**Investigate the role of ephrin-A3 in Tonotopic Map Formation of the Mouse Cochlear Nucleus**

Yazan Altarshan*, Ahmad Alzein*, Natalia Hoshino, Amali Fernando, Michael William Rochlin Wei-Ming Yu

Department of Biology, Loyola University Chicago

(* equal contribution)

---

**Introduction**

Sound information is transmitted from cochlea, a structure within the inner ear, to the cochlear nucleus (CN) via spiral ganglion neurons (SGN). Both structures feature a tonotopic gradient as the neurons therein are organized by frequency response from high to low frequencies. SGN innervate the CN in a tonotopic fashion, preserving the tonotopic gradient as sound information flows from the inner ear to the brain (Fig. 1) (Yu, 2014). The purpose of this study is to determine what molecules regulate SGN axon guidance and thereby create a tonotopic gradient of information processing within the auditory system.

**Methods**

The first step determining if Ephs and ephrins are involved in the tonotopic map formation of the cochlear nucleus, is detecting if these proteins are present during neurodevelopment. To identify which Ephs and ephrins are present during development, RNAscope technology, a modification of in situ hybridization, was conducted on E15.5 and E17.5 embryos. To obtain cochlear nucleus samples, the head of the embryo was fixed; subsequently, 20 μm coronal sections of whole heads are prepared. mRNA signals were detected using RNAscope Detection Reagents. Finally, the chromogenic signals of mRNA were visualized under a microscope.

Once the ephrins present within the CN during tonotopic map formation were identified, stripe assays were conducted to determine the effect of the guidance cue on the SGN. A silicone matrix was used to apply a solution of the guidance cue to a cover glass in a striped pattern. The stripes containing the guidance cue were fluorescently labeled. Between these stripes, a control strip was created with solution lacking the ephrin molecule. Expiants of the CN were placed on the coverglass and subsequent outgrowth was measured. Ephrin-A3 mutant mice were also imported and tested for hearing functions using recordings of auditory brainstem responses (ABRs). ABRs are electrophysiological responses of neurons in the cochlea and auditory brainstem after providing sound stimuli to mice. It provides an objective quantification of hearing and can detect even subtle changes of sound detection in the cochlea and sound processing in the brainstem.

---

**Results**

Ephrins and ephrins are a family of signaling proteins that are known to be involved in contact-dependent axon guidance. Previous studies have shown the involvement of Eph receptors and ephrin ligands in the development of topographic gradients within other sensory systems. This study seeks to determine the role of ephrin-A3 in tonotopic map formation.

**Figure 1. Schematic drawing of tonotopic map within the auditory circuit.** On the VCN is shown: DCN also features the same tonotopic gradient.

**Figure 2. Schematic of Eph receptors and ephrin ligands.**

Ephs and ephrins are a family of signaling proteins that are known to be involved in contact-dependent axon guidance. In this study, the ephrin-A3 gene is null mutant and the control littermates were prepared. Ephrin-A3 is a known molecule that is involved in axon guidance and synaptic plasticity within the auditory system. In the control cultures, the ephrin-A3 gene was not null, and the control cultures were used as a reference for the ephrin-A3 mutant cultures.

**Figure 3. Expression of ephrin-A in the CN from E15.5 and E17.5 mouse embryos.** (A-F) The expression levels of ephrin-A2, -A3, and -A5 in the CN were detected by RNAscope® in situ hybridization using a non-AR-based chromogen (blue) or an AP-based chromogen (red). Cochlear nuclei are outlined with red dashed lines. For E15.5, the CN anlage is shown, and for E17.5, the VCN anlage is shown. F9: eighth nerve root. LV4: lateral recess of 4th ventricle.

The RNAscope data indicates that ephrin A1 and A4 are not present in the developing CN and ephrins A2 and A5 present at low levels. Expression of ephrin-A3 is particularly interesting in that it is expressed at high levels with a regional difference within the cochlear nucleus. This graded expression of the contact-dependent guidance cues allows for the possibility that ephrin A3 is involved in the tonotopic map creation. Thus, stripe assays were conducted using this transmembrane guidance cue. In these experiments, the cochlear nucleus was dissected and placed on the coverglass containing the stripes.

**Figure 4 Ephrin-A3 stripe assays.** In control cultures both stripes contained pre-clustered unconjugated human-IgG-Fc but the first stripe was pre-clustered with an Alexa-488-goat anti-human IgG-Fc (shown in pseudo-colored magenta). In ephrin-A3 cultures, the first stripe contained ephrin-A3-Fc pre-clustered with an Alexa-488-goat anti-human IgG-Fc (shown in pseudo-colored magenta) and the second stripe was the same as in the control cultures. A subset of SGN fibers are repelled by ephrin-A3-Fc stripes. (D) High magnification of the boxed region in C shows the hair cells labeled by Myosin-6 staining (shown in green) and the SGN peripheral fibers labeled by neurofilament/GAP43 (red). Arrowheads (in C) indicate hair cells in the sensory epithelium labeled by Myosin-6 staining (shown in green). Neurites were labeled in red by neurofilament/GAP43 staining.

Using the explant culture system, we found that a subset of auditory nerve fibers were repelled by stripes containing ephrin-A3-Fc (40 μg/ml, shown in magenta stripes in Fig. 4B, C). These observations lead us to suggest that ephrin-A3 forward signaling plays a role in controlling the growth response of auditory nerve fibers in tonotopic map formation in the CN and the populations of auditory nerve fibers are differentially sensitive to ephrin molecules.

**Figure 5 ABR analysis of Ephrin-A3 mutants.** Representative ABR recordings from a 6-week old control (blue traces) and ephrin-A3 null mutant (red traces) exposed to 8 kHz, 11.3 kHz, 16 kHz, or 22.6 kHz pure tone stimuli at intensity of 90 dB sound pressure level (SPL). Roman numerals mark the ABR waves. ABRs are delayed in the ephrin-A3 mutant compared to its control littermate.

**Future Directions**

We will assess sound discrimination ability of ephrin-A3 mutant mice using an acoustic startle response-based assay.

We will also evaluate tonotopic map precision of the auditory brainstem in ephrin-A3 mutants by c-fos induction after auditory stimulation.

Ephrin ligands interact with Eph receptors. Currently, we are studying the ephrins of interest within the CN, yet to determine the molecular mechanism of tonotopic map formation, we must also examine the presence and the significance of Eph receptors on the SGN. We posit that the Eph receptor expression varies with the frequency response of the neuron, and thus that Eph-ephrin signaling can mediate tonotopic map formation. To determine if Eph receptors are present on SGN, a RNAscope assay will be conducted using a whole mount cochlea.

**References**

