhibit characteristic layered arrangement where the afferent inputs form distinct bands or laminae in the ipsilateral and contralateral IC, beginning at birth. The presence of the
Figure 11. EphA4<sup>lacZ/+</sup> mutant LSO-IC projections and subsequent pattern formation at P4. Medial LSO injection (A) results in labeling in both ipsilateral and contralateral CNIC at postnatal day 4 in an EphA4 heterozygous mutant. Arrowhead in (A) indicates labeling of the medial nucleus of the trapezoid body, confirming that the injection was made in LSO. Axons are seen growing into the corresponding mid-frequency region of the ipsilateral CNIC (B). Arrowheads in (C) highlight multiple layers in the mid-frequency aspect of contralateral CNIC. Dashed lines represent the ventromedial borders of CNIC. Scale bars in A=500μm; B, C=200μm.
Figure 12. Bilateral LSO-IC projections and presence of prominent axonal layers in EphA4lacZ/+ at P4.
Mid-frequency NeuroVue placement in LSO (not pictured here) yields labeling in mid-frequency aspects of ipsilateral and contralateral CNIC. (A) Low magnification digital merge illustrating bilateral projections from LSO in the IC. Insets in (A) are shown at higher magnifications in (B) and (C). Several layers are seen in ipsilateral CNIC (B) as distinguished by arrowheads. Arrowheads in (C) point out the two layers present in contralateral CNIC. Scale bars in A=500μm; B, C=200μm.
contralateral labeling and organization demonstrates that in EphA4^{+/+} and EphA4^{lacZ/+} pioneer axons are able to cross the midline to reach the contralateral CNIC, and retain the ability to arrange themselves in the described banded pattern. These findings across the developmental period from birth to onset of hearing indicate that these projections begin organizing themselves as early as P0, and continue to refine their layering at P4 in wild-types and heterozygous mutants.
Discussion

Processing and encoding auditory stimuli is complex and requires precise circuitry in order to preserve stimulus attributes such as frequency, intensity, and location. This process begins peripherally at the external ear and cochlea, and the information is received by different nuclei in the central auditory system through neuronal pathways to eventually reach cortex. The pathways involved must be fully developed and functional by the onset of hearing, and there are many influences on which this development depends.

While there has been much research conducted on the organization of the auditory system, relatively little is known about how the circuitry and patterns are established during development. The Eph-ephrin family of receptor tyrosine kinases and their ligands has been shown to be involved in neuronal guiding and map formation in other sensory systems (Flanagan and Vanderhaeghen, 1998). Recent findings in our lab have shown several members of this family to be expressed in the IC and LSO during the critical developmental period of projection shaping prior to hearing (Gabriele, et al., 2011). The current study focused on one of these members, EphA4, and discovering its possible role in guiding neuronal targeting and organization of projections from the LSO to the CNIC. Anterograde tract-tracing studies were performed in EphA4 wild-type and heterozygous mutant mice to assess how input is ordered in the IC.

The results of this study revealed the bilateral patterned arrangement of LSO to IC input in the form of layers in both the wild-type and heterozygous mutant mice. EphA4+/+ cases showed the presence of these layers at the time of birth (Figure 9), and its refinement continued until the onset of hearing at postnatal day 12 (Figure 8). Layers were seen forming in both the ipsilateral and contralateral CNIC, implicating that the projections could cross the midline as well. After confirming this development of layered input in the wild-type mice, similar
anterograde experiments conducted in EphA4\textsuperscript{lacZ/+} mice yielded similar results in seeing the emergence of layered organization in the CNIC. At birth, the axons can be seen growing into the CNIC and beginning to differentiate themselves into layers (Figure 10). Further along in development at postnatal day 4, we continue to see labeling in the CNIC and further refinement into individual layers (Figures 11, 12). The projections in the mutant were seen bilaterally as well, demonstrating that the axons are able to cross the midline.

The results seen in the heterozygous mutant suggest that EphA4 reverse signaling may not be necessary for afferent projections from LSO to segregate themselves into layers within the CNIC. The results further suggest that EphA4 reverse signaling may not be necessary in axonal midline decisions, since labeling was consistently seen and evident in the contralateral CNIC in the mutant cases. However, the mutants tested here still retain a functional copy of the EphA4 gene, and in order to be able to fully understand the role EphA4 may play in circuitry development, future experiments involving EphA4 homozygous mutant mice would need to be accomplished in order to address its functionality in making these path-finding and targeting decisions.

The data presented in this study is concerned with pattern formation and did not specifically look at LSO to IC projections topographically. Therefore, future quantification of these findings is necessary to directly correlate aspects of LSO-IC frequency mapping in wild-type and EphA4 mutants. This would be accomplished by calculating the spread and location of dye in the LSO and then seeing the spread and location of labeling in the IC. While these studies suggest that EphA4 may not be needed in pattern formation, future results could determine whether or not it is necessary in topographic map formation.
In addition to the described future studies, investigating the physiological and behavioral responses in EphA4 wild-type and heterozygous mutant mice would also be important, especially if the homozygous mutants demonstrated a system with less refinement and accuracy in projection development. Some studies have already been conducted in addressing this aspect by looking at auditory brainstem responses in EphA4 deficient mice. Auditory brainstem responses are used to observe several aspects of hearing including response threshold latencies. EphA4\textsuperscript{lacZlacZ} mice have been reported to exhibit elevated ABR thresholds, increased latencies, and reduced peak amplitudes. This evidence indicates the presence of hearing deficits in EphA4 homozygous mutant mice, which further emphasizes the potentially important role EphA4 plays in forming functional auditory pathways (Miko, et al., 2007).

Although the specific mechanism of how EphA4 works in regulating afferent projections from LSO to IC during neuronal development is still unclear, the findings of this study do help in creating a better understanding of how input is organized in EphA4 mice before the onset of hearing. Its role in mice completely devoid of a functional EphA4 allele in the arrangement of the seen layers is yet to be determined, and could be an aim of a future student’s research question in the lab. The role of EphA4 in tonotopy establishment in both heterozygous and homozygous EphA4 mutants is a point of interest for future experiments as well. The value of this knowledge is especially helpful in the design of therapeutic interventions for people who suffer from hearing loss or tinnitus. The results add to the fundamental goal of better understanding the development and organization of auditory circuitry as a whole so that hearing loss problems associated with these different pathways can be addressed.
References


Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. Neuron. 17(1): 9-19.


topography but not pattern formation of lateral superior olivary inputs to the inferior colliculus.

Neurosci. 2(3): 155-164.