Spiral Ganglion Neurons with Distinct Preferred Frequency Response
Employ Different Strategies to Innervate the Cochlear Nucleus

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Introduction
Sound information begins from hair cells (HCs) in the inner ear and travels to the brain through spiral ganglion neurons (SGNs) (Figure 1). The peripheral processes of SGN auditory nerve fibers (ANFs) extend toward the HCs and the central processes of ANFs bifurcate and project into the cochlear nucleus. The ascending branch goes into the anteroventral cochlear nucleus (AVCN) and the descending branch extends toward the dorsal cochlear nucleus (DCN) via the posteroventral cochlear nucleus (PVCN). When SGN innervate neurons in the DCN, the endings of SGN AFNs form a small button-like synapse. In contrast to the DCN, the auditory nerve fibers connect to the bushy cell neurons in the AVCN with an extraordinary large synaptic ending called the Endbulb of Held. (Figure 1)

Methods
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). Therefore, a small subset of SGNs can be genetically labeled using the Ngn1-CreERT2 mouse line and a Cre-dependent reporter upon Cre induction by a single low dose tamoxifen administration. This allows us to reproducibly label SGNs and their ANFs that respond to different sound frequencies by providing tamoxifen at a specific time point between E9.5 (start of neurogenesis, label high-frequency-responsive neurons and their fibers) and E12.5 (end of neurogenesis, label low-frequency-responsive neurons and their fibers) (Figure 2 and 3). Two Cre-dependent reporter mouse lines were used. R26αAP Cre reporter expresses alkaline phosphatase after Cre-mediated recombination and allows us to examine overall innervation patterns of ANFs in the CN. Ai14 tdTomato Cre reporter expresses tdTomato fluorescence proteins after Cre-mediated recombination and allows us to visualize individual ANFs and their synaptic endings.

Results

Figure 2 Schematic of auditory circuits in the cochlea and the CN. (A-H') Innervation of high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) fibers in the AVCN on P0, P5, P10, and P20. Red dotted lines outline the AVCN. High-frequency fibers initially innervate a widespread area but later confine to a small region. In contrast, low-frequency fibers are more accurate in initial targeting and stay in a small area from P0 to P20. ChP: choroid plexus.

Figure 3 Genetic labeling of high- or low-frequency-responsive SGNs and their fibers. By treating Ngn1-CreERT2/R26αAP mice with a single dose of tamoxifen on either E9.5 or E12.5, we can respectively label the high- (tamoxifen on E9.5) or low-frequency (tamoxifen on E12.5) SGNs and their afferent fibers. Labeled neurons and fibers were revealed by staining of alkaline phosphatase (AP) activity in the cochlea. (A-B) AP staining of apical or basal turns of P0 cochlea with tamoxifen on E9.5 (A) or E12.5 (B). (B,B) is the angular spread of AP staining in the cochlear turn. Green arrowhead indicates that the labeled central fibers from the apical SGNs can be seen in the basal turn.

Figure 4 Tonotopic innervation of high- or low-frequency fibers in the CN. (A-F) Innervation of high- (tamoxifen on E9.5) or low-frequency (tamoxifen on E12.5) fibers in the CN on E15.5 and P0 or in three subdivisions of the CN on P20. Red dotted lines outline the CN or CN subdivisions (DCN, PVCN, or AVCN). AP staining of CN sections revealed that afferent inputs from the cochlea were tonotopically distinct by E15.5 (A-D), and high- or low-frequency fibers target dorsal or ventral portion respectively in the CN subdivisions on P20 (C, C', F, and F'). ChP: choroid plexus.

Figure 5 High- and low-frequency fibers show differential innervation patterns in the CN during development. (A-H) Innervation of high- (tamoxifen on E9.5) and low-frequency (tamoxifen on E12.5) fibers in the AVCN on P0, P5, P10, and P20. Red dotted lines outline the AVCN. High-frequency fibers initially innervate a widespread area but later confine to a small region. In contrast, low-frequency fibers are more accurate in initial targeting and stay in a small area from P0 to P20. ChP: choroid plexus.

Figure 6 Quantification of normalized surface fraction of AVCN innervated by AP-labeled fibers (Sv/Smax). To normalize the amount of SGN labeling between samples, we measured the angular spread of AP staining from the cochlear turn (θ) to be normalized (e.g. Fg. 3A and B) and normalized this value to the average angular spread of staining of all cochlear turns (θav). High-frequency fibers initially innervated ~68% AVCN surface area (Smax) on P0 but gradually confined to ~16% Smax by P20. In contrast, low-frequency fibers underwent minimal target sampling, and the percentage of Smax targeted by low-frequency fibers refined only slightly from ~24% to ~13% from P0 to P20. n = 3 animals per group per age. ***: P<0.005, ns: not statistically significant. Means ± SEMs are shown.

Conclusions
Functionally distinct SGN populations employ different strategies to target and innervate CN neurons during tonotopic map formation.

1. High-frequency fibers initially overshoot and sample a large area of different targets before refining their connections to correct targets, while low-frequency SGNs are more accurate in initial targeting and undergo minimal target sampling.
2. The central fibers of SGNs also show a gradient of development along the tonotopic axis, with outgrowth and branching of high-frequency fibers initiate two days earlier than low-frequency fibers.
3. The processes of synaptogenesis are similar between high- and low-frequency fibers but a higher proportion of low-frequency fibers form smaller endbulb endings with multiple terminal branching.

Figure 7 Bifurcation and outgrowth of high- or low-frequency fibers in the CN. (A-H') Individual high- (tamoxifen on E9.5) or low-frequency (tamoxifen on E12.5) fibers were genetically labeled in vibratome sections from P0 to P20. Ngn1-CreERT2, Ai14 tdTomato mice. Neurons were counter-stained by NeuN (green). (A-C and E-G) Branching of high-frequency fibers (E15.5) and low-frequency fibers (E12.5) fiber and its synaptic terminals (red) were genetically labeled in vibratome sections from P0 to P20. Ngn1-CreERT2, Ai14 tdTomato mice. Neurons were counter-stained by NeuN staining (green). (A-H') Both high- and low-frequency auditory nerve fibers in the AVCN initially form puncta and small swellings (white arrows in P0) but progressively grow to form a highly branched and intricate endbulb synaptic terminals (white arrows in P10 and P20).

Figure 8 Synaptogenesis of high- or low-frequency fibers in the CN subdivision. (A-H') Individual high- (tamoxifen on E9.5) or low-frequency (tamoxifen on E12.5) fibers and its synaptic terminals (red) were genetically labeled in vibratome sections from P0 to P20. Ngn1-CreERT2, Ai14 tdTomato mice. Neurons were counter-stained by NeuN staining (green). (A-H') Both high- and low-frequency auditory nerve fibers in the AVCN initially form puncta and small swellings (white arrows in P0) but progressively grow to form a highly branched and intricate endbulb synaptic terminals (white arrows in P10 and P20).

Figure 9 Endbulb synaptic terminals of high- or low-frequency fibers in the AVCN. (A-B) Individual high- (tamoxifen on E9.5) or low-frequency (tamoxifen on E12.5) fiber and its endbulb terminals were genetically labeled in vibratome sections from P0 to P20. Ngn1-CreERT2, Ai14 tdTomato mice. Post-synaptic bushy cells were labeled by Nlsl staining (green). (A-D') 3D reconstruction of Endbulbs of Held (red) and bushy cells (green) using Amira 3D software. Most high-frequency fibers terminated on bushy cells unbranchedly as a single large Endbulb of Held (Endbulb size: 351 μm3 in A'), while a higher proportion of low-frequency fibers showed multiple terminal branching and small Endbulb endings (white arrows; Endbulb size: 151 μm3 and 96 μm3 in B').

References