Supplementary Information for

Molecular Signatures Define Subtypes of Auditory Afferents with Distinct Peripheral Projection Patterns and Physiological Properties

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Supplementary Methods

Materials and Methods

Mouse lines

All animals were handled in accordance with animal protocols approved by the Johns Hopkins University Animal Care and Use Committee. *Calb2-CreERT2* mice were obtained from The Jackson Laboratory (B6(Cg) *Calb2tm2.1(cre/ERT2)Zjh/J*, Stock No: 013730). Briefly, these mice have a knock-in allele at the *Calb2* locus, which abolishes *Calb2* gene function and expresses a CreERT2 fusion protein in CALB2 positive neurons from the endogenous *Calb2* genetic locus. To avoid potential issues arising from lack of CALB2, we exclusively used mice heterozygous for *CreERT2*. Lypd1-*CreERT2* mice were generated by the Johns Hopkins transgenic core. CRISPR/Cas9 genomic editing was used to insert a 2TA cleavage peptide followed by *CreERT2* directly in front of the endogenous *Lypd1* stop codon. The target site was chosen to allow for continued expression of *Lypd1* as well as expression of the *CreERT2*. Target specific crRNA and tracrRNA were ordered from Integrated DNA Technologies (IDT). The ssDNAs containing desired mutations were designed containing 36 bp homology arms flanking the region of interest 91 and contained silent mutations to PAM sites to prevent excessive cleavage by CAS9 post integration. Using standard microinjection techniques and a mix of CAS9 protein (30ng.ul, PNABio), tracrRNA (0.6uM, Dharmacon), cRNA (0.6uM, IDT) and ssDNA oligo (10ng/μL, IDT) diluted in RNAse free injection buffer (10 mM Tris-HCl, pH 7.4, 0.25 mM EDTA), pronuclear injection of one-cell C57B/6J embryos (Jackson Laboratory) was performed. Injected embryos were transferred into the oviducts of pseudopregnant ICR females (Envigo). Offspring were screened using PCR and sequencing to determine the presence of the *CreERT2* allele. Founder mice were bred withC57BL/6J (RRID:IMSR JAX:000664) mice for several generations and offspring were genotyped to verify germ-line transmission of mutations.

Labeling of nerve fibers using Ai9/Ai14 reporter mice

Tamoxifen induction in *Calb2-CreERT2;Ai9/Ai14* and *Lypd1-CreERT2;Ai9/Ai14* mice was performed by single intraperitoneal injection of tamoxifen (Sigma, T5648-1G) or 4-hydroxy-tamoxifen (Sigma, H6278-50MG) dissolved in corn oil (Sigma, C8267). For analysis of the percentage of cell bodies labeled by tdTomato at different ages, 0.1 mg tamoxifen per 1 g body weight were injected at postnatal days 1 (P2) or 21 (P21) and animals were sacrificed at P28. This approach was used to permit comparison of labeling at P1 versus P21 without imperiling pup survival. As an alternative approach, 2 doses of 4-hydroxy-tamoxifen (0.05 mg per 1 g body weight) were also used. To achieve sparse labeling for SGN projections, animals were injected with lower doses of tamoxifen (50 μg/animal). Embryonic labelling of SGNs in Lypd1-CreERT2; Ai14 mice was achieved by a single intraperitoneal injection of 4-hyrdoxy-tamoxifen (0.25 mg) in pregnant dams.

Labeling of nerve fibers by using AAV vectors

To produce very sparse labeling, we combined injection of Cre-dependent reporter viruses with tamoxifen-induction. Pups were anesthetized on ice and gently poked to verify anesthesia. An incision was made behind the left ear, the posterior semicircular canal was exposed, and low volumes of a Cre-dependent reporter virus (pAAV.synP.DIO.EGFP.WPRE.hGH (AAV9 – Addgene 100043 Lot : v25058, Titer: $4.3*10^{13}$ GC/mL) were injected using the Nanoject II (Drummond). Fast green dye was added to aliquots of virus to facilitate monitoring of the injection. Following surgery, pup walking and survival were monitored.

Calb2-CreERT2 pups were injected at P2 with 276 nL of a mixture of 4 parts virus to 1 part fast green dye. Animals were treated with intraperitoneal injection of 30 μg tamoxifen dissolved in corn oil at P21, sacrificed at P28, and tissue was processed as described below. *Lypd1-CreERT2* pups were injected at P2 with 276 nL of a mixture of 4 parts virus to 1 part fast green dye. This was followed by intraperitoneal injection of 30 μg tamoxifen dissolved in corn oil after pups woke up. Animals were sacrificed at P7 or P28.

Immunofluorescence

For immunohistochemistry, cochleas were dissected from mice perfused with 20 mL PBS, followed by 20 mL fixative (4% paraformaldehyde(vol/vol)). After post-fixation either at room temperature for 1 hr or overnight at 4°C, tissues were decalcified in 250 mM EDTA (pH 8.0) for 2-3 hrs at room temperature.

For analysis of gene expression in SGN cell bodies, tissue was cryoprotected in 30% sucrose (weight/vol), embedded in OCT and sectioned at 14 μm. Sections were stored at -80°C, dried at room temperature for 30 min, and fixed with 4% paraformaldehyde at room temperature for 10 min. Tissues on

slides were permeabilized for 30 minutes (0.6% Triton-X100), then pre-incubated in blocking solution (10% heat inactivated goat serum, 0.6% Triton-X100, 0.4% sodium azide in PBS (pH 7.4)) for 1 hr. For staining using goat primary antibodies, an alternative blocking solution was used (10% normal donkey serum, 1% BSA, 0.6% Triton-X100, 0.4% sodium azide). Slides were incubated overnight at 4°C with primary antibodies in blocking solution, secondary antibody incubation was performed at room temperature for 1 hr. After washing with PBS, samples were mounted using ProLong Gold antifade reagent and coverslipped. For POU4F1 staining, antigen retrieval was performed on slides overnight at 60°C in 10 mM sodium citrate buffer prior to the blocking step.

For whole-mount analysis, decalcified cochleas were microdissected in PBS (pH 7.4). The cochlear capsule, stria, Reissner's membrane and the tectorial membrane were carefully removed in order to expose the cochlear epithelium. The epithelium was separated into apical, middle, and basal sections. Tissue was permeabilized and blocked as described above. Whole-mount cochleas were incubated with primary antibodies overnight either at 4°C on a shaker or at 37°C.

For primary antibodies, we used rabbit α-RFP (Abcam ab62341, 1:200), goat α-mCherry (SicGen AB0040-200, 1:100), rat α-RFP (Chromotek 5F8, 1:300), mouse α-PV (Swant PV235 1:250), chicken α-GFP (Aves GFP-1020, 1:100), rabbit α-MYO7A (Proteus 25-6790, 1:500), rabbit α-CALB1 (Cell Signaling Technology 13176S, 1:200), mouse α-POU4F1 (Millipore MAB1585, 1:20), mouse α-CALB2 (Millipore MAB1568, 1:1000), guinea pig α-CALB1 (Synaptic Systems 214-004 ,1:500) and rabbit α-GFP (Invitrogen, A-11122, 1:1000). For secondary antibodies, we used goat α-rabbit (Invitrogen A11070, A21430, and A21246), goat α-mouse (Invitrogen A11017 and A32728), goat α-rat (Invitrogen A21434), donkey α-goat Jackson ImmunoResearch 705-546-147 and Invitrogen A32816), donkey α-mouse (Jackson ImmunoResearch 715-606-151 and Invitrogen A32766), donkey α-rabbit (Jackson ImmunoResearch 711-166-152 and 711-606-152 and Invitrogen A21206), and goat α-guinea pig (Jackson ImmunoResearch 106-606-003). Goat secondary antibodies were diluted 1:1000 in blocking solution, donkey secondary antibodies were diluted 1:500 in blocking solution.

Imaging

Sections were imaged using a Zeiss 800 Confocal Laser Microscope System. For analysis of gene expression in SGN cell bodies, serial sections from at least three animals for each genotype were imaged at 40X and analyzed for each experiment. For analysis of innervation of hair cells by SGN peripheral projections, whole-mount cochleas from at least three animals for each genotype were analyzed per experiment. Each whole-mount cochlea was imaged at multiple positions across the middle turn at 63X.

Image analysis

Images were processed using Imaris software (version 9.6 – 9.7.1). Images were imported into Imaris software as 2-D maximum intensity projections. For quantification of colocalization of subtype markers, all stainings and imaging were completed in batches to keep parameters consistent and reduce variability. For semi-automatic quantification, 3D volumes of confocal z-stacks were generated in Imaris software (Bitplane). Briefly, individual SGN cell bodies were determined positive for individual subtype markers or reporter expression via the 'Detect Spots' function in Imaris. The default Imaris creation parameters algorithm was used for all individual channels. Automatic background subtraction was turned on for estimated cell body diameter of 10 μm. Quality threshold filtering post-detection was manually assessed for each image stack. Only cells in which full cell bodies were present in image stacks were included for subsequent analysis. Cells were quantified for colocalization of reporter expression and subtype markers via the 'Colocalize Spots' function in Imaris. A default distance threshold of 1 was used for all analysis. Tracing of individual SGN peripheral projections was achieved using the Filament Tracer tool in Imaris.

Quantification of tdTomato+ SGNs

To quantify the number of tdTomato+ SGNs, sections were stained with goat α-mCherry and mouse α-TUJ1. We determined the number of tdTomato+ SGNs in the middle turn of the cochlea and then normalized that number against the total number of $TUJ1^+$ SGNs in the middle turn to obtain an averaged percentage of tdTomato+ SGNs.

Analysis of innervation location

We analyzed the position at which peripheral SGN projections innervated IHCs using 3D volumes in Imaris. We determined for each innervating fiber the 'Normalized Basal Position' (NBP) as described (1). Briefly, we used Imaris to draw a bisecting plane from the basal pole through the nucleus to the cuticular plate of individual inner hair cells in the XZ plane of the confocal scan. This defined the length of the hair cell (L) and bisected the hair cells into modiolar and pillar halves. We then determined the contact position of a given SGN along the radial axis of the hair cell starting from the basal pole (c) and normalized it to L. The resulting fractional distance of the contact position from the basal pole of the hair cell was multiplied by +1 or -1 for innervations on the modiolar or pillar sides of the bisecting plane, respectively. If there were multiple branches of a single SGN, each contact position was analyzed. For the experiments reported here, only SGNs innervating the middle turn of the cochlea were analyzed.

Loose patch recordings to determine spontaneous firing rates in SGNs in acutely excised cochlear coils

Loose-patch recordings from SGN bouton endings were performed to monitor spontaneous firing rates (2). To achieve sparse labeling of SGN projections, mice were injected with lower doses of tamoxifen (50 μg/animal) at P21 as described above. Recordings were performed at P28 for *Calb2-CreERT2* mice and *Lypd1-CreERT2* mice of either sex. Mice were deeply anesthetized by isoflurane inhalation (Forane, Baxter Healthcare Corporation, USA) and decapitated. The apical cochlear coil was dissected from the temporal bone in cold extracellular solution (in mM): 5.8 KCl, 144 NaCl, 0.9 $MgCl_2$, 1.3 $CaCl_2$, 0.7 NaH_2PO_4, 5.6 glucose, and 10 HEPES (pH 7.4 adjusted with 1 M NaOH; 300 mOsm), pinned on a coverslip and transferred to the recording chamber containing extracellular solution. Cochlear tissue from both ears was used simultaneously, by two experimenters in two recording setups. The two recording setups were equipped with different components. Setup 1 was equipped with a differential interference contrast (DIC) upright microscope (Examiner D1 microscope or Axioskop 2 FS plus, Zeiss, Germany) with 4x or 10X and 40X magnification, and an ORCA flash 4.0 V3 digital CMOS Hamamatsu camera (Hamamatsu, Japan). Setup 2 was equipped with a Nikon A1R-MP confocal unit adapted on an eclipse Ni-E upright microscope controlled by the Nis element software (Nikon company). Samples were visualized by DIC with a 40x water immersion objective (CFI60 Apochromat, N.A. 0.8, W.D. 3.5mm).

Patch pipettes were pulled from 1 mm glass capillary tubes (borosilicate #1B100F-4, World Precision Instruments) with a multistep horizontal puller (Sutter instruments), fire polished and filled with extracellular solution. Pipette resistances were 9-13 MΩ. The loose-patch was obtained by applying a small negative pressure to the membrane of the bouton ending from the patch pipette. A successful loose-patch was indicated by an increase in the series resistance two- to five- times of the pipette resistance and the appearance of action potentials, recorded in current clamp mode (I=0). In setup 1, recordings were performed with a Multiclamp 700B amplifier and digitized with either a Digidata 1322A or a Digidata 1440A (Molecular Devices, USA), using pCLAMP 10.2 software. In setup 2, recordings were done with the double patch-clamp Heka amplifier and Patchmaster software (Heka Elektronik). Recordings were sampled between 20-50 kHz and low pass filtered at 10 kHz.

Event detection and determination of the spontaneous firing rate

Spontaneous firing rates (SRs) were monitored for 1 min after establishing the loose-patch recording to allow the seal to stabilize. To determine the SR of a bouton ending, the spike rate was averaged for the subsequent two minutes. Spike detection was performed in MiniAnalysis software (Synaptosoft; RRID:SCR_014441) and double-checked by eye. Analysis of spike-timing was performed blinded to the type of fiber recorded (pillar/modiolar; fluorescent/ non-fluorescent), by the experimenter from the other setup.

Verification of presynaptic release driven firing rate in loose-patch recordings

We used loose-patch recordings for reporting spontaneous firing rates in SGN bouton endings induced by presynaptic transmitter release. However, sometimes such recordings also exhibited AP firing intrinsically generated by SGNs (2) which could be artifactual, for example due to mechanical stretching of the bouton ending during the recording. Such recordings showed preferred interevent intervals in their spike timing rather than the expected near Poisson distribution that has been found in *in vivo* recordings of auditory nerve fibers (3-5). To identify and exclude such recordings, hazard functions were performed on the spike-timing data of all recordings using a MATLAB routine written by Dr. Eric Young (2). The hazard

function shows the probability of an event at a particular interval, given that there has been no event so far. For values larger than the absolute and relative refractory period of the AP, hazard functions are approximately constant or continue to increase slowly at longer intervals (6, 7), reflecting near Poisson distribution of spike timing. However, recordings exhibiting preferred intervals show a peak in the hazard function, here defined as >1.6x steady state value. In recordings with a peak in the hazard function, auditory nerve fiber activity cannot completely be eliminated when blocking synaptic transmission with cadmium, but it can be completely eliminated in recordings without a peak in the hazard function, as has been shown in rat (2). Secondly, in ~ 1 month old C57bl6/J mice, when using the AMPA receptor antagonist NBQX (20 μM) to block synaptic transmission in recordings without a peak in the hazard function, AP firing was blocked by a median value of 92.86 % (n = 11; 25% percentile was 100% and 75% percentile was 50.73 %), whereas recordings with a peak in the hazard function were only blocked by a median value of 0.37% (n = 7; 25% percentile was 54.22% and 75% percentile was 55.58%; Mann-Whitney test, $p<0.05$). Therefore, recordings with no peak in the hazard function were selected to assure that the spontaneous firing rate was due to presynaptic transmitter release.

Discrimination between pillar vs. modiolar, and fluorescent vs. non-fluorescent type I SGNs for bouton recordings

The SGN bouton endings chosen for recording were judged to be contacting the IHC on the pillar or the modiolar side before forming a loose-patch seal. To judge the side of the IHC the bouton endings contacted, the focus of the DIC image was scrolled through the IHC from top to bottom and then the IHC was cut by an imaginary longitudinal axis going from the base of the stereocilia passing through the nucleus. All bouton endings above this imaginary line were judged as modiolar whereas the bouton endings below this imaginary line were judged as pillar. Because CALB2-positive bouton endings contacted IHCs deep in the tissue around the base of the IHC, DIC optics were not sufficient to unambiguously determine whether the CALB2-positive bouton endings contacted the IHC on either the modiolar or pillar side and therefore, their side of IHC contact was not judged. To determine for each bouton ending whether they were LYPD1- or CALB2-positive, the bouton endings were assessed for fluorescence in response to epifluorescent light. Subsequent to the recording of spontaneous rate, the tissue was exposed to an epifluorescent light centered either at 565 nm and 488 nm and snapshots were taken under both DIC and epifluorescent light conditions. The DIC and epifluorescent snapshots were overlaid offline and those bouton endings where fluorescent signal overlapped the tip of the recording pipette were judged to be either LYPD1- or CALB2-positive. For some bouton endings, confocal images and z-stacks were taken for higher resolution examples.

Statistics

The Shapiro-Wilk test was used to determine that SRs from electrophysiological recordings were not normally distributed. Therefore, the SRs between groups were compared using a Kruskal-Wallis test followed by a Dunn's multiple comparison. Results are reported as mean ± standard deviation (SD). Statistical tests are named in Results or Figure Legends with the statistical significance (p) and the number of cells (n). In the figures, the whiskers represent the minimum and maximum value, and p is defined as: n.s. (not significant) $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Graphing of results

Graphs of results were made in Prism 9 for MacOS and windows (version 9.1.2).

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