

Gene therapy restores auditory and vestibular function in a mouse model of Usher syndrome type 1c

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Because there are currently no biological treatments for hearing loss, we sought to advance gene therapy approaches to treat genetic deafness. We focused on Usher syndrome, a devastating genetic disorder that causes blindness, balance disorders and profound deafness, and studied a knock-in mouse model, *Ush1c c.216G>A*, for Usher syndrome type IC (USH1C). As restoration of complex auditory and balance function is likely to require gene delivery systems that target auditory and vestibular sensory cells with high efficiency, we delivered wild-type *Ush1c* into the inner ear of *Ush1c c.216G>A* mice using a synthetic adeno-associated viral vector, Anc80L65, shown to transduce 80–90% of sensory hair cells. We demonstrate recovery of gene and protein expression, restoration of sensory cell function, rescue of complex auditory function and recovery of hearing and balance behavior to near wild-type levels. The data represent unprecedented recovery of inner ear function and suggest that biological therapies to treat deafness may be suitable for translation to humans with genetic inner ear disorders.

Hearing loss affects ~250 million people worldwide, with ~50% of cases due to inherited genetic mutations. Recent studies in animal models suggest that gene therapy in the inner ear may correct genetic deafness in humans. However, progress has been limited and has failed to restore the exquisite sensitivity of the healthy mammalian inner ear. A major hurdle is the lack of efficient vectors that target large numbers of sensory hair cells in the inner ear. For example, AAV2/8 vectors that encode wild-type whirlin rescued inner hair cell (IHC) but not outer hair cell (OHC) morphology or hearing function¹. IHCs account for ~25% of total auditory hair cells. The other 75% are OHCs, which enhance the sensitivity and frequency selectivity of the inner ear. In a study using AAV2/1 vectors injected in *Tmc1* mutant mice, the gene therapy targeted 80–90% of IHCs and enabled moderate auditory rescue, with minimal auditory brainstem response thresholds of ~80 dB (8 kHz)². A similar viral capsid and a promoter that restricted expression to IHCs partially restored auditory function (thresholds ~60 dB at 8 kHz) in mice deficient in the IHC gene *Vglut3* (ref. 3). While these studies have demonstrated exogenous gene delivery into auditory IHCs, gene delivery to OHCs has remained a challenge. Because many genes that cause deafness are expressed in both IHCs and OHCs, gene delivery methods that target both cell types are needed to restore complex auditory function. Among the genes expressed in both cell types are those that encode Usher syndrome proteins.

Usher syndrome (USH) is a rare genetic condition that affects 16,000–20,000 people in the United States and is responsible for 3–6%

of early childhood deafness^{4–6}. Of three subtypes, Usher type I (USH1) is the most severe form. USH1 patients suffer profound sensorineural hearing loss, vestibular areflexia and progressive blindness. Unless fitted with a cochlear implant, USH1 patients do not recover auditory function or develop the ability to speak. Six genes are associated with USH1: *MYO7A* (myosin 7a)^{7–10}, *USH1C* (harmonin)^{11,12}, *CDH23* (cadherin 23)^{13,14}, *PCDH15* (protocadherin 15)^{15–17}, *SANS* (sans; also known as *USH1G*)¹⁸ and *CIB2* (calcium and integrin binding protein 2)¹⁹. USH1 proteins are involved in morphogenesis of sensory hair bundles and are localized to the apex of hair cells. Harmonin resides at the core of an USH1 interactome, where it binds to other USH1 proteins. The *USH1C* gene includes 28 exons, which code for ten alternative splice forms^{11,12} grouped in three different subclasses (a, b and c), depending on the domain composition of the protein. In hair cells, harmonin is expressed during early postnatal stages but expression declines around postnatal day 30 (P30). The harmonin-b splice form is found at the tips of stereocilia near the tip-link insertion point in mouse hair cells^{20–22}, where it has a structural role and is likely required for sensory transduction in both auditory and vestibular hair cells^{20,23}. Harmonin-a is localized at the synapse, where it associates with Ca_v1.3 Ca²⁺ channels and limits channel availability through a ubiquitin-dependent pathway^{24,25}.

Of several mouse models for Usher syndrome characterized over the past decade, only one, *Ush1c c.216G>A*, reproduces both auditory and retinal deficits typical of human Usher I syndrome²⁶.

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The *Ush1c* c.216G>A mutation affects expression of all conventional harmonin isoforms due to a point mutation similar to one found in a cohort of French-Acadian USH1C patients²⁷. The mutation introduces a cryptic splice site at the end of exon three in the *Ush1c* gene resulting in a severely truncated protein²⁸. Homozygous *Ush1c* c.216G>A mice (c.216AA) suffer from severe hearing loss at 1 month of age²⁶, while heterozygous c.216GA mice are phenotypically normal. Cochlear histology in c.216AA mice reveals disorganized hair bundles and loss of both IHCs and OHCs in the middle and basal turns of the cochlea at P30 (ref. 26).

To identify a therapeutic window for treatment, we studied the function and maintenance of hair cells in newborn c.216AA mice. Our data show that a large majority of c.216AA hair cells survive and remain mechanosensitive during the first postnatal week. Since reduced expression of full-length harmonin is the most likely cause of deafness in *USH1C* c.216G>A patients²⁷, we investigated whether early reintroduction of full-length harmonin in c.216AA hair cells would preserve hair cell and auditory function in homozygous c.216AA mice. We hypothesized that expression of exogenous harmonin in larger percentages of sensory hair cells would be required for complex auditory and vestibular function. Since Anc80L65, a synthetic adeno-associated viral vector, efficiently transduces both auditory and vestibular hair cells *in vivo*²⁹, we designed Anc80L65 vectors encoding harmonin-a1 or harmonin-b1. The vectors successfully transduced large numbers of IHCs, OHCs and vestibular cells and drove harmonin expression and correct protein localization. Early postnatal round window membrane injection of harmonin vectors successfully restored auditory and vestibular function to near wild-type levels in otherwise deaf and dizzy c.216AA mice. Our results demonstrate that early reintroduction of wild-type harmonin with vectors that efficiently target IHCs and OHCs can improve auditory sensitivity by over a thousand-fold relative to first-generation inner ear gene therapy approaches.

RESULTS

Hair cell survival in *Ush1c* mice

Homozygous c.216AA mice are deaf and show circling and head tossing behaviors characteristic of vestibular dysfunction²⁶. At 1 month of age, there is pronounced IHC and OHC degeneration at the base of the cochlea and degeneration and hair cell death in the middle turn, while the apical portion of the organ is better preserved²⁶. To assess hair cell survival at earlier stages, we performed scanning electron microscopy (SEM) analysis along the organ of Corti at P8 and P18. OHCs and IHCs of heterozygous c.216GA mice were preserved and their hair bundles were properly oriented (Fig. 1a–c,g,i and Supplementary Fig. 1a–c,k). However, disorganized hair bundles were evident along the entire length of the organ of Corti in homozygous c.216AA mice at both ages analyzed (Fig. 1d–f,h,j–l and Supplementary Fig. 1d–j,l). At P8, IHC bundles were mildly disorganized at the base, mid and apical regions (Fig. 1d–f,j). While many OHCs of c.216AA mice possessed well-preserved hair bundles (Fig. 1h,k), fragmented and disorganized hair bundles were evident sporadically along the organ (Fig. 1d–f,l). Disruption was more pronounced at P18, although the majority of hair cells were still present as previously reported³⁰ (Supplementary Fig. 1d–f).

To assess hair cell function at early stages, we analyzed uptake of FM1-43, a compound that permeates functional mechanosensitive channels^{31–33}, in inner ear organs at P4. Uniform FM1-43 uptake was observed in hair cells of c.216GA mice (Fig. 2a), but uptake varied among OHCs of c.216AA mice, suggesting that some, but not all, cells

retained functional channels (Fig. 2b). FM1-43 uptake decreased in IHCs of c.216AA mice during the first postnatal week (data not shown). In hair cells of the utricle of c.216AA mice, uptake was restricted to the extra-striola region at P6, suggesting that hair cells of the striola region lacked mechanosensitive channels open at rest (Fig. 2c,d).

Sensory transduction in *Ush1c* hair cells

Next, we assessed mechanotransduction currents in IHCs, OHCs and vestibular hair cells (VHCs) of c.216GA and c.216AA mice during the first postnatal week. Mechanotransduction currents were recorded in whole-cell voltage-clamp configuration in response to mechanical displacements of hair bundles by stiff glass probes. In c.216AA mice, recordings were obtained from the middle and apical turns of the cochlea from P3 to P6. OHCs retained mechanosensitivity, although the amplitudes of the responses were reduced by ~63% to 170 ± 80 pA ($n = 24$; $P < 0.001$, Fig. 2e–g). Severely disorganized bundles had smaller currents than better preserved hair bundles (120 ± 65 pA, $n = 9$ and 201 ± 74 pA, $n = 15$, respectively). Despite the reduction in current amplitude, c.216AA OHC sensitivity to mechanical displacements was similar to that of heterozygous c.216GA hair cells (Fig. 2f and Supplementary Fig. 2b).

Similarly, while hair bundles from IHCs of c.216AA mice appeared only mildly disrupted under the differential interference contrast (DIC) microscope, transduction currents were markedly reduced at P6 (Fig. 2e–g). 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. c.216AA IHCs showed a significant ($P < 0.01$) reduction in sensitivity (Supplementary Fig. 2g). Adaptation, or the decline in transduction current in the presence of constant bundle deflection, was also present, but the rate and extent of adaptation was reduced in c.216AA hair cells relative to c.216GA cells (Supplementary Fig. 2c–e,h–j). These results demonstrate that mechanosensitivity is mildly compromised in IHCs and OHCs 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.

Vestibular hair cells of c.216AA mice also showed a reduction in mechanotransduction currents. In the extra-striola region, 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 (Fig. 2e,f,h). Very small or no currents were evoked from hair cells of the striola region (6 ± 13 pA, $n = 6$, P5–P7), in agreement with the absence of FM1-43 uptake in that region (Fig. 2c,d). In conclusion, while hair bundles of the utricle appeared grossly well-preserved, transduction currents were significantly reduced in the extra-striola and absent in the striola.

Rescue of sensory transduction in *Ush1c* hair cells

To assess whether AAV vectors can drive expression of exogenous harmonin splice forms, we exposed P1 utricles and organs of Corti from neonatal c.216AA, c.216GA and wild-type (C57BL/6J) mice to AAV2/1 vectors encoding EGFP fused to the N terminus of harmonin-b1 (EGFP::harmonin-b1) or tdTomato fused to the N terminus of harmonin-a1 (tdTomato::harmonin-a1). Hair cells of wild-type, c.216GA and c.216AA mice were successfully transduced *in vitro* (Fig. 3a–c,e). EGFP::harmonin-b1 signal was evident at the tips of the stereocilia in VHCs (Fig. 3a), IHCs and OHCs (Fig. 3b,c). EGFP signal was also detected at P60 in OHCs and IHCs in the basal portion of the cochlea of mice injected at P1 (Fig. 3d). TdTomato::harmonin-a1 was detected at the base of auditory hair cells (Fig. 3e). Co-staining with a ribbon synapse marker CTBP2 frequently revealed colocalization in P7 IHCs

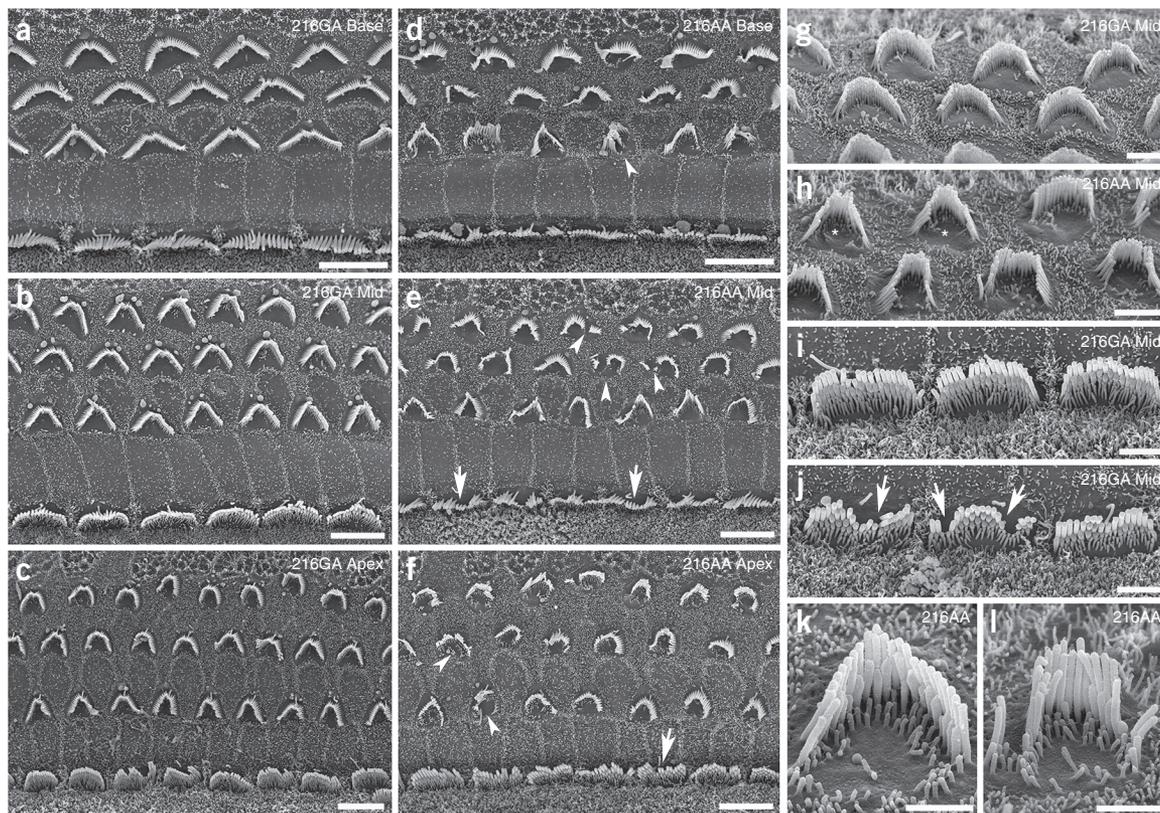


Figure 1 SEM of the organ of Corti in *Ush1c* c.216G>A mice at P8. (a–f) Basal, middle and apical regions of the organ of Corti were imaged in P8 c.216GA ($n = 3$ mice) and c.216AA ($n = 4$ mice) mice. OHC and IHC hair bundles were preserved in heterozygous mice but some hair bundles appeared disorganized along the organ of Corti in homozygous c.216AA mice. (g–l) High-magnification images revealed fragmented and disorganized bundles with disruptions in the staircase array in many but not all OHCs (g,h) and IHCs (i,j). Examples of OHC hair bundles imaged in the middle region of the organ at P8 illustrate a preserved (k) and a disorganized hair bundle (l) present in the same preparation. Stars indicate preserved hair bundles; arrowhead, disorganized hair bundles; and arrows, wavy IHC bundles. Scale bars, low mag., 5 μm (a–f); high mag., 2 μm (g), 3 μm (h), 2 μm (i–j) and 1 μm (k,l).

(Fig. 3e) but not in P7 utricles (data not shown). Localization of our exogenous fusion constructs was consistent with previous work that localized harmonin-b to the distal end of stereocilia, near the tip-link insertions^{20–22}, and harmonin-a to the synapse^{24,25}.

Next, we assessed function in c.216AA hair cells exposed to AAV vectors driving harmonin expression. To enhance the likelihood of functional rescue, we packaged untagged harmonin-a1 or harmonin-b1 coding sequences driven by a cytomegalovirus (CMV) promoter into AAV2/Anc80L65 (ref. 34). We performed round window membrane (RWM) injections at P1 of AAV2/Anc80L65.CMV.harmonin-b1 and separately a mixture of AAV2/Anc80L65.CMV.harmonin-a1 + AAV2/Anc80L65.CMV.harmonin-b1 and assessed mechanotransduction responses 2 weeks after treatment. Although mature OHCs (>P10) do not survive *ex vivo* recording experiments, robust electrophysiological recordings were obtained from IHCs at the equivalent of P14–P16 (Fig. 4). While IHCs from uninjected mice displayed severely reduced transduction currents at P16 (79 ± 43 pA, $n = 8$), significant recovery ($***P < 0.001$) was observed in mice injected at P1 with harmonin-b1 or a combination of harmonin b1 and a1, with respective average maximal transduction currents of 388 ± 66 pA ($n = 15$) and 352 ± 28 pA ($n = 7$; Fig. 4c). Transduction current amplitudes in IHCs after treatment with harmonin-b1 or the combination of harmonin b1 and a1 were not significantly different from those of control c.216GA mice. These results suggest that delivery of exogenous harmonin-b1 via RWM injection at early stages can restore mechanotransduction in IHCs.

Rescue of auditory brainstem responses in *Ush1c* mice

To investigate rescue of auditory and balance function in *Ush1c* mice, we performed P0–P1 RWM injections of AAV2/Anc80L65.CMV.harmonin-a1 and/or AAV2/Anc80L65.CMV.harmonin-b1 and later assessed auditory brainstem responses (ABRs), distortion product otoacoustic emissions (DPOAEs), acoustic startle reflexes, open field and rotarod behavior. Mice were assessed at 6 weeks, a stage at which c.216AA mice show 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/Anc80L65.CMV.harmonin-a1 recovered auditory function at 6 weeks (Fig. 5a–c), suggesting exogenous expression of harmonin-a1 was insufficient for auditory rescue. However, 19 of 25 mice injected with AAV2/Anc80L65.CMV.harmonin-b1 recovered considerable auditory function (ABR thresholds < 80 dB) at 6 weeks. The reason for lack of rescue in 6 of 25 mice was not clear, but may have been due to failed injections, clogged injection pipettes or injections that missed the targeted perilymphatic space. At low frequencies (5.6–16 kHz), the lowest ABR thresholds in AAV2/Anc80L65.CMV.harmonin-b1 injected ears were 25–30 dB, remarkably similar to thresholds of wild-type mice (Fig. 5a,b). Partial rescue was observed at 22.6 kHz and little to none at 32 kHz. Rescue of DPOAE thresholds was also evident (Fig. 5c) at low frequencies, consistent with rescue of OHC function. Eight of the mice that possessed auditory thresholds < 45 dB 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 ABR threshold

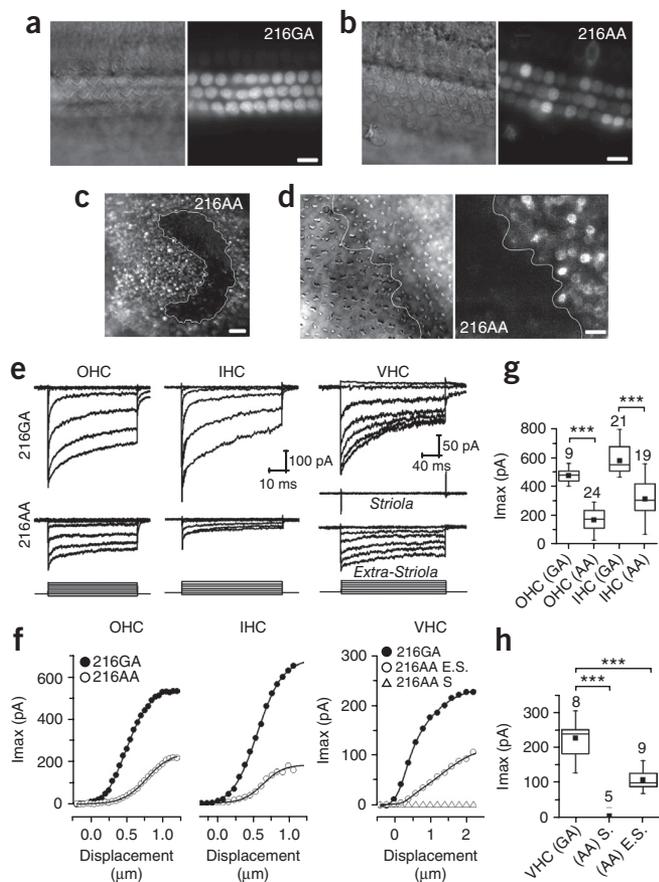


Figure 2 Mechanotransduction in hair cells of *Ush1c.c.216G>A* neonatal mice. (a–d) The permeable styryl dye FM1-43 was used to assess the presence of open transduction channels in hair cells of c.216GA and c.216AA mice. FM1-43 uptake was reduced in sensory hair cells of c.216AA mice at P4 (a,b, mid-base). Note that IHC FM1-43 fluorescence appears dimmer as IHCs are in a different focal plane. Left: DIC, right: FM1-43; scale bar, 10 μ m. In the utricle, FM1-43 uptake was restricted to the extra-striola region in c.216AA mutants at P6 (c; scale bar, 50 μ m) while utricular hair cells retained gross normal bundle morphology as assessed by DIC (d; scale bar, 10 μ m). The white lines on c and d delineate the striola (no uptake) and extra-striola regions (uptake). The experiment was repeated three times. (e–h) Mechanotransduction was assessed in OHCs, IHCs and VHCs in neonatal c.216GA and c.216AA mice (number of mice recorded from are: $n = 7$, 6 for OHCs, $n = 2$, 4 for IHCs and $n = 2$, 6 for VHCs, number of cells are indicated above the bar graph; holding potential: -60 mV). Representative transduction currents (e), their associated current/displacement plots fitted with a second order Boltzmann function (f) and average peak transduction current are plotted (g,h). In the cochlea, recordings were obtained in the middle and mid-apical turn of the organ at P3–P6. In the utricle transduction currents were recorded from VHCs of the extra-striola (E.S.) and striola (S.) region between P5 and P7 (e,f). While hair bundles appeared well preserved under DIC microscopy, smaller average transduction currents were evoked in c.216AA mutants (h). Average peak transduction was significantly different between the two genotypes in OHCs, IHCs and VHCs (*** $P < 0.01$, one-way ANOVA).

shifts were observed in the low frequency range and ~ 30 dB in the high frequency range (Fig. 5d). A similar shift was also observed in the DPOAEs thresholds (Fig. 5e). After this time point, ABR and DPOAE thresholds remained stable for up to 6 months (Fig. 5d–e), 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

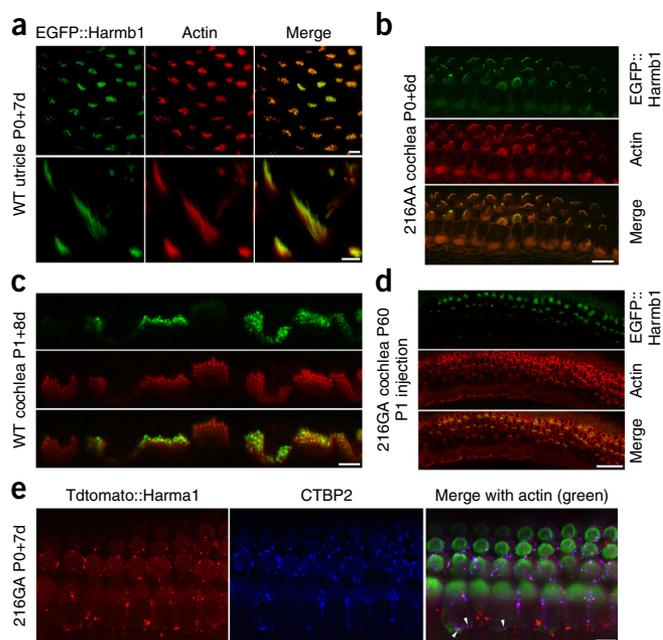


Figure 3 Expression and localization of fluorescently labeled harmonin in tissues exposed to adeno-associated viral vectors *in vitro* and *in vivo*. (a–c) Acutely dissected P0–P1 inner ear tissue was exposed to AAV2/1 vectors for 24 h, kept in culture for 7–8 d before being fixed, counterstained (Alexa Fluor phalloidin, Invitrogen) and imaged with a Zeiss LSM confocal microscope. A large number of sensory hair cells were infected in wild-type utricle and expression of harmonin-b1 fused to EGFP was evident in most hair cells with specific localization at the apex of the sensory hair bundle (a, scale bar, 10 μ m, upper panels; 5 μ m, lower panels). Similarly expression of EGFP::harmonin-b1 was evident at the tip of the stereocilia in OHCs and IHCs of c.216AA and wild-type mice (b, scale bar, 10 μ m; c, scale bar, 3 μ m). (d) When 1 μ l AAV2/1.CMV.EGFP::harmonin-b1 was injected at P1, EGFP signal was detected in some IHCs and OHCs at P60 in the injected ear (scale bar, 30 μ m). (e) Exogenous tdTomato::harmonin-a1 was detected in the cell body of IHCs and OHCs in P7 organotypic cultures exposed to AAV2/1.CMV.tdTomato::harmonin-a1 for 24 h at P0 (scale bar, 5 μ m). Some harmonin-a1 puncta were colocalized with CTBP2 (blue; mouse anti-CTBP2 1/200) in particular at the base of the sensory cells presumably near the ribbon synapse (arrowheads). No expression was observed in the stereociliary bundle.

end, we co-injected two vectors, each driving expression of one splice form of harmonin. To confirm that both were targeted to the hair cells, we co-injected AAV2/Anc80L65.CMV.tdTomato::harmonin-a1 and AAV2/Anc80L65.CMV.EGFP::harmonin-b1 at P0–P1 (0.5 μ l + 0.5 μ l). 65% of the hair cells expressed both harmonin-a1 and harmonin-b1, as evident from cells positive for both fluorescent tags (Supplementary Fig. 3). Fluorescently labeled harmonin-a1 was occasionally observed in the stereocilia of mice exposed to AAV2/Anc80L65.CMV.tdTomato::harmonin-a1, perhaps due to overexpression. ABR and DPOAE thresholds in mice co-injected with unlabeled harmonin-a1 and harmonin-b1 vectors (Fig. 5) were similar to those of mice injected with harmonin-b1 alone, suggesting that harmonin-a1 may be dispensable for auditory function. Notably, harmonin-b1 alone was sufficient for significant restoration of auditory thresholds at low frequencies (Fig. 5).

To further characterize the rescue, we analyzed and compared ABR waveforms from eight control c.216GA mice and five c.216AA mice, injected with AAV2/Anc80L65.CMV.harmonin-b1, with thresholds ≤ 45 dB. The analysis of waveforms at 8, 11.3 and 16 kHz revealed

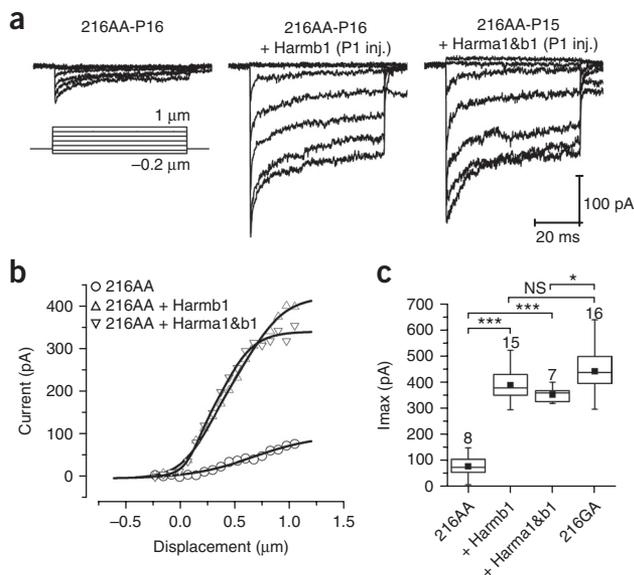


Figure 4 Recovery of mechanotransduction in hair cells of mice injected with Anc80L65 harmonin vectors. (a–c) Mechanotransduction currents were recorded in IHCs of c.216AA un.injected control mice ($n = 8$ cells, one mouse) and c.216AA mice injected at P1 with AAV2/Anc80L65.CMV.harmonin-b1 (0.8 μ l, 1.9×10^{12} gc/ml, $n = 15$ cells, one mouse) or a combined injection of the AAV2/Anc80L65.CMV.harmonin-a1 (1.7×10^{12} gc/ml) and AAV2/Anc80L65.CMV.harmonin-b1 (0.5 μ l + 0.5 μ l, $n = 7$ cells, one mouse). Tissue was extracted at P5–P6, before the cochlea became ossified, and was maintained in culture for 9 to 10 d. While small mechanotransduction currents could be induced by hair bundle stimulations of c.216AA mice, larger currents were evoked in c.216AA mice injected with vectors driving harmonin-b1 or dual harmonin-a1/b1 expression (a). Corresponding I/X curve for each data set fit with a double Boltzmann function. Respective maximal mechanotransduction current $I_{max} = 102.1$ pA (c.216AA); 424.3 pA (c.216AA + harmonin-b1) and 341.1 pA (c.216AA + harmonin-a1 and harmonin-b1) (b). Average responses (mean \pm s.d.) show significant recovery of transduction ($***P < 0.001$) for harmonin-b1 and harmonin-a1 + harmonin-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. One-way ANOVA (c).

normal wave 1 amplitudes, relative (non-significant differences, $P > 0.2$, Student's t -test) and longer peak 1 latencies ($P < 0.001$) (Supplementary Fig. 4), 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 at 11.3 kHz (harmonin-b1: average 59.7 ± 5.3 dB, $n = 15/25$; harmonin-a1 + harmonin-b1: average 76.2 ± 10.3 dB, $n = 4/6$). Diffusion of AAV vectors to the contralateral ear has been previously observed³⁵ and likely occurs via the cochlear aqueduct.

To determine whether injections at a later developmental stage might lead to partial auditory rescue, we performed RWM injections of AAV2/Anc80L65.CMV.harmonin-b1 (0.8 μ l) at P10–P12 and assessed auditory thresholds at 6 weeks. None of these mice had detectable DPOAEs, and their ABR thresholds did not differ from the uninjected c.216AA control mice ($n = 10$; data not shown).

To address the concern that truncated harmonin, a consequence of the c.216G>A mutation, may disrupt function by competing with

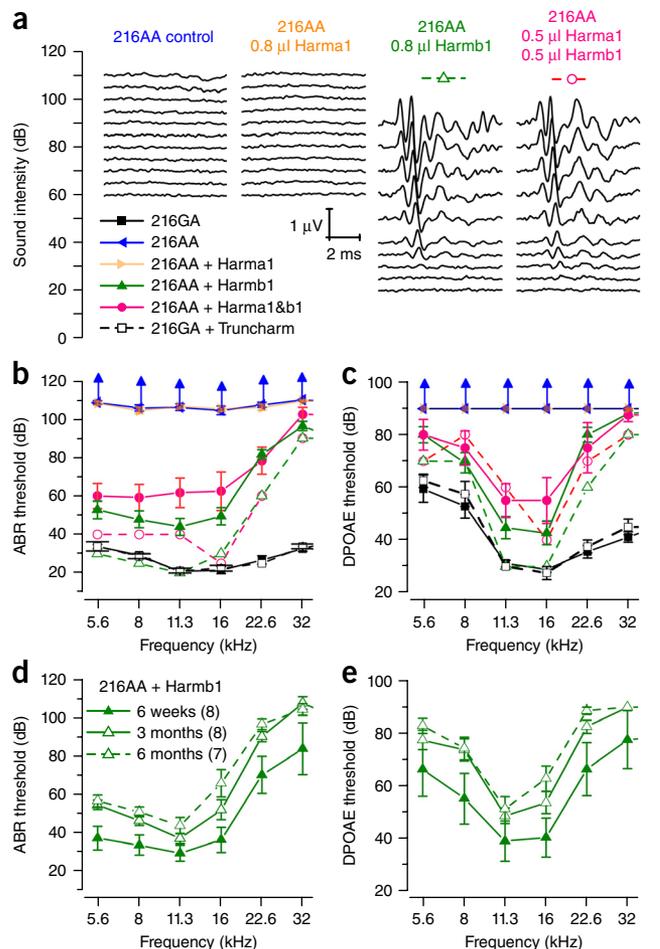


Figure 5 ABR and DPOAE threshold recovery in mice injected at P1 with AAV2/Anc80L65.CMV.harmonin-b1. (a) Representative ABR responses for 16 kHz tones in 6-week-old c.216AA control mice and c.216AA mice injected at P1 via RWM injection of vectors encoding harmonin-a1 (0.8 μ l, 1.7×10^{12} gc/ml), harmonin-b1 (0.8 μ l, 1.9×10^{12} gc/ml) or a combination of the two (0.5 μ l + 0.5 μ l). Recovered ABR thresholds near 30 dB were measured in mice injected with harmonin-b1 alone or harmonin-a1/b1 together. (b) Mean ABR responses obtained for: c.216 AA ($n = 13$); c.216GA ($n = 12$); c.216GA + trunc-harmonin ($n = 4$); c.216AA + harmonin-a1 ($n = 12$); c.216AA + harmonin-b1 ($n = 19$ with rescued ABR thresholds < 80 dB of 25 tested); c.216AA + harmonin-a1/b1 ($n = 6$ rescued with ABR thresholds < 80 dB/11 tested). Mean \pm s.e.m., continuous lines. Dotted lines: ABR thresholds for the entire frequency range in mice whose 16 kHz recordings are shown in a. (c) Mean DPOAE responses obtained for: c.216AA ($n = 13$); c.216GA ($n = 12$); c.216GA + trunc-harmonin ($n = 4$); c.216AA + harmonin-a1 ($n = 12$); c.216AA + harmonin-a1/b1 ($n = 15$ rescued with DPOAE thresholds < 70 dB/25 tested); c.216AA + harmonin-a1/b1 ($n = 4$ rescued with DPOAEs < 70 dB/11 tested). Mean \pm s.e.m., continuous lines. Dotted lines: DPOAEs thresholds for the four mice whose recordings are illustrated in a. Arrows indicate that the thresholds are higher than the maximal stimulus level tested. (d,e) ABR (d) and DPOAE (e) responses obtained at 6 weeks and 3 months in eight mice that showed initial ABR thresholds under or equal to 45 dB SPL. Seven of the eight mice were kept for 6 months (one taken for histology) and had ABRs and DPOAEs assessed (dotted line). Mean \pm s.e.m. While ABRs and DPOAEs thresholds shifts were evident over the first 3 months, hearing rescue was still prominent at 6 months of age in the lower frequency range.

full-length harmonin for endogenous binding partners^{26,30}, we generated Anc80L65.CMV.trunc-harm vectors that overexpressed the truncated protein, and injected them via RWM into the inner ears of

c.216GA mice. ABR and DPOAES measurements at 4, 6 and 12 weeks revealed no difference in thresholds between injected and uninjected c.216GA mice over the entire frequency range (Fig. 5b,c). These data, which also serve as a control for the injection technique and the vector, suggest that exogenous truncated harmonin does not compete with endogenous full-length harmonin.

Rescue of auditory and vestibular behavior in Ush1c mice

To assess whether the ABR/DPOAE recovery yielded behaviorally relevant recovery of auditory function, we measured acoustic startle responses in subsets of mice injected with AAV2/Anc80L65.CMV.harmonin-a1, AAV2/Anc80L65.CMV.harmonin-b1, or both vectors. Startle response to white noise showed partial rescue in 6-week-old mice injected with AAV2/Anc80L65.CMV.harmonin-b1 and in mice co-injected with both vectors (Fig. 6a). Mice that received harmonin-a1 alone were similar to uninjected c.216AA mice and did not recover startle responses.

Since the perilymphatic space is continuous between the cochlea and vestibular labyrinth, AAV vectors injected via RWM may transduce vestibular sensory organs. To assess vestibular behavior, we tested performance of subsets of mice on a rotarod over five trials. While poor rotarod performance was observed in c.216AA and c.216AA mice injected with AAV2/Anc80L65.CMV.harmonin-a1 (latency to fall <22 s on average), c.216AA mice injected with AAV2/Anc80L65.CMV.harmonin-b1 and those co-injected with both vectors maintained balance function on the rotarod for 60–120 s, consistent with control c.216GA mice (Fig. 6b). We also observed mice in a 42-cm wide open-field chamber for 5 min. Recovery in open-field behavior was evident in harmonin-b1 and dual harmonin-a1/b1-injected c.216AA mice (Fig. 6c). 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 (Fig. 6d,e). Surprisingly, while no ABR rescue was observed in mice injected with AAV2/Anc80L65.CMV.harmonin-a1, open field data demonstrated recovery of normal behavior to the level of the control mice.

Hair bundle morphology in rescued Ush1c mice

We assessed hair bundle morphology with SEM in 6-week-old harmonin-b1-treated and untreated mice (Fig. 7e). c.216AA mice displayed severe hair cell loss at the basal and middle regions of the organ (Fig. 7e,f). 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, the first row of OHCs was also largely absent. Less disabling phenotypes were observed at the apical end. High-magnification SEM also revealed severely disorganized hair bundles along the entire length of the organ. Notably, no hair bundles were observed that retained the typical staircase structure with all three rows of stereocilia (Fig. 7h). Instead, hair bundles were disorganized, with retracted stereocilia along the first row, abnormal second row and fairly preserved tallest row.

In contrast, treated c.216AA mice that showed functional recovery had more hair cells and normal hair bundle morphologies (Fig. 7i–l,n–p). Hair cell counts were estimated from the presence or absence of hair bundles in representative fields of view. The data revealed pronounced preservation of hair cell numbers in injected mice from the base to the apex of the organ, 40–79% in the base, 68–95% in the middle and 93–99% in the apex ($n = 1,824$ cells from $n = 4$ c.216AA cochleas and $n = 792$ from $n = 2$ rescued cochleas of c.216AA mice) (Fig. 7m). Although abnormal hair bundles were still

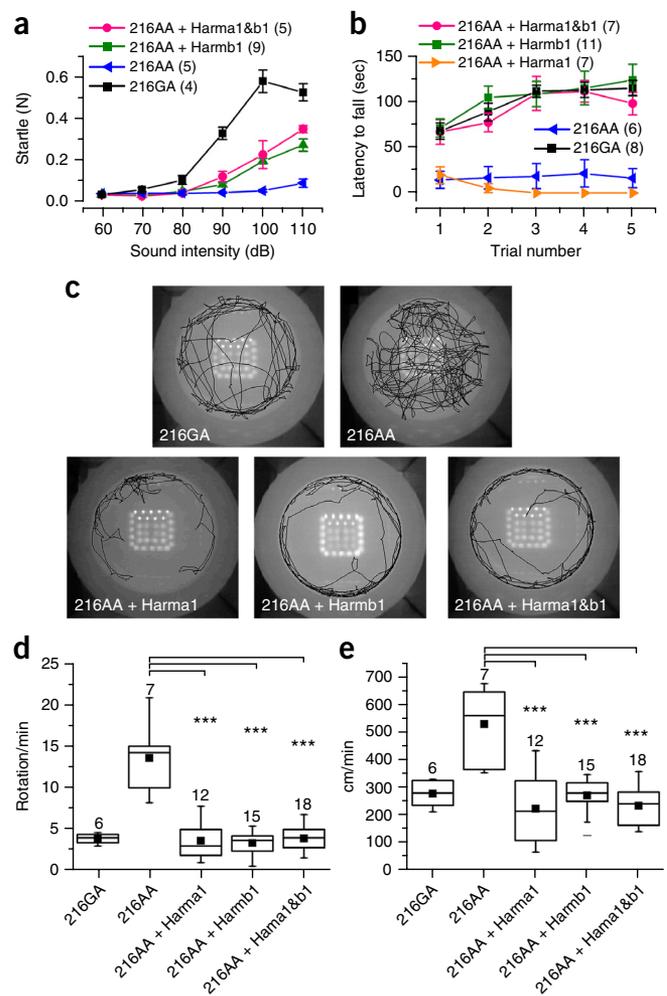


Figure 6 Startle response, rotarod performance and open field behavior recovery in mice injected at P1 with AAV2/Anc80L65.CMV.harmonin-a1 and AAV2/Anc80L65.CMV.harmonin-b1. (a) Startle response to white noise stimuli was recorded in 6-week-old control c.216GA, c.216AA mice and injected mice. Partial but significant startle rescue was evident in mice injected with harmonin-b1 but not harmonin-a1 (data overlap with control c.216AA mice). Averages \pm s.e.m. are shown for all mice tested. Statistical analysis: 216AA relative to 216AA + harmonin-a1/b1 (Harna1&b1): $P = 0.008$ at 100 dB; $P = 0.005$ at 110 dB; 216AA relative to 216AA + Harn b1: $P = 0.007$ at 100 dB; 0.001 at 110 dB, Student's *t*-test. (b) Rotarod performance was recorded between 4 and 6 weeks in control c.216GA, c.216AA and injected mice. Full recovery was observed in all mice tested that were injected with harmonin-b1 and harmonin-a1/b1. No recovery was observed for harmonin-a1 alone. Averages \pm s.e.m. are shown for all mice tested. (c–e) Open field observations were performed in 42-cm-wide arena for 5 min in 6-week-old control c.216GA, c.216AA and injected mice. Representative tracks over 2.5 min are shown (c). While c.216AA 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. Mean \pm s.d. for the number of rotations (d) and distance covered (e) per minute for all mice tested. Number of mice tested are indicated in parenthesis (a,b) and above box plots (d,e). No mice were excluded. Significant recovery $***P < 0.001$ was observed between the uninjected and injected mice. Statistical analysis by one-way ANOVA.

evident, most hair bundles possessed three rows of stereocilia and had morphology almost indistinguishable from that of heterozygous controls (Fig. 7i,n,p).

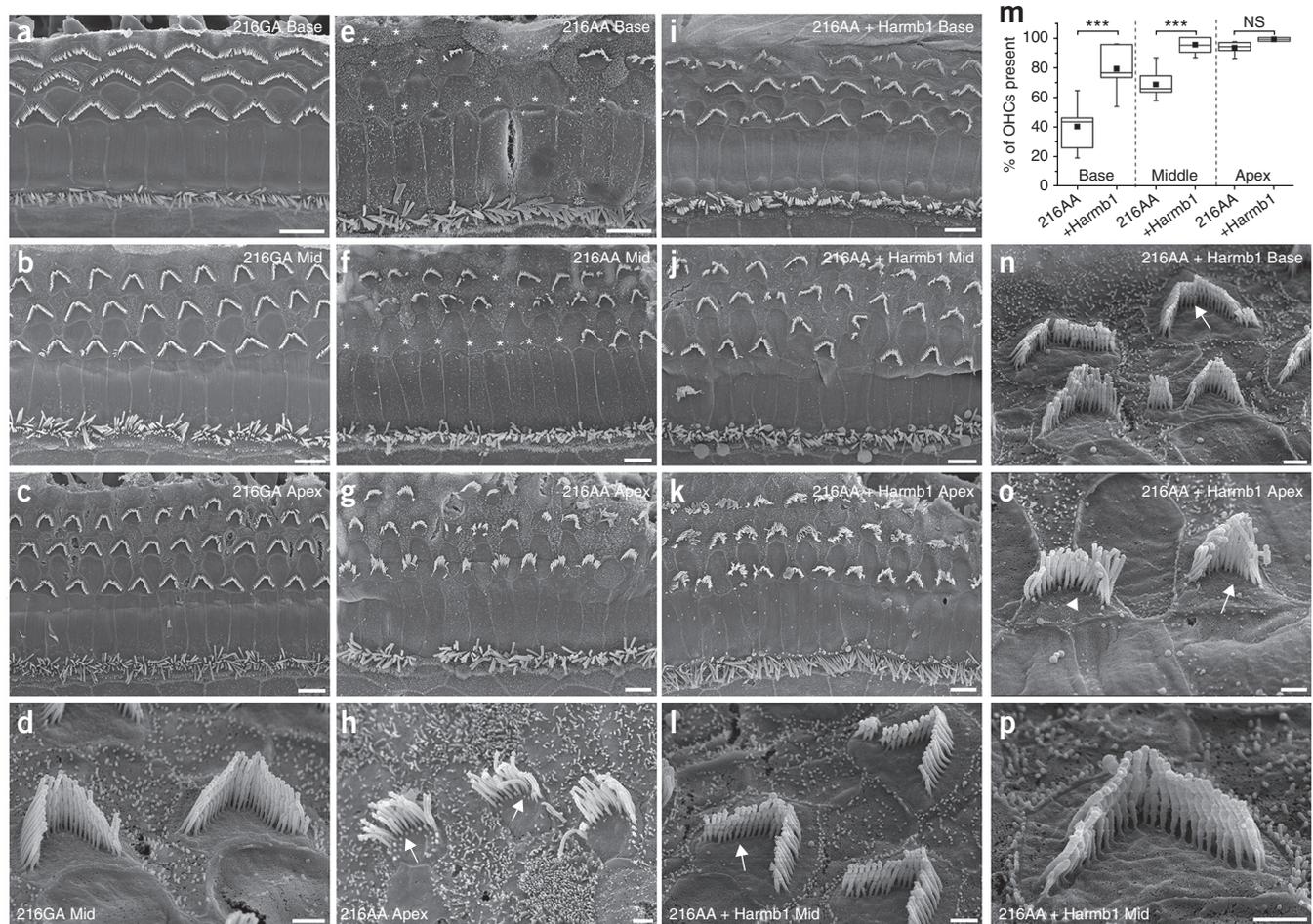


Figure 7 SEM of the organ of Corti in mice treated with harmonin-b1 and untreated mice. Basal, middle and apical regions of the organ of Corti were imaged at 6 weeks in c.216GA mice (a–d), c.216AA mice (e–h) and c.216AA mice injected at P1 (RMW injection 0.8 μ l AAV2/Anc80L65.CMV.harmonin-b1) (i–l, n–p). OHC and IHC hair bundles were preserved in c.216GA mice but appeared disorganized along the organ of Corti in c.216AA mice. c.216AA mice exhibited noticeable hair cell loss (asterisks) and hair bundle disorganization; more pronounced degeneration at the basal end; and lack of normal stereocilia rows, with retraction of shorter rows and maintenance of the tallest rows (arrows). 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 1,824 cells were counted in c.216AA mice (4 ears) and 792 in rescued c.216AA mice (2 ears). Mean \pm s.e.m. 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 μ m; high magnification, 1 μ m.

Harmonin expression correlates with hair cell survival and ABR thresholds

To confirm expression of AAV2/Anc80L65.CMV.harmonin-b1 and explore the relationship between viral expression level and ABR thresholds, we quantified DNA and RNA isolated from individual injected and contralateral cochleas. Expression was assessed in 6-week-old c.216GA and AAV2/Anc80L65.CMV.harmonin-b1-injected and non-injected c.216AA mice. Mouse *Ush1c* RNA expressed from the AAV2/Anc80L65.CMV.harmonin-b1 vector (Supplementary Fig. 5a) and AAV2/Anc80L65.CMV.harmonin-b1 DNA (Supplementary Fig. 5b) were detected in all of the injected cochleas and, to a lesser extent, in the contralateral cochleas of injected animals. There was variability between animals in ABR thresholds and the amount of DNA and RNA expressed (Supplementary Fig. 5c). However, we found a strong correlation between AAV2/Anc80L65.CMV.harmonin-b1 DNA levels, the amount of mRNA encoding the correctly spliced form of harmonin and ABR threshold levels, which suggests that the variability in ABR data may be a direct result of Anc80L65 transduction. Absent or poor Anc80L65 transduction likely

resulted from failed RWM injections. To confirm that harmonin was expressed in rescued ears, we performed harmonin immunostaining in rescued cochleas as well as cochleas harvested from wild-type, c.216AA and c.216AA mice with failed rescued. Specific harmonin labeling was observed at stereocilia tips in wild-type and rescued ears but was absent in uninjected c.216AA ears and injected but not rescued c.216AA ears (Supplementary Fig. 5d).

To assess long-term hair cell survival in mice that had successful recovery of ABR thresholds, we prepared tissue and counted the number of IHCs and OHCs at 6 months of age from five mice (Supplementary Fig. 6). 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 (Supplementary Fig. 6).

DISCUSSION

While previous work has shown that homozygous knock-in c.216AA mice have significant hair cell degeneration at P18 and P30 (refs. 27,28), in early postnatal c.216AA mice, we found altered hair bundle

morphology but functionally viable hair cells, revealing a potential therapeutic window. During the first postnatal week, auditory and vestibular epithelia retained mechanosensitive hair cells, including some with relatively normal morphology (Fig. 1). Transduction currents were diminished in amplitude, but many auditory hair cells retained characteristics within the normal range for adaptation and sensitivity (Supplementary Fig. 2). In the vestibular system, c.216AA hair cells in the extra-striola region had reduced current amplitudes, whereas cells in the striola region did not take up FM1-43 and did not respond to bundle displacements (Fig. 2). Therefore, with the exception of the striola region, our results suggest that the transduction apparatus can be correctly assembled and targeted in neonatal, mutant mice, although the number of functional complexes appears to be reduced. In contrast, previous work showed that full knockouts of *Ush1c* have transduction currents that were nearly absent by P7 ref. 23), which is expected to limit the window for intervention.

Since a small percentage of correct splicing still occurs in c.216AA mice³⁰, we tested the therapeutic efficacy of wild-type harmonin expression in this model during early postnatal stages. AAV2/1 vectors successfully targeted hair cells, and harmonin fusion constructs were correctly localized (Fig. 3). EGFP::harmonin-b1 was targeted to the tips of stereocilia in wild-type or mutant auditory or vestibular tissues, consistent with prior localization studies²³. The harmonin-a1 variant is localized near the ribbon synapse where it interacts with Cav1.3 channels to alter exocytosis^{24,25}. While expression of tdTomato::harmonin-a1 packaged in AAV2/1 was observed at the base of the IHCs and was absent from stereocilia, higher expression levels obtained with AAV2/Anc80L65 led to diffuse localization throughout the cell and in the stereociliary bundle. TdTomato::harmonin-a1 was also observed throughout the utricle, was excluded from stereocilia and showed little colocalization with ribbon synapse markers at P7. While this may be a consequence of the tagged fluorescent marker, it is also possible that harmonin-a1 plays a different role in the utricle or that localization differs in more mature epithelia.

We studied whether expression of multiple harmonin isoforms are needed for full functional rescue in auditory and vestibular organs. Since harmonin-b plays an important role in the stereocilia and harmonin-a has been shown to alter exocytosis, we suspected that both splice forms would be necessary to rescue inner ear function. However, co-injection of harmonin-b1 and harmonin-a1 did not enhance recovery. Thus, harmonin-b1 alone was sufficient to restore partial function. ABR responses in rescued mice showed normal wave 1 amplitudes and longer peak 1 latencies relative to normal hearing controls (Supplementary Fig. 4), the latter perhaps due to an incomplete functional recovery at the synapse.

Auditory rescue was prominent at low, but not high, frequencies (Fig. 5), while hair bundles with normal morphology were observed along the entire organ at 6 weeks of age (Fig. 7). The absence of rescue at high frequencies is unlikely to have been caused by damage from the injection as high-frequency hearing loss was not observed in any injected c.216GA mice (Fig. 5b,c). Poor transduction efficiency at the base²⁶ is also an unlikely explanation given AAV targeting along the entire length of the cochlea²⁹. It is possible that other harmonin isoforms, such as 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, by P0 these hair cells may have matured beyond the point of repair. If so, prenatal intervention^{36,37} may be more effective.

In behavioral assays, harmonin-a1 mediated partial vestibular rescue (indicated by reduced circling behavior), whereas harmonin-b1 enabled more complete functional recovery in both vestibular tests (reduced

circling and enhanced rotarod performance; Fig. 6). The absence of transduction and FM1-43 uptake in the striola regions argues that hair cells of the striola region and perhaps type I cell function depend on proper harmonin expression (Fig. 2). Further studies are needed to clarify the role of harmonin in the vestibular system. Additional assays of vestibular function, such as vestibular evoked potentials, may provide a physiological measure of the extent of rescue.

A previous study³⁰ restored function in early postnatal *Ush1c* mice using intra-peritoneal injection of antisense oligonucleotides targeting c.216AA. Another report corrected *Ush1c* gene expression in mice with the same antisense oligonucleotide delivered to the amniotic cavity³⁸. An advantage of the antisense approach is that all splice forms may be expressed; however, repeated injections may be needed to maintain long-term rescue. AAV vectors, on the other hand, can provide long-term transgene expression in post-mitotic tissue even in the absence of genome integration^{39,40}. Our results obtained at 12 and 24 weeks suggest that this may indeed be the case in inner ear hair cells. Both strategies have now provided compelling evidence that gene therapy can be further developed to treat Usher syndrome type 1C for patients who have the 216G>A splice site mutation and possibly other *USH1C* mutations.

In summary, our data provide further support for development of viral gene transfer in the inner ear to treat disorders of sensory hair cells. Using a vector with high transduction efficiency in both IHCs and OHCs, we show the most complete rescue of auditory function for any inner ear gene therapy application to date, with over a thousand-fold improvement in sound pressure level sensitivity (thresholds ~25 dB at 8 kHz) relative to prior studies^{2,3}. Furthermore, we show the most complete gene therapy recovery of vestibular function in a genetic model of combined auditory and vestibular dysfunction. Translation of these successes to the clinic will likely necessitate optimization of the delivery vectors, promoters and coding sequences, validation of the viral targeting and efficacy in larger animal models, and perhaps in human tissue *in vitro*, as well as a better understanding of the therapeutic window in humans. Finally, while conventional AAV vectors have a good safety profile, next-generation AAVs, such as Anc80L65, have not yet been evaluated in humans and thus will require careful scrutiny.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

The project was conceived and designed by G.S.G. C.A. performed RWM injections with AAV2/1 vectors, electrophysiological recordings and analysis of OHCs, FM1-43 imaging of the organ of Corti; B.P. carried out RWM injections with AAV2/Anc80L65 vectors, electrophysiological recordings and analysis of IHCs mechanotransduction currents. G.S.G. performed electrophysiological recordings of vestibular hair cells. A.G., S.H.-A. and G.S.G. performed ABR experiments. Semi-quantitative radiolabeled PCR and RT-PCR was performed by F.M.J. and M.L.H. ABR and behavioral data analysis was performed by A.G. and G.S.G. Y.A. designed and prepared the plasmid constructs. SEM was performed by J.J.L. (P18), and A.A.I. (P8 and 6 weeks). DIC, immunostaining and confocal imaging was performed by G.S.G. L.H.V. assisted with production of AAV vectors. J.R.H. assisted with the design of the experiments, preparation of figures and manuscript. G.S.G. analyzed, interpreted the results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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ONLINE METHODS

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^{41,42}. All procedures used for this work met the NIH guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees at Boston Children's Hospital (Protocols# 12-02-2146, #14-03-2659R and #15-01-2878R). Mice were genotyped using toe clip (before P8) or ear punch (after P8) and PCR was performed as described previously²⁶. For all studies, both male and female mice were used in approximately equal proportions. No randomization paradigm was otherwise applied.

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 previously⁴³. 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 the presence of 1% FBS. Cultures were maintained for 7 to 10 d and the media was replaced every 2 to 3 d for experiments that involved viral vectors infection *in vitro*.

Scanning electron microscopy (SEM). SEM was performed at P7, P18 and ~P42 (6 weeks) along the organ of Corti of control and mutant mice. P18 SEM was performed in collaboration with 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 0.1M 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-week stages by G.S.G. and A.A.I. Organ of Corti explants were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (Electron Microscopy Sciences) supplemented with 2 mM CaCl₂ for 1 h at room temperature. Specimens were dehydrated in a graded series of acetone, critical-point dried from liquid CO₂, 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).

Electrophysiological recording. Recordings were performed in standard artificial perilymph solution containing (in mM): 144 NaCl, 0.7 NaH₂PO₄, 5.8 KCl, 1.3 CaCl₂, 0.9 MgCl₂, 5.6 D-glucose, and 10 HEPES-NaOH, adjusted to pH 7.4 and 320 m Osmol/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 63× water immersion objective with differential interference contrast optics. Recording pipettes (3–5 MΩ) were pulled from borosilicate capillary glass (Garner Glass, Claremont, CA) and filled with intracellular solution containing (in mM): 135 KCl, 5 EGTA-KOH, 10 HEPES, 2.5 K₂ATP, 3.5 MgCl₂, 0.1 CaCl₂, pH 7.4. Currents were recorded under whole-cell voltage-clamp at a holding potential of -64 mV at room temperature. Data were acquired using an Axopatch Multiclamp 700A or Axopatch 200A (Molecular devices, Palo Alto, CA) filtered at 10 kHz with a low pass Bessel filter, digitized at ≥20 kHz with a 12-bit acquisition board (Digidata 1322) and pClamp 8.2 and 10.5 (Molecular Devices, Palo Alto, CA). Data were analyzed offline with OriginLab software and are presented as means ± s.d. unless otherwise noted.

Mechanical stimulation. *OHCs* and *IHCs*. Mechanical stimuli were transmitted via a stiff glass probe mounted on a one-dimensional PICMA chip piezo actuator (Physik Instruments, Waldbronn, Germany) driven by a 400 mA

ENV400 Amplifier (Piezosystem Jena Germany)⁴⁴. The tip of the probe was fired polished (Fire polisher, H602, World Precision Instruments Inc., Sarasota, FL) to fit stereociliary bundle⁴⁴. Deflections were evoked by applying voltage steps filtered with an 8-pole Bessel filter (Khron-Hite, 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 an 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 before the experiments.

Viral vector generation. Harmonin-a1 and harmonin-b1 plasmids were prepared in our laboratory from EGFP-tagged, labeled constructs graciously provided by Lily Zheng and James Bartles⁴⁵ (Department of Cell and Molecular Biology, Northwestern University, Feinberg School of medicine, Chicago, IL). Harmonin-a1 was originally obtained from mouse kidney and harmonin-b1 from isolated mouse cochlea sensory epithelium. We further modified the harmonin-a1 construct to replace the EGFP tag with tdTomato at its N-terminal end. Fluorescently labeled and unlabeled constructs were packaged into AAV vectors. Viral vectors were generated by the viral core facility at Boston Children's Hospital and the Gene Transfer Vector Core at the Massachusetts Eye and Ear Infirmary. The following vectors were generated: AAV2/1.CMV.tdTomato::harmonin-a1 4.3 × 10¹³ gc/ml (BCH); AAV2/1.CMV.EGFP::harmonin-b1 2.7 × 10¹⁴ gc/ml (BCH); AAV2/1.CMV.EGFP::harmonin-a1: 2.8 × 10¹² gc/ml (MEEI); AAV2/Anc80L65.CMV.harmonin-a1: 1.9 × 10¹² gc/ml (MEEI); AAV2/Anc80L65.CMV.harmonin-b1: 1.7 × 10¹² gc/ml (MEEI); AAV2/Anc80L65.CMV.tdTomato::harmonin-a1: 4.1 × 10¹² gc/ml (MEEI); AAV2/Anc80L65.CMV.EGFP::Harmonin-b1: 3.0 × 10¹² gc/ml (MEEI).

Cloning of the truncated harmonin was performed from total RNA isolated from cochleas of *c.216AA* 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 GAGGTACCATGGACCGGAAGGTGGCCCGAG

Trunc-harmonin.RV CAGGATCCGGACAATTCATCCCTAC

The 387-bp PCR product was cloned into a shuttle vector with AAV2 inverted terminal repeats (ITRs) where the transgene cassette was driven by a CMV promoter. Custom vector was packaged into the Anc80L65 capsid: AAV2/Anc80L65.CMV.trunc-harm.WPRE: 9.0 × 10¹² gc/ml (MEEI).

FM1-43 imaging. FM1-43 (Invitrogen) was diluted in extracellular recording solution (5 μM) and applied to tissues for 10 s and then washed 3 times in extracellular recording solution to remove excess dye and prevent uptake via endocytosis. After 5 min 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 20×, 40× and 63× 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.

Confocal imaging. To prepare the tissue for confocal imaging from postnatal mice P0–P8, fixation was performed for 15 min with 4% paraformaldehyde (PFA). Permeabilization with 0.01% triton and counterstaining with Alexa Fluor phalloidin (Invitrogen, 1/200) was used to label 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 h, followed by decalcification for 24 to 36 h with 120 mM EDTA. The sensory epithelium was then dissected out and treated as above for immunostaining. Mouse anti-CTBP2 (BD bioscience #612044, 1DB ID for anti-CTBP2 #1DB-001-0000869740, 1/200) or anti-harmonin (Novus Biologicals, LLC #NBP1-89189, 1/100) were applied for 48 h and incubated with secondary antibody (1/200) and Alexa Fluor Phalloidin to label F-actin (1/200) 3–12 h at 4 °C. 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.

RT-PCR. cDNA was prepared from six 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' CTCATTGAAAATGACGCAGAGAAGG 3', Reverse mUsh1c_Ex5R: 5' TCTCACTTTGATGGACACGGTCTT 3'. These primers are specific for mouse *Ush1c* sequences and will amplify both endogenous and AAV2-derived *Ush1c*, as the target sequence is outside the region of the human knocked-in portion of the *Ush1c.c.216A* allele. DNA and RNA levels were also assessed from mouse tissue collected at 6 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'-GAACCCAACCGCTGCCG) and mUsh1c_Ex4WTR (5'-TGCAGACGGTCCAAGCGT-3') were used. These primers only amplify the viral *Ush1c* DNA because the homozygous *Ush1c.216AA* mice have the human *USH1C.c.216A* gene knocked-in at exons 3 and 4, replacing the mouse sequence (32). For cDNA amplification of full-length (450 bp) and aberrantly spliced/truncated harmonin (415 bp), the same primers as above were used (mUsh1c_Ex2F and mUsh1c_Ex5R). Gapdh primers were: mGapdh_Ex3F (5'-GTGAGGCCGGTCTGAGTATG-3') and mGapdh_Ex4R (5'-GCCAAAGTTGTCATGGATGAC-3'). Products were separated on a 6% nondenaturing polyacrylamide gel and quantified using a Typhoon 9400 phosphorimager (GE Healthcare).

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 μ l–1.0 μ l 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 micromanipulator⁶. The volume of the injected materials was controlled at an ~0.02 μ l/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.

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-msec 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 1,024 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 \pm s.d. 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 in 10-dB increments. DPOAE threshold was defined from the average spectra as the L2-level eliciting a DPOAE of magnitude 5 dB above the noise floor. The mean noise floor level was under 0 dB 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.

Acoustic startle responses. The acoustic startle responses (ASR) were measured using the Startle Monitor (Kinder Scientific). Mice were placed in a small-sized, nonrestrictive, cubical Plexiglas recording chamber (27 cm \times 10 cm \times 12.5 cm) fixed on a piezo/plexiglass sensing assembly and allowed to acclimate for 5 min with a 60-dB background white noise. Each session consisted of 35 trials, during which a single noise pulse ranging in 10-dB intensities from 60–120 dB 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 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.

Vestibular assessment. Vestibular function was assessed using open field and rotarod balance test. The open field test was conducted using a circular frame measuring 42 cm in diameter, placed inside a sound chamber with overhead LED lighting, set to 30 lux at the center, inside a dimmed room. Mice were placed one at a time inside the circular open field, and allowed to explore for 5 min. Behavior was recorded and tracked using Ethovision XT, enabling measures of distance traveled and velocity. Open field assessments were all conducted blind. The rotarod performance involved placement of mice on a rod in an enclosed housing that began rotating at 4 r.p.m. and accelerated at a rate of 0.1 r.p.m. 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.

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 < 80 dB SPL. Statistical analyses were performed with Origin 2016 (OriginLab Corporation). Data are presented as means \pm s.d. or s.e.m. as noted in the text and figure legends. One-way analysis of variance (ANOVA) or Student's *t*-test was used to determine significant differences between the means. In the box plot graphs, the ends of whisker is defined by maximum and minimum values. Central rectangle spans from first quartile to third quartile. The segment in the rectangle indicates median and the square dot indicates the mean.

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