Spatiotemporal Analysis of Cochlear Nucleus Innervation by Spiral Ganglion Neurons that Serve Distinct Regions of the Cochlea

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Abbreviations:

SGNs, spiral ganglion neurons; ANFs, auditory nerve fibers; CN, cochlear nucleus; AVCN, anteroventral cochlear nucleus; PVCN, posteroventral cochlear nucleus; DCN, dorsal cochlear nucleus; pHF and pLF, prospective high- and low-frequency; Ngn1, Neurogenin1; E, embryonic day; P, postnatal day; PFA, paraformaldehyde; PBS, phosphate buffered saline; ANOVA, one-way analysis of variance; PLAP, placental alkaline phosphatase; CC3, cleaved caspase 3; RGC, retinal ganglion cell
Abstract

Cochlear neurons innervate the brainstem cochlear nucleus in a tonotopic fashion according to their sensitivity to different sound frequencies (known as the neuron's characteristic frequency). It is unclear whether these neurons with distinct characteristic frequencies use different strategies to innervate the cochlear nucleus. Here, we use genetic approaches to differentially label spiral ganglion neurons (SGNs) and their auditory nerve fibers (ANFs) that relay different characteristic frequencies. We found that SGN populations that supply distinct regions of the cochlea employ different cellular strategies to target and innervate neurons in the cochlear nucleus during tonotopic map formation. ANFs that will exhibit high-characteristic frequencies initially overshoot and sample a large area of targets before refining their connections to correct targets, while fibers that will exhibit low-characteristic frequencies are more accurate in initial targeting and undergo minimal target sampling. Moreover, similar to their peripheral projections, the central projections of ANFs show a gradient of development along the tonotopic axis, with outgrowth and branching of prospective high-frequency ANFs initiated about two days earlier than those of prospective low-frequency ANFs. The processes of synaptogenesis are similar between high- and low-frequency ANFs, but a higher proportion of low-frequency ANFs form smaller endbulb synaptic endings. These observations reveal the diversity of cellular mechanisms that auditory neurons that will become functionally distinct use to innervate their targets during tonotopic map formation.

Keywords: auditory system, spiral ganglion neuron, auditory nerve fiber, cochlear nucleus, tonotopy, innervation
INTRODUCTION

The sense of hearing allows humans and animals to distinguish different sound stimuli they are exposed to, not only in the strength and pitch of the sound, but also in the direction and duration. To accomplish this complicated task, the auditory system is organized with precisely wired circuits and specialized synaptic structures (Yu & Goodrich, 2014). The auditory circuit first arises from spiral ganglion neurons (SGNs) in the cochlea extending the peripheral processes of their auditory nerve fibers (ANFs) to receive inputs from hair cells (Appler & Goodrich, 2011; Rubel & Fritzsch, 2002). SGNs then transmit sound information to the auditory brainstem through the central projections of ANFs. Upon entering the brainstem, each individual ANF bifurcates and innervates the three subdivisions of the cochlear nucleus (CN) (Fig. 1A) (Fekete, Rouiller, Liberman, & Ryugo, 1984). The ascending branch projects toward the anteroventral cochlear nucleus (AVCN) and elaborates a large synaptic ending, known as the endbulb of Held, on the bushy cell (Ryugo & Fekete, 1982). By comparison, the descending branch travels through the posteroventral cochlear nucleus (PVCN), terminates in the dorsal cochlear nucleus (DCN), and innervates a variety of target neurons along the way with conventional bouton-type synapses (Rouiller, Cronin-Schreiber, Fekete, & Ryugo, 1986).

In each of the three subdivisions of the CN, the innervation by ANFs forms tonotopic maps where neuronal connectivity is organized in an orderly arrangement according to frequency responses (Fekete et al., 1984; Kandler, Clause, & Noh, 2009; Muniak et al., 2013; Ryugo & May, 1993; Ryugo & Parks, 2003). Each SGN is most sensitive to a particular sound frequency, which is known as the neuron’s characteristic frequency (Kiang & Moxon, 1972). SGNs with high-characteristic frequencies in the base of the cochlea send their ANFs to dorsal regions of the CN subdivision, while SGNs having low-characteristic frequencies at the apical end of the cochlea project their ANFs to ventral portions of the CN subdivision (Fekete et al., 1984). The axon terminal arbors of ANFs with similar characteristic frequencies then form isofrequency bands to activate nearby target CN neurons (Young & Rubel, 1983). This tonotopic arrangement allows animals to separate a complex sound into its frequency components, which forms the basis of sound discrimination. Despite the importance of tonotopy in auditory functions, how auditory circuits assemble to form tonotopic maps remains largely unknown.

While the gross organization of the tonotopic projections has been examined (Fekete et al., 1984; Koundakjian, Appler, & Goodrich, 2007; Leake, Snyder, & Hradek, 2002; Molea & Rubel, 2003), the cellular events (e.g., initial mapping precision/targeting/pruning) of how ANFs with different characteristic frequencies innervate the CN have not yet been determined. It is also
unclear whether SGN populations that serve distinct regions of the cochlea use different cellular strategies to assemble the circuit during tonotopic map formation. One obstacle to address these questions is the lack of a reliable way to differentially label these populations of SGNs. Although traditional histology approaches using dye labeling in anatomic tracing studies have provided valuable insight on how cochlear ganglion neurons innervate the CN during tonotopic map formation (Leake et al., 2002; Snyder & Leake, 1997), they have several limitations. As a surgical intervention is required to inject the dye into the cochlea, it is technically challenging to perform this procedure in embryonic stages. Consequently, these studies only assessed postnatal development, long after the initial establishment of the circuit. Additionally, since dye injections are made through the round window in the inner ear, only a limited subset of SGNs from the relatively high-frequency region at the base of the cochlea can be labeled. Therefore, it is difficult to use this approach to compare the cellular strategy used by SGNs that supply high-versus low-frequency regions of the cochlea.

SGNs originate from a neurogenic domain of the otic vesicle by transiently expressing the transcription factor Neurogenin1 (Ngn1) in a basal to apical progression along the length of the cochlea between E9.5 and E12.5 in mice (Koundakjian et al., 2007; Ma, Chen, del Barco Barrantes, de la Pompa, & Anderson, 1998). A small subset of SGNs can be genetically labeled using the \textit{Ngn1-creER}^{T2} mouse line and a Cre-dependent reporter upon induction of Cre recombination by a single low-dose tamoxifen administration (Koundakjian et al., 2007). Using this approach, Koundakjian et al. were able to reproducibly label SGNs and their ANFs that ultimately respond to different sound frequencies by providing tamoxifen at a specific time point between E9.5 (start of neurogenesis) and E12.5 (end of neurogenesis). For simplicity, although characteristic frequencies of SGNs develop after innervation of the CN, we will refer to SGNs that will ultimately have high- or low-characteristic frequencies as “prospective high- or low-frequency (pHF or pLF) SGNs” and refer to them as “functionally distinct.” In this study, we employed the same genetic strategy to respectively label pHF and pLF SGNs and their ANFs and investigate how distinct populations of SGNs explore and innervate the CN. We first used the \textit{Ngn1-creER}^{T2} line and the \textit{R26iAP} Cre reporter to label pHF and pLF ANFs and compared their overall innervation patterns in the CN at different stages. We then used the \textit{Ngn1-creER}^{T2} line and the \textit{Ai14-tdTomato} Cre reporter to trace individual ANFs at single-synapse resolution throughout development to determine if pHF and pLF ANFs synapse differently on CN neurons. We found that functionally distinct SGN populations employ different cellular mechanisms to target and innervate CN neurons during tonotopic map formation.
EXPERIMENTAL PROCEDURES

Animals

All animal experiments in this study have been performed in compliance with institutional and National Institutes of Health guidelines approved by the Institutional Animal Care and Use Committee at Loyola University Chicago (Protocol 1926). All efforts were made to minimize the number of mice used and their suffering. The following mouse strains were used: Ngn1-creERT2 mice (Koundakjian et al., 2007), R26iAP mice (Stock Number 009253; Jackson Laboratories, Bar Harbor, ME), and Ai14-RCL-tdTomato mice (Stock Number 007908; Jackson Laboratories, Bar Harbor, ME). All mice were maintained on the CD1 background. For timed pregnancies, embryonic day 0.5 (E0.5) was defined as noon on the day of the presence of a vaginal plug.

Tamoxifen administration

Tamoxifen (J63509; Alfa Aesar, Haverhill, MA) was dissolved in sunflower seed oil (S5007; Sigma-Aldrich, St. Louis, MO) at a concentration of 5 mg/ml by shaking at 37°C for three hours with vortexing every 30 minutes. For oral gavage of tamoxifen, each pregnant dam was given a single low-dose of tamoxifen at 0.5 mg per 40 g maternal bodyweight on E9.5 or E12.5 using a 20G disposable feeding needle (D-001; Braintree Scientific, Braintree, MA). To alleviate the estrogen agonist effects of tamoxifen during fetal development (Lizen, Claus, Jeannotte, Rijli, & Gofflot, 2015), β-estradiol (E8875; Sigma-Aldrich) at 1/1000th concentration to the tamoxifen was co-administrated in all experiments. However, delayed delivery of pups due to dystocia was still observed in some dams older than 6 months of age. To circumvent this problem, female mice younger than 4 months old were used. Alternatively, E19 pups were collected from the tamoxifen-treated pregnant dam by caesarian section and nursed by a CD1 foster mother.

Immunohistochemistry and placental alkaline phosphatase (PLAP) staining

Tissue processing of embryo heads, mouse brains, and inner ears were performed as previously described (Yu et al., 2013). Briefly, embryonic and postnatal day (P) 0 mouse heads were fixed directly in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4°C. P5, P10, or P20 mice were anesthetized with ketamine and xylazine and then transcardially perfused with 4% PFA in PBS. Inner ears and brains were collected and post-fixed in 4% PFA for two hours or overnight at 4°C. Inner ears from P10 or P20 mice were decalcified in 120 mM EDTA in PBS at room temperature for 2 days after post-fixation.
For whole-mount PLAP staining, cochleae were incubated at 67°C for 1 h to heat inactivate endogenous alkaline phosphatase activity. Tissues were then incubated in a diluted PLAP staining solution containing 0.25 mg/ml nitroblue–tetrazolium– chloride (N6876, Sigma-Aldrich), 0.025 mg/ml 5-bromo-4-chloro-indolyl-phosphate (B6149, Sigma-Aldrich), 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50mM MgCl₂ for 30 minutes to overnight. Stained cochleae were dehydrated in 100% methanol to reduce background, rehydrated in PBS, and cleared in 80% glycerol. For cryosectioning, tissues were steeped through 10, 20, and 30% sucrose in PBS, and embedded in NEG 50 (Richard-Allan Scientific, San Diego, CA). Coronal brain sections through the cochlear nucleus were prepared at 30 µm and heat inactivated for endogenous alkaline phosphatase activity at 65°C for 40 minutes. Sections were stained in diluted PLAP staining solution for 4 to 6 hours, dehydrated in methanol, rehydrated, and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL).

Individual ANFs were visualized in mice carrying the Ngn1-creERT² and Ai14-tdTomato alleles upon tamoxifen administration. The Ai14-tdTomato Cre reporter line is much more sensitive to low levels of Cre than many other Cre reporter lines (Alvarez-Aznar et al., 2020), including the R26iAP Cre reporter. Basal CreERT² leakage would result in sparse recombination of the Ai14-tdTomato allele even without tamoxifen administration. Accumulation of low levels of tdTomato proteins would eventually result in random, sparse background labeling of a few ANFs in postnatal stages. Therefore, for samples from P5, P10, or P20 mice, only fibers with highly intense tdTomato fluorescence were analyzed. The embryonic heads and postnatal brains were embedded in 4% low-melting agarose (IBI Scientific, Dubuque, IA) in PBS. Sagittal head and brain sections through all subdivisions of the cochlear nucleus were prepared at 60 µm using a vibratome (VT1000s; Leica Biosystems, Wetzlar, Germany). Free-floating sections were blocked for one hour at room temperature in a solution containing 5% normal goat serum and 0.3% Triton X-100 in PBS. Sections were then counterstained with an anti-NeuN antibody (ab177487; Abcam, Cambridge, MA) diluted in the blocking solution overnight at 4°C, followed by Alexa 488-conjugated secondary antibodies at room temperature for one hour to label the cochlear nucleus neurons.

**Quantification of AVCN innervation by ANFs**

Quantification of AVCN innervation was done by a previously described method (Karmakar et al., 2017) with minor modifications. ImageJ software (National Institutes of Health, Bethesda, MD) was used to measure the angle of spread of PLAP staining in the spiral ganglion of each cochlear turn and the area of the respective PLAP-stained regions in the AVCN, where θₓ is the
angle of spread of PLAP staining in a particular cochlear turn (see examples in Figs. 1B1 and C; θα: angle of spread of the apical turn, θβ: angle of spread of the basal turn), θav is the average angle of spread of PLAP staining from all cochlear turns, SAP is the AVCN area innervated by PLAP-positive fibers from a particular cochlear turn, and SAVCN is the total AVCN area. For measurement of the θx, the origin of the angle of spread was determined as the point where PLAP-positive ANFs are converging on the auditory nerve. The boundaries of the angle of spread were demarcated by the sector containing PLAP-positive peripheral processes of ANFs. For measurement of SAP and SAVCN, the section containing the largest SAVCN was chosen for analysis in each animal. SAP and SAVCN were outlined manually using Freehand selections in ImageJ. The AVCN border was demarcated from the small granule cell region by comparing the adjacent section stained with an anti-NeuN antibody to label CN neurons. The fraction of AVCN innervated by PLAP-positive fibers from a particular cochlear turn was calculated as (SAP/SAVCN) and normalized to (θx/θav). The values obtained from the apical and basal turns were then compared between each other by statistical analysis.

**Apoptosis analysis of SGNs**

Apoptosis analysis of SGNs was performed as previously described (Brooks et al., 2020). Briefly, P5 and P10 cochleae were subjected to whole-mount immunofluorescence with a mouse anti-HuD antibody (sc-48421; Santa Cruz Biotechnology, Dallas, Texas) and a rabbit anti-cleaved caspase-3 (Cat# 9664; Cell Signaling Technology, Danvers, MA) to label the SGN cell body and the apoptotic cell. Z-stack confocal images of whole-mount cochleae were obtained using a 60x (NA:1.40) oil-immersion objective. Three or four different optical fields were counted for each cochlear turn and each image of the z-stack was counted individually without being compressed. Cells were considered apoptotic SGNs if they were stained positive for cleaved caspase-3 and the cleaved caspase-3 staining was inside the SGN cell body. The total number of SGNs and cleaved caspase-3-positive SGNs were acquired to calculate the percentage of apoptotic SGNs.

**Confocal imaging, 3D reconstruction and quantitative analyses of bifurcation and the endbulb of Held**

Confocal z-stacks from the selected cochlear nucleus subdivisions were obtained on an Olympus FluoView FV1000 (Tokyo, Japan) using a 20X (NA:0.75) or a 60X (NA:1.40) oil-immersion objective. A 1600 × 1600 image was acquired at optimal step size (automatically calculated by the software as half of the z resolution; z resolution = 1.4 λη / NA^2, λ = the
wavelength of the light, $\eta$ = the refractive index of the medium between the lens and the specimen, $NA$ = the numerical aperture of the objective; the optimal step size for 20X/0.75 objective = 1.16 $\mu$m and 60X/1.4 = 0.44 $\mu$m) in the Z axis. For quantification of ANF bifurcation, the zone containing fluorescent labeling of two branches and the zone innervated by all ANFs were demarcated manually using Freehand selections in ImageJ and the fluorescence intensity inside the two zones was measured respectively. The two-branch zone was defined as the CN region innervated by all ANFs subtracting the central region containing the auditory nerve root. The percentage of fluorescence intensity of the two branches in total fluorescence intensity of ANFs in the CN was then calculated. Only the section with the largest auditory nerve root was selected for analysis in each animal and three animals were analyzed in each group. For confocal imaging of the endbulb of Held for 3D reconstruction, z-stacks from the selected AVCN region were obtained using a 60X (NA:1.40) oil-immersion objective and 2X digital zoom. Because of the random and sparse background labeling of a few auditory nerve fibers in P20 cochlear nuclei, only endbulbs located in one-third of the dorsal or ventral region (for HF or LF ANFs, respectively) with intense tdTomato fluorescence were chosen for 3D reconstruction and analysis. Additionally, the confocal stacks were carefully inspected to ensure that the chosen endbulbs did not have cut branches and no other structures were overlapped with the endbulbs to interfere with the analysis. Confocal image stacks were imported into Amira imaging-processing software (Thermo Fisher Scientific, Waltham, MA), where three-dimensional reconstructions of endbulb synaptic terminals and the cell body of postsynaptic bushy cells were produced. The three-dimensional images were first visualized using the Isosurface function in Amira to determine the location of endbulbs. Endbulbs were isolated by using the Volume Editing function in Amira to subtract other regions and reconstructed using the LabelVoxel function. The size of endbulbs was then measured using the MaterialStatistics function in Amira.

**Statistics**

All statistical analyses, including the two-factor analysis of variance (ANOVA), multiple t-tests with the Holm-Sidak correction for multiple comparisons, Welch's unequal variances t-test, and Mann–Whitney U test were carried out in Excel software (Microsoft, Redmond, WA) and GraphPad Prism (GraphPad, La Jolla, CA) to determine if sets of data are significantly different from each other. The difference between means or medians was considered significant if $p < 0.05$. Data were plotted using GraphPad Prism (GraphPad, La Jolla, CA). The results are expressed as means ± SDs unless otherwise noted.
RESULTS

A genetic approach using the tamoxifen-inducible Cre-loxP system allows us to label populations of SGNs that are tuned to different sound frequencies

SGNs are born in a basal to apical progression (Ruben, 1967). Basal SGNs supply auditory hair cells that respond to high frequencies (HF), apical SGNs supply auditory hair cells that respond to low frequencies (LF). Although most of the studies described in this work were conducted prior to stages at which the SGNs are responding to sound (around P12 to P14 in mice), for simplicity we will refer them as prospective HF or LF (pHF or pLF) SGNs and their auditory nerve fibers (ANFs) as pHF or pLF ANFs. At P20, high- or low-frequency SGNs and ANFs will be referred to as HF or LF SGNs and ANFs. In the mouse cochlea, pHF SGNs at the base are generated early around E9.5 and pLF SGNs at the apex are born later around E12.5 (Koundakjian et al., 2007; Matei et al., 2005; Ruben, 1967; Shepard, Scheffel, & Yu, 2019). This basal-to-apical wave of neurogenesis is induced by transient expression of a proneural basic helix-loop-helix transcription factor Neurogenin1 (Ngn1) in neural precursors (Ma et al., 1998). It has been demonstrated that SGNs responding to different sound frequencies can be reproducibly labeled by providing a single low-dose of tamoxifen to mice carrying the Ngn1-creERT² and a Cre-dependent reporter between E9.5 and E12.5 (Koundakjian et al., 2007).

We crossed Ngn1-creERT² mice with mice carrying the R26iAP Cre reporter, which express axonal marker placental alkaline phosphatase (PLAP) upon Cre-mediated recombination. By treating Ngn1-CreERT²; R26iAP pregnant dams with a single dose of tamoxifen (0.5 mg/40 g mouse bodyweight) on either E9.5 or E12.5 and performing PLAP staining on the pups at postnatal stages, we confirmed that this approach allowed us to label a small subset of SGNs and their ANFs (~80° to 120° sector in a two and a half turn cochlea) at a specific tonotopic position along the basal-apical axis of the cochlea (Figs. 1B-C1). Administering tamoxifen on E9.5 induced labeling of pHF SGNs and their fibers in the basal turn of the cochlea (Fig. 1B1). It should be noted that a few labeled cells could also be found in nonsensory regions of the cochlea (green arrows in Figs. 1B and B1) when Cre recombination of Ngn1-creERT² is induced on E9.5. This labeling of nonsensory cells has also been reported in the previous study (Koundakjian et al., 2007). Nevertheless, we did not observe any SGNs being labeled in the cochlear apical turn when Cre recombination was induced by tamoxifen on E9.5 (Fig. 1B). By comparison, treatment of tamoxifen on E12.5 labeled pLF SGNs and their fibers in the apex of the cochlea (Fig. 1C), with no labeled SGNs found in the basal region (Fig. 1C1). The amount
of SGN labeling between samples can then be normalized according to the angular spread of PLAP staining in the cochlear basal or apical turn (θ_b in Fig. 1B1 or θ_a in Fig. 1C).

**Tonotopic segregation of pHF and pLF ANFs in the CN throughout development**

We next examined the distribution of pHF and pLF ANFs at multiple time points to compare their overall innervation patterns in the CN throughout development. A single low-dose of tamoxifen was given to pregnant dams on either E9.5 or E12.5 to label pHF or pLF ANFs. Embryonic heads and postnatal brains were collected at E15.5, P0, P5, P10, or P20. Labeled fibers in the CN were revealed by conducting PLAP staining on sections containing the CN.

At E15.5, SGNs are still at an early stage actively extending their ANFs to assemble the circuits (Angulo, Merchán, & Merchán, 1990; Koundakjian et al., 2007; C. C. Lu, Appler, Houseman, & Goodrich, 2011). Meanwhile, the anlage of the CN is just beginning to emerge, while neurogenesis is complete in VCN but still ongoing in the DCN (Ivanova & Yuasa, 1998; Martin & Rickets, 1981; Pierce, 1967; Shepard et al., 2019). Nevertheless, afferent innervation in the CN was found to already be tonotopically distinct by this stage, in agreement with previous findings (Koundakjian et al., 2007), and pHF or pLF ANFs respectively targeted different regions in the immature CN anlage (Figs. 2A and E). At P0, the tonotopic organization of ANFs was apparent in the three subdivisions of the CN. The descending and ascending branches of pHF ANFs innervated the majority of the PVCN and AVCN and some dorsal portions of the DCN (Figs. 2B and I), whereas the descending and ascending branches of pLF ANFs targeted ventral domains of the PVCN, DCN (Fig. 2F), and AVCN (Fig. 2M). This tonotopic segregation of ANFs into distinct regions of the CN subdivisions became more evident when the CN is further developed at P10 and P20 (Figs. C-D, G-H, K-L, and O-P).

**pHF and pLF ANFs show distinct innervation patterns in the CN during development**

We noticed that at early ages (E15.5 and P0), pHF ANFs explored a widespread area, whereas pLF ANFs innervated only a small confined region in the CN (compare Figs. 2A, B, I to Figs. 2E, F, M). By P20, a time after hearing onset (P12-14 in mice), endings of fibers from both groups were restricted to a specific zone within each CN subdivision (Figs. 2D, H, L, P). To further explore the possibility that pHF ANFs may initially innervate a larger area and later retract to a small region, we assessed innervation patterns of the ascending branch from pHF and pLF ANFs in the AVCN throughout postnatal development. We chose to analyze innervation of the ascending branch in the AVCN for two reasons. First, the afferent projections from the ANF descending branch in the posterodorsal quadrant of the PVCN are tonotopically
organized in a rostral-to-caudal, but not in the conventional dorsal-to-ventral orientation (see Fig. 1A) (Muniak et al., 2013; Oertel, Bal, Gardner, Smith, & Joris, 2000), which would require another set of animals sectioned horizontally for the analysis. Second, the proportion of the area between the DCN and PVCN in the coronal sections varies posteriorly to anteriorly (DCN area to PVCN area is larger in more posterior sections but smaller in more anterior sections), making it more challenging to analyze the ANF-targeted area accurately. We prepared coronal brain sections through the AVCN from P0, P5, P10, or P20 mice and stained them for PLAP activity to visualize the distribution of pHF and pLF ANFs. At P0, pHF ANFs not only projected to the dorsal half of the AVCN, but also innervated a significant portion of the ventral AVCN (Fig. 2I), whereas projections from pLF ANFs were concentrated in the ventral domain of the AVCN (Fig. 2M). At P5, the innervation patterns of pHF and pLF ANFs in the AVCN were similar to what was observed at P0 (Figs. 2J and 2N). Interestingly, at P10 the projections of pHF ANFs started to diminish from the ventral AVCN and eventually confined to a small region in the dorsal AVCN by P20 (Figs. 2K and L). By comparison, endings from pLF ANFs were restricted to a small area of the ventral AVCN throughout development (Figs. 2M-P).

We then investigated whether the proportion of the AVCN explored by pHF ANFs differed from that of pLF ANFs. To normalize the amount of SGN labeling between samples, we used a previously described method (Karmakar et al., 2017) with slight modifications. We correlated the angular spread of PLAP staining in the cochlea to the corresponding area targeted by PLAP-positive ANFs in the AVCN. The angular spread of PLAP staining in each particular cochlear turn was measured as $\theta_x$. We then averaged the angular spread of PLAP staining from all cochlear turns of each labeling to acquire $\theta_{av}$. The area of the AVCN innervated by PLAP-positive ANFs from a particular cochlear turn and the total AVCN area were also determined as $S_{AP}$ and $S_{AVCN}$, respectively. For each animal treated with tamoxifen to induce genetic labeling, the percentage of AVCN area innervated by PLAP-positive fibers from a particular cochlear turn was calculated as $S_{AP}/S_{AVCN}$ and normalized to $\theta_x/\theta_{av}$ to obtain the value (Fig. 3). We found that pHF ANFs initially innervated ~55.5% AVCN area ($S_{AVCN}$) at P0, but later confined their projections to ~16% $S_{AVCN}$ by P20. In contrast, pLF ANFs underwent minimal target sampling and the percentage of $S_{AVCN}$ targeted by pLF ANFs only slightly refined from ~25.4% at P0 to ~13.3% at P20 (Fig. 3C). A two-factor analysis of variance (ANOVA) was conducted to examine the effect of the fiber’s characteristic frequencies and animal ages on ANF innervation in the CN (Fig. 3B). The pHF ANFs innervated a significantly larger portion of the AVCN than pLF ANFs, $F(1, 16) = 114.25$, $p = 1.08 \times 10^{-8}$, and ANFs targeted a higher fraction of $S_{AVCN}$ when the animal
is at a younger age than at an older age, $F(3, 16) = 34.82, p = 3.04 \times 10^{-7}$. There was also a statistically significant interaction between the effects of frequency and age on ANF innervation in the AVCN, $F(3, 16) = 9.85, p = 0.00064$. Post-hoc analysis using multiple t-tests with the Holm-Sidak correction for multiple comparisons showed that at younger ages (P0, P5, and P10) the fraction of $S_{AVCN}$ innervated by the pHF ANFs was significantly larger than the fraction targeted by the pLF ANFs and the significance declined with age (P0, $p = 0.0069$; P5, $p = 0.0090$; P10, $p = 0.0149$). At P20, the percentage of $S_{AVCN}$ occupied by the HF and LF ANFs was similar ($p = 0.2144$) (Fig. 3C). These findings suggest that pHF ANFs initially overshoot and sample a large target area before refining their connections to the correct region, while pLF ANFs are more accurate in initial targeting and undergo minimal target sampling.

To determine whether elimination of pHF ANFs from the ventral AVCN reflects programmed cell death, we compared SGN apoptosis between the basal and apical turns of the cochlea at P5 or P10 by identifying cells containing the apoptosis marker cleaved caspase 3 (CC3). We found that CC3-positive SGNs were rarely detected in cochlear turns from either P5 or P10 mice (arrows in Figs. 4B-E). Statistical analyses showed that there was no difference in SGN apoptosis between basal and apical cochlear turns at either P5 ($p = 0.19$, Welch's unequal variances t-test), or P10 ($p = 0.23$, Welch's unequal variances t-test), and less than 1-2% of SGNs underwent apoptosis at these ages (Fig. 4A). Indeed, previous work has demonstrated that SGN apoptosis during early postnatal development in mice occurs predominantly in the type II SGNs, which only comprise ~5% of the SGN population, and there is no significant cell death of the remaining 95% type I SGNs in these sages (Barclay, Ryan, & Housley, 2011). Thus, removal of pHF ANFs from the ventral AVCN during postnatal development were not attributable to SGN apoptosis.

**ANFs innervate the CN in a developmental gradient according to their tonotopic locations**

Having established the overall innervation patterns of pHF and pLF ANFs in the CN, we then asked how individual ANFs with different characteristic frequencies extend their central processes to target and synapse on postsynaptic neurons. To track individual ANFs in the CN, we generated mice carrying both $Ngn1$-creERT2 and $Ai14$-tdTomato alleles and treated the pregnant animals with tamoxifen on E9.5 or E12.5 to induce labeling of pHF and pLF ANFs with the red fluorescent protein tdTomato. Sagittal brain sections containing the three subdivisions of the CN were prepared on E13.5, E15.5, E17.5, P0, P5, P10, or P20. These stages encompass the axon outgrowth/bifurcation (E13.5-E15.5), axon pathfinding and targeting of postsynaptic
cells (E15.5-P0), the initial stage of synaptogenesis (P0-P5), and the formation and maturation of the synapse during the pre- and post-hearing stages (P5-P20). Sections were also counterstained with an anti-NeuN antibody to mark the postsynaptic CN neurons.

At E13.5, pHF ANFs already bifurcated with apparent ascending and descending branches (arrows in Figs. 5A and A1), while pLF ANFs invaded the developing auditory brainstem but have not yet started to branch (arrowheads in Figs. 5B and B1). At E15.5, pHF ANFs showed more branched axons (arrows in Figs. 5C and C1) whereas pLF ANFs began bifurcating with discernible ascending and descending branches (arrows in Figs. 5D and D1). We quantified the percentage of fluorescence intensity of the two branches in total fluorescence intensity of ANFs in the CN and compared these percentages between pHF and pLF ANFs (Fig. 5G). We found that the percentage of branch intensity in total ANF intensity is significantly lower in pLF ANFs than in pHF ANFs at E13.5 (low vs. high, 29.16% ± 2.21% vs. 70.45% ± 4.73%, means ± SDs; p = 0.0008, Welch's unequal variances t-test) indicating that pLF ANFs have barely started bifurcation at this age. At E15.5, the percentage of branch intensity in total ANF intensity became similar between pHF and pLF ANFs (high: 68.48% ± 1.61% vs. low: 67.39% ± 6.63%, means ± SDs; p = 0.81, Welch's unequal variances t-test). By E17.5, bifurcation of pHF and pLF ANFs was essentially completed with ascending and descending branches clearly visible in both groups (Figs. 5E, E1, F, and F1). These observations suggest pHF ANFs initiate outgrowth and branching about two days earlier than pLF ANFs.

Next, we examined the axon pathfinding and targeting of ANFs in the CN. During axon pathfinding in both invertebrate and vertebrate systems, developing axons which follow similar growth trajectories usually bundle with each other en route to their targets (Araujo & Tear, 2003; Raper & Mason, 2010). Pioneer axons respond first to guidance cues and establish a scaffold of axon pathways on which subsequent axons can join and follow pioneers to the target area. Once reaching the appropriate choice point in the target area, individual axons then defasciculate from the bundle and begin their final journey to select postsynaptic targets. In the developing mouse cochlea, the peripheral processes of ANFs from SGNs fasciculate with one another to form radial bundles where they can extend together toward the sensory epithelium (Appler et al., 2013; Bruce, Kingsley, Nichols, & Fritzsch, 1997; Druckenbrod & Goodrich, 2015). This fasciculation is regulated by a Pou3f4/EphA4 signaling pathway from otic mesenchyme cells (Coate et al., 2012). To examine whether central processes of ANFs also fasciculate as they advance through the CN during axon guidance, we visualized tdTomato-labeled ANFs at high magnification at E17.5. Indeed, bundles of growing axons from the ascending and
descending branches of ANFs were observed in the AVCN and DCN (Figs. 6A-F). By P0, axon guidance for both pHF and pLF ANFs was essentially finished. Most developing axons of pHF and pLF ANFs had defasciculated and transformed their growth cones to small terminal boutons to contact postsynaptic CN neurons, forming reticulated puncta surrounding CN neurons (Figs. 6G-L).

Altogether, these results suggest that similar to what happens in their peripheral counterparts (Bruce et al., 1997; Koundakjian et al., 2007), outgrowth and bifurcation of central processes of ANFs occur in a gradient of development according to tonotopy, with pHF ANFs developing ahead of pLF ANFs at any given time during these processes.

**Synaptogenesis is similar between pHF and pLF ANFs but a higher proportion of LF ANFs develop smaller endbulb synaptic endings**

During synaptogenesis in the CN, ascending branches of ANFs form one or several large endbulb of Held synaptic endings on the soma of bushy cells in the AVCN, whereas descending branches of ANFs make small boutons *en passant* and conventional synaptic boutons with various target neurons in the PVCN and DCN (Rouiller et al., 1986). The endbulb of Held is a highly specialized synapse that allows fast and precise synaptic transmission between the SGNs and the bushy cells and is one of the largest synapses in the mammalian brain (15-30 μm in diameter) (T. Lu & Trussell, 2007; O'Neil, Connelly, Limb, & Ryugo, 2011; Yu & Goodrich, 2014). In mice, the endbulb starts to develop perinatally in the AVCN as a small swelling similar to the synaptic bouton at the terminal of ANFs (Limb & Ryugo, 2000; Ryugo & Parks, 2003). Over the first postnatal week, the swelling becomes larger but still appears as a simple structure with no apparent terminal branching. By the time of hearing onset (P12-P14 in mice), the ending of ANFs grows 10 to 15 times larger to form a nascent prototype of the endbulb, characterized as a cup-shaped terminal with a few branches. Afterward, the endbulb continues to mature and gradually acquires a more complex morphology. At P20, the young endbulb already has an adult-like appearance and appears as a calyceal terminal with multiple branches and extensions. Maturation of the endbulb in mice can then persist up until ~2 months of age.

We compared synaptogenesis of the ascending and descending branches between pHF and pLF ANFs. In the DCN, bud-like swellings at the terminals of the descending branches resembling synaptic boutons were identified at P5 (Figs. 7A1 and D1). The swellings slightly enlarged and thickened over time and became more obvious at P10 (Figs. 7B1 and E1). By P20, clear well-defined terminal boutons were present surrounding the target neurons (Figs. 7G-L).
We did not notice any apparent differences in morphogenesis of synaptic boutons of the descending branches between pHF and pLF ANFs. Next, we examined the development of endbulb synapses in the AVCN. A bulbous enlargement emerging from the axon endings of both pHF and pLF ANFs could be recognized at P5 (arrows in Figs. 7A and D). At P10, the endings of ANFs had undergone substantial morphological changes and developed into identifiable nascent endbulbs, characterized as a large cup-shaped terminal swelling with a few filopodia arising from the primary swelling (arrows in Figs. 7B and E). At P20, the endbulbs further evolved into a more complex tree-like structure, similar to the adult morphology, where many branches arborized from the primary trunk to form a complicated network wrapping around the soma of the bushy cells (arrows in Figs. 7C and F). Through the qualitative comparison of endbulb morphology, we did not observe any notable differences in synaptogenesis of endbulbs between pHF and pLF ANFs.

To reveal whether there were any subtle quantitative differences between HF and LF endbulbs, we carried out a 3D reconstruction of endbulbs with their postsynaptic bushy cells (Figs. 8A-B2) and measured the endbulb size. We quantified the size of 144 HF and 164 LF endbulbs from 4 mice in each group at P20 and found no statistically significant difference (high vs. low, 476.4 \( \mu \)m\(^3\) vs. 445.6 \( \mu \)m\(^3\), medians; \( p = 0.11 \), Mann-Whitney U test, \( n = 4 \) in each group) (Fig. 8C). Most endbulbs from both groups had a size range between 300 and 800 \( \mu \)m\(^3\). However, we noticed that there were more small endbulbs present in LF ANFs. Some of these small endbulbs appeared to be formed from multiple terminal branches of LF ANF collaterals (arrows in Figs. 8B, B1, and B2). We categorized all endbulbs into three different bins based on size, endbulbs smaller than 300 \( \mu \)m\(^3\), endbulbs between 300-600 \( \mu \)m\(^3\), and endbulbs larger than 600 \( \mu \)m\(^3\) (Fig. 8D). We calculated the percentage of endbulbs in each group and compared these percentages between HF and LF ANF. We found that LF ANFs have a significantly higher percentage of small endbulbs (< 300 \( \mu \)m\(^3\)) than HF ANFs (low vs. high, 27.88% ± 3.19% vs. 11.25% ± 1.78%, means ± SDs; \( P = 0.0002 \), Welch's unequal variances t-test).

In summary, the process of synaptogenesis in ascending and descending branches is similar between pHF and pLF ANFs, but a higher percentage of LF ANFs have endbulbs smaller than 300 \( \mu \)m\(^3\).

**DISCUSSION**

In this study, we used inducible genetic labeling to trace innervation of ANFs with distinct characteristic frequencies within the CN. This approach offers several advantages over
traditional histological dye labeling (Bruce et al., 1997; Leake et al., 2002; Snyder & Leake, 1997). First, most anatomic tracing studies using dye labeling are carried out on fixed tissues from a specific developmental stage and can only reveal discrete cellular events. Genetic labeling is achieved \textit{in vivo}, allowing labeled cells to continue to develop and undergo morphogenesis. Therefore, it can track a single cell in the circuit from its birth to its integration into the circuit, providing us the opportunity to investigate dynamic and coordinated cellular events during circuit assembly. Second, application or injection of the dye into the tissues usually requires time-consuming and technically challenging surgical procedures and laborious dissections. As a consequence, most dye labeling studies are conducted in postnatal stages but not on tiny tissues from embryonic stages. Genetic labeling is quick and easy to establish, even in small embryos. This allows us to track the neurons when they first originate from neural progenitor cells and study the circuit formation from the very beginning. Finally, another benefit of genetic labeling is that the labeled cells under study are chosen based on spatial and temporal expression of a specific marker gene. Therefore, it is a powerful method to mark a defined subset of neurons with specific characteristics at single-synapse resolution in highly heterogeneous environments such as the cochlea and the CN. By taking advantage of the genetic labeling approach, we generated a comprehensive map of how pHF and pLF ANFs innervate postsynaptic CN cells. This spatiotemporal map of auditory circuit assembly presents an important resource for future research. Examples include identifying relevant developmental stages for molecular profiling of component pHF and pLF neurons in the circuit or providing a reference to better characterize any cellular defects in mutant animals that affect the circuit assembly or tonotopic map formation. Moreover, through analysis of this map, we also uncovered that functionally distinct SGNs employ different strategies to target and synapse on CN neurons.

We found that at early developmental stages, pHF ANFs innervate a much larger area in the AVCN than pLF ANFs innervate at any time. A similar observation has also been made in a previous study using lipophilic fluorescent dyes to label ANFs from distinct positions in the cochlear middle and basal turns from E18.5 mouse embryos (Karmakar et al., 2017). In that study, the author showed that ANFs from the basal turn innervated around 40% AVCN area, whereas ANFs from the middle turn targeted only about 20% AVCN area [please refer to Figures 2C and D in (Karmakar et al., 2017)]. However, they did not examine ANF innervation at later time points beyond E18.5 to determine whether pHF ANFs from the cochlear base turn gradually refine their projections to correct target areas in the AVCN over postnatal development. In our study, we found that pHF ANFs initially innervated not only the dorsal
AVCN but also an extensive area of the ventral AVCN at P0. With development, pHF ANFs progressively eliminate their projections and restrict their inputs to appropriate target regions in the dorsal AVCN. This type of target selection strategy has also been observed in the developing visual system (Osterhout, El-Danaf, Nguyen, & Huberman, 2014). In the retina, the cellular mechanisms used by retinal ganglion cell (RGC) axons for target sampling are determined by the birthdate and outgrowth timing of the axons. Early-born RGC axons initially innervate large targeting areas and refine their connections afterward, while later-arriving RGC axons have fewer targets to choose and are more accurate in their initial targeting. It is possible that SGN axons also use a similar mechanism for target sampling. Early-born pHF ANFs are pioneer axons, arriving in the CN around two to three days earlier than pLF ANFs, so they may initially overshoot and sample a large area of different targets before refining their connections to correct targets. In contrast, later-arriving pLF ANFs (from late-born SGNs) have fewer remaining targets from which to select and are more accurate in their initial targeting and undergo minimal target sampling.

Several possible mechanisms could account for significant refinement of pHF ANFs during development, programmed cell death, pruning, and growth of the CN. The removal of pHF ANFs is unlikely to result from programmed cell death as we only observed minimal SGN apoptosis in the first and second postnatal weeks. Another plausible mechanism is axon pruning. Using histological staining, electrophysiological recording, and anatomic tracing, it has been shown that axonal pruning of ANF branches occurs in the avian AVCN (nucleus magnocellularis) (Jackson & Parks, 1982; Jhaveri & Morest, 1982). Elimination of axon branching during postnatal refinement of ANFs could also take place in our case, with both pre-hearing spontaneous activity and post-hearing sensory experience likely to play a role in this process (Babola et al., 2018; Clause et al., 2014; Connelly, Ryugo, & Muniak, 2017; Leake et al., 2002; Lee, Cahill, & Ryugo, 2003; Muller, Sonntag, Maraslioglu, Hirtz, & Friauf, 2019; Singer, Panford-Walsh, & Knipper, 2014; Wang & Bergles, 2015). However, the extent of ANF axon pruning in mice is still an open question and requires future work using retrograde dye tracing of SGN axons to address the question. Finally, Leake and Snyder et al. carried out a series of anatomic tracing studies in cats using dye injection to label a small subset of SGNs and demonstrated that the tonotopic organization of ANF projections in all three CN subdivisions undergoes significant refinement during early postnatal development (Leake et al., 2002; Snyder & Leake, 1997). This refinement of the tonotopic map is caused by disproportionate growth of the CN relative to the increased width of the isofrequency bands formed by terminal arbors of ANFs with similar frequency responses. Over development, CN
subdivisions grow in size and isofrequency bands increase in width as well. However, CN grows more than the expansion of the isofrequency bands, resulting in decreased relative width of the bands and increased precision of the tonotopic map. Whether a similar mechanism is also employed in the mouse CN to sharpen the isofrequency bands is unclear and remains to be elucidated.

Similar to the wiring events of their peripheral projections in the cochlea (Bruce et al., 1997; Koundakjian et al., 2007), outgrowth and innervation of ANF central projections also happen in a developmental gradient from high to low frequency regions. This is expected as SGNs exit the cell cycle and start to differentiate in a basal to apical progression along the cochlear axis from E9.5 to E12.5 in mice (Koundakjian et al., 2007; Matei et al., 2005; Ruben, 1967; Shepard et al., 2019). As a result, the pHF ANFs from the basal SGNs grow into the CN about two to three days earlier than the pLF ANFs from the apical SGNs.

We observed that a higher percentage of small endbulb synapses were present in LF ANFs. Some of these small endbulbs are probably the modified endbulbs of Held (Bruce et al., 1997; Koundakjian et al., 2007), which are smaller endbulbs frequently found near the auditory nerve root where they synapse on globular bushy cells located in the posterior region of the AVCN (Wu & Oertel, 1984). However, some of these small endbulbs do not appear to be the modified endbulbs of Held. They are usually made from multiple terminal branches of LF ANF collaterals. An analogous example has been documented in the barn owl AVCN (nucleus magnocellularis), where the collaterals arise from LF ANFs show several terminal branches with small en passant or terminal bouton-like swellings, whereas HF ANF collaterals usually terminate without branching in a single large endbulb (Koppl, 1994). It has also been reported in the chicken nucleus magnocellularis that synaptic terminals of HF and LF ANFs show differences in endbulb size and convergence (Fukui & Ohmori, 2004). Synaptic terminals of ANFs form a single large endbulb of Held around the neuronal soma in the high- and middle-frequency regions of nucleus magnocellularis, whereas neurons in the low-frequency region receive multiple axonal inputs from ANFs. In our study, we do not know whether all of these small endings form functional synapses. Assuming that these small endings indeed form functional endbulb synapses, it has been suggested that the difference of endbulb size between HF and LF ANFs may simply reflect different temporal precision needed by the fibers to transmit sound information. HF ANFs typically demand larger endbulb synapses in order to ensure high temporal fidelity of synaptic transmission for phase locking to high-frequency sounds (Oertel, 1985). The exact functional
significance of these small endbulb synapses is not clear and will require future electrophysiological studies to clarify.

In summary, our study provides a detailed spatiotemporal analysis of ANF innervation in the CN. These findings will broaden our knowledge of how ANFs with different characteristic frequencies target and synapse on postsynaptic CN neurons during tonotopic map formation. As perturbations of central auditory circuit assembly often result in auditory processing disorders with no known cause, the current work may also shed some light on pathogenesis of some of these disorders.
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DECLARATIONS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

REFERENCES


Highlights

1. Functionally distinct cochlear neurons use different schemes to innervate targets

2. Axons of cochlear neurons show a gradient of development based on tonotopy

3. Low-frequency auditory nerve fibers form more small endbulb synaptic endings
FIGURE CAPTIONS

FIGURE 1 Tonotopy of neuronal projections in the cochlea and the CN. (A) A schematic of the tonotopic organization of neuronal connectivity between the cochlea and three subdivisions of the CN. The ascending branch endings of SGN axons give rise to the endbulbs of Held in the AVCN. (B–C1) By treating Ngn1-CreERT2; R26iAP mice with a single dose of tamoxifen on either E9.5 or E12.5, the prospective high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) SGNs and their afferent fibers were respectively labeled. Labeled SGNs and fibers were revealed by PLAP staining in the P0 cochlea. PLAP staining can be observed in the cochlear basal but not apical turn with tamoxifen on E9.5 (B, B1) or in the cochlear apical but not basal turn with tamoxifen on E12.5 (C, C1). Nonsensory regions of the cochlea contain some labeled cells (green arrows in B and B1) when Cre recombination is induced on E9.5. θx (θa or θb) is the angular spread of PLAP staining in the particular cochlear (apical or basal) turn. Green arrowhead indicates that the PLAP-labeled ANF central projections from the apical SGNs can be seen in the basal turn. pp: peripheral processes of ANFs. sg: the region between two arcs indicate where the spiral ganglion is located. Scale bar, 200 μm.

FIGURE 2 Distinct tonotopic innervation of prospective high- and low-frequency ANFs in the CN or CN subdivisions during development. (A–H) Innervation of prospective high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) ANFs in the CN anlage at E15.5 or in the PVCN and DCN at P0, P10 or P20. Magenta dotted lines outline the CN anlage or the PVCN and DCN. PLAP staining of CN sections revealed that afferent inputs from the cochlea were tonotopically distinct by E15.5 (A, E), and prospective high- and low-frequency ANFs respectively target different regions in the DCN and PVCN at P0 (B, F), P10 (C, G) or P20 (D, H). (I–P) Innervation of prospective high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) ANFs in the AVCN at P0, P5, P10, or P20. Magenta dotted lines outline the AVCN. Prospective high-frequency ANFs initially innervate a widespread area but later retract their endings to a small region. Prospective low-frequency ANFs are more accurate in initial targeting, their endings initially (P0) target only the ventral AVCN, their ultimate (P20) target.
ChP: choroid plexus. The axis in (A) indicates the orientation of all the sections in the figure. D, dorsal; L, lateral. Scale bar in (A, E), 100 μm; (B, F, I, J, M, and N), 150 μm; (C, G, K, and O), 200 μm; (D, H, L, and P), 300 μm.

FIGURE 3 Quantification of the normalized surface fraction of the AVCN innervated by PLAP-labeled ANFs (SAP/SAVCN) reveals that prospective high- and low-frequency ANFs use different strategies to innervate the AVCN during development. To normalize the
amount of SGN labeling between samples, we measured the angular spread of PLAP staining from the cochlear turn ($\theta_x$) to be normalized (e.g. Figs. 1B1 and C) and normalized this value to the average angular spread of staining of all cochlear turns ($\theta_m$). (A) A plot of normalized $S_{AP}/S_{AVCN}$ at different ages. Means ± SDs are shown. n= 3 animals per group per age. Blue and magenta dots indicate individual data points from each animal in each group. (B) A two-factor ANOVA indicates that the prospective high-frequency ANFs innervated a significant large portion of the AVCN than prospective low-frequency ANFs and ANFs targeted a higher fraction of $S_{AVCN}$ when the animal is at a younger age than at an older age. The effects of frequency and age on ANF innervation in the AVCN show a statistically significant interaction. (A and C)

Prospective high-frequency ANFs initially innervated ~55.5% AVCN surface area ($S_{AVCN}$) at P0 but gradually confined to ~16% $S_{AVCN}$ by P20. In contrast, prospective low-frequency ANFs underwent minimal target sampling, and the percentage of $S_{AVCN}$ targeted by prospective low-frequency ANFs refined only slightly from ~25.4% to ~13.3% from P0 to P20. **: P < 0.01, *: P <0.05, ns: not statistically significant, multiple t-tests with the Holm-Sidak correction for multiple comparisons.

FIGURE 4 Quantification of SGN apoptosis. (A) Quantification of the percentage of apoptotic SGNs in apical and basal cochlear turns from P5 or P10 mice. ns: not statistically significant, Welch's unequal variances t-test. n= 4 animals per group per age. Means ± SDs are shown. (B-E) Cochlear whole mounts were stained with anti-HuD to mark SGNs (green) and anti-cleaved caspase 3 (CC3) to reveal the apoptotic cells (magenta). There is no extensive SGN apoptosis in apical or basal cochlear turns from P5 or P10 mice. White arrows indicate occasional CC3-positive apoptotic SGNs. Scale bar, 20 μm.

FIGURE 5 Bifurcation of prospective high- and low-frequency ANFs in the CN. (A-F1)
Individual prospective high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) ANFs (magenta) were genetically labeled with the red fluorescent protein tdTomato in sagittal head vibratome sections from E13.5, E15.5, or E17.5 Ngn1-CreER122; Ai14-tdTomato mice. Sections were counterstained with an anti-NeuN antibody to label neurons (green). Axon bifurcation starts about two days earlier in prospective high- than in prospective low-frequency ANFs (E13.5 v.s E15.5). White arrows point out the ascending and descending branches of ANFs and white arrowheads in (B, B1) indicate the unbranched prospective low-frequency ANFs at E13.5. AB: the ascending branch of ANFs. DB: the descending branch of ANFs. ANR: auditory nerve root. SG: spiral ganglion. 4V: fourth ventricle. The axis in (A) indicates the orientation of all the sections in the figure. D, dorsal; A, anterior. Scale bar, 100 μm. (G) Quantification of the
percentage of branch fluorescence intensity to total fluorescence intensity of ANFs in the CN. The percentage is significantly lower for prospective low-frequency ANFs than for prospective high-frequency ANFs at E13.5 but not at E15.5. N = 3 animals per group per age. Means ± SDs are shown. ***: P < 0.001 ( = 0.0008), ns: not statistically significant (p = 0.81), Welch’s unequal variances t-test.

FIGURE 6 Axon pathfinding and targeting of prospective high- and low-frequency ANFs in the CN subdivisions. (A-L) Individual prospective high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) ANFs (magenta) were genetically labeled with the red fluorescent protein tdTomato in sagittal head vibratome sections from E17.5 or P0 Ngn1-CreERT²; Ai14-tdTomato mice. Sections were counterstained with an anti-NeuN antibody to label neurons (green). (A-F) At E17.5, both prospective high- and low-frequency ANFs fasciculate to form axon bundles which may facilitate axon pathfinding in the CN subdivisions. 

B, C, E, and F are high magnifications of dashed boxed areas in A and D. (G-L) At P0, both prospective high- and low-frequency ANFs defasciculate, transform their growth cones to small terminal boutons, and target postsynaptic cells, forming reticulated puncta surrounding these cells. H, I, K, and L are high magnifications of dashed boxed areas in G and J. ChP: choroid plexus. ANR: auditory nerve root. 4V: fourth ventricle. Scale bar in (B, C, E, F, H, I, K, and L), 20 μm; (A and D), 120 μm; (G and J), 240 μm.

FIGURE 7 Synaptogenesis of prospective high- and low-frequency ANFs in the CN subdivision. (A-F) Individual prospective high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) ANFs and their synaptic terminals (magenta) were genetically labeled with the red fluorescent protein tdTomato in sagittal brain vibratome sections from P5, P10, or P20 Ngn1-CreERT²; Ai14 tdTomato mice. Sections were counterstained with an anti-NeuN antibody to label neurons (green). (A-F) In the AVCN, endings of ascending branches from prospective high- and low-frequency ANFs initially form a bulbous terminal swelling at P5 but progressively grow larger and transform to become a highly branched and intricate endbulb synaptic ending by P20. Arrows indicate the developing endbulb synaptic endings. (A1-F1) In the DCN, descending branches of prospective high- and low-frequency ANFs make standard bouton-type synapses in the DCN. Scale bar in (A, A1, D, and D1), 16 μm; (B-C1 and E-F1), 20 μm.

FIGURE 8 3D reconstruction and quantification of endbulb synaptic endings from high- and low-frequency ANFs. (A, B) Individual high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) ANFs and its endbulb terminals (magenta) were genetically labeled with the red fluorescent protein tdTomato in sagittal brain vibratome sections from P20 Ngn1-
CreER\textsuperscript{T2}; Ai14 tdTomato mice. Sections were counterstained with anti-NeuN to label postsynaptic bushy cells (green). (A1-B2) 3D reconstruction of the endbulb of Held synapses (magenta) and bushy cells (green) using Amira software. Most high-frequency ANF collaterals terminate without branching in a single large endbulb, whereas collaterals of low-frequency ANFs tend to have smaller endbulb endings that appear to be made from multiple terminal branches. White arrows indicate the two terminal branches forming a single small endbulb. Scale bar, 10 μm. (C) Scatter plot of the size of endbulbs from each individual P20 mice with genetically labeled high- (blue) or low-frequency (magenta) ANFs. There is no significant difference in the size of endbulbs between high- and low-frequency ANFs. Middle bars represent medians. High vs. low, 476.4 μm\textsuperscript{3} vs. 445.6 μm\textsuperscript{3}. ns: not statistically significant, P = 0.11, Mann-Whitney U test. n = 4 in each group. 1 to 4 below the x-axis indicate the four different mice in each experimental group. (D) A stacked bar graph shows the percentage of endbulbs of three different size categories (< 300 μm\textsuperscript{3}, 300-600 μm\textsuperscript{3}, and > 600 μm\textsuperscript{3}) in each individual P20 mice with genetically labeled high- (blue) or low-frequency (magenta) ANFs. Low-frequency ANFs show a significantly higher percentage of small endbulbs (< 300 μm\textsuperscript{3}) than high-frequency ANFs. Low vs. high, 27.88% ± 3.19% vs. 11.25% ± 1.78%, means ± SDs. ***: P < 0.001 ( = 0.0002), Welch's unequal variances t-test. n = 4 in each group. 1 to 4 below the x-axis indicate the four different mice in each experimental group.
Figure 1

A

Dorsal (High F)

Ventral (Low F)

DCN

AVCN

cochlear nucleus

cochlea

Basal (High F, born ~E9.5)

Apical (Low F, born ~E12.5)

Apex

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endbulb

PVCN

DCN

PVCN

AVCN

cochlear nucleus

endbulb

sg

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</tbody>
</table>

| Tam on E12.5 (Low Freq) |  |  |  |  |
|-------------------------|  |  |  |  |
| P0                      |  |  |  |  |
| E15.5                   |  |  |  |  |
| I                       | ![Image](I) | ![Image](J) | ![Image](K) | ![Image](L) |
| J                       | ![Image](J) | ![Image](K) | ![Image](K) | ![Image](L) |
| K                       | ![Image](K) | ![Image](K) | ![Image](K) | ![Image](L) |
| L                       | ![Image](L) | ![Image](L) | ![Image](L) | ![Image](L) |

<table>
<thead>
<tr>
<th>Tam on E9.5 (High Freq)</th>
<th>AVCN</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E15.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td><img src="M" alt="Image" /></td>
<td><img src="N" alt="Image" /></td>
<td><img src="O" alt="Image" /></td>
<td><img src="P" alt="Image" /></td>
</tr>
<tr>
<td>N</td>
<td><img src="N" alt="Image" /></td>
<td><img src="O" alt="Image" /></td>
<td><img src="O" alt="Image" /></td>
<td><img src="P" alt="Image" /></td>
</tr>
<tr>
<td>O</td>
<td><img src="O" alt="Image" /></td>
<td><img src="O" alt="Image" /></td>
<td><img src="O" alt="Image" /></td>
<td><img src="P" alt="Image" /></td>
</tr>
<tr>
<td>P</td>
<td><img src="P" alt="Image" /></td>
<td><img src="P" alt="Image" /></td>
<td><img src="P" alt="Image" /></td>
<td><img src="P" alt="Image" /></td>
</tr>
</tbody>
</table>

| Tam on E12.5 (Low Freq) |  |  |  |  |
|-------------------------|  |  |  |  |
| P0                      |  |  |  |  |
| E15.5                   |  |  |  |  |
| M                       | ![Image](M) | ![Image](N) | ![Image](O) | ![Image](P) |
| N                       | ![Image](N) | ![Image](O) | ![Image](O) | ![Image](P) |
| O                       | ![Image](O) | ![Image](O) | ![Image](O) | ![Image](P) |
| P                       | ![Image](P) | ![Image](P) | ![Image](P) | ![Image](P) |
Two-Factor Anova With Replication

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p-value</th>
<th>significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (High vs. Low)</td>
<td>0.2615</td>
<td>1</td>
<td>0.2615</td>
<td>114.25</td>
<td>1.08 x 10^-8</td>
<td>yes</td>
</tr>
<tr>
<td>Age</td>
<td>0.2391</td>
<td>3</td>
<td>0.0797</td>
<td>34.82</td>
<td>3.04 x 10^-7</td>
<td>yes</td>
</tr>
<tr>
<td>Interaction (Frequency × Age)</td>
<td>0.0677</td>
<td>3</td>
<td>0.0226</td>
<td>9.85</td>
<td>0.00064</td>
<td>yes</td>
</tr>
<tr>
<td>Within</td>
<td>0.0366</td>
<td>16</td>
<td>0.0023</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.6049</td>
<td>23</td>
<td>0.0000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: SS, sum of squares; df, degrees of freedom; MS, mean Square

Multiple t-tests with the Holm-Sidak correction

<table>
<thead>
<tr>
<th></th>
<th>P0</th>
<th>P5</th>
<th>P10</th>
<th>P20</th>
</tr>
</thead>
<tbody>
<tr>
<td>High frequency (Mean ± SD)</td>
<td>55.52 ± 4.50%</td>
<td>49.18 ± 2.31%</td>
<td>39.32 ± 8.42%</td>
<td>16.06 ± 2.70%</td>
</tr>
<tr>
<td>Low frequency (Mean ± SD)</td>
<td>25.44 ± 5.34%</td>
<td>23.90 ± 6.41%</td>
<td>13.96 ± 2.50%</td>
<td>13.27 ± 1.86%</td>
</tr>
<tr>
<td>P-value (high vs. low; two-tailed)</td>
<td>0.0069</td>
<td>0.0090</td>
<td>0.0149</td>
<td>0.2144</td>
</tr>
<tr>
<td>Significant?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 4

(A) Graph showing the percentage of apoptotic cells at different ages (P5 and P10) and locations (apical and basal). The graph indicates no significant difference (ns) between the two locations.

(B) Image of apical region at P5.

(C) Image of basal region at P5.

(D) Image of apical region at P10 with an arrow indicating a specific feature.

(E) Image of basal region at P10.

The scale bar represents 100 μm.
Figure 5

<table>
<thead>
<tr>
<th>Tam on E9.5 (High Freq)</th>
<th>E13.5</th>
<th>NeuN/Ngn1creER^{T2}-tdT</th>
<th>Ngn1creER^{T2}-tdT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>AB</td>
<td>ANR</td>
<td>SG</td>
</tr>
<tr>
<td>B</td>
<td>AB</td>
<td>ANR</td>
<td>SG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tam on E12.5 (Low Freq)</th>
<th>E15.5</th>
<th>NeuN/Ngn1creER^{T2}-tdT</th>
<th>Ngn1creER^{T2}-tdT</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>AB</td>
<td>DB</td>
<td>4V</td>
</tr>
<tr>
<td>D</td>
<td>AB</td>
<td>DB</td>
<td>4V</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tam on E9.5 (High Freq)</th>
<th>E17.5</th>
<th>NeuN/Ngn1creER^{T2}-tdT</th>
<th>Ngn1creER^{T2}-tdT</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>AB</td>
<td>DB</td>
<td>4V</td>
</tr>
<tr>
<td>F</td>
<td>AB</td>
<td>DB</td>
<td>4V</td>
</tr>
</tbody>
</table>

| Tam on E12.5 (Low Freq) |  |
|-------------------------|  |
| E1                       | AB    | DB                      | 4V                  |
| F1                      | AB    | DB                      | 4V                  |

G: Branch intensity Total ANF intensity in CN

- **High frequency fibers**
- **Low frequency fibers**

***: p < 0.001
ns: not significant

E13.5 vs. E15.5:
- **High frequency fibers**
- **Low frequency fibers**
### Figure 6

**NeuN/Ngn1creER\textsuperscript{T2}-tdT**

<table>
<thead>
<tr>
<th>E17.5</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>CN</td>
<td>AVCN</td>
<td>DCN</td>
</tr>
<tr>
<td><img src="image" alt="A" /></td>
<td><img src="image" alt="B" /></td>
<td><img src="image" alt="C" /></td>
</tr>
<tr>
<td>Tam on E9.5 (High Freq)</td>
<td>Tam on E12.5 (Low Freq)</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="D" /></td>
<td><img src="image" alt="E" /></td>
<td><img src="image" alt="F" /></td>
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</table>

<table>
<thead>
<tr>
<th>P0</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>AVCN</td>
<td>DCN</td>
</tr>
<tr>
<td><img src="image" alt="G" /></td>
<td><img src="image" alt="H" /></td>
<td><img src="image" alt="I" /></td>
</tr>
<tr>
<td>Tam on E9.5 (High Freq)</td>
<td>Tam on E12.5 (Low Freq)</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="J" /></td>
<td><img src="image" alt="K" /></td>
<td><img src="image" alt="L" /></td>
</tr>
</tbody>
</table>
Figure 7

<table>
<thead>
<tr>
<th>Tam on E9.5 (High Freq)</th>
<th>P5</th>
<th>P10</th>
<th>P20</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVCN</td>
<td>DCN</td>
<td>AVCN</td>
<td>DCN</td>
</tr>
<tr>
<td>A</td>
<td>A1</td>
<td>B</td>
<td>B1</td>
</tr>
<tr>
<td>D</td>
<td>D1</td>
<td>E</td>
<td>E1</td>
</tr>
</tbody>
</table>

NeuN/Ngn1creER<sup>T2</sup>-tdT
Figure 8

A. Tamoxifen on E9.5
   High freq

B. Tamoxifen on E12.5
   Low freq

C. ns

D. <300 μm  300-600 μm  >600 μm
   High Freq (Tam on E9.5)
   Low Freq (Tam on E12.5)