Sensorineural hearing loss affects the quality of life and communication of millions of people, but the underlying molecular mechanisms remain elusive. Here, we identify mutations in Gipc3 underlying progressive sensorineural hearing loss (age-related hearing loss 5, ahl5) and audiogenic seizures (juvenile audiogenic monogenic seizure 1, jams1) in mice and autosomal recessive deafness DFNB15 and DFNB95 in humans. Gipc3 localizes to inner ear sensory hair cells and spiral ganglion. A missense mutation in the PDZ domain has an attenuating effect on mechanotransduction and the acquisition of mature inner hair cell potassium currents. Magnitude and temporal progression of wave I amplitude of afferent neurons correlate with susceptibility and resistance to audiogenic seizures. The Gipc3343A allele disrupts the structure of the stereocilia bundle and affects long-term function of auditory hair cells and spiral ganglion neurons. Our study suggests a pivotal role of Gipc3 in acoustic signal acquisition and propagation in cochlear hair cells.
related pathology suggested that the hearing loss in the Frings and BUB / BnJ mouse strains 13,14 . However, the majority of the genetic variation underlying susceptibility to SNHL remains unknown. Progressive SNHL in mice displays a histopathology similar to that in humans4,5. Extensive natural variation in mouse strains has caused a remarkable phenotypic and genetic heterogeneity of SNHL, providing experimental model systems to systematically decipher the underlying risk factors6.

We recently identified an early-onset hearing loss in Black Swiss (BLSW) mice that is controlled by the quantitative trait loci (QTL) age-related hearing loss 5 (ahl5) and ahls (ref. 11), whereby the ahls QTl accounts for ~60% of the variation observed. The 95% confidence interval for ahls is localized to a 25 Mbp region on chromosome 10 (D10Mit20-D10Mit95; 66, 475–91, 965 kbp). BLSW mice are also sensitive to acoustic stimulus, responding to loud noise with seizures, characterized by wild running, and tonic and clonic convulsions12. Sensitivity to audiogenic seizures is highest at 2–3 weeks of age, but then susceptibility gradually decreases and mice become resistant at 6 weeks of age. The underlying locus, juvenile audiogenic monogenic seizure (jams1), localizes to a 1.6 cM region that overlaps with the ahls interval13. The genetic location and their related pathology suggested that the ahls and jams1 phenotypes are caused by the same allele as previously shown for the 6748delC mutation on the Gpc9 gene causing both audiogenic seizures and hearing loss in the Frings and BUB/BnJ mouse strains14,14.

The Gipc (GAIP interacting protein, C terminus) genes encode a small family of proteins characterized by a single, centrally located PDZ domain15. GIPC1 was first identified through its interaction with regulator of G-protein signalling 19 (Rgs19) and has now been shown to interact with a host of proteins that are involved in signal transduction, vesicular trafficking, endocytosis and neurotransmitter release16–20. Using its carboxy (C) terminal domain, GIPC1 was demonstrated to interact with Myosin VI, which is associated with SNHL in both mice and humans21–23. Loss of Gipc1 function (Gipc1tm1Mec) results in a reduction in pre- and postsynaptic transmission in hippocampal neurons24.

Here, we identified a sequence polymorphism in the PDZ domain of Gipc3 as the cause of SNHL (ahl5) and audiogenic seizure susceptibility (jams1) in BLSW mice and we implicate mutations in Gipc3 in human recessive SNHL (DFNB15 and DFNB95). Our study suggests a critical role of Gipc3 in signal acquisition and propagation in auditory hair cells.

Results Positional cloning of ahls. To genetically refine the ahls region, we generated two congeneric lines, 10.8 and 10.2, by serial backcrossing normal hearing BLSW .CAST-/+ ahls congenic lines allowing us to evaluate the effect of ahls on the progression of the hearing loss. BLSW .CAST-/+ mice showed normal hearing at 2 and 12 months of age with mean thresholds of 12±4 and 16±5 dB SPL for the 16 kHz stimulus, respectively (Fig. 2b). BLSW .CAST-/+ ahls heterozygotes had normal thresholds at 12 months of age (21±6 dB SPL), but exhibited significantly elevated thresholds at 12 months. This increase was more prominent in males (57±23 dB SPL) than in females (25±16 dB SPL; P<0.001, analysis of variance (ANOVA)). The threshold increase in 12-month-old female heterozygotes was modest with large standard deviations, but the difference compared with 2-month-old females was not significant. Last, the elevated thresholds of male heterozygotes were lower than the thresholds in 12-month-old BLSW mice (87±7 dB SPL). These results suggest that a gender-ahls interaction controls the progression of hearing loss in BLSW .CAST-/+ ahls heterozygotes.

We next measured distortion product otoacoustic emissions (DPOAEs), which are an indicator of outer hair cell (OHC) function. At 4 weeks of age, DPOAEs were reduced at the 5–20 kHz frequencies (L2 = 65 dB SPL) and were absent between 20 and 55 kHz compared with normal hearing C3HeB/FeJ mice at the same age (Fig. 2c). To test for a defect in the stria vascularis, we measured the endocochlear potential (EP). In 10-week-old BLSW mice, the EP was 104±18 mV, which was comparable with potentials obtained from C3HeB/FeJ mice (105±7 mV; Fig. 2d). Together, the data suggest that BLSW mice exhibit progressive SNHL.

To test whether jams1 localized to the ahls critical region, we tested BLSW .CAST congenic mice for audiogenic seizure susceptibility. An acoustic stimulus of 90–100 dB SPL for the duration of 61±20 s readily induced a seizure response in 3-week-old BLSW mice. Age-matched C3HeB/FeJ mice showed no seizing activity after 180 s at 110 dB SPL (P<0.001). Likewise, BLSW .CAST-/+ ahls homozygotes were highly susceptible to white noise stimulation showing a rapid seizure response (27±31 s). In contrast, BLSW .CAST-/+ showed no signs of audiogenic seizures (180 s) exhibiting a response identical to that of C3HeB/FeJ mice (180 s, P>0.05; Fig. 2e). In addition, although five BLSW .CAST-/+ ahls responded with convulsions at the highest 110 dB SPL stimulus (132±4 s), most of the BLSW .CAST-/+ ahls mice were resistant to seizure induction (180 s, n = 18) and the mean response latency between BLSW .CAST-/+ and BLSW .CAST-/+ ahls was not different. Together, these data indicate that the jams1 locus resides within the ahls critical interval.

Gipc3 transgenic rescue. To further test that the 343G>A mutation in Gipc3 causes the ahls and jams1 phenotypes, we generated transgenic animals expressing Gipc3. Transgenic mice that were backcrossed to BLSW (N2–N3) were phenotyped using ABR at 6–8 weeks of age. Gipc3tm1Mec homozygotes had impaired hearing (95±7 dB SPL at 16 kHz), whereas NIH Swiss mice homozygous at the wild-type allele (Gipc3tm2Hk) exhibited normal hearing (16±5 dB SPL at 16 kHz; Fig. 1e). The missense mutation replaced a glycine with an arginine at position 115 (Gly115Arg) located within the PDZ domain (aa107–174). The Gly115 residue is highly conserved, showing 100% identity in all published GIPC protein sequences in humans and plants (Fig. 1f).

SNHL and audiogenic seizures in BLSW. At 4 weeks of age, BLSW mice showed a moderate hearing impairment with mean thresholds significantly elevated for the click, 8, 16 and 32 kHz stimuli (68±7, 41±6, 41±5 and 85±5 dB SPL) compared with thresholds of 4-week-old normal hearing C3HeB/FeJ mice (P<0.001). At 52 weeks of age, BLSW mice exhibited a profound hearing loss that progressed from higher to lower test frequencies. The increase in hearing thresholds was most pronounced at 16 kHz with a shift of 48 dB SPL over the threshold of 4-week-old BLSW mice (Fig. 2a).

The availability of the BLSW .CAST-/+ ahls congenic lines allowed us to evaluate the effect of ahls on the progression of the hearing loss. BLSW .CAST-/+ mice showed normal hearing at 2 and 12 months of age with mean thresholds of 12±4 and 16±5 dB SPL for the 16 kHz stimulus, respectively (Fig. 2b). BLSW .CAST-/+ ahls heterozygotes had normal thresholds at 12 months of age (21±6 dB SPL), but exhibited significantly elevated thresholds at 12 months. This increase was more prominent in males (57±23 dB SPL) than in females (25±16 dB SPL; P<0.001, analysis of variance (ANOVA)). The threshold increase in 12-month-old female heterozygotes was modest with large standard deviations, but the difference compared with 2-month-old females was not significant. Last, the elevated thresholds of male heterozygotes were lower than the thresholds in 12-month-old BLSW mice (89±7 dB SPL). These results suggest that a gender-ahls interaction controls the progression of hearing loss in BLSW .CAST-/+ ahls heterozygotes.

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Gipc3 transgenic rescue. To further test that the 343G>A mutation in Gipc3 causes the ahls and jams1 phenotypes, we generated transgenic animals expressing Gipc3. Transgenic mice that were backcrossed to BLSW (N2–N3) were phenotyped using ABR at 6–8 weeks of age. Gipc3tm1Mec homozygotes had impaired hearing (95±7 dB SPL at 16 kHz), whereas NIH Swiss mice homozygous at the wild-type allele (Gipc3tm2Hk) exhibited normal hearing (16±5 dB SPL at 16 kHz; Fig. 1e). The missense mutation replaced a glycine with an arginine at position 115 (Gly115Arg) located within the PDZ domain (aa107–174). The Gly115 residue is highly conserved, showing 100% identity in all published GIPC protein sequences in humans and plants (Fig. 1f).
Figure 1 | Positional cloning of the ah15 locus. (a) ABR thresholds at click (orange), 8 (green), 16 (purple) and 32 kHz (red) of congenic BLSW.CAST-+/ahl5 (n = 83) and BLSW.CAST-ahl5/ahl5 (n = 59) mice at 8 weeks of age. Hearing thresholds (dBSPL) were significantly elevated for the BLSW.CAST-ahl5/ahl5 animals compared with BLSW.CAST-+/ahl5 (ANOVA, P < 0.001). Data are given as mean ± s.d. (b) The ah15 95% confidence interval (CI) on chromosome 10 (MMU10) defined by markers D10Mit20 and D10M1095 is shown. Refined genotyping of two congenic lines 10.R and 10.2 delimited the ah15 critical interval in a 2.19 Mbp region (red box) defined by markers D10Ntra205 and D10Ntra222. Blue bars at the bottom of the map represent the genomic segments from CAST/EiJ introgressed onto the BLSW genetic background (red lines). The jans1 locus delimited by the gene Basigin (Bsg) and marker D10Mit140 on MMU10 is shown (orange box) [12]. Physical location of Gipc3 within the ah15 interval is given. Position of the markers is given in kilobase pairs (kbp). (c) Gipc3 has six exons and is predicted to encode a 297 amino-acid protein with a central PDZ domain (blue box) flanked by amino- and C-terminal GIPC homologous domains GH1 and GH2 (grey boxes). Position of the mutation (red annotation) and polyclonal antisera Pb867, Pb869 and Pb877 (green boxes) is shown. (d) Sequencing chromatograms demonstrating the 343G→A transition in wild-type (left) and mutant (right) alleles. Homozygous (343A/343A, red and blue) and heterozygotes (343G/343A, purple) show a complete resistance (Fig. 3b). To test whether mutations in GIPC3 also underlie human SNHL, we screened panels of families of Dutch and Indian origin presenting with autosomal recessive nonsyndromic hearing impairment. In one Dutch family, W98-042,
a homozygous region at 19p13.11-p13.3 encompassing GIPC3 was shared by the two affected siblings (rs11880407–rs6512152; assigned locus symbol: DFNB95). GIPC3 mutation analysis revealed a homozygous nucleotide substitution in exon 6, c.903G > A that segregated with the disease in the family (Fig. 4a,b and Supplementary Table S2). The c.903G > A change is predicted to truncate the protein after residue 300 (p.Trp301X) causing the deletion of the C-terminal 12 amino acids of the protein. The mutation was not found in 156 (312 chromosomes) ethnically matched control individuals.

The hearing loss in family W98-042 is bilateral and sensorineural with an early onset. Brainstem-evoked response audiometry for individual II.1 at 11 months of age revealed a threshold of ~70 dB hearing levels (HL). For individual II.3 at the age of 3 months, brainstem-evoked response audiometry demonstrated a threshold of ~80 dB HL.

**Figure 2 | Sensorineural hearing loss and audiogenic seizures in BLSW.** (a) ABR thresholds (dB SPL) of BLSW at 4 (orange symbol, n = 18) and 52 (red, n = 20) weeks age compared with 4-week-old C3HeB/FeJ (blue, n = 9) are given as mean ± s.d. (b) ABR thresholds (dB SPL) at the 16k Hz stimulus in 2- (n = 6) and 12-months-old (n = 17) BLSW.CAST-+/+ (blue), and in 2-month-old BLSW.CAST-+/ahl5 (green, n = 52), and 2- (n = 9) and 12-months-old (n = 20) BLSW (red) are given as mean ± s.d. ABR thresholds in 12-month-old females (12f, n = 30) and males (12m, n = 30) are given. NS P > 0.05, ***P < 0.001 (ANOVA). (c) DPOAE levels (dB SPL) at the 2f1-f2 frequency are plotted against the f2 frequency (kHz) for 55 dB SPL (left), 65 dB SPL (middle) and 75 dB SPL (right) L2 levels. Emission levels relative to noise floor of C3HeB/FeJ (blue, n = 25) and BLSW (red, n = 16) mice at 4 weeks of age are given as mean ± s.e.m. (d) Endocochlear potential measurements of C3HeB/FeJ (blue, n = 5) and BLSW (red, n = 15) mice. Each circle refers to one measurement and the lines indicate the mean in each strain. Values are given in millivolt (mV). (e) Audiogenic seizure susceptibility of C3HeB/FeJ (blue, n = 5), BLSW (dark red, n = 14) and congenic BLSW.CAST-+/+ (blue, n = 9), BLSW.CAST-+/ahl5 (green, n = 23), BLSW.CAST-ahl5/ahl5 (light red, n = 34) at 3 weeks of age using white noise stimuli of 90, 100 and 110 dB SPL (dotted lines). Each circle represents one animal and lines indicate the mean of the seizure latency of each strain in seconds. NS P > 0.05; ***P < 0.001. NS, not significant.
natal day 3 (P3) onwards, OHC bundles were slightly disoriented and the hair bundle of IHC appeared less rigid and sparse (Fig. 5a,b) and at P21 stereocilia were thinner and at times also shorter (Fig. 5c). Stereocilia bundles at the base and mid-apical region were present and were affected to a similar degree. Histological sections of 8-week-old BLSW ears revealed a normal morphology of the organ of Corti and the spiral ganglion. At 52 weeks of age, there was a significant degeneration of the organ of Corti starting at the base and progressing towards the apex. Concomitantly, the spiral ganglion exhibited a severe loss of neurons that was most obvious at the base and the mid-apical region of the cochlea. The organ of Corti and spiral ganglion at the apex were of normal appearance (Fig. 5d).

Together, these data suggest that Gipc3 is required for postnatal maturation of the hair bundle and long-term survival of hair cells and spiral ganglion.

Reduced Gipc3 protein levels in the BLSW cochlea. We reasoned that the Gly115Arg mutation might alter the expression or stability of Gipc3. To test this hypothesis, we probed western blots with polyclonal antisera raised against Gipc3-specific peptides. In protein extracts derived from HEK293T cells transiently expressing wild-type Gipc3, the Pab867, Pab869 and Pab877 antisera detected a single ~40 kDa immunoreactive band (Fig. 6a and Supplementary Fig. S2). When we probed western blots of cochlea and testis protein extracts from BLSW and C3HeB/FeJ we detected a reduction in intensity of the ~40 kDa band in BLSW cochlea extracts by 82±7% (n=3) and in testis extracts by 42±9% (n=3) compared with wild-type C3HeB/FeJ extracts (Fig. 6b). As an additional test of the expression level differences, we generated a mutant Gipc3 (G343A) cDNA construct for expression in HEK293T cells. Quantification, after normalizing against actin expression, revealed a reduction in expression levels by 69% of the mutant Gipc3 (115Arg) protein compared with that for wild-type Gipc3 (115Gly) (Fig. 6c,d). TaqMan qPCR analyses revealed no significant differences in Gipc3 mRNA expression in HEK293T cells transfected with wild-type (Gipc3115Gly) or mutant (Gipc3115Arg) or mutant (Gipc3115Arg) construct (data not shown). In addition to the ~40 kDa band, Pab867 recognizes a ~37 kDa band in both cochlea and testis extracts. Both bands can be blocked by the Gipc3-specific peptide used for generating the Pab867 antibody and are also detected by the Pab867/Pab877 antisemur (Supplementary Fig. S2). In addition, Pab867 is specific for Gipc3, showing strong reactivity with human Gipc3 and only faint cross-reactivity with mouse Gipc1 and Gipc2 (Supplementary Fig. S2). Thus, the ~37 kDa band may represent either an alternate isoform or partially cleaved product of the ~40 kDa protein. Together, these data indicate that the Gly115Arg substitution causes a reduction of the 40 kDa Gipc3 protein in BLSW cochleae.

Gipc3 localization in hair cells and ganglion neurons. Using the Pab867 antibody on cochlear sections, we detected Gipc3-specific immunoreactivity in IHCs and OHCs, as well as in cochlear spiral ganglion neurons in C3HeB/FeJ mice. Similar expression domains were observed in the BLSW cochlea (Fig. 6e,f). Staining was also present in the cytoplasm of vestibular hair cells and vestibular ganglion neurons (Supplementary Fig. S2). On whole-mount organ of Corti preparations, we noted abundant staining in the cytoplasm of IHCs and OHCs characterized by fine punctate staining apparent at the base, around the nucleus and throughout the apical part of the hair cells (Fig. 6g-i). There was no obvious staining at the stereocilia bundle (Fig. 6j). Immunostaining with Pab877 revealed a similar expression and localization profile, showing staining of the cytoplasm of vestibular and cochlear hair cells and spiral ganglion neurons (Supplementary Fig. S2). Staining with antibodies against Vglut3 and Myosin VI showed localizations similar with Gipc3 in BLSW cochleae (Fig. 6k,l). Incubation with nonspecific rabbit IgG antiserum produced no staining (Supplementary Fig. S2).

In individual II.1, the hearing loss progressed slowly to a loss of 110 dB at 12 years of age and individual II.3, who is now 14 years old, has a stable hearing loss of 90 dB HL (Fig. 4c).

In an Indian family, segregating autosomal recessive non-syndromic hearing impairment previous linkage analyses detected a 32 cM region of homozygosity by descent at 19p13 (D19S209–D19S411; assigned locus symbol: DFNB15) that included the GIPC3 gene. Sequencing of the six GIPC3 exons identified a homozygous missense mutation, c.785T>G (p.Leu262Arg), in exon 5 that segregated with the deafness in the family (Fig. 4d,e and Supplementary Table S3). Sequence alignments of GIPC3 proteins indicated that the Leu262 residue is highly conserved (Fig. 4f). The mutation was not observed in 161 (322 chromosomes) control individuals.

Hair bundle defects and sensorineural degeneration in BLSW. The high-to-low frequency progression of the hearing loss suggested a histopathology in the organ of Corti. By confocal and scanning electron microscopy we observed an irregular structure of the stereocilia bundle of OHC and inner hair cells (IHC). Starting from postnatal day 3 (P3) onwards, OHC bundles were slightly disoriented and smaller, with bent lateral edges and a rounded apical pole (Fig. 5a,b). The hair bundle of IHC appeared less rigid and sparse (Fig. 5a,b) and at P21 stereocilia were thinner and at times also shorter (Fig. 5c). Stereocilia bundles at the base and mid-apical region were present and were affected to a similar degree. Histological sections of 8-week-old BLSW ears revealed a normal morphology of the organ of Corti and the spiral ganglion. At 52 weeks of age, there was a significant degeneration of the organ of Corti starting at the base and progressing towards the apex. Concomitantly, the spiral ganglion exhibited a severe loss of neurons that was most obvious at the base and the mid-apical region of the cochlea. The organ of Corti and spiral ganglion at the apex were of normal appearance (Fig. 5d).

Together, these data suggest that Gipc3 is required for postnatal maturation of the hair bundle and long-term survival of hair cells and spiral ganglion.

Figure 3 | Analysis of Gipc3 transgenic mice. (a) ABR thresholds (dB SPL) for click and pure tone pips at 8, 16 and 32 kHz are given in bars as mean±s.d. for backcross progeny tg(+/−) G/A (green, n=20), tg+A/A (blue, n=36) and tg–A/A (red, n=37). Green bars represent pooled data from backcross mice that were either positive or negative (+/−) for the transgene. (b) Audiogenic seizure susceptibility of tg(+/−) G/A (green symbol, n=10), tg+A/A (blue, n=14) and tg–A/A (red, n=16) was assessed at 3 weeks of age using white noise stimuli (90, 100 and 110 dB). Seizure latency (sec) and testis latency (sec) were assessed at 3 weeks of age using white noise stimuli (90, 100 and 110 dB) and individual II.3, who is now 14 years old, has a stable hearing loss of 90 dB HL (Fig. 4c).
Mechanotransduction and $K^+$ currents in BLSW. The compromised structure of the stereocilia bundle suggested a defect in mechanotransduction. At P6, we found no difference in transduction currents recorded from OHCs and IHCs between BLSW and Swiss Webster control mice. The mean maximal amplitudes of the transduction currents operating range and adaptation time constants were not significantly different between BLSW and control hair cells. However, by P12, we observed a significant reduction in the maximal transduction current amplitude in both IHCs and OHCs of P12-P14 BLSW mice were reduced by 57 and 83%, respectively, compared with control C3HeB/FeJ mice (3.9 ± 1.5 μV, n = 12). These high amplitudes declined rapidly to 0.55 ± 0.25 μV (n = 10) at 7 weeks of age. In particular, decreases in wave I amplitudes were significant between 3 (3.5 ± 1.5 μV, n = 18) and 5 (2.1 ± 1.5 μV, n = 24) weeks of age and between 4 (3.3 ± 1.5 μV, n = 33) and 6 (1.2 ± 0.9 μV, n = 32) weeks of age (Fig. 8b). In contrast, the wave I amplitude in control mice remained constant over the 8-week test period, showing no difference between 2- and 10-week-old mice (2.9 ± 0.8 μV, n = 10, P > 0.05). Amplitudes of waves II through V were not different compared with C3HeB/FeJ controls (Fig. 8c,d).

We next analysed the latency of the ABR wave I. Latencies of 3- to 10-week-old BLSW mice were on average 0.14 ± 0.05 ms higher than those in control mice (P < 0.001), but did not change significantly over the test period (P > 0.05). Specifically, no significant delays were observed between 3 (0.4 ± 0.04 ms, n = 10) and 5 (0.4 ± 0.03 ms, n = 8) and between 4 (0.4 ± 0.05 ms, n = 9) and 6 (0.4 ± 0.04, n = 17) weeks of age (Fig. 8e).

Last, we correlated ABR thresholds at the click stimulus with seizure progression. ABR thresholds significantly increased in BLSW mice between 2 and 12 weeks of age. However, there were no significant increases in thresholds in mice between 3 (65 ± 6 dB SPL, n = 33) and 6 weeks of age.

**Figure 4 | GIPC3 mutations and human deafness.** (a) Sequencing chromatograms indicating the guanine (G) and adenine (A) in family W98-042 (top) and control (bottom) at nucleotide position c.903 changing the tryptophan (Trp) residue at position p.301 to a stop codon (p.Trp301X). (b) Shown is the two-generation pedigree of consanguineous family W98-042 with first-cousin parents. Hearing-impaired brothers homozygous for the mutation (II.1 and II.3; A/A) are indicated by filled black boxes and normal hearing relatives are indicated by open symbols. (c) Mean pure-tone audiograms of the two affected individuals II.1 and II.3 of family W98-042 at different ages are given in years (y). Note the threshold shifts of 80–120 dB HL above normal hearing levels. (d) Sequence chromatogram showing c.785T > G (p.Leu262Arg) mutation in GIPC3 in individual II-5. (e) Pedigree of the Indian family with prelingual, profound autosomal recessive non-syndromic hearing impairment. The c.785 genotype is shown. Open symbols unaffected; filled black symbols affected; double line consanguineous event. (f) Multi-sequence alignment of GIPC family proteins showing the high conservation of the Leu262 residue.

**ABR amplitudes and audiogenic seizure progression.** To investigate the cause of the audiogenic seizure susceptibility and resistance, we correlated various ABR output parameters with seizure progression. Acoustic stimulation showed that BLSW mice were most susceptible to seizures between 2 and 4 weeks of age with mean seizure latencies of 40 ± 42 s (n = 19) and 89 ± 32 s (n = 21), respectively. Thereafter, latencies increased until animals became completely resistant at 6 weeks of age (180 s, n = 15). Increases in seizure latencies were significant between 2 and 3 weeks and between 4 and 6 weeks of age (Fig. 8a).

Amplitudes of the first (I) ABR wave (click at 100 dB SPL) were significantly higher in 2-week-old BLSW mice (6.8 ± 2.6 μV, n = 24) compared with control C3HeB/FeJ mice (3.9 ± 1.0 μV, n = 12). These high amplitudes declined rapidly to 0.55 ± 0.25 μV (n = 10) at 7 weeks of age. In particular, decreases in wave I amplitudes were significant between 3 (3.5 ± 1.5 μV, n = 18) and 5 (2.1 ± 1.5 μV, n = 24) and between 4 (3.3 ± 1.5 μV, n = 33) and 6 (1.2 ± 0.9 μV, n = 32) weeks of age (Fig. 8b). In contrast, the wave I amplitude in control mice remained constant over the 8-week test period, showing no difference between 2- and 10-week-old mice (2.9 ± 0.8 μV, n = 10, P > 0.05). Amplitudes of waves II through V were not significantly different between BLSW and controls at 2 weeks of age. At 6 weeks of age the amplitude of wave II was reduced in BLSW, but the amplitudes of waves III through V were not different compared with C3HeB/FeJ control (Fig. 8c,d).

We next analysed the latency of the ABR wave I. Latencies of 3- to 10-week-old BLSW mice were on average 0.14 ms higher than those in control mice (P < 0.001), but did not change significantly over the test period (P > 0.05). Specifically, no significant delays were observed between 3 (0.4 ± 0.04 ms, n = 10) and 5 (0.4 ± 0.03 ms, n = 8) and between 4 (0.4 ± 0.05 ms, n = 9) and 6 (0.4 ± 0.04, n = 17) weeks of age (Fig. 8e).
5 (67±6 dBSPL, n = 27) and between 4 (69±8 dBSPL, n = 32) and 6 weeks of age (71±7 dBSPL, n = 24; Fig. 8f). Together, these data suggest that the elevated and subsequent decline of the wave I amplitude correlated with the susceptibility and resistance to audiogenic seizures, respectively.

**Discussion**

Despite significant advances in the understanding of monogenic deafness, the molecular genetics of the more common and complex forms of hearing loss is poorly characterized. Nine QTLs controlling oligogenic hearing impairment in the mouse have been reported,
but, thus far, only two loci have been molecularly characterized (Cdh23\textsuperscript{753A} and Fscn\textsuperscript{326A})\textsuperscript{26,27}. Here, we identified the \textit{ahl5}/\textit{jams1} and DFNB15/DFNB95 loci uncovering a function for Gipc3 in peripheral auditory signal transmission in mouse and human.

Gipc3 distribution in hair cells is reminiscent of vesicular staining and shows a similar localization as Vglut3 and Myosin VI, proteins that regulate synaptic glutamate release and \(\text{Ca}^{2+}\)-dependent exocytosis at the IHC ribbon synapse, respectively\textsuperscript{28–30}. GIPC1 was shown to associate with membranous and cytosolic fractions and also localizes to synaptic vesicles at presynaptic axon terminals of hippocampal neurons\textsuperscript{19,31}. Interestingly, glutamate release from presynaptic terminals in the hippocampus was shown to depend on GIPC1/Myo6 interactions\textsuperscript{32}. Given the hair bundle defect, reduced mechanotransduction and \(\text{K}^{+}\) currents as well as abnormal ABR wave I amplitude in BLSW mice, we hypothesize that Gipc3 may be part of a protein complex involved in vesicle trafficking that transports factors critical for signal acquisition and propagation in cochlear hair cells.

The Gly115Arg change occurring in the loop connecting the \(\beta\)B with the \(\beta\)C strand of the PDZ domain is positioned closely to the peptide recognition pocket\textsuperscript{32}. Our western blot data indicate that the Gly115Arg substitution leads to a reduction in Gipc3 protein levels. It is possible that a conformational change or loss of stable interactions may target Gipc3(115Arg) for early degradation. The human

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**Figure 6 | Protein blot analysis and immunolocalization of Gipc3.** (a) Western blot analysis of Gipc3\textsuperscript{343G} transfected (+) and untransfected (−) HEK293T cell lysates using Gipc3-specific polyclonal antiserum Pb867 detecting a single ~40 kDa band (black arrow). (b) Immunoblot analysis of protein extracts from cochlea and testis from BLSW and C3HeB/FeJ (C3H) mice with anti-Gipc3 antiserum Pb867. Lysates from HeLa cells transfected with the wild-type Gipc3\textsuperscript{343G} construct were included as control. The ~40 kDa band (black arrow) was detected in both tissues from C3HeB/FeJ wild-type mice. The similar ~40 kDa band was detected with reduced intensity in mutant BLSW cochlea and testis. The ~37 kDa band (arrow head) and ~29 kDa band (dot, presumably nonspecific) are indicated. (c) Quantitative analysis of wild-type Gipc3(115Gly) and mutant Gipc3(115Arg) expression in lysates from transiently transfected HEK293T cells with varying amounts of protein loaded (10 and 30 µg) for wild-type Gipc3\textsuperscript{343G} and mutant Gipc3\textsuperscript{343A}. Protein blots were probed with the anti-Gipc3 antiserum Pb867and anti-\(\alpha\)-actin antibody. (d) Densitometric analysis of Gipc3 expression from wild-type Gipc3(115Gly) and mutant Gipc3(115Arg)-transfected HEK293T cells. The data are represented as mean ± s.e.m., **\(P<0.01\) (n = 6, t-test). Confocal images of cryosections of organ of Corti (e) and spiral ganglion (f) stained with Pb867 antibody (red) demonstrating Gipc3 localization in IHCs (arrow head) and OHCs (arrow) and spiral ganglion (arrow). Confocal images of organ of Corti of C3HeB/FeJ (g, h, j) and BLSW (i, k, l) demonstrating Gipc3 (g-i) and Vglut3 (k) and myosin VI (l) localization; (i-l) counterstained with phalloidin (green); arrows point to positive staining. (e-k) Counterstained with 4,6-diamidino-2-phenylindole (blue). (l) No Pb867-staining is seen in stereocilia of IHC (arrow head); scale bar, 5 µm (e-l).
Trp301X mutation truncates a peptide that shows 42% sequence identity with the GIPC1 C-terminus. It was recently shown that truncation of 29 residues of the C-terminus of GIPC1 abolishes interaction with Myosin VI suggesting that the full-length C-terminus is required to form stable interactions. In addition, both Leu262 and Trp301 residues are highly conserved among metazoan GIPC proteins. These data indicate that Trp301X and Leu262Arg are pathogenic mutations.

It was recently suggested that the transient audiogenic seizures in BLSW mice occur independent of the hearing loss. Indeed, our data do not support a correlation between hearing thresholds and audiogenic seizure progression. Furthermore, the reduced mechanosensitivity seems to be at odds with audiogenic seizure susceptibility. However, our results implicate the ABR wave I amplitude, which represents the summed activity of the cochlear nerve fibres projecting from IHCs to the cochlear nucleus, in seizure susceptibility and resistance. The high wave I amplitude at 2 weeks of age may result from the stunted developmental acquisition of IHC K⁺ currents. Whereas the normal acquisition of potassium currents attenuates the IHC receptor potential, lack of those currents in BLSW allows greater depolarization, which may in turn enhance Ca²⁺-dependent neurotransmission, perhaps leading to synchronized cochlear nerve activity, enhanced ABR wave I amplitudes and seizure susceptibility. The subsequent decline in the ABR wave I amplitude and seizure resistance in BLSW mice may be indicative of a neuropathy at the IHC spiral ganglion neuron synapse. In the cochlea, glutamate receptors mediate fast synaptic transmission at the IHC afferent synapse and glutamate excitotoxicity is central to the neural damage during acoustic trauma.

Clinically, the audiogenic seizure pathology in BLSW mice is a form of reflex epilepsy, in which external sensory stimuli induce seizures. In humans, acoustic reflex epilepsies, although rare, include startle and musicogenic epilepsies and overt sensitivity to sound also underlies hyperacusis and hyperacute hearing in autism.
syndrome\textsuperscript{37–40}. The pathogeneses underlying these sensitivities are not understood or is it clear whether they originate in the central or peripheral auditory system. BLSW mice and perhaps other mutant alleles of Gipc3 may prove critical to better understand the cause of acoustic hypersensitivity.

Our analysis of the hearing loss in BLSW mice has interesting implications for the genetics of human progressive SNHL. GIPC3 and its paralogs are excellent candidate genes for ARHI. It can be argued that ahls5 is a monogenic trait not modelling the degree of genetic complexity presumed to underlie AHRI. Although this argument assumes the common variant–common disease hypothesis, it is also conceivable that rare alleles with strong effects in homozygous or heterozygous state may be strong contributors to ARHI. Furthermore, a mutation in the graninhead like 2 gene (GRHL2) causes progressive hearing loss in humans and a candidate gene approach subsequently associated a GRHL2 variant with ARHI\textsuperscript{41,42}. Susceptibility to metabotropic glutamate receptor 7 (mGlur7)-mediated excitotoxicity was suggested as risk factor for ARHI\textsuperscript{43}. Interestingly, mGlur7 and Gipc3 show identical localization domains in the mouse cochlea and also share functional similarities\textsuperscript{3,18}. Primary neuronal degeneration, which causes hearing impairment because of spiral ganglion degeneration in the absence of hair cell loss, was recently shown to underlie the long-term effect of impairment because of spiral ganglion degeneration in the absence of hair cell loss. Together, these observations suggest a prominent role for variations in genes affecting spiral ganglion function in sensorineural progressive hearing loss.

**Methods**

**Mice and crosses.** Black Swiss mice were obtained from Taconic and C3HeB/FeJ mice were purchased from The Jackson Laboratory. BLSW mice were originally derived male pronuclei. Transgenic animals were serially backcrossed to BLSW and sequence and human β-globin polyadenylation sequence was injected into FVB/N-derived male pronuclei. Transgenic animals were serially backcrossed to BLSW and genotyped by PCR. Institutional review boards at NIH and University of Virginia approved the animal studies.

**Auditory tests in mice.** For ARB, acoustic stimuli were generated from a high frequency transducer and presented at varying intensity at a rate of 19.1 times per second for a total of 350 presentations. Neuronal responses were captured through subdermal needle electrodes inserted at the vertex, filtered, amplified and displayed. Thresholds, latencies and amplitudes were determined using NIH software.

DPOAEs were measured using National Instruments (NI) LabView 8.6 software, operating an NI PCI-4461 Dynamic Signal Analyzer sound card (National Instruments), to generate two pure tones, f1 and f2, at the fixed f2/f1 ratio of 1.25, which were emitted separately by two Clarion SRU310H high frequency dome tweeters (Clarion) placed in the outer ear canal at the presentation level of f2 = f1. Emissions were measured using an E tymetric-ER-10B+ microphone. The amplitude of the 2f1-f2 distortion product was plotted in dB SPL against the f2 frequency where the distortion product is generated.

To measure the EP, a silver/chloride electrode bathed in 0.1 M KCl was inserted through the round window into the endolymph using a remote controlled motorized Micromanipulator (Piezo World Precision Instrument). The electrode was connected to a Warner Dual Channel Differential Electrometer (HIZ-223; Warner Instruments), which amplified and routed the voltage difference (subdermal 1 M KCl reference electrode) to the data acquisition system (Digidata 1440A, Axon Instruments) using Axoscope software (Axon) at a sampling rate of 10 kHz for 60 s.
To induce audiogenic seizures, mice were exposed to white noise of 90, 100 and 110 dB SPL consecutively for 60 s each stimulus. Acoustic stimuli were generated by a Bose loudspeaker (Bose) operated by ToneGen software. Speaker levels were calibrated with SoundProDL (Clarke Analytical Instruments). The mouse was placed in a clear polycarbonate box container with a lid with the loud speaker mounted into the lid. Each stimulus was maintained for 60 s and latency was measured as evidenced by wild running, flexing and tonic-clonic convulsions\(^{44}\). Seizure latency (sec) was defined as the time of the first stimulus onset to the time of seizure onset. Animals resistant to the three consecutive sound stimuli were scored as having a latency of 180 s.

To measure mechanotransduction, the hair bundles were stimulated with a fire polished glass pipette that fit the shape of the bundles. The stimulator was cemented to a PICMA ultra fast piezo actuator (Physik Instruments), driven by a ENV-400 amplifier (Piezosystem Jena). Transduction currents were recorded using the whole-cell, tight-seal technique. Recording pipettes were filled with intracellular solution. Membrane potential was clamped to ~64 mV and controlled using an Axopatch Multiclamp 700A amplifier (Molecular Devices). pClamp 8.2 (Molecular Device) and Origin 7.5 (OriginLab) were used to record and analyse the currents.

**Antibodies and immunoblot analysis.** Rabbit polyclonal antisera (Covance) were raised against Gipc3-specific peptides (Princeton Biomolecules). Sera were affinity-purified (Covance). Antiserum Pb867 was raised against EPPARPRPLY-FRTQLAHGS (aa 13–32), Pb869: APSDVEAAARVDVDL (aa 22–32); Pb877 VSSGRETLLRSGGAATVEEA (aa 199–220). Mouse Gipc3 protein reference sequence: NP_683753, GenBank protein sequence database. Additional antibodies used are Anti-Mycos V1 (Sigma) and Anti-V-glut3 (SynapticSystem).

Cochlear and tectis were homogenized in RIPA buffer containing 1 mM phenylmethylsulphonyl fluoride and complete protease inhibitor cocktail (Roche). Confuent HEK293 or HeLa cells were lysed on ice in 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 10 mM iodoacetamide, 1% Triton X-100 with complete protease inhibitor cocktail (Roche). For immunoblotts, the protein lysate was resolved by 4–12% SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, incubated with the primary antibody, complemented with a secondary goat anti-rabbit or goat anti-mouse antibody conjugated to horsedarish peroxidase (Kirkegaard and Perry Laboratories) and detected using enhanced Chemiluminescence (Pierce Laboratories). The data were quantified by measuring total protein band intensities ratios using UVP multispectral imaging system and normalized to control α-actin band.

**Histology and immunohistochemistry.** For gross morphology, the ear was fixed with 4% paraformaldehyde, dehydrated with a graded series of ethanol, and infiltrated with JB-4 polymer. Serial sections were cut at 4 mm thickness and stained with 1% Tolidine Blue O. For cryosections, ears were fixed, equilibrated in a graded series of sucrose in PBS, transferred to a 2:1 mixture of 20% Sucrose in PBS (4°C) (diluted 1:4), from -10°C in 20% sucrose, –12°C, at 4°C in 1% OsO\(_4\) dehydrated in ethanol, critical point dried, sputter coated with platinum, dehydrated in ethanol, critical point dried, sputter coated with platinum and imaged in a Field emission scanning electron microscope (Hitachi, S-4500).

**Plasmid constructions and transfections.** Full-length mouse Gipc3 (ref. sequence: NM_148951, GenBank Nucleotide Core) was amplified from CBA/CaJ cochlea cDNA by PCR and cloned into pc4-TOPO vector (Invitrogen) to generate clone pClu2. For the transgene construct, a 932-bp wild-type cDNA clone pU2. For the transgene construct, a 932-bp NM_148951, GenBank Nucleotide Core) was amplified from CBA/CaJ cochlea and imaged in a Field emission scanning electron microscope (Hitachi, S-4500).

**Human patients and mutation analysis.** The local medical ethics committees at the Radboud University Nijmegen, the Netherlands, the University of Iowa, USA and the University of Madras, India approved the studies. Informed consent was obtained from the participating subject or, in the case of children, from their parents. For the DFN95 family, patients were examined by otoscopy and pure-tone audiometry. One sound-treated room in accordance with current clinical standards. Individual II.1 received a cochlear implant after the audiogram taken at 11.5 years of age. High-resolution single-nucleotide polymorphism (SNP) genotyping and mutation analysis was performed as described\(^{44}\). Briefly, genome-wide linkage scans were performed using the Affymetrix mapping 250K SNP array (Affymetrix, Santa Clara, CA). Genotypes and regions of homozygosity were defined using the Genotyping Console software (Affymetrix). Primers for amplification of exons and exon–intron boundaries of GIPC3 were designed with PrimerExperimenter according to the reference sequences NM_133261.2 and NT_011255.14 (Supplementary Table S2). To determine the segregation of the c.903G>A variant in the family and the presence in normal controls, exon 6 was amplified and the PCR product was digested with Sau96I (New England Biosciences).

The DFN95 locus segregating in a two-generation consanguineous Indian family with prelingual, profound autosomal recessive hearing impairment was reported previously\(^{44}\). The GIPC3 gene was amplified using gene-specific primers using a standard protocol on a GeneMate Genius thermocycler (ISC Bioexpress), sequenced and analysed as described above.

**Statistical analyses.** Unless otherwise indicated groups of data were compared using one-way ANOVA followed by Bonferroni post-tests to correct for multiple testing. GraphPad Prism 4.0b software (GraphPad) was used to perform the arithmetic, statistics and to plot the data.

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Author contributions

N.C. (Figs 1a–f, 2a,b,e, 3a,b and 8a–c) conceived, performed and analysed the experiments. J.R.L. (Figs 2c,d, 5a and 6g–l) performed and analysed the experiments. J.R.H. conceived and analysed experiments (Figs 5c, 7a–f) and A. L. (Fig. 7a–f), J.O., K.R. and J.K.N. (Fig. 6a–d) performed and analysed experiments. H.K. conceived experiments; R.J.C.A. contributed material and analysed data (Fig. 4a–c). M.H., A.R., C.R.S.S. and R.H.S. performed experiments, contributed reagents and analysed data (Fig. 4d–f). N.C., J.R.H. and K.N.-T. wrote the paper with comments from M.H., M.S.H., K.R. and H.K. on 2004-512063), and the Oticon Foundation. C.R.S.S. was supported by the University Grants Commission Research Scientist Scheme in New Delhi, India. M.S.H. was supported by an Australian NHMRC Overseas Biomedical Postdoctoral Training Fellowship.

Additional information

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