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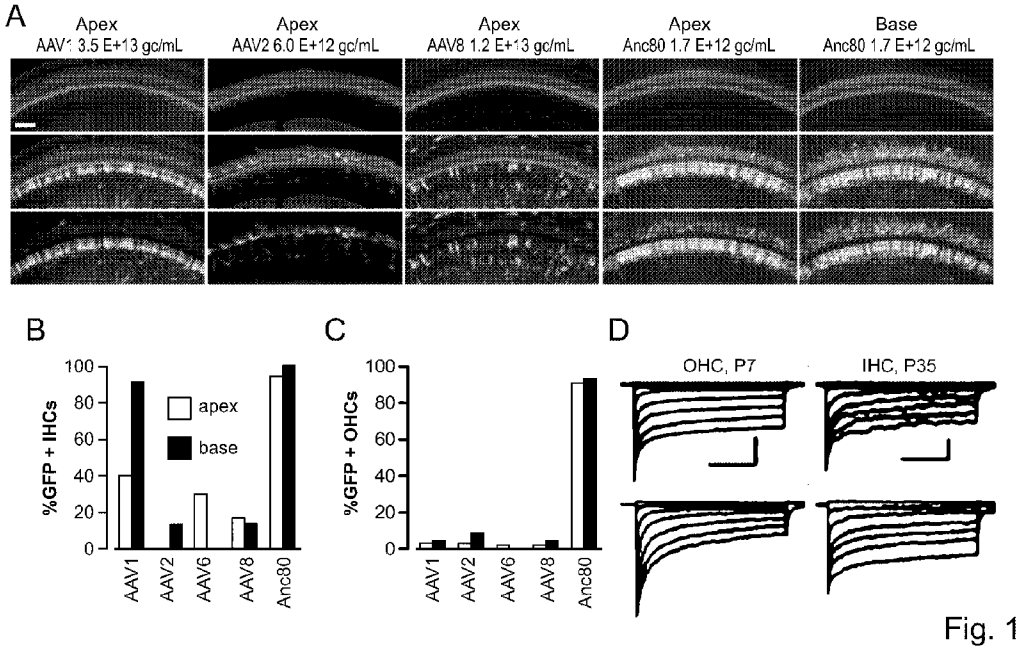

Fig. 1

(57) Abstract: Provided herein are materials and methods for efficiently delivering nucleic acids to cochlear and vestibular cells, and methods of treating sensory transduction disorders associated with a genetic defect.

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MATERIALS AND METHODS FOR DELIVERING NUCLEIC ACIDS TO COCHLEAR AND VESTIBULAR CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to and the benefit of U.S. Provisional Application No. 62/455,197, filed February 6, 2017, the disclosure of which is incorporated herein by reference in its entirety.

BACKGROUND

Genetically-based hearing loss is a significant problem with few therapeutic options other than cochlear implants. Inherited hearing problems are often due to single gene defects. Prelingual deafness is diagnosed in 1/500 infants, of which about 50% have a genetic etiology. Usher syndrome, which is associated with a number of different clinical subtypes, each of which can be caused by a mutation in any of a number of different genes, is responsible for 3 to 6% of early childhood deafness. One of the more prevalent genetic defects, estimated to be 1-2% of all genetic deafness, occurs in the TMC1 gene. The most severe form of Usher Syndrome, USH1, is associated with defects in six genes: *USH1*, *MYO7A* (myosin 7a), *USH1C* (harmonin), *CDH23*(cadherin 23), *PCDH15* (protocadherin 15), *SANS* (sans; also known as USH1G) and *CIB2* (calcium and integrin binding protein2).

The inner ear, e.g., cochlea, particularly the inner and outer hair cells (IHCs and OHCs) in the cochlea, is an attractive target for polynucleotide therapy approaches to intervene in hearing loss and deafness of various etiologies, most immediately monogenic forms of inherited deafness. However, it has been a challenge to efficiently target and transduce IHCs and OHCs as well as other inner ear cells that may be relevant to gene therapy approaches.

SUMMARY

In one particular aspect, the invention encompasses a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, wherein the vector: encodes a capsid

having at least about 85% sequence identity to Anc80 of SEQ ID NO:1, comprises a promoter selected from the group consisting of an Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter, and comprises a polynucleotide, wherein the promoter directs expression of the polynucleotide, and wherein the polynucleotide encodes transmembrane channel-like 1 (TMC1).

In one other aspect, the invention encompasses a use of the synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector of a preceding aspect for preparing a medicament for treating hearing loss and/or vestibular dysfunction in a subject, wherein the hearing loss and/or vestibular dysfunction is associated with a defect in a TMC1 gene.

In one other aspect, the invention encompasses a use of the synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector of a preceding aspect for preparing a medicament for providing a wild-type form of a defective gene to a subject in need thereof, wherein the wild-type gene is a TMC1 gene.

In one other aspect, the invention encompasses a method of treating hearing loss and/or vestibular dysfunction in a subject, the method comprising contacting a cell of the subject with a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, wherein the vector: encodes a capsid having at least about 85% sequence identity to Anc80 of SEQ ID NO:1, comprises a promoter selected from the group consisting of an Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter, and comprises a polynucleotide, wherein the promoter directs expression of the polynucleotide, wherein the polynucleotide encodes human TMC1, and wherein the hearing loss and/or vestibular dysfunction is associated with a defect in a TMC1 gene.

General aspects of the present disclosure are also provided herein. These are set out below and in the description that follows.

The present disclosure provides compositions and methods for targeting a cell of the inner ear of a subject (e.g., inner or outer hair cell) and expressing a transgene encoding a polypeptide of interest (e.g., TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7 USH1C (e.g., harmonin-a, b, or c)). In one embodiment, an Inner Ear Hair Cell Targeting AAV is administered to the inner ear of a subject having a genetic defect in auditory and/or vestibular mechanosensation.

As shown herein, an adeno-associated viruses (AAV) containing an ancestral scaffold capsid protein referred to as Anc80 or a specific Anc80 capsid protein (e.g., Anc80-0065) efficiently target various cells in the inner ear, such as IHCs and OHCs *in vivo*.

Gene transfer to inner ear cells in animal models has had limited efficacy due to limited transduction of one or more types of cells, e.g., the outer hair cells. However, the novel gene delivery modalities described herein, which include new compositions and methods based on an AAV containing an Anc80 capsid protein, provide highly efficient gene transfer to inner ear cells including both IHCs and OHCs.

In one aspect, the present disclosure provides a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, where the vector contains a polynucleotide encoding a human USH1 polypeptide, where the USH1 polypeptide is myosin 7a, harmonin (e.g., harmonin-a, harmonin-b, or harmonin-c), cadherin 23, protocadherin 15, SANS and calcium and integrin binding protein 2, or any other polypeptide described herein.

In another aspect, the present disclosure provides a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, where the vector encodes a capsid having at least about 85% sequence identity to Anc80L65, and contains a promoter that directs expression of a human TMC1 or TMC2 polynucleotide.

In another aspect, the present disclosure provides a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, where the vector contains a promoter that is an Espin promoter, a PCDH15 promoter, a PTPRQ promoter or a TMHS (LHFPL5) promoter that directs expression of a downstream polynucleotide.

In another aspect, the present disclosure provides a cell containing the synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector of previous aspect.

In another aspect, the present disclosure provides a method of expressing a polypeptide in the inner ear of a subject, the method involving contacting a cell of the inner ear with a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector encoding a polypeptide of interest, where the AAV vector transfects at least about 70% of cells of the inner ear and contains ancestral AAV sequences.

In another aspect, the present disclosure provides a method of expressing a polypeptide in the inner ear of a subject, the method involving contacting a cell of the inner ear with a synthetic adeno-associated virus (AAV) vector encoding a human polypeptide of interest, where the AAV vector encodes a capsid having at least about 85% sequence identity to Anc80L65.

In another aspect, the present disclosure provides a method of treating a sensory transduction defect in a subject, the method involving contacting a cell of the subject with a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, where the vector contains a polynucleotide encoding a human USH1 polypeptide, where the USH1 polypeptide is any one or more of myosin 7a, harmonin, cadherin 23, protocadherin 15, SANS and calcium and integrin binding protein 2.

In another aspect, the present disclosure provides a method of treating a sensory transduction defect in a subject, the method involving contacting a cell of the subject with a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, where the vector contains a promoter is any of an Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter.

In another aspect, the present disclosure provides a method of treating a sensory transduction defect in a subject, the method involving contacting a cell of the subject with a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, where the vector encodes a capsid having at least about 85% sequence identity to Anc80L65, and contains a promoter operably linked to a polynucleotide encoding an USH1 polypeptide that is myosin 7a, harmonin, cadherin 23, protocadherin 15, SANS and calcium or integrin binding protein 2.

In various embodiments of the above-aspects or any other aspect of the present disclosure described herein, the sensory transduction defect is a genetic disorder associated with a genetic alteration in a polypeptide expressed in the inner ear. In other embodiments of the above aspects, the promoter is any one or more of an Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter. In other

embodiments of the above aspects, the vector transduces inner and outer hair cells with at least about 70% or greater efficiency. In other embodiments of the above aspects, the harmonin polypeptide is harmonin-a, harmonin-b, or harmonin-c. In other embodiments of the above aspects, the cell is an outer or inner hair cell. In other embodiments of the above aspects, the vector contains a promoter directing expression of a downstream polynucleotide, and the promoter is an Espin promoter, a PCDH15 promoter, a PTPRQ promoter or a TMHS (LHFPL5) promoter. In other embodiments of the above aspects, the downstream polynucleotide is TMC1, TMC2 or an USH1 polypeptide that is myosin 7a, harmonin, cadherin 23, protocadherin 15, SANS and calcium or integrin binding protein 2. In particular embodiments of the above aspects, the harmonin polypeptide is harmonin-a, harmonin-b, or harmonin-c. In other embodiments of the above aspects, the synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector targets inner and outer hair cells with at least about 70%, 80%, 90%, 95% or greater efficiency, even as high as 100% efficiency. In other embodiments of the above aspects, the human polypeptide is TMC1, TMC2, harmonin-a, harmonin-b, or harmonin-c. In other embodiments of the above aspects, the sensory transduction defect is a hearing disorder or vestibular disorder. In other embodiments of the above aspects, the sensory transduction defect is Usher Syndrome.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this disclosure belongs. The following references provide one of skill with a general definition of many of the terms used in this present disclosure: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

By "Ancestral AAV sequence" is meant a designed sequence that is the product of genetic engineering arising from an analysis of naturally existing AAVs and prediction of an evolutionary ancestor.

By "Anc80 polypeptide" is meant a capsid polypeptide having at least about 85% amino acid identity to the following polypeptide sequence:

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGLDKGEPVNAADA
AALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQAKKRVLEPLGLVEEGAKTAP
GKKRPVEQSPQEPDSSSGIGKKGQQPARKRLNFGQTGDSESVPDPQPLGEPPAAPSGVGSNTMAAGGGAP
MADNNEGADGVGNASGNWHCDSTWLGDRVITTSTRT ALPTYNNHLYKQISSQSGGSTNDNTYFGYSTPW
GYFDFNRFHCHFSPRDWQRLINNNWGFRPKKLNFKLFNIQVKEVTTNDGTTTIANNLTSTVQVFTDSEYQ
LPYVLGSAHQGCLPPFPADVFMI PQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYTFEDVP
FHSSYAHSQSLDRLMNPLIDQYLYYLSRTQTTSGTAGNRTLQFSQAGPSSMANQAKNWLPGPCYRQQRVS
KTTNQNNNSNFAWTGATKYHLNGRDSLVNPGPAMATHKDDEDKFFPMSGVLIFGKQGAGNSNVDLDNVMI
TNEEEIKTTNPVATEEYGTVATNLQSANTAPATGTVNSQGALPGMVWQDRDVYLQGPIWAKIPHTDGHFH
PSPLMGGFGLKHPPPQILIKNTPVPANPPTTFSPAKFASFITQYSTGQVSVEIE ELQKENSKRWNPEIQ
YTSNYNKSTNVDFAVDTNGVYSEPRPIGTRYLTRNL

By "Anc80 polynucleotide" is meant a nucleic acid molecule encoding a Anc80 polypeptide.

By "Seroprevalence" is meant the number of persons in a population who test positive for a specific disease based on serology (blood serum) specimens. In one embodiment, seroprevalence is characterized as a percentage of the total specimens tested or as a proportion per 100,000 persons tested.

By "Inner Ear Hair Cell Targeting AAV" is meant an adeno-associated virus that transfects at least 70% of inner hair cells and 70% of outer hair cells following administration to the inner ear of a subject or contact with a cell derived from an inner ear *in vitro*. Preferably, an Inner Ear Hair Cell targeting AAV is an AAV that transfects at least 90% of inner hair cells and 90% of outer hair cells after injecting to the cochlea *in vivo*. The transfection efficiency may be assessed using a gene encoding GFP in a mouse model.

By "mechanosensation" is meant a response to a mechanical stimulus. Touch, hearing, and balance of examples of the conversion of a mechanical stimulus into a neuronal signal. Mechanosensory input is converted into a response to a mechanical stimulus through a process termed "mechanotransduction."

By "TMC1 polypeptide" is meant a polypeptide having at least about 85% or greater amino acid sequence identity to NCBI Reference Sequence: NP_619636.2 or a fragment thereof having mechanotransduction channel activity. An exemplary amino acid sequence of TMC1 is provided below:

1 mspkkvqikv eekedetees sseeeeeved klprreslrp krkrtrdvin eddpepeped

61 eetrkareke rrrrlkrgae eeeideeele rlkaeldekr qiiatvkckp wkmekkievl
121 keakkfvsen egalgkgkgk rwfafkmmma kkwakflrdf enfkaacvpw enkikaiesq
181 fgssvasyfl flrwmygvnm vlfiltfsli mlpeylwglp ygslprktvp raeeasaanf
241 gvlydfngla qysvlfygyy dnkrtigwmn frlplsyflv gimcigysfl vvlkamtkni
301 gddgggddnt fnfswkvfts wdylignpet adnkfnsitm nfkeaiteek aaqveenvhl
361 irflrflanf fvfltlggsg ylifwavkrs qefaqqdpdt lgwweknemn mvmsllgmfc
421 ptlfdlfael edyhplialk wllgrifall lgnlyvfila lmdeinnkie eeklvkanit
481 lweanmikay nasfsenstg ppffvhpadv prgpcwetmv gqefvrltvs dvlttyvtil
541 igdflracfv rfcnycwcwd leygypsyte fdisgnvlal ifnqgmiwmg sffapslpgi
601 nilrlhtsmy fqcwavmccn vpearvfkas rsnnfylgml llilflstmp vlymivslpp
661 sfdcgpfsgk nrmfeviget lehdfpswma kilrqlsnpg lviavilvmv laiyylnata
721 kgqkaanldl kkkmkmqale nkmrnkkmaa araaaaagrq

By "TMC1 polynucleotide" is meant a polynucleotide encoding a TMC1 polypeptide. The sequence of an exemplary TMC1 polynucleotide is provided at NCBI Reference Sequence: NM_138691.2, which is reproduced below:

1 cagaaactat gagggcagaa cccagcaatc tgtgctttct ttcacaagcc ctccaggagt
61 tgctgaaatt taggaatcat tgccccaaaa agtggccctc ataatgatgc cagatgggat
121 cttactctgt tgcccaggct ggagtgcagt ggtgcgatct cggctctctg caacctccgc
181 ctcccaggtt caagtgattc tcctgcctcg gcctcctgag tagctgggat ttcaggccat
241 gaaagatcac tgttttagtc tgcgtggtgc agtggaacag atagacctcg gtttgaatct
301 cagctctact gtttactaga catgaaatgg ggaaatctaa aatgagatgc cagaagcctc
361 aaaaatggaa aacccccctgt gcttcacatc tgaaaatctc tgctgggggc agcaactttg
421 agcctgtggg gaaggaactg tccacgtgga gtggtctggt gaatgcttaa ggagctgcag
481 aagggaagtc cctctccaaa ctagccagcc actgagacct tctgacagga caccccccagg
541 atgtcaccca aaaaagtaca aatcaaagtg gaggaaaaag aagacgagac tgaggaaagc
601 tcaagtgaag aggaagagga ggtggaagat aagctacctc gaagagagag cttgagacca
661 aagaggaaac ggaccagaga tgttatcaat gaggatgacc cagaacctga accagaggat
721 gaagaaacaa ggaaggcaag agaaaaagag aggaggagga ggctaaagag aggagcagaa
781 gaagaagaaa ttgatgaaga ggaattggaa agattgaagg cagagttaga tgagaaaaga
841 caaataattg ctactgtcaa atgcaaacca tggaagatgg agaagaaaat tgaagttctc
901 aaggaggcaa aaaaatttgt gagtgaaaat gaaggggctc ttgggaaagg aaaaggaaaa
961 cggtggtttg catttaagat gatgatggcc aagaaatggg caaaattcct ccgtgatttt
1021 gagaacttca aagctgcgtg tgtcccatgg gaaaataaaa tcaaggctat tgaaagtcag
1081 tttggctcct cagtggcctc atacttcctc ttcttgagat ggatgtatgg agtcaatatg
1141 gttctcttta tcctgacatt tagcctcatc atgttgccag agtacctctg gggtttgcca
1201 tatggcagtt tacctaggaa aaccgttccc agagccgaag aggcatcggc agcaaacttt
1261 ggtgtgttgt acgacttcaa tggtttggca caatattccg ttctctttta tggctattat
1321 gacaataaac gaacaattgg atggatgaat ttcaggttgc cgctctccta ttttctagtg
1381 gggattatgt gcattggata cagctttctg gttgtcctca aagcaatgac caaaaacatt
1441 ggtgatgatg gaggtggaga tgacaacact ttcaatttca gctggaaggt ctttaccagc
1501 tgggactacc tgatcggcaa tcctgaaaca gcagacaaca aatttaattc tatcacaatg
1561 aactttaagg aagctatcac agaagaaaaa gcagcccaag tagaagaaaa cgtccacttg
1621 atcagattcc tgaggtttct ggctaacttc ttcgtgtttc taacacttgg agggagtgga

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1681 tacctcatct tttgggctgt gaagcgatcc caggaatttg cacagcaaga tcctgacacc
1741 cttgggtggt gggaaaaaaa tgaaatgaac atggttatgt ccctcctagg gatgttctgt
1801 ccaacattgt ttgacttatt tgctgaatta gaagactacc atcctctcat cgctttgaaa
1861 tggctactgg gacgcatttt tgctcttctt ttaggcaatt tatacgtatt tattcttgca
1921 ttaatggatg agattaacaa caagattgaa gaggagaagc tagtaaaggc caatattacc
1981 ctttgggaag ccaatatgat caaggcctac aatgcatcat tctctgaaaa tagcactgga
2041 ccacccctttt ttgttcaccc tgcagatgta cctcgaggac cttgctggga aacaatggtg
2101 ggacaggagt ttgtgaggct gacagtctct gatgttctga ccacctacgt cacaatcctc
2161 attggggact ttctaagggc atgttttgtg aggttttgca attattgctg gtgctgggac
2221 ttggagtatg gatatccttc atacaccgaa ttcgacatca gtggcaacgt cctcgctctg
2281 atcttcaacc aaggcatgat ctggatgggc tccttctttg ctcccagcct cccaggcatc
2341 aatatccttc gactccatac atccatgtac ttccagtgct gggccgttat gtgctgcaat
2401 gttcctgagg ccagggtctt caaagcttcc agatcaaata acttctacct gggcatgcta
2461 ctgctcatcc tcttcctgtc cacaatgcct gtcttgtaca tgatcgtgtc cctcccacca
2521 tcttttgatt gtggtccatt cagtggcaaa aatagaatgt ttgaagtcat tggagagacc
2581 ctggagcacg atttcccaag ctggatggcg aagatcttga gacagctttc aaaccctggg
2641 ctggtcattg ctgtcatttt ggtgatggtt ttggccatct attatctcaa tgctactgcc
2701 aagggccaga aggcagcgaa tctggatctc aaaaagaaga tgaaaatgca agctttggag
2761 aacaaaatgc gaaacaagaa aatggcagct gcacgagcag ctgcagctgc tggtcgccag
2821 taataagtat cctgagagcc cagaaaaggt acactttgcc ttgctgttta aaagtaatgc
2881 aatatgtgaa cgcccagaga acaagcactg tggaactgct attttcctgt tctacccttg
2941 atggattttc aaggtcatgc tggccaatta aggcatcatc agtcctacct gagcaacaag
3001 aatctaaact ttattccaag tcagaaactg tttctgcaga gccactctct cccctgctcc
3061 atttcgtgac ttttttttt ttttaacaa attgagttta gaagtgagtg taatccagca
3121 atacagttta ctggtttagt tggtgggtta attaaaaaaa atttgctcat atgaactttc
3181 attttatatg tttcttttgc c
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By "TMC2 polypeptide" is meant a polypeptide having at least about 85% or greater amino acid sequence identity to NCBI Reference Sequence: NP_542789 or a fragment thereof that functions in mechanosensation. An exemplary amino acid sequence of TMC2 is provided below:

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  1 mshqvkglke earggvkgrv ksgsphtgdr lgrrssskra lkaegtpgrr gaqrsqkera
 61 ggspspgspr rkqtgrrrhr eelgeqerge aertcegrrk rderasfqer taapkrekei
121 prreekskrq kkprssslas sasggeslse eelaqileqv eekkkliatm rskpwpmakk
181 ltelreaqef vekyegalgk gkgkqlyayk mlmakkwvkf krdfdnfktq cipwemkikd
241 ieshfgssva syfiflrwmy gvnlvlfgli fglviipevl mgmpygsipr ktvpraeeek
301 amdfsvlwdf egyikysalf ygyynnqrti gwlryrlpma yfmvgvsvfg ysliivirsm
361 asntqgstge gesdnftfsf kmftswdyli gnsetadnky asittsfkes ivdeqesnke
421 enihltrflr vlanfliicc lcgsgyliyf vvkrsqqfsk mqnvswyern eveivmsllg
481 mfcpplfeti aalenyhprt glkwqlgrif alflgnlytf llalmddvhl klaneetikn
541 ithwtlfnyy nssgwnesvp rpplhpadvp rgscwetavg iefmrltvsd mlvtyitill
601 gdflracfvr fmnycwcwdl eagfpsyaef disgnvlgli fnqgmiwmgs fyapglvgin
661 vlrlltsmyf qcwavmssnv phervfkasr snnfymglll lvlflsllpv aytimslpps
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721 fdcgpfsgkn rmydvlqeti endfptflgk ifaflanpgl iipaillmfl aiyylnsvsk
781 slsranaqlr kkiqvlreve kshksvkgka tardsedtpk sssknatqlq ltkeettpps
841 asqsqamdkk aqgpgtsnsa srttlpasgh lpisrppgig pdsghapsqt hpwrsasgks
901 aqrpph
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By "harmonin" polypeptide is meant a polypeptide having at least about 85% amino acid sequence identity to Q9Y6N9-1 (isoform 1), Q9Y6N9-2, Q9Y6N9-3, Q9Y6N9-4, Q9Y6N9-5 or a fragment thereof that functions in mechanosensation or that interacts with any one or more of USH1C, USH1G, CDH23 and MYO7A. The sequence of an exemplary harmonin-a polypeptide (isoform 1) is provided below:

```
>sp|Q9Y6N9|USH1C_HUMAN Harmonin OS=Homo sapiens GN=USH1C PE=1 SV=3
MDRKVAREFRHKVDFLIENDAEKDYLYDVLRMYHQTMDVAVLVGDLKLVINEPSRLPLFD
AIRPLIPLKHQVEYDQLTPRRSRKLKEVRLDRLHPEGLGLSVRGGLEFGCGLFISHLIKG
GQADSVGLQVGDEIVRINGYSISSCTHEEVINLIRTKKTVSIKVRHIGLIPVKSSPDEPL
TWQYVDQFVSESGGVRGSLGSPGNRENKEKKVFISLVGSRGLGCSISSGPIQKPGIFISH
VKPGSLSAEVGLEIGDQIVEVNGVDFSNLDHKEAVNVLKSSRSLTISIVAAAGRELFMTD
RERLAEARQRELQRQELLMQKRLAMESNKILQEQQEMERQRRKEIAQKAAEENERYRKEM
EQIVEEEEKFKKQWEEDWGSKEQLLLPKTITAEVHPVPLRKPKYDQGVEPELEPADDLDG
GTEEQGEQDFRKYEEGFDPYSMFTPEQIMGKDVRLLRIKKEGSLDLALEGGVDSPIGKVV
VSAVYERGAAERHGGIVKGDEIMAINGKIVTDYTLAEAEAALQKAWNQGGDWIDLVVAVC
PPKEYDDELTFF
```

By "Ush1C polynucleotide" is meant a nucleic acid molecule encoding a harmonin polypeptide. The sequence of exemplary Ush1C polynucleotide NM_005709 is provided below:

```
   1 agctccgagg gcggctggcc cggtcgcggt cgcggctctt tccagctcct ggcagccggg
  61 cacccgaagg aacgggtcgt gcaacgacgc agctggacct ggcccagcca tggaccgaaa
 121 agtggcccga gaattccggc ataaggtgga ttttctgatt gaaaatgatg cagagaagga
 181 ctatctctat gatgtgctgc gaatgtacca ccagaccatg gacgtggccg tgctcgtggg
 241 agacctgaag ctggtcatca atgaacccag ccgtctgcct ctgtttgatg ccattcggcc
 301 gctgatccca ctgaagcacc aggtggaata tgatcagctg acccccggc gctccaggaa
 361 gctgaaggag gtgcgtctgg accgtctgca ccccgaaggc ctcggcctga gtgtgcgtgg
 421 tggcctggag tttggctgtg ggctcttcat ctcccacctc atcaaaggcg gtcaggcaga
 481 cagcgtcggg ctccaggtag gggacgagat cgtccggatc aatggatatt ccatctcctc
 541 ctgtacccat gaggaggtca tcaacctcat tcgaaccaag aaaactgtgt ccatcaaagt
 601 gagacacatc ggcctgatcc ccgtgaaaag ctctcctgat gagcccctca cttggcagta
 661 tgtggatcag tttgtgtcgg aatctggggg cgtgcgaggc agcctgggct cccctggaaa
 721 tcgggaaaac aaggagaaga aggtcttcat cagcctggta ggctcccgag gccttggctg
 781 cagcatttcc agcggcccca tccagaagcc tggcatcttt atcagccatg tgaaacctgg
 841 ctccctgtct gctgaggtgg gattggagat aggggaccag attgtcgaag tcaatggcgt
 901 cgacttctct aacctggatc acaaggaggc tgtaaatgtg ctgaagagta gccgcagcct
 961 gaccatctcc attgtagctg cagctggccg ggagctgttc atgacagacc gggagcggct
1021 ggcagaggcg cggcagcgtg agctgcagcg gcaggagctt ctcatgcaga agcggctggc
1081 gatggagtcc aacaagatcc tccaggagca gcaggagatg gagcggcaaa ggagaaaaga
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1141 aattgcccag aaggcagcag aggaaaatga gagataccgg aaggagatgg aacagattgt
1201 agaggaggaa gagaagttta agaagcaatg ggaagaagac tggggctcaa aggaacagct
1261 actcttgcct aaaaccatca ctgctgaggt acacccagta ccccttcgca agccaaagta
1321 tgatcaggga gtggaacctg agctcgagcc cgcagatgac ctggatggag gcacggagga
1381 gcagggagag caggatttcc ggaaatatga ggaaggcttt gacccctact ctatgttcac
1441 cccagagcag atcatgggga aggatgtccg gctcctacgc atcaagaagg agggatcctt
1501 agacctggcc ctggaaggcg gtgtggactc ccccattggg aaggtggtcg tttctgctgt
1561 gtatgagcgg ggagctgctg agcggcatgg tggcattgtg aaaggggacg agatcatggc
1621 aatcaacggc aagattgtga cagactacac cctggctgag gctgaggctg ccctgcagaa
1681 ggcctggaat cagggcgggg actggatcga ccttgtggtt gccgtctgcc ccccaaagga
1741 gtatgacgat gagctgacct tcttctgaag tccaaaaggg gaaaccaaat tcaccgttag
1801 gaaacagtga gctccggccc cacctcgtga acacaaagcc tcggatcagc cttgagagag
1861 gccacactac acacaccaga tggcatcctt gggacctgaa tctatcaccc aggaatctca
1921 aactcccttt ggccctgaac cagggccaga taaggaacag ctcgggccac tcttctgaag
1981 gccaacgtgg aggaaaggga gcagccagcc atttgggaga agatctcaag gatccagact
2041 ctcattcctt tcctctggcc cagtgaattt ggtctctccc agctctgggg gactccttcc
2101 ttgaacccta ataagacccc actggagtct ctctctctcc atccctctcc tctgccctct
2161 gctctaattg ctgccaggat tgtcactcca aaccttactc tgagctcatt aataaaatag
2221 atttattttc cagctta
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Other Exemplary harmonin sequences are provided below:

Harmonin-B

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>XM_011519832.2 PREDICTED: Homo sapiens USH1 protein network component
harmonin (USH1C), transcript variant X3, mRNA
AGCTCCGAGGGCGGCTGGCCCGGTCGCGGTCGCGGCTCTTTCCAGCTCCTGGCAGCCGGGCACCCGAAGG
AACGGGTCGTGCAACGACGCAGCTGGACCTGGCCCAGCCATGGACCGAAAAGTGGCCCGAGAATTCCGGC
ATAAGGTGGATTTTCTGATTGAAAATGATGCAGAGAAGGACTATCTCTATGATGTGCTGCGAATGTACCA
CCAGACCATGGACGTGGCCGTGCTCGTGGGAGACCTGAAGCTGGTCATCAATGAACCCAGCCGTCTGCCT
CTGTTTGATGCCATTCGGCCGCTGATCCCACTGAAGCACCAGGTGGAATATGATCAGCTGACCCCCCGGC
GCTCCAGGAAGCTGAAGGAGGTGCGTCTGGACCGTCTGCACCCCGAAGGCCTCGGCCTGAGTGTGCGTGG
TGGCCTGGAGTTTGGCTGTGGGCTCTTCATCTCCCACCTCATCAAAGGCGGTCAGGCAGACAGCGTCGGG
CTCCAGGTAGGGGACGAGATCGTCCGGATCAATGGATATTCCATCTCCTCCTGTACCCATGAGGAGGTCA
TCAACCTCATTCGAACCAAGAAAACTGTGTCCATCAAAGTGAGACACATCGGCCTGATCCCCGTGAAAAG
CTCTCCTGATGAGCCCCTCACTTGGCAGTATGTGGATCAGTTTGTGTCGGAATCTGGGGGCGTGCGAGGC
AGCCTGGGCTCCCCTGGAAATCGGGAAAACAAGGAGAAGAAGGTCTTCATCAGCCTGGTAGGCTCCCGAG
GCCTTGGCTGCAGCATTTCCAGCGGCCCCATCCAGAAGCCTGGCATCTTTATCAGCCATGTGAAACCTGG
CTCCCTGTCTGCTGAGGTGGGATTGGAGATAGGGGACCAGATTGTCGAAGTCAATGGCGTCGACTTCTCT
AACCTGGATCACAAGGAGGCTGTAAATGTGCTGAAGAGTAGCCGCAGCCTGACCATCTCCATTGTAGCTG
CAGCTGGCCGGGAGCTGTTCATGACAGACCGGGAGCGGCTGGCAGAGGCGCGGCAGCGTGAGCTGCAGCG
GCAGGAGCTTCTCATGCAGAAGCGGCTGGCGATGGAGTCCAACAAGATCCTCCAGGAGCAGCAGGAGATG
GAGCGGCAAAGGAGAAAAGAAATTGCCCAGAAGGCAGCAGAGGAAAATGAGAGATACCGGAAGGAGATGG
AACAGATTGTAGAGGAGGAAGAGAAGTTTAAGAAGCAATGGGAAGAAGACTGGGGCTCAAAGGAACAGCT
ACTCTTGCCTAAAACCATCACTGCTGAGGTACACCCAGTACCCCTTCGCAAGCCAAAGTATGATCAGGGA
GTGGAACCTGAGCTCGAGCCCGCAGATGACCTGGATGGAGGCACGGAGGAGCAGGGAGAGCAGAAAGGAA
AAGATAAGAAGAAAGCCAAGTATGGCAGCCTGCAGGACTTGAGAAAGAATAAGAAAGAACTGGAGTTTGA
GCAAAAGCTTTACAAAGAGAAAGAGGAAATGCTGGAGAAGGAAAAGCAGCTAAAGATCAACCGGCTGGCC
CAGGAGGATTTCCGGAAATATGAGGAAGGCTTTGACCCCTACTCTATGTTCACCCCAGAGCAGATCATGG
GGAAGGATGTCCGGCTCCTACGCATCAAGAAGGAGGGATCCTTAGACCTGGCCCTGGAAGGCGGTGTGGA
CTCCCCCATTGGGAAGGTGGTCGTTTCTGCTGTGTATGAGCGGGGAGCTGCTGAGCGGCATGGTGGCATT
GTGAAAGGGGACGAGATCATGGCAATCAACGGCAAGATTGTGACAGACTACACCCTGGCTGAGGCTGAGG
CTGCCCTGCAGAAGGCCTGGAATCAGGGCGGGGACTGGATCGACCTTGTGGTTGCCGTCTGCCCCCCAAA
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GGAGTATGACGATGAGCTGACCTTCTTCTGAAGTCCAAAAGGGGAAACCAAATTCACCGTTAGGAAACAG
TGAGCTCCGGCCCCACCTCGTGAACACAAAGCCTCGGATCAGCCTTGAGAGAGGCCACACTACACACACC
AGATGGCATCCTTGGGACCTGAATCTATCACCCAGGAATCTCAAACTCCCTTTGGCCCTGAACCAGGGCC
AGATAAGGAACAGCTCGGGCCACTCTTCTGAAGGCCAACGTGGAGGAAAGGGAGCAGCCAGCCATTTGGG
AGAAGATCTCAAGGATCCAGACTCTCATTCCTTTCCTCTGGCCCAGTGAATTTGGTCTCTCCCAGCTCTG
GGGGACTCCTTCCTTGAACCCTAATAAGACCCCACTGGAGTCTCTCTCTCTCCATCCCTCTCCTCTGCCC
TCTGCTCTAATTGCTGCCAGGATTGTCACTCCAAACCTTACTCTGAGCTCATTAATAAAATAGATTTATT
TTCCA

Harmonin-B Polypeptide

MDRKVAREFRHKVDFLIENDAEKDYLYDVLRMYHQTMDVAVLVG
DLKLVINEPSRLPLFDAIRPLIPLKHQVEYDQLTPRRSRKLKEVRLDRLHPEGLGLSV
RGGLEFGCGLFISHLIKGGQADSVGLQVGDEIVRINGYSISSCTHEEVINLIRTKKTV
SIKVRHIGLIPVKSSPDEPLTWQYVDQFVSESGGVRGSLGSPGNRENKEKKVFISLVG
SRGLGCSISSGPIQKPGIFISHVKPGSLSAEVGLEIGDQIVEVNGVDFSNLDHKEAVN
VLKSSRSLTISIVAAAGRELFMTDRERLAEARQRELQRQELLMQKRLAMESNKILQEQ
QEMERQRRKEIAQKAAEENERYRKEMEQIVEEEEKFKKQWEEDWGSKEQLLLPKTITA
EVHPVPLRKPKSFGWFYRYDGKFPTIRKKGKDKKKAKYGSLQDLRKNKKELEFEQKLY
KEKEEMLEKEKQLKINRLAQEVSETEREDLEESEKIQYWVERLCQTRLEQISSADNEI
SEMTTGPPPPPPSVSPLAPPLRRFAGGLHLHTTDLDDIPLDMFYYPPKTPSALPVMPH
PPPSNPPHKVPAPPVLPLSGHVSASSSPWVQRTPPPIPIPPPPSVPTQDLTPTRPLPS
ALEEALSNHPFRTGDTGNPVEDWEAKNHSGKPTNSPVPEQSFPPTPKTFCPSPQPPRG
PGVSTISKPVMVHQEPNFIYRPAVKSEVLPQEMLKRMVVYQTAFRQDFRKYEEGFDPY
SMFTPEQIMGKDVRLLRIKKEGSLDLALEGGVDSPIGKVVVSAVYERGAAERHGGIVK
GDEIMAINGKIVTDYTLAEAEAALQKAWNQGGDWIDLVVAVCPPKEYDDELASLPSSV
AESPQPVRKLLEDRAAVHRHGFLLQLEPTDLLLKSKRGNQIHR"

Harmonin-C

>NM_001297764.1 Homo sapiens USH1 protein network component harmonin (USH1C), transcript variant 3, mRNA
AGCTCCGAGGGCGGCTGGCCCGGTCGCGGTCGCGGCTCTTTCCAGCTCCTGGCAGCCGGGCACCCGAAGG
AACGGGTCGTGCAACGACGCAGCTGGACCTGGCCCAGCCATGGACCGAAAAGTGGCCCGAGAATTCCGGC
ATAAGGTGGATTTTCTGATTGAAAATGATGCAGAGAAGGACTATCTCTATGATGTGCTGCGAATGTACCA
CCAGACCATGGACGTGGCCGTGCTCGTGGGAGACCTGAAGCTGGTCATCAATGAACCCAGCCGTCTGCCT
CTGTTTGATGCCATTCGGCCGCTGATCCCACTGAAGCACCAGGTGGAATATGATCAGCTGACCCCCCGGC
GCTCCAGGAAGCTGAAGGAGGTGCGTCTGGACCGTCTGCACCCCGAAGGCCTCGGCCTGAGTGTGCGTGG
TGGCCTGGAGTTTGGCTGTGGGCTCTTCATCTCCCACCTCATCAAAGGCGGTCAGGCAGACAGCGTCGGG
CTCCAGGTAGGGGACGAGATCGTCCGGATCAATGGATATTCCATCTCCTCCTGTACCCATGAGGAGGTCA
TCAACCTCATTCGAACCAAGAAAACTGTGTCCATCAAAGTGAGACACATCGGCCTGATCCCCGTGAAAAG
CTCTCCTGATGAGCCCCTCACTTGGCAGTATGTGGATCAGTTTGTGTCGGAATCTGGGGGCGTGCGAGGC
AGCCTGGGCTCCCCTGGAAATCGGGAAAACAAGGAGAAGAAGGTCTTCATCAGCCTGGTAGGCTCCCGAG
GCCTTGGCTGCAGCATTTCCAGCGGCCCCATCCAGAAGCCTGGCATCTTTATCAGCCATGTGAAACCTGG
CTCCCTGTCTGCTGAGGTGGGATTGGAGATAGGGGACCAGATTGTCGAAGTCAATGGCGTCGACTTCTCT
AACCTGGATCACAAGGAGGGCCGGGAGCTGTTCATGACAGACCGGGAGCGGCTGGCAGAGGCGCGGCAGC
GTGAGCTGCAGCGGCAGGAGCTTCTCATGCAGAAGCGGCTGGCGATGGAGTCCAACAAGATCCTCCAGGA
GCAGCAGGAGATGGAGCGGCAAAGGAGAAAAGAAATTGCCCAGAAGGCAGCAGAGGAAAATGAGAGATAC
CGGAAGGAGATGGAACAGATTGTAGAGGAGGAAGAGAAGTTTAAGAAGCAATGGGAAGAAGACTGGGGCT
CAAAGGAACAGCTACTCTTGCCTAAAACCATCACTGCTGAGGTACACCCAGTACCCCTTCGCAAGCCAAA
GTATGATCAGGGAGTGGAACCTGAGCTCGAGCCCGCAGATGACCTGGATGGAGGCACGGAGGAGCAGGGA
GAGCAGGATTTCCGGAAATATGAGGAAGGCTTTGACCCCTACTCTATGTTCACCCCAGAGCAGATCATGG
GGAAGGATGTCCGGCTCCTACGCATCAAGAAGGAGGGATCCTTAGACCTGGCCCTGGAAGGCGGTGTGGA
CTCCCCCATTGGGAAGGTGGTCGTTTCTGCTGTGTATGAGCGGGGAGCTGCTGAGCGGCATGGTGGCATT
GTGAAAGGGGACGAGATCATGGCAATCAACGGCAAGATTGTGACAGACTACACCCTGGCTGAGGCTGAGG
CTGCCCTGCAGAAGGCCTGGAATCAGGGCGGGGACTGGATCGACCTTGTGGTTGCCGTCTGCCCCCCAAA
GGAGTATGACGATGAGCTGACCTTCTTCTGAAGTCCAAAAGGGGAAACCAAATTCACCGTTAGGAAACAG
TGAGCTCCGGCCCCACCTCGTGAACACAAAGCCTCGGATCAGCCTTGAGAGAGGCCACACTACACACACC
AGATGGCATCCTTGGGACCTGAATCTATCACCCAGGAATCTCAAACTCCCTTTGGCCCTGAACCAGGGCC
AGATAAGGAACAGCTCGGGCCACTCTTCTGAAGGCCAACGTGGAGGAAAGGGAGCAGCCAGCCATTTGGG

AGAAGATCTCAAGGATCCAGACTCTCATTCCTTTCCTCTGGCCCAGTGAATTTGGTCTCTCCCAGCTCTG
GGGGACTCCTTCCTTGAACCCTAATAAGACCCCACTGGAGTCTCTCTCTCTCCATCCCTCTCCTCTGCCC
TCTGCTCTAATTGCTGCCAGGATTGTCACTCCAAACCTTACTCTGAGCTCATTAATAAAATAGATTTATT
TTCCAGCTTA

Harmonin-C Polypeptide

MDRKVAREFRHKVDFLIENDAEKDYLYDVLRMYHQTMDVAVLVG
DLKLVINEPSRLPLFDAIRPLIPLKHQVEYDQLTPRRSRKLKEVRLDRLHPEGLGLSV
RGGLEFGCGLFISHLIKGGQADSVGLQVGDEIVRINGYSISSCTHEEVINLIRTKKTV
SIKVRHIGLIPVKSSPDEPLTWQYVDQFVSESGGVRGSLGSPGNRENKEKKVFISLVG
SRGLGCSISSGPIQKPGIFISHVKPGSLSAEVGLEIGDQIVEVNGVDFSNLDHKEGRE
LFMTDRERLAEARQRELQRQELLMQKRLAMESNKILQEQQEMERQRRKEIAQKAAEEN
ERYRKEMEQIVEEEEKFKKQWEEDWGSKEQLLLPKTITAEVHPVPLRKPKYDQGVEPE
LEPADDLDGGTEEQGEQDFRKYEEGFDPYSMFTPEQIMGKDVRLLRIKKEGSLDLALE
GGVDSPIGKVVVSAVYERGAAERHGGIVKGDEIMAINGKIVTDYTLAEAEAALQKAWN
QGGDWIDLVVAVCPPKEYDDELTFF"

By “Espin promoter” is meant a regulatory polynucleotide sequence derived from NCBI Reference Sequence: NG_015866.1 that is sufficient to direct expression of a downstream polynucleotide in a cochlear cell. In one embodiment, the Espin promoter comprises at least about 350, 500, 1000, 2000, 3000, 4000, 5000, or more base pairs upstream of an Espin coding sequence.

By “protocadherin related 15 (PCDH15) promoter” is meant a regulatory polynucleotide sequence derived from NCBI Reference Sequence: NG_009191 that is sufficient to direct expression of a downstream polynucleotide in a cochlear cell. In one embodiment, the PCDH15promoter comprises at least about 350, 500, 1000, 2000, 3000, 4000, 5000, or more base pairs upstream of an PCDH15 coding sequence.

By “protein tyrosine phosphatase, receptor type Q (PTPRQ) promoter” is meant a regulatory polynucleotide sequence derived from GeneID: 374462 that is sufficient to direct expression of a downstream polynucleotide in a cochlear cell. In one embodiment, the PTPRQ promoter comprises at least about 350, 500, 1000, 2000, 3000, 4000, 5000, or more base pairs upstream of an PTPRQ coding sequence.

By “lipoma HMGIC fusion partner-like 5 (LHFPL5) promoter” also termed “TMHS promoter” is meant a regulatory polynucleotide sequence derived from NCBI Reference Sequence: GeneID: 222662 that is sufficient to direct expression of a downstream polynucleotide in a cochlear cell. In one embodiment, the TMHS promoter comprises at least about 350, 500, 1000, 2000, 3000, 4000, 5000, or more base pairs upstream of an PCDH15 coding sequence.

By “agent” is meant a polypeptide, polynucleotide, or small compound.

By “ameliorate” is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease or disorder.

By "alteration" is meant a change (increase or decrease) in the expression levels or activity of a gene or polypeptide as detected by standard art known methods such as those described herein. As used herein, an alteration includes a 10% change in expression levels, preferably a 25% change, more preferably a 40% change, and most preferably a 50% or greater change in expression levels. "

The terms “comprises” and “comprising” are to be construed as being inclusive and open ended rather than exclusive. Specifically, when used in this specification, including the claims, the terms “comprises” and “comprising” and variations thereof mean that the specified features, steps, or components are included. The terms are not to be interpreted to exclude the presence of other features, steps, or components.

Therefore, in this disclosure, "comprises," "comprising," "containing" and "having" and the like have the meaning "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

“Detect” refers to identifying the presence, absence or amount of the analyte to be detected.

By “disease” is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include genetic disorders characterized by a loss of function in a protein that functions in mechanosensory transduction that is expressed, for example, in the inner ear of a subject. In another embodiment, the disease is Usher Syndrome (e.g., USH1) or age-related hearing loss. In one embodiment, a disease is an auditory disorder associated with a genetic defect, such as a defect in TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7, USH1C (e.g., harmonin-a, b, or c).

By "effective amount" is meant the amount of an agent required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active agent(s) used to practice the methods of the present disclosure for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and

general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.

"Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleobases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds.

The terms "isolated," "purified," or "biologically pure" refer to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation. A "purified" or "biologically pure" protein is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid or peptide of this disclosure is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high performance liquid chromatography. The term "purified" can denote that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated proteins, which can be separately purified.

By "isolated polynucleotide" is meant a nucleic acid (e.g., a DNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the present disclosure is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a

prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By an "isolated polypeptide" is meant a polypeptide of the present disclosure that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, a polypeptide of the present disclosure. An isolated polypeptide of the present disclosure may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding such a polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By “marker” is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

As used herein, “obtaining” as in “obtaining an agent” includes synthesizing, purchasing, or otherwise acquiring the agent.

By "promoter" is meant a polynucleotide sufficient to direct transcription of a downstream polynucleotide.

By “reduces” is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

By “reference” is meant a standard or control condition.

A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least

about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or therebetween.

Nucleic acid molecules useful in the methods of the present disclosure include any nucleic acid molecule that encodes a polypeptide of the this disclosure or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. Nucleic acid molecules useful in the methods of the present disclosure include any nucleic acid molecule that encodes a polypeptide of this disclosure or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule.

By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) Methods Enzymol. 152:399; Kimmel, A. R. (1987) Methods Enzymol. 152:507).

For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, more preferably of at least about 37° C, and most preferably of at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred: embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur

at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 μg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 μg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42 C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group,

University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

By "transgene" is meant any piece of DNA that is inserted by artifice into a cell and becomes part of the genome of the organism that develops from that cell or, in the case of a nematode transgene, becomes part of a heritable extrachromosomal array. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

As used herein, the terms “treat,” treating,” “treatment,” and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless

otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods and compositions of matter belong. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods and compositions of matter, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

DESCRIPTION OF DRAWINGS

Part 1: Highly Efficient Cochlear Gene Transfer

Figure 1A-1G provides a series of micrographs (A) and six panels of graphs (B-G). *In vivo* cochlear transduction of natural AAV serotypes and Anc80. (A) Confocal images of mouse organs of Corti, counterstained with Alexa-546-phalloidin (red) and imaged for eGFP (green—shown throughout this document as bright staining in middle and bottom panels). Mice were injected with 1 μL of AAV stock solution at the titer indicated above each panel. Scale bar = 50 μm. (B) Quantification of eGFP-positive IHCs in the base and apex of AAV-eGFP injected cochleae. (C) Quantification of eGFP-positive OHCs in the base and apex of AAV-eGFP injected cochleae. One ear per C57BL/6 mouse was injected in 5 (AAV1), 4 (AAV2), 2 (AAV8), 1 (AAV6), 3 (Anc80) animals. (D) Families of sensory transduction currents recorded at P7 (left) from eGFP-negative OHCs (black) and eGFP-positive OHCs (green). Hair bundles were deflected between -0.1 and 1 μm in 0.1 μm increments. Vertical scale bar indicates 200 pA; horizontal indicates 20 msec. Currents from eGFP negative (black) and eGFP-positive

(green) P35 IHCs are shown on the right. Vertical scale bar indicates 100 pA; horizontal indicates 20 msec. (E) Sensory transduction current amplitudes plotted for 103 IHCs and OHCs at the ages indicated at the bottom. Data from eGFP-negative (black) and eGFP-positive (green) are shown. The numbers of cells in each group are shown on the graph. All mice were injected at P1. (F) Mean ± standard deviation (SD). ABR thresholds plotted for four Anc80-injected ears (green) and four uninjected ears (black) together with data from one injected ear that had no eGFP fluorescence due to injection-related damage (red). (G) Mean ± SD. DPOAE thresholds are plotted for four Anc80-injected ears (green) and four uninjected ears (black) and one negative control ear with injection damage without eGFP fluorescence (red). Injection titers for data points in B-G are as in A.

Figure 2A-2D are images showing Anc80-eGFP transduction in vestibular sensory epithelia. (A) Mouse utricle from a P1 mouse injected with 1 µL Anc80-eGFP (1.7×10^{12} GC/mL). The tissue was harvested at P10 fixed and stained with Alexa546-phalloidin (red) and imaged for eGFP (green). Morphological assessment across multiple focal planes of eGFP-positive cells demonstrated transduction of stereotypical flask-shaped morphology of type I cells and the cylinder morphology of type II cells in every sample examined (not shown). Scale bar = 100 µm. (B) The crista of the posterior semicircular canal from the same mouse described for panel A. Scale bar = 50 µm. (C) The sensory epithelium of a human utricle. The tissue was exposed to 10^{10} GC Anc80.CMV.eGFP.WPRE for 24 hours, cultured for 10 days, fixed, stained with Alexa546-phalloidin (red) and imaged for eGFP fluorescence (green). Scale bar = 100 µm. (D) High magnification view of a human epithelium in the utricle stained with Alexa546-phalloidin (red) and Myo7A (blue) and imaged for eGFP (green) transduced in identical conditions as in C. White arrows in the overlay panel indicate selected eGFP-positive/Myo7A-positive cells. Scale bar = 20 µm.

Figure 3A-3E are images showing extensive Inner and Outer Hair Cell transduction in murine cochleae with Anc80. (A) Low-magnification image of the entire apical portion of a mouse cochlea injected at P1 with 1 µL of Anc80-eGFP at 1.7×10^{12} GC/mL. The cochlea was harvested at P10 and stained with Alexa546-phalloidin (red) and imaged for eGFP (green). Scale bar = 100 µm. (B) High magnification view of a basal portion from a different mouse cochlea injected at P1 with 1 µL of Anc80-493 eGFP at 1.7×10^{12} GC/mL. The cochlea was harvested at P10 and stained with

Alexa546-phalloidin (red) and imaged for eGFP (green). Scale bar = 20 μm. (C, D) Quantitative comparison of inner and outer hair cell transduction efficiency at an equal dose for all serotypes following round window injection of P1-2 C57BL/6 mice. Mice cochleae in C,D were injected with 1.36 x 10^{12} of AAV1, AAV2, AAV8, and Anc80 and harvested at 7-9 days for live-cell imaging and quantitation by epifluorescent microscopy (n=8 per group). (E) Dose-dependency of Anc80 hair cell transduction. Cochleae exposed to two different Anc80-eGFP titers (1.8 x 10^{12} versus 1.36 x 10^{12} GC) were fixed and stained with Alexa546-phalloidin (red) and imaged for eGFP (green). Scale bar = 20 μm

Figures 4A and 4B are an image and a graph, respectively, showing vestibular function following Anc80 cochlear transduction. Mice were injected at P1 with Anc80.CMV.eGFP via the RWM and evaluated for expression and balance function on the rotarod device. Expression of eGFP (green) in the vestibular tissue is via confocal microscopy with immunofluorescent staining for Myo7A (red) (A). Rotarod data revealed no difference between injected and uninjected controls. The mean time until the mice fell off the device +/- SEM is plotted. N=3 animals, 5 trials each (injected) and 2 animals 5 trials each (control) (B). Scale bar = 50 μm

Part 2: Gene Therapy Restores Auditory and Vestibular Function in a Mouse Model of Usher Syndrome, Type 1c

Figures 5A-5L are images showing scanning electron microscopy of the organ of Corti in Ush1c c.216G>A mutant mice at P8. (A-F) Basal, middle and apical regions of the organ of Corti were imaged in P8 c.216GA (n= 3 mice) and c.216AA (n=4 mice) mutant mice. OHC and IHC hair bundles were preserved in heterozygous mice but some hair bundles appeared disorganized along the organ of Corti in homozygous 216AA mice. (G-L) High magnification images revealed fragmented and disorganized bundles with disruptions in the staircase array in many but not all OHC (G-H) and IHCs (I-J). Examples of OHC hair bundles imaged in the middle region of the organ at P8 illustrate a preserved (K) and a disorganized hair bundle (L) present in the same preparation. Stars indicate preserved hair bundles; arrowhead, disorganized hair bundles; and arrows, wavy IHC bundles. Scale bars low mag.: 5 μm (A-F); high mag.: 2 μm (G), 3 μm (H), 2 μm (I-863 J) and 1 μm (K,L).

Figures 6A-6H are images showing mechanotransduction in hair cells of Ush1c c.216G>A neonatal mutant mice. (A-D) The permeable styryl dye FM1-43 was used to assess the presence of open transduction channels in hair cells of c.216GA and c.216AA mice. In the organ of Corti, FM uptake was reduced in sensory hair cells of c.216AA mice at P4 (A-B, mid base). Note that IHC FM1-43 fluorescence appears dimmer as IHCs are in a different focal plan. Left: DIC, Right: FM1-43; Scale bar 10 μm. In the utricle, FM1-43 uptake was restricted to the extra-striola region in c.216AA mutants at P6 (C; scale bar 50 μm) while utricular hair cells retained gross normal bundle morphology as assessed by DIC (D; scale bar 10μm). The white line on panel D delineate the striola (no uptake) and extra-striola regions (uptake). Experiment was repeated three times. (E-H) Mechanotransduction was assessed in OHCs, IHCs and VHCs in neonatal c.216GA and c.216AA mice (number of mice recorded from respectively are: n= 7, 6 for OHCs, n= 2, 4 for IHCs and n= 2, 6 for VHCs, number of cells are indicated above the bar graph). Representative transduction currents (E), their associated current/displacement plots fitted with a second order Boltzmann function (F) and average peak transduction current are plotted (G-H). In the cochlea, recordings were obtained in the middle and mid-apical turn of the organ at P3-P6. In the utricle transduction currents were recorded from VHCs of the extra-striola and striola region between P5 and P7 (E-F). While hair bundles appeared well preserved under DIC, smaller average transduction currents were evoked in c.216AA mutants (H). Average peak transduction was significantly different between the two genotypes in OHCs, IHCs and VHCs (***$P < 0.01$, One-way ANOVA).

Figures 7A-7E are images showing expression and localization of fluorescently labeled harmonin in tissues exposed to adeno-associated viral vectors *in vitro* and *in vivo*. (A-C) Acutely dissected P0-P1 inner ear tissue were exposed to AAV2/1 vectors for 24 h, kept in culture for 7 to 8 days before being fixed, counterstained (Alexa Fluor phalloidin, Invitrogen) and imaged with a Zeiss LSM confocal microscope. A large number of sensory hair cells were infected in wild-type utricle and expression of harmonin-b1 fused to EGFP was evident in most hair cells with specific localization at the apex of the sensory hair bundle (A, scale bar: 10 μm- upper panels; 5 μm- lower panels). Similarly expression of EGFP::harmonin-b1 was evident at the tip of the stereocilia in OHCs and IHCs of c.216AA and wild-type mice (B, scale bar: 10 μm; C, scale bar: 3 μm). When AAV2/1.CMV.EGFP::harmonin-b1 vectors were injected at P1, EGFP signal was detected in some IHCs and OHCs at P60 in the left injected ear (D, scale bar: 30 μm).

Exogenous tdTomato::harmonin-a1 was detected in the cell body of IHCs and OHCs in P7 organotypic cultures exposed to AAV2/1.CMV.tdTomato::harmonin-a1 for 24h at P0 (E, scale bar: 5μm). Some harmonin-a1 puncta were colocalized with CTBP2 (blue; mouse anti-CTBP2 1/200, BD bioscience) in particular at the base of the sensory cells presumably near the ribbon synapse. No expression was observed in the stereociliary bundle.

Figures 8A-8C are images showing recovery of mechanotransduction in hair cells of mice injected with Anc80 harmonin vectors. (A-C) Mechanotransduction currents were recorded in IHCs of c.216AA uninjected control mice (n=8 cells, one mouse) and c.216AA mice injected at P1 with AAV2/Anc80.CMV.harmonin-b1 (0.8 μl, n=15 cells, one mouse) or a combined injection of the AAV2/Anc80.CMV.harmonin-b1 and AAV2/Anc80.CMV.harmonin-a1 (0.5 μl + 0.5 μl, n=7 cells, one mouse). Organotypic cultures were prepared at P6 and recordings were performed between P15 and P16 (9 to 10 DIV). While small mechanotransduction currents could be induced by hair bundle stimulations of c.216AA mice, larger currents were evoked in c.216AA mice injected with vectors driving harmonin-b1 or dual harmonin-a1 and -b1 expression (A). Corresponding I/X curve for each dataset and double Boltzmann fitting function. Respective maximal mechanotransduction current Imax= 102.1 pA (c.216AA); 424.3 pA (c.216AA + harmonin-b1) and 341.1 pA (c.216AA + harmonin-a1&-b1) (B). Average responses (Mean ± S.D.) show significant recovery of transduction (***P < 0.001) for harmonin-b1 and harmonin-a1 + -b1 injected relative to unjected mice. Average transduction currents were not significantly different in harmonin-b1 injected mice and c.216GA control mice (N.S. P>0.5). Recovery of mechanotransduction was also not significantly improved when harmonin-a and harmonin-b were combined. (C), one-way ANOVA.

Figure 9A-9E are images showing ABR and DPOAE threshold recovery in mice injected at P1 with AAV2/Anc80.CMV.harmonin-b1. (A) Representative ABR responses for 16 kHz tones in 6 weeks old c.216AA control mice and c.216AA mice injected at P1 via RWM injection of vectors encoding harmonin-a1, harmonin-b1 or a combination of the two. Recovered ABR thresholds near 30 dB SPL were measured in mice injected with harmonin-b1 alone or harmonin-a1 and b1 together. (B) Mean ABR responses obtained for: c.216 AA (n=13); c.216GA (n=12); c.216AA + harmonin-a1 (n=12); c.216AA + harmonin-b1 (n=19 rescued /25 tested); c.216AA + harmonin-a1&-b1 (n=6

rescued /11 tested). Mean ± S.E, continuous lines. Dotted lines: ABR thresholds for the entire frequency range in mice whose 16 kHz recordings are shown in panel A. (C) Mean DPOAEs responses obtained for: c.216AA (n=13); c.216GA (n=12); c.216AA + harmonin-a1 (n=12); c.216AA + harmonin-b1 (n=15 rescued- DPOAEs <70dB SPL /25 tested); c.216AA + harmonin-a1&-b1 (n=4 rescued DPOAEs <70dB SPL /11 tested). Mean ± S.E, continuous lines. Dotted lines: DPOAEs thresholds for the four mice whose recordings are illustrated in panel A. Arrows indicate that the thresholds are higher than the maximal stimulus level tested. (D-E) ABRs and DPOAEs responses obtained at 6 weeks and 3 months in eight mice that showed initial ABR thresholds under or equal to 45dB. Six of the eight mice were kept for 6 months and had ABRs and DPOAEs assessed (dotted line). Mean ± S.E. While ABRs and DPOAEs thresholds shifts were evident over the first three month, hearing rescue was still prominent at 6 months of age in the lower frequency range.

Figure 10A-10E are images showing startle response, rotarod performance and open field behavior recovery in mice injected at P1 with AAV2/Anc80.CMV.harmonin-a1 and AAV2/Anc80.CMV.harmonin-b1. (A) Startle response to white noise stimuli was recorded in 6 weeks old control c.216GA, c.216AA and c.216AA injected mice. Partial startle rescue was evident in mice injected with harmonin-b1 but not harmonin-a1 (data overlapping with control c.216AA mice). Averages are shown ± S.E. (B) Rotarod performance was recorded between 4 and 6 weeks in control c.216GA, c.216AA and c.216AA injected mice. Full recovery was observed in mice injected with harmonin-b1 and harmonin-a1/b1. No recovery was observed with harmonin-a1 alone. Averages are shown ± S.E. (C-E) Open field observations were performed for 5 min in 6 weeks old control c.216GA, c.216AA and c.216AA and c.216GA injected mice. Representative tracks over 2.5 min are shown (B). While c.216AA mutant mice explore the entire field and perform repetitive full body rotations, c.216AA mice injected at P1 with harmonin-a1, harmonin-b1 or the combination of the two vectors demonstrate normal behavior similar to their heterozygous c.216GA counterparts or c.216GA mice injected with the truncated vector. (C). Graphs illustrate the mean ± S.D. for the number of rotations and distance covered per minute. Significant recovery ***$P < 0.001$ was observed between the uninjected and injected mice. Statistical analysis by one-way ANOVA.

Figure 11 are scanning electron microscopy images of the organ of Corti in mice injected with AAV2/Anc80.CMV.harmonin-b1. Basal, Middle and Apical regions of the

organ of Corti were imaged at six weeks in c.216GA, c.216AA and c.216AA mice injected at P1 (RMW injection 0.8 µl AAV2/Anc80.CMV.harmonin-b1). OHC and IHC hair bundles were preserved in c.216GA mice but appeared disorganized along the organ of Corti in c.216AA mice. Noticeable hair cell loss (asterisk) and hair bundle disorganization was observed in c.216AA mice with more pronounced degeneration in the basal end of the organ. Hair bundles of c.216AA mice lacked normal stereocilia rows. The shorter rows appeared to be retracted while the tallest rows were maintained in c.216AA mice (arrow). While hair cell loss and bundle disorganization were still evident in rescued c.216AA mice, hair cell survival was noticeably higher in the basal and middle regions of the Organ. Hair cell counts are summarized in the bar graph. A total of 1824 cells were counted in c.216AA mice (4 ears) and 792 in rescued c.216AA mice (2 ears). Mean ± S.E. High magnification imaging reveals rescue of the staircase array in injected c.216AA mice (arrow) in many but not all cells (arrowhead). Scale bar low magnification: 5µm; high magnification: 1µm.

Figures 12A-12L are images showing an analysis of hair bundle morphology in Ush1c c.216G>A mice at P18 by SEM. (A-C) Heterozygous c.216GA mice displayed normal hair bundle morphology at P18. (D-I) Disorganized hair bundles were observed along the organ of P18 Homozygous c.216AA mutant mice. (J-L) IHCs hair bundle were mildly disrupted in c.216AA mice. Distance measured from apex tip: Base 3.5-4 mm; Mid 1.8-2.2 mm; Apex 0.6-0.8 mm. Scale bar low magnification: 5µm; high magnification: 1µm.

Figures 13A-13J are images showing mechanotranduction properties in c.216AA mutant mice. (A-E) Analysis of mechanotransduction in neonatal OHCs from middle and mid-apical turns of the cochlea, P3-P6. Representative current traces from ~Po= 0.5 were fit with a double exponential decay function to assess adaptation in c.216GA and c.216AA mutant (A). Fits were used to generate fast (C) and slow (D) time constants as well as the extent of adaptation (E). The 10-90% operating range was not significantly altered (B). Extent of adaptation in c.216AA mice was significantly less than or heterozygous OHCs as shown in this scatter plot (E). (F-J) Analysis of mechanotransduction in neonatal IHCs. 10-90% operating range values were smaller in c.216GA versus c.216AA IHCs (G). Adaptation was always present albeit slightly slower and with a significant lesser extent in c.216AA IHCs (H-J). Statistical analysis is indicated in each plot: *P<0.05, **P<0.01 and ***P<0.001, one-way ANOVA.

Figures 14A-14C are images showing expression of fluorescently labeled harmonin-a and harmonin-b Anc80 vectors at 6 weeks in c.216AA organ of Corti after P1 dual vector injection. (A-C) Confocal images of the basal turn in 6 weeks old c.216AA mice after P1 co-injection of AAV2/Anc80.CMV.tdTomato::harmonin-a1 (0.5 µl; 4.11E^12 gc/ml) and AAV2/Anc80.CMV.eGFP::harmonin-b1 (0.5 µl; 2.99E^12 gc/ml). 69% and 74% of the total number of cells respectively expressed eGFP (A) and tdTomato (C) and 65% expressed both markers demonstrating successful co-transduction. Scale bar: 20 µm.

Figures 15A-15F are data showing an analysis of ABR response in 6 weeks old control c.216GA and injected rescued c.216AA mice. (A, D) Example of ABR responses at 8 and 16 kHz for control c.216GA and rescued c.216AA mice. (B-C, E-F) Average peak 1 amplitude (B-D) and latency (C-D) at 8-11.3 and 16 kHz in 6 weeks old mice with comparable thresholds (n=8 c.216GA, n=5 c.216AA + Harmonin-b1 RWM P1). Mean ± S.E.: One-way ANOVA.

Figures 16A-16D show the mutant form of harmonin expressed in Ush1c c.216G>A mice does not alter hair cell or auditory function. (A) Sequence alignment between the wild-type harmonin-b1 protein and the truncated harmonin that is secreted as a result of the cryptic splicing and frame shift associated with the acadian G>A mutation in exon 3 of the Ush1c gene. (B) Semi-quantitative RT-PCR from auditory organs of P2-P3 wild-type mice, c.216GA and c.216AA mutant mice confirms expression of the wild-type (450 bp) and truncated (-35 bp) harmonin in c.216GA and c.216AA mice. (C-D) Auditory brainstem responses (ABR, C) and Distortion products (DPOAEs, D) were measured at 6 weeks old c.216GA injected mice and control c.216GA and c.216AA mice. Plots are shown as averages ± S.E. No threshold shift was observed in injected versus control 216GA mice.

Figures 17A-17C are images showing recovery of correct Ush1c splicing in the inner ear of 6 weeks old mice injected with AAV2/Anc80.CMV.harmonin-b1. (A) Semi-quantitative RT-PCR quantification of correctly spliced (450 bp) and aberrant (415 bp) mRNA from the Ush1c.216A allele shows recovery of correct Ush1c splicing in injected (I) and contralateral ears (C) of c.216AA rescued mice #1 and #2 (35 dB SPL response at 11.3 kHz from injected ears). Mouse #3 with poor ABR response (90 dB SPL at 11.3 kHz) shows modest recovery of correct mRNA expression and mouse #4 (100 dB SPL at 11.3 kHz) shows none. While the correct splice form is not detected in uninjected

c.216AA mice (mice #5,6), both the correct and truncated splice forms are detected in c.216GA mice (mice #7,8,9). Corresponding mouse Gapdh shown in the bottom panel was amplified to confirm the relative amount of material. (B) Semi-quantitative radiolabeled PCR analysis confirms the presence AAV-mUsh1c in injected and contralateral ears of Ush1c.216AA mice. Relative levels of AAV-mUsh1c DNA were present but reduced in mice #3 and #4. (C) Relative amount of AAV-mUsh1c correlates with ABR thresholds. Analysis for 11.3 and 16 kHz are illustrated. Linear regressions show high correlations between the two.

Figures 18 is a graph showing long term ABR threshold recovery correlates with OHCs survival in the mid to apical region of the auditory organ. Hair cell count across the entire Organ of Corti was performed post-mortem in left ears of three uninjected c.216AA and five injected c.216AA (P1 RWM injection, 0.8 µl AAV2/Anc80.CMV.harmonin-b1) at 6 months of age. Insert: While two of the mice (#1 and #2) showed poor ABR response thresholds across the entire range tested (≥95 dB SPL), three (#3-5) responded with thresholds ranging 35 and 55 dB SPL for sound stimuli between 5.6 and 16 kHz. The total number of IHC and OHCs hair cells was increased in injected mice. Comparison of rescued injected mice with those injected that had poor rescue shows that the number of IHCs was not different but a significant number of OHCs were noted in the rescued mice. Analysis across the entire length of the organ showed the difference can be accounted for as an increase in hair cell survival from the mid to apical regions of the organ.

Part 3: Gene Therapy of Additional Mutations Involved in Hearing Loss

Figures 19A-19D are representative confocal images of a cochlea from an Ush1c mutant mouse injected through the RWM with 1 ml of Anc80-Harmonin::GFP (i.e., the GFP is fused to the Harmonin polypeptide) (6×10^{12} gc/ml) at P2, harvested at P9, and stained with actin (red; 19A), Myo7a (blue; 19B), and imaged for GFP (green; 19C). A merged image of (19A), (19B), and (19C) is shown in (19D).

Figure 20 is a graph showing ABR threshold plotted as a function of sound frequency for an Ush1c mutant mice (squares) and an Ush1c mutant mice injected with an Anc80-Harmonin::GFP vector (circles).

Figures 21A-21C show representative confocal images of a KCNQ4 -/- cochlea injected through the RWM with 1 µl of Anc80-KCNQ4 (6×10^{12} gc/ml) at P2, harvested

at P9, and stained with Alexa 546–phalloidin (red) and an antibody against KCNQ4 (green) at low magnification (21A) or high magnification (21B) relative to uninjected cochlea at high magnification (21C).

Figure 22A-22C are a series of graphs that show the KCNQ4 current in a P10 wild type mouse (22A), a P10 KCNQ4 -/- mouse (22B), and a P10 KCNQ4 -/- mouse injected via the RWM (2.4×10^{13} gc/ml) with Anc80- KCNQ4 (22C). Cochleas were harvested 8 days after injection.

Figure 23 is a series of three images of FM1-43 uptake (FM1-43 only permeates functional Tmc1 channels) in Tmc1 -/- tissue injected with the Anc80-CMV-Tmc1 vector. P2 Tmc1 -/- mice were injected via the RWM with the Anc80-CMV-Tmc1 vector (2.4×10^{13} gc/ml), and cochleas were harvested 6 to 7 days after injection.

Figure 24A shows representative families of sensory transduction currents recorded from IHCs of a P10 wild type mouse (left), a P10 Tmc1 -/- mouse (middle), and a P10 Tmc1 -/- mouse injected at P2 via the RWM (2.4×10^{13} gc/ml) with Anc80-CMV-Tmc1 (right). Cochleas were harvested 8 days after injection.

Figure 24B is a graphical representation of the recovery rate of the mice shown in Figure 24A. The graph in Figure 24B indicates the percentage of functional cells in a wild type mouse (left), a Tmc1 -/- mouse (middle), and a Tmc1 -/- mouse injected with Anc80-CMV-Tmc1 (right).

Figure 25 is a graph showing the Distortion Product Otoacoustic Emissions (DPOAE) thresholds as a function of stimulus frequency for wild type, Tmc1 -/- mice, and Tmc1 -/- mice injected with Anc80-CMV-Tmc1.

Figures 26A-26C show the effect of various promoters on expression. Figure 26A shows Anc80-Pcdh15-GFP expression in hair cells and supporting cells GFP and Myosin7a are shown in as indicated and hair cells are stained in red for Myosin7a, a hair cell marker. Figure 26B shows Anc80-Myo6-GFP with inner and outer hair cells showing GFP expression. There was no counter stain for this experiment. Figure 26C shows Anc80-KCNQ4-GFP with just outer hair cells showing GFP expression. The tissue was counter stained with phalloidin to illuminate hair cells.

DETAILED DESCRIPTION

The present disclosure provides compositions and methods for delivering and expressing a protein (e.g., TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS,

CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7 USH1C (e.g., harmonin-a, b, or c)) required for mechanosensation, including hearing, and/or vestibular function, in a cell of the inner ear of a subject, such as a cochlear cell (e.g., inner or outer hair cell), wherein the subject has a loss or reduction in the level or activity of that protein.

The present disclosure is based, at least in part, on the discoveries that a rationally designed synthetic vector encoding an Anc80 capsid ("Anc80 vector") is useful for efficient transgene delivery to the cochlea; and that this vector could be used to deliver wild-type Ush1c into the inner ear of a murine model of Usher Syndrome. Round window membrane injection resulted in highly efficient transduction of inner and outer haircells in mice, a substantial improvement over conventional adeno-associated virus (AAV) vectors. Anc80 round window injection was well tolerated, as indicated by sensory cell function, hearing and vestibular function, and immunologic parameters. The ability of Anc80 to target outer hair cells at high rates, a requirement for restoration of complex auditory function, may enable future gene therapies for hearing and balance disorders. The mice injected with the Anc80 vector encoding Ush1c demonstrated recovery of gene and protein expression, restoration of sensory cell function, rescue of complex auditory function and recovery of hearing and balance behavior to near wild-type levels. The data represent unprecedented recovery of inner ear function and indicate that biological therapies to treat deafness may be suitable for translation to humans with genetic inner ear disorders..

Usher Syndrome

Human Usher syndrome (USH) is a rare genetic condition responsible for combined deafness and blindness. Inherited as an autosomal recessive trait, it affects 16,000 to 20,000 people in the United States and is responsible for 3 to 6% of early childhood deafness. Usher syndrome is classified under three clinical subtypes (USH-1, -2 and -3) according to the severity of the symptoms. USH1 is the most severe form. Patients who are affected by USH1 suffer congenital bilateral profound sensorineural hearing loss, vestibular areflexia and pre-pubertal retinitis pigmentosa (a progressive, bilateral, symmetric degeneration of rod and cone function of the retina). Unless fitted with a cochlear implant, individuals do not typically develop the ability to generate speech. While no biological treatments currently exist for Usher patients, early

reintroduction of the wild-type form of the defective gene may allow for reversal of the disease.

Six Usher genes are associated with USH1: MYO7A (myosin 7a), USH1C (harmonin), CDH23 (cadherin 23), PCDH15 (protocadherin 15), SANS (sans) and CIB2 (calcium and integrin binding protein 2). These genes encode proteins that are involved in hair bundle morphogenesis in the inner ear and are part of an interactome (see, for example, Mathur & Yang, 2015, *Biochim. Biophys. Acta*, 1852:406-20). Harmonin resides at the center of the USH1 interactome where it binds to other Usher 1 proteins. Because of its PDZ (PSD-59 95/Dlg/ZO-1) interaction domains, harmonin has been proposed to function as a scaffolding protein. *In vitro* binding studies have shown that all other known USH1 proteins bind to PDZ domains of harmonin as do two of the USH2 proteins, usherin, and VLGR1. The USH1C gene consists of 28 exons, which code for 10 alternative splice forms of harmonin, grouped into three different subclasses (a, b and c) depending on the domain composition of the protein. The three isoforms differ in the number of PDZ protein-protein interaction domains, coiled-coiled (CC) domains, and proline-serine-threonine (PST) rich domains.

USH1 proteins are localized to the apex of hair cells in mechanosenosory hair bundles, which are composed of hundreds of stereocilia interconnected by numerous extracellular links. Cadherin 23 and Protocadherin 15, products of Usher genes (USH1D and USH1E, respectively) form tip-links located at the distal end of the stereocilia. Harmonin-b binds to CDH23, PCDH15, F-actin and itself. It is found at the tips of the stereocilia near the tip-link insertion point in hair cells where it is thought to play a functional role in transduction and adaptation in hair cells. Harmonin-b is expressed during early postnatal stages but its expression diminishes around postnatal day 30 (P30) in both the cochlea and vestibule. Harmonin-a also binds to cadherin 23 and is found in the stereocilia. Recent reports reveal an additional role for harmonin-a at the synapse where it associates with Cav1.3 Ca2+ channels to limit channel availability through an ubiquitin-dependent pathway.

Several mouse models for Usher syndrome have been identified or engineered over the past decade, seven of which affect harmonin. Of these, only one model, the Ush1c c.216G>A model, reproduces both auditory and retinal deficits that characterize human Usher Syndrome. Ush1c c.216G>A is a knock-in mouse model that affects expression of all conventional harmonin isoforms due a point mutation similar to the one

found in a cohort of French-Acadian USH1C patients. The mutation introduces a cryptic splice site at the end of exon three of the Ush1c gene. Use of this cryptic splice site produces a frame-shifted transcript with a 35 bp deletion and results in translation of a severely truncated protein lacking PDZ, PST and CC domains. Homozygous c.216AA knock-in mice suffer from severe hearing loss at 1 month of age while heterozygous c.216GA mice do not present any abnormal phenotype. Cochlear histology in c.216AA mice shows disorganized hair bundles, abnormal cell rows and loss of both inner and outer hair cells in middle and basal turns at P30.

It is demonstrated herein that an AAV containing an ancestral AAV capsid protein successfully transduce hair cells and drive expression and correct localization of harmonin splice forms. Furthermore, it is demonstrated herein that early postnatal round window membrane injection of an AAV containing an ancestral AAV capsid protein as described herein successfully restore auditory and vestibular function in homozygous c.216AA mice. Recovery of auditory function in injected mice is associated with recovery of mRNA expression encoding for wild-type harmonin as well as preservation of hair bundle morphology and mechanotransduction. The results provided herein demonstrate that early re-introduction of wild-type harmonin using an AAV containing an ancestral AAV capsid protein as described herein may be useful for treating USH1C.

TMC1/TMC2

Over 40 distinct mutations have been identified in TMC1 that cause deafness. These are subdivided into 35 recessive mutations and 5 dominant mutations. Most of the recessive mutations cause profound, congenital hearing loss (e.g., DFNB7/11) though a few cause later onset, moderate to severe hearing loss. All of the dominant mutations cause progressive hearing loss (e.g., DFNA36), with onset in the mid-teen years. In particular, an AAV vector that includes an Anc80 capsid protein as described herein can be used to deliver a non-mutant (e.g., wild type) TMC1 sequence or TMC2 sequence, thereby preventing hearing loss (e.g., further hearing loss) and/or restoring hearing function.

Therapeutic Strategies for the Treatment of Hearing Loss

Since the sensory cells of the adult mammalian cochlea lack the capacity for self-repair, current therapeutic strategies (depending on the level and exact position of

impairment) rely on amplification (hearing aids), better transmission of sound (middle ear prostheses/active implants), or direct neuronal stimulation (cochlear implants) to compensate for permanent damage to primary sensory hair cells or spiral ganglion neurons which form the auditory nerve and relay acoustic information to the brain. While these approaches have been transformative, they remain far from optimal in restoring complex human hearing function important for modern life. Specifically, major problems still include limited frequency sensitivity, unnatural sound perception, and limited speech discrimination in noisy environments.

Therapeutic gene transfer to the cochlea has been considered to further improve upon the current standard of care ranging from age-related and environmentally induced hearing loss to genetic forms of deafness. More than 300 genetic loci have been linked to hereditary hearing loss with over 70 causative genes described (Parker & Bitner-Glindzicz, 2015, *Arch. Dis. Childhood*, 100:271-8). Therapeutic success in these approaches relies significantly on the safe and efficient delivery of exogenous gene constructs to the relevant therapeutic cell targets in the organ of Corti (OC) in the cochlea.

The OC includes two classes of sensory hair cells: IHCs, which convert mechanical information carried by sound into electrical signals transmitted to neuronal structures and OHCs which serve to amplify and tune the cochlear response, a process required for complex hearing function. Other potential targets in the inner ear include spiral ganglion neurons, columnar cells of the spiral limbus, which are important for the maintenance of the adjacent tectorial membrane or supporting cells, which have protective functions and can be triggered to trans-differentiate into hair cells up to an early neonatal stage.

Injection to the cochlear duct, which is filled with high potassium endolymph fluid, could provide direct access to hair cells. However, alterations to this delicate fluid environment may disrupt the endocochlear potential, heightening the risk for injection-related toxicity. The perilymph-filled spaces surrounding the cochlear duct, scala tympani and scala vestibuli, can be accessed from the middle ear, either through the oval or round window membrane (RWM). The RWM, which is the only non-bony opening into the inner ear, is relatively easily accessible in many animal models and administration of viral vector using this route is well tolerated. In humans, cochlear implant placement routinely relies on surgical electrode insertion through the RWM.

Previous studies evaluating AAV serotypes in organotypic cochlear explant and *in vivo* inner ear injection have resulted in only partial rescue of hearing in mouse models of inherited deafness. Unexpectedly, an adeno-associated virus (AAV) containing an ancestral AAV capsid protein transduces OHCs with high efficiency. This finding overcomes the low transduction rates that have limited successful development of cochlear gene therapy using conventional AAV serotypes. An AAV containing an ancestral AAV capsid protein as described herein provides a valuable platform for inner ear gene delivery to IHCs and OHCs, as well as an array of other inner ear cell types that are compromised by genetic hearing and balance disorders. In addition to providing high transduction rates, an AAV containing an ancestral AAV capsid protein as described herein was shown to have an analogous safety profile in mouse and nonhuman primate upon systemic injection, and is antigenically distinct from circulating AAVs, providing a potential benefit in terms of pre-existing immunity that limits the efficacy of conventional AAV vectors.

Compositions and methods are described herein, however, that allow for highly efficient delivery of nucleic acids (e.g., AAV vectors, such as an ANC80 vector comprising a promoter (e.g., CMV, Espin, PCDH15, a PTPRQ, a TMHS (LHFPL5)) directing expression of a polynucleotide encoding one or more of TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7 USH1C (e.g., harmonin-a, b, or c) to cells, particularly cells within the inner ear, e.g., in the cochlea (or cells of the cochlea or cochlear cells). As used herein, inner ear cells refer to, without limitation, inner hair cells (IHCs), outer hair cells (OHCs), spiral ganglion neurons, stria vascularis, vestibular hair cells, vestibular ganglion neurons, and supporting cells. Supporting cells refer to cells in the ear that are not excitable, e.g., cells that are not hair cells or neurons. An example of a supporting cell is a Schwann cell.

Delivery of one or more of the nucleic acids described herein to inner ear cells can be used to treat any number of inherited or acquired hearing disorders, which are typically defined by partial hearing loss or complete deafness. The methods described herein can be used to treat a hearing disorder such as, without limitation, recessive deafness, dominant deafness, Usher syndrome, and other syndromic deafness, as well as hearing loss due to trauma or aging.

Methods of Making Viruses Carrying Specific Transgenes

As described herein, an adeno-associated virus (AAV) containing an ancestral AAV capsid protein are particularly efficient at delivering nucleic acids (e.g., transgenes, including but not limited to a polynucleotide encoding one or more of TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7 USH1C (e.g., harmonin-a, b, or c)) to inner ear cells, and a particularly effective class of ancestral AAV capsid proteins is designated by an ancestral scaffold capsid protein designated Anc80, which is shown in SEQ ID NO:1. The Anc80 vector is an example of an Inner Ear Hair Cell Targeting AAV that advantageously transduced greater than about 60%, 70%, 80%, 90%, 95%, or even 100% of inner or outer hair cells. One particular ancestral capsid protein that falls within the class of Anc80 ancestral capsid protein is Anc80-0065 (SEQ ID NO:2), however, WO 2015/054653, which is incorporated herein in its entirety, describes a number of additional ancestral capsid proteins that fall within the class of Anc80 ancestral capsid proteins.

In particular embodiments the adeno-associated virus (AAV) contains an ancestral AAV capsid protein that has a natural or engineered tropism for hair cells. In some embodiments, the virus is an Inner Ear Hair Cell Targeting AAV, which delivers a transgene (e.g., a polynucleotide encoding one or more of TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7 USH1C (e.g., harmonin-a, b, or c)) to the inner ear in a subject. In some embodiments, the virus is an AAV that comprises purified capsid polypeptides. In some embodiments, the virus is artificial. In some embodiments, the virus contains ancestral AAV sequences. In some embodiments, the virus is an AAV that has lower seroprevalence than AAV2. In some embodiments, the virus is an exome-associated AAV. In some embodiments, the virus is an exome-associated AAV1. In some embodiments, the virus comprises a capsid protein with at least 95% amino acid sequence identity or homology to Anc80 capsid proteins.

The viruses described herein that contain an Anc80 capsid protein can be used to deliver a variety of nucleic acids to inner ear cells. In one embodiment, an Inner Ear Hair Cell Targeting AAV (e.g., ANC80 vector) comprising a promoter (e.g., CMV, Espin, PCDH15, a PTPRQ, a TMHS (LHFPL5)) directing expression of a polynucleotide encoding one or more of TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7 USH1C (e.g., harmonin-a, b, or c). A nucleic acid sequence delivered to a cell for the purpose of expression oftentimes is

referred to as a transgene. Representative transgenes that can be delivered to, and expressed in, inner ear cells include, without limitation, a transgene encoding a polypeptide that functions in auditory and/or vestibular mechanosensation (e.g., TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7 (e.g., harmonin-a, b, or c)), a transgene that encodes a neurotrophic factor (e.g., GDNV, BDNF, or HSP70), an immunomodulatory protein or an anti-oncogenic transcript. In addition, representative transgenes that can be delivered to, and expressed in, inner ear cells also include, without limitation, a transgene that encodes an antibody or fragment thereof, an antisense, silencing or long non-coding RNA species, or a genome editing system (e.g., a genetically-modified zine finger nuclease, transcription activator-like effector nucleases (TALENs), or clustered regularly interspaced short palindromic repeats (CRISPRs)). Further, representative transgenes that can be delivered to, and expressed in, inner ear cells include nucleic acids designated ACTG1, ADCY1, ATOHI, ATP6V1B1, BDNF, BDP1, BSND, DATSPER2, CABP2, CD164, CDC14A, CDH23, CEACAM16, CHD7, CCDC50, CIB2, CLDN14, CLIC5, CLPP, CLRN1, COCH, COL2A1, COL4A3, COL4A4, COL4A5, COL9A1, COL9A2, COL11A1, COL11A2, CRYM, DCDC2, DFNA5, DFNB31, DFNB59, DIAPH1, EDN3, EDNRB, ELMOD3, EMOD3, EPS8, EPS8L2, ESPN, ESRRB, EYA1, EYA4, FAM65B, FOXI1, GIPC3, GJB2, GJB3, GJB6, GPR98, GRHL2, GPSM2, GRXCR1, GRXCR2, HARS2, HGF, HOMER2, HSD17B4, ILDR1, KARS, KCNE1, KCNJ10, KCNQ1, KCNQ4, KITLG, LARS2, LHFPL5, LOXHD1, LRTOMT, MARVELD2, MCM2, MET, MIR183, MIRN96, MITF, MSRB3, MT-RNR1, MT-TS1, MYH14, MYH9, MYO15A, MYO1A, MYO3A, MYO6, MYO7A, NARS2, NDP,NF2, NT3, OSBPL2, OTOA, OTOF, OTOG, OTOGL, P2RX2, PAX3, PCDH15, PDZD7, PJVK, PNPT1, POLR1D, POLR1C, POU3F4, POU4F3, PRPS1, PTPRQ, RDX, S1PR2, SANS, SEMA3E, SERPINB6, SLC17A8, SLC22A4, SLC26A4, SLC26A5, SIX1, SIX5, SMAC/DIABLO, SNAI2, SOX10, STRC, SYNE4, TBC1D24, TCOF1, TECTA, TIMM8A, TJP2, TNC, TMC1, TMC2, TMIE, TMEM132E, TMPRSS3, TRPN, TRIOBP, TSPEAR, USH1C, USH1G, USH2A, USH2D, VLGR1,WFS1, WHRN, and XIA. In particular embodiments, the transgene is one or more of MYO7A, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7, USH1C (e.g., harmonin-a, b, or c).

Expression of a transgene may be directed by the transgene's natural promoter (i.e., the promoter found naturally with the transgenic coding sequence) or expression of a

transgene may be directed by a heterologous promoter (e.g., CMV promoter, Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter). For example, any of the transgenes described herein can be used with its natural promoter. Alternatively, any of the transgenes described herein can be used with a heterologous promoter. As used herein, a heterologous promoter refers to a promoter that does not naturally direct expression of that sequence (i.e., is not found with that sequence in nature). Representative heterologous promoters that can be used to direct expression of any of the transgenes indicated herein include, for example, a CMV promoter, a CBA promoter, a CASI promoter, a P promoter, and a EF-1 promoter, an alpha9 nicotinic receptor promoter, a prestin promoter, a Gfi1 promoter, and a Vglut3 promoter. In addition, a promoter that naturally directs expression of one of the above-referenced transgenes (e.g., a KCNQ4 promoter, a Myo7a promoter, a Myo6 promoter or an Atoh1 promoter) can be used as a heterologous promoter to direct expression of a transgene. In other embodiments, the promoter is an Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter.

Methods of making a transgene (e.g., TMC1, TMC2, USH1C (e.g., harmonin-a, b, or c), MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7) for packaging into a virus that contains an Anc80 capsid protein are known in the art, and utilize conventional molecular biology and recombinant nucleic acid techniques. In one embodiment, a construct that includes a nucleic acid sequence encoding an Anc80 capsid protein and a construct carrying the transgene flanked by suitable Inverted Terminal Repeats (ITRs) are provided, which allows for the transgene to be packaged within the Anc80 capsid protein.

The transgene can be packaged into an AAV containing an Anc80 capsid protein using, for example, a packaging host cell. The components of a virus particle (e.g., rep sequences, cap sequences, inverted terminal repeat (ITR) sequences) can be introduced, transiently or stably, into a packaging host cell using one or more constructs as described herein. The viruses described herein contain at least an Anc80 capsid protein; the other components of a virus particle (e.g., rep sequences, ITR sequences) can be based on an ancestral sequence or a contemporary sequence. In some instances, for example, the entire virus particle can be based on ancestral sequences. Such viruses can be purified using routine methods.

In general, as used herein, "nucleic acids," can include DNA and RNA, and also can include nucleic acids that contain one or more nucleotide analogs or backbone modifications. Nucleic acids can be single-stranded or double-stranded, which usually depends upon its intended use. Nucleic acids that can be used in the methods described herein can be identical to a known nucleic acid sequence, or nucleic acids that can be used in the methods described herein can differ in sequence from such known sequences. Simply by way of example, nucleic acids (or the encoded polypeptides) can have at least 75% sequence identity (e.g., at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity) to a known sequence.

In calculating percent sequence identity, two sequences are aligned and the number of identical matches of nucleotides or amino acid residues between the two sequences is determined. The number of identical matches is divided by the length of the aligned region (i.e., the number of aligned nucleotides or amino acid residues) and multiplied by 100 to arrive at a percent sequence identity value. It will be appreciated that the length of the aligned region can be a portion of one or both sequences up to the full-length size of the shortest sequence. It also will be appreciated that a single sequence can align with more than one other sequence and hence, can have different percent sequence identity values over each aligned region.

The alignment of two or more sequences to determine percent sequence identity is performed using the computer program ClustalW and default parameters, which allows alignments of nucleic acid or polypeptide sequences to be carried out across their entire length (global alignment). Chenna et al., 2003, Nucleic Acids Res., 31(13):3497-500. ClustalW calculates the best match between a query and one or more subject sequences, and aligns them so that identities, similarities and differences are determined. Gaps of one or more residues can be inserted into a query sequence, a subject sequence, or both, to maximize sequence alignments. For pairwise alignment of nucleic acid sequences, the default parameters are used (i.e., word size: 2; window size: 4; scoring method: percentage; number of top diagonals: 4; and gap penalty: 5); for an alignment of multiple nucleic acid sequences, the following parameters are used: gap opening penalty: 10.0; gap extension penalty: 5.0; and weight transitions: yes. For pairwise alignment of polypeptide sequences, the following parameters are used: word size: 1; window size: 5; scoring method: percentage; number of top diagonals: 5; and gap penalty: 3. For multiple

alignment of polypeptide sequences, the following parameters are used: weight matrix: BLOSUM (blocks substitution matrix); gap opening penalty: 10.0; gap extension penalty: 0.05; hydrophilic gaps: on; hydrophilic residues: Gly, Pro, Ser, Asn, Asp, Gln, Glu, Arg, and Lys; and residue-specific gap penalties: on. ClustalW can be run, for example, at the Baylor College of Medicine Search Launcher website or at the European Bioinformatics Institute website on the World Wide Web.

Changes can be introduced into a nucleic acid sequence, which can lead to changes in the amino acid sequence of the encoded polypeptide if the nucleic acid sequence is a coding sequence. For example, changes can be introduced into nucleic acid coding sequences using mutagenesis (e.g., site-directed mutagenesis, PCR-mediated mutagenesis) or by chemically synthesizing a nucleic acid molecule having such changes. Such nucleic acid changes can lead to conservative and/or non-conservative amino acid substitutions at one or more amino acid residues. A "conservative amino acid substitution" is one in which one amino acid residue is replaced with a different amino acid residue having a similar side chain (see, for example, Dayhoff et al. (1978, in Atlas of Protein Sequence and Structure, 5(Suppl. 3):345-352), which provides frequency tables for amino acid substitutions), and a non-conservative substitution is one in which an amino acid residue is replaced with an amino acid residue that does not have a similar side chain.

A nucleic acid can be contained within a construct, which also can be referred to as a vector or a plasmid. Constructs are commercially available or can be produced by recombinant techniques routine in the art. A construct containing a nucleic acid can have expression elements that direct and/or regulate expression of such a nucleic acid, and also can include sequences such as those for maintaining the construct (e.g., origin of replication, a selectable marker). Expression elements are known in the art and include, for example, promoters, introns, enhancer sequences, response elements, or inducible elements.

Pharmaceutical Compositions

An Inner Ear Hair Cell Targeting AAV (e.g., Anc805 vector) comprising a promoter (e.g., CMV, Espin, PCDH15, a PTPRQ, a TMHS (LHFPL5)) and a polynucleotide that is one or more of USH1, MYO7A, USH1C (harmonin-a, b, c), CDH23, PCDH15, SANS and CIB2, usually suspended in a physiologically

compatible excipient, can be administered to a subject (e.g., a human or non-human mammal) by injection to the inner ear of a subject through the round window. Suitable carriers include saline, which may be formulated with a variety of buffering solutions (e.g., phosphate buffered saline), lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, and water. The Inner Ear Hair Cell Targeting AAV is administered in sufficient amounts to transduce or infect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects.

The dose of the Inner Ear Hair Cell Targeting AAV administered to a subject will depend primarily on factors such as the condition being treated, and the age, weight, and health of the subject. For example, a therapeutically effective dosage of an Inner Ear Hair Cell Targeting AAV to be administered to a human subject generally is in the range of from about 0.1 ml to about 10 ml of a solution containing concentrations of from about 1 x 10' to 1 x 10^{12} genome copies (GCs) of AAVs (e.g., about 1 x 10^{3} to 1 x 10^{9} GCs).

Methods of Delivering Nucleic Acids to Inner Ear Cells

Methods of delivering nucleic acids to cells generally are known in the art, and methods of delivering viruses (which also can be referred to as viral particles) containing a transgene to inner ear cells *in vivo* are described herein. As described herein, about 10^{8} to about 10^{12} viral particles can be administered to a subject, and the virus can be suspended within a suitable volume (e.g., 10 μL, 50 μL, 100 μL, 500 μL, or 1000 μL) of, for example, artificial perilymph solution.

A virus containing a promoter (e.g., CMV, Espin, PCDH15, a PTPRQ, a TMHS (LHFPL5)) and a transgene (e.g., TMC1, TMC2, USH1C (e.g., harmonin-a, b, or c), MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7) as described herein can be delivered to inner ear cells (e.g., cells in the cochlea) using any number of means. For example, a therapeutically effective amount of a composition including virus particles containing one or more different types of transgenes as described herein can be injected through the round window or the oval window, typically in a relatively simple (e.g., outpatient) procedure. In some embodiments, a composition comprising a therapeutically effective number of virus

particles containing a transgene, or containing one or more sets of different virus particles, wherein each particle in a set can contain the same type of transgene, but wherein each set of particles contains a different type of transgene than in the other sets, as described herein can be delivered to the appropriate position within the ear during surgery (e.g., a cochleostomy or a canalostomy).

In one embodiment, an Inner Ear Hair Cell Targeting AAV (e.g., Anc80 vector) comprising a promoter (e.g., CMV, Espin, PCDH15, a PTPRQ, a TMHS (LHFPL5)) and a polynucleotide that is one or more of USH1, MYO7A, USH1C (harmonin-a, b, c), CDH23, PCDH15, SANS and CIB2 is injected through the round window of a subject in need thereof.

In addition, delivery vehicles (e.g., polymers) are available that facilitate the transfer of agents across the tympanic membrane and/or through the round window, and any such delivery vehicles can be used to deliver the viruses described herein. See, for example, Arnold et al., 2005, *Audiol. Neurootol.*, 10:53-63.

The compositions and methods described herein enable the highly efficient delivery of nucleic acids to inner ear cells, e.g., cochlear cells. For example, the compositions and methods described herein enable the delivery to, and expression of, a transgene in at least 80% (e.g., at least 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99%) of inner hair cells or delivery to, and expression in, at least 80% (e.g., at least 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99) of outer hair cells.

As demonstrated herein, expression of a transgene delivered using an AAV containing an Anc80 capsid protein can result in regeneration of inner hair cells (IHCs), outer hair cells (OHCs), spiral ganglion neurons, stria vascularis, vestibular hair cells, and/or vestibular ganglion neurons (e.g. Atoh1, NF2) such that hearing or vestibular function is restored for an extended period of time (e.g., months, years, decades, a life time).

As discussed in WO 2015/054653, an AAV containing an Anc80 capsid protein can be characterized by its seroprevelance and/or the extent it is neutralized relative to conventional AAVs (i.e., an AAV not containing an Anc80 capsid protein). Seroprevalence is understood in the art to refer to the proportion of subjects in a population that is seropositive (i.e., has been exposed to a particular pathogen or immunogen), and is calculated as the number of subjects in a population who produce an antibody against a particular pathogen or immunogen divided by the total number of

individuals in the population examined. Determining the seroprevalence of a virus is routinely performed in the art and typically includes using an immunoassay to determine the prevalence of one or more antibodies in samples (e.g., blood samples) from a particular population of individuals. In addition, several methods to determine the extent of neutralizing antibodies in a serum sample are available. For example, a neutralizing antibody assay measures the titer at which an experimental sample contains an antibody concentration that neutralizes infection by 50% or more as compared to a control sample without antibody. See, also, Fisher et al. (1997, *Nature Med.*, 3:306-12) and Manning et al. (1998, *Human Gene Ther.*, 9:477-85). Representative conventional AAVs include, without limitation, AAV8 (or a virus comprising an AAV8 capsid protein) and/or AAV2 (or a virus comprising an AAV2 capsid protein).

Kits

The present disclosure also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of this disclosure (e.g., Inner Ear Hair Cell Targeting AAV (e.g., Anc80 vector) comprising a promoter (e.g., CMV, Espin, PCDH15, a PTPRQ, a TMHS (LHFPL5)) and a polynucleotide that is one or more of USH1, MYO7A, USH1C (harmonin-a, b, c), CDH23, PCDH15, SANS and CIB2). Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The present disclosure also provides kits for treatment or prevention of a disease or disorder (or symptoms) thereof associated with a defect in auditory and/or vestibular mechanosensation. In one embodiment, the kit includes an effective amount of an Inner Ear Hair Cell Targeting AAV (e.g., Anc80 vector) comprising a promoter (e.g., CMV, Espin, PCDH15, a PTPRQ, a TMHS (LHFPL5)) and a polynucleotide that is one or more of USH1, MYO7A, USH1C (harmonin-a, b, c), CDH23, PCDH15, SANS and CIB2 in unit dosage form, together with instructions for administering the angiogenesis-inhibiting compound to a subject suffering from or susceptible to a disease or disorder or symptoms thereof associated with angiogenesis, wherein the effective amount of an angiogenesis-inhibiting compound is less than 500 mg of the compound. In preferred embodiments, the kit comprises a sterile container which contains the angiogenesis-inhibiting compound; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container form known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments. The instructions will generally include information about the use of the angiogenesis-inhibiting compound for treatment of a disease or disorder or symptoms thereof associated with angiogenesis; in preferred embodiments, the instructions include at least one of the following: description of the angiogenesis-inhibiting compound; dosage schedule and administration for treatment of a disease or disorder or symptoms thereof associated with angiogenesis; precautions; warnings; indications; counter-indications; overdosage information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container

(when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

Conventional molecular biology, microbiology, biochemical, and recombinant DNA techniques within the skill of the art can be used in accordance with the present disclosure. Such techniques are explained fully in the literature. This disclosure will be further described in the following examples, which do not limit the scope of the methods and compositions of matter described in the claims.

EXAMPLES

Example 1—An Adeno-Associated Virus (AAV) Containing an Ancestral AAV Capsid Protein Results in Safe and Efficient Cochlear Gene Transfer

The following methods and materials were used in Example 1.

Viral Vectors

AAV2/1, 2/2, 2/6, 2/8, 2/9 and AAV2/Anc80 with a CMV-driven eGFP transgene and the Woodchuck hepatitis virus Post-transcriptional Regulatory Element (WPRE) cassette were prepared at Gene Transfer Vector Core (vector.meei.harvard.edu) at Massachusetts Eye and Ear as previously described. AAV2/Anc80 plasmid reagents are available through addgene.com.

Animal Models and General Methods

All experiments were approved by Boston Children's Hospital (protocol #12-02-2146) as well as the Institutional Biosafety Committee (protocol #IBC-P00000447). Wild-type C57BL/6J and CBA/CaJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and animals of either sex were used for experimentation in an estimated 50/50 ratio. Group sizes per experiment for the *in vitro* and *in vivo* transduction assays and subsequent endpoints were determined by access to specimen and technical feasibility. Reported observations on Anc80 transduction were qualitatively validated in subsequent experiments with various vector lots (except for the human vestibular tissue transduction due to the unique and limited nature of access to specimen). No statistical analysis between serotype transduction efficiencies was performed due to the limited access to specimen and qualitative nature of the reported findings.

Example 1A—*In Vivo* Injections

Mouse pups (P0 to P2) were injected via the round window membrane (RWM) using beveled glass microinjection pipettes. Pipettes were pulled from capillary glass (WPI) on a P-2000 pipette puller (Sutter Instrument, Novato, CA) and were beveled (~20 μm tip diameter at a 28° angle) using a micropipette beveler (Sutter Instrument, Novato, CA). EMLA cream (lidocaine 2.5% and prilocaine 2.5%) was applied externally for analgesia using sterile swabs to cover the surgical site (left mastoid prominence). Body temperature was maintained on a 38°C warming pad prior to surgery. Pups were anesthetized by rapid induction of hypothermia into ice/water for 2-3 minutes until loss of consciousness, and this state was maintained on a cooling platform for 5-10 minutes during the surgery. The surgical site was disinfected by scrubbing with Betadine and wiping with 70% Ethanol in repetition three times. A post-auricular incision was made to expose the transparent otic bulla, a micropipette was advanced manually through the bulla and overlying fascia, and the RWM was penetrated by the tip of the micropipette. Approximately 1 μL of virus was injected unilaterally within 1 min into the left ear manually in 5 (AAV1), 4 (AAV2), 2 (AAV8), 1 (AAV6), 3 (Anc80) C57BL/6 animals. In order to control for factors related to the specific vector preparation such as quality and purity, Anc80 results were confirmed in subsequent studies with different vector lots from independent preparation which were confirmatory of our qualitative findings presented here (data not shown). Injections were performed per group in a non-blinded fashion. Occasionally, the injection needle was inserted too deep, too shallow or at the wrong angle. If there was visible damage to the middle or inner ear structures, the samples were excluded from further analysis. Success rates of injection ranged between ~50% to ~80% depending on the experience level of the injector. After the injection, the skin incision was closed using a 6-0 black monofilament suture (Surgical Specialties, Wyomissing, PA). Pups were subsequently returned to the 38°C warming pad for 5-10 min and then put back to their mother for breeding.

Consistent with prior reports, AAV1 transduced IHCs with moderate to high efficiency (Figure 1A, 1B). These studies indicate AAV2, 6, and 8 targeted low numbers of IHCs, with only AAV8 demonstrating roughly equivalent transduction in apex and base (Figure 1B). Also, consistent with prior reports, there was minimal OHC transduction (<5%) for all conventional AAV serotypes tested. However, Anc80 transduced nearly 100% of IHCs and ~90% of OHCs (Figure 2A-2C) at a 20- (for AAV1)

to 3-fold (for AAV2) lower dose. Transduction at equal dose of 1.36 x 10^{12} GC for all serotypes resulted in substantial IHC and OHC transduction for Anc80, but minimal IHC targeting for AAV1, 2, and 8, and none noted in OHCs as observed by live-cell imaging by epifluorescent microscopy (Figure 8C, 8D).

The Anc80-transduced samples were subsequently fixed, stained and imaged by confocal microscopy, revealing a dose-dependency of hair cell transduction (Figure 1E). The unparalleled OHC targeting (Figure 1C, Figure 3) illustrates qualitatively distinct transduction biology of Anc80 compared to other AAVs. Similar levels of Anc80 transduction were found throughout the cochlea from base to apex in a total of three Anc80-injected mice (Figure 1A, B, C). Low magnification views of the cochlear apex (Figure 3A) showed strong eGFP expression far from the injection site. High magnification images of the base reveal 100% IHC and 95% OHC transduction (Figure 3B).

Since some forms of genetic deafness also cause vestibular dysfunction, Anc80 may be a useful vector for gene delivery into human vestibular organs. To investigate this possibility, human vestibular epithelia were harvested from four adult patients undergoing resection of vestibular schwannoma tumors; the sensory epithelium was placed in culture as previously described. For AAV transduced samples, Figures 3C reveal strong eGFP fluorescence throughout the human vestibular epithelium in both hair cells and supporting cells. A high-magnification view in an epithelium counterstained with Myo7A in Figure 3D revealed that 83% (19/23) of Myo7A-positive hair cells were also eGFP-positive, suggesting that Anc80 can transduce both mouse and human hair cells efficiently.

Example 1B—Hair Cell Electrophysiology

Cochleae were excised, mounted on glass coverslips and viewed on an Axio Examiner.A1 upright microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 63x water-immersion objective and differential interference contrast optics. Electrophysiological recordings were performed at room temperature (22°C - 24°C) in standard solutions containing (in mM): 137 NaCl, 5.8 KCl, 10 HEPES, 0.7 NaH2PO4, 1.3 CaCl2, 0.9 MgCl2, and 5.6 D-glucose, vitamins (1:100), and amino acids (1:50) as in MEM (Life Technologies, Carlsbad, CA) (pH 7.4; ~310 mOsm/kg). Recording electrodes (3–4 MΩ) were pulled from R-6 glass (King Precision Glass, Claremont, CA)

and filled with intracellular solution containing (in mM): 140 CsCl, 5 EGTA-KOH, 5 HEPES, 2.5 Na2ATP, 3.5 MgCl2, and 0.1 CaCl2 (pH 7.4; ~280 mOsm/kg). The whole-cell, tight-seal technique was used to record mechanotransduction currents using an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Hair cells were held at –84 mV. Currents were filtered at 5 kHz with a low-pass Bessel filter, digitized at ≥ 20 kHz with a 12-bit acquisition board (Digidata 1440A, Molecular Devices, Sunnyvale, CA), and recorded using pCLAMP 10 software (Molecular Devices, Sunnyvale, CA). Hair bundles from IHCs and OHCs were deflected using stiff glass probes mounted on a PICMA chip piezo actuator (Physik Instrumente, Karlsruhe, Germany) driven by an LVPZT amplifier (E-500.00, Physik Instrumente, Karlsruhe, Germany) and filtered with an 8-pole Bessel filter (Model 3384 filter, Krohn-Hite Corporation, Brockton, MA) at 40 kHz to eliminate residual pipette resonance. Stiff glass probes were designed to fit into the concave aspect of the array of hair cell stereocilia for whole-bundle recordings (3-4 μm diameter for OHCs and 4-5 μm diameter for IHCs). For the whole cell electrophysiology recording at >P10, cochlea tissues were dissected at P5-7 and incubated in MEM(1X) + GlutaMAXTM-I medium with 1% FBS at 37°C, 5% CO2 for up to 30 days.

Representative currents evoked by hair bundle deflections from P7 OHCs and P35 IHCs revealed no differences in amplitude, sensitivity or kinetics, between eGFP positive and eGFP-negative control cells (Figure 1D). 51 eGFP positive and 52 eGFP-negative hair cells were recorded from all regions of the cochlea and from ages between one and five weeks after exposure to Anc80. Responses were indistinguishable from wild-type in all cases (Figure 1E), which confirmed that Anc80 transduction had no detrimental effects on sensory cell function.

Example 1E—Hearing Tests

Auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) data were collected as described previously. DPOAE is an assay for proper cochlear amplification and tuning and is a sensitive measure of outer hair cell viability. Stimuli tested in anesthetized mice varied between 10 and 90 dB sound pressure level at frequencies of 5.6, 8, 11.3, 16, 22.6, and 32 kHz. Four Anc80-injected ears and four uninjected ears and one negative control ear with injection damage without eGFP fluorescence were analyzed at P28-P30.

Minimal sound thresholds required to evoke ABRs were plotted (Figure 1F) and revealed no difference in threshold between injected and uninjected ears. Histological analysis revealed strong eGFP fluorescence in all four injected ears (data not shown). In one case, there were no eGFP-positive cells and ABR thresholds were elevated (Figure 1F), suggesting the injection failed and that the needle may have breached the cochlear duct and caused permanent damage. Despite robust outer hair cell transduction by Anc80-eGFP, no difference was found in DPOAE thresholds relative to uninjected control ears (Figure 1G). Thus, data from ABRs and DPOAEs indicate that RWM injection, Anc80 transduction and transgene expression in IHCs and OHCs are all safe for auditory function.

Example 1F—Rotarod Test

Five C57BL/6 mice were tested for balance behavior on the rotarod device. Mice with impaired vestibular function are known to perform poorly on the rotarod device. Previous studies highlighted the ability of this rotarod test to detect balance dysfunction when only one ear is affected. Three mice injected at P1 and tested at P36 and two uninjected control mice at P79. All mice were tested using the following rotarod protocol. On day one, mice were trained to balance on a rod that was rotating at four RPM for five minutes. On day two, the mice were tested in five trials with each trial separated by five minutes. For each trial, the rod accelerated one RPM from a starting rate of two RPM. The time (in seconds) was recorded until the mice fell off the device.

Since the perilymphatic solutions of the cochlea are continuous with those of the vestibular labyrinth, it was evaluated whether Anc80-eGFP injected via the cochlear RWM would transduce vestibular sensory organs. Indeed, whole-mounts of vestibular epithelia revealed robust eGFP expression in both type I and type II hair cells of the utricle, a vestibular organ sensitive to gravity and linear head movements and in the semicircular canals, which are sensitive to rotational head movements (Figure 2A, 2B). Thus, to address the safety concern that Anc80 transduction may affect balance, injected mice with confirmed vestibular expression performed the rotarod test for vestibular function similarly to uninjected controls (Figure 4).

Part 2: Gene Therapy Restores Auditory and Vestibular Function in a Mouse Model of Usher Syndrome, Type 1c

Example 2—Mouse Model of Usher Syndrome

The following methods and materials were used in Example 2.

Tissue Preparation

Utricle and organ of Corti from Ush1c c.216G>A heterozygous or homozygous mutant mice were harvested from postnatal day 0 to 8 (P0 to P8) for electrophysiological studies. Postnatal mouse pups were killed by rapid decapitation. The temporal bones were excised and bathed in MEM (Invitrogen, Carlsbad, CA) supplemented with 10mM HEPES (pH 7.4). The organ of Corti was dissected away without the use of enzyme as described previously (53). Utricles were removed after 10 min protease treatment (Protease XXIV, Sigma) at 0.1 mg/ml. The excised organs were mounted on round glass coverslips. A pair of thin glass fibers previously glued to the coverslip was placed on the edge of the tissue to stabilize it in a flat position. Tissues were either used acutely or kept in culture in presence of 1% Fetal Bovine Serum. Cultures were maintained for 7 to 8 days and the media was replaced every 2 to 3 days for experiments that involved viral vectors infection *in vitro*.

Animals

Ush1c c.216G>A knock-in mice were obtained from Louisiana State University Health Science Center. The imported strain while on a C57BL6 background were previously bred out of the Cdh23 (Ahl) mutation causing age related hearing loss (48, 49). All procedures used for this work met the NIH guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees at Boston Children's Hospital (Protocols# 12-02-2146, #14-03-2659R and #15-01-2878R). Mice were genotyped using toe clip (before P8) or ear punch (after P8) and PCR was performed as described previously (32). For all studies, both male and female mice were used in approximately equal proportions. No randomization paradigm was otherwise applied.

Viral Vector Generation

Total RNA was isolated from cochleae of c.216AA mutant mice (RNAqueous micro kit, Ambion) and reverse transcribed with QuantiTect Reverse Transcription kit (Qiagen). The cDNA of trunc-harmonin was amplified by PCR with Platinum Taq DNA

polymerase High Fidelity (Invitrogen) and primers: Trunc-harmonin.F(KpnI) GAG GTA CCA TGG ACC GGA AGG TGG CCC GAG; Trunc-harmomin.RV(BamHI) CAG GAT CCG GAC AAT TTC ATC CCC TAC. The 387 bp PCR product was cloned with TA cloning kit (Invitrogen), and confirmed by sequencing. To generate a GFP fusion construct, the truncated harmonin fragment was subcloned into pEGFP-C1 with KpnI and BamHI. The NheI-XbaI EGFP::trunc-harmonin cDNA was transferred into an AAV shuttle vector. Custom vectors were packaged with AAV2 inverted terminal repeats (ITRs) into the AAV1 capsid where the transgene cassette was driven by a CMV promoter (AAV2/1.CMV.EGFP::trunc-harmomin.hGH, 1.92 E14gc/m, BCH).

Harmonin-a1 and harmonin-b1 plasmid were prepared in our laboratory from EGFP tagged labeled constructs graciously provided by Lily Zheng and James Bartles (52) (Department of Cell and Molecular Biology, Northwestern University, Feinberg School of medicine, Chicago, IL). Harmonin-a1 was originally obtained from mouse kidney and harmonin-b1 from isolated mouse cochlea sensory epithelium. We further modified the harmonin-a1 construct to replace the EGFP tag with tdTomato at its N terminal end. Fluorescently labeled and unlabeled constructs were packaged into AAV vectors. Viral vectors were generated by the viral core facility at Boston Children's Hospital and the Gene Transfer Vector Core at the Massachusetts Eye and Ear Infirmary. The following vectors were generated: AAV2/1.CMV.tdTomato::harmonin-a1 4.33 10^13gc/ml (BCH); AAV2/1.CMV.EGFP::harmonin-b1 2.73 564 10^14 gc/ml (BCH); AAV2/1.CMV.EGFP-harmonin-a1: 2.81 10^12 gc/ml (MEEI); AAV2/1.CMV.EGFP-trunc-harmonin; 1.92 10^14 gc/ml (BCH); AAV2/Anc80.CMV.harmonin-a1: 1.93 10^12 gc/ml (MEEI); AAV2/Anc80.CMV.harmonin-b1: 1.74 10^12 gc/ml (MEEI); AAV2/Anc80.CMV.trunc-harm.WPRE: 9.02 567 10^12 gc/ml (MEEI); For *in vitro* experiments, 10 µl of concentrated vector was applied to 1ml MEM supplemented media on acutely dissected tissue in presence of 1% Fetal Bovine Serum for 24h. Cultures were subsequently maintained for up to 10 days.

Round Window Membrane (RWM) Injection

RWM injections were performed as approved by the Institutional Animal Care and Use Committees at Boston Children's Hospital animal protocol #15-01-2878R. 0.8 µl-1ul of AAV vectors were injected in neonatal mice P0-P1 and P10-P12. P0-P1 mice were first anesthetized using hypothermia exposure while P10-P12 mice were anesthetized with isoflurane. Upon anesthesia, post-auricular incision was made to

expose the otic bulla and visualize the cochlea. Injections were done through the RWM with a glass micropipette controlled by a micromanipulator (Askew et al. 2015). The volume of the injected materials was controlled at an approximately 0.02 µl/min for 10 min. Standard post-operative care was applied. Sample size for *in vivo* studies were determined on a continuing basis to optimize the sample size and decrease the variance.

Electrophysiological Recording

Recordings were performed in standard artificial perilymph solution containing (in mM): 144 NaCl, 0.7 NaH2PO4, 5.8 KCl, 1.3 CaCl2, 0.9 MgCl2, 5.6 D-glucose, and 10 HEPES-NaOH, adjusted to pH 7.4 and 320mOsmol/kg. Vitamins (1:50) and amino acids (1:100) were added from concentrates (Invitrogen, Carlsbad, CA). Hair cells were viewed from the apical surface using an upright Axioskop FS microscope (Zeiss, Oberkochen, Germany) equipped with a 63X water immersion objective with differential interference contrast optics. Recording pipettes (3-5 MΩ) were pulled from borosilicate capillary glass (Garner Glass, Claremont, CA) and filled with intracellular solution containing (in mM): 135 KCl, 5 EGTA-KOH, 10 HEPES, 2.5 K2ATP, 3.5 MgCl2, 0.1 CaCl2, pH 7.4. Currents were recorded under whole-cell voltage-clamp at a holding potential of -64 mV at room temperature. Data were acquired using an Axopatch Multiclamp 700A or Axopatch 200A (Molecular devices, Palo Alto, CA) filtered at 10 kHz with a low pass Bessel filter, digitized at ≥20 kHz with a 12-bit acquisition board (Digidata 1322) and pClamp 8.2 and 10.5 (Molecular Devices, Palo Alto, CA). Data were analyzed offline with OriginLab software and are presented as means ± standard deviations unless otherwise noted.

Statistical Analyses

Test and control vectors were evaluated in at least three mice per group at each time point to ensure reproducibility. Sample sizes are noted in figure legends. All animals with successful RWM injection were included in the study analysis. Those animals with unsuccessful injection were excluded from the mean but included in the legend for full disclosure. Injection success was determined according to ABR recovery with thresholds >90 dB SPL. Statistical analyses were performed with Origin 2016 (OriginLab Corporation). Data are presented as means ± standard deviations (S.D) or standard error of the mean (S.E.M) as noted in the text and figure legend. One-way analysis of variance (ANOVA) was used to determine significant differences between the means.

Example 2A—Scanning Electron Microscopy (SEM)

SEM was performed at P7, P18 and ~P42 (6 weeks) along the organ of Corti of control and mutant mice. P18 SEM was performed in collaboration with Dr. Edwin Rubel at the University of Washington. Inner ears were fixed in 4% glutaraldehyde in 0.1 M sodium phosphate at 4°C overnight. The next day specimens were rinsed three times in 0.1 M sodium phosphate buffer (PB) and post-fixed in 1% osmium tetroxide in 0.1 M PB for 30 min in an ice bath. Specimens were then rinsed in 0.1 M PB and dehydrated through a graded ethanol series: 35%, 70%, 95%, and 100% (x2). Samples were critical point dried, mounted on SEM stubs, and sputter coated with Au/Pd. SEM was performed using a JEOL JSM-840A scanning electron microscope. A similar preparation was performed for P8 and 6 weeks stages by Dr. Géléoc and Dr. Indzhykulian. Organ of Corti explants were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (Electron Microscopy Sciences) supplemented with 2 mM $CaCl_2$ for 1 h at room temperature. Specimens were dehydrated in a graded series of acetone, critical-point dried from liquid CO_2, sputter-coated with 4–5 nm of platinum (Q150T, Quorum Technologies, United Kingdom), and observed with a field emission scanning electron microscope (S-4800, Hitachi, Japan).

Homozygous c.216AA mutant mice are deaf and show circling and head tossing behaviors characteristics of vestibular dysfunction. Previous work from Lentz et al. (34) described pronounced inner and outer hair cell degeneration at the base of the cochlea at P30. Degeneration and hair cell death was also observed in the middle turn, while the apical portion of the organ was better preserved at 1 month of age. It was hypothesized that hair cell degeneration occurs progressively during development of the inner ear organs and, to assess hair cell survival at earlier stages, SEM analysis was performed along the organ of Corti at P8 and P18. Outer hair cells (OHCs) and inner hair cells (IHCs) of heterozygous c.216GA mice were preserved and their bundles were properly oriented at these ages (Figure 5A-5C, 5G, 5I and Figure 12A-12C, 12K). However, disorganized hair bundles were evident along the entire length of the organ of Corti in homozygous c.216AA mice at both ages analyzed (Figure 12D-12F, 12H, 12J-12L and Figure 19D-19J, 19L). At P8, IHC bundles were mildly disorganized at the base, mid and apical regions (Figure 12D-12F, 12J). Numerous IHC bundles displayed a wavy pattern and mild disorganization of the stereocilia rows (Figure 12J). While many OHCs of

c.216AA mutant mice possessed well-preserved hair bundles (Figure 12H, 12K), fragmented and disorganized hair bundles were evident sporadically along the organ (Figure 12D-12F, 12L). Disruption was more pronounced at P18, though the majority of hair cells were still present as previously reported (35) (Figure 12D-12F).

To assess hair bundle morphology in mice that have undergone gene therapy with harmonin-b1, temporal bones of 6-week old untreated (or uninjected) and treated (or injected) mice were prepared for SEM analysis. Untreated c.216AA mice displayed severe hair cells loss at the basal and middle regions of the organ (Figure 11). In the basal region, OHCs were mostly absent in the first row and present sporadically in the second and third rows. In the middle region of the organ, the first row of OHCs was also largely absent. Milder phenotypes were observed in the apical end. High magnification SEM also revealed severely disorganized hair bundles along the entire length of the organ of c.216AA mutant mice. Remarkably, in 6 weeks old c.216AA mice, no hair bundles were observed that retained the typical staircase structure with all three rows of stereocilia. Instead, hair cells from c.216AA mice displayed disorganized hair bundles with retracted stereocilia along the first row, abnormal second row and fairly preserved tallest row. In contrast, reduced hair cell loss and normal hair bundles were observed in c.216AA mice after treatment with harmonin-b1. Hair cells counts were estimated from the presence or absence of hair bundles in representative fields of view. The data revealed pronounced preservation of hair cell number in injected mice from the base to the apex of the organ, from 40 to 79% in the base, 68 to 95% in the middle and 93 to 99% in the apex (n=1824 cells from n=4 c.216AA mice ears and n=792 from n=2 rescued c.216AA ears). Although abnormal hair bundles were still evident in harmonin-b1 injected mice, most hair bundles possessed three rows of stereocilia and had morphology almost indistinguishable from their heterozygous controls (Figure 11).

Example 2B—FM1-43 Imaging

5 micromolar FM1-43 (Invitrogen) was diluted in extracellular recording solution and applied to tissues for 10 seconds and then washed 3 times in extracellular recording solution to remove excess dye and prevent uptake via endocytosis. After 5 minutes the intracellular FM1-43 was imaged using an epifluorescence light source, differential interference contrast optics, and an FM1-43 filter set (Chroma Technologies) on a Zeiss Axioscope FS plus with water immersion 20x, 40x, and 63x objectives. Images were

captured at 16-bit with a CCD camera and Argus-20 image processor (Hamamatsu) using background fluorescence subtraction. The same gain and contrast settings were maintained for the acquisition of all images and analyzed offline with Adobe Photoshop or Image-J software.

To assess hair cell function at earlier stages, FM1-43 uptake in acutely dissected inner ear organs was analyzed at P4. Upon brief applications (< 10 s), FM1-43 permeates hair cells that possess functional mechanosensitive channels (36, 37, 38). Uniform FM1-43 uptake was observed in hair cells of c.216GA mice (Figure 6A), but the level of uptake varied among OHCs of c.216AA mice, suggesting that some, but not all, cells retained functional transduction channels (Figure 6B). Similar observations were made along the entire length of the cochlea. No tonotopic differences were noted. FM1-43 uptake also decreased in IHCs of c.216AA mice during the first postnatal week (data not shown). FM1-43 uptake also was assessed in utricle hair cells of mutant mice. Interestingly, in c.216AA mutant mice, uptake was restricted to the extra-striola region at P6, suggesting that hair cells of the striola region lack mechanosensitive channels open at rest (Figure 6C, 6D).

Example 2C—Mechanical Stimulation

OHCs and IHCs: Mechanical stimuli were transmitted via a stiff glass probe mounted on a one-524 dimensional PICMA chip piezo actuator (Physik Instruments, Waldbronn, Gernamy) driven by a 400 mA ENV400 Amplifier (Piezosystem Jena Germany, 54). The tip of the probe was fired polished (Fire polisher, H602, World Precision Instruments Inc., Sarasota, FL) to fit stereociliary bundle (51). Deflections were evoked by applying voltage steps filtered with an 8-pole Bessel filter (Khron-Hite, 528 Brockton, MA) at 50 kHz to eliminate residual pipette resonance. Hair bundle deflections were monitored using a C2400 CCD camera (Hamamatsu, Japan). Voltage steps were used to calibrate the motion of the stimulus probe around ± 2μm of its rest position. Video images of the probe were recorded to confirm absence of off-axis motion and calibrate the probe motion (spatial resolution of ~ 4 nm). The 10-90% rise-time of the probe was ~20 μsec.

VHCs: Mechanical stimuli were transmitted via a stiff glass probe mounted on a piezoelectric bimorph element. Coupling was performed by gentle suction of the kinocilium into the stimulus pipette. Deflections were evoked by applying voltage steps

to the piezoelectrical device which consisted of two bimorphs mounted in series and directly coupled to the stimulus probe. Voltage steps were controlled by pClamp 8.0 software and filtered with a 8 pole Bessel filter at 1 kHz (Khron-Hite, Brockton, MA). Hair bundle deflections were monitored using a C2400 CCD camera (Hamamatsu, Japan). The motion of the stimulus probe was calibrated around (± 2 μm) its rest position prior to the experiments.

During the first postnatal week, auditory and vestibular epithelia retain mechanosensitive hair cells, including some with relatively normal morphology (Figure 5). In the organ of Corti, recordings were obtained from the middle and apical turns of the cochlea from P3 to P6 c.216AA mice from hair cells with bundles that appeared normal and those with more severely disrupted hair bundles. In c.216AA mutants, OHCs retained mechanosensitivity, although the amplitudes of the responses were significantly reduced by ~63% to 170 ± 80 pA (n=24; $p<0.001$, Figure 6E, 6F, 6G). A wide range of response amplitudes was observed in OHCs, between 31 and 292 pA in c.216AA mice. Significant difference ($p<0.01$) was observed when data were grouped according to hair bundle morphology: currents evoked in mutant hair cells that possessed severely disorganized bundles were smaller than those evoked in mutant cells that had more preserved hair bundles, 120 ± 65 pA (n=9) and 201 ± 74 pA (n=15), respectively. Despite the reduction in current amplitude, hair cell responses to mechanical displacements retained similar properties to those of heterozygous c.216GA mice. Stimulus response [I(X)] curves were fitted using a second-order Boltzmann equation (Figure 6F) and the fit was used to determine the 10-90% operating range (Figure 13B). No significant difference ($p= 0.054$) in operating range was observed between OHCs recorded from c.216GA and c.216AA. Similarly, while hair bundles from IHCs of c.216AA mutant mice appeared mildly disrupted under the DIC microscope, transduction currents were significantly reduced at P6 (Figure 13E, 13F, 13G). At a holding potential of -64 mV, maximum transduction currents in heterozygous c.216GA IHCs (P6-P7) averaged 587 ± 96 pA (n=21) but were reduced by 46% to 316 ± 127 pA (n=19; $p<0.001$) in c.216AA IHCs. A significant ($p<0.01$) reduction in the operating range was measured in IHCs of c.216AA mutant mice (Figure 13G).

Adaptation, defined as a decline in the transduction current in the presence of a constant bundle deflection, was also present in the c.216AA mutant mice. Adaptation kinetics were analyzed using double exponential fits to determine fast and slow

components. While both components were slower in IHCs and OHCs from c.216AA mutant mice the difference was only significant for the slow component ($p<0.05$ in OHCs, and $p<0.001$ in IHCs; Figure 13C, 13D, 13H, 13I). On the other hand, the extent of adaptation measured at Popen= 0.5 was significantly less in OHCs and IHCs of c.216AA than c.216GA hair cells (Figure 20E, 20J; $p<0.001$). Together, these results demonstrate that mechanosensitivity is mildly compromised in inner and outer hair cells of c.216AA mice and importantly that both cell types survive throughout the first postnatal week, a prerequisite for gene therapy and restoration of cellular function.

In vestibular hair cells, a reduction in mechanotransduction currents also was observed in c.216AA mice. In the extra-striola region, c.216AA currents were significantly ($p<0.001$) reduced to 109 ± 30 pA (n=9, P5-P7) versus 231 ± 53 pA (n=8, P6-P7) for c.216GA currents (Figure 6E, 6F, 6H). Very small or no currents were recorded from hair cell of the striola region (6 ±13 pA, n=6, P5-P7), in agreement with the absence of FM1-43 uptake in that region (see below; Figure 6C, 6D). While utricle hair bundles appeared grossly well-preserved by DIC microscopy, transduction currents were significantly reduced or absent from hair cells in the extra-striola and striola, respectively. Thus, with the exception of the striola region, these results suggest that the transduction apparatus is correctly assembled and targeted in mutant mice but that the number of functional complexes is reduced in neonatal mice.

Next, function in c.216AA hair cells exposed to AAV vectors driving harmonin expression was assessed. To enhance the likelihood of functional rescue with exogenous harmonin, untagged harmonin-a1 or harmonin-b1 coding sequences driven by a CMV promoter were packaged into an AAV capsid known as Anc80 (39). The Anc80 capsid has recently been shown to transduce 100% of IHCs and 80-90% of OHCs *in vivo* (40). We hypothesize that harmonin-b is required for mechanotransduction in both IHCs and OHCs and is necessary for auditory function in both cell types. RWM injections of AAV2/Anc80.CMV.harmonin-b1 (0.8 µl, 1.9×10^12 gc/ml) and separately a mixture of AAV2/Anc80.CMV.harmonin-a1 (1.7×10^12 gc/ml) + AAV2/Anc80.CMV.harmonin-b1 (0.5 µl +0.5 µl) were performed and mechanotransduction responses assessed 2 weeks after treatment. Tissue was extracted at P5-P6, before the cochlea became ossified and was maintained in culture for 10 days. Although mature OHCs (>P10) do not survive *ex-vivo* recording paradigms, robust electrophysiological recordings were obtained from IHCs at the equivalent of P14-P16. Results are presented in Figure 8. While IHCs from

uninjected mice displayed severely reduced transduction currents at P16 (79 ± 43 pA, n=8), recovery of sensory transduction was evident in mice that received the AAV treatment. Significant recovery (***P<0.001) was observed in mice injected at P1 with harmonin-b1 or a combination of both b1 and a1 with respective average maximal transduction currents of 388 ± 66 pA (n=15) and 352 ± 28 pA (n=7; Figure 8C). Transduction current amplitudes in IHCs after treatment with harmonin-b1 were not significantly different from control c.216GA mice. The level of recovery was not significantly altered by co-injection of harmonin-b1 and harmonin-a1. These results suggest that delivery of exogenous harmonin-b1 via RWM injection at early stages can restore mechanotransduction in IHCs.

Example 2D—Confocal Imaging

To prepare the tissue for confocal imaging from postnatal mice P0-P8, fixation was performed for 15 min with 4% Paraformaldehyde (PFA). Permeabilization with 0.01% triton and counterstaining with Alexa Fluor phalloidin (Invitrogen, 1/200) was used to labeled actin filaments. Images were obtained on a LSM700 Zeiss confocal microscope. In older mice (4 to 8 weeks), temporal bones were removed after euthanasia and placed in 4% PFA for 1 hour, followed by decalcification for 24 to 36 hours with 120 mM EDTA. The sensory epithelium was then dissected out and injected as above for immunostaining. Mouse anti-CTBP2 (BD bioscience #612044, 1/200) was applied for 48 hours and counterstained with Alexa Fluor goat anti-mouse (1/200) overnight at 4°C to label ribbon synapses. Images were acquired on a Zeiss LSM 710 laser confocal microscope (IDDRC Imaging Core grant P30 HD18655) and processed with Zeiss LSM image viewer 4.2.

Previous work revealed expression of two alternative splice forms of harmonin in sensory hair cells. To assess the ability of AAV vectors to drive expression of exogenous harmonin splice forms, utricles and organs of Corti from neonatal c.216AA and wild-type (C57BL/6J) mice were exposed to AAV2/1 vectors coding for eGFP fused to the N-terminus of harmonin-b1 (eGFP::harmonin-b1) or tdTomato fused to the 181 N-terminus of harmonin-a1 (tdTomato::harmonin-a1). The vectors were applied either *in vitro* or *in vivo* through RWM injection (1 µl) at P1. When applied *in vitro*, P0-P1 tissues were incubated in the presence of the vectors for 24 hours and maintained in culture for one week. Confocal images show that hair cells of wild-type, c.216GA and c.216AA mice

were successfully transduced (Figure 7A-7C, 7E). EGFP::harmonin-b1 signal was evident at the tips of the stereocilia in VHCs (Figure 7A), IHCs and OHCs (Figure 7B, 7C). EGFP signal was also detected at P60 in OHCs and IHCs in the basal portion of the cochlea of mice injected at P1 (Figure 14D). TdTomato::harmonin-a1 was detected at the base of auditory hair cells (Figure 14E). Co-staining with a ribbon synapse marker CTBP2 frequently revealed colocalization in P7 IHCs (Figure 14E) but not in P7 utricles (data not shown). Localization of exogenous fusion constructs was consistent with previous work that localized harmonin-b to the distal end of stereocilia, near the tip-link insertions (26, 27, 28) and harmonin-a to the synapse (30, 31).

Example 2E—Auditory Brainstem Responses (ABRs) and Distortion Products (DPOAEs)

ABRs and DPOAEs were recorded from mice anesthetized with xylazine (5-10 mg/kg i.p.) and ketamine (60 – 100 mg/kg i.p.). Subcutaneous needle electrodes were inserted into the skin a) dorsally between the two ears (reference electrode); b) behind the left pinna (recording electrode); and c) dorsally at the rump of the animal (ground electrode). The meatus at the base of the pinna was trimmed away to expose the ear canal. For ABR recordings the ear canal and hearing apparatus (EPL Acoustic system, MEEI, Boston) were presented with 5-millisec tone pips. The responses were amplified (10,000 times), filtered (0.1–3 kHz), and averaged with an analog-to-digital board in a PC based data-acquisition system (EPL, Cochlear function test suite, MEEI, Boston). Sound level was raised in 5 to 10 dB steps from 0 to 110 dB sound pressure level (decibels SPL). At each level, 512 to 1024 responses were averaged (with stimulus polarity alternated) after "artifact rejection". Threshold was determined by visual inspection. Data were analyzed and plotted using Origin-2015 (OriginLab Corporation, MA). Thresholds averages ± standard deviations are presented unless otherwise stated. For DPOAEs, f1 and f2 primary tones (f2/f1 = 1.2) were presented with f2 varied between 5.6 and 45.2 kHz in half-octave steps and L1–L2 = 10dB SPL. At each f2, L2 was varied between 10 and 80 dB SPL in 10 dB SPL increments. DPOAE threshold was defined from the average spectra as the L2-level eliciting a DPOAE of magnitude 5 dB SPL above the noise floor. The mean noise floor level was under 0dB SPL across all frequencies. Stimuli were generated with 24-bit digital I–O cards (National Instruments PXI-4461) in a PXI-1042Q chassis, amplified by an SA-1 speaker driver (Tucker–Davis Technologies,

Inc.), and delivered from two electrostatic drivers (CUI CDMG15008-03A) in our custom acoustic system. An electret microphone (Knowles FG-23329-P07) at the end of a small probe tube was used to monitor ear-canal sound pressure. The majority of these experiments were not performed under blind conditions.

To determine if truncated harmonin interfered with normal auditory function, Anc80.CMV.trunc-harm vectors were generated to over-express the truncated protein. The vectors were injected via RWM into the inner ears of c.216GA mice. ABR and DPOAES were measured at 4, 6 and 12 weeks and found no difference in thresholds between injected and uninjected c.216GA mice (recordings from 6 weeks old mice shown in Figure 16C-16D). The data serve as a control for the injection technique, the vector and importantly, argue that exogenous truncated harmonin does not compete with endogenous full-length harmonin, implying that the endogenous truncated form in c.216AA hair cells is unlikely to interfere with exogenous full-length harmonin expressed via gene therapy vectors.

To determine if harmonin gene augmentation can rescue auditory and balance function in Ush1c mice, P0-P1 RWM injections of AAV2/Anc80.CMV.harmonin-a1 (0.8 µl, 1.7×10^12 gc/ml) or AAV2/Anc80.CMV.harmonin-b1 (0.8 µl, 1.9×10^12 gc/ml) were performed and auditory brainstem responses (ABRs), distortion product otoacoustic emissions (DPOAEs), acoustic startle reflexes, open field and rotarod behavior assessed. Mice were assessed at six weeks, a stage at which c.216AA mice suffer from profound hearing loss and vestibular dysfunction. Some of the mice were further tested at 3 and 6 months. None of the 12 mice injected with AAV2/Anc80.CMV.harmonin-a1 recovered auditory function at 6 weeks (Figure 9A-9C), suggesting exogenous expression of harmonin-a1 was insufficient for auditory rescue. However, 19 of 25 mice injected with AAV2/Anc80.CMV.harmonin-b1 recovered significant auditory function at 6 weeks. At low frequencies (5.6 to 16 kHz), best ABR thresholds in AAV2/Anc80.CMV.harmonin-b1 injected ears were at 25-30 dB SPL, remarkably similar to thresholds of wild-type mice (Figure 16A-16B). Partial rescue was observed at 22.6 kHz and little to none at 32 kHz. Rescue of DPOAE thresholds was also evident, consistent with rescue of function in OHCs (Figure 16C). Eight of the mice that possessed auditory thresholds <45dB SPL for stimuli 8-11.3 kHz were tested at later stages to assess the longevity of the rescue. From 6 weeks to 3 months, ~10 dB SPL ABR threshold shifts were observed in the low frequency range and ~30 dB SPL in the high frequency range (Figure 16D). A similar

shift was also observed in the DPOAEs thresholds (Figure 16E). After this time point, ABR thresholds and DPOAEs remained stabled up to 6 months of age (Figure 16D-16E), the latest time point tested.

To assess whether both harmonin-a1 and harmonin-b1 are required for more complete auditory rescue, particularly at the high frequency end, AAV2/Anc80.CMV.tdTomato::harmonin-a1 (0.5 µl; 238 4.1E^12 gc/ml) and AAV2/Anc80.CMV.eGFP::harmonin-b1 (0.5 µl; 3.0E^12 gc/ml) were co-injected. 65% of the hair cells expressed both harmonin-a1 and harmonin-b1, as evident from cells positive for both fluorescent tags (Figure 14). Fluorescently labeled harmonin-a1 was occasionally observed in the stereocilia of mice exposed to AAV2/Anc80.CMV.tdTomato::harmonin-a1, perhaps due to over expression. ABR and DPOAE thresholds in mice co-injected with unlabeled harmonin-a1 and harmonin-b1 vectors (Figure 9) were similar to those injected with harmonin-b1 alone and did not provide further improvement, suggesting that harmonin-a1 may be dispensable for auditory function. Importantly, the data demonstrate that harmonin-b1 alone is sufficient for significant restoration of auditory thresholds at low frequencies (Figure 9).

To further evaluate the extent of the rescue, ABR waveforms, from mice with thresholds $\leq$ 45 dB SPL, were analyzed and compared between eight control c.216GA mice and five c.216AA mice injected with AAV2/Anc80.CMV.harmonin-b1. The analysis for responses at 8-11.3 kHz and 16 kHz revealed normal wave 1 amplitudes (non-significant differences, P>0.2, Student t-test) and longer peak 1 latencies (P>0.001) (Figure 15), suggesting a possible lag in neurotransmission at the synapse. In many animals, auditory rescue was also observed in the contralateral ear, with ABR thresholds as low as 20 dB SPL at 11.3 kHz (harmonin-b1: average 59.7 ± 5.3 dB SPL, n=15/25; harmonin-a1+ -b1: 255 average 76.2 ± 10.3 dB SPL, n=4-6). Diffusion of AAV vectors to the contralateral ear has been previously observed (37) and likely occurs via the perilymphatic duct that remains continuous with the subarachnoid space in newborn mice.

We also wondered whether injections at later developmental stages might lead to partial auditory rescue. RWM injections of AAV2/Anc80.CMV.harmonin-b1 (0.8 µl) at P10-P12 were performed and auditory thresholds assessed at 6 weeks. None of the P10-P12 injected mice had detectable DPOAEs and their ABR thresholds did not differ from the uninjected c.216AA control mice (n=10; data not shown), suggesting the window of opportunity for intervention may be limited to early postnatal stages, possibly due to low

viral transduction efficiency in older tissue or degeneration of the organ of Corti at later development stages.

Example 2F—RT-PCR in the Usher Mouse Model

cDNA was prepared from 6 auditory organs of P2-P3 wild-type, heterozygous and homozygous Ush1c c.216G>A mice using QuantiTect Revese Transcription Kit (Qiagen). cDNA encoding full length (450bp) or truncated harmonin (-35bp) was amplified using the following primers: Forward primer mUsh1c_Ex2F: 5' CTC ATT GAA AAT GAC GCA GAG AAG G 3', Reverse mUsh1c_Ex5R: 5' TCT CAC TTT GAT GGA CAC GGT CTT 3'. These primers are specific for mouse Ush1c sequences and will amplify both endogenous and AAV2-derived Ush1c as the target sequence is outside the region of the human knocked in portion of the Ush1c c.216A allele. DNA and RNA levels were also assessed from mouse tissue collected at six weeks post-treatment. DNA and RNA were isolated from the cochlea using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. RNA was reverse transcribed using GoScript reverse transcription system (Promega, Madison, WI). Radiolabeled PCR was carried out using GoTaq Green Master Mix (Promega, Madison, WI). For viral DNA amplification, primers specific for mouse Ush1c: mUsh1c_Ex3F (5'-GAA CCC AAC CGC CTG CCG) and mUsh1c_Ex4WTR (5'-TGC AGA CGG TCC AAG CGT-3') were used. These primers will only amplify the viral Ush1c DNA because the homozygous Ush1c.216AA mice have the human USH1C c.216A gene knocked in to exon 3 and 4, replacing the mouse sequence (32). For cDNA amplification of full-length (450 bp) and aberrantly spliced/truncated harmonin (415 bp), the same primers as above were used (mUsh1c_Ex2F and mUsh1c_Ex5R). Gapdh primers were: mGapdh_Ex3F (5'-611 GTG AGG CCG GTG CTG AGT ATG-3') and mGapdh_Ex4R (5'-GCC AAA GTT GTC ATG GAT GAC-3'). Products were separated on a 6% nondenaturing polyacrylamide gel and quantified using a Typhoon 9400 phosphorimager (GE Healthcare).

Since previous studies raised the possibility that truncated harmonin may disrupt function by competing with full-length harmonin for endogenous binding partners (34, 35), we wondered whether persistent expression of the truncated protein may limit recovery in c.216AA mice injected with vectors that express exogenous full-length harmonin (Figure 16A). To address this concern, expression of Ush1c transcripts in c.216GA and c.216AA mice was examined using an RT-PCR assay. Consistent with

previous reports, Ush1c transcripts that encoded full-length and truncated harmonin were detected in c.216GA cochleas and only transcripts that encoded truncated harmonin were detected in c.216AA cochleas (Figure 16B).

To confirm expression of AAV2/Anc80.CMV.harmonin-b1 and explore the relationship between viral expression level and ABR thresholds, DNA and RNA were isolated from injected and contralateral cochleae and quantified by PCR and RT-PCR, respectively. Expression was assessed in six-week old c.216GA and AAV2/Anc80.CMV.harmonin-b1 (0.8 µl; 1.93 10^12 gc/ml)-injected and non-injected c.216AA mice. Samples included two injected mice with good ABR rescue (thresholds ≤ 35 dB SPL at 11.3 kHz) and two with poor ABR rescue (thresholds ≥ 90 dB SPL at 11.3 kHz). RNA encoding the correct splice form of harmonin (Figure 18A) and AAV2/Anc80.CMV.harmonin-b1 DNA (Figure 18B) were detected in all of the injected cochleae and, to a lesser extent, in the contralateral cochleae of all animals tested. There was variability between animals in ABR thresholds and amount of DNA and RNA expressed (Figure 18C). However, a strong correlation was found between AAV2/Anc80.CMV.harmonin-b1 DNA levels, the amount of RNA encoding for the correct splice form of harmonin and ABR threshold levels, which suggests that the variability in ABR data may be a direct result of AAV expression. To assess long term hair cell survival in mice that had successful recovery of ABR thresholds, tissue was prepared and the number of IHCs and OHCs counted at 6 months of age from 5 mice (Figure 17). While the number of IHCs did not vary in the two cohorts, 50% or more OHCs remained in the three mice that showed long term ABR rescue. OHC survival was observed along the entire organ with the exception of the basal turn (Figure 17).

Example 2G—Acoustic Startle Responses

The acoustic startle responses (ASR) were measured using the Startle Monitor (Kinder Scientific). Mice were placed in a small-sized, nonrestrictive, cubical Plexiglas recording chamber (27cm × 10cm × 652 12.5cm) fixed on a piezo/plexiglass sensing assembly and allowed to acclimate for 5 min with a 60 dB SPL background white noise. Each session consisted of 35 trials, during which a single noise pulse ranging in 10 dB SPL intensities from 60-120 db SPL was delivered with an inter-trial interval averaging 30s (25-35s range). Pulses were arranged in a pseudorandom order, on a constant 60 dB SPL background noise to limit external noise interference. The Startle Monitor system

reduced the response to each pulse into measurements of first N, max N, and max time of the response (ms), for calculations of peak startle response (ASR amplitude) and time from stimulus to peak startle response (ASR latency). ASR were all conducted blind.

To assess whether the ABR / DPOAE recovery yielded behaviorally relevant recovery of auditory function, acoustic startle responses was measured in mice injected with AAV2/Anc80.CMV.harmonin-a1, AAV2/Anc80.CMV.harmonin-b1 and those injected with both vectors. Analysis of the startle response to white noise showed partial rescue of the response in 6 weeks old mice injected with AAV2/Anc80.CMV.harmonin-b1 and in mice that were co-injected with both vectors (Figure 10A). Mice that received harmonin-a1 alone were similar to uninjected c.216AA mice and did not recover startle responses.

Example 2H—Vestibular Assessment

Vestibular function was assessed using open field and rotarod balance test. The open field test was conducted using a circular frame measuring 42 cm in diameter, placed inside a sound chamber with overhead LED lighting, set to 30 lux at the center, inside a dimmed room. Mice were placed one at a time inside the circular open field, and allowed to explore for 5 min. Behavior was recorded and tracked using Ethovision XT, enabling measures of distance traveled and velocity. Open field assessments were all conducted blind. The rotarod performance involved placement of mice on a rod in an enclosed housing that began rotating at 4 rpm and accelerated at a rate of 0.1 rpm s−1. The mice were placed on the rods on day one for 5 min to get familiarized with the equipment. The next day, the animals were placed on the rods for a total of 5 trials. A 5 min resting period was imposed between trials. The length of time the animals were able to remain on the device before dropping onto the instrumented floor of the housing was displayed on a timer and recorded after each test run.

Since the perilymphatic space is continuous between the cochlea and vestibular labyrinth, AAV vectors injected via RWM may transduce vestibular sensory organs as well. To assess vestibular behavior, mice were tested for their performance on a rotarod. While poor rotarod performance was observed in c.216AA and c.216AA mice injected with AAV2/Anc80.CMV.harmonin-a1 mice (latency to fall <22 sec on average), c.216AA mice injected with AAV2/Anc80.CMV.harmonin-b1 and those co-injected with

harmonin-a1 and -b1 vectors maintained balance function on the rotarod for 60-120 seconds, consistent with control c.216GA mice (Figure 10B).

Recovery in open field behavior was also observed in harmonin-b1 and dual harmonina1 and b1 injected c.216AA mice. Representative open-field exploration traces are plotted in Figure 17C. c.216GA mice explored the border of the field and displayed minimal full body rotations, whereas c.216AA mice displayed more activity throughout the entire chamber with increased full body rotations quantified as rotations/min (Figure 10D-10E). Surprisingly, while no ABR rescue was observed in mice injected with AAV2/Anc80.CMV.harmonin-a1, open field data demonstrated recovery of vestibular function to the level of the control mice. Behavior of c.216GA mice injected with AAV2/Anc80.CMV.trunc-harmonin did not differ from the control c.216GA mice, again indicating a lack of interference between truncated and wild-type harmonin (Figure 10C-10E).

Example 3—Polynucleotide Therapy of Additional Mutations Involved in Hearing Loss

Example 3A—*In Vivo* Experiments

Behavioral assays demonstrated partial vestibular rescue with harmonin-a1, as circling behavior was abolished but harmonin-a1 injected mice failed the rotarod test. Mice injected with harmonin-b1, on the other hand, had functional recovery in both tests (Figure 10). The absence of transduction and FM1-43 uptake in the striola regions indicates that hair cells of the striola region and perhaps type I cell function depends on proper harmonin expression (Figure 6).

While auditory rescue was prominent at low but not high frequencies (Figure 9), preservation of hair bundle morphology at 6 weeks was observed along the entire organ (Figure 11). The absence of rescue at high frequencies is unlikely due to damage caused by the injection. High frequency hearing loss was not observed in any of the c.216GA injected with AAV vectors (Figure 16C-16D). AAV targeting along the entire length of the cochlea argues against a lack of transduction efficiency at the base as an explanation. One possibility is that other harmonin isoforms, such as the short harmonin-c, may be necessary for rescue of function in the basal high frequency end of the cochlea. Alternatively, since cochlear development begins at the basal end, it is possible that by P0, hair cells from the basal high frequency end have matured beyond the point of repair.

If this is the case embryonic intervention may allow better rescue in the high frequency region.

Anc80 vectors carrying the coding sequence for mouse TMC1 driven by a modified CMV promoter were generated using a helper virus free system and a double transfection method as described previously (Grimm et al., 2003, Mol. Ther., 7:839:50). A triple flag-tag (FLAG) sequence was fused to the C-terminal end of the TMC coding sequence to enable visualization of the expressed protein. Anc80-CMV-Tmc vector was purified using an iodixanol step gradient followed by ion exchange chromatography. Titers ranged from 1×10^{12} to 1×10^{13} gc/ml as measured by quantitative PCR using primer sets specific for the human beta-globin intronic element. Virus aliquots were stored at −80°C and thawed just prior to use.

Mice, age P0-P2, were used for *in vivo* delivery of viral vectors as described below according to protocols approved by the Institutional Animal Care and Use Committee (protocols #2659, #2146) at Boston Children's Hospital. C57BL/6J (Jackson Laboratories) or Swiss Webster mouse lines (Taconic) were used for wild-type control mice, and mice that carried TMC1 mutant alleles (TMC1Δ/Δ or Tmc1 -/-) were on a C57BL/6J background as described previously (Kawashima et al., 2011, J. Clin. Invest., 121:4796-809).

To prepare tissue for evaluation, temporal bones were harvested from mouse pups at P0-P10. Pups were euthanized by rapid decapitation and temporal bones were dissected in MEM (Invitrogen) supplemented with 10 mM HEPES, 0.05 mg/ml ampicillin, and 0.01 mg/ml ciprofloxacin at pH 7.40. The membranous labyrinth was isolated under a dissection scope, Reissner's membrane was peeled back, and the tectorial membrane and stria vascularis were mechanically removed. Organ of Corti cultures were pinned flatly beneath a pair of thin glass fibers adhered at one end with Sylgard to an 18-mm round glass coverslip. The tissue was used acutely for electrophysiological studies. For mice older than P10, temporal bones were harvested after euthanizing the animal with inhaled CO_2, and cochlear whole mounts were generated.

All mean values and error bars presented in the figures represent mean ± SD. Comparisons for statistical significance between injected ears and uninjected ears were performed using a two-tailed paired t test. $P < 0.05$ was considered significant.

Example 3B—*In Vivo* Injection of Viral Vectors

Mouse pups (P0-P2) were injected via the round window membrane (RWM) using beveled glass microinjection pipettes. Pipettes were pulled from capillary glass on a P-2000 pipette puller (Sutter Instruments) and were beveled (~20 μm tip diameter at a 28° angle) using a micropipette beveler (Sutter Instruments). EMLA cream (lidocaine 2.5% and prilocaine 2.5%) was applied externally for analgesia using sterile swabs to cover the surgical site (left mastoid prominence). Body temperature was maintained on a 37°C warming pad for 30-60 minutes prior to surgery.

Pups were anesthetized by rapid induction of hypothermia for 2-3 minutes until loss of consciousness, and this state was maintained on a cooling platform for 10-15 minutes during the surgery. The surgical site was disinfected by scrubbing with Betadine and wiping with 70% Ethanol in repetition three times. A post-auricular incision was made to expose the transparent otic bulla, a micropipette was advanced by micromanipulator (MP-30, Sutter Instrument Company) through the bulla and overlying fascia, and the RWM was penetrated by the tip of the micropipette.

Approximately 1 μl of virus at titers between 10^{12} and 10^{14} gc/mL (10^{9} and 10^{11} total viral particles) was injected unilaterally at 0.1 μl/min into the left ear using a pneumatic microinjector (WPI Nanoliter 2010). The skin incision was closed using a 6-0 monofilament suture (Ethicon). Pups were then returned to the warming pad for recovery.

Example 3C—Immunofluorescence

Immunostaining was performed to determine the distribution of expression of a transgene delivered by a viral vector. To do so, immunostaining was performed on freshly dissected organs of Corti, immersion fixed for 1 h at room temperature with 4% paraformaldehyde diluted in PBS. The tissue was then rinsed in PBS, permeabilized in 0.01-0.1% Triton X-100 for 30 minutes, and counterstained for 1 h with AlexaFluor546-phalloidin (Molecular Probes, 1:200 dilution) to label filamentous actin.

For localization of exogenously expressed TMC::FLAG fusion proteins, the tissue was blocked for 1 hour using 2% BSA and 5% Normal Goat Serum, and was incubated overnight at 4°C with an antibody to the FLAG motif (BD Biosciences, 1:200 dilution). For hair cell counts, tissue was blocked in Normal Goat Serum for 1 hour, stained with a rabbit anti-Myosin VIIa primary antibody (Proteus Biosciences, 1:1000 dilution) at 4°C

overnight, and labeled with goat anti-rabbit antibody conjugated to AlexaFluor488 (Life Technologies, 1:200 dilution) for 1 h. Samples were mounted on glass coverslips with Vectashield mounting medium (Vector Laboratories), and imaged at 10X-63X magnification using a Zeiss LSM700 confocal microscope.

Figure 13 shows immunofluorescence that demonstrates uniform Anc80 delivery of Harmonin to Ush1c mutant mice, and Figure 28 shows immunofluorescence that demonstrates Anc80 delivery of KCNQ4 to cells in KCNQ4 mutant mice.

Example 3D—Hair Cell Electrophysiology

Organotypic cochlear cultures were bathed in standard artificial perilymph containing 137 mM NaCl, 0.7 mM NaH_2PO_4, 5.8 mM KCl, 1.3 mM $CaCl_2$, 0.9mM $MgCl_2$, 10 mM Hepes, and 5.6 mM D-glucose. Vitamins (1:50) and amino acids (1:100) were added to the solution from concentrates (Invitrogen), and NaOH was used to adjust the final pH to 7.40 (310 mosmol/kg). Recording pipettes (3 to 5 megohms) were pulled from R6 capillary glass (King Precision Glass) and filled with intracellular solution containing 135 mM CsCl, 5 mM Hepes, 5 mM EGTA, 2.5 mM $MgCl_2$, 2.5 mM Na_2–adenosine triphosphate, and 0.1 mM $CaCl_2$, where CsOH was used to adjust the final pH to 7.40 (285 mosmol/kg). Whole-cell, tight-seal voltage-clamp recordings were done at −84 mV at room temperature (22° to 24°C) using an Axopatch 200B amplifier (Molecular Devices). Sensory transduction currents were filtered at 10 kHz with a low-pass Bessel filter and digitized at ≥20 kHz with a 16-bit acquisition board (Digidata 1440A) and pCLAMP 10 software (Molecular Devices). Data were stored for offline analysis using OriginPro 8 (OriginLab).

Figure 23 shows recovery of potassium currents to near wild type levels (Figure 23A) in KCNQ4 -/- cells transfected with Anc80-KCNQ4 relative to the mutant mice (Figure 23B).

Example 3E—Auditory Brainstem Responses (ABR)

ABR recordings were conducted as described previously (Maison et al., 2010, J. Neurosci., 30:6751-62). Briefly, P25-P30 mice were anesthetized via IP injection (0.1 ml/10 g–body weight) with 50 mg of ketamine and 5 mg of xylazine diluted into 5 ml of 0.9% saline. ABR experiments were performed at 32°C in a sound-proof chamber. To test hearing function, mice were presented pure tone stimuli of 5.6 kHz, 8 kHz, 11.3k Hz,

16 kHz, 22.6 kHz, or 32 kHz at sound pressure levels between 10 and 115 dB in 5 dB steps until a threshold intensity that evoked a reproducible ABR waveform (peaks I-IV) was detected. Using an alternating polarity stimulus, 512 to 1024 responses were collected and averaged for each sound pressure level. Waveforms with amplitude larger than 15 μV (peak-to-trough) were discarded by an "artifact reject" function. Prior to the onset of ABR testing, the flap of skin and cartilage that typically obscures the entrance of the external auditory meatus was trimmed away with dissecting scissors, and sound pressure at the entrance of the ear canal was calibrated for each individual test subject at all stimulus frequencies. Acoustic stimuli were delivered directly to the studied ear through a custom probe tube speaker/microphone assembly (EPL PXI Systems) consisting of two electrostatic earphones (CUI Miniature Dynamics) to generate primary tones and a Knowles miniature microphone (Electret Condenser) to record ear-canal sound pressure. Sound stimuli consisted of 5-ms tone bursts (0.5 ms rise–fall with a $\cos^2$ onset, delivered at 40/s). ABR signals were collected using subcutaneous needle electrodes inserted at the pinna (active electrode), vertex (reference electrode), and rump (ground electrode). ABR potentials were amplified (10,000x), pass-filtered (0.3-10 kHz), and digitized using custom data acquisition software (LabVIEW). Sound stimuli and electrode voltage were sampled at 40-μs intervals using a digital I-O board (National Instruments) and stored for offline analysis. Threshold was defined visually as the lowest decibel level at which any wave (I-IV) could be detected and reproduced with increasing sound intensities. ABR thresholds were averaged within each experimental group and used for statistical analysis.

Figure 20 graphically demonstrates that delivery of an Anc80 viral vector encoding and expressing Harmonin can provide nearly complete recovery of auditory function, particularly at lower frequencies (e.g., about 5 to about 22 kHz).

Example 3F—Quantitative RT-PCR Analysis

Experiments were performed to evaluate the amount of virus present in the cochlea following *in vivo* administration. Two TMC1 -/- mice were injected in the left ear at P1. Cochlea were excised from left and right ears and maintained in culture for 3 days, the equivalent of P10. RNA was extracted and quality was confirmed using an Agilent Bioanalyzer (Agilent Technologies), and reverse transcribed into cDNA for quantitative RT-PCR analysis with efficient primer sets specific to TMC1 with SYBR

GreenER qPCR reagent (Invitrogen) as previously described (Kawashima et al., 2011, J. Clin. Invest., 121:4796-809).

To amplify a fragment of TMC1, the following primers were used: 5'-CAT CTG CAG CCA ACT TTG GTG TGT-3' (SEQ ID NO:9) and 5'-AGA GGT AGC CGG AAA TTC AGC CAT-3' (SEQ ID NO:10). Expression levels were normalized to those of Actb (encoding β-actin) amplified with 5'-TGA GCG CAA GTA CTC TGT GTG GAT-3' (SEQ ID NO:11) and 5'-ACT CAT CGT ACT CCT GCT TGC TGA-3' (SEQ ID NO:12). All primers were designed to span introns, and validated using melt curve analysis and negative controls. Data were analyzed using the ΔΔCT method, relative to Actb and the difference between injected and uninjected ears. In injected ears, TMC1 mRNA expression was 12-fold higher than in uninjected ears.

Example 3G—FM1-43 Labeling

FM1-43 dye loading experiments were performed as described previously (Gale et al., 2001, J. Neurosci., 21:7013-25; Meyers et al., 2003, J. Neurosci., 23:4054-65; and Géléoc & Holt, 2003, Nat. Neurosci., 10:1019-20). Coverslips with adherent cochlear cultures were placed under an upright microscope (Zeiss Axioscope FS Plus) on a glass-bottomed chamber. Five-μM FM1-43FX (Invitrogen) diluted in artificial perilymph was applied for 10 sec and the tissue was washed three times in artificial perilymph to remove dye from the outer leaflet of the cell membrane. After 5 minutes, intracellular FM1-43 was imaged using an FM1-43 filter set and an epifluorescence light source with a 63X water immersion objective. The tissue was fixed and processed for immunofluorescence as described above.

Figure 23 are the immunostaining images showing uptake of FM1-43 dye by cells exposed to an Anc80 viral vector as described herein, and Figure 28 graphically demonstrates that TMC1 delivered by an Anc80 viral vector as described herein restores sensory transduction in Tmc1-deficient hair cells *in vivo*.

Example 3H—Distortion Product Otoacoustic Emissions (DPOAE)

DPOAE data were collected under the same conditions, and during the same recording sessions as ABR data. Primary tones were produced at a frequency ratio of 1.2 (f2/f1) for the generation of DPOAEs at 2f1-f2, where the f2 level was 10 dB sound pressure level below f1 level for each f2/f1 pair. The f2 levels were swept in 5-dB steps

from 20 to 80 dB. Waveform and spectral averaging were used at each level to increase the signal-to-noise ratio of the recorded ear-canal sound pressure. The amplitude of the DPOAE at 2f1-f2 was extracted from the averaged spectra, along with the noise floor at nearby points in the spectrum. Iso-response curves were interpolated from plots of DPOAE amplitude versus sound level. Threshold was defined as the f2 level required to produce DPOAEs at 0 dB.

Figure 25 graphically demonstrates that TMC1 delivered using an Anc80 viral vector as described herein rescues outer hair cell function in TMC1 -/- mice, particularly at lower frequencies (e.g., about 5 to about 16 kHz).

Figure 26 demonstrates the effect of promoters Pcdh15, Myo6 and KCNQ4 on GFP expression in hair cells.

OTHER EMBODIMENTS

It is to be understood that, while the methods and compositions of matter have been described herein in conjunction with a number of different aspects, the foregoing description of the various aspects is intended to illustrate and not limit the scope of the methods and compositions of matter. Other aspects, advantages, and modifications are within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

Disclosed are methods and compositions that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that combinations, subsets, interactions, groups, etc. of these methods and compositions are disclosed. That is, while specific reference to each various individual and collective combinations and permutations of these compositions and methods may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular composition of matter or a particular method is disclosed and discussed and a

number of compositions or methods are discussed, each and every combination and permutation of the compositions and the methods are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed.

In this specification, where reference has been made to external sources of information, including patent specifications and other documents, this is generally for the purpose of providing a context for discussing the aspects of the present disclosure. Unless stated otherwise, reference to such sources of information is not to be construed, in any jurisdiction, as an admission that such sources of information are prior art or form part of the common general knowledge in the art.

The description herein may contain subject matter that falls outside of the scope of the claimed invention. This subject matter is included to aid understanding of the invention.

Sequence Listing

Anc80 capsid protein (SEQ ID NO:1)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGLDKGEPV
NAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQAKKRVLEPLG
LVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKKGQQPAX$_1$KRLNFGQTGDSESVPDPQPLGEPPAAP
SGVGSNTMX$_2$AGGGAPMADNNEGADGVGNASGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQI
SSQSGX$_3$STNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKX$_4$LNFKLFNIQVKEV
TTNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVG
RSSFYCLEYFPSQMLRTGNNFX$_5$FSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQTTSG
TAGNRX$_6$LQFSQAGPSSMANQAKNWLPGPCYRQQRVSKTX$_7$NQNNNSNFAWTGATKYHLNGRDSLV
NPGPAMATHKDDEDKFFPMSGVLIFGKQGAGNSNVDLDNVMITX$_8$EEEIKTTNPVATEX$_9$YGTVAT
NLQSX$_{10}$NTAPATGTVNSQGALPGMVWQX$_{11}$RDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPP
QILIKNTPVPANPPTTFSPAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYNKSTN
VDFAVDTNGVYSEPRPIGTRYLTRNL

where X$_1$ = K/R; X$_2$ = A/S; X$_3$ = A/G; X$_4$ = R/K; X$_5$ = E/Q; X$_6$ = T/E; X$_7$ = A/T; X$_8$ = S/N; X$_9$ = Q/E; X$_{10}$ = S/A; X$_{11}$ = N/D

Anc80-L0065 capsid protein (SEQ ID NO:2)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGLDKGEPV
NAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQAKKRVLEPLG
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NTPVPANPPTTFSPAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYNKSTNVDFAV
DTNGVYSEPRPIGTRYLTRNL

pAAV-TMC2 (SEQ ID NO:3)

Left-inverted terminal repeat (L-ITR): 1-130 nt
Cytomegalovirus (CMV) promoter: 206-799 nt
Simian virus 40 (SV40) misc intron: 831-963 nt
Transmembrane channel-like 1 (TMC1ex1): 982-3,267 nt
Post-transcriptional regulatory element from Woodchuck hepatitis virus (WPRE): 3,268-3,821 nt
Bovine growth hormone (bGH) polyA signal: 3,822-4,086 nt
Right-inverted terminal repeat (R-ITR): 4,124-4,253 nt

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pAAV-TMC1ex2 (SEQ ID NO:4)
L-ITR: 1-130
CMV promoter: 206-799
SV40 misc intron: 831-963
TMC1ex2: 982-3,255
WPRE: 3,256-3,809
bGH polyA signal: 3,810-4,074
R-ITR: 4,112-4,241

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CAACTTCTACATGGGCCTGCTGCTGTTGGTGCTCTTCCTCAGCCTCCTGCCTGTGGCCTACACTG
TCATGTCTCTCCCACCCTCGTTTGACTGTGGCCCCTTCAGTGGGAAAAACAGAATGTACGATGTC
CTCCATGAGACCATCGAGAACGATTTCCCTAAGTTCCTGGGCAAGATCTTTGCGTTCCTTGCCAA
CCCAGGCCTGATCATTCCAGCCATCCTGCTAATGTTTCTGGCCATTTACTACCTGAACTCAGTTT
CAAAAAGTCTTTCCAGAGCTAATGCCCAGCTGCGAAAGAAGATCCAAGCGCTCCGTGAAGTTGAG
AAGAACCATAAATCCATCAAGGGAAAAGCCATAGTCACATATTCAGAGGACACAATCAAGAACAG
CTCCAAAAATGCCACCCAGATACATCTTACTAAAGAAGAGCCCACATCTCACTCTTCCAGCCAAA

TCCAGACCCTGGACAAGAAAGCGCAGGGCCCCCACACCTCCAGTACTGAGGGTGGGGCCTCGCCG
TCTACCTCCTGGCACCATGTTGGGTCTCAACCACCGAGAGGCAGACGAGATTCTGGCCAACCCCA
GTCTCAGACTTATACAGGCAGGTCACCTTCTGGAAAGAGAACCCAGAGGCCTCACAACTGAGCGG
CCGCTCGAGCCTAAGCTTCTAGAagatctacgggtggcatccctgtgacccctccccagtgcctc
tcctggccctggaagttgccactccagtgcccaccagccttgtcctaataaaattaagttgcatc
attttgtctgactaggtgtccttctataatattatggggtggaggggggtggtatggagcaaggg
gcaagttgggaagacaacctgtagggcctgcggggtctattgggaaccaagctggagtgcagtgg
cacaatcttggctcactgcaatctccgcctcctgggttcaagcgattctcctgcctcagcctccc
gagttgttgggattccaggcatgcatgaccaggctcagctaatttttgtttttttggtagagacg
gggtttcaccatattggccaggctggtctccaactcctaatctcaggtgatctacccaccttggc
ctcccaaattgctgggattacaggcgtgaaccactgctcccttccctgtccttctgattttgtag
gtaaccacgtgcggaccgagcggccgcaggaacccctagtgatggagttggccactccctctctg
cgcgctcgctcgctcactgaggccgggcgaccaaaggtcgcccgacgcccgggctttgcccgggc
ggcctcagtgagcgagcgagcgcgcagctgcctgcaggggcgcctgatgcggtattttctcctta
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cccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctct
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atttgggtgatggttcacgtagtgggccatcgccctgatagacggtttttcgccctttgacgttg
gagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaaccctatctcggg
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ctgacgggcttgtctgctcccggcatccgcttacagacaagctgtgaccgtctccgggagctgca
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tttttataggttaatgtcatgataataatggtttcttagacgtcaggtggcacttttcggggaaa
tgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagac
aataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgt
gtcgcccttattcccttttttgcggcattttgccttcctgtttttgctcacccagaaacgctggt
gaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaaca
gcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagtt
ctgctatgtggcgcggtattatcccgtattgacgccgggcaagagcaactcggtcgccgcataca
ctattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatggcatga
cagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctg
acaacgatcggaggaccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcg
ccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcgtgacaccacgatgc
ctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccgg
caacaattaatagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttcc
ggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcag
cactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaact
atggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtc
agaccaagtttactcatatatactttagattgatttaaaacttcatttttaatttaaaaggatct
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gcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttttctgcgcgtaatctg
ctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaa
ctcttttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtag
ccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcct
gttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagt
taccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcga
acgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagg
gagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttc
cagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcga
tttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttttacg
gttcctggccttttgctggccttttgctcacatgt

WHAT IS CLAIMED IS:

1. A synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, wherein the vector:

encodes a capsid having at least about 85% sequence identity to Anc80 of SEQ ID NO:1,

comprises a promoter selected from the group consisting of an Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter, and

comprises a polynucleotide,

wherein the promoter directs expression of the polynucleotide, and

wherein the polynucleotide encodes transmembrane channel-like 1 (TMC1).

2. The synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector of claim 1, wherein the vector transduces inner and outer hair cells with at least about 70% or greater efficiency.

3. A cell comprising the synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector of claim 1 or claim 2.

4. The cell of claim 3, wherein the cell is an outer or inner hair cell.

5. Use of the synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector of claim 1 for preparing a medicament for treating hearing loss and/or vestibular dysfunction in a subject, wherein the hearing loss and/or vestibular dysfunction is associated with a defect in a TMC1 gene.

6. Use of the synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector of claim 1 for preparing a medicament for providing a wild-type form of a defective gene to a subject in need thereof, wherein the wild-type gene is a TMC1 gene.

7. A method of treating hearing loss and/or vestibular dysfunction in a subject, the method comprising contacting a cell of the subject with a synthetic inner ear hair cell targeting adeno-associated virus (AAV) vector, wherein the vector:

encodes a capsid having at least about 85% sequence identity to Anc80 of SEQ ID NO:1,

comprises a promoter selected from the group consisting of an Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter, and

comprises a polynucleotide,

wherein the promoter directs expression of the polynucleotide,

wherein the polynucleotide encodes human TMC1, and

wherein the hearing loss and/or vestibular dysfunction is associated with a defect in a TMC1 gene.

8. The method of claim 7, wherein the vector is administered by injection.

9. The method of claim 7 or claim 8, wherein the contacting reverses the hearing loss.

10. The method of claim 9, wherein the hearing loss is partial hearing loss or complete deafness.

11. The method of claim 9, wherein the method provides recovery of auditory function which is associated with preservation of hair bundle morphology and/or restoration of mechanotransduction.

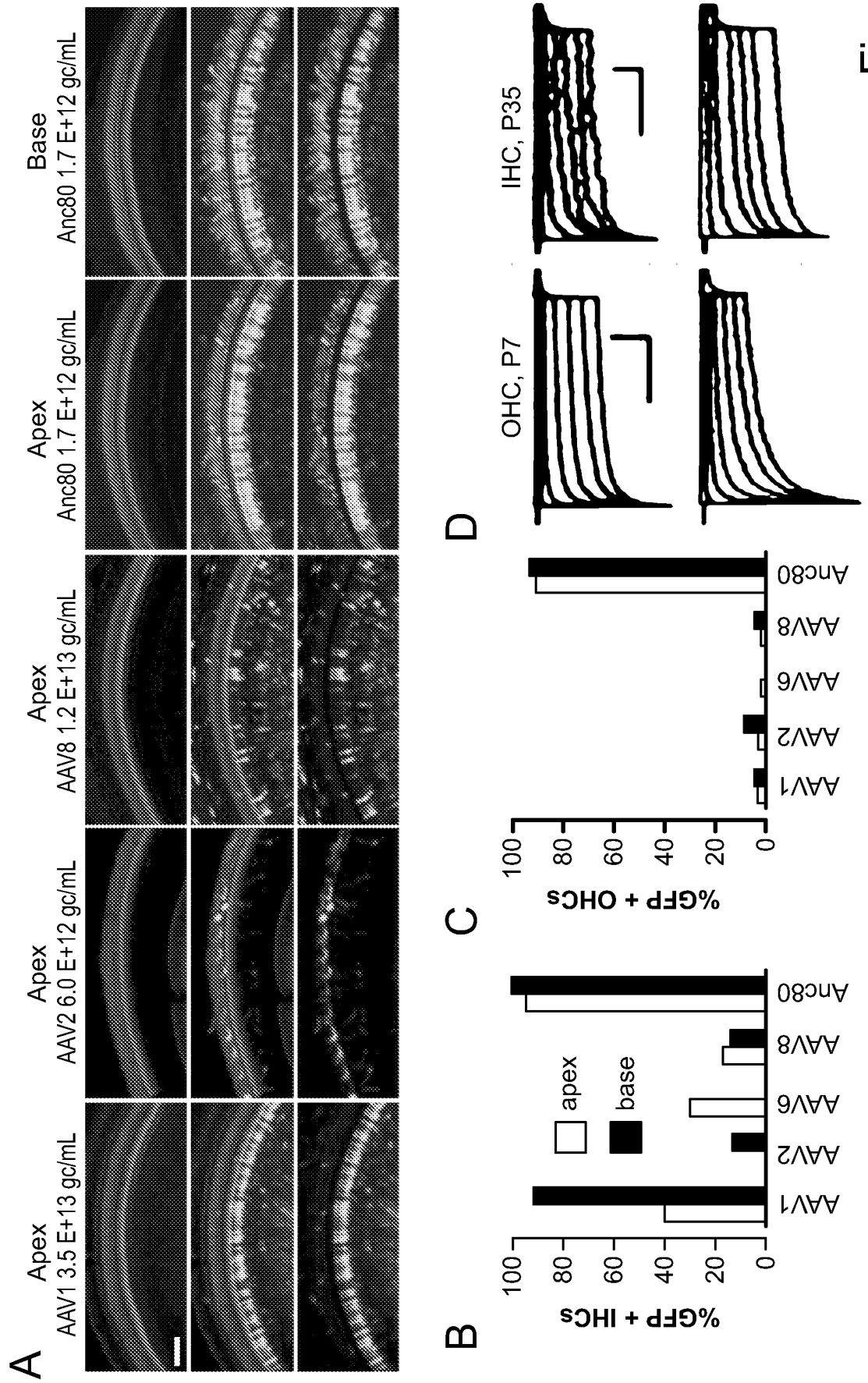

A
Apex
AAV1 3.5 E+13 gc/mL
Apex
AAV2 6.0 E+12 gc/mL
Apex
AAV8 1.2 E+13 gc/mL
Apex
Anc80 1.7 E+12 gc/mL
Base
Anc80 1.7 E+12 gc/mL
B
%GFP + IHCs
100
80
60
40
20
0
apex
base
AAV1
AAV2
AAV6
AAV8
Anc80
C
%GFP + OHCs
100
80
60
40
20
0
AAV1
AAV2
AAV6
AAV8
Anc80
D
OHC, P7
IHC, P35

Fig. 1

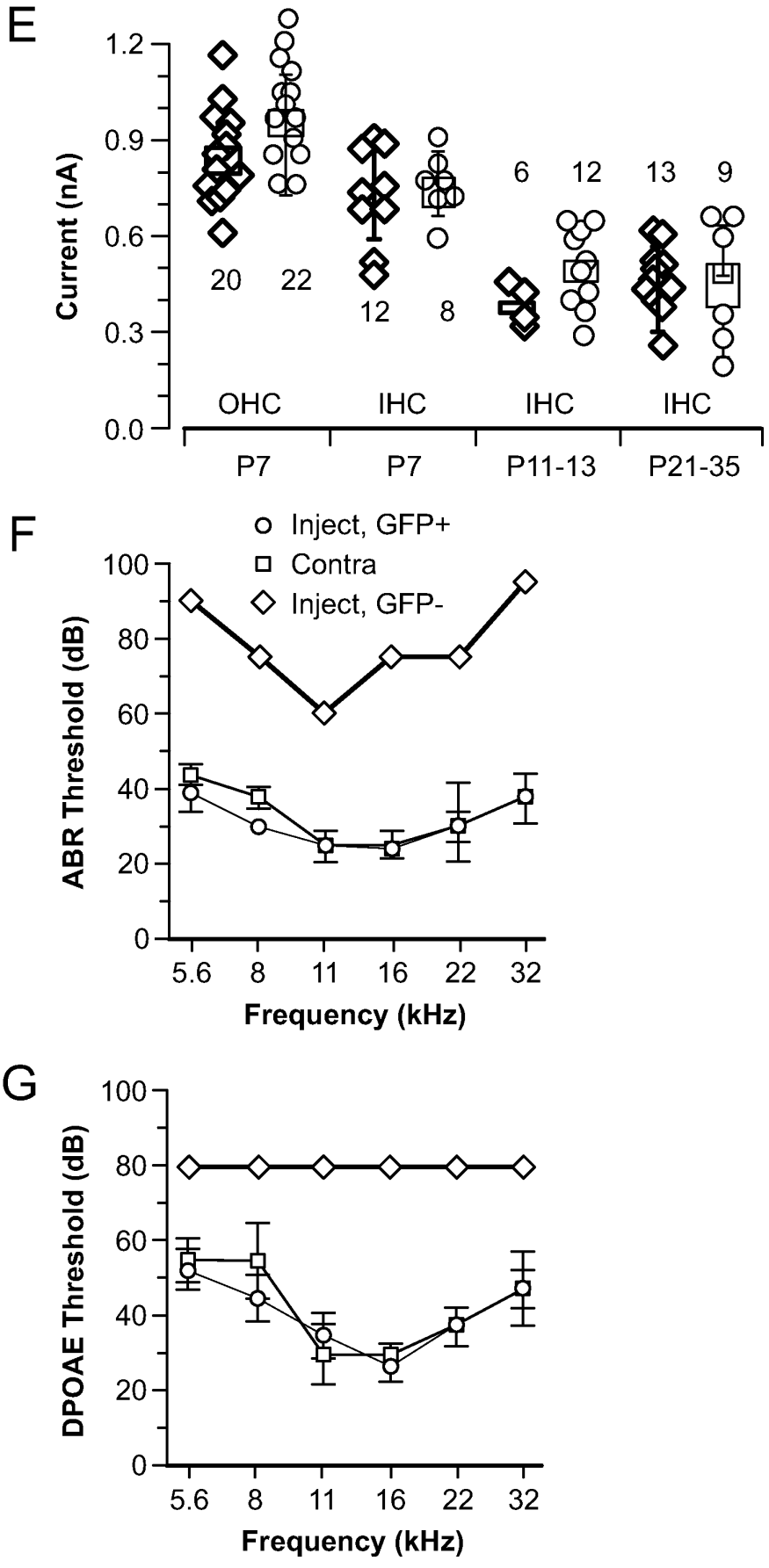

E
Current (nA)
1.2
0.9
0.6
0.3
0.0
20
22
12
8
6
12
13
9
OHC
IHC
IHC
IHC
P7
P7
P11-13
P21-35
F
Inject, GFP+
Contra
Inject, GFP-
ABR Threshold (dB)
100
80
60
40
20
0
5.6
8
11
16
22
32
Frequency (kHz)
G
DPOAE Threshold (dB)
100
80
60
40
20
0
5.6
8
11
16
22
32
Frequency (kHz)

Fig. 1

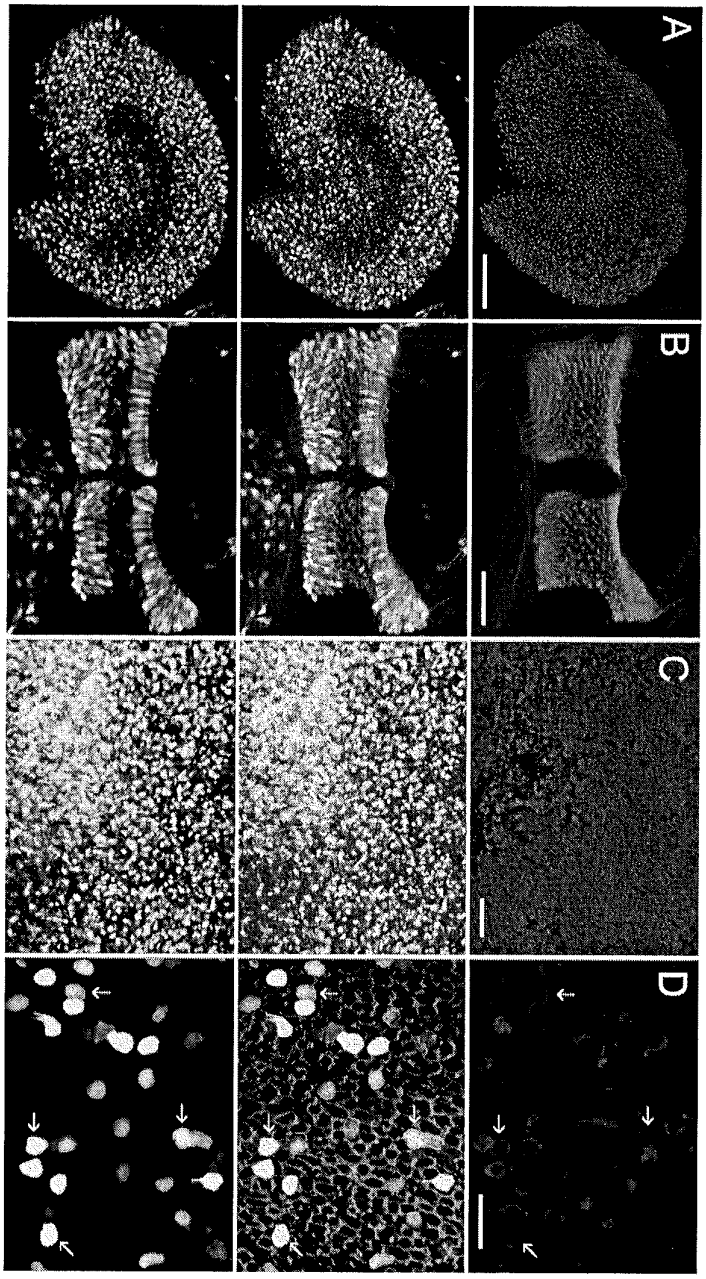


Fig. 2

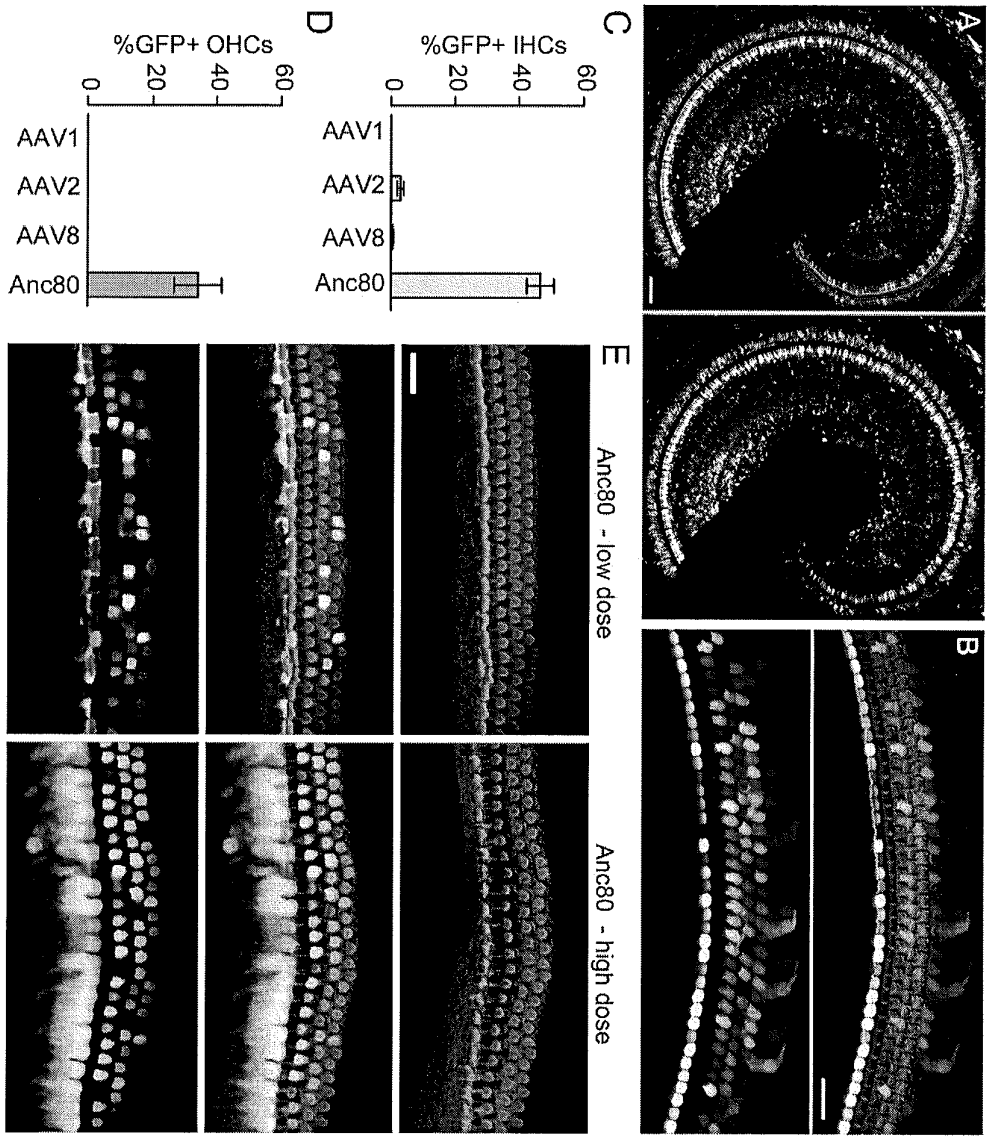

A
B
C
%GFP+ IHCs
0
20
40
60
AAV1
AAV2
AAV8
Anc80
D
%GFP+ OHCs
0
20
40
60
AAV1
AAV2
AAV8
Anc80
E
Anc80 - low dose
Anc80 - high dose

Fig. 3

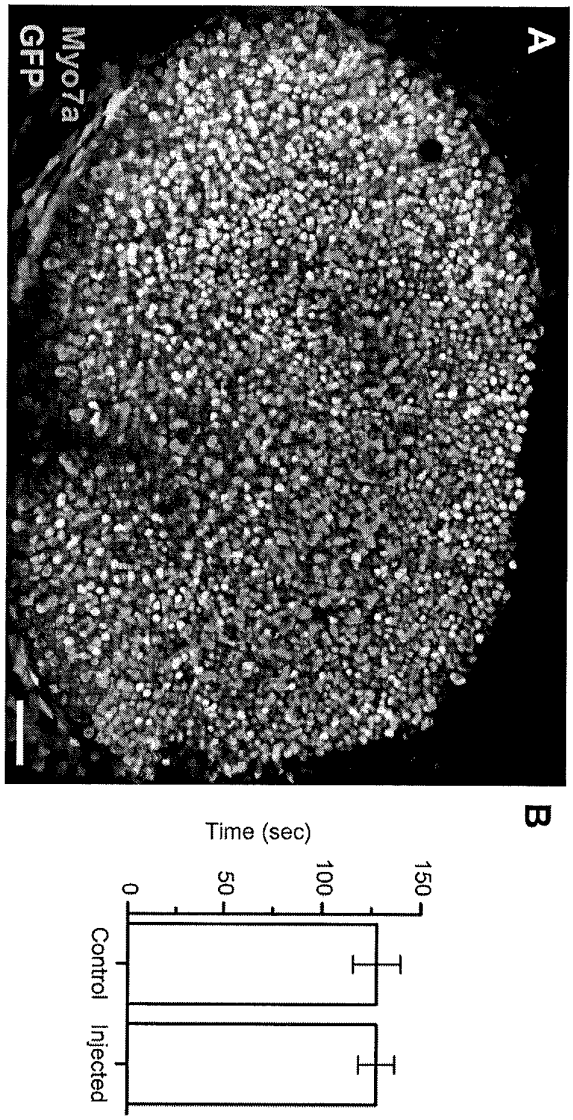


Fig. 4

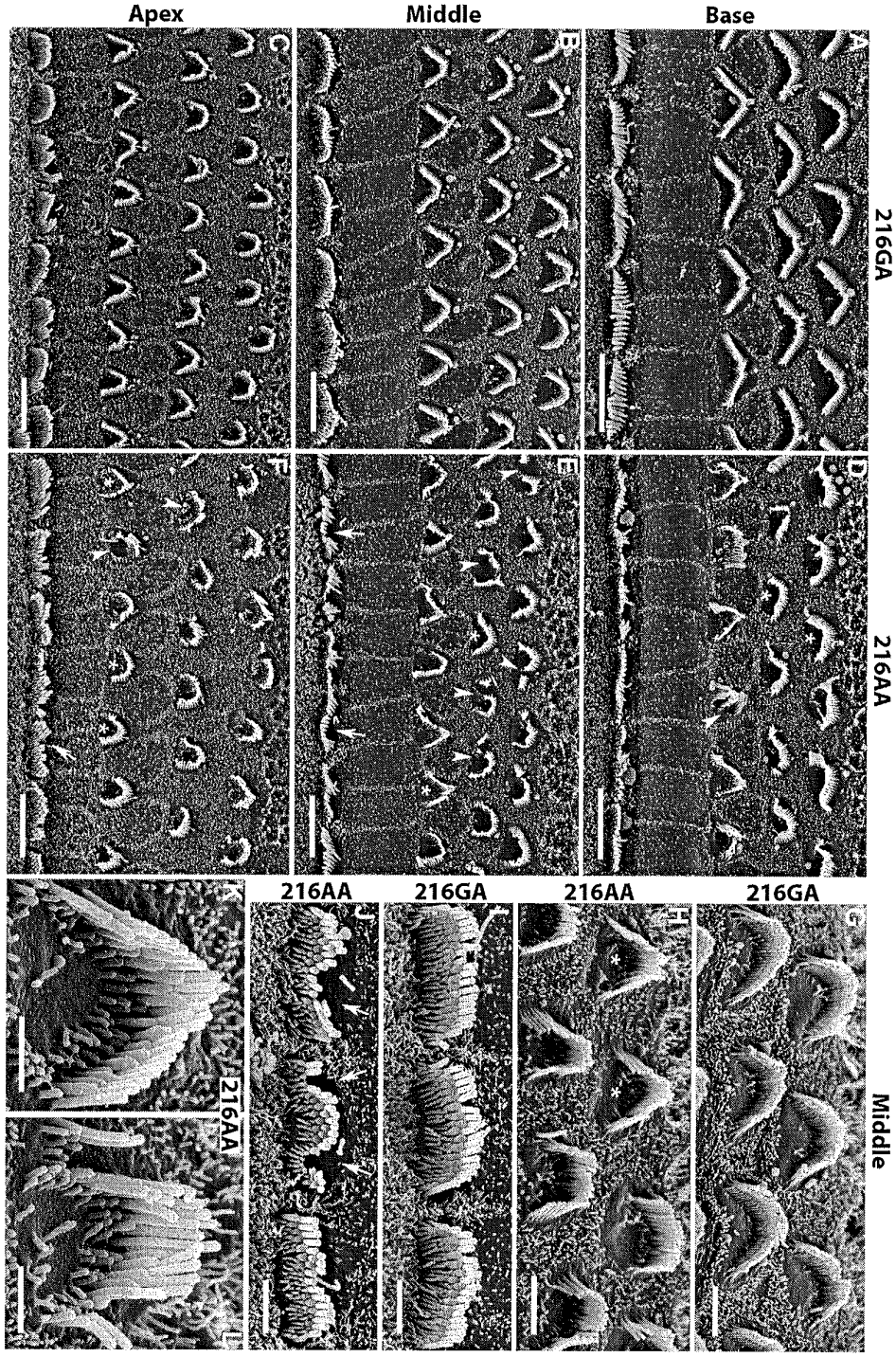

Apex
Middle
Base
216GA
216AA
216AA
216GA
216AA
216GA
216AA
Middle

Fig. 5

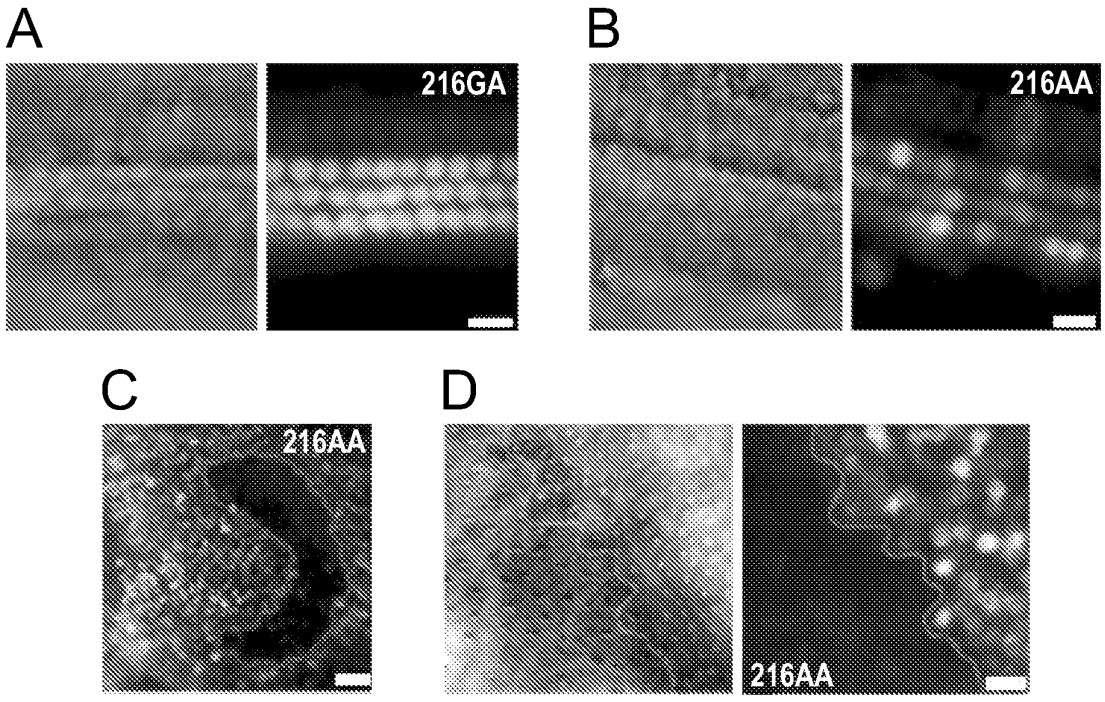


Fig. 6

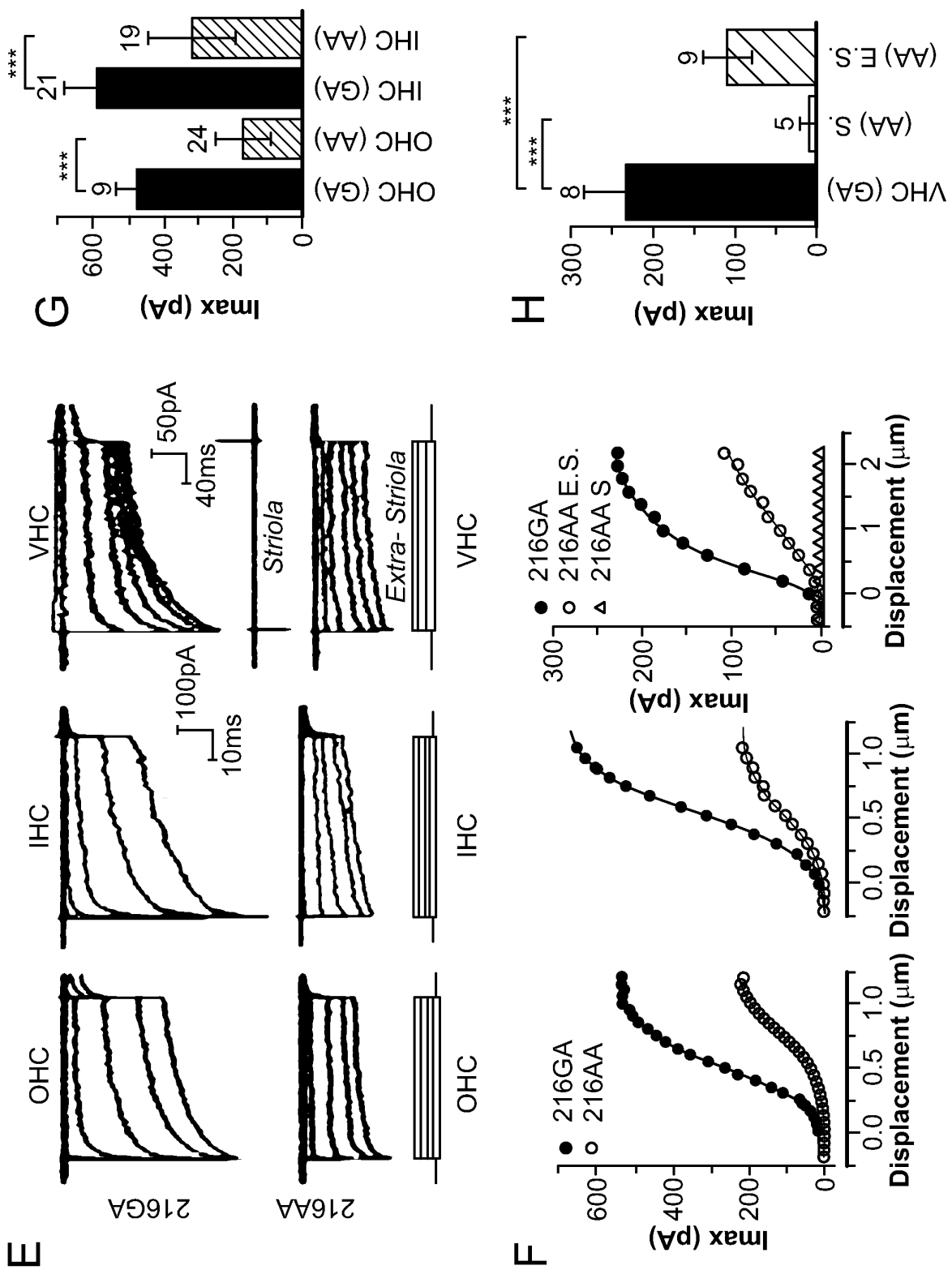

E
216GA
216AA
OHC
IHC
VHC
100pA
10ms
50pA
40ms
Striola
Extra- Striola
F
216GA
216AA
216AA E.S.
216AA S
Imax (pA)
Displacement (μm)
G
Imax (pA)
OHC (GA)
OHC (AA)
IHC (GA)
IHC (AA)
***
H
Imax (pA)
VHC (GA)
(AA) S.
(AA) E.S.
***

Fig. 6

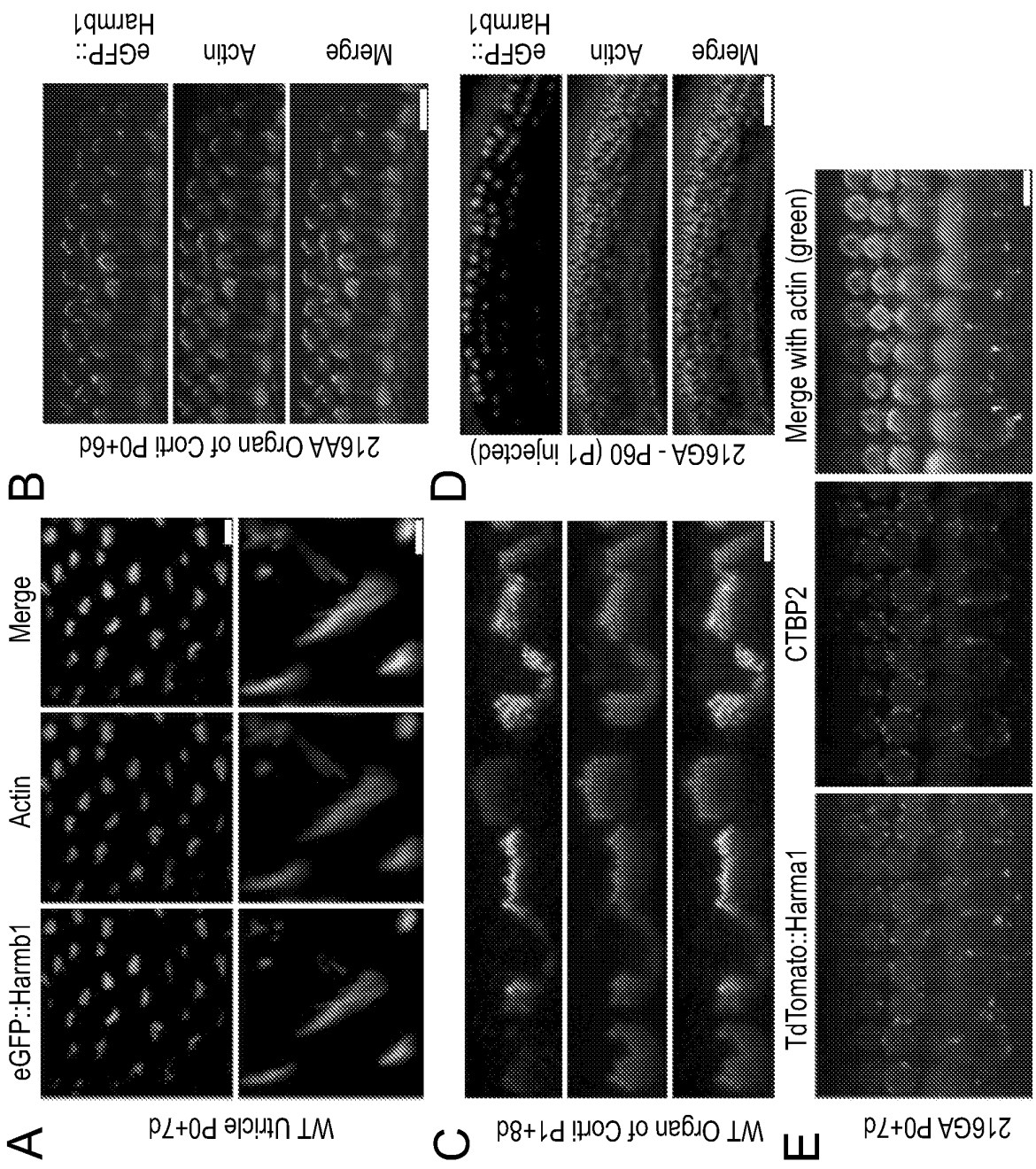

A
eGFP::Harmb1
Actin
Merge
WT Utricle P0+7d
B
Merge
Actin
eGFP::
Harmb1
216AA Organ of Corti P0+6d
C
WT Organ of Corti P1+8d
D
Merge
Actin
eGFP::
Harmb1
216GA - P60 (P1 injected)
E
TdTomato::Harma1
CTBP2
Merge with actin (green)
216GA P0+7d

Fig. 7

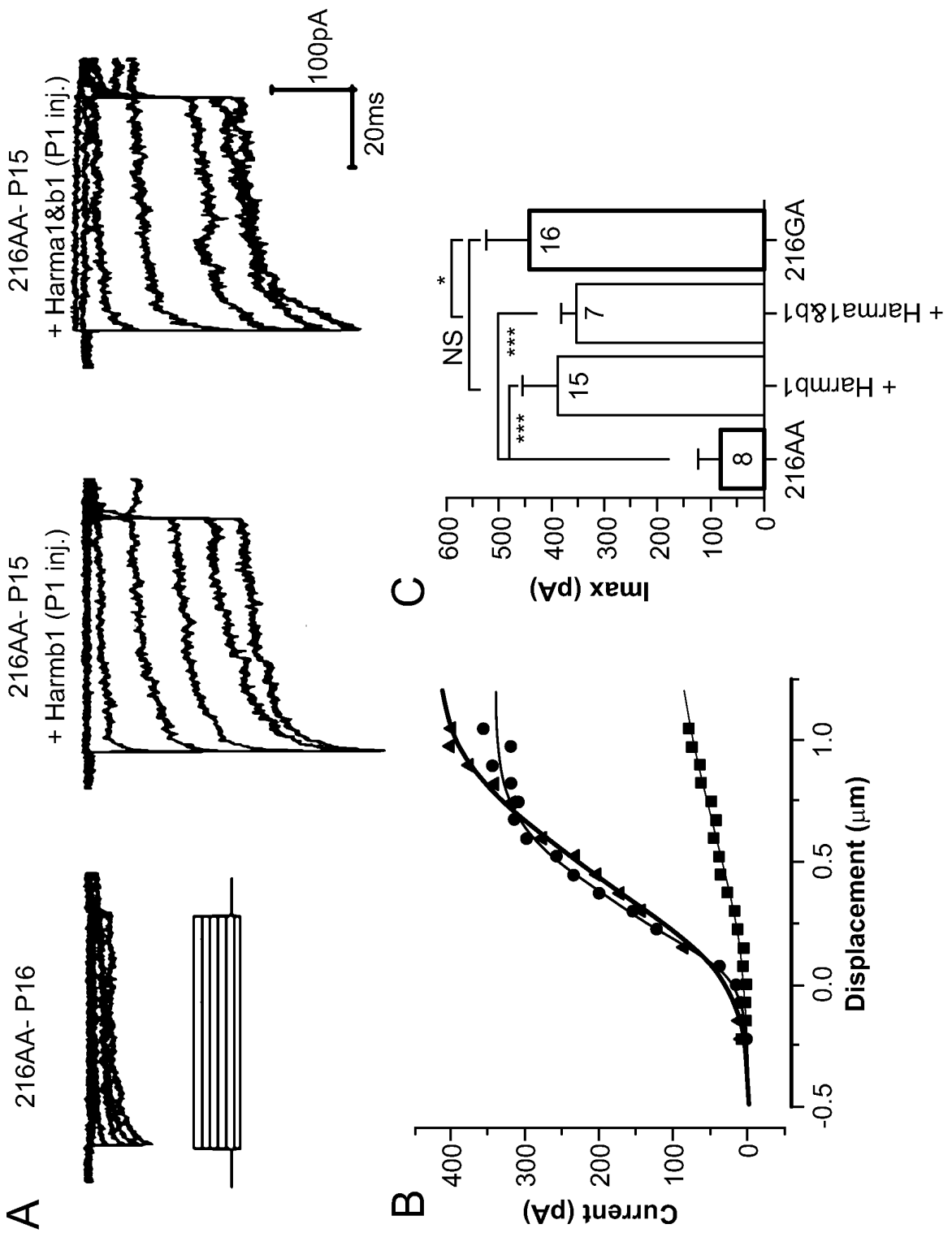
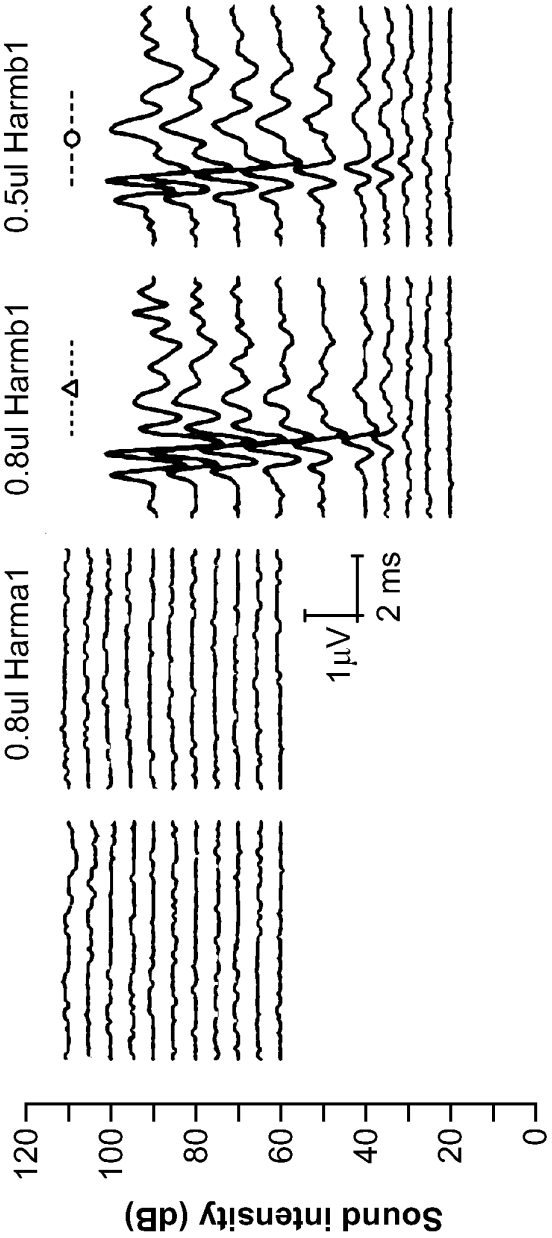

A
216AA- P16
216AA- P15
+ Harmb1 (P1 inj.)
216AA- P15
+ Harma1&b1 (P1 inj.)
100pA
20ms
B
Current (pA)
Displacement (μm)
C
Imax (pA)
NS
*
***
***
8
15
7
16
216AA
+ Harmb1
+ Harma1&b1
216GA

Fig. 8

Fig. 9
A


216AA control
216AA
0.8ul Harma1
216AA
0.8ul Harmb1
216AA
0.5ul Harma1
0.5ul Harmb1
1μV
2 ms
Sound intensity (dB)
120
100
80
60
40
20
0

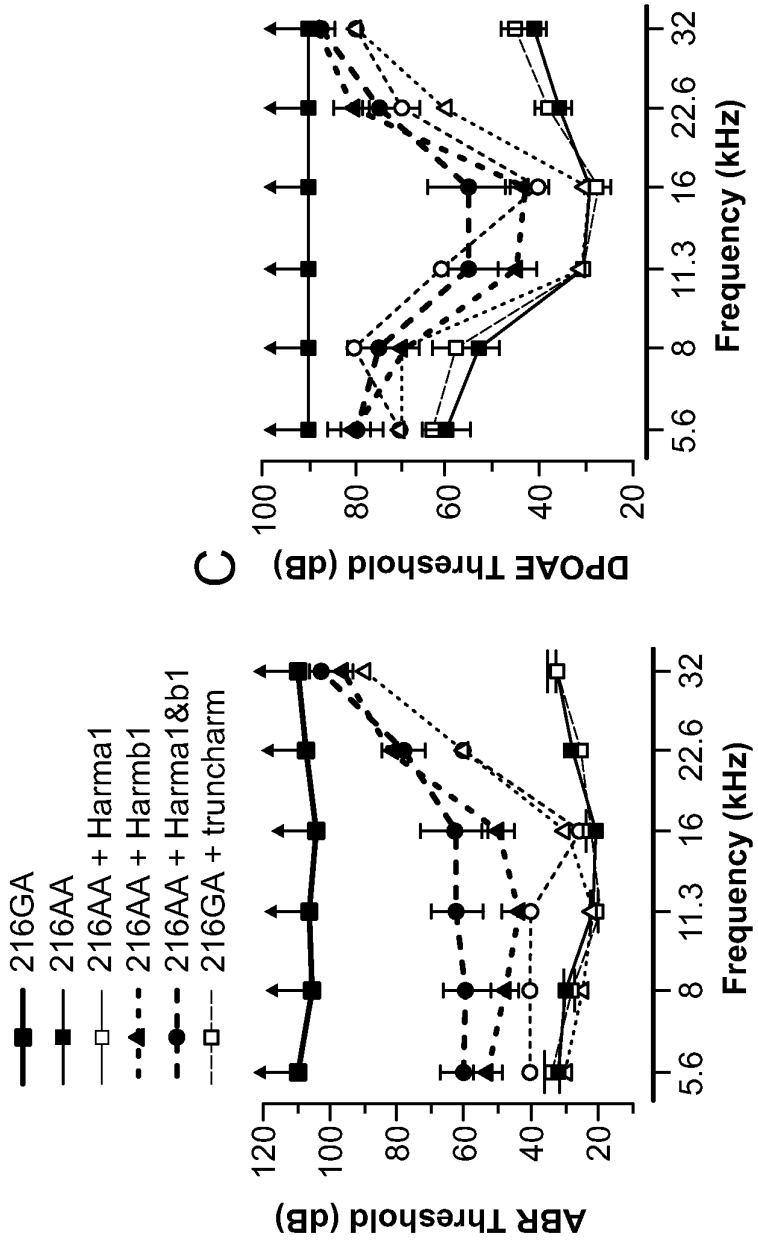

B
ABR Threshold (dB)
120
100
80
60
40
20
5.6
8
11.3
16
22.6
32
Frequency (kHz)
216GA
216AA
216AA + Harma1
216AA + Harmb1
216AA + Harma1&b1
216GA + truncharm
C
DPOAE Threshold (dB)
100
80
60
40
20
5.6
8
11.3
16
22.6
32
Frequency (kHz)

Fig. 9

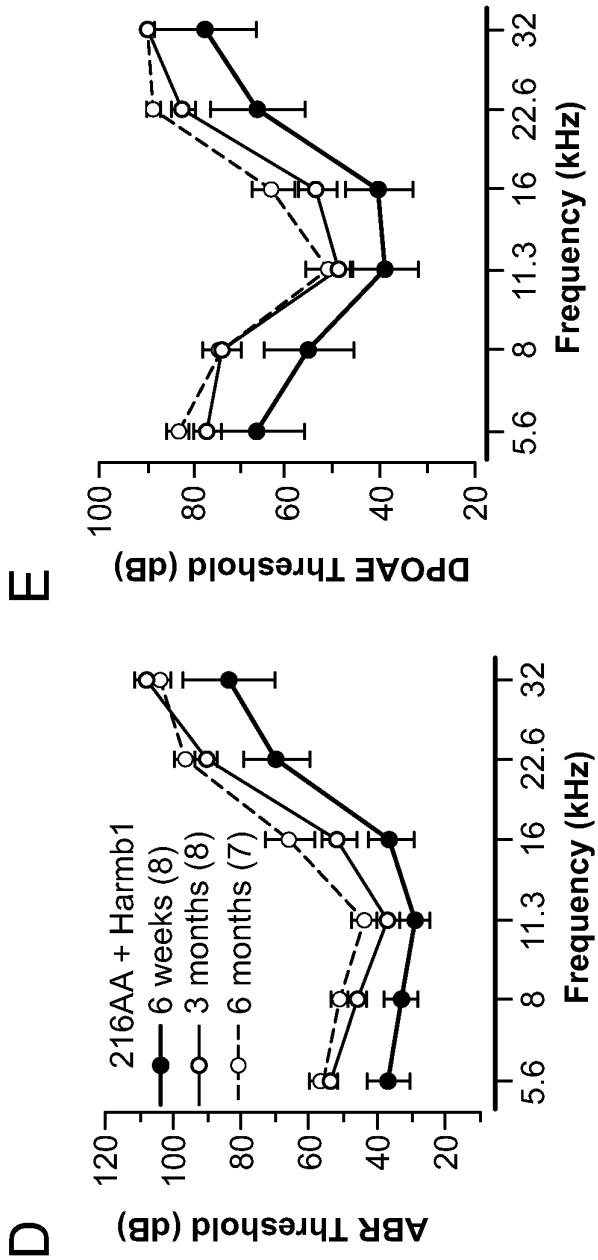

D
ABR Threshold (dB)
120
100
80
60
40
20
5.6
8
11.3
16
22.6
32
Frequency (kHz)
216AA + Harmb1
6 weeks (8)
3 months (8)
6 months (7)
E
DPOAE Threshold (dB)
100
80
60
40
20
5.6
8
11.3
16
22.6
32
Frequency (kHz)

Fig. 9

Fig. 10

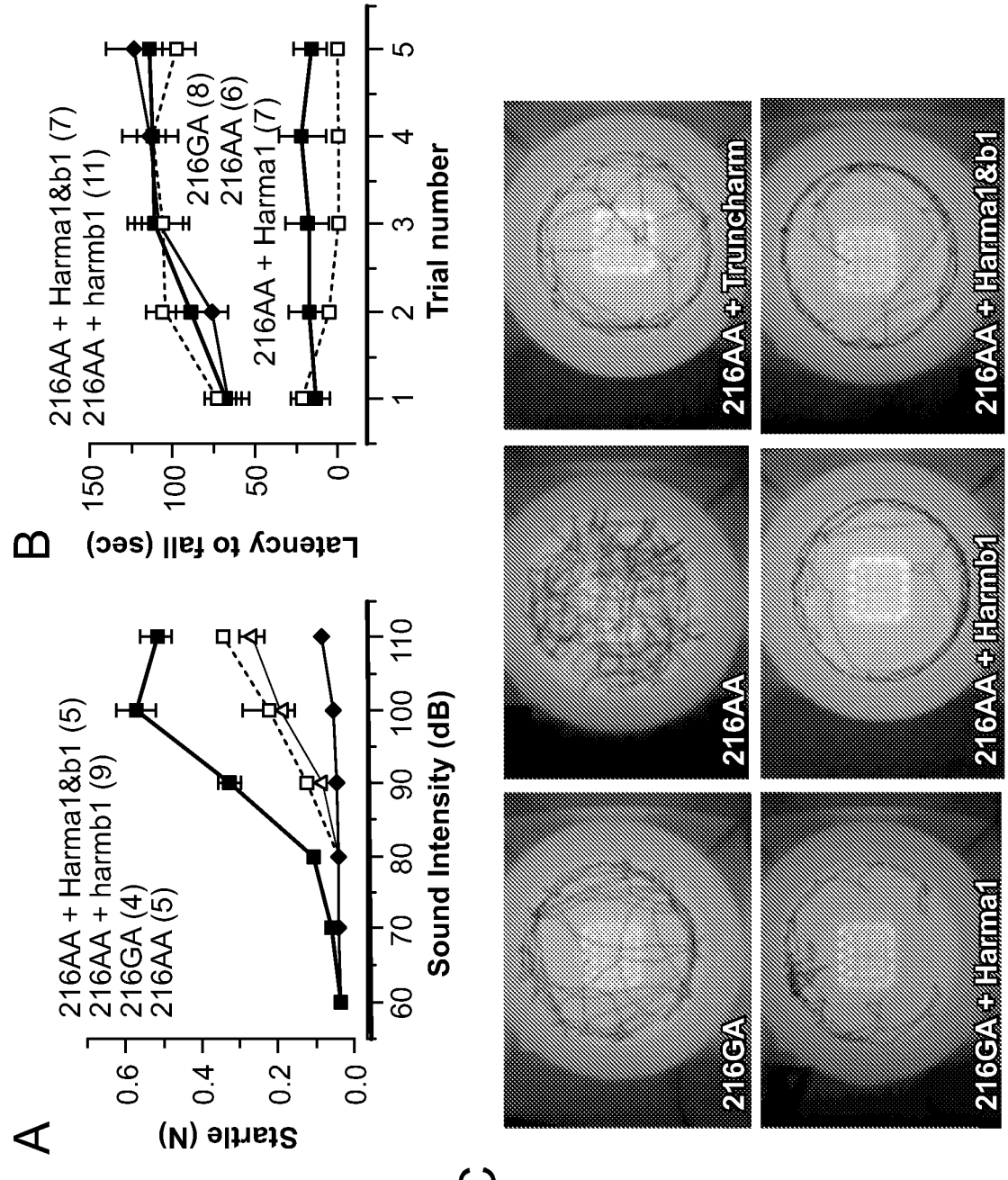
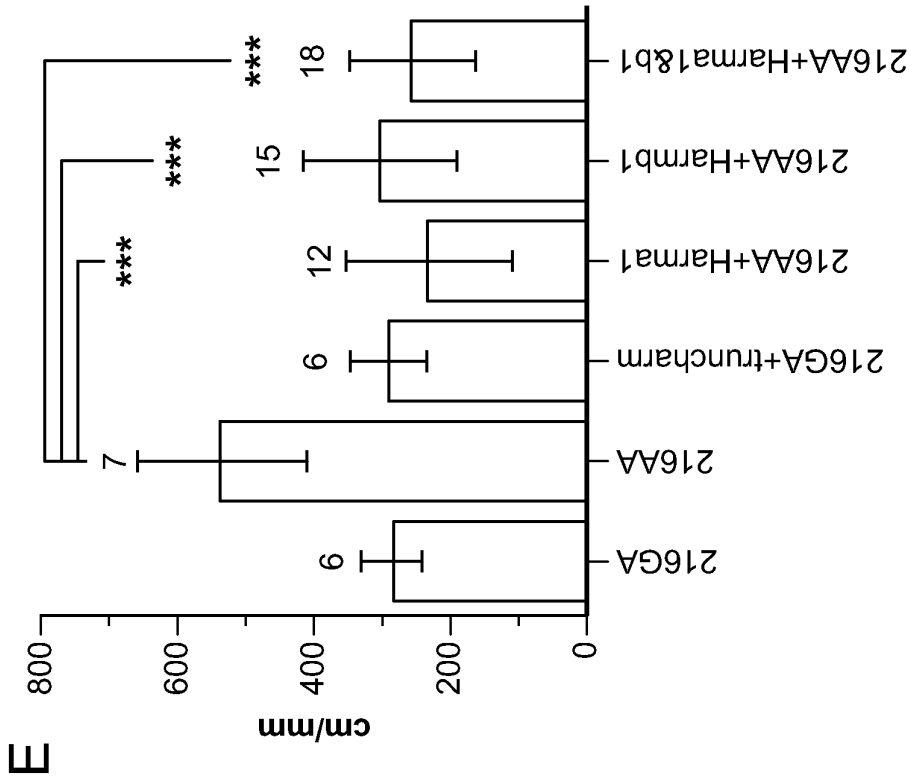
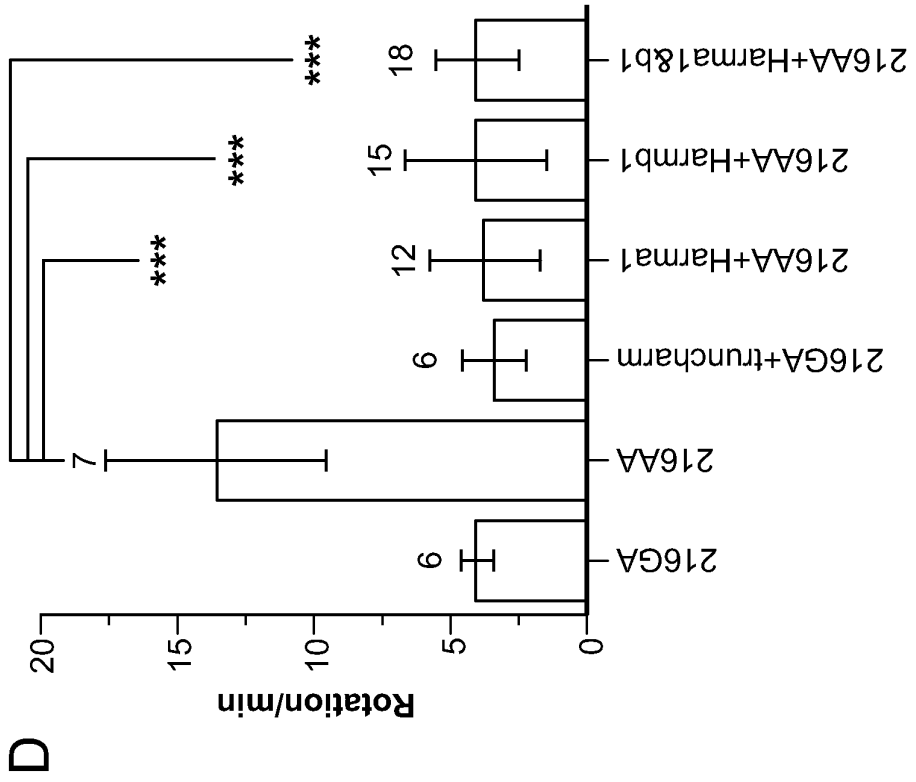

A
Startle (N)
0.0
0.2
0.4
0.6
60
70
80
90
100
110
Sound Intensity (dB)
216AA + Harma1&b1 (5)
216AA + harmb1 (9)
216GA (4)
216AA (5)
B
Latency to fall (sec)
0
50
100
150
1
2
3
4
5
Trial number
216AA + Harma1&b1 (7)
216AA + harmb1 (11)
216GA (8)
216AA (6)
216AA + Harma1 (7)
C
216GA
216AA
216AA + Truncharm
216GA + Harma1
216AA + Harmb1
216AA + Harma1&b1

Fig. 10


E
cm/mm
0
200
400
600
800
6
7
6
12
15
18
***
***
***
***
216GA
216AA
216GA+truncharm
216AA+Harma1
216AA+Harmb1
216AA+Harma1&b1


D
Rotation/min
0
5
10
15
20
6
7
6
12
15
18
***
***
***
***
216GA
216AA
216GA+truncharm
216AA+Harma1
216AA+Harmb1
216AA+Harma1&b1

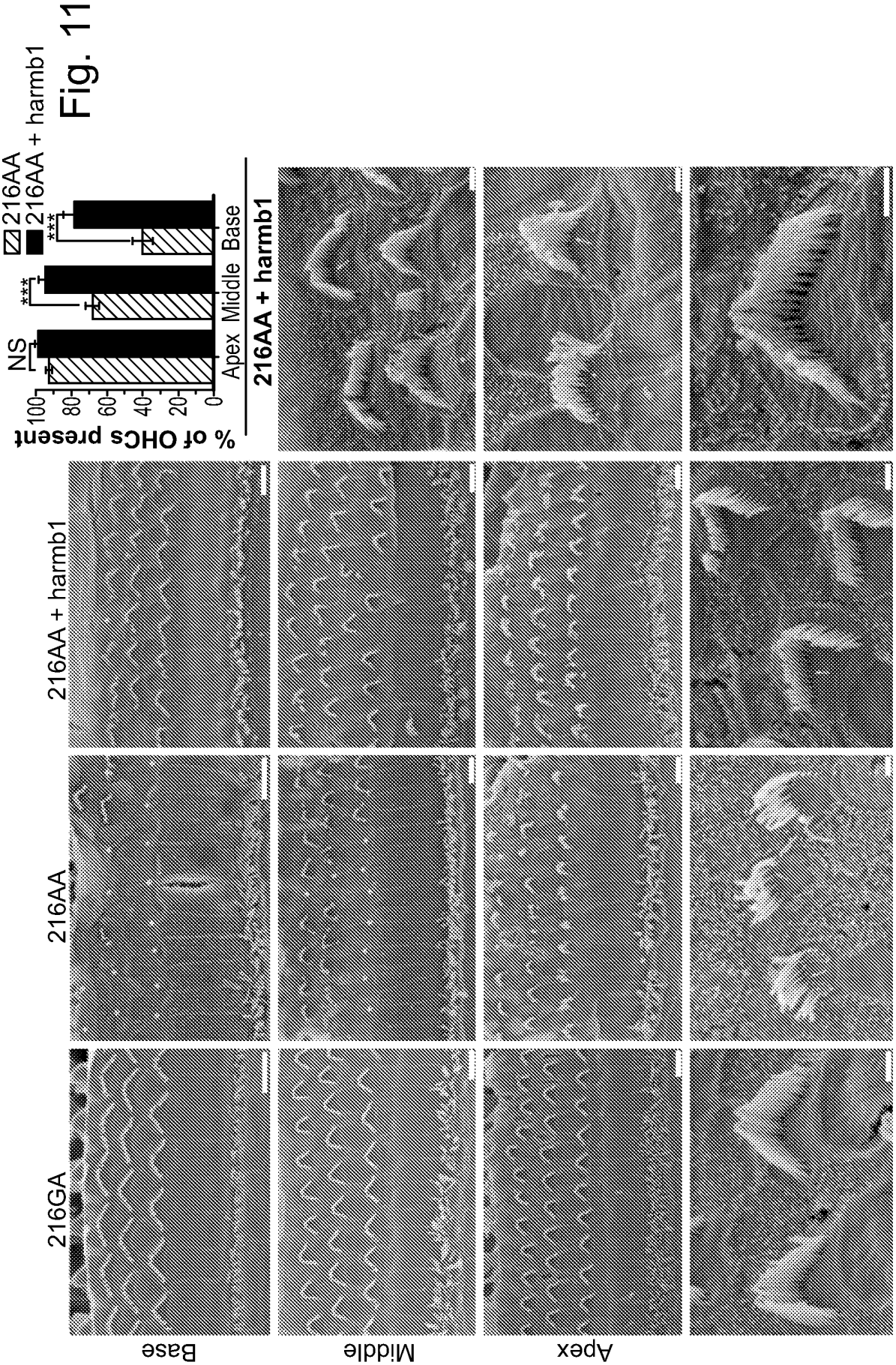

216GA
216AA
216AA + harmb1
Base
Middle
Apex
% of OHCs present
NS
***
***
216AA
216AA + harmb1
Apex Middle Base
216AA + harmb1

Fig. 11

Fig. 12

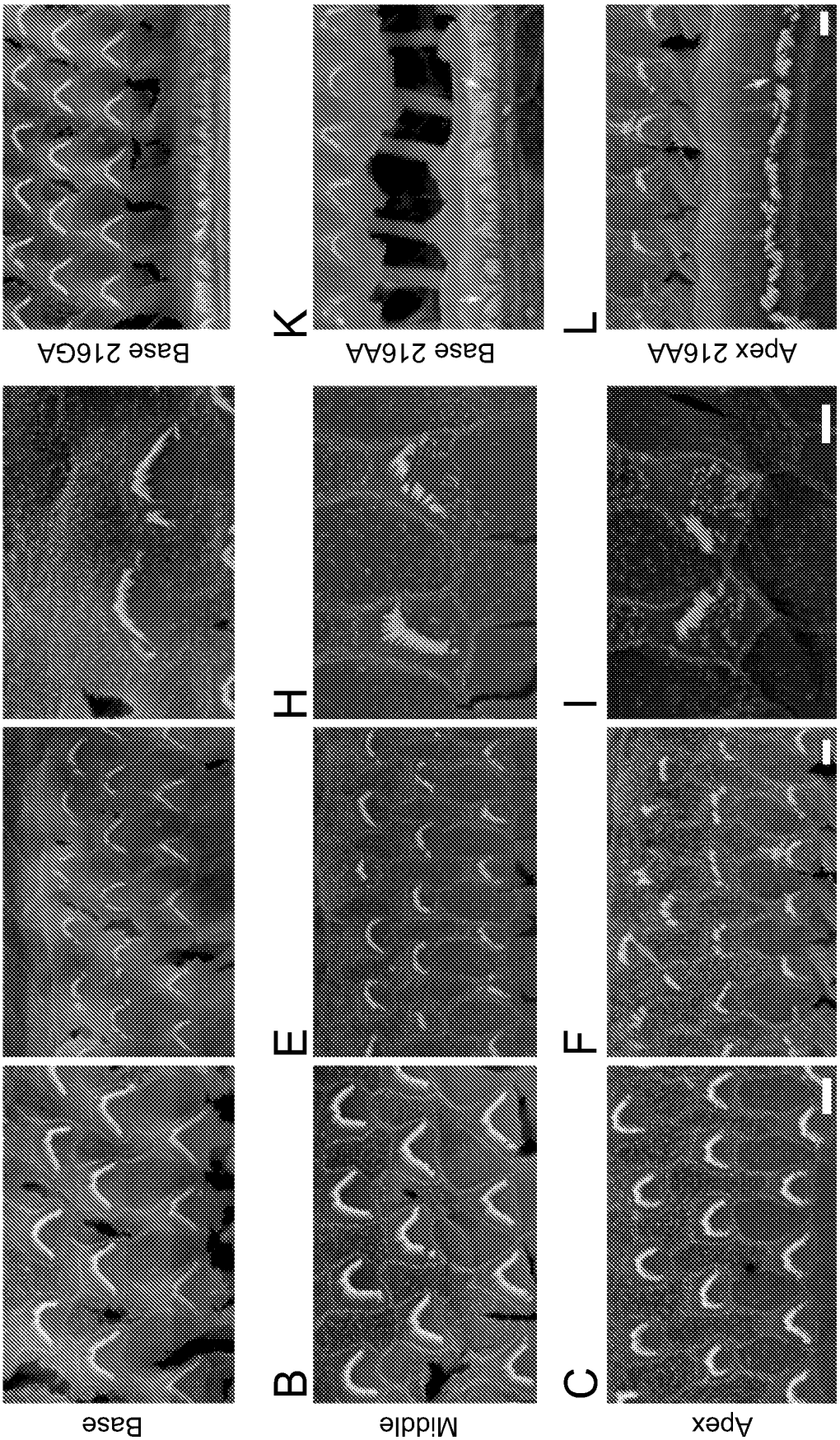
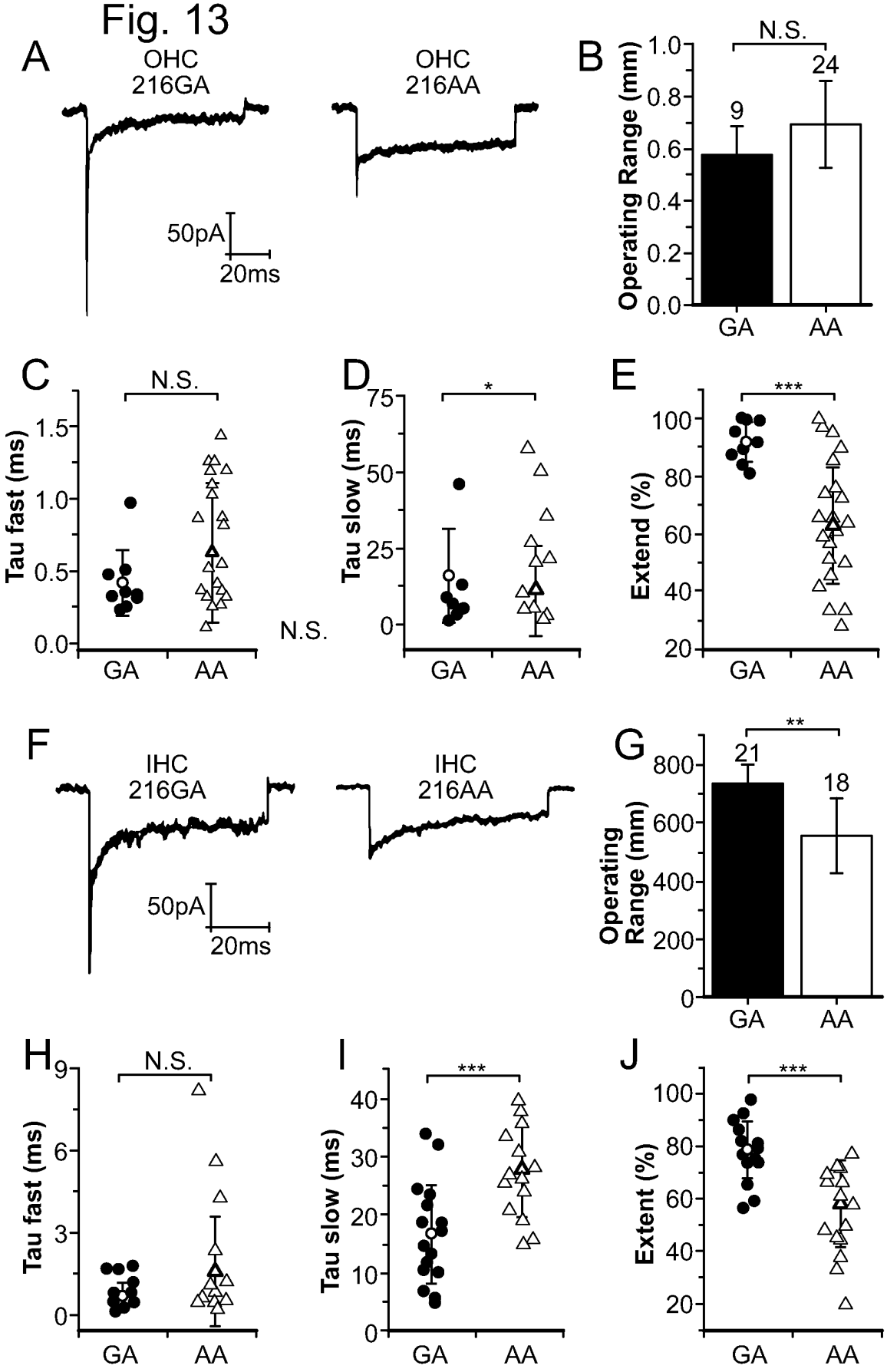

A
216GA
D
216AA
G
216AA
J
Base 216GA
K
Base 216AA
L
Apex 216AA
Base
B
E
H
Middle
C
F
I
Apex

Fig. 13


A
OHC 216GA
OHC 216AA
50pA
20ms
B
N.S.
9
24
Operating Range (mm)
1.0
0.8
0.6
0.4
0.2
0.0
GA
AA
C
N.S.
Tau fast (ms)
1.5
1.0
0.5
0.0
GA
AA
N.S.
D
*
Tau slow (ms)
75
50
25
0
GA
AA
E
***
Extend (%)
100
80
60
40
20
GA
AA
F
IHC 216GA
IHC 216AA
50pA
20ms
G
**
21
18
Operating Range (mm)
800
600
400
200
0
GA
AA
H
N.S.
Tau fast (ms)
9
6
3
0
GA
AA
I
***
Tau slow (ms)
40
30
20
10
0
GA
AA
J
***
Extent (%)
100
80
60
40
20
GA
AA

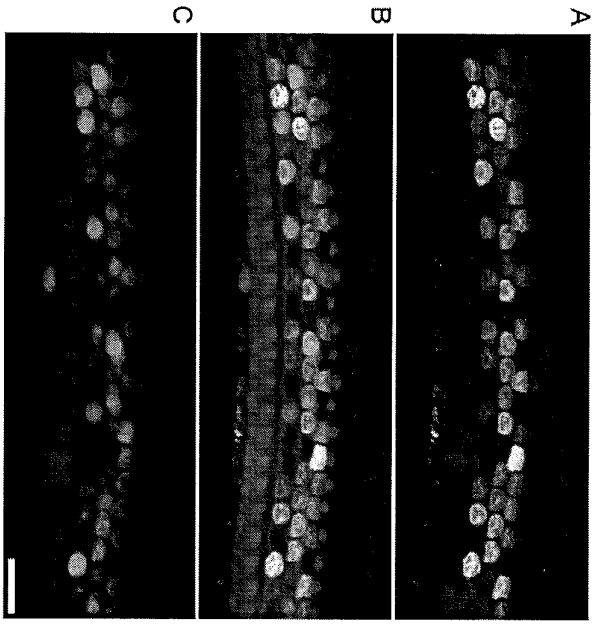


Fig. 14

Fig. 15

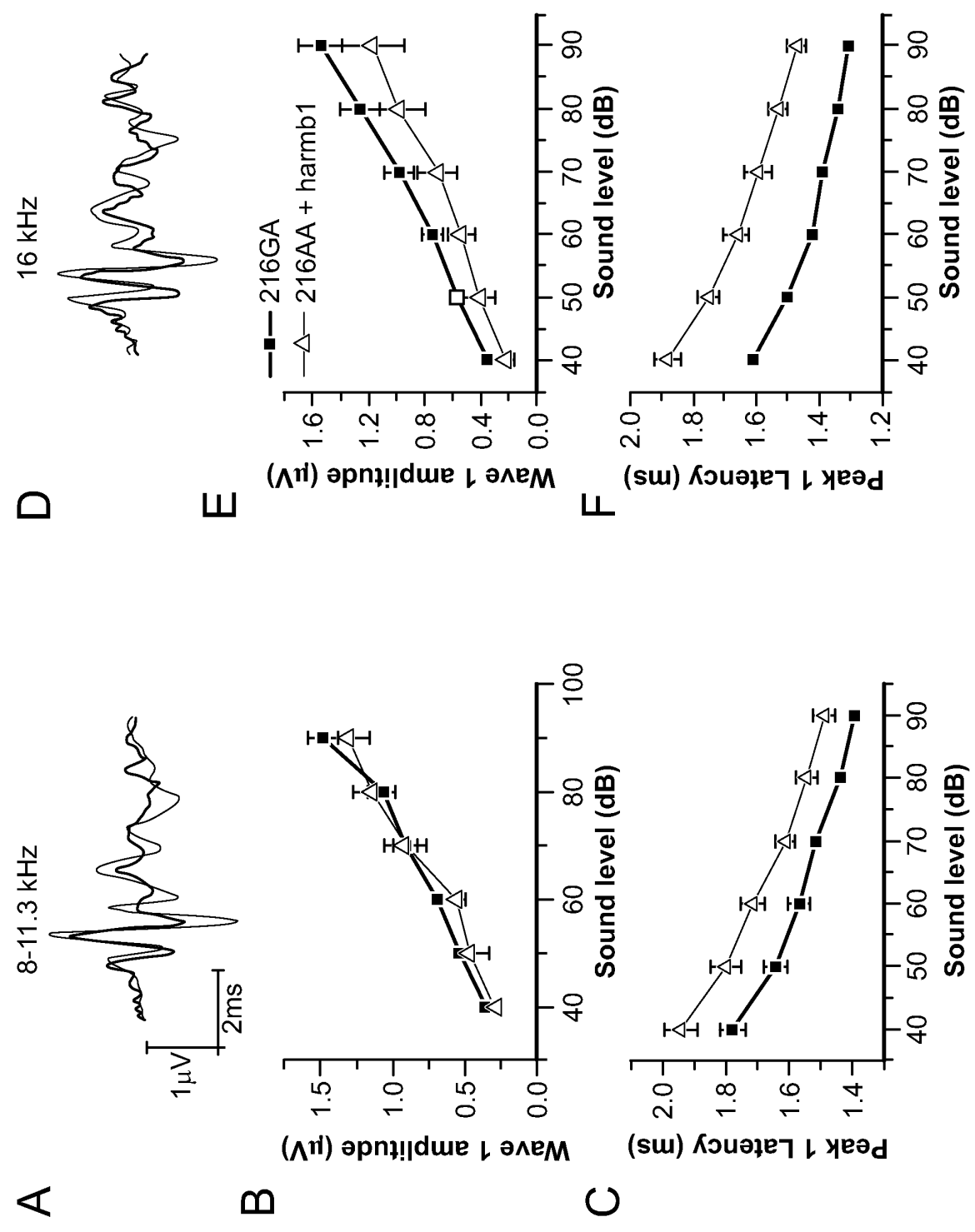

A
8-11.3 kHz
1μV
2ms
B
Wave 1 amplitude (μV)
Sound level (dB)
C
Peak 1 Latency (ms)
Sound level (dB)
D
16 kHz
E
216GA
216AA + harmb1
Wave 1 amplitude (μV)
Sound level (dB)
F
Peak 1 Latency (ms)
Sound level (dB)

Fig. 16A-1

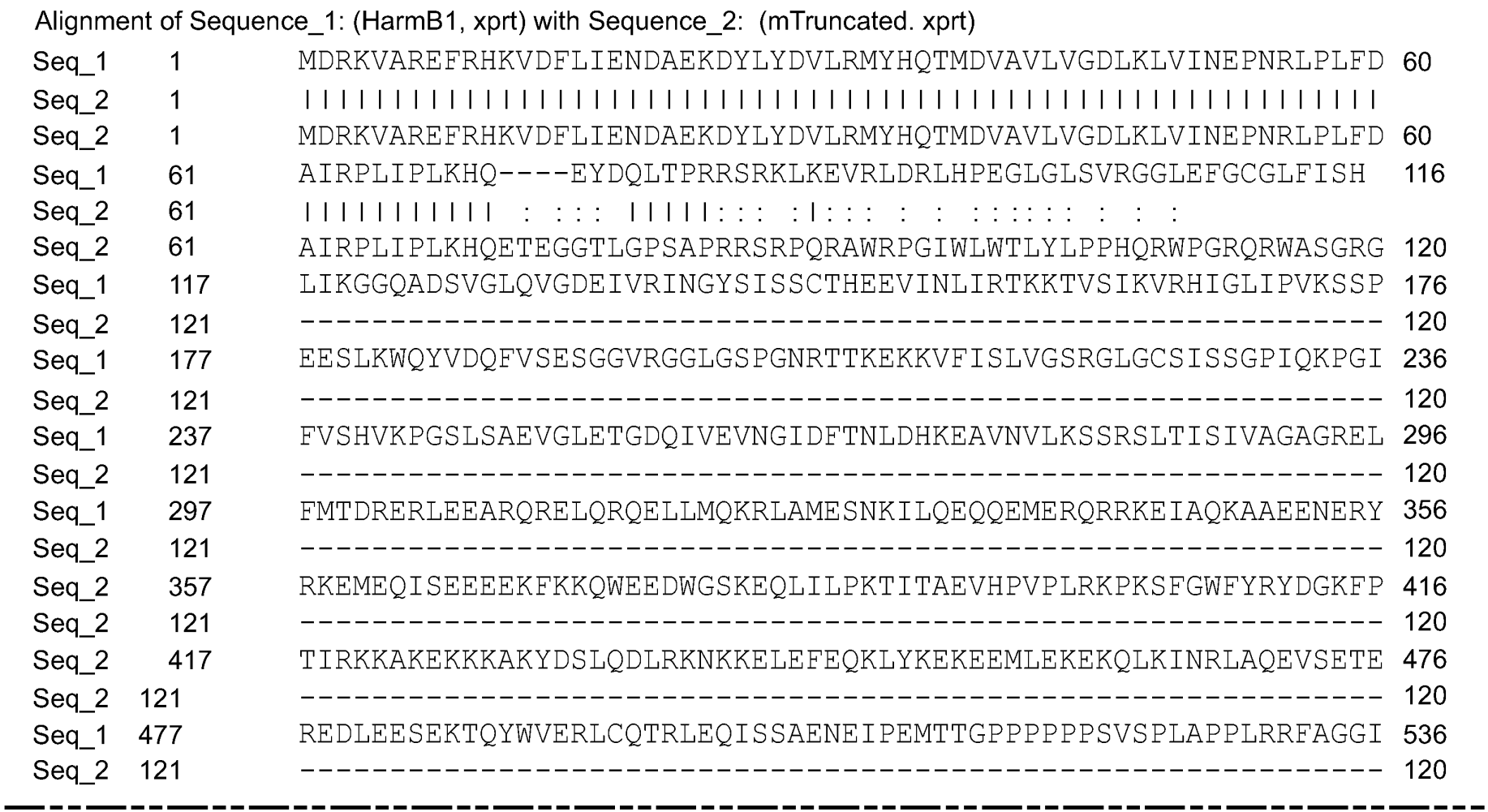

Alignment of Sequence_1: (HarmB1, xprt) with Sequence_2: (mTruncated. xprt)
Seq_1 1 MDRKVAREFRHKVDFLIENDAEKDYLYDVLRMYHQTMDVAVLVGDLKLVINEPNRLPLFD 60
Seq_2 1 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2 1 MDRKVAREFRHKVDFLIENDAEKDYLYDVLRMYHQTMDVAVLVGDLKLVINEPNRLPLFD 60
Seq_1 61 AIRPLIPLKHQ----EYDQLTPRRSRKLKEVRLDRLHPEGLGLSVRGGLEFGCGLFISH 116
Seq_2 61 |||||||||||| : ::: ||||| ::: :|::: : : ::::::: : : :
Seq_2 61 AIRPLIPLKHQETEGGTLGPSAPRRSRPQRAWRPGIWLWTLYLPPHQRWPGRQRWASGRG 120
Seq_1 117 LIKGGQADSVGLQVGDEIVRINGYSISSCTHEEVINLIRTKKTVSIKVRHIGLIPVKSSP 176
Seq_2 121 -- 120
Seq_1 177 EESLKWQYVDQFVSESGGVRGGLGSPGNRTTKEKKVFISLVGSRGLGCSISSGPIQKPGI 236
Seq_2 121 -- 120
Seq_1 237 FVSHVKPGSLSAEVGLETGDQIVEVNGIDFTNLDHKEAVNVLKSSRSLTISIVAGAGREL 296
Seq_2 121 -- 120
Seq_1 297 FMTDRERLEEARQRELQRQELLMQKRLAMESNKILQEQQEMERQRRKEIAQKAAEENERY 356
Seq_2 121 -- 120
Seq_2 357 RKEMEQISEEEEKFKKQWEEDWGSKEQLILPKTITAEVHPVPLRKPKSFGWFYRYDGKFP 416
Seq_2 121 -- 120
Seq_2 417 TIRKKAKEKKKAKYDSLQDLRKNKKELEFEQKLYKEKEEMLEKEKQLKINRLAQEVSETE 476
Seq_2 121 -- 120
Seq_1 477 REDLEESEKTQYWVERLCQTRLEQISSAENEIPEMTTGPPPPPPSVSPLAPPLRRFAGGI 536
Seq_2 121 -- 120

Fig. 16A-2

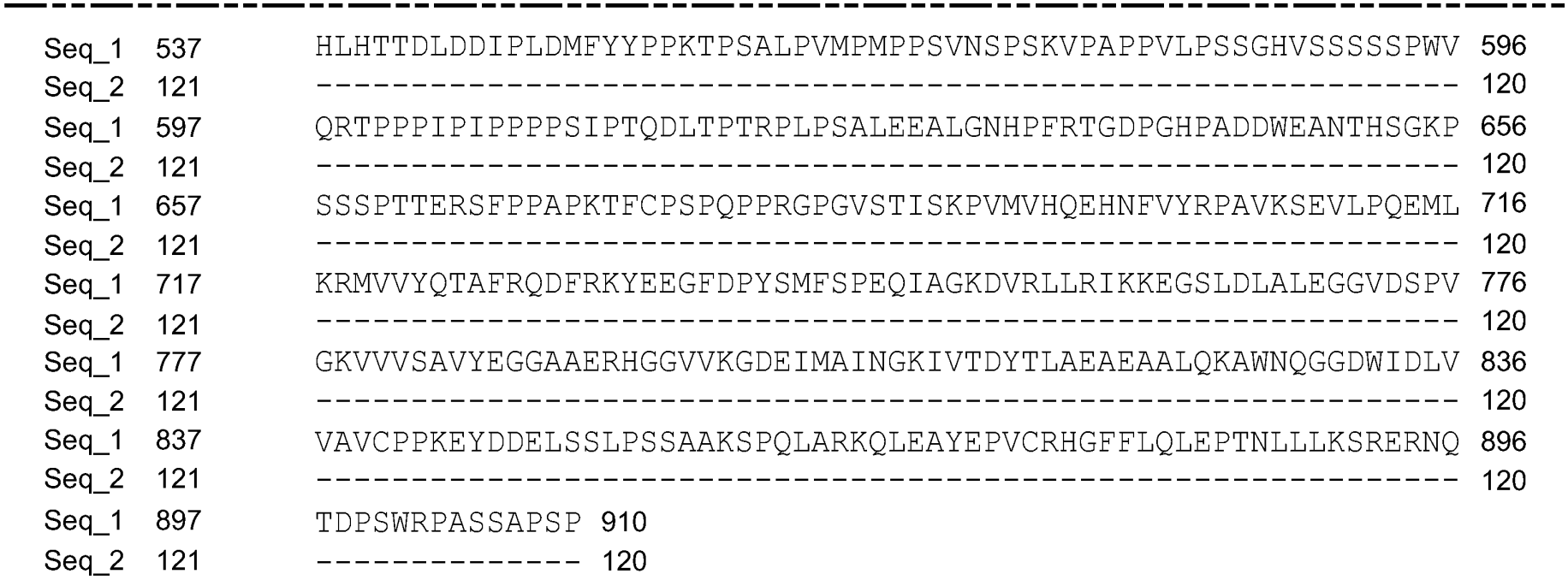


FIG. 16

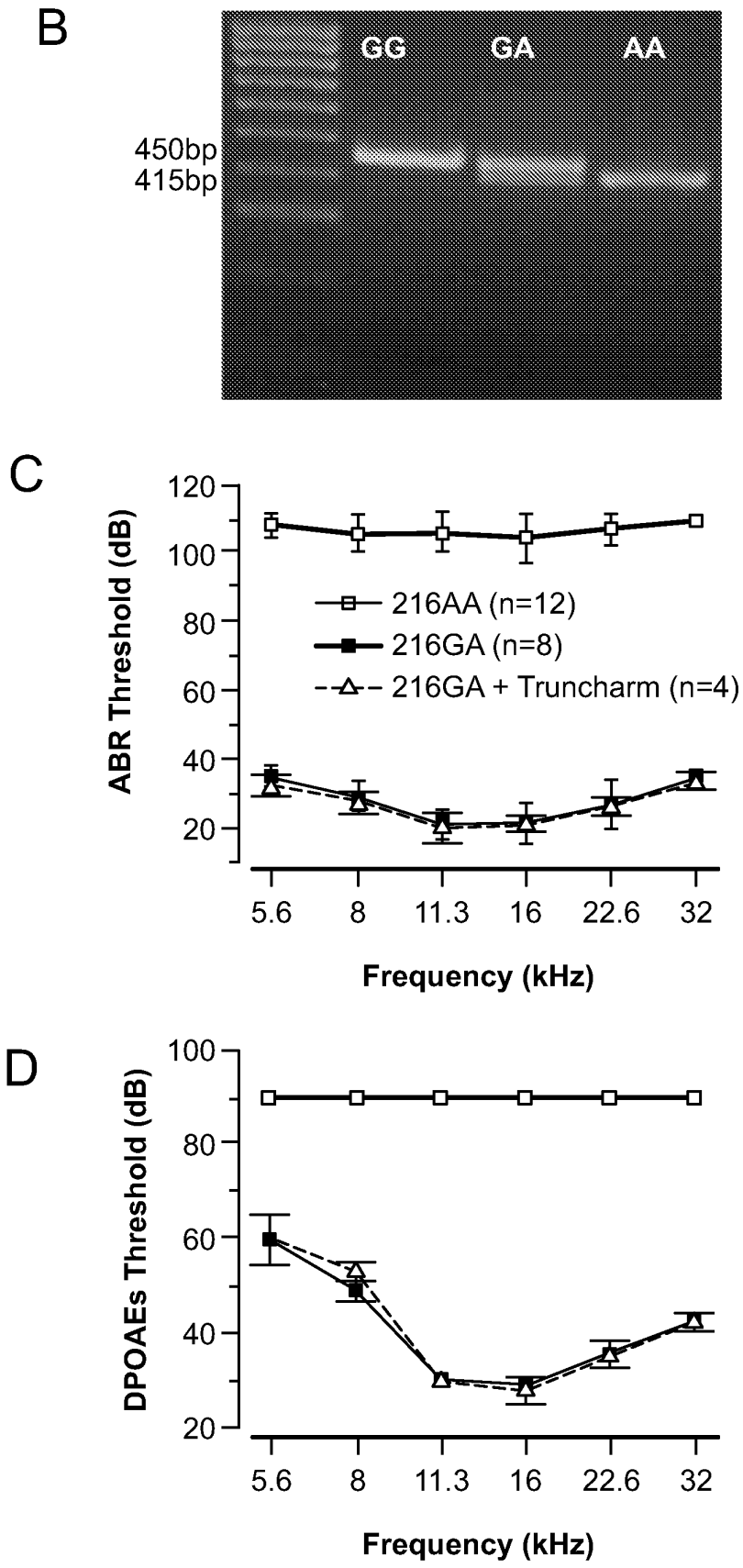
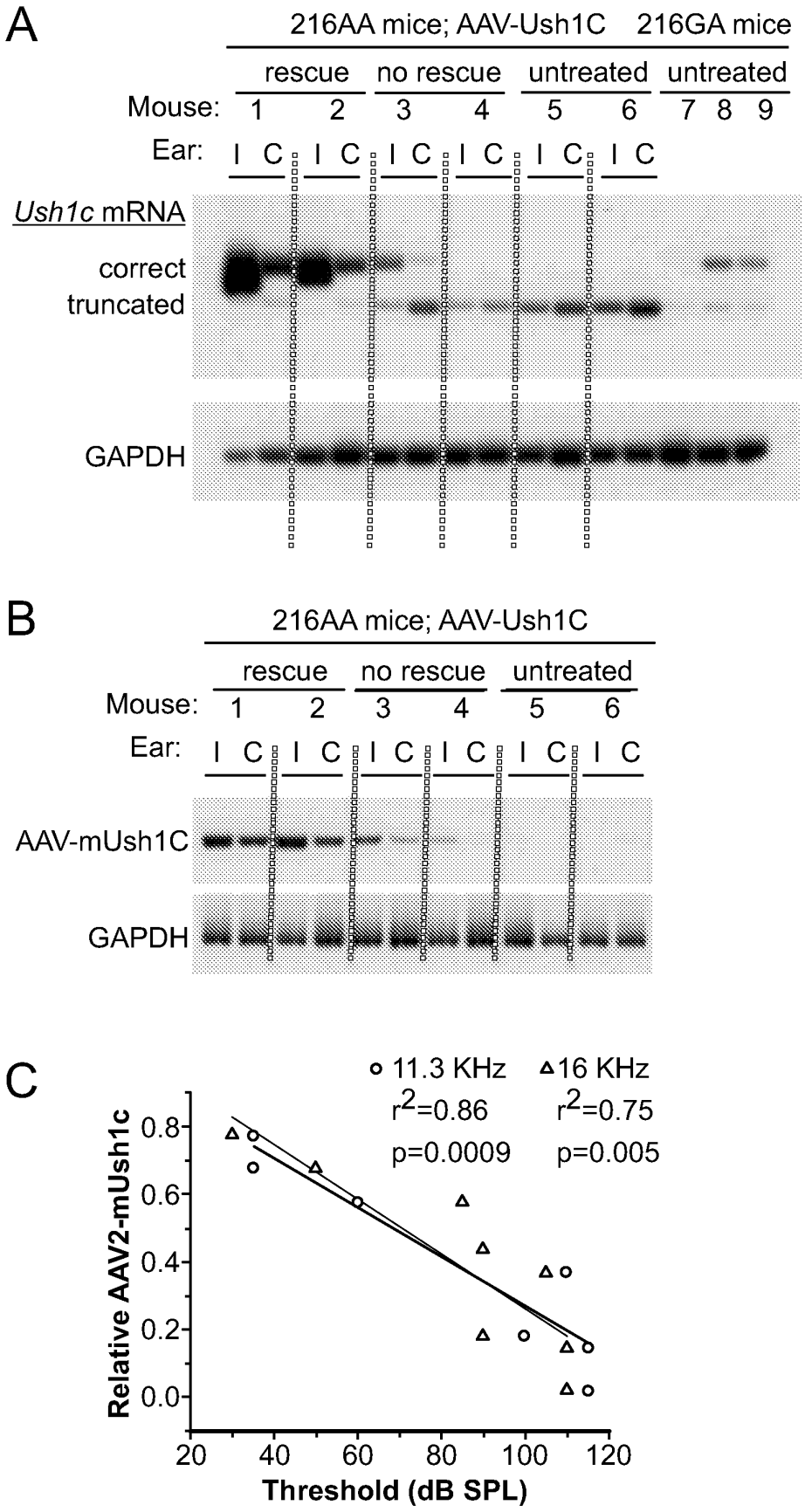

B
GG
GA
AA
450bp
415bp
C
ABR Threshold (dB)
120
100
80
60
40
20
216AA (n=12)
216GA (n=8)
216GA + Truncharm (n=4)
5.6
8
11.3
16
22.6
32
Frequency (kHz)
D
DPOAEs Threshold (dB)
100
80
60
40
20
5.6
8
11.3
16
22.6
32
Frequency (kHz)

FIG. 17


A
216AA mice; AAV-Ush1C
216GA mice
rescue
no rescue
untreated
untreated
Mouse: 1 2 3 4 5 6 7 8 9
Ear: I C I C I C I C I C I C
Ush1c mRNA
correct
truncated
GAPDH
B
216AA mice; AAV-Ush1C
rescue
no rescue
untreated
Mouse: 1 2 3 4 5 6
Ear: I C I C I C I C I C I C
AAV-mUsh1C
GAPDH
C
11.3 KHz
16 KHz
r2=0.86
r2=0.75
p=0.0009
p=0.005
Relative AAV2-mUsh1c
0.0
0.2
0.4
0.6
0.8
20 40 60 80 100 120
Threshold (dB SPL)

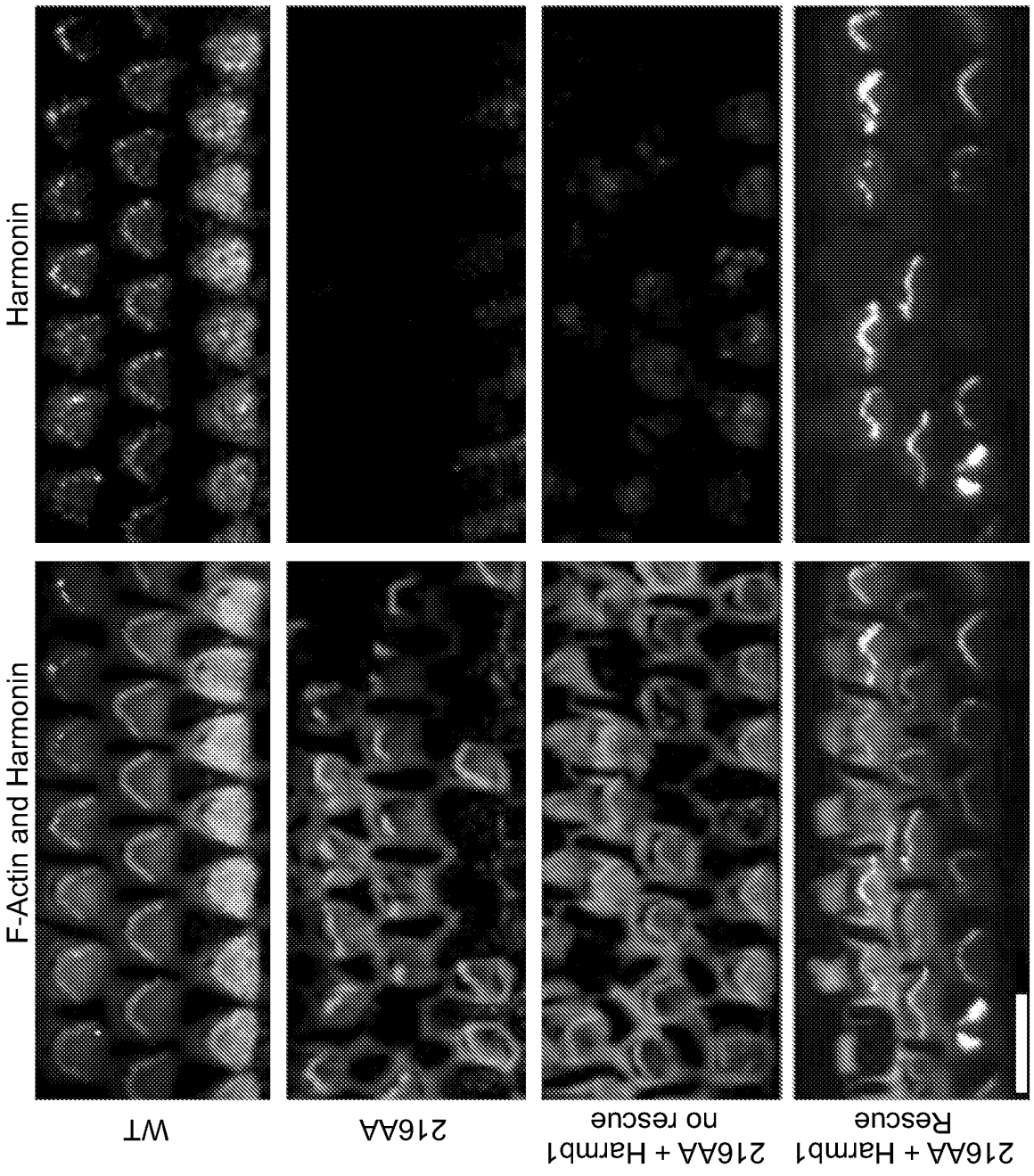

Harmonin
F-Actin and Harmonin
WT
216AA
216AA + Harmb1
no rescue
216AA + Harmb1
Rescue

FIG. 18 d

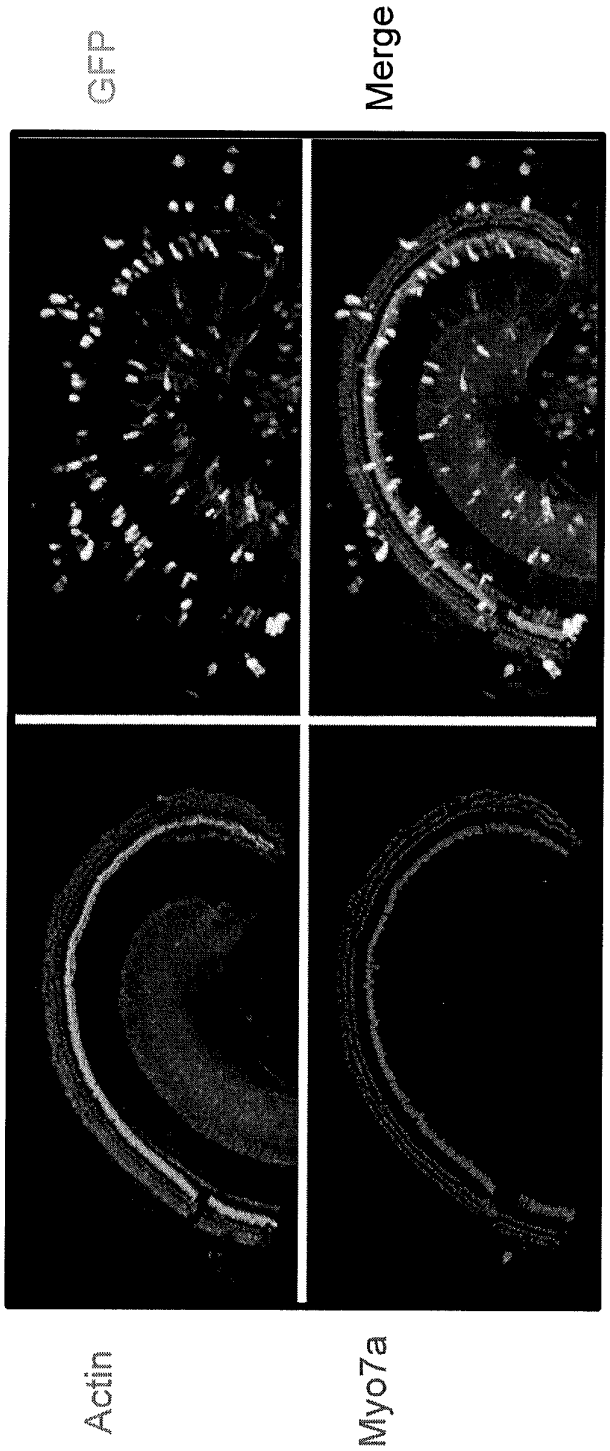


Figure 19

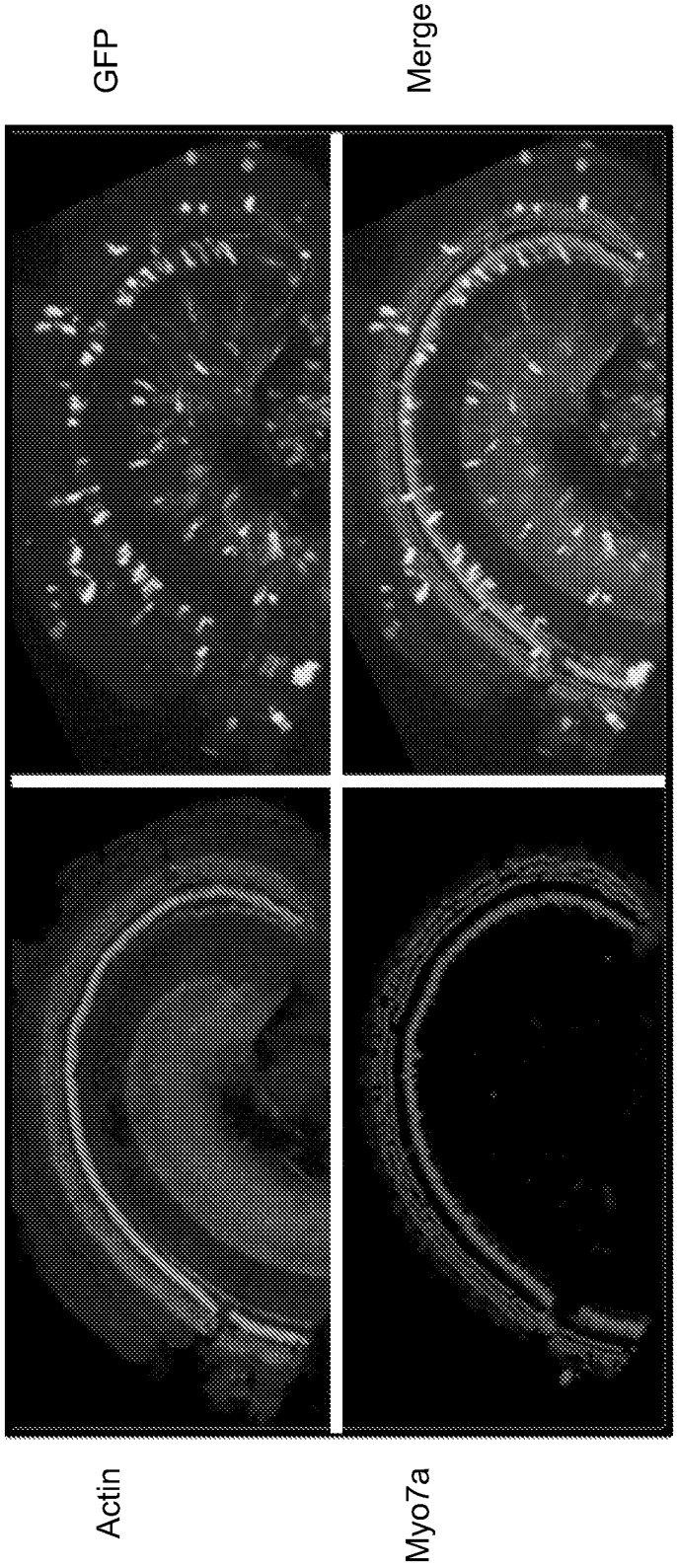


FIG. 20

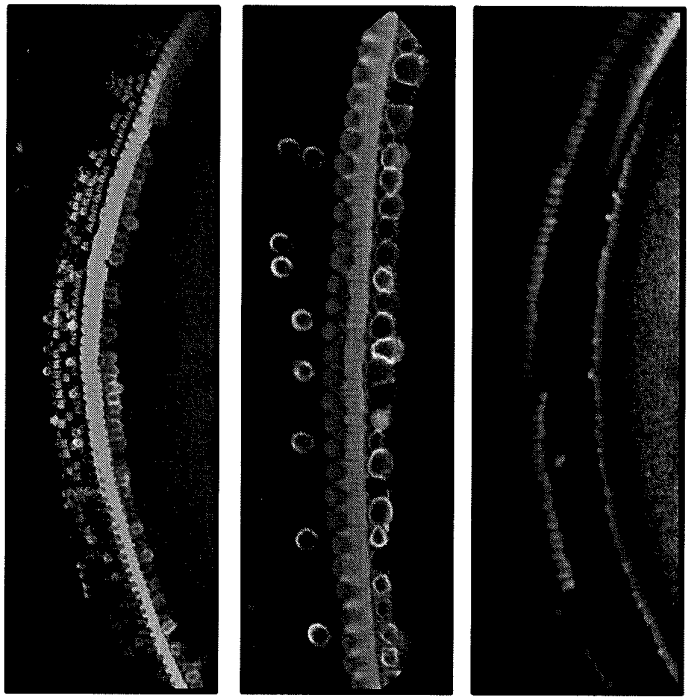


Figure 21

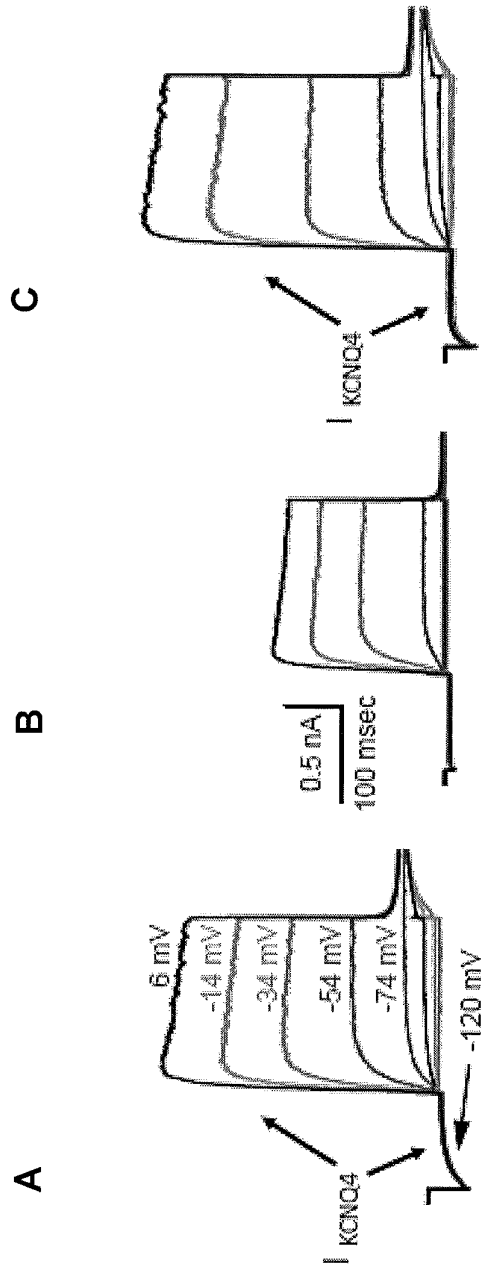

A
B
C
6 mV
-14 mV
-34 mV
-54 mV
-74 mV
-120 mV
I KCNQ4
0.5 nA
100 msec
I KCNQ4

Figure 22

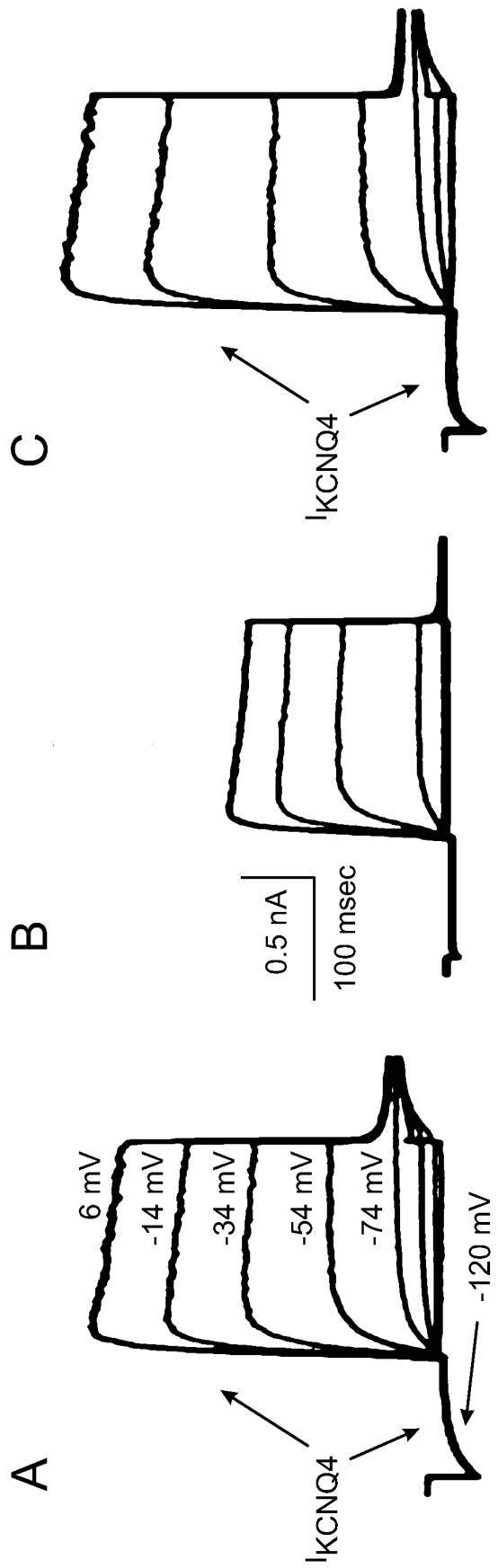

A
B
C
6 mV
-14 mV
-34 mV
-54 mV
-74 mV
-120 mV
I_{KCNQ4}
I_{KCNQ4}
0.5 nA
100 msec

FIG. 23

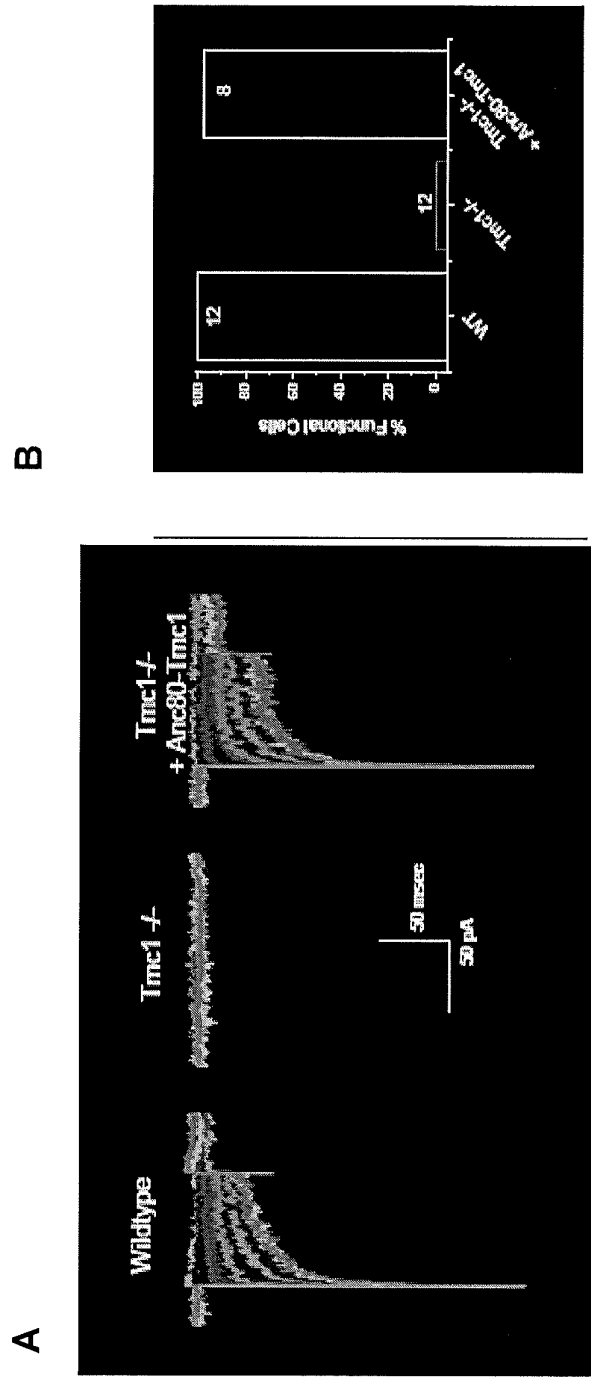

A
Wildtype
Tmc1 -/-
Tmc1-/-
+ Anc80-Tmc1
50 msec
50 pA
B
% Functional Cells
100
80
60
40
20
0
12
12
8
WT
Tmc1-/-
Tmc1-/-
+ Anc80-Tmc1

Figure 24

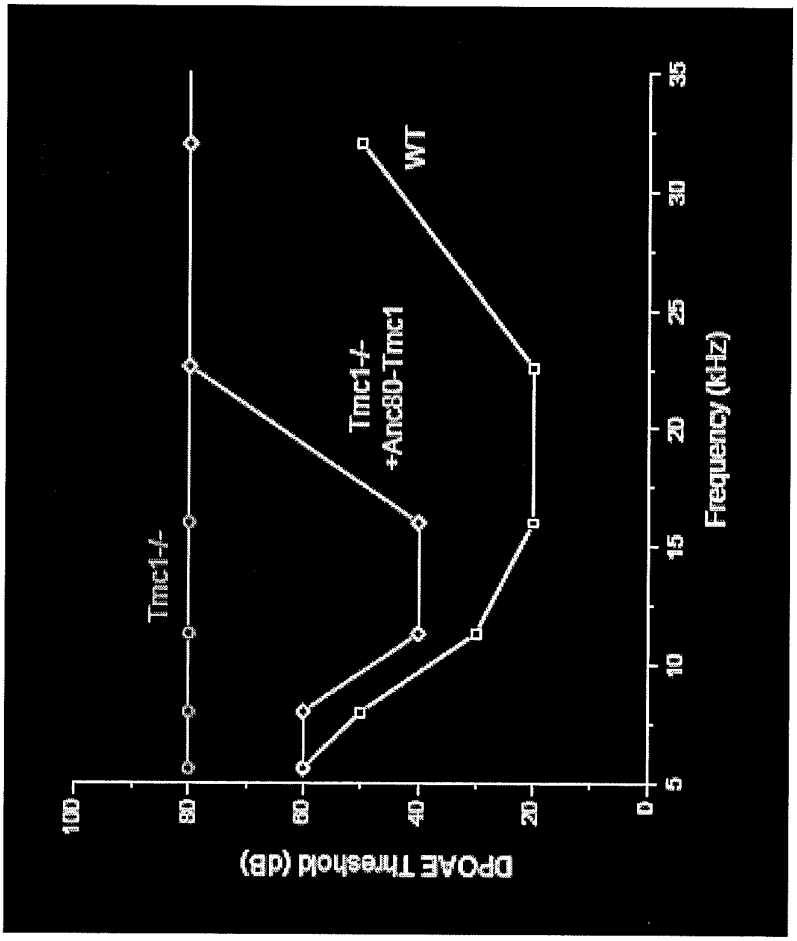

DPOAE Threshold (dB)
100
80
60
40
20
0
5
10
15
20
25
30
35
Frequency (kHz)
Tmc1-/-
Tmc1-/-
+Anc80-Tmc1
WT

Figure 25

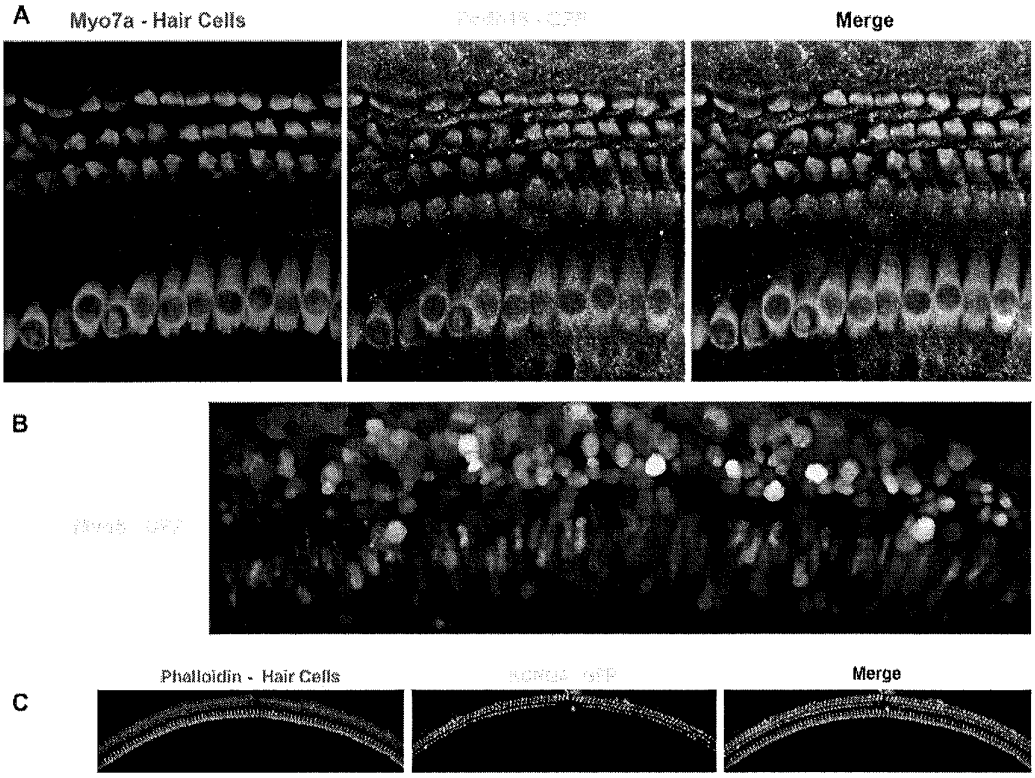


FIGURE 26

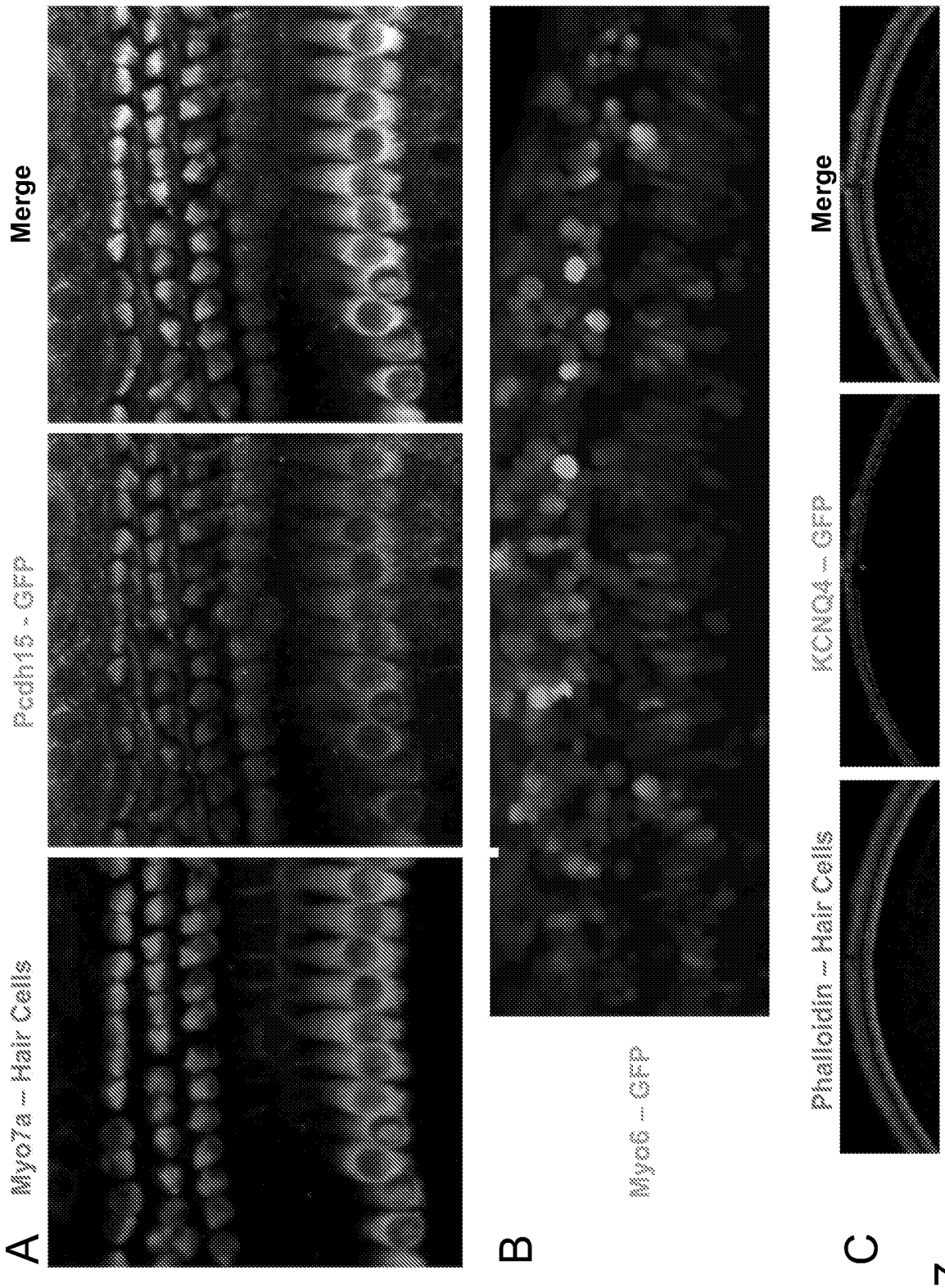

A
Myo7a – Hair Cells
Pcdh15 - GFP
Merge
B
Myo6 – GFP
C
Phalloidin – Hair Cells
KCNQ4 – GFP
Merge

FIG. 27