A targeted Coch missense mutation: a knock-in mouse model for DFNA9 late-onset hearing loss and vestibular dysfunction

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Mutations in COCH (coagulation factor C homology) are etiologic for the late-onset, progressive, sensorineural hearing loss and vestibular dysfunction known as DFNA9. We introduced the G88E mutation by gene targeting into the mouse and have created a CochG88E/G88E mouse model for the study of DFNA9 pathogenesis and cochlin function. Vestibular-evoked potential (VsEP) thresholds of CochG88E/G88E mice were elevated at all ages tested compared with wild-type littermates. At the oldest ages, two out of eight CochG88E/G88E mice had no measurable VsEP. Auditory brainstem response (ABR) thresholds of CochG88E/G88E mice were substantially elevated at 21 months but not at younger ages tested. At 21 months, four of eight CochG88E/G88E mice had absent ABRs at all frequencies tested and two of three Coch¹/¹ mice had absent ABRs at three of four frequencies tested. Distortion product otoacoustic emission amplitudes of CochG88E/G88E mice were substantially lower than Coch¹/¹ mice and absent in the same CochG88E/G88E mice with absent ABRs. These results suggest that vestibular function is affected beginning as early as 11 months when cochlear function appears to be normal, and dysfunction increases with age. Hearing loss declines substantially at 21 months of age and progresses to profound hearing loss at some to all frequencies tested. This is the only mouse model developed to date where hearing loss begins at such an advanced age, providing an opportunity to study both progressive age-related hearing loss and possible interventional therapies.

INTRODUCTION

COCH (coagulation factor C homology) and its encoded protein, cochlin, are expressed at high levels in the inner ear as initially shown by northern blot, tissue in situ hybridization, and immunohistochemistry (1,2). Cochlin is detected by proteomic analysis as the most abundant protein in the bovine (3), as well as mouse and human inner ears (4). A growing number of mutations in COCH, causing late-onset, progressive hearing loss and vestibular dysfunction characteristic of DFNA9 are being diagnosed (Fig. 1 and Table 1). In addition to the original mutations clustered in the FCH(factor C homology)/LCCL(limulus factor C, cochlin, lung gestational protein) domain (5–8), there are now three mutations reported in the vWFA2 domain (von Willebrand factor A-like domain) (9,10), as well as another recently reported mutation (in the FCH/LCCL domain) in a Dutch kindred (11).

Prevalence of COCH mutations is not known, as there is no systematic genetic screening worldwide in individuals with late-onset hearing loss. However, the finding of COCH mutations on four continents (Table 1), and the observation of four distinct mutations in the Netherlands alone, suggests...
that the true prevalence of COCH mutations may be substantially higher than currently recognized. In addition, the possibility of COCH playing important roles in presbycusis and disorders of imbalance has been considered (6,7), and cochlin has also been shown as a major target antigen for autoimmune sensorineural hearing loss by both antibody and T-cell-mediated mechanisms (12–14).

Temporal bones from individuals with DFNA9 exhibit an intriguing and characteristic histopathology. The number of fibrocytes in the spiral ligament and in the spiral limbus is reduced and a homogeneous eosinophilic material is present in these structures that stain with anti-cochlin antibodies (4). The relationship between the finding of cochlin-staining eosinophilic material and lack of cellularity remains to be elucidated. In an effort to understand the function of COCH in the inner ear and its role in pathogenesis of DFNA9 hearing and balance disorder, we created a mouse knock-in model designated CochG88E/G88E. Because all mutations known to date in COCH are either missense or an in-frame deletion, and none lead to premature termination and truncation of the protein, we chose to create the targeted missense mutation in the mouse rather than a null mutant.

The G88E mutation was initially found in a US family (5) and subsequently in the Netherlands with likely different founders (15). In vitro studies of the FCH/LCCL domain of bacterially expressed cochlin with the G88E mutation (as well as several others of the mutations) have revealed misfolding of this domain by nuclear magnetic resonance (NMR) structural analyses (16). Herein, we report the generation of a mouse model bearing the G88E mutation, and describe the results of functional hearing and vestibular testing as well as histological findings. With age-related hearing loss being the most common sensory problem in humans, our mouse model provides potentially an excellent system for its study; this is the only model to date with onset of hearing loss at this advanced age.

**RESULTS AND DISCUSSION**

**Verification of the CochG88E/G88E mouse model**

**RT–PCR analysis.** To confirm successful transcription of mutant Coch, we performed reverse transcription polymerase chain reaction (RT–PCR) of ribonucleic acid (RNA) isolated from a CochG88E/þ mouse. An expected product of 1.7 kb representing the full-length coding region of Coch was obtained. Sequencing of the PCR product was performed, showing presence of the introduced Coch mutation as well as correct splicing of introns 4–6, confirming that the remaining loxP site in intron 5 after deletion of neo (neomycin) does not disrupt accurate splicing.

**Immunohistochemistry.** To confirm translation of the mutant CochG88E/G88E transcript, we performed immunohistochemistry on cochlear and vestibular sections from a 3-month-old CochG88E/G88E mouse, using an anti-cochlin antibody, which showed intense and specific cochlin staining in the structures where cochlin expression is normally seen in the wild-type inner ear (4) (Fig. 2). Specifically, cochlin immunostaining was prominent throughout the spiral ligament and spiral limbus in the cochlea and in the area of stromal fibrocytes in the crista ampullaris of a semi-circular canal. All immunostaining

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**Table 1. COCH mutations in DFNA9**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Exon with mutation</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Protein domain</th>
<th>Reference</th>
</tr>
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<tr>
<td>Belgium and The Netherlands</td>
<td>4</td>
<td>C207T</td>
<td>P51S</td>
<td>FCH/LCCL</td>
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<td>V66G</td>
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<td>G87W</td>
<td>FCH/LCCL</td>
<td>(28)</td>
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<tr>
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<td>FCH/LCCL</td>
<td>(5,15)</td>
</tr>
<tr>
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<td>V104del</td>
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<td>(11)</td>
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represents mutant cochlin, confirming successful translation of the mutant cochlin protein. Of note, inner ear sections from Coch$^{+/+}$ immunostained with the same antibody show complete lack of staining (4).

**Functional studies: VsEP, ABR and DPOAE measurements**

Vestibular-evoked potential (VsEP) measurements are a reflection of vestibular function, more specifically of gravity receptor or otolithic organs, the saccule and utricle. VsEP thresholds for Coch$^{G88E/G88E}$, Coch$^{G88E/+}$ and Coch$^{+/+}$ mice were obtained at five ages spanning 11–21 months (Fig. 3). VsEP thresholds of Coch$^{G88E/G88E}$ mice were elevated at all ages tested compared with the Coch$^{+/+}$ littermates. At 19 and 21 months, two of the Coch$^{G88E/G88E}$ mice had no measurable VsEP. VsEP thresholds for Coch$^{G88E/G88E}$ and Coch$^{+/+}$ mice at 11, 17, 19 and 21 months were analyzed using a two-factor analysis of variance (ANOVA, Age X Genotype). The two factor ANOVA revealed no significant interaction; however significant main effects were seen for age \([F(3,35) = 4.349, P = 0.01]\) and genotype \([F(1,35) = 15.749, P < 0.001]\). VsEP thresholds for Coch$^{G88E/G88E}$ mice were significantly higher than for Coch$^{+/+}$ mice and the oldest age groups (17, 19 and 21 months) each had significantly higher VsEP thresholds than 11-month-old mice (post-hoc least significant difference, \(P = 0.015, 0.006\) and 0.0001, respectively). These results suggest that gravity receptor function declines with advancing age for both genotypes, but Coch$^{G88E/G88E}$ mice have a greater deficit at all ages tested.

Interestingly, VsEP thresholds for the Coch$^{G88E/+}$ mice were similar to the Coch$^{+/+}$ mice at the youngest and oldest ages tested.

Although gravity receptor (otolithic organs) function for the Coch$^{G88E/G88E}$ mice appeared to be affected as early as 11 months, auditory function at 11 months was identical for all genotypes (Figs 4 and 5). Distortion product otoacoustic emission (DPOAE) amplitudes of Coch$^{G88E/G88E}$ mice at 19 months were slightly reduced for the mid-frequencies (5–10 dB smaller) (Fig. 5). One 19-month-old Coch$^{G88E/G88E}$ mouse had much higher ABR thresholds than the other Coch$^{G88E/G88E}$ mice (data not shown) and had essentially absent DPOAEs indicating that it was more substantially affected with hearing loss. This mouse also had absent VsEPs, indicating concomitantly more severe phenotypes in both the hearing and gravity receptor systems. By 21 months of age, DPOAE amplitudes for the Coch$^{G88E/G88E}$ mice were...
substantially smaller than for Coch$^{+/+}$ mice (up to 30 dB smaller) and ABR thresholds were substantially higher than for wild-types. Indeed, six of the eight Coch$^{G88E/G88E}$ mice tested at 21 months had absent DPOAEs and absent ABRs at the maximum stimulus levels of 32 and 41 kHz frequencies, and four of eight Coch$^{G88E/G88E}$ mice tested had absent ABRs at all frequencies; therefore, the ABR mean thresholds shown in Figure 4 likely underestimate actual auditory thresholds. Coch$^{G88E/G88E}$ mice also show decline in auditory function at a level similar to that of the Coch$^{G88E/G88E}$ mice.

Overall, the functional results suggest that vestibular and cochlear dysfunction occur in the DFNA9 mouse model, but not simultaneously. Vestibular function, particularly gravity receptor function, is affected beginning as young as 11 months when cochlear function appears to be normal. Vestibular deficit increases with advancing age, including profound deficits identified in two elderly Coch$^{G88E/G88E}$ mice. Cochlear function begins to deteriorate at advanced ages. Since few studies have systematically evaluated vestibular function in patients with DFNA9, the extent of vestibular dysfunction and age of onset are not well characterized. However, some clinical reports of DFNA9 patients do indicate onset of vestibular malfunction to precede hearing loss, similar to the situation observed in this mouse model. Studies on a Dutch family bearing this mutation revealed start of hearing deterioration at age 46–49 and onward, and onset of vestibular dysfunction at ~46 years of age (15). Similarly, a large study of

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**Figure 4.** Means and standard errors for ABR thresholds at 8, 16, 32, and 41.2 kHz. Data for all genotypes at two ages are shown. At 21 months, four of eight Coch$^{G88E/G88E}$ mice had absent ABRs at all frequencies tested, and two of three Coch$^{G88E/+}$ mice had absent ABRs in three of four frequencies tested; therefore, symbols for these groups represent a conservative estimate of ‘average’ thresholds. Arrows denote that true thresholds may be higher than estimated. Overall, the data demonstrate an age-related decline in hearing among all genotypes, but the most substantial declines were seen in the Coch$^{G88E/G88E}$ mice where half of the animals tested had profound hearing loss at all frequencies. Coch$^{G88E/+}$ mice also show decline in auditory function at a level similar to that of the Coch$^{G88E/G88E}$ mice.

**Figure 5.** Average DPOAE amplitudes are shown at 19 and 21 months. Error bars represent standard errors. Primaries ($f_2/f_1 = 1.25$) from 5 to 55 kHz were presented at 65 dB SPL. At 19 months, DPOAEs were present for Coch$^{G88E/G88E}$ and Coch$^{+/+}$ mice although emissions for Coch$^{G88E/G88E}$ mice were 10–15 dB smaller than those of the Coch$^{+/+}$ mice. At 21 months, smaller DPOAE amplitudes were recorded for all genotypes suggesting age-related changes; however, emissions for the Coch$^{G88E/G88E}$ mice decreased by up to 20 dB while Coch$^{+/+}$ mice emissions dropped only by ~10 dB. Emission amplitudes for Coch$^{G88E/G88E}$ mice were substantially smaller than those of Coch$^{+/+}$ mice at 21 months. Consistent with the ABR results, half of the Coch$^{G88E/G88E}$ mice (i.e. those with absent ABRs) also had absent DPOAEs (i.e. DPOAE amplitudes indistinguishable from the noise floor).
74 individuals in the Netherlands bearing the P51S mutation, in the same domain of cochlin as the G88E mutation, also showed the onset of vestibular malfunction (34 years of age) to precede onset of hearing deterioration (43 years of age, onwards) (17). Another study of DFNA9 patients, with the C542F mutation (9), demonstrates that VEMPs (vestibularevoked myogenic potentials) were absent in two of three DFNA9 patients tested, indicating saccular otolithic deficits. These results are in agreement with our mouse VsEP results, which also demonstrate otolithic deficits. In this study (9), canal dysfunctions in some subjects [bilateral weakness for calorics and reduced VOR (vestibular ocular reflex) gains in rotary chair testing] were implicated. We cannot rule out canal dysfunction in the mouse model at this time, as VOR testing has not been performed.

Onset of hearing loss in patients with DFNA9 is quite late, up to the fifth decade. Late-onset hearing loss in this mouse model is consistent with findings reported in human patients. Earlier onset of vestibular malfunction in this mouse model, as well as the human VOR data (15,17), strongly indicate that vestibular testing, including measures that evaluate ampullar and macular end organs, should be considered in the clinical evaluation of patients with DFNA9.

Histopathology

Initial examination of CochG88E/G88E mice at 1 year of age revealed some deterioration of type IV fibrocytes of the spiral ligament seen as loss of nuclei (data not shown). However, at more advanced ages, the loss of type IV fibrocytes observed in the CochG88E/G88E were similar to changes seen in the Coch+/+ mice. In CochG88E/G88E mice of 21 (n = 8) and 26 (n = 2) months of age, histology did not reveal obvious deterioration of cochlear and vestibular sensory epithelia (Fig. 6). Interestingly, however, CochG88E/G88E mice at this age have pronounced hearing and vestibular symptoms.

In addition, our mouse model did not show the eosinophilic deposits characteristic of DFNA9 suggesting that there is no detectable cochlin aggregation in the mouse at the ages tested. It is possible that these microfibrillar deposits have not yet formed to the degree of visibility and are at a lower level than detectable at the time of analysis in the mouse. It is also possible that the histopathology in the mouse exhibits different expressivity from that in the human where eosinophilic deposits are observed in end-stage DFNA9.

MATERIALS AND METHODS

Construction of the targeting vector

To construct a mutant Coch targeting vector (Fig. 7A), we screened a mouse 129 genomic library in EMBL3 phage (gift of Dr R. Maas, Boston, MA, USA) (18), using radiolabeled Coch cDNA probes by standard methods. Coch genomic clones were isolated and characterized by restriction mapping. A 5.58 kb fragment spanning introns 3 to 8 of Coch were subcloned into pBluescript II SK vector (Stratagene, La Jolla, CA, USA). A nucleotide change in exon 5 (A to G), translating into the p.G88E mutation was introduced using QuikChange site-directed mutagenesis following the manufacturer’s protocol (Stratagene). A unique AvrII site was introduced into intron 5 by the same method, to allow subsequent cloning of a neomycin-resistant gene, pgk-neo, flanked by loxP sites, for positive selection of embryonic stem (ES) cells transfected with the construct. The 5.58 kb Coch genomic fragment containing the mutation was cloned into a vector containing the thymidine kinase (tk) gene for negative selection (gift of Dr R. DePinho, Boston, MA, USA), with the tk gene positioned upstream of the 5‘ end of the Coch fragment. A 2 kb loxP-neo-loxP cassette was then cloned into intron 5. The resulting targeting vector (Fig. 7A) has a shorter homologous recombinant arm of 1.68 kb derived from the 5‘ end of the Coch fragment, upstream of the neo cassette, and a longer homologous recombinant arm of 3.9 kb from the 3‘ side of the Coch fragment, downstream of neo.
Generation of Coch-G88E/G88E mutant mice

The targeting construct was linearized with a unique AscI site, 3’ of the Coch construct and electroporated into J1 (129-derived) ES cells. Homologous recombinants were identified by both PCR and Southern blot analysis. For PCR analysis (Fig. 7A), a forward primer upstream of the 5’ Coch homology arm in the targeting construct (5’-GTG CTG CCT TCC ATA TTC CTG-3’) and a reverse primer in neo (5’-CGC ATT GTT AGA TTT CAT ACA CGG-3’) were used, yielding a 2.26 kb fragment. For the long homologous arm, a forward primer in neo (5’-TAT ACG AAG TTA TTA GGT CCA CGG-3’) and a reverse primer in the 3’ region external to the homologous arm of the targeting construct (5’-CAT GTA AAG AGA GCT AGT GTG ATG-3’) were used, yielding a 4.2 kb fragment. The amplified products were sequenced to confirm homologous recombination of the construct into the genomic Coch targeted locus, as well as presence of the targeted mutation and accuracy of all exons, exon–intron junctions and neo sequence.

Southern blot analysis (Fig. 7B) using probes external to the genomic DNA in the targeting vector was performed for both the 5’ and 3’ sides of the homologous regions. Southern blots of BamHI and HindIII digests of the ES cell genomic DNA probed with a 5’ probe yielded 4.57 and 7.8 kb bands, respectively, in the wild-type, and 3.77 and 6.45 kb band, respectively, in the targeted allele. BglII and PstI genomic digests probed with a 3’ probe yielded 9.16 and 7.5 kb bands, respectively, in the wild-type, and 11.2 and 5.0 kb bands, respectively, in the targeted allele.

Two rounds of transfections of the mutant Coch targeting construct into J1 ES cells were performed, yielding ~240 ES cell clones, which were screened for homologous recombination. A total of four ES cell clones were found to be homologous recombinants. Integrity of all exons, the introduced mutation,loxP sites, and accurate recombination of homologous arms were screened by PCR, Southern blots and sequencing. One homologous recombinant clone had a small insertion from the vector at the end of one homologous arm,
and another had not incorporated the mutant base pair; therefore these clones were not used for blastocyst injections. The third clone, which appeared not to have any aberrations in homologous recombination, yielded very few chimera, none of which showed germine transmission of the mutant allele. Subsequently this ES cell clone was karyotyped and shown to be mosaic for trisomy 8, which may have been the cause of unsuccessful germine transmission. The fourth ES cell clone showed homologous recombination and no other defects as verified further by sequencing of the mutation, exons and borders of the homologous arms. This ES cell clone was used for microinjection into C57/6 blastocysts, which were implanted into pseudopregnant mice and resulted in several chimera which showed germine transmission of the Coch G88E allele.

Male chimeric mice with high levels of ES cell contribution were backcrossed to C57BL/6 females. F1 heterozygous offspring with agouti coat color were further tested by genotyping using tail DNA for PCR and Southern blot analysis to confirm germine transmission of the mutant Coch construct. For PCR analysis of F1 genotyping, we employed a forward primer in exon 5 (5'-TTC GTC GAG AGA ACT AC-3') and two reverse primers, one in neo (5'-CGC ATT GTA TTA TTA ACA CGG-3') and the other in exon 6 (5'-CIT CCT GGG TAC TGC TTT GCG-3'), yielding a 400 and a 550 bp amplified product in the targeted and wild-type alleles, respectively.

The neo cassette flanked by loxP sites was then excised from the targeted locus by breeding F1 heterozygotes with a transgenic mouse carrying EIIa-cre (19), leaving a loxP site in intron 5 of Coch (Fig. 7A). Deletion of the neo gene in the F1 offspring was assayed by PCR of tail DNA using forward and reverse primers (5'-TCAACCATGGGACA GAGTTACC-3' and 5'-TTGATGTATTTGAAAAGCAC-3') flanking the remaining loxP site, yielding a 276 and a 176 bp amplified product in the targeted and wild-type alleles, respectively (Fig. 7A). Heterozygotes with excised neo were then crossed with the CBA/CaJ strain, and those with germine transmission of the excised neo and loss of the cre transgene were identified. Breeding to the CBA/CaJ strain for at least 10 generations was continued, because this strain does not exhibit significant hearing loss at advanced ages, and therefore does not confound hearing analysis of the mutant mouse at advanced ages. Intercrosses from the first and third generations of CBA/CaJ crosses were performed to generate cohorts of mice with all three genotypes for functional and third generations of CBA/CaJ crosses were performed to generate cohorts of mice with all three genotypes for functional and histological analyses.

**Tissues**

Mouse tissues were obtained according to guidelines and protocols approved by the Harvard Medical School Standing Committee on Animals (Boston, MA, USA). For RNA isolation, spleen tissue was dissected from a Coch G88E/+ mouse and processed as described in the following section. For histology and immunohistochemistry, mice were perfused intracardially and tissues fixed in 4% paraformaldehyde. After removal of the stapes from the oval window, and piercing of the round window, 4% paraformaldehyde fixative was perfused gently through the cochlea. Inner ears were immersed in fixative for 24 h, followed by decalcification in 120 mM ethylenediaminetetraacetic acid (EDTA) for 1 week at room temperature, and embedded in paraffin by standard histologic procedures. Serial sections were obtained at 5–8 microns thickness and used for staining with hematoxylin and eosin (H&E) and for immunohistochemistry.

Also for histologic evaluation, osmium tetroxide staining and embedding in Araldite resins (Polysciences, Warrenton, PA, USA) was performed, as this technique yields better morphology of tissues. Mice were perfused intracardially with 2.5% glutaraldehyde and 1.5% paraformaldehyde in a 65 mM phosphate buffer. Both petrous temporal bones were extracted and the round and oval windows were opened to allow intralabyrinthine perfusion of fixative. After overnight postfixation in the same fixative at room temperature, cochleas were osmicated (1% OsO4 in dH2O) for 1 h and decaledified (100 mM EDTA with 0.4% glutaraldehyde) for 3 days. After decalcification, cochleas were dehydrated in ethanol and propylene oxide and then embedded in Araldite resins and sectioned at 40 μm with a carbide steel knife. A total of 10 Coch G88E/G88E mice (eight at 21 months old, and two at 26 months old), and seven Coch +/+ mice (five at 21 months old, and two at 26 months old) were analyzed for histology.

**RNA isolation and RT–PCR**

Total cellular RNAs were isolated (20) from a Coch G88E/+ mouse spleen and reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. PCR of the cDNA was performed for 30 cycles under standard conditions, with a 53°C annealing, using forward (5'-GCC AGG ATG TTC GAC ATG-3') and reverse (5'-TCC AGT TCC GGA GGT GAG-3') primers in exons 2 and 12, respectively. The amplified product was gel isolated and sequenced.

**Immunohistochemistry**

Immunostaining was performed using an anti-cochlin antibody generated against a peptide in the vWF A1 domain of cochlin (Fig. 1), corresponding to amino acid residues 163–181 of human cochlin, identical to the residues in murine and bovine cochlin (21). Anti-serum was purified through a protein A sepharose column, followed by peptide-affinity chromatography. This antibody (anti-cochlin/vWF A1 domain) recognizes all three different-sized isoforms of cochlin.

Immunohistochemistry was performed as previously described (4). Briefly, paraffin-embedded sections from postnatal Coch G88E/G88E and Coch +/+ mice, were incubated with anti-cochlin/vWF A1 domain antibody overnight at room temperature, washed, and incubated with a secondary biotinylated anti-rabbit IgG (Vector Labs, Burlingame, CA, USA). Immunostaining was visualized by incubation with the Vectastain ABC reagent (Vector Labs) followed by 3,3′-diaminobenzidine (DAB). Sections were not counterstained.

**VsEP, ABR and DPOAE measurements**

*Animals and animal preparation*. The use of animals for these studies was approved by the Institutional Animal Care and Use Committee at East Carolina University. Homozygous,
Coch\(^{G88E/G88E}(n = 17)\), heterozygous, Coch\(^{G88E/+}(n = 14)\) and Coch\(^{+/+}\) wild-type littermates \((n = 15)\) were studied at five ages: 11, 13, 17, 19 and 21 months. Mice were anesthetized with a ketamine \((18 \text{ mg/ml})\) and xylazine \((2 \text{ mg/ml})\) solution \((5–7 \mu\text{l} \text{ per gram body weight injected intraperitoneally})\). Core body temperature was maintained at 37.0 ± 0.1°C using a homeothermic heating blanket system (FHC, Inc., Bowdoin, ME, USA). VsEPs were measured at all ages, auditory brainstem responses (ABRs) are reported at 11 and 21 months of age, and DPOAEs were measured at 19 and 21 months only. Six animals were measured at two or more ages.

Vestibular stimulus and stimulus coupling. VsEP recordings were based on methods for mice published by Jones and Jones (22) and Jones et al. (23–25), and are briefly described later. The published methods were modified to utilize a non-invasive coupling system for securing the head to the mechanical shaker. Linear acceleration pulses, 2 ms duration, were presented to the cranium in the naso-occipital axis using two stimulus polarities, normal (+Gx axis) and inverted (−Gx axis). Stimuli were presented at a rate of 17 pulses/s. Stimulus amplitudes ranged from +6 to −18 dB re: 1.0 g/ms (where 1 g = 9.8 m/s\(^2\)) adjusted in 3 dB steps.

Stimuli were delivered to the head using a voltage-controlled mechanical shaker. The head was coupled to a custom platform with a custom head clip, a lightweight plastic spring clip with tines modified to encircle the head anterior to the pinnae. The spring clip was screwed to the custom platform mounted to the mechanical shaker. Although previously published reports using VsEPs utilized an invasive surgical approach to couple the cranium to the shaker, the coupling used here was non-invasive.

ABR stimulus and stimulus coupling. For ABR testing, tone burst stimuli were generated by a signal generator (Agilent HP 23201A or Stanford Research Systems SRS785 signal analyzer) and controlled using custom software and Tucker Davis Technologies (TDT, Gainesville, FL, USA) modules (TG6, SW2, PA4). Tone bursts at 8, 16, 32 and 41.2 kHz had 1.0 ms rise–fall times with 1.0 ms plateau (3 ms total duration). Stimuli for ABR testing were calibrated using a Brüel & Kjaer 1/4 in. microphone and Nexus amplifier. Stimuli were calibrated in dB peSPL and were presented via high frequency transducers (TDT ED1 driver, EC1 speakers) coupled at the left ear via PE tubing. Auditory stimuli were presented at a rate of 17 stimuli/s.

VsEP and ABR recording parameters. Stainless steel wire was placed subcutaneously at the nuchal crest to serve as the non-inverting electrode. Needle electrodes were placed posterior to the left pinnae and at the ventral neck for inverting and ground electrodes, respectively. Traditional signal averaging was used to resolve responses in electrophysiological recordings. Ongoing electroencephalographic activity was amplified \((×200 \text{,000})\), filtered \((300 \text{ to } 3000 \text{ Hz, } −6 \text{ dB amplitude points})\) and digitized \((1024 \text{ points, } 10 \mu\text{s/pt})\). Two hundred and fifty six primary responses were averaged for each VsEP or ABR response waveform. All responses were replicated. VsEP recordings began at the maximum stimulus intensity \((\text{i.e. } +6 \text{ dB re: } 1.0 \text{ g/ms})\) with and without acoustic masking, then intensity was dropped to −18 dB and raised in 3 dB steps to complete an intensity profile. A broad band forward masker \((50–50 000 \text{ Hz, } 97 \text{ dB SPL})\) was presented during VsEP measurements to verify absence of cochlear responses (22). ABR intensity series was collected with a descending series of stimulus intensities \((12 \text{ dB steps})\) beginning at \(≈100 \text{ dB peSPL}\).

DPOAE stimulus and recording. Methods for recording DPOAEs were similar to those previously described (26,27). Stimuli for DPOAEs were generated and controlled with modules from TDT. Pure tone frequencies \((f1, f2, f2/f1 \text{ ratio } = 1.25)\), at equal levels \((L1 = L2 = 60 \text{ dB SPL})\), 150 ms duration, were generated with independent sources (Agilent HP 23201A signal generators) and routed through separate drivers to mix acoustically in the ear canal (via plastic tubing placed securely at the external acoustic meatus). Stimuli were calibrated in a 0.1 ml coupler, which simulated the mouse ear canal volume. Stimulus frequencies for the primaries were such that geometric mean \([\text{GM } = (f1 \times f2)^{0.5}]\) frequencies ranged from 6.0 to 48.5 kHz \((\text{at least eight frequencies per octave})\). Ear canal sound pressure levels were recorded with a low noise probe microphone (E tymotic ER 10B+). The microphone output was amplified and routed to a dynamic signal analyzer (Stanford Research Systems SRS785) for sampling \((\text{at } 200 \text{ kHz})\) and fast Fourier transform (FFT). The amplitude of f1, f2, and the 2f1−f2 distortion product were measured from the FFT waveform. The noise floor was measured from the amplitudes in the five frequency bins above and below the 2f1−f2 component. The recording system was also tested periodically in the 0.1 ml coupler to assess the presence of artifact distortion products. Frequencies where distortion products were present in the test cavity were excluded from further analyses; however no artificial distortion was identified.

Data analysis. The first three positive and negative response peaks were scored for VsEPs. Response peak latencies \((\text{measured in milliseconds, ms})\), peak to peak amplitudes \((\text{measured in microvolts, } \mu\text{V})\) and thresholds \((\text{measured in dB re: } 1.0 \text{ g/ms})\) were quantified for VsEPs. Threshold \((\text{in dB peSPL})\) were obtained for ABR waveforms and amplitudes were quantified for DPOAEs. Descriptive statistics were calculated for each measure of each genotype. Animals with absent-evoked potentials contributed to an estimated mean of the group by replacing the ‘no response’ data points with the maximum possible stimulus level \((+9 \text{ dB for VsEP and } 100 \text{ dB peSPL for ABR})\). All DPOAE amplitudes contributed to the DPOAE mean data.

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Conflict of Interest statement. None to declare.

REFERENCES


