Gαi Proteins are Indispensable for Hearing


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Key Words
Heterotrimeric G-proteins • Gαi3/GNAI3 • Stereocilia bundle • Cochlear hair cell maturation • Neural gain • Deafness gene

Abstract
Background/Aims: From invertebrates to mammals, Gαi proteins act together with their common binding partner Gpsm2 to govern cell polarization and planar organization in virtually any polarized cell. Recently, we demonstrated that Gαi3-deficiency in pre-hearing murine cochleae pointed to a role of Gαi for asymmetric migration of the kinocilium as well as the orientation and shape of the stereociliary (“hair”) bundle, a requirement for the progression of mature hearing. We found that the lack of Gαi3 impairs stereociliary elongation and hair bundle shape in high-frequency cochlear regions, linked to elevated hearing thresholds for high-frequency sound. How these morphological defects translate into hearing phenotypes is not clear.

Methods: Here, we studied global and conditional Gnaia3 and Gnaia2 mouse mutants deficient for either one or both Gαi proteins. Comparative analyses of global versus Foxg1-driven conditional mutants that mainly delete in the inner ear and telencephalon in combination with functional tests were applied to dissect essential and redundant functions of different Gαi isoforms and to assign specific defects to outer or inner hair cells, the auditory nerve, satellite...
cells or central auditory neurons. **Results:** Here we report that lack of Gαq, but not of the ubiquitously expressed Gαq, elevates hearing threshold, accompanied by impaired hair bundle elongation and shape in high-frequency cochlear regions. During the crucial reprogramming of the immature inner hair cell (IHC) synapse into a functional sensory synapse of the mature IHC deficiency for Gαq, or Gαq, had no impact. In contrast, double-deficiency for Gαq and Gαq isoforms results in abnormalities along the entire tonotopic axis including profound deafness associated with stereocilia defects. In these mice, postnatal IHC synapse maturation is also impaired. In addition, the analysis of conditional versus global Gαq-deficient mice revealed that the amplitude of ABR wave IV was disproportionally elevated in comparison to ABR wave I indicating that Gαq is selectively involved in generation of neural gain during auditory processing. **Conclusion:** We propose a so far unrecognized complexity of isoform-specific and overlapping Gαq protein functions particular during final differentiation processes.

**Introduction**

Heterotrimeric G-proteins are grouped into four subfamilies, i.e. Gαq, Gα12, Gα13, and Gα12/13, according to the nature of their α subunit, which also reflects their specificity for distinct G-protein-coupled receptors and sets of effector proteins [1, 2]. The three mammalian Gαq isoforms, i.e. Gαq', Gαq', and Gαq', are best described as being the inhibitory Gα subunits that suppress adenylyl cyclase activity resulting in decreased cellular cAMP levels [3-5]. In addition, specific binding partners such as members of the activators of G-protein signaling (AGS) family have emerged as crucial intracellular regulators that can control G-protein activity even in the absence of GPCR-stimulation. Until now, Gαq isoforms together with Gpsm2 (also known as AGS5, LGN), a group II AGS/guanine nucleotide dissociation inhibitor (GDI) [6, 7], have been implicated in signaling pathways that control mitotic spindle dynamics and thereby asymmetric cell division, polarity, growth and differentiation in invertebrates up to mammalian cells [8-12]. Initially, Gpsm2 was found in functional screens as a receptor-independent activator of G-protein-signaling interacting with GDP-bound Gαq through its four Gαq' -loco motifs [13, 14]. Subsequently, this interaction was demonstrated for all three Gαq isoforms at least in vitro [15]. Inhibiting Gαq protein function with the pan-Gαq-blocker PTX or via expression of an Atoh1-PTX construct demonstrated that Gαq and Gpsm2 control kinocilium migration and planar organization of stereociliary bundles in the hair cells of the murine cochlea [16, 17]. Gpsm2 dysfunction has been linked to mutations in the GPSM2 gene in patients, formerly known as the deafness locus DFNB82, responsible for the autosomal recessive disorder Chudley-McCullough-syndrome [18, 19] that is characterized by severe to profound congenital hearing loss in humans [20-22]. Moreover, knock-out mice revealed that the deletion of the Gαq isoform was sufficient to mimic the PTX phenotype in the immature pre-hearing organ by disrupting the migration of the kinocilium at the surface of hair cells and affecting hair bundle orientation and shape [16]. So far, Gαq per se has not been associated with hereditary deafness because of an assumed functional Gαq isoform redundancy for hearing. Nevertheless, we and others recently reported hearing loss in Gαq cKO and Atoh1-PTX mice, correlating with stereociliary elongation defects in IHCs [19, 23]. It remains open whether the ubiquitously expressed Gpsm2-binding partner Gαq has also an impact on cochlear architecture and auditory function and whether both Gαq proteins play specific and/or redundant roles.

Here, we examined the physiological role of Gαq isoforms for hearing by using gene-targeted mouse lines for Gαq, Gαq, and Gαq/Gαq, and transgenic reporter mice expressing Gαq-GFP. Comparative analyses of global versus conditional mutants that mainly delete in the inner ear and the telencephalon in combination with functional tests allowed us to dissect essential and redundant functions of different Gαq isoforms and to assign specific defects to outer or inner hair cells, the auditory nerve, satellite cells or central auditory neurons. We show that both Gαq and Gαq act in a spatially and functionally complex manner not only on planar organization of hair cell bundles, but are also required for final differentiation
of the first auditory synapse (the inner hair cell), and central neural gain during auditory processing. Therefore, hearing loss associated with dysfunction of specific $G\alpha_i$ isoforms is not only caused by abnormal stereociliary bundle or mitotic spindle organization but may also be a consequence of the disability for postnatal neuronal maturation and central adaptation.

**Materials and Methods**

**Animals**

Adult (1- to 8-month-old) mice weighing 15-35 g were included in this study. For all mouse lines, male and female animals were used, because we did not observe sex-dependent differences. The use, treatment and care of the animals and the experimental protocols were based on the institutional guidelines of the Veterinary Care Unit, University of Tübingen and were approved by the animal welfare commissioner of the regional board for scientific animal experiments in Tübingen (PH7/14). Experiments were performed according to the European Union Directive 86/609/EEC for the protection of animals used for experimental and other scientific purposes. Mice were kept according to national guidelines for animal care in an SPF-pathogen free (SPF) animal facility.

**Gene targeting and mice**

For generation of $G\alpha_i^{3/\circ}$ mice, E14.1 embryonic stem (ES) cells from 129/Ola mice were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine (Seromed, Wien, Austria), leukemia inhibitory factor, 100 U/ml penicillin, 100 µg/ml streptomycin (Seromed), 50 µM 2-mercaptoethanol (Invitrogen) and 15% heat-inactivated fetal bovine serum (Pan Biotech, Aidenbach, Germany). Genomic fragments flanking the murine $G\alpha_3$ gene as well as $TK$-neomycin and $diphtheria$ $toxin$ $A$ ($DTA$) were cloned into pBluescript (Agilent technologies, Santa Clara, CA, USA) and fully sequenced. The targeting vector was designed according to the $G\alpha_3$ knockout construct, where exon 6 is deleted [24]. Therefore, exon 6 of the $G\alpha_3$ gene was flanked by loxP-sequences. At the 3’ site, the loxP-flanked $TK$-$Neo$ resistance cassette was inserted in reverse orientation in intron 6 of the $G\alpha_3$ gene without deleting any endogenous sequence. Additionally, after the short arm, which encodes exon 7, a DTA cassette was inserted for the negative selection of ES cell clones (for all online suppl. material, see www.karger.com/doi/10.1159/000490867, suppl. Fig. 1). E14.1 ES cells were electroporated with the NotI-linearized targeting vector, and the transfected clones were subsequently subjected to $G418$ and Ganciclovir selection. Clones carrying the correct homologous recombination were identified by Southern blot hybridization with the 5’ flanking probe (5´fp) indicated in (see online suppl. material) suppl. Fig. 1 after digestion with PstI (data not shown). Single integration was verified by digestion with BamHI and probing the Southern blot with the internal probe P2 (3´fp2, see online suppl. material, suppl. Fig. 1A) after digestion with PstI (data not shown). Correctly targeted ES cell clones were injected into C57BL/6 blastocysts, which were transferred into pseudopregnant foster mice. Resulting chimeric mice were backcrossed to C57BL/6 mice, and germ line transmission of the targeted allele was again confirmed by Southern blot analysis. $G\alpha_i^{3/\circ}$ mice were backcrossed to the C57BL/6 genetic background for at least five generations. Genotyping of $G\alpha_i^{3/\circ}$ mice was performed by PCR with the following primers: 5’-GGAATTCTTTTGGTGGC-3’ (Ex6-F); 5’-ATGCTGTCAAACATTTGCACGT-3’ (Ex7-R); 5’-TTAGCATTTGTCACTGGTTCC-3’ (Int-F) (see also online suppl. material, suppl. Table 1). The recombined allele results in a band of 350 bp (see online suppl. material, suppl. Fig. 1 B and C). To check successful deletion of $G\alpha_3$, mice were crossed with $Ella$-$Cre$ mice and PCR analysis confirmed deletion of $G\alpha_3$ (see online suppl. material, suppl. Fig. 1B and C). A specific deletion of $G\alpha_3$ or $G\alpha_2$ in the inner ear and telencephalon was achieved by crossing $G\alpha_i^{3/\circ}$ or $G\alpha_i^{2/\circ}$ mice and $Foxg1$-$Cre$ mice. $G\alpha_i^{2/\circ}$ mice were provided by the Comparative Medicine Branch of the National Institute of Environmental Health Sciences, North Carolina 27709 [25], backcrossed on a C57BL/6N background [26], and the $Foxg1$-$Cre$ mouse line [27] was purchased from The Jackson Laboratory (Bar Harbor, ME, USA), on a C57BL/6N background. A tissue-specific mutant mouse line was generated where both $G\alpha_i^{2/\circ}$
genes were disrupted by crossing with the FoxG1-Cre mouse line. Gnai2\textsuperscript{fl/fl}, Gnai3\textsuperscript{fl/fl}, Foxg1-Cre\textsuperscript{+/+} littermates were used as controls. Genotyping of Gnai2\textsuperscript{fl/fl}, Gnai3\textsuperscript{fl/fl}, Foxg1-Cre\textsuperscript{+/-} littermates was performed by PCR with the following primers: for: 5'-GGCTATGATCCCAAAACTCCCCG-3' and rev: 5'-GTGGTAAGCCTGTGTTTGTGAGAG-3'. The recombined allele results in a band of 420 bp [data not shown].

Gnai3-GFP mutant mice were generated in collaboration with Cyagen Biosciences and a more detailed description will be described elsewhere (Beer-Hammer et al., in preparation). Briefly, the eGFP-cDNA was inserted in Exon 8 of Gnai3 right after the TGA stop codon. To achieve equal expression of Ga\textsubscript{i3} and eGFP, the construct contains an IRES (internal ribosomal entry site). With this we achieve an equivalent expression of Ga\textsubscript{i3} and GFP. This expression was successfully detectable via flow cytometry (data not shown) and immunohistochemistry (this study). Gnai3-GFP mutant mice are born and grow without any obvious signs of abnormalities.

Organismal ("global") Gnai-deficient mice were originally provided by the Comparative Medicine Branch of the National Institute of Environmental Health Sciences, North Carolina 27709 on a genetic SV129 background [28]. Subsequently, the mouse strains were backcrossed on a C57BL/6N background for at least nine generations [26, 29]. The Gnai2 mutant mouse line was kept in Individually Ventilated Cages (IVC), while the Gnai3 mutant mouse line was kept under SPF conditions. Generation and initial characterization of global and conditional Gnai2- or global Gnai3-deficient mice is described in detail in [16, 24, 30].

**Hearing tests**

Hearing was tested on anesthetized animals through auditory brainstem response (ABR) measurements of the distortion product otoacoustic emissions (DPOAE), and electrocochleography. ABR to click and pure tone stimuli and the cubic 2*\textit{f1}–\textit{f2} DPOAE for \textit{f2}=1.24*\textit{f1} and \textit{L2}=L1–10 dB were recorded in adult 1- to 8-month-old anesthetized mice. From click-evoked ABRs, consecutive wave components that reflect the summed neural activity along the ascending auditory pathway were analyzed as described [31-33]. In vivo electrocochleography (compound action potentials of the auditory nerve, CAP; summing potential of IHC receptor potentials, SP; receptor potential of OHCs, cochlear microphonic, CM) was performed in anesthetized mice as described [34]. From the waveform signal of the CAP and CM recordings, peak amplitudes (in mV) were calculated and plotted as input/output (I/O) functions with increasing stimulus level (0–100 dB SPL). For CM I/O function, peak-to-peak amplitudes were calculated for the stimulus frequency of 16 kHz, the stimulus frequency giving the best response. For SP, amplitude was defined as average DC potential minus baseline for stimulus frequency at 11.3 kHz.

**SEM and measurements**

The inner ear (cochlear and vestibular system) of mice aged P21 were harvested and immersed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.35, with 3mM CaCl\textsubscript{2} for 24 h or more. The tissues were postfixed in 1% OsO\textsubscript{4} in the same buffer, dissected and double processed with thiocarbohydrazide followed by OsO\textsubscript{4} [35] before dehydration through an alcohol series and critical point drying with CO\textsubscript{2} [36]. After mounting on specimen support stubs, samples were sputter coated with platinum. Samples were examined with a JEOL 6700 F cold field emission scanning electron microscope operating at 3 or 5 kV. Measurements were made on the longest row of stereocilia closest to the kinocilium.

Stereocilia length was estimated in 20 (control) and 11 (Gnai3 cKO) independent experiments in tissue samples from 4 control and 4 Gnai3 cKO mice, respectively. Only if the length of at least 4 stereocilia could be determined, the value was considered for the statistical analysis. Stereocilia length could be derived from a total of 64 hair cells of control mice and 64 hair cells of Gnai3 cKO mice. A total of 272 stereocilia and 305 stereocilia could be measured for control and Gnai3 cKO mice, respectively. Stereocilia number per hair cell was counted in 12 and 14 independent experiments (countings) for 5 control and 4 Gnai3 cKO mice, respectively. Each stereocilia counting included at least 4 hair cells. From a total of 87 and 106 hair cells from control and Gnai3 cKO mice, a total of 8299 and 7621 stereocilia could be counted, respectively.

Images were collected from the basal or middle turn of the cochlea, defined as B20/30% and 50/60% of the total length of the organ of Corti from the base. At each location, the hair bundles were viewed both from behind the longest row of stereocilia to view the height of the hair bundle as well as approximately perpendicular to the apical surface of the HC or toward the inner aspect of the bundle to examine its overall morphology and composition. To estimate bundle height, measurements were made from images at calibrated instrument magnifications of 20, 000 or occasionally 10, 000. Although we might have underestimated the
actual stereocilia length, all efforts were taken to minimize the effect of parallax. Measurements were taken from bundles viewed from the laterale side toward the medial side (from the stria toward the modiolus), so that the row of longest stereocilia were imaged from the ‘rear.’ Samples were tilted and rotated so that the row of longest stereocilia was approximately perpendicular to the direction of view. Stereo-imaging was used in a few cases to gain an indication of possible errors in length measurements from 2D images. From the anaglyphs generated by a pair of images separated by 8 degrees of tilt, height measurements were obtained using anlySIS software. These revealed little difference in the height measurement from that obtained from the 2D image of a stereocilium at close to perpendicular to its long axis. Measurements were made using ImageJ software.

**Tissue preparation and Immunohistochemistry**

Murine and rat cochlear tissues were prepared as whole-mounts or cryosections for the purpose of immunohistochemistry. Cochleae were isolated, fixed by immersion in 2% paraformaldehyde, 125 mM sucrose in 100 mM phosphate buffered saline, pH 7.4, for 2 h and then decalcified for 45 min in RDO rapid decalcifier (Apex Engineering Products Corporation, Aurora, IL, USA) as described [31, 36, 37], cryosectioned at 10 µm and mounted on SuperFrost® plus microscope slides (R. Langenbrinck Labor- u. Medizintechnik, Emmendingen, Germany) at –20°C. For whole-mount preparation, we dissected cochlear turns and mounted them on slides with the tissue adhesive CelI-Tek (BD Bioscience, Schwerte, Germany) in PBS and performed phalloidin labeling as described [38]. For immunohistochemistry and LacZ staining, mouse cochlear sections were stained as previously described [31, 37, 39]. BK (KCa1.1) (rb, Alomone Labs, Jerusalem, Israel), αtBP2/RIBEYE (rb, American Research Products, Waltham, MA, USA), KCNQ4 (rb, [40, 41]), NZ200 (ms), SK2 (rb), Kir 4.1 (ms) all three from Sigma, otoferlin (ms, LifeSpan Biosciences, Seattle, WA, USA), prestin (gt, Santa Cruz Biotechnology, Dallas, TX, USA), Sox10 (gt, Santa Cruz Biotechnology, Dallas, TX, USA), VAMP2 and brevican (ms) from BD Bioscience and vGLUT1 (rb) both from Synaptic Systems (Gottingen, Germany), espin (ms, Fisher Scientific, Schwerte, Germany), Gαi1,2/3 and Gαi3-specific antibodies [29], and GFP (rb, Novus Biologicals, Littleton, CO, USA) were used as primary antibodies for localizing the respective proteins in the inner ear, see also online suppl. material, suppl. Table 1. All commercial antibodies were used as recommended by the manufacturers. Gαi1,2/3 and Gαi3-specific antibodies have been routinely validated with immunoblot and immunohistochemistry on wildtype and appropriate Gαi-deficient tissues [19, 29, 42]. For double labeling studies, both antibodies were simultaneously incubated for identical time periods. Hair cell stereocilia bundles were visualized by fluorescent phalloidin labeling of F-actin (Alexa Fluor 568 Phalloidin, Molecular Probes, Eugene, OR, USA). Sections and whole-mounts were viewed as described [43] using an Olympus BX61 microscope (Olympus, Hamburg, Germany) equipped with epifluorescence illumination. Images were acquired using an Olympus XM10 CCD monochrome camera and analyzed with cellSens Dimension software (OSIS GmbH, Münster, Germany). To increase spatial resolution, slices were imaged over a distance of 15 µm within an image-stack along the z-axis (z-stack) followed by 3-dimensional deconvolution using cellSens Dimension built in algorithm.

**Tissue preparation and Immunoblot**

Liquid-frozen cerebellum and brain stem were homogenized in 500 µl protein lysis buffer (20 mmol/l Tris, pH 8.3; 0.67% SDS; 238 mmol/l 2-mercaptoethanol; 0.2 mmol/l PMSF). To achieve electrophoretical separation of Gαi isoforms, separation was performed in gels containing 6 M urea. The proteins were visualized by immunodetection using the following primary antibodies described elsewhere [29] rabbit anti-Gαi1,2/3 (7.2 ng/ml), rabbit anti-Gαi3 (50 ng/ml). Finally, to visualize equal loading membranes were stained with Amido black.

**Experimental Design and Statistical analysis**

Data are presented as mean with standard deviation (SD) or mean with standard error of the mean (SEM). In the Figures, error bars are presented with and without caps for SD and SEM, respectively. Details for all statistical analyses are provided (see online suppl. material) in suppl. Table 2. In brief: differences of the mean were compared for statistical significance pair-wise by student’s t-test (click-ABR thresholds in Fig. 1F and see online suppl. material, suppl. Fig. 2; ABR slope in Fig. 7B-C) and see online suppl. material, suppl. Fig. 3B and C; loss of ABR threshold over age in Fig. 7D) or multiple comparisons 2-way ANOVA for factor “genotype” followed by post-hoc comparisons with Bonferroni’s multiple comparison test (f-ABR in...
Fig. 1A–E; DPOAE thresholds and amplitudes in Fig. 2; electrocochleography in Fig. 5; ribbon counting in Fig. 6D; ABR amplitude I/O and latency I/O in Fig. 7B and C and (see online suppl. material) suppl. Fig. 3B and C; ABR wave IV/I ratios in Fig. 7E and (see online suppl. material) suppl. Fig. 3D. Fisher’s exact test for associations was performed for 2x2 contingency tables (Fig. 1F, and stereocilia orientation in Fig. 3B). Non-parametric Mann-Whitney test was used to compare for statistical significance of stereocilia number and length in Fig. 3E and (see online suppl. material) suppl. Table 3. Unless otherwise noted, statistical significance was tested at the level of 5%. Level of significance is illustrated in the Figures with symbols or shaded areas (n.s. *p < 0.05; **p < 0.05; ***p < 0.001) and depicted in (see online suppl. material) suppl. Table 2. For Student’s t-test, 2-way ANOVA Graph by Mann-Whitney test, and Kruskal-Wallis test Pad Prism 6.01 (GraphPad Software, Inc. La Jolla, CA, USA) was used. For the Fisher’s exact test the online tool of vassarstats (http://vassarstats.net/tab2x2.html) was used.

**Results**

Isoform-specific and redundant roles of Gαi proteins for high- and low-frequency hearing and hair bundle organization

The functional consequences of deficiency for different Gαi isoforms for auditory responses was explored by special hearing tests. The auditory brainstem response (ABR) was measured in response to click and pure tone auditory stimuli in 1- to 3-month-old mice with global (g) or conditional (c) absence of Gαi2 and/or Gαi3. Conditional mouse mutants lacking one or both isoforms were generated by Cre-driven recombination using the Foxg1 promoter [27]. Gna12 gKO (Fig. 1A) and Gna12 cKO (Fig. 1B) mice both displayed tone-burst evoked ABR thresholds (f-ABR) and click-evoked ABR thresholds (Fig. 1F; click-ABR) similar to controls, suggesting that Gαi2 is either dispensable for hearing or may be functionally substituted by remaining Gαi isoforms. In contrast, Gna13 gKO (Fig. 1C) and Gna13 cKO (Fig. 1D) mice demonstrated a significant elevation of f-ABR and click-ABR thresholds towards higher frequency regions. This suggests a selective role of Gαi13 particularly for these frequencies, which cannot be rescued by Gαi2. In order to identify
overlapping functions between $\mathrm{G}_{\alpha i2}$ and $\mathrm{G}_{\alpha i3}$, we next analyzed mice defective for both genes. As mice with a global deficiency for both $\mathrm{G}_{\alpha i2}$ and $\mathrm{G}_{\alpha i3}$ are embryonic lethal [29] we generated conditional $\mathrm{G}_{\alpha i2}/\mathrm{G}_{\alpha i3}$ cKO mice that were viable and allowed inspection of hearing functions in the adult animal. Rather unexpectedly, $\mathrm{G}_{\alpha i2}/\mathrm{G}_{\alpha i3}$ cKO mice were profoundly deaf (Fig. 1E) with click-ABR thresholds that consistently exceeded 100 dB sound pressure level (SPL, root mean square weighted for click stimuli, SPL$_{\text{rms}}$) (Fig. 1F and see online suppl. material, suppl. Fig. 2). Indeed, in comparison to $\mathrm{G}_{\alpha i2}/\mathrm{G}_{\alpha i3}$ cKO mice the hearing thresholds of global and conditional $\mathrm{G}_{\alpha i3}$ mutants were only significantly elevated while that of global or conditional $\mathrm{G}_{\alpha i2}$ mutants were normal (Fig. 1F and see online suppl. material, suppl. Fig. 2).

![Fig. 2. DPOAE thresholds and DPOAEs amplitude I/O function in $\mathrm{G}_{\alpha i2}$ and $\mathrm{G}_{\alpha i3}$ gene-targeted mice. (A,B) OHC function in global and tissue-specific $\mathrm{G}_{\alpha i2}$ KO is not impaired. Mean±SD DPOAE thresholds (left panels) and DPOAEs amplitude I/O function evoked by stimulus f2=11.3 kHz (right panels) above noise floor (crosses) for $\mathrm{G}_{\alpha i2}$ gKO (A, KO) and $\mathrm{G}_{\alpha i2}$ cKO mice (B, KO) and their respective controls (Ctr) are similar. (C,D) Impairment of OHC function at high-frequency regions in global and tissue-specific $\mathrm{G}_{\alpha i3}$ KO mice. Mean±SD DPOAE thresholds (left panels) and DPOAEs amplitude I/O function (right panels) for $\mathrm{G}_{\alpha i3}$ gKO (C) and $\mathrm{G}_{\alpha i3}$ cKO mice (D) and their respective controls. DPOAE thresholds for stimulation frequencies above 8 kHz are significantly elevated and DPOAE amplitudes were significantly reduced in KO mice compared to the respective controls. (E) OHC function is lost in $\mathrm{G}_{\alpha i2}/\mathrm{G}_{\alpha i3}$ cKO mice. Mean±SD DPOAE thresholds (left panels) and DPOAEs amplitude I/O function evoked by stimulus f2=11.3 kHz (right panels) for $\mathrm{G}_{\alpha i2}/\mathrm{G}_{\alpha i3}$ cKO and their respective controls. Remarkable loss of thresholds at most testing frequencies and reduction of amplitudes are evident. Grey-shaded areas represent statistically significant post-hoc comparisons with $p<0.05$ after Bonferroni corrections. "n.s." and "***" indicate level of statistical significance $p>0.05$ and $p<0.001$, respectively. Data points represent the mean of 6 to 14 ears (numbers in brackets close to legend key) from 4 to 7 mice (A-D) and 3 to 4 ears from 2 mice (E).
To validate the specific contribution of outer hair cells (OHCs) to this hearing threshold difference in Gnai mutant mice, distortion product otoacoustic emissions (DPOAE) thresholds were measured as a metric of intact OHC mechanoelectrical response [44]. As expected from normal ABR thresholds seen in Gnai2 gKO and Gnai2 cKO mouse models (Fig. 1A and B) normal input/output (I/O) functions of emission amplitudes evoked by stimulus frequency f2=11.3 kHz were also observed in both Gnai2 KO lines (Fig. 2A and B). Global (Fig. 2C) and conditional (Fig. 2D) Gnai3 KO mice had significantly elevated DPOAE thresholds at test frequencies from 8 to 22.6 kHz (Fig. 2C and D, left panel) and exhibited reduced emission amplitudes at f2=11.3 kHz (Fig. 2C and D, right panel). Both observations indicate that the elevated hearing thresholds in these Gnai mouse mutants (Fig. 1C and D) are related to impaired mechanoelectrical OHC properties in high-frequency cochlear regions.

In contrast to single Gnai2 KO and Gnai3 KO, Gnai2/i3 cKO mice displayed residual DPOAEs only for the highest stimulus intensities at f2=11.3 kHz with significantly reduced emission amplitudes (Fig. 2E). This indicates that both Gαi2 and Gαi3 are essential for hearing and that Gαi2 can functionally replace Gαi3 in low frequency but not in high frequency regions.

Furthermore, to specify the role of Gαi for hair bundle organization we next examined the shape of OHC hair bundles in young Gnai3 gKO mice around hearing onset (P11-12) by using phalloidin staining to visualize actin in stereocilia (Fig. 3A) and categorized their shape in terms of the angle between the hair bundles arms (Fig. 3B). A significantly higher number of OHCs with wider V-shaped stereociliary bundles exceeding 110° was found in basal (high-frequency) cochlear turns of Gnai3 gKO compared to control mice whereas the visible differences were statistically insignificant in apical (low-frequency) cochlear turns. The quantification is shown in lower panel of Fig. 3B. Scanning electron microscopy, performed in cochlear samples obtained at P21 from Gnai3 cKO confirmed abnormal bundles in basal but no obvious anomaly in apical (low-frequency) cochlear turns were found (Fig. 3C and D). Additionally, there were shorter and fewer stereocilia on OHCs in basal cochlear turns of Gnai3 cKOs (Fig. 3E and see online suppl. material, suppl. Table 3). Gnai2/i3 double-deficiency induced hair bundle shape abnormalities also in the apical cochlear turn (Fig. 3F) as shown by fluorescence microscopy using phalloidin and espin as stereocilia markers.

Next, we examined if the hair bundle abnormalities are linked to expression profiles of the different Gαi isoforms. These abnormalities were restricted to higher frequency regions in Gnai3 cKO mice whereas they extended over high and low frequency regions in Gnai2/i3 cKO mice. The expression profiles of Gαi2 (Fig. 4A and B) and Gαi3 (Fig. 4C and D) were analyzed by immunohistochemistry in whole-mount preparations of the rat cochlea. At P2, Gαi3 was detected in inner hair cell (IHC) and OHC hair bundles throughout the cochlear length, while Gαi2 immunoreactivity was restricted to the apical turn (Fig. 4A and C). At P10, both Gαi2 and Gαi3 expression was evident in the apical turn, while Gαi3 dominated mainly in the basal turn (Fig. 4B and D). The strong expression of Gαi3 in hair bundles in basal high-frequency cochlear turns and the co-expression of Gαi2 and Gαi3 in hair bundles in apical lower-frequency cochlear turns thus support the idea that Gαi3 does not substitute for Gnai3-deficiency in basal cochlear turn, which may explain the distinct hearing deficits in Gnai3 mouse mutants.

Abnormal hair bundle elongation in the Gnai3-deficient mice may lead to hearing deficits through dysfunctional mechanoelectrical transduction (MET). The functionality of MET and the electrical potential from the subsequent initiated synchronous activation of the auditory nerve fibers can be captured by the steepness with which sound-induced compound action potentials of the auditory nerve (CAP) increase to escalating sound intensities and CAP threshold [45, 46]. To address if a disruption of stereociliary bundle elongation in Gnai3 KO would impact the functionality of MET channels, we measured sound-induced CAP responses in age-matched Gnai3 cKO and control mice. Best CAP thresholds of about 20 dB SPL at 11.3 kHz were found in control mice, and the growth functions for this stimulus frequency are shown in Fig. 5A. Gnai3 cKO mice had significantly elevated CAP thresholds of >35 dB SPL and the CAP amplitude growth points to disturbed transduction of sound-induced mechanical signals (Fig. 5A). On average, the CAP amplitude reduction in Gnai3 cKO mice was larger at
Fig. 3. Stereocilial configuration of OHCs in apical and basal cochlear turns from gene-targeted Gna1 KO mice. (A) Phalloidin staining of control (Ctr, left panel) and Gnaβ gKO (right panel) OHC stereocilia bundles in P11-12 whole-mount cochlea for both cochlear turns. Scale bars 10µm. One representative animal of n=4-8 is shown. (B) Gaussian distribution of OHC stereocilia spreading angle for control (black) and Gnaβ gKO (red). Gnaβ gKO have significantly shallowed V-shaped bundles (Phi>110°) in basal turn OHCs, depicted by shaded area and encircled widespread chevron icons. Upper panel, OHCs from apical cochlear turn. Middle panel, OHCs from basal cochlear turn. Curves illustrate a fit of normal distribution for either group, ordinate gives absolute number of cells. Lower panel, quantification of OHCs with Phi>110° (%) of total cells analyzed (Gnaβ gKO 32-36 cells; Gnaβ ctr 26-29 cells). (C,D) Scanning electron microscopy of apical and basal stereocilia from control and Gnaβ cKO mice confirms normal hair bundle shape in apical cochlear turn (C) and the disorganized ultrastructure of OHC bundles from basal turns of Gnaβ cKO (D). Hair bundle shape is outlined in yellow. Blue arrows point to asymmetrically shaped bundles. Scale bars, 1µm. One representative animal of n=3 is shown. (E) Significantly reduced stereocilia length (left panel) and number (right panel) in basal cochlear turn of Gnaβ cKO mice. Stereocilia length was assessed in 20 (Ctr) and 11 (Gnaβ KO) independent experiments (depicted as individual dots) in tissue samples from 4 control and 4 Gnaβ KO mice. Stereocilia length was derived from a total of 64 hair cells of control mice and 64 hair cells of Gnaβ KO mice. A total of 272 stereocilia and 305 stereocilia could be measured for control and KO mice, respectively. Stereocilia number per hair cell was counted in 12 and 14 independent experiments (countings depicted as dots) for 5 control and 4 Gnaβ KO mice, respectively. From 87 and 106 hair cells from control and Gnaβ KO mice, a total of 8299 and 7621 stereocilia could be counted, respectively. Stereocilia number ranged from 72-119 in control mice and 46-86 in Gnaβ KO mice. For both parameters median and single values are depicted. *** and **** indicate level of statistical significance p= 0.0107 and p< 0.0001, respectively. (F) Phalloidin (red) and espin (green) staining of Gnaβ I/3 cKO OHC indicate abnormal bundle shape in apical low-frequency turns. Scale bars 10µm.
**Fig. 4.** Gα\(_i2\) and Gα\(_i3\) expression in the inner ear. (A-D) Surface view of whole-mount preparations of rat cochleae at P2 and P10. (A, B) Gα\(_i2\) and (C, D) Gα\(_i3\) labeling (green in upper panel and white in middle panel) in the actin-rich hair bundles labeled by phalloidin (purple in upper panel and white in lower panel). (AC) At P2, Gα\(_i2\) (A) and Gα\(_i3\) (C) localizes at the tip of the OHC hair bundles in apical but only Gα\(_i3\) in basal cochlear turns, whereas (BD) at P10 Gα\(_i2/i3\) (B) and Gα\(_i3\) antibody (D) stains in IHCs in the apical but only Gα\(_i3\) in the basal turn. Scale bars 7 μm. One representative animal of n=5 is shown.
frequencies ≥ 16 kHz (~80%) than at lower frequencies ≤ 4 kHz (~50%) in comparison to age-matched controls (these growth functions are not shown as extra Figures). Moreover, the CAP responses were (right-)shifted to higher sound pressure levels in \( \text{Gnai}^3 \text{cKO} \) mice, albeit with similar steepness of amplitude growth (slope) from thresholds up to 50 dB SPL compared to control mice (Fig. 5A, CAP, encircled). Hence, mechanoelectrical currents in \( \text{Gnai}^3 \text{cKO} \) are likely to be functional in a limited response range but their sensitivity for activation seems to be impaired in high-frequency regions.

Failed activation of intact MET currents in high-frequency regions of \( \text{Gnai}^3 \text{cKO} \) mice was also confirmed by a barely detectable summation potential (SP) amplitude, that was recorded in \( \text{Gnai}^3 \text{cKO} \) mice at the round window for 11.3 kHz tone bursts as an estimate of whether MET channel currents are impaired [47] (Fig. 5B, SP). Again, SP amplitude reduction was larger at higher (~70% at 16 kHz) than at lower or middle frequencies (~50% at 4-8 kHz) (the growth function is not shown as extra Figure). This assumption is supported by a failure to generate cochlear microphonic (CM) amplitudes upon increasing sound pressure levels in \( \text{Gnai}^3 \text{cKO} \) mice (Fig. 5C, CM). CM amplitudes are assumed to be proportional to the number of functional phasic MET currents from mainly basal-coil OHCs [48]. Overall, these findings support hampered activation of functional MET currents in high-frequency cochlear turns of \( \text{Gnai}^3 \text{cKO} \) mice to be causally related to hearing and hair bundle abnormalities.

In conclusion, a close structural/functional relationship between impaired stereocilia elongation upon \( \text{Gnai} \) deletion and impaired auditory responses is supported by (i) an overlap of hearing function deficit and \( \text{G} \alpha_3 \) expression patterns in either higher frequency (\( \text{G} \alpha_{i3} \)) or lower frequency cochlear regions (\( \text{G} \alpha_{i2}/\text{G} \alpha_{i3} \)), and (ii) impaired CAP, SP and CM function in \( \text{Gnai}^3 \text{cKO} \) towards higher frequencies.

**Specific \( \text{G} \alpha_{i2/3} \) functions during maturation of IHC synapses**

To obtain insight if the presumably impaired activation of MET currents might influence the maturation process of the hair cell phenotype in the high-frequency cochlear turn (\( \text{Gnai}^3 \text{cKO} \)) or in all cochlear turns (\( \text{Gnai}^2/\text{Gnai}^3 \text{cKO} \)) we inspected key biomarkers for hair cell maturation (Fig. 6). The motor protein prestin (Fig. 6A, left panel, green) and
the voltage-gated K⁺-channel KCNQ4 (Fig. 6A, left panel, red) are typically upregulated in OHCs before hearing onset [49-52] and remain expressed in the mature cochlea. In mature Gna13 mutants’ OHCs both marker proteins were expressed as expected suggesting normal development (Fig. 6A, right panel). Analyzing GFP fluorescence in Gna13-GFP reporter mice indicated that Gαi3 protein should be expressed in IHCs (Fig. 6B). Nevertheless, Gna13 cKO mice showed a normal expression profile of the Ca²⁺-sensor protein otoferlin within the IHC [53] (Fig. 6C, green) with a regular distribution pattern (Fig. 6C, red) and no indication for
Fig. 7. ABR amplitudes, latencies, wave IV to wave I amplitude ratios, and ABR thresholds change over age in global and conditional Gnai3 KO mice. (A) Individual ABR waveform from control (Ctr, black) and Gnai3 KO (red and purple line) 1- to 3-month-old mice with indicated wave I and wave IV peak-to-peak amplitude (amplitude) and leading peak latency (latency). (B,C) ABR wave I (upper panels) and IV (lower panels) amplitude I/O functions (left panels) with slope (insets) and wave I and IV leading peak latency I/O function (right panels) for Gnai3 gKO (B), Gnai3 cKO (C) and respective control mice. Decrease of amplitudes, amplitude slope and increase of wave I latencies are evident in the KO mice (shaded areas, p< 0.05). (D) Age-related loss of ABR thresholds to 11.3 kHz stimuli for Gnai3 gKO and Gnai3 cKO mice at the age of 1-3 months (triangles) and 5-7 months (circles). Progression of threshold loss occurs in both KO mouse lines (dashed lines). The loss of ABR thresholds per month of age in Gnai3 KO mice significantly exceeded the loss in control mice (graph bars). The loss of ABR threshold per month was significantly larger in Gnai3 gKO mice than in Gnai3 cKO (star, p< 0.05), and not significantly different between both control groups (n.s.). Thresholds for individual ears are denoted by small symbols. (E) ABR wave IV/I ratios for Gnai3 gKO, Gnai3 cKO and respective control mice. Significant elevation of wave IV/I ratios at middle to high stimulation levels is found only in Gnai3 cKO mice. Mean±SEM (straight error bars) or SD (error bars with caps) from 4 to 9 mice (number of ears in brackets).

an altered number (Fig. 6D) of CtBP2/RIBEYE protein-labeled ribbon structures, though the statistical conclusion may be limited by the small sample size. Furthermore, a characteristic feature of immature IHCs during normal development is the formation of an axosomatic...
contact of efferent nerve fibers with the small conductance Ca^{2+}-activated K^-channel type 2 (SK2) at the base of IHCs [54-56] (Fig. 6E). With hearing onset (P11-12), a reorganization of axosomatic efferent nerves on IHCs occurs that is characterized by downregulation of SK2 and upregulation of BK, both Ca^{2+}- and voltage-gated K^-channels (Fig. 6F). BK channels carry I_{k,i} currents that are essential to transform immature IHCs (exhibiting spontaneous Ca^{2+}-spikes) to mature functional cells that are then able to respond to sound [57, 58]. Gnai3 cKO IHCs innervated by VAMP 2 positive efferent nerves mature normally, i.e. lacking SK2 at the base of otoferlin-positive cells (Fig. 6G, upper panel) and normal BK expression in supranuclear position on IHCs (Fig. 6H, lower panel). In contrast, in Gnai2/i3 cKO IHCs an immature phenotype was observed along the entire tonotopic axis, characterized by a persistent expression of SK2 (Fig. 6H, upper panel) and a lack of BK expression (Fig. 6H, lower panel) at the basal pole of IHCs. Since Gnai2 KO mice show no IHC maturation defects and IHCs from Gnai3 cKO mice develop normally in spite of hair bundle defects, whereas IHCs from Gnai2/i3 cKO remain in an immature stage, we conclude that Gnai_{i2} or Gnai_{i3} function is required for final maturation of IHC synapses prior to hearing onset.

**Function of Gnai_{i} in the central auditory pathway correlates with its expression in satellite cells and brainstem auditory nuclei**

Although IHCs mature normally in Gnai3 KO mice their hair bundles are defective (see Fig. 3B-E) and functionally impaired (see Fig. 1C,D,F), and the cochlear output is diminished (see Fig. 5). Therefore, we were interested whether central auditory processing is also affected and studied supra-threshold ABR wave amplitudes, which reflect the summed activity of the auditory nerve independent of OHC contribution to hearing thresholds.

Whereas Gnai2 cKO mice showed normal early (ABR wave I) and late (ABR wave IV) amplitudes and latencies in response to click sound stimuli (see online suppl. material, suppl. Fig. 3), supra-threshold ABR wave I amplitudes were significantly reduced in both Gnai3 gKO (Fig. 7A and B) and Gnai3 cKO mice (Fig. 7A and C) in comparison to controls. In addition, the slopes of the amplitude growth functions were significantly weaker as compared to controls (Fig. 7B and C, inset left panels). Moreover, a significant delay of the leading peak of ABR wave I that recovered towards ABR wave IV became apparent (Fig. 7B and C, right panels). Reduced amplitude and response delay are consistent with the observed diminished cochlear output as we think is a consequence of impaired activation of IHC MET channels. This was accompanied by an accelerated threshold loss over age observed in ABRs at a stimulation frequency of 11.3 kHz (and thresholds of DPOAE at f2=11.3 kHz, not shown) as seen in individual mice at different age, i.e. 1.5-3 months vs. 5.4-7.5 months (Fig. 7D). The rate of loss of ABR thresholds per month of age in Gnai3 KO mice exceeded significantly the minor loss seen in control mice (Fig. 7D, bar graphs). Furthermore, the progression of ABR threshold loss was significantly stronger in global Gnai3 KO mice when compared to mice with conditional Gnai3 deletion (Fig. 7D, bar graph) while no significant difference was found when comparing the progression for the respective controls (Fig. 7D, bar graph).

Strikingly, the amplitude of ABR wave IV was disproportionally elevated in comparison to ABR wave I in Gnai3 cKO mice but not in Gnai3 gKO (Fig. 7E), indicating that conditional deletion of Gnai3 mainly restricted to the inner ear preserves a capacity to generate neural gain, while this function is mostly lost in global Gnai3 KO mice. In Gnai2 KO mice showing normal hearing and in Gnai2/i3 cKO being almost deaf this effect was not further analyzed. The different capabilities of global and conditional Gnai3 KO to generate central neural gain suggested that distinct Gnai_{i} expression patterns might be causally involved in the progression of the deviating functional phenotypes. Isoform-specific antibodies detected Gnai_{i} proteins in the brainstem and cerebellar tissues from control but not from Gnai3 gKO mice (see online suppl. material, suppl. Fig. 4, upper panel), whereas in the same tissues Gnai_{i2} immunoreactivity was unaffected (see online suppl. material, suppl. Fig. 4, middle panel).

We next searched for differences in the expression pattern of Gnai_{i} between Gnai3 gKOs and Gnai3 cKOs in retrocochlear regions possibly involved in neural gain generation at the level of the auditory nerve or its target neurons in the cochlear nucleus [59, 60].
**Fig. 8.** Expression of \(G_{\alpha^i_3}\) in the spiral ganglion. (A) Sections through the spiral ganglion stained with \(G_{\alpha^i_3}\) antibodies reveal its presence in control (Ctr) and \(Gna_i^3\) cKO mice but not in \(Gna_i^3\) gKO mutants. One representative animal of \(n=2\) is shown. Scale bars, 5\(\mu\)m. (B) Schematic illustration of the expected Nf200, Kir4.1, Sox10, brevican, and \(G_{\alpha^i_3}\) immunoreactivity in the soma of a single spiral ganglion neuron and the ensheathing satellite (glia) cell, and the surrounding perineural net matrix. Lac Z staining of Foxg1\(^{cre/+}\) ROSA 26 reveals activity of Cre in the cytoplasm of spiral ganglion neurons (middle panel, some somas are indicated with arrows) but not in neighboring Sox10-labelled satellite cells (right panel, some cells are indicated with white outlines). Scale bars, 15\(\mu\)m. One representative animal of \(n=2\) is shown. (C, D) Sections through the spiral ganglion co-labelled with \(G_{\alpha^i_3}\) and Kir4.1 antibodies confirm its presence in satellite cells surrounding Nf200 positive spiral ganglion neurons. \(G_{\alpha^i_3}\) itself is surrounded by the perineuronal marker brevican. Scale bars, 5\(\mu\)m. One representative animal of \(n=2\) is shown.

**Fig. 9.** \(G_{\alpha^i_3}\) and vGLUT1 expression in the ventral cochlear nucleus. (AB) Left panels: Overview of cross sections showing dorsal (DCN) and ventral (VCN) cochlear nucleus of \(Gna_i^3\) gKO (A) and \(Gna_i^3\) cKO (B) and corresponding controls (Ctr). Scale bars 200\(\mu\)m. Middle and right panels: stained with \(G_{\alpha^i_3}\)-specific antibodies (red) and the vesicular-glutamate-transporter-1 (vGLUT1, green). Note the absence of \(G_{\alpha^i_3}\) labeling contacted by vGLUT1 in \(Gna_i^3\) gKO (A) but not cKO (B). One representative animal of \(n=2\) is shown. Scale bars 5\(\mu\)m.
At the level of the spiral ganglion neurons (SGN) Gαi3 immunoreactivity was absent in Gnai3 gKO but not in Gnai3 cKO mice (Fig. 8A). Also in target neurons of the auditory nerve in the cochlear nucleus (e.g. the bushy cells in the ventral cochlear nucleus, VCN), which are identified through vGLUT1 positive presynaptic contacts (Fig. 9, green) [61], Gαi3 antibody staining was detected in Gnai3 cKO (Fig. 9B, red) but not in Gnai3 gKO mice (Fig. 9A, red). Moreover, preliminary findings revealed reduced vGLUT1 staining intensity in Gnai3 gKOs (Fig. 9A, green) in comparison to Gnai3 cKO mice and control mice (Fig. 9B, green). As previously described reduced vGLUT1 expression goes along with reduced activity [61], we suggest differences in vGLUT1 activity of auditory nerves between both Gnai3 mutants.

To search for additional differences between Gnai3 gKO and Gnai3 cKO mice we compared the expression pattern of Gαi3 with that of the Cre activity generated by the Foxg1cre/+ line used to generate Gnai3 cKO mice. Lac Z staining of Foxg1cre/+ROSA 26 animals showed Cre activity in the ball-shaped SGNs (Fig. 8B, middle panel) which correspond to the previously described expression of Foxg1 [27]. Gαi3 expression was assigned to the bean-shaped supporting satellite cells, which were characterized by Sox10 expression (Fig. 8B, right panel) and colabelling with the marker Kir4.1 [62, 63] (Fig. 8B and C) surrounding the Nf200 positive SGNs (Fig. 8B and D, left panel). Furthermore, Gαi3 expression itself was encircled by the perineuronal net marker brevican [64] (Fig. 8B and D, right panel). Importantly, expression of Gαi3 in satellite cells is evident in Gnai3 cKO but not in Gnai3 gKO mice and hence could contribute to auditory neural gain only seen in Gnai3 cKO.

Taken together, our findings have various implications on the role of Gαi proteins as regulators of auditory functions and can be summarized by four key observations (Fig. 10). (1) Hair bundles in low-frequency regions are shaped by Gαi2 and/or Gαi3, whereas their correct formation in high-frequency regions only depends on Gαi3 (Fig. 10A). (2) At least one Gαi isoform is required to trigger the final maturation of IHC synapses (Fig. 10B). (3) In low-frequency regions the deletion of either Gαi2 or Gαi3 has no hearing phenotype, whereas in high-frequency regions Gαi3 is required for appropriate hearing (Fig. 10C). (4) Expression of Gαi3 in satellite cells of the cochlea and/or auditory neurons in the brainstem associates with neural gain adjustment (Fig. 10D).

**Discussion**

The present study unravels specific auditory functions of different Gαi proteins, the binding partners of Gpsm2, which are known as evolutionary highly conserved determinants of cell polarity, asymmetric cell division, or planar cell polarity [11, 42]. The present data provide evidence to discriminate between so far elusive functions of Gαi2 and Gαi3 for (1) shaping polarity and length of hair bundles in different cochlear regions, and (2) also uncover new functionally relevant actions of Gαi2 and Gαi3 for final differentiation of inner hair cell (IHC) synapses, and (3) suggest Gαi3 to be important for central auditory adaptation processes.

**Gαi2 and Gαi3 shape hair bundles**

Using various Gnai mutant mouse lines we demonstrate that deficiency for Gαi3 but not Gαi2 produces high-frequency hearing loss, whereas the absence of both isoforms results in profound deafness resembling the phenotype seen in Gpsm2 mouse mutants [19, 23].

Lower numbers and reduced elongation of stereocilia in high frequency cochlear turns in outer hair cells (OHCs) of Gnai3 cKO mice and stereociliary elongation defects in IHC in basal turns [19] correlated with the deficiency for Gnai3 in high frequency cochlear regions and high frequency hearing threshold loss. In contrast, due to an overlapping expression profile of Gαi2 and Gαi3 proteins we observed reduced numbers and impaired elongation of stereocilia together with low-frequency hearing threshold loss exclusively in the Gnai2/3 cKO mice which are deficient for both proteins. These findings uncover an unexpected difference of Gαi2 and Gαi3 functions for shaping size and length of hair cell stereociliary bundles specifically along the tonotopic axis and suggest selective Gαi3 functions for hearing.
**Fig. 10.** Schematic illustration of supposed roles of $G_{\alpha_i}$ and $G_{\alpha_3}$ for hearing. (A) Hair bundles in low-frequency cochlear regions are shaped by $G_{\alpha_2}$ and $G_{\alpha_3}$ activity and hair bundles in high-frequency cochlear regions are shaped by specific activity of $G_{\alpha_3}$ that cannot be replaced by $G_{\alpha_2}$. (B) During the maturation process the reorganization of the axosomatic efferent nerves is described by the downregulation of SK2 and upregulation of BK channels. $G_{\alpha_2}$ and $G_{\alpha_3}$ exhibit mutual redundant activity to trigger the final maturation of IHC synapses, whereas upon deletion of both $G_{\alpha}$ proteins IHCs remain in an immature stage characterized by a sustained SK2 expression and a failure of BK upregulation. (C) For hearing in low-frequency regions expression of either $G_{\alpha_2}$ or $G_{\alpha_3}$ is indispensable, whereas in high-frequency regions $G_{\alpha_3}$ expression is crucial for proper hearing. (D) Central neural gain adjustment in response to impaired auditory input is presumably shaped by $G_{\alpha_3}$ activity in satellite cells or brainstem auditory neurons, as $G_{\alpha_3}$ is absent in $Gna13$ gKO but not in $Gna13$ cKO mice in these cells. n.d., not determined. LF, low-frequency. HF, high-frequency.
low and high pitch sounds. In this context the existence of frequency-specific complexes of either Gαi2 or Gαi3, together with actin regulatory proteins (such as espin, radixin, TRIOBP or twinfilin) [65-67] or with hair cell-specific myosins (Myosin 1c, 3a, 6, 7a and 15a) [65, 68] or proteins such as Ep8 (actin-binding protein epidermal growth factor receptor pathway substrate 8) [55, 69], whirlin [19] and proteins associated with the mechanoelectrical transduction (MET) [70] may be considered.

The failure of proper activation of functionally intact MET may be explained through structural abnormalities of stereocilia in Gna13 KO mice, such as shorter hair bundles. Indeed, we have several implications that MET are functional: (i) normal slopes but reduced CAP amplitudes in Gna13 cKO mice point to functional MET currents [71] (ii) reduced summed potential (SP) amplitudes indicating functional MET currents although the activation of those seems to be hampered [47] (iii) the deficient growth of cochlear microphonics (CM) typically seen upon insufficient responses of MET currents to sound caused by an inappropriate activation of the MET channels [72] and (iv) the shallower slopes of the amplitude growth functions of early and late ABR waves at moderate sound intensities observed in global and conditional Gna13 KO mice point to reduced sensitivity to sound [73]. The overall findings thus suggest a failure of functionally intact MET activation in Gna13 KO mice following e.g. inappropriate coupling of shorter or disorganized stereocilia to the tectorial membrane. Reduced length of stereocilia despite normal biophysical properties of MET currents is also discussed for Eps8 mutants [55]. Eps8 shapes stereocilia length together with Usher protein candidates such as whirlin or myosin 15 along the entire tonotopic axis [74]. Deletion of the Eps8L2, a member of the Eps8-like protein family, causes deafness [55, 69] similarly to Gna12/13 cKO. Strikingly, the deletion of Eps8 induced progressive high-frequency hearing loss [75] similar to the phenotype observed here in Gna13 KO mice. Further studies will clarify whether Gαi protein expression and/or signaling depends on Eps8 or vice versa. Moreover, it remained open in which signaling pathways Gαi integrates in the inner ear. Aside from classical GPCR-driven canonical pathways non-canonical mechanisms should be considered in future studies.

**Gαi2 and Gαi3 trigger maturation of IHC synapses**

IHCs of Gna12/13 cKO mice remain immature along the entire tonotopic axis as judged from a failure to upregulate BK and downregulate SK2 channels. Since Gna13 mutants develop a normal post-hearing IHC structural phenotype, the impairment of stereocilia elongation per se is unlikely the cause of the developmental block of IHC maturation. Deafness by itself does not prevent IHC maturation of Gna12/13 cKO mice, as can be concluded from situations of hereditary deafness that do not prevent proper IHC maturation; for example the absence of thyroid hormone leads to deafness, but IHCs gradually develop after 2 months [76-78], whereas IHC maturation is still absent in 4- to 5-month-old Gna12/13 cKO mice. The immature IHC phenotype of Gna12/13 cKO mice may rather be considered in the context of a redundant Gαi1/Gαi2 activity that acts together with myosin 6 e.g. on pruning of connected fibers. Accordingly a timely elimination of axosomatic efferent contacts [54-56], coincides with the initiation of biophysical maturation in IHCs [79] (see for a review [58]). Myosin 6 mutants show not only a defective structural integrity of the hair bundle but also an impaired post-hearing IHC maturation that includes the failure to properly target BK channels [80, 81], similar to Gna12/13 cKO.

**Gαi3 is essential for central auditory processing**

An unexpected finding was a reduced compensatory neural gain in the Gna13 gKO but not in the Gna13 cKO mice that came along with a significantly accelerated worsening of hearing thresholds over age. For the auditory system, it was shown that the peripheral loss of response to sensory stimuli due to traumatic events, aging processes or genetic defects among others triggers central gain adjustment in order to re-stabilize the network through a disproportional elevation of the neuronal firing rate [31, 82, 83]. How target neurons of auditory nerve fibers, likely to be present in the dorsal [59] or ventral [84] cochlear nucleus
(DCN, VCN), sense nerve deprivation and re-stabilize their firing rate proportional to sensory decline is entirely elusive.

The finding that central compensation fails in Gnai3 gKO but not in Gnai3 cKO, despite comparable hearing threshold deficits, is surprising. It is tempting to speculate that Gαi3 expressing auditory neurons that may correspond to target cells of auditory nerves via their vGLUT1 positive contacts [85] may participate in generation of compensating central hyperactivity. Indeed, Gα-Gβγ signaling has been shown to modulate basic G protein-activated K⁺-channel GIRK [86] in response to e.g. GABA B R mediated (GABA "spillover") activation from neighboring sites and thereby triggers elevated firing rates through shunting of excitatory currents [87]. Interestingly, following noise exposure elevated firing rates in the cochlear nucleus (CN) are accompanied by a modification of GABA B R [88, 89]. In this context preliminary findings of lower vGLUT1 immunoreactivity in Gnai3 gKO than Gnai3 cKO mice may be indicative for less active transmitter release sites, as previously suggested [61]. Additionally, the absence of Gαi3 expression in satellite cells of the cochlea in global but not conditional Gnai3 mutants (Fig. 8A) may lead to a differential excitability of cochlear neuronal cells as has been observed for the brain [90, 91]. Indeed, dysfunction of satellite cells has been shown to accelerate hearing loss over age and delays ABR wave I responses in high-frequency hearing loss [92] as seen here for Gnai3 KO.

Gαi proteins and in particular the Gαi3 isoform have been previously linked to control asymmetric cell division and hair cell bundle polarity in the inner ear [16, 17, 19, 23]. Beyond that, our present findings indicate redundant functions of Gαi2 and Gαi3 for final maturation of synapses and low frequency hearing, whereas Gαi3 is indispensable for basal cochlea hair bundle shaping, high frequency hearing, and homeostatic excitability changes in the brain. As a consequence, Gαi cell functions are not only pathophysiologically important for hearing, but should also be considered in the context of novel functions for the sensory synapse and neuronal differentiation processes.

**Conclusion**

From invertebrates to mammals, Gαi proteins are involved in regulation of mitotic spindle dynamics, cell division, polarity, growth, and differentiation. We show for the sensory cells in the cochlea that the action of Gαi members is not limited to cilia movements or regulation of mitotic spindle dynamics in early development, but that distinct isoforms differentially account for early and later developmental steps. In particular Gαi proteins (i) shape stereociliary bundles isoform-specifically, (ii) act on differentiation of synapses during critical rewiring periods and (iii) allow for central adaptive neural gain responses.

**Abbreviations**

ABR (auditory brainstem response); AGS (activators of G-protein signaling); BK (Ca²⁺-and voltage-activated K⁺-channels of the BK type); CAP (compound action potential); CN (cochlear nucleus); CM (cochlear microphonic potential); cKO (conditional deletion); dB (decibel); DCN (dorsal cochlear nucleus); DPOAE (distortion product otoacoustic emissions); ES (embryonic stem cells); Eps8 (actin-binding protein epidermal growth factor receptor pathway substrate 8); f1 (DPOAE primary tone 1 frequency); f2 (DPOAE primary tone 2 frequency); GFP (green fluorescent protein); gKO (global deletion); Gα (α subunit of G-proteins); GDI (guanine nucleotide dissociation inhibitor); GIRK (G protein-coupled inwardly-rectifying potassium channels); Gnai (gene coding for Gα subunit); GPCR (G-protein-coupled receptors); Gpsm2 (G protein signaling modulator 2); IHC (inner hair cells); I/O (input/output function); Kir4.1 (inward rectifying potassium channel); MET (mechanoelectrical transduction); Nf200 (neurofilament 200); n.s., not (significant); OHC (outer hair cell(s)); PTX (Pertussis Toxin); SD (standard deviation); SEM (standard error of the mean); SGN (spiral ganglion neuron); SK2 (small conductance Ca²⁺-activated K⁺-channel type 2); SP (summation potential); SPL (sound pressure level); VAMP 2 (vesicle-associated protein 2).
membrane protein 2); VCN (ventral cochlear nucleus); vGLUT 1 (vesicular glutamate transporter 1).

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Disclosure Statement

The authors declare no competing financial interest.

References

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