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BASIC AND TRANSLATIONAL RESEARCH

Optimizing the simultaneous identification of the three type I spiral ganglion neuron subtypes in models of genetic forms of deafness.

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ABSTRACT

Auditory neuropathies disrupt the transmission of sound information from the ear to the brain by affecting type I spiral ganglion neurons (SGNs) or the synapses they form with sensory inner hair cells. Afferent type I SGNs form three physiologically and molecularly distinct subtypes, which have been defined based on their spontaneous rates (SRs) and on differential gene expression, respectively. The heterogeneity of type I SGNs is crucial in allowing for the encoding of sound over a wide range of intensities, and these three subtypes have shown differences in their susceptibility to aging. However, little else is known regarding the impact that genetic, molecular, or pathological factors might have on the differentiation of type I SGNs, in part due to the technically challenging nature of simultaneously identifying the three molecularly defined populations. Here, we optimized a triple immunohistochemistry protocol (Wang et al., 2020) to simultaneously identify all three type I SGN subtypes using antibodies targeting Calb1, Calb2, and Pou4f1. With this method, we characterized type I SGN distributions in *Otof* $^{+/+}$ and *Otof* $^{-/-}$ mice, thus revealing how deafness may differentially affect type I SGN subtypes. This protocol can also be applied more widely across mouse models for auditory research, making it a valuable tool for understanding the roles that type I SGN subtypes play in the pathophysiological mechanisms of auditory neuropathies.

MATERIALS & METHODS

Immunohistochemistry:

- *Otof* $^{+/+}$ and *Otof* $^{-/-}$ mice were sacrificed between P35 and P39
- Cochleae were stepped through 10, 20, 30% sucrose, then embedded in OCT and frozen
- Samples were sectioned and then underwent **antigen retrieval**: slides were submerged in 1% SDS for 10 minutes
- Sections were stained with **chicken-anti-Calb1**, **rabbit-anti-Calb2**, **mouse-anti-Pou4f1**, and DAPI

Imaging and Quantification:

- Confocal images were taken at 20x using a Zeiss LSM900
- Three segmentation models (one per subtype marker) were trained on confocal images using Cellpose 2.0 (Pachitariu & Stringer, 2022) to automatically identify positive cells
- Models were integrated with ImageJ and Matlab to count cells as positive to Calb1, Calb2, and Pou4f1, and to quantify the overlap between subtype groups for each genotype

CONCLUSIONS

1. Molecularly defined type I SGN subtypes can be reliably identified using triple immunostaining for Calb1, Calb2, and Pou4f1 with a 1% SDS antigen retrieval step. When combined with trained identification models from Cellpose 2.0, this protocol can serve as a valuable tool for investigating Type I SGN differentiation in mouse models across auditory research.

2. *Otof* $^{-/-}$ mice show altered Type I SGN distributions at the apex, mid, and base, as has been previously shown with *Vglut3-ko* mice (Sun et al., 2018). Future investigations will explore how type I SGN differentiation may be affected in other models of genetic forms of deafness.

RESULTS

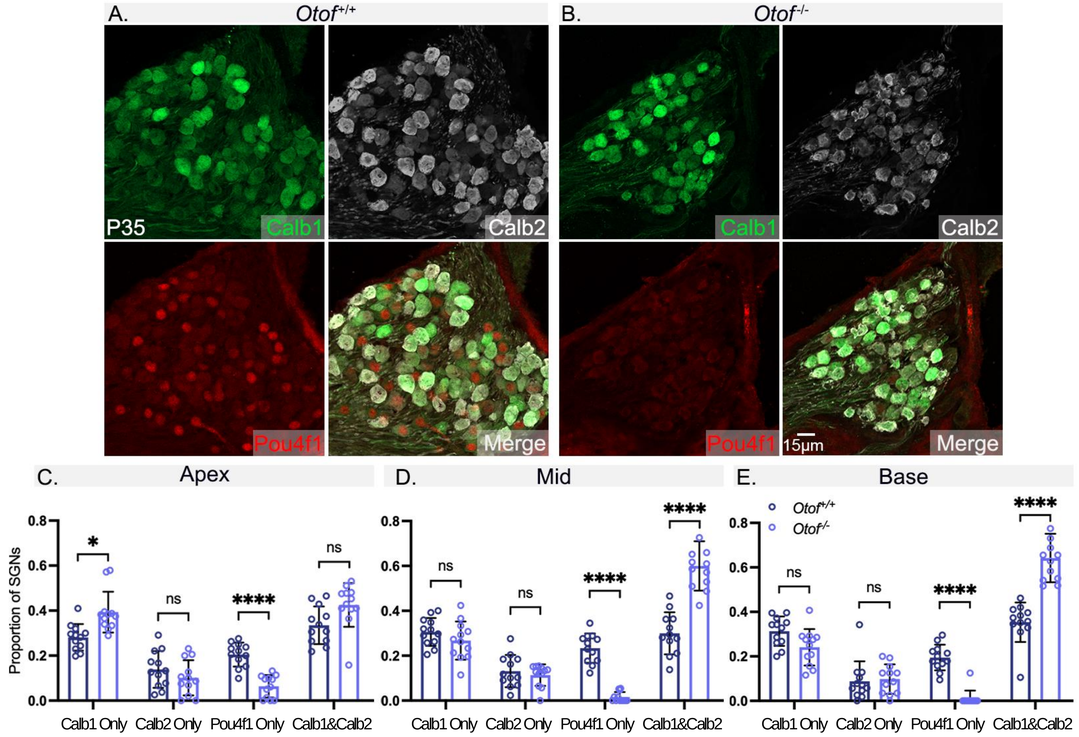

Figure 1. Type I SGN subtypes show altered distribution in *Otof* $^{-/-}$ mice. (A, B) Representative triple-staining of *Otof* $^{+/+}$ and *Otof* $^{-/-}$ P35 cochleae labeled with Calb1 (green), Calb2 (white), and Pou4f1 (red). (C-E) Quantification of the proportion of SGNs expressing each individual or combination of subtype marker(s) at the apex, mid, and base of P35-P39 mice (N=6 cochleae from 3 mice for each genotype). Data presented as mean ± SEM, ns = not significant, *p < 0.5, ****p<0.0001.

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