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

TECHNICAL FIELD

This disclosure generally relates to materials and methods for delivering nucleic acids to cochlear and vestibular cells.

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, while one of the more prevalent genetic defects, estimated to be 1-2% of all genetic deafness, occurs in the TMC1 gene.

The inner ear, e.g., cochlea, particularly the inner and outer hair cells (IHCs and OHCs) in the cochlea, is an attractive target for gene 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

Hearing loss is the most common sensory disorder worldwide, with half of pre-lingual deafness due to genetic causes. Nonetheless, translation of cochlear gene therapy to the clinic has been slowed by lack of safe, clinically relevant, and efficient delivery modalities. The novel gene delivery modalities described herein, however, which include new compositions and methods based on an adeno-associated virus (AAV) containing an Anc80 capsid protein, provide highly efficient gene transfer to inner ear cells including both IHCs and OHCs. As shown herein, an adeno-associated virus (AAV) containing an

ancestral scaffold capsid protein referred to as Anc80 or a specific Anc80 capsid protein (e.g., Anc80-0065) is surprisingly efficient at targeting various cells in the inner ear *in vivo*, including both IHCs and OHCs.

In one aspect, an AAV vector is provided that includes an Anc80 capsid protein and a TMC1 or TMC2 transgene. In another aspect, an AAV vector is provided that includes an Anc80 capsid protein and one or more transgenes selected from the group consisting of MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7. In one embodiment, the AAV vector further comprises a heterologous promoter.

In yet another aspect, a method of delivering a transgene to one or more cells in the inner ear in a subject is provided. Such a method typically includes administering an adeno-associated virus (AAV) to the inner ear in a subject, wherein the AAV comprises an Anc80 capsid protein and a transgene.

In still another aspect, a method of treating a hearing disorder (e.g., hearing restoration) or preventing hearing loss (or further hearing loss) in a subject is provided. Such a method typically includes administering an AAV to the subject, wherein the AAV comprises an Anc80 capsid protein and a transgene that, when expressed in one or more cells in the inner ear, restores hearing to the subject.

In one embodiment, the one or more cells in the inner ear are selected from the group consisting of inner hair cells (IHCs) and outer hair cells (OHCs). In some embodiments, the transgene is delivered to at least 80% of inner hair cells and at least 80% of outer hair cells. In some embodiments, the one or more cells in the inner ear are selected from the group consisting of spiral ganglion neurons, vestibular hair cells, vestibular ganglion neurons, supporting cells, and cells in the stria vascularis.

In some embodiments, the transgene is selected from the group consisting of 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 XIAP.

In some embodiments, the transgene encodes a neurotrophic factor (e.g., GDNF, BDNF, NT3, and HSP70). In some embodiments, the transgene encodes an antibody or fragment thereof. In some embodiments, the transgene encodes an immunomodulatory protein. In some embodiments, the transgene encodes an anti-oncogenic transcript. In some embodiments, the transgene encodes for an antisense, silencing, or long non-coding RNA species. In some embodiments, the transgene encodes a genome editing system selected from the group consisting of a genetically-engineered zinc finger nuclease, TALEN, and CRISPR.

In some embodiments, the Anc80 capsid protein has the sequence shown in SEQ ID NO:1. In some embodiments, the Anc80 capsid protein has the sequence shown in SEQ ID NO:2. In some embodiments, the transgene is under control of a heterologous promoter sequence. Representative heterologous promoter sequences include, without limitation, a CMV promoter, a CBA promoter, a CASI promoter, a PGK promoter, a EF-1 promoter, an alpha9 nicotinic receptor promoter, a prestin promoter, a KCNQ4 promoter, a Myo7a promoter, a Myo6 promoter, a Gfi1 promoter, a Vglut3 promoter, and an Atoh1 promoter.

In some embodiments, the administering step includes injecting the Anc AAV through the round window. In some embodiments, the Anc AAV is administered via injection through the round window. In some embodiments, the Anc AAV is administered during a cochleostomy or during a canalostomy. In some embodiments, the Anc AAV is administered to the middle ear and/or the round window via one or more drug delivery vehicles.

In some embodiments, expression of the transgene results 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), thereby restoring hearing or vestibular function.

In one aspect, an article of manufacture is provided that includes an AAV vector and a pharmaceutical composition. In such an article of manufacture, the AAV vector comprises an Anc80 capsid protein and a transgene operably linked to a promoter. In some embodiments, the transgene is selected from the group consisting of 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 XIAP.

In another aspect, a method of delivering a TMC1 or TMC2 transgene to one or more cells in the inner ear in a subject is provided. Such a method typically includes administering an adeno-associated virus (AAV) to the inner ear in a subject, wherein the AAV comprises an Anc80 capsid protein and a transgene. In still another aspect, a method of treating a hearing disorder in a subject is provided. Such a method typically includes administering an AAV to the subject, wherein the AAV comprises an Anc80 capsid protein and a TMC1 or TMC2 transgene that, when expressed in one or more cells in the inner ear, restores hearing to the subject or prevents hearing loss in the subject (e.g., further hearing loss).

In yet another aspect, a method of delivering an Usher transgene to one or more cells in the inner ear in a subject is provided. Such a method typically includes

administering an adeno-associated virus (AAV) to the inner ear in a subject, wherein the AAV comprises an Anc80 capsid protein and a transgene. In still another aspect, a method of treating a hearing disorder in a subject is provided. Such a method can include administering an AAV to the subject, wherein the AAV comprises an Anc80 capsid protein and an Usher transgene that, when expressed in one or more cells in the inner ear, restores hearing to the subject. Representative Usher transgenes include, without limitation, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7.

In one embodiment, the one or more cells in the inner ear are selected from the group consisting of inner hair cells (IHCs) and outer hair cells (OHCs). In one embodiment, the transgene is delivered to at least 80% of inner hair cells and at least 80% of outer hair cells. In one embodiment, the one or more cells in the inner ear are selected from the group consisting of spiral ganglion neurons, vestibular hair cells, vestibular ganglion neurons, supporting cells and cells in the stria vascularis.

In one embodiment, the Anc80 capsid protein has the sequence shown in SEQ ID NO:1. In one embodiment, the Anc80 capsid protein has the sequence shown in SEQ ID NO:2. In one embodiment, the transgene is under control of a heterologous promoter sequence. Representative heterologous promoter sequences include, without limitation, a CMV promoter, a CBA promoter, a CASI promoter, a PGK promoter, a EF-1 promoter, an alpha9 nicotinic receptor promoter, a prestin promoter, a KCNQ4 promoter, a Myo7a promoter, a Myo6 promoter, a Gfi1 promoter, a Vglut3 promoter, and an Atoh1 promoter.

In one embodiment, the administering step comprises injecting the Anc AAV through the round window. In one embodiment, the Anc AAV is administered via injection through the round window. In one embodiment, the Anc AAV is administered during a cochleostomy or during a canalostomy. In one embodiment, the Anc AAV is administered to the middle ear and/or the round window via one or more drug delivery vehicles.

In one embodiment, expression of the transgene results 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), thereby restoring hearing or vestibular function and/or preventing hearing loss (e.g., further hearing loss).

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

Figures 1A-1G are images showing representative confocal projections of an *in vitro* comparison of several AAV serotypes for eGFP transgene expression in cochlear explants of C57BL/6 mice. Figures 1A-1G show expression at the cochlear base for all serotypes, and apex and base for the indicated AAV. Scale bar = 100 μM. Top: Myo7A+TuJ1; bottom: eGFP only; middle: overlay; S. Cells: supporting cells, OHC: outer hair cell, IHC: inner hair cell. Figures 1H-1K are graphs showing the percentage of eGFP-positive hair cells per 100 μM after 48 h or 48h+5 days of incubation. N=3 for 48 h, and N=2 for 48h+5d. Error bars represent standard error of the mean (SEM).

Figures 2A-2G are images showing *in vivo* cochlear transduction of the indicated AAV serotype at the titer indicated above each panel. Figure 2A are confocal images of mouse organs of Corti, counterstained with Alexa-546-phalloidin (red) and imaged for eGFP (green). Scale bar = 50 μm. Figure 2B is a graph showing quantification of eGFP-positive IHCs in the base and apex of AAV-eGFP injected cochleae. Figure 2C is a graph showing quantification of eGFP-positive OHCs in the base and apex of AAV-eGFP injected cochleae. Figure 2D are images showing families of sensory transduction currents recorded at P7 (left) from eGFP-negative OHCs (black) and eGFP-positive OHCs (green). Vertical scale bar indicates 200 pA; horizontal scale bar 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 scale bar indicates 20 msec. Figure 2E is a graph showing 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. Figure 2F is a graph showing the 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). Figure 2G is a graph showing the 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 Figures 2B-2G are as in Figure 2A.

Figures 3A-3D are images showing Anc80-eGFP transduction in vestibular sensory epithelia. Figure 3A are images showing mouse utricle from a P1 mouse injected with 1 μL Anc80-eGFP (1.7×10^{12} GC/mL). The tissue was harvested, fixed and stained with Alexa546-phalloidin (red) and imaged for eGFP (green). Scale bar = 100 μm. Figure 3B are images showing the crista of the posterior semicircular canal from the same mouse described in Figure 3A. Scale bar = 50 μm. Figure 3C are images showing the sensory epithelium of a human utricle. The tissue was exposed to the Anc80-eGFP vector, cultured, fixed, stained with Alexa546-phalloidin (red) and imaged for eGFP fluorescence (green). Scale bar = 100 μm. Figure 3D are images showing 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 Figure 3C. White arrows in the overlay panel indicate selected eGFP-positive/Myo7A-positive cells. Scale bar = 20 μm.

Figures 4A-4J are representative images of an *in vitro* comparison of several AAV serotypes regarding eGFP expression in cochlear explants of CBA/CaJ mice. Figures 4A-4F are images showing results after incubation at equal doses of the AAV serotype. Scale bar = 200 μm. The error bars shown in Figures 4G-4J represent SEM.

Figures 5A-5H are images showing a eGFP qualitative expression scoring system ranging from 0 (Figures 5D, 5H) (lowest expression) to 3 (Figures 5A, 5E) (highest level of expression) to illustrate the range of expression in terms of intensity and number of infected cells with "0" representing no noted expression (Figures 5D, 5H), "1" select number of cells expressing dimly (Figures 5C, 5G), "2" low to moderate levels of expression in significant numbers of cells per microscopic field (Figures 5B, 5F), and "3" high percentage of cell expressing eGFP at levels ranging from moderate to high (Figures 5A, 5E). Scale bar shown in Figure 5A (for Figures 5A-5D) and Figure 5E (for Figures 5E-5H) = 20 μm.

Figure 6 are graphs showing eGFP expression in limbus, supporting cells, and spiral ganglion neurons of C57BL/6 mice using an eGFP scoring system detailed above in Figure 5. Error bars represent SEM. SGN transduction was evaluated by eGFP-positive cell counts per microscopic field.

Figure 7 are graphs showing eGFP expression in limbus, supporting cells, and spiral ganglion neurons of CBA/CaJ mice using an eGFP scoring system detailed above in Figure 5. Error bars represent SEM. SGN transduction was evaluated by eGFP-positive cell counts per microscopic field.

Figures 8A-8E are images showing extensive inner and outer hair cell transduction in murine cochleae with Anc80. Figure 8A is an image showing low-magnification of the entire apical portion of a mouse cochlea injected with Anc80-eGFP. The cochlea was harvested and stained with Alexa546-phalloidin (red) and imaged for eGFP (green). Scale bar = 100 μm. Figure 8B is an image showing high magnification view of a basal portion from a different mouse cochlea injected with Anc80-eGFP. The cochlea was harvested and stained with Alexa546-phalloidin (red) and imaged for eGFP (green). Scale bar = 20 μm. Figures 8C and 8D are graphs showing quantitative comparison of inner and outer hair cell transduction efficiency following round window injection of C57BL/6 mice. Figure 8E are images showing dose-dependency of Anc80 hair cell transduction. Cochleae were exposed to two different Anc80-eGFP titers, fixed, stained with Alexa546-phalloidin (red) and imaged for eGFP (green). Scale bar = 20 μm

Figures 9A-9H are images showing bilateral cochlear transduction from base to apex in mouse cochleae evaluated for eGFP transgene expression in histological sections stained for TuJ1 (Red) and Myo7A (Mangenta). Efficient Anc80 transduction was observed in the injected cochlea extending up to apex (Figures 9A/9F) and also in the contralateral uninjected ear (Figures 9B-9E=from apex to base). Close-up image of eGFP-positive and TuJ1-positive spiral ganglion neurons (Figure 9G). Reconstructed 3D image for SGN evaluation of Anc80 transduction (Figure 9H). Scale bars 100 μm (Figures 9A-9E) and 20 μm (Figures 9F/9G).

Figures 10A-10B are representations of microscopic images showing an axial section of a mouse brain after unilateral cochlear injection with Anc80 (Figure 10A). Predominant expression was observed in the cerebellum, in particular Purkinje cells (white arrowheads) (Figure 10B). Scale bars 1 mm (Figure 10A) and 300 μm (Figure 10B). Figure 10C is an image showing anti-AAV neutralizing antibody (NAB) titers in

serum and cerebrospinal fluid (CSF) in uninjected and Anc80 RWM-injected animals. Titers reflect the dilution of serum or CSF at which 50% inhibition of transduction was observed in the NAB assay. Due to sample volume limitations, the limit of sensitivity for serum NAB was 1/4 and for CSF was 1/52.5.

Figures 11A-11B are an image and a graph, respectively, showing vestibular function following Anc80 cochlear transduction. Mice were injected with Anc80-eGFP and evaluated for expression and balance function on the rotarod device. Figure 11A is an image showing expression of eGFP (green) in the vestibular tissue via confocal microscopy with immunofluorescent staining for Myo7A (red). Figure 11B is a graph showing the mean time until the mice fell off the device +/- SEM. Scale bar = 50 μm

Part 2—Gene Therapy Restores Function in a Mouse Model of Usher Syndrome

Figures 12A-12L are images showing scanning electron microscopy of the organ of Corti in Ush1c c.216G>A mutant mice. Figures 12A-12F are images showing basal, middle and apical regions of the organ of Corti imaged in c.216GA and c.216AA mutant mice. Figures 12G-12L are high magnification images of OHCs (Figures 12G-12H) and IHCs (Figures 12I-12J). Stars indicate preserved hair bundles; arrowhead, disorganized hair bundles; and arrows, wavy IHC bundles. Scale bars low mag.: 5 μm (Figures 12A-12F); high mag.: 2 μm (Figure 12G), 3 μm (Figure 12H), 2 μm (Figures 12I-12J) and 1 μm (Figures 12K, 12L).

Figures 13A-13H are images showing mechanotransduction in hair cells of Ush1c c.216G>A neonatal mutant mice. Figures 13A-13D are images showing FM1-43 staining to assess the presence of open transduction channels in hair cells of c.216GA and c.216AA mice. IHC FM1-43 fluorescence appears dimmer as IHCs are in a different focal plan. Left: DIC, Right: FM1-43; Scale bar 10 μm; Figure 13C, scale bar 50 μm; Figure 13D, scale bar 10 μm. The white line on Figure 13D delineate the striola (no uptake) and extra-striola regions (uptake). Figures 13E-13H are graphs showing mechanotransduction assessed in OHCs, IHCs and VHCs in neonatal c.216GA and c.216AA mice. Representative transduction currents (Figure 13E), their associated current/displacement plots fitted with a second order Boltzmann function (Figure 13F) and average peak transduction current are plotted (Figures 13G-13H). Average peak transduction was significantly different between the two genotypes in OHCs, IHCs and VHCs (***$P < 0.01$, one-way ANOVA).

Figures 14A-14E are images showing expression and localization of fluorescently labeled harmonin in tissues exposed to adeno-associated viral vectors *in vitro* and *in vivo*. Figures 14A-14C show acutely dissected inner ear tissue exposed to AAV2/1 vectors, cultured, fixed, counterstained (Alexa Fluor phalloidin, Invitrogen) and imaged with a confocal microscope. Figure 14A scale bar: 10 μm- upper panels; 5 μm- lower panels; Figure 14B scale bar: 10 μm; Figure 14C scale bar: 3 μm; Figure 14D scale bar: 30 μm; Figure 14E scale bar: 5μm.

Figures 15A-15C are images showing recovery of mechanotransduction in hair cells of mice injected with Anc80 harmonin vectors. Figures 15A-15C show mechanotransduction currents recorded in IHCs of c.216AA uninjected control mice and c.216AA mice injected with Anc80 harmonin-b1 or a combined injection of the Anc80 harmonin-b1 and Anc80 harmonin-a1. Organotypic cultures were prepared and recordings were performed. 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) (Figure 15B). Average responses (mean ± SD) show significant recovery of transduction (***$P < 0.001$) for harmonin-b1 and harmonin-a1 + -b1 injected relative to uninjected mice. Average transduction currents were not significantly different in harmonin-b1 injected mice and c.216GA control mice (NS $P>0.5$). Recovery of mechanotransduction was also not significantly improved when harmonin-a and harmonin-b were combined. Figure 15C shows one-way ANOVA.

Figures 16A-16E are images showing ABR and DPOAE threshold recovery in mice injected with Anc80 harmonin-b1. Figure 16A is an image showing representative ABR responses for 16 kHz tones in c.216AA control mice and c.216AA mice injected with 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. Figure 16B is an image showing mean ABR responses obtained for: c.216 AA; c.216GA; c.216AA + harmonin-a1; c.216AA + harmonin-b1; c.216AA + harmonin-a1&-b1. Mean ± SE, continuous lines. Dotted lines: ABR thresholds for the entire frequency range in mice whose 16 kHz recordings are shown in Figure 16A. Figure 16C shows the mean DPOAEs responses obtained for: c.216AA; c.216GA; c.216AA + harmonin-a1; c.216AA + harmonin-b1; c.216AA + harmonin-a1&-b1. Mean ± SE, continuous lines. Dotted lines: DPOAEs

thresholds for the four mice whose recordings are illustrated in Figure 16A. Arrows indicate that the thresholds are higher than the maximal stimulus level tested. Figures 16D-16E show ABRs and DPOAEs responses obtained at 6 weeks and 3 months in mice that showed initial ABR thresholds under or equal to 45 dB. Six of the eight mice were kept for 6 months and had ABRs and DPOAEs assessed (dotted line). Mean ± SE. 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.

Figures 17A-17E are images showing startle response, rotarod performance and open field behavior recovery in mice injected with Anc80 harmonin-a1 and Anc80 harmonin-b1. Figure 17A shows startle response to white noise stimuli recorded in 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. Averages are shown ± SE. Figure 17B show rotarod performance 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 ± SE. Figures 17C-17E show open field observations performed for 5 min in control c.216GA, c.216AA and c.216AA and c.216GA injected mice. Representative tracks over 2.5 min are shown (Figure 17B). 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. Figure 17C show graphs illustrating the mean ± SD 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 18 are scanning electron microscopy images of the organ of Corti in mice injected with Anc80 harmonin-b1. Basal, Middle and Apical regions of the organ of Corti were imaged in c.216GA, c.216AA and c.216AA mice. 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 and 792 in rescued c.216AA mice. Mean ± SE. 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: 5 μm; high magnification: 1 μm.

Figures 19A-19L are images showing analysis of hair bundle morphology in Ush1c c.216G>A mice by SEM. Figures 19A-19C show heterozygous c.216GA mice displayed normal hair bundle morphology. Figures 19D-19I show disorganized hair bundles observed along the organ of homozygous c.216AA mutant mice. Figures 19J-19L show IHCs hair bundle 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: 5 μm; high magnification: 1 μm.

Figures 20A-20J are images showing mechanotranduction properties in c.216AA mutant mice. Figures 20A-20E show analysis of mechanotransduction in neonatal OHCs from middle and mid-apical turns of the cochlea. 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 (Figure 20A). Fits were used to generate fast (Figure 20C) and slow (Figure 20D) time constants as well as the extent of adaptation (Figure 20E). The 10-90% operating range was not significantly altered (Figure 20B). Extent of adaptation in c.216AA mice was significantly less than or heterozygous OHCs as shown in this scatter plot (Figure 20E). Figures 20F-20J show analysis of mechanotransduction in neonatal IHCs. 10-90% operating range values were smaller in c.216GA versus c.216AA IHCs (Figure 20G). Adaptation was always present albeit slightly slower and with a significant lesser extent in c.216AA IHCs (Figures 20H-20J). Statistical analysis is indicated in each plot: *P<0.05, **P<0.01 and ***P<0.001, one-way ANOVA.

Figures 21A-21C are data 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. Figures 21A-21C show 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 (Figure

21A) and tdTomato (Figure 21C) and 65% expressed both markers demonstrating successful co-transduction. Scale bar: 20 μm.

Figures 22A-22F are data showing analysis of ABR response in control c.216GA and injected rescued c.216AA mice. Figures 22A and 22D show example of ABR responses at 8 and 16 kHz for control c.216GA and rescued c.216AA mice. Figures 22B-22C and 22E-22F show average peak 1 amplitude (Figures 22B-22D) and latency (Figures 22C-22D) 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 ± SE: One-way ANOVA.

Figures 23A-23D show the mutant form of harmonin expressed in Ush1c c.216G>A mice does not alter hair cell or auditory function. Figure 23A is 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. Figure 23B shows semi-quantitative RT-PCR from auditory organs of 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. Figures 23C-23D show auditory brainstem responses (ABR, Figure 23C) and distortion products (DPOAEs, Figure 23D) were measured in c.216GA injected mice and control c.216GA and c.216AA mice. Plots are shown as averages ± SE.

Figures 24A-24C are images showing recovery of correct Ush1c splicing in the inner ear of 6 weeks old mice injected with AAV2/Anc80.CMV.harmonin-b1. Figure 24A shows 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 (Figure 24C) 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. Figure 24B is an image showing that semi-quantitative radiolabeled PCR analysis confirmed the presence AAV-mUsh1c in injected and contralateral ears of Ush1c.216AA

mice. Relative levels of AAV-mUshlc DNA were present but reduced in mice #3 and #4. Figure 24C shows relative amount of AAV-mUshlc correlated with ABR thresholds. Analysis for 11.3 and 16 kHz are illustrated. Linear regressions showed high correlations between the two.

Figure 25 is a graph showing long term ABR threshold recovery correlated 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. 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. Insert: while two of the mice (mice #1,2) showed poor ABR response thresholds across the entire range tested (≥95 dB SPL), three (mice #3,4,5) responded with thresholds ranging 35 and 55 dB SPL for sound stimuli between 5.6 and 16 kHz.

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

Figures 26A-26D are representative confocal images of a cochlea from an Ushlc mutant mouse injected through the RWM with of Anc80-Harmonin::GFP (i.e., the GFP is fused to the Harmonin polypeptide), harvested, and stained with actin (red; Figure 26A), Myo7a (blue; Figure 26B), and imaged for GFP (green; Figure 26C). A merged image of Figures 26A, 26B, and 26C is shown in Figure 26D.

Figure 27 is a graph showing ABR threshold plotted as a function of sound frequency for an Ushlc mutant mice (squares) and an Ushlc mutant mice injected with an Anc80-Harmonin::GFP vector (circles).

Figures 28A-28C show representative confocal images of a KCNQ4 -/- cochlea injected through the RWM with Anc80-KCNQ4, harvested, and stained with Alexa 546–phalloidin (red) and an antibody against KCNQ4 (green) at low magnification (Figure 28A) or high magnification (Figure 28B) relative to uninjected cochlea at high magnification (Figure 28C).

Figures 29A-29C are a series of graphs that show the KCNQ4 current in a wild type mouse (Figure 29A), a P10 KCNQ4 -/- mouse (Figure 29B), and a P10 KCNQ4 -/-

mouse injected with Anc80- KCNQ4 (Figure 29C). Cochleas were harvested 8 days after injection.

Figure 30 is a series of three images showing FM1-43 uptake (FM1-43 only permeates functional Tmc1 channels) in Tmc1 -/- tissue injected with the Anc80 Tmc1 vector.

Figure 31A is an image that shows representative families of sensory transduction currents recorded from IHCs of a wild type mouse (left), a Tmc1 -/- mouse (middle), and a Tmc1 -/- mouse injected with Anc80 Tmc1 (right). Cochleas were harvested 8 days after injection.

Figure 31B is a graphical representation of the recovery rate of the mice shown in Figure 31A. The graph in Figure 31B indicates the percentage of functional cells in a wild type mouse (left), a Tmc1 -/- mouse (middle), and a Tmc1 -/- mouse injected with Anc80 Tmc1 (right).

Figure 32 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 Tmc1.

DETAILED DESCRIPTION

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 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, vestibular hair cells, vestibular ganglion neurons, supporting cells and cells in the stria vascularis. 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 deafnesses, dominant deafnesses, Usher syndrome, and other syndromic deafnesses 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) 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. One particular ancestral capsid protein that falls within the class of Anc80 ancestral capsid proteins is Anc80-0065 (SEQ ID NO:2), however, WO 2015/054653 describes a number of additional ancestral capsid proteins that fall within the class of Anc80 ancestral 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. 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 that encodes a neurotrophic factor (e.g., glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), or heat shock protein (HSP)-70), 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 XIAP. Descriptions and definitions of the nomenclature used herein can be found at hereditaryhearingloss.org/ on the World Wide Web.

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. 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 cytomegalovirus (CMV) promoter, a chicken beta actin (CBA) promoter, a synthetic CASI promoter, a phosphoglycerate kinase (PGK) promoter, and a elongation factor (EF)-1 promoter, an alpha9 nicotinic

receptor promoter, a prestin promoter, a growth factor independent (GFI1) promoter, and a vesicular glutamate transporter 3 (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.

Methods of making a transgene 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.

It would be appreciated that one or more than one transgene can be delivered to the inner ear. It also would be appreciated that more than one transgene can be delivered to the inner ear using a single AAV vector that includes an Anc80 capsid protein or using multiple AAV vectors that includes an Anc80 capsid protein.

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.

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 transgene as described herein can be delivered to inner ear cells (e.g., cells in the cochlea) using any number of mechanisms. 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 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).

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 to 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.

In particular, one can treat a patient diagnosed with Usher Syndrome-related deafness, e.g., USH1C-related deafness, using the ancestral AAV capsid proteins described herein combined with a harmonin transgene to successfully transduce hair cells and drive expression and correct localization of harmonin splice forms, thereby re-introducing wild-type harmonin. 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 restored 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.

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.

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 and are exemplified in the certain of the Examples below. The invention 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

Part 1: Highly Efficient Cochlear Gene Transfer

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/Anc80L65 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 (Zinn et al., 2015, Cell Reports, 12:1056-68). AAV2/Anc80L65 plasmid reagents are available through addgene.com.

In Vitro Explant Cultures

A total of 156 cochlear explant cultures from mouse pups of both strains were prepared on postnatal day 4 in order to assess as described in an earlier publication (Dilwali et al., 2015, Scientific Reports, 5:18599). In brief, murine temporal bones were harvested after decapitation and the cochlea was dissected to culture as organotypic explants connected to the spiral ganglion neuron region. Two specimens were obtained per cochlea, one ("apical") consisting of the lower apical and one ("basal") of the upper basal turn. For each serotype, a minimum of 4 (CBA/CaJ, 48h), 2 (CBA/CaJ, 48h+5d), 3 (C57BL/6, 48h), 2 (C57BL/6, 48h+5d) basal and apical specimen were inoculated. Specimens were excluded if cochlear morphology was not retained during the culture. Sample numbers were chosen to inform on the variability of transduction and to provide a basis for selection for further *in vivo* evaluation. Explants were incubated with culture medium (98% Dulbecco's Modified Eagle Medium (DMEM), 1% ampicillin, and 1% N2 supplement during the first 12 hours, plus 1% fetal bovine serum (FBS)) and 10^{10} GC of AAV for 48 h in 50 µl. For the 48h+5d condition, the medium with AAV was replaced with fresh media without AAV for an additional 5 days. Human vestibular epithelia from utricles obtained from four consented, adult patients undergoing vestibular schwannoma tumor resection were cultured as previously described (Kesser et al., 2007, Gene Ther., 14:1121-1131), exposed to 10^{10} GC AAV for 24 hours and maintained in culture for 10 days after which the tissue was fixed and stained with phalloidin and imaged. Studies were approved by the Surrey Borders NRES Committee London (Health Research Authority) under reference number 11/LO/0475.

Animal Models and General Methods

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.

CSF and Blood Sampling

Cerebrospinal fluid (CSF) sampling from the cisterna magna (Lui & Duff, 2008, J. Visualized Exp., 21:e960) and intracardiac blood collection with thoracotomy were performed in a terminal procedure. Through the microcapillary tube, the maximum amount (up to 5 μL) of clear CSF per animal was collected in a volume of 60 μL PBS, leading to slightly different starting dilutions that subsequently were standardized with additional control PBS prior to the start of the experiment. After obtaining the blood sample in a 1.1 mL Z-Gel micro tube (Sarstedt, Nümbrecht, Germany), it was spun down at 8,000 rpm for 8 minutes and serum was stored together with the CSF sample (in PBS) at -80°C until further use.

Example 1A—Histological Analysis

After a follow-up period of 5 to 29 days, animals were sacrificed and cochlear whole mounts were prepared as previously reported (Sergeyenko et al., 2013, J. Neurosci., 33:13686-94). Both cochlear whole-mounts and explants were stained with antibodies against myosin 7A (Myo7A, #25-6790 Proteus Biosciences, Ramona, CA, 1:400) and beta-tubulin (TuJ1, #MMS-435P Biolegend, San Diego, CA, 1:200), together with corresponding secondary antibodies (Alexa Fluor 555 anti-mouse and Alexa Fluor 647 anti-rabbit, #A-21422 and #A-21245 Thermo Fisher Scientific, Waltham, MA, 1:1000) (Dilwali et al., 2015, Scientific Reports, 5:18599). Mounting of the specimens was followed by confocal microscopy. Every image of a given experimental series was obtained with the same settings, with laser intensity being chosen based on the specimen with the strongest eGFP signal to prevent fluorescence saturation. Z-stacks for overview images and zoomed-in pictures for the organ of Corti and spiral ganglion neuron (SGN) areas were obtained. 3D reconstruction with AMIRA was used to determine SGN transfection more accurately.

The results in Figure 1 and Figure 4 illustrate the tropism of the 5 serotypes as monitored by expression of the AAV encoding eGFP in C57BL/6 and CBA/CaJ

respectively. Notably, eGFP expression was qualitatively brighter in cochlear cultures exposed to Anc80, with expression apparent in many cochlear cell types.

Example 1B—Quantification of eGFP-Expression

For *in vitro* data, the percentage of eGFP-positive inner (IHCs) and outer hair cells (OHCs) was manually quantified along the cochlea, by dividing the number of eGFP positive cells by the total number of outer or inner hair cells per one or two 100 μm sections per base and apex sample for each specimen. All visible SGNs in a cochlear explant were evaluated regarding their eGFP expression. The areas of the spiral limbus and supporting cells were assessed with a qualitative approach (as explained above, adjusted for each experimental series) by means of a scale from 0 (no expression) to 3 (strongest signal). Control samples without AAV were used to exclude autofluorescence. The data demonstrated that Anc80 targeted IHCs and OHCs at efficiencies between 60 and 100% in apex and base in both mouse strains tested. Anc80 demonstrated consistently and qualitatively brighter IHC and OHC eGFP expression as compared to AAV2 (Figure 1 and Figure 4).

To control for potential differences in the onset of expression between the different AAVs that may lead to an underestimate of expression at a 2-day (early) time point, a longer experiment was conducted. A new set of cochleae were transduced at identical conditions, yet following 48 h incubation with AAV the vector containing medium of the explant culture was removed and replaced with fresh media to maintain the culture viable for an additional 5 days (referred to as 48h+5d). A similar pattern of expression was observed in this longer-term study for AAV2 and Anc80. Moderate increases in expression for AAV6, 8, and 9 in CBA/CaJ mice, particularly at the basal turn (Figure 1J, 1K, and Figure 4I, 4J) were noted. Other cell types were targeted by all serotypes with limbus being more permissive than supporting cells followed by SGN (Figure 5, 6, and 7). Consistently, Anc80 transduction yielded higher efficiencies and stronger expression, evidenced by brighter eGFP fluorescence.

Example 1C—*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 2A, 2B). These studies indicate AAV2, 6, and 8 targeted low numbers of IHCs, with only AAV8 demonstrating roughly equivalent transduction in apex and base (Figure 2B). 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 1.36×10^{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 8E). The unparalleled OHC targeting (Figure 2C, Figure 8) 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 2A, B, C). Low magnification views of the cochlear apex (Figure 8A) showed strong eGFP expression far from the injection site. High magnification images of the base reveal 100% IHC and 95% OHC transduction (Figure 8B).

In some animals, robust eGFP expression was found in the contralateral uninjected ear (Figure 9). In mice, the cochlear aqueduct is patent providing a fluid path from the cochlear perilymph into the CSF, the contralateral aqueduct and into the contralateral cochlea. As such, it was also investigated whether Anc80-eGFP injected via the RWM was capable of transducing neurons in the brain. Indeed, cross-sections of the cerebellum revealed strong eGFP expression in cerebellar Purkinje neurons (Figure 10A, 10B).

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 (Kesser et al., 2007, Gene Ther., 14:1121-31). 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 1D—Immunological Assays

Antibody titers against Anc80 in CSF and serum were determined through neutralization assays (Zinn et al., 2015, Cell Reports, 12:1056-68). Using a 96-well format, heat-inactivated CSF or serum samples (collected as described above) were serially diluted in serum free medium (Life Technologies, Carlsbad, CA), and then treated with Anc80-luciferase (10^6 GC/well) for 1 hour at 37°C. The sample/Anc80-luciferase

mix was then transferred onto HEK293 cells, which were treated with adenovirus (MOI 20) the day before. After 1 hour at 37°C, diluted serum medium (1 part serum-free, 2 parts with serum) was added to each well.

Two days later, the cells were treated with lysis buffer (Promega, Madison, WI) and frozen at -80°C for 30 minutes. The cells were then thawed at 37°C for 15 minutes before being treated with substrate buffer (Tris-HCl, MgCl2, ATP (Life Technologies, Carlsbad, CA), D-Luciferin (Caliper Life Sciences, Hopkinton, MA)). Luminescence output was read using the Synergy BioTek Plate Reader (BioTek, Winooski, VT).

A low level of neutralization against the vector was detectable in serum of injected mice, but not in CSF, at the level of sensitivity of the assay and sampling (Figure 10C).

Example 1E—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 NaH_2PO_4, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 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 Na_2ATP, 3.5 $MgCl_2$, and 0.1 $CaCl_2$ (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% CO_2 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 2D). 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 2E), which confirmed that Anc80 transduction had no detrimental effects on sensory cell function.

Example 1F—Hearing Tests

Auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) data were collected as described previously (Askew et al., 2015, Science Translational Med., 7:285ra108). DPOAE is an assay for proper cochlear amplification and tuning and is a sensitive measure of outer hair cell viability (Guinan et al., 2012, Hearing Res., 293:12-20). 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 2F) 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 2F), 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 2G). 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 1G—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 (Parker & Bitner-Glindzicz, 2015, Archives Dis. Childhood, 100:271-8). Previous studies highlighted the ability of this rotarod test to detect balance dysfunction when only one ear is affected (Fukui & Raphael, 2013, Hearing Res., 297:99-105; Geleoc & Holt, 2014, Science, 344:1241062). 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 (Fukui & Rapheal, 2013, Hearing Res., 297:99-105) 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 3A, 3B). 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 11).

Part 2—Gene Therapy Restores Function in a Mouse Model of Usher Syndrome

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 10 mM HEPES (pH 7.4). The organ of Corti was dissected away without the use of enzyme as

described previously. 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. Mice were genotyped using toe clip (before P8) or ear punch (after P8) and PCR was performed as described previously (Lentz et al., 2007, Mutat. Res., 616:139-44). 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 (SEQ ID NO:9); Trunc-harmomin.RV(BamHI) CAG GAT CCG GAC AAT TTC ATC CCC TAC (SEQ ID NO:10). 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

(Zheng et al., 2010, J. Neurosci., 30:7187-201) (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. The harmonin-a1 construct was further modified 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 24 h. 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-1 µl 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, Sci. Transl. Med., 7:295ra108). 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 NaH_2PO_4, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 5.6 D-glucose, and 10 HEPES-NaOH, adjusted to pH 7.4 and 320 mOsmol/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 K_2ATP, 3.5 $MgCl_2$, 0.1 $CaCl_2$, 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 (SD) or standard error of the mean (SEM) 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) in the Mouse Usher Model

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. 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 (Lentz et al., 2010, Dev., Neurobiol., 70:253-67) 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 12A-12C, 12G, 12I and Figure 19A-19C, 19K). 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 (Lentz et al., 2013, Nat. Med., 19:345-50) (Figure 19D-19F).

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 18). 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 18).

Example 2B—FM1-43 Imaging in the Usher Mouse Model

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. Uniform FM1-43 uptake was observed in hair cells of c.216GA mice (Figure 13A), but the level of uptake varied among OHCs of c.216AA mice, suggesting that some, but not all, cells retained functional transduction channels (Figure 13B). 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 13C, 13D).

Example 2C—Mechanical Stimulation in the Usher Mouse Model

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). The tip of the probe was fired polished (Fire polisher, H602, World Precision Instruments Inc., Sarasota, FL) to fit stereociliary bundle (Stauffer & Holt, 2007, J. Neurophysiol., 98:3360-9). 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 ± 2 μm 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 12). 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 13E, 13F, 13G). 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 13F) and the fit was used to determine the 10-90% operating range (Figure 20B). 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 20G).

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 20C, 20D, 20H, 20I). 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 13E, 13F, 13H). 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 13C, 13D). 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 (Zinn et al., 2015, Cell Rep., 12:1056-68). As shown herein, the Anc80 capsid transduces 100% of IHCs and 80-90% of OHCs *in vivo*. It was hypothesized 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 15. 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 15C). 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 in the Usher Mouse Model

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 14A-14C, 14E). EGFP::harmonin-b1 signal was evident at the tips of the stereocilia in VHCs (Figure 14A), IHCs and OHCs (Figure 14B, 14C). 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 and harmonin-a to the synapse.

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 = 10 dB 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 0 dB 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 23C-23D). 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 16A-16C), 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 <45 dB 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 21). 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 16) 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 16).

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 22), 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 and likely occurs via the perilymphatic duct that remains continuous with the subarachnoid space in newborn mice.

We also examined 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® Reverse Transcription Kit (Qiagen). cDNA encoding full length (450 bp) or truncated harmonin (-35 bp) was amplified using the following primers: Forward primer mUsh1c_Ex2F: 5' CTC ATT GAA AAT GAC GCA GAG AAG G 3' (SEQ ID NO:11), Reverse mUsh1c_Ex5R: 5' TCT CAC TTT GAT GGA CAC GGT CTT 3' (SEQ ID NO:12). 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 (SEQ ID NO:13)) and mUsh1c_Ex4WTR (5'-TGC AGA CGG TCC AAG CGT-3' (SEQ ID NO:14)) 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 (Lentz et al., 2007, Mutat. Res., 616:139-44). 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' (SEQ ID NO:15)) and mGapdh_Ex4R (5'-GCC AAA GTT GTC ATG GAT GAC-3' (SEQ ID NO:16)). 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, it was explored whether persistent expression of the truncated protein may limit recovery in

c.216AA mice injected with vectors that express exogenous full-length harmonin (Figure 23A). 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 23B).

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 24A) and AAV2/Anc80.CMV.harmonin-b1 DNA (Figure 24B) 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 24C). 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 25). 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 25).

Example 2G—Acoustic Startle Responses in the Usher Mouse Model

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 (27 cm × 10 cm × 12.5 cm) 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 30 s (25-35 s 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 17A). Mice that received harmonin-a1 alone were similar to uninjected c.216AA mice and did not recover startle responses.

Example 2H—Vestibular Assessment in the Usher Mouse Model

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 17B).

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 17D-17E). 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 17C-17E).

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 17). 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 13).

While auditory rescue was prominent at low but not high frequencies (Figure 16), preservation of hair bundle morphology at 6 weeks was observed along the entire organ (Figure 18). 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 23C-23D). 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.

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

Example 3A—*In Vivo* Experiments

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 26 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. Thus, Anc80 is an effective vector for treating a number of different genetic defects (at a number of different genetic loci) that result in hearing loss.

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 29 shows recovery of potassium currents to near wild type levels (Figure 29A) in KCNQ4 -/- cells transfected with Anc80-KCNQ4 (Figure 10C) relative to the mutant mice (Figure 29B), thereby demonstrating that gene therapy using Anc80 is capable of restoring function.

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 27 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:17) and 5'-AGA GGT AGC CGG AAA TTC AGC CAT-3' (SEQ ID NO:18). 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:19) and 5'-ACT CAT CGT ACT CCT GCT TGC TGA-3' (SEQ ID NO:20). 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.

These results demonstrate that, in injected cars, 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 30 are the immunostaining images showing uptake of FM1-43 dye by cells exposed to an Anc80 viral vector as described herein, and Figure 31 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 32 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).

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.

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.

WHAT IS CLAIMED IS:

1. Use of an adeno-associated virus (AAV) vector comprising an Anc80 capsid protein having an amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 and a nucleic acid molecule comprising one or more transgenes selected from the group consisting of TMC1, TMC2, MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, and PDZD7 in the manufacture of a pharmaceutical composition for the treatment of a hearing disorder associated with a genetic defect, or for the prevention of hearing loss associated with a genetic defect, wherein the one or more transgenes are to be delivered to an inner ear of a subject.

2. Use of an adeno-associated virus (AAV) vector comprising an Anc80 capsid protein having an amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 and a nucleic acid molecule comprising one or more transgenes in the manufacture of a pharmaceutical composition for the treatment of a hearing disorder associated with a genetic defect, or for the prevention of hearing loss associated with a genetic defect, wherein the AAV is to be administered to an inner ear of a subject, and wherein the one or more transgenes include one or more of 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, or XIAP,

thereby delivering the transgene to one or more cells in the inner ear of the subject.

3. The use of claim 1 or 2, wherein the transgene is to be delivered to at least 80% of inner hair cells (IHCs) of the inner ear and to at least 80% of outer hair cells (OHCs) of the inner ear.

4. The use of claim 1 or 2, wherein the transgene is a TMC1 or TMC2 transgene.

5. The use of claim 1 or 2, wherein the transgene is an Usher transgene.

6. The use of claim 5, wherein the Usher transgene is selected from the group consisting of MYO7A, USCH1C, CDH23, PCDH15, SANS, CIB2, USH2A, VLGR1, WHRN, CLRN1, PDZD7.

7. The use of any one of claims 1 to 6, wherein the transgene is further delivered to one or more cells in the inner ear selected from the group consisting of spiral ganglion neurons, vestibular hair cells, vestibular ganglion neurons, supporting cells, and cells in the stria vascularis.

8. The use of any one of claims 1 to 6, wherein the Anc80 capsid protein has the sequence shown in SEQ ID NO:1.

9. The use of any one of claims 1 to 6, wherein the Anc80 capsid protein has the sequence shown in SEQ ID NO:2.

10. The use of any one of claims 1 to 6, wherein the transgene is under control of a heterologous promoter sequence.

11. The use of claim 10, wherein the heterologous promoter sequence is selected from the group consisting of a CMV promoter, a CBA promoter, a CASI promoter, a PGK promoter, a EF-1 promoter, an alpha9 nicotinic receptor promoter, a prestin promoter, a KCNQ4 promoter, a Myo7a promoter, a Myo6 promoter, a Gfi1 promoter, a Vglut3 promoter, and an Atoh1 promoter.

12. The use of claim 2, wherein the transgene encodes a neurotrophic factor.

13. The use of claim 12, wherein the neurotrophic factor is selected from the group consisting of GDNF, BDNF, NT3, and HSP70.

14. The use of claim 2, wherein the transgene encodes an antibody or fragment thereof.

15. The use of claim 2, wherein the transgene encodes an immunomodulatory protein.

16. The use of claim 2, wherein the transgene encodes an anti-oncogenic transcript.

17. The use of claim 2, wherein the transgene encodes an antisense, silencing, or long non-coding RNA species.

18. The use of claim 2, wherein the transgene encodes a genome editing system selected from the group consisting of a genetically-engineered zinc finger nuclease, TALEN, and CRISPR.

19. The use of any one of claims 1 to 6, wherein the AAV vector is to be administered via injection through the round window.

20. The use of any one of claims 1 to 6, wherein the AAV vector is to be administered during a cochleostomy or during a canalostomy.

21. The use of any one of claims 1 to 6, wherein the AAV vector is to be administered to the middle ear and/or the round window via one or more drug delivery vehicles.

22. The use of any one of claims 1 to 6, wherein expression of the transgene results in regeneration of inner hair cells (IHCs), outer hair cells (OHCs), spiral ganglion neurons, stria vascularis, vestibular hair cells, and/or vestibular ganglion neurons, thereby restoring hearing or vestibular function.

23. An article of manufacture comprising an adeno-associated virus (AAV) vector and a pharmaceutical composition, wherein the AAV vector comprises an Anc80 capsid protein having an amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 and a nucleic acid molecule comprising one or more transgenes operably linked to a promoter, for use in a method of delivering the transgene to at least 80% of inner hair cells (IHCs) and to at least 80% of outer hair cells (OHCs) in a subject's inner ear to treat a hearing disorder associated with a genetic defect, wherein the one or more transgenes are selected from the group consisting of 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 XIAP.

24. The use of claim 1, wherein the one or more transgenes are to be delivered to at least 80% of inner hair cells (IHCs) of the inner ear.

25. The use of claim 1, wherein the one or more transgenes are to be delivered to at least 90% of inner hair cells (IHCs) of the inner ear.

26. The use of claim 1, wherein the one or more transgenes are to be delivered to at least 95% of inner hair cells (IHCs) of the inner ear.