Orphan Glutamate Receptor δ1 Subunit Required for High-Frequency Hearing

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The function of the orphan glutamate receptor delta subunits (GluRδ1 and GluRδ2) remains unclear. GluRδ2 is expressed exclusively in the Purkinje cells of the cerebellum, and GluRδ1 is prominently expressed in inner ear hair cells and neurons of the hippocampus. We found that mice lacking the GluRδ1 protein displayed significant cochlear threshold shifts for frequencies of >16 kHz. These deficits correlated with a substantial loss of type IV spiral ligament fibrocytes and a significant reduction of endolymphatic potential in high-frequency cochlear regions. Vulnerability to acoustic injury was significantly enhanced; however, the efferent innervation of hair cells and the classic efferent inhibition of outer hair cells were unaffected. Hippocampal and vestibular morphology and function were normal. Our findings show that the orphan GluRδ1 plays an essential role in high-frequency hearing and ionic homeostasis in the basal cochlea, and the locus encoding GluRδ1 represents a candidate gene for congenital or acquired high-frequency hearing loss in humans.

Ionotropic glutamate receptors include three major families, N-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA) receptors, and a fourth orphan family of delta receptors (GluRδ1 and GluRδ2). GluRδ1 and GluRδ2 share 36% amino acid identity with each other but only 17 to 28% identity with other ionotropic glutamate receptors (25, 45). Neither GluRδ1 nor GluRδ2 can be activated by AMPA, kainate, NMDA, glutamate, or any other ligands when expressed alone or in combination with other subunits in heterologous expression systems (46). Sequence analysis suggests that both GluRδ1 and GluRδ2 are more homologous to non-NMDA receptors; an analysis of GluRδ2 with the Lurcher mutation in the third transmembrane domain suggests that GluRδ2 functions as an AMPA-like receptor (21, 44, 48). It is expressed exclusively in Purkinje cells of the cerebellum. Targeted disruption of GluRδ2 causes motor coordination impairment, Purkinje cell maturation, and long-term depression of synaptic transmission (20). Subsequently, it was found that the appropriate transport of GluRδ2 to the Purkinje cell surface is required for the function of the receptor in synaptic transmission (15, 46). Recently, it has been suggested that GluRδ2 is the receptor for cerebellin 1, a glycoprotein of the Clq and tumor necrosis factor family that is secreted from cerebellar granule cells (16).

In contrast to GluRδ2, GluRδ1 is expressed in many areas in the developing central nervous system, including the hippocampus and the caudate putamen, but is absent in the cerebellum in guinea pigs, rats, and mice (24–26). In the adult, GluRδ1 is expressed at high levels in hippocampal neurons (24–26), cochlear inner hair cells (IHCs), and spiral ganglia and their satellite cells as well as vestibular hair cells and vestibular ganglia in guinea pigs and rats (36). GluRδ1 is also weakly expressed in Claudius cells in the basal cochlear turns and in vestibular supporting cells (36). In IHCs, GluRδ1 immunostaining appears over the entire cell surface rather than localized to the synaptic sites at the base of the cell (36). Despite these expression patterns, no functional role of GluRδ1 in vivo has been reported.

To investigate its role, we created and characterized a null allele of GluRδ1 in mice. The GluRδ1−/− mice displayed a significant auditory phenotype, demonstrating that GluRδ1 is required for high-frequency hearing and suggesting that it has a role in cochlear ion homeostasis. Function in other cells expressing GluRδ1, such as vestibular hair cells, vestibular ganglia, and hippocampal neurons, was not significantly affected in GluRδ1−/− mice. The locus encoding GluRδ1 thus represents a candidate gene for congenital or acquired high-frequency hearing loss in humans.

MATERIALS AND METHODS

Table 1 summarizes the procedures used in these studies as well as the age and number of mice used for each procedure. Details for each procedure follow.

Construction of the GluRδ1 targeting vector and generation of GluRδ1 mutant mice. We screened a bacterial artificial chromosome library (Research Genetics) containing mouse 129/Sv genomic DNA and obtained overlapping
TABLE 1. The age, genotype, and number of mice used for each procedure

| Procedure                     | Age (wk) | GluR61+/+ | GluR61+/- | GluR61-/- |
|-------------------------------|----------|-----------|-----------|-----------|
| Western blot                  | 8        | 4         | 4         | 4         |
| Immunostaining                |          |           |           |           |
| Histopathology                | 8        | 13        | 0         | 12        |
| Laser capture                 | 8        | 2         | 0         | 2         |
| microdissection               |          |           |           |           |
| ABR                           | 6–8      | 4         | 6         | 4         |
| DPOAE                         |          |           |           |           |
| EP                            | 8        | 7         | 7         | 7         |
| ABR post-noise exposure       | 6–8      | 6         | 0         | 6         |
| DPOAE post-noise exposure     | 6–8      | 6         | 0         | 6         |
| DPOAE suppression             | 6–8      | 3         | 6         | 3         |
| Rotarod                       |          |           |           |           |
| Swim test                     | 8        | 9         | 9         | 9         |
| VatEP                         | 11–12    | 10        | 12        | 10        |
| Hippocampal morphology        |          |           |           |           |
| Electrophysiology             | 10–11    | 4         | 0         | 4         |
| Water maze test               | 8        | 8         | 10        | 11        |

Western blot analyses. To confirm the ablation of the GluR61 protein in GluR61−/− mice, we performed Western blot analysis. Extracts from mouse inner ears, hippocampus, and cerebella containing 50 to 150 μg of protein were separated in a 3 to 8% NuPAGE Tris-acetate polyacrylamide gel (Novex) containing sodium dodecyl sulfate. After transfer, the polyvinylidene fluoride membrane (Immobilon) was treated with primary antibodies (rabbit GluR61/2 polyclonal antibody from Chemicon [catalog no. AB1514] and β-actin antibody from Sigma [catalog no. A5441]), hors eradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech), and SuperSignal (Pierce).

Immunostaining and histologic analysis. For the evaluation of molecular and morphological changes in GluR61−/− mice, mice were anesthetized with Avertin (500 mg/kg of body weight) or ketamine and xylazine (0.72/0.46 mg/30 g of body weight), followed by intracardial perfusions of 0.1 M phosphate-buffered saline and subsequently 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3). Cochleas were postfixed overnight and then decalcified in EDTA for 1 to 3 days. For whole-mount immunolabeling, cochleas were dissected, permeablized with 1.0% Triton X-100 for 10 min, and incubated overnight in primary antibodies. The next day, the samples were placed in biotin-labeled secondary antibody, a complex consisting of avidin, biotin, and horseradish peroxidase (ABC kit; Vector Laboratory), and then incubated in peroxidase substrate. For immunostaining of sections, decalcified cochleas were embedded in paraffin and sectioned into 12-μm thicknesses. Slides were deparaffinized. Nonspecific binding of secondary antibody was blocked by incubation with 10% goat serum in phosphate-buffered saline for 30 min at room temperature. Samples were then incubated in primary and secondary antibodies as described above. Samples were observed under a microscope (Olympus BX60). The primary antibodies used were Chemicon AB1514 for GluR61/2, Abnova H00002894-A01, specific for GluR61 (for hippocampal immunostaining), Santa Cruz SC22926 for Kir3.1, Sigma P6610 for Kv4.1, Santa Cruz SC16053 for the Na+−K+ ATPase α1 subunit (Atpa1b1), Santa Cruz SC21547 for the Na+−K+−2Cl− cotransporter (Ncc1e), Chemicon MAB392 for synaptophysin, Sigma V5387 for the vesicular acetylcholine transporter (VAT), Chemicon AB197 for the calretinin gene-related peptide (CGRP), and Chemicon AB8511P for SNAP25. For the anti-GluR61/2 antibody from Chemicon, lots produced before 2002 worked well in our immunostaining and Western blot analyses, but recently produced lots failed in immunostaining. In our hands, the various GluR61-specific antibodies (Abnova H00002894-A01 and Abnova H00002894-M01; kindly provided by R. Wenthold) did not result in immunostaining signals that were consistent and different between GluR61+/+ and GluR61−/− cochlear sections despite numerous attempts with a variety of conditions, including antigen retrieval.

For the immunostaining of SNAP25, synaptophysin, CGRP, or VAT, each dissected cochlear piece was measured by computerized planimetry and the cochlear location was converted to the frequency that is normally processed at that location (8). To quantify immunopositive terminals, outlines were traced via a drawing tube using high-numerical-aperture objective lenses (total magnification, ×2,000). During tracing, fine focus was continually adjusted to optimize focus.
imaging of each terminal cluster. Traces were digitized, and areas were computed using NIH Image software. For the outer hair cell (OHC) area, all immunopositive terminals were traced and values from each row were averaged within bins corresponding to 100 μm of cochlear length.

For an assessment of histopathology, animals were anesthetized, followed by intracardial perfusion with 2.5% glutaraldehyde and 1.5% paraformaldehyde in phosphate buffer. Temporal bones were extracted, and round and oval windows opened for intralabyrinthine perfusion of fixative. Cochleas were then osmicated (1% OsO4 in dH2O2, decalcified (0.1 M EDTA with 0.4% glutaraldehyde), dehydrated in ethanol and propylene oxide, embedded in Araldite resins, and sectioned at 40 μm on a Histostage with a carbide steel knife. Sections were mounted on slides and coverslipped.

Laser capture microdissection of cochlear sections and reverse transcriptase PCR (RT-PCR) analysis. For detailed expression analysis of GluR6i in the inner ear, laser capture microdissection was performed using the PixCell II system (Arcturus). We used a previously described method (32) with some modifications. Briefly, the mice were anesthetized and intracardially perfused with 4% paraformaldehyde in phosphate buffer. Temporal bones were removed, and oval windows were opened for the injection of fixative. Cochleas were then postfixed overnight and decalcified in 0.1 M EDTA for 1 to 3 days. The cochleas were dehydrated in ascending concentrations of alcohol and embedded in paraffin. The embedded cochleas were sectioned into 12-μm thicknesses, and sections were mounted on uncharged slides (six sections on each slide). The sections were deparaffinized in xylene and dried at room temperature. We captured inner hair cells, outer hair cells, spiral ganglion cells, type I and IV fibrocytes, Deiters’ cells, Claudius cells, Boettcher cells, inner sulcus cells, marginal cells, and vestibular hair cells from eight slides from each mouse. We pooled all of the cells in each category from different slides into a single tube. As a control, we scraped the hair cells from eight slides from each mouse. We pooled all of the cells in each category from different slides into a single tube.

For linear vestibular evoked potential (VsEP) measurements, experimenters were blinded to the genotype during data collection and analysis. Animals were weighed and monitored (Equi-izer) as a control. At intraperitoneally, each skull was prepared with a head mount. A threensure was secured at the midline, and two additional electrodes were placed behind the left and right pinnae with a ground at the ventral neck. The animals were placed supine, and each head was secured to an electromechanical shaker with the naso-occipital axis oriented vertically. Stimuli were linear acceleration pulses (2 ms duration; 16 pulses/s) presented in two directions, normal and inverted. Normal polarity was used as a control, while inverted polarity was used to invert the platform downward. Stimulus amplitude was measured in jerk (i.e., g/ms, where 1.0 g = 9.8 m/s² and 1.0 g/ms = 9.8 μm/s³ [18]) using a calibrated accelerometer attached to the shaker platform. To monitor the jerk component of the stimulus, the output of the accelerometer was differentiated electronically. Stimulus amplitude was recorded in db re: 1 g/ms and ranged from −18 to +6 db re: 1.0 g/ms, adjusted in 3-dB steps. Electrophysiologic activity was amplified (200,000-fold) and filtered (300 to 3,000 Hz), and VsEPs to normal and inverted stimuli polarities (1,024 points, 10 μs/pixel, 128 responses per averaged waveform) were recorded. Four waveforms were obtained at each stimulus intensity level, two for normal polarity stimuli and two for the inverted polarity. Averaging of responses to normal and inverted polarities was completed offline to produce the final averaged waveforms used for data analysis. Three parameters were quantified: threshold, peak latencies, and peak-to-peak amplitudes. The threshold was defined in dB re: 1.0 g/ms was defined as the stimulus amplitude midway between which produced a discernible VsEP and that which failed to produce a response. Thresholds, latencies, and amplitudes were compared among the three groups of animals using one-way analysis of variance (ANOVA) (thresholds) and multivariate ANOVA (latencies and amplitudes) with a significance level of P less than 0.05.

Hippocampal electrophysiology. For the evaluation of the role of GluR6i in synaptic transmission and synaptic plasticity, hippocampal slices were prepared from GluR6i+/− and GluR6i+/+ male mice without prior knowledge of mouse genotype. Slices were continuously superfused with artificial cerebrospinal fluid containing 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 2 mM MgSO4, 1.25 mM NaH2PO4, 26 mM NaHCO3, and 10 mM glucose, with 95% O2 and 5% CO2 at 30 to 31°C (2 ml/min). Schaffer collateral synapses were stimulated with a bipolar tungsten electrode in CA1 stratum radiatum placed 100 to 150 μm from the recording pipette, and field excitatory postsynaptic potentials (fEPSPs) were collected using a MultiClamp 700B amplifier (Molecular Devices). To ensure equivalent activation of postsynaptic neurons in all experiments, stimulation intensities were chosen to evoke an fEPSP with a slope of approximately 1 mV/ms. In long-term potentiation (LTP) experiments, Schaffer collateral were stimulated at 0.033 Hz before and after the induction of LTP. LTP was induced by a 200-Hz pulse protocol consisting of 10 trains of 200 ms of stimulation at 20 Hz delivered every 5 s at the baseline stimulation intensity. Data were analyzed using Clampfit 9.0 software (Molecular Devices). Results were grouped according to mouse genotype.
Morris water maze test. For an examination of defects in LTP in GluR1/−/− mice in vivo, mice of three genotypes were tested in a water maze that consisted of a circular blue plastic tank, 160 cm in diameter and 38 cm deep. The maze was located in a large test room surrounded by external cues that could be used for spatial navigation. The tank was filled to 30 cm with water at 21°C made opaque by the addition of a small quantity of nontoxic white paint (tempura). The platform, a 10-cm square of Plexiglas covered with a rough green plastic scouring pad, was mounted on a solid column 1 cm below the surface such that it could not be seen from water level. Four equally spaced points around the edge of the tank were used as start positions and divided the maze into four quadrants. During the acquisition of the place task, the platform was in the middle of one quadrant, equidistant between the center and the outer wall of the maze. Mice were trained for one block of four trials on each of 10 consecutive days. Within each block of trials, all four start positions were used once each in a pseudorandom sequence.

For each trial, a mouse was placed in the water facing the wall at the start position. The time required to find the escape platform was recorded. Any mouse failing to find the platform within 60 s was placed on the platform. Approximately 10 min separated the individual trials in each day’s block of tests.

RESULTS

Generation of GluR61/−/− mice. To create GluR61/−/− mice, we designed a targeting construct that deleted exons 11 and 12 of the GluR61 gene (Fig. 1A). This targeted disruption ensured the removal of three of the four transmembrane domains and introduced a frameshift after exon 12. We screened 380 ES cell colonies by genomic Southern blot analysis using an external probe, and one underwent homologous recombination. Using Neo as a probe, we confirmed that there were no other random integrations in this ES cell line (data not shown). We performed karyotyping to determine cytogenetic normality. After blastocyst injection, high chimeras were obtained, and germ line transmission was achieved. The crosses between GluR61/−/− mice yielded offspring with an approximately 1:2:1 ratio of the GluR61+/+/ (63 offspring), GluR61+/−/ (126 offspring), and GluR61−/−/ (65 offspring) genotypes, suggesting no embryonic lethality in the GluR61−/−/ mice. The correct targeting of GluR61 gene was further confirmed by genomic Southern blot analysis of mice with germ line transmission (Fig. 1B). In the PCR analysis, no 220-bp wild-type bands (in the deleted region) were detected in the homozygous mice (Fig. 1C).

To confirm the ablation of the GluR61 protein in GluR61−/− mice, we performed Western blot analysis with a polyclonal antibody against the C termini of both the GluR61 and GluR62 proteins. As a positive control for this antibody, we used the cerebellum, where GluR62 is predominantly expressed and is unchanged in either GluR61+/+ or GluR61−/− mice (Fig. 2A); because GluR62 is not normally expressed in the inner ear or hippocampus (36), this antibody can be used to assay GluR61 expression in these tissues from mutant mice. Both GluR61+/+ and GluR61−/− mice expressed the expected ~115-kDa GluR61 protein, and no GluR61 protein with a mass of 115-kDa or any other size was detected in the inner ear or hippocampus of GluR61−/− mice (Fig. 2A). It remains possible that a small N-terminal peptide is made in GluR61−/−; however, it is likely that this peptide would be degraded due to the lack of proper transmembrane domains and a C terminus, so GluR61−/− is therefore an effective null allele.

In addition, we performed immunofluorescence on hippocampus and cerebellum from GluR61+/+ and GluR61−/− mice using a GluR61-specific antibody (Fig. 2B; data not shown). GluR61 is absent in cerebellum but present in hippocampus of GluR61+/+ mice; however, it is absent in both regions of GluR61−/− mice (Fig. 2B; data not shown), consistent with Western blot results (Fig. 2A). Furthermore, our wild-type immunostaining results are consistent with in situ hybridization results reported recently for developing and adult mouse brains (24, 26). Our Western blotting and immunostaining results also demonstrated that GluR62 is absent in the inner ear and hippocampus and that no obvious up-regulation of GluR62 occurs in the cerebellum, hippocampus, or inner ear of GluR61−/− mice.
GlurR1\(^{-/-}\) mice showed no obvious developmental or behavioral abnormality, except for overall weight reductions by 4 months of age. In all of the groups at 2 months of age or younger, weight differences were not significant (data not shown). At 4 months, the weights of male GlurR1\(^{-/-}\) mice were approximately 89% of those of the GlurR1\(^{+/+}\) males, and the weights of female GlurR1\(^{-/-}\) mice were approximately 87% of those of the GlurR1\(^{+/+}\) females; there was no significant difference between the weights of GlurR1\(^{+/+}\) and GlurR1\(^{-/-}\) mice of either sex.

**Cochlear GlurR1 expression.** To independently verify the expression of GlurR1 in specific single cell types of the inner ear of GlurR1\(^{+/+}\) mice and to confirm the deletion of GlurR1 in the inner ear of GlurR1\(^{-/-}\) mice, we used laser capture microdissection and RT-PCR (Fig. 2C). Prestin (an OHC-specific marker), p27 (a supporting cell marker), and β-actin (a ubiquitous marker) were used as controls. GlurR1 was expressed in IHCs, OHCs, spiral ganglia, and vestibular HCs. However, it was not expressed in type I and IV fibrocytes, Deiters’ cells, Claudius cells, inner sulcus cells, Boettcher cells, or marginal cells in stria vascularis (Fig. 2C). Our results are largely consistent with those of a previous report (36); however, some differences exist: in previous studies, OHCs were negative in both in situ and immunostaining analyses for both guinea pigs and rats, whereas Claudius cells in basal turns were negative in rat cochlea by in situ analysis but weakly positive in guinea pig cochlea by immunostaining analysis with only anti-GluR1 antibody (see Fig. 2 and 5 in reference 36). Such differences are subtle and can be subject to differences between species or in sensitivities of detection methods. In the inner ears of GlurR1\(^{-/-}\) mice, the corresponding portion of GlurR1 mRNA was indeed deleted in all cell types analyzed (Fig. 2C). These results were reproduced in three independent experiments using different GlurR1\(^{+/+}\) and GlurR1\(^{-/-}\) mouse cochlear sections.

**Cochlear responses.** Given the strong cochlear expression of GlurR1, we examined cochlear function in GlurR1\(^{-/-}\) mice. The three assays used were (i) ABR, the summed sound-evoked activity of the auditory nerve and ascending auditory neural pathways; (ii) DPOAE, a sound-evoked preneural signal generated and amplified by the OHCs and transmitted back to the ear canal; and (iii) the magnitude of the EP, the potential generated by the stria vascularis and measured inside the endolymphatic space, which generates the transepithelial electric driving force necessary to drive transduction currents through the hair cell stereocilia when they are deflected by acoustic stimulation.

At 6 to 8 weeks of age, ABR thresholds in GlurR1\(^{-/-}\) mice were elevated compared with those in GlurR1\(^{+/+}\) mice by 20 to 45 dB for frequencies of >16 kHz (Fig. 3A) (differences between GlurR1\(^{+/+}\) and GlurR1\(^{-/-}\) mice were significant by two-way ANOVA; \(P = 0.003; F = 21.77\)). At lower frequencies (<16 kHz), threshold elevation was <10 dB. Thresholds in GlurR1\(^{-/-}\) mice were intermediate between those in GlurR1\(^{+/+}\) and GlurR1\(^{-/-}\) mice. DPOAEs showed similar patterns of threshold elevation (Fig. 3B): differences between GlurR1\(^{-/-}\) and GlurR1\(^{+/+}\) mice for test frequencies of >16 kHz were significant by two-way ANOVA (\(P = 0.01; F = 10.43\)). Suprathreshold ABR and DPOAE amplitudes were also significantly reduced in GlurR1\(^{-/-}\) mice at frequencies of >16 kHz (data not shown); however, there were no significant changes in response waveforms.

EP measured in the basal turn (Fig. 3C), a cochlear location corresponding to the 45-kHz tonotopic location (17), was lower in GlurR1\(^{-/-}\) mice (77.1 ± 8.2 mV [mean ± standard error of the mean (SEM)]; \(n = 7\)) than in GlurR1\(^{+/+}\) (100.7 ± 3.2 mV [mean ± SEM]; \(n = 7\)) and GlurR1\(^{-/-}\) mice (90.0 ± 6.2 mV [mean ± SEM]; \(n = 7\)). The difference between GlurR1\(^{-/-}\) and GlurR1\(^{+/+}\) mice was significant (\(P < 0.01\) by Student’s \(t\) test; \(P < 0.05\) by one-way ANOVA).

FIG. 3. Cochlear thresholds are elevated at high frequencies in GlurR1\(^{-/-}\) (−/−) mice (A and B), and the EP is reduced in the basal turn (C). GlurR1\(^{+/−}\) (+/−) animals show intermediate values by all measures. Panels A and B show ABR and DPOAE data, respectively, for the same cohort of animals; panel C is from a separate group. Means and standard errors (error bars) are shown; numbers of animals (\(n\)) in each group are given. Statistical analyses are described in the text. Arrows on DPOAE points from GlurR1\(^{-/-}\) mice show no obvious developmental or behavioral abnormality, except for overall weight reductions by 4 months of age. In all of the groups at 2 months of age or younger, weight differences were not significant (data not shown). At 4 months, the weights of male GlurR1\(^{-/-}\) mice were approximately 89% of those of the GlurR1\(^{+/+}\) males, and the weights of female GlurR1\(^{-/-}\) mice were approximately 87% of those of the GlurR1\(^{+/+}\) females; there was no significant difference between the weights of GlurR1\(^{+/+}\) and GlurR1\(^{-/-}\) mice of either sex.

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Cochlear morphology and immunostaining. To evaluate morphological changes in the GluRδ1−/− mice, we examined plastic sections of osmium-stained cochleas. Histologic staining in GluRδ1−/− mice (8 weeks old) showed a pathological pattern consisting of variable and scattered OHC loss in the basalmost region of the cochlea (Fig. 4E) and consistent and substantial loss of type IV fibrocytes in the spiral ligament (Fig. 4D) throughout much of the basal turn (Fig. 4E). As shown in high-power micrographs (Fig. 4B and D), the nuclei of type IV fibrocytes are normally visible interspersed among a complex fibrous network visible in differential interference contrast optics (Fig. 4D). In the mutant, all cell nuclei are absent in this region of the spiral ligament, and only the fibrous network remains (Fig. 4B). According to a mouse cochlear frequency map (8), the OHC loss was restricted to cochlear frequency regions well above that where the threshold shift was seen; however, the region of spiral ligament pathology correlated well with the region of threshold shift. Quantitative results from one ear of each genotype are shown in Fig. 4E to F: similar results for the loss of fibrocytes were seen in the other ears evaluated (n = 3 of each genotype). There was no loss of IHCs or cochlear neurons, and all other structures of the cochlear duct, including the stria vascularis, appeared normal. To further evaluate the condition of remaining cells of the spiral ligament and stria vascularis, we performed immunostaining for markers normally expressed in these areas and implicated in ionic homeostasis and therefore EP generation: Kv3.1b, Kir4.1, Atp1b1, and Nkcc1 (7, 10, 14, 38). There was little evidence of down-regulation of these key channels/pumps, except that Kv3.1b staining was reduced or absent in regions where type IV fibrocytes were missing in the basal turns but remained present in the type IV fibrocytes in the apical and middle turns and even in adjacent cells in the basal turns (Fig. 5A and B; data not shown). Similarly, Kir4.1 and Atp1b1 appeared normal in marginal cells of the stria vascularis (Fig. 5C, D, G, H). Kir4.1 expression also appeared normal in the Deiters’ cells, the supporting cells for OHCs, in both GluRδ1−/− and GluRδ1+/+ mice (data not shown). No significant change in Nkcc1 staining was observed in marginal cells, type IV fibrocytes, and other cochlear cells of GluRδ1−/− or GluRδ1+/+ mice (Fig. 5E and F; data not shown).

FIG. 4. There is a loss of type IV fibrocytes from the spiral ligaments in the high-frequency regions of ears of GluRδ1−/− mice. Panels A and C show place-matched views of the upper basal turn (~30-kHz region) from an ear of a GluRδ1−/− mouse and an ear of a GluRδ1+/+ mouse, respectively. Arrows point to OHCs; dotted boxes show the region of the spiral ligament where type IV fibrocytes are normally found. Panels B and D enlarge these regions. The white arrow in panel D indicates the nucleus of a type IV fibrocyte in the ear of the GluRδ1+/+ mouse; the arrow in panel B shows the absence of this cell type in the ear of the GluRδ1−/− mouse, which leaves characteristic gaps in the extracellular matrix. Panels E and F show the estimated fractional survival of IHCs, OHCs, and type IV fibrocytes in an ear of a GluRδ1−/− mouse and an ear of a GluRδ1+/+ mouse, respectively, as a function of cochlear location (converted to frequency).
Cochlear vulnerability to noise damage. Synaptic transmission between the IHC and its afferent innervation is glutamatergic, and acoustic overstimulation produces a type of glutamate excitotoxicity that can contribute to temporary noise-induced threshold shifts after acoustic overstimulation. In search of a functional role for the GluR\(\alpha_{1}\)/H9254 receptor in the IHC area, we examined the vulnerability of GluR\(\alpha_{1}\)/H9254 mice to temporary acoustic injury. Twelve hours after a 2-h exposure to a noise band (8 to 16 kHz) at 89 dB SPL, GluR\(\alpha_{1}\)/H9254 mice displayed threshold shifts in both ABR (Fig. 6A) and DPOAE (Fig. 6B) responses that recovered within 1 week (data not shown). In GluR\(\alpha_{1}\)/H9254 mice, the temporary threshold shift was much larger for the same exposure, and the increased dysfunction was seen in both ABR and DPOAE (Fig. 6A and B). The symmetry of the threshold shifts in both the neural (ABR) and the preneural, OHC-based measures (DPOAE) suggests that the shifts are well explained by OHC dysfunction and, thus, that the increased vulnerability did not arise exclusively in the IHC area, i.e., it is not due to enhanced excitotoxicity at the IHC/afferent synapse (29). In contrast, when the efferent innervation of the IHCs is selectively destroyed, the increased vulnerability is seen only in the ABRs and not the DPOAEs, consistent with changes in synaptic transmission associated with excitotoxicity (5).

FIG. 5. Immunostaining of Kv3.1b (A and B) was reduced and lost in type IV fibrocytes (arrows) but normal in other fibrocytes of spiral ligament in the basal turn. Immunostaining of Kir4.1 (C and D), Nkcc1 (E and F), and Atp1b1 (G and H) appeared unaffected in marginal cells of stria vascularis. The bar in panel B (20 μm) applies to all panels. +/+; GluR\(\delta_{1}\) +/+; −/−; GluR\(\delta_{1}\) −/−.

FIG. 6. GluR\(\delta_{1}\) −/− (−/−) mice are more vulnerable to acoustic injury. Temporary threshold shifts were measured in both ABR (A) and DPOAE (B) 12 h after exposure to an octave band noise at 8 to 16 kHz at 89 dB SPL for 2 h. Means and standard errors (error bars) are shown; numbers of animals in each group are in the key in panel B, which applies to both panels. Threshold shifts are not shown for the two highest test frequencies because some of the GluR\(\delta_{1}\) −/− mice showed preexposure thresholds at or near the sound pressure ceiling of our functional assay. +/+; GluR\(\delta_{1}\) +/+.
Cochlear efferent innervation and efferent inhibition. The vulnerability of the cochlea to acoustic injury is controlled, in part, by a cholinergic feedback inhibitory circuit to the OHCs, the medial olivocochlear pathway (27). Given the enhanced vulnerability of the GluR_δ1−/− mice to acoustic injury, we evaluated the integrity of the efferent innervation, both morphologically and functionally.

To assess the density of efferent innervation, we immuno-stained cochlear whole mounts for SNAP25 (Fig. 7A) or synaptophysin (synaptic vesicle-associated proteins abundant in efferent terminals) or CGRP or VAT (markers for neurotransmitters found in efferent terminals; data not shown). Qualitative analysis suggested no abnormalities in the efferent innervation of the GluR_δ1−/− ears. In one cochlea from each genotype, we quantified SNAP25 immunostaining in both IHC and OHC areas and found no significant differences (Fig. 7B and C).

To assess the function of the OHC efferent pathway, we measured the suppression of the DPOAEs elicited by electrical stimulation of the efferent bundle at the floor of the IVth ventricle (Fig. 7D). There were no significant differences among the three genotypes in the mean magnitude of this suppression at different test frequencies obtained from a cohort of animals using the assay illustrated in panel D. Means and standard errors (error bars) are shown. +/+, GluR_δ1+/−; −/−, GluR_δ1−/−; +/−, GluR_δ1+/−.

FIG. 7. There is no change in the density of efferent innervation (A to C) or in the strength of shock-evoked efferent effects (D and E) in GluR_δ1−/− mice. Panel A shows SNAP25 immunostained efferent terminals in the ear of a GluR_δ1+/+ mouse. The white arrow marks a terminal in the OHC area, the black-rimmed arrow shows a terminal in the IHC area. Panels B and C show the total silhouette area of immunostained terminals as a function of cochlear location (converted to frequency) in one ear from each genotype. In the OHC area (C), values are averaged over all three OHC rows. Panel D shows representative data for each genotype from the assay used to measure cochlear efferent suppression. DPOAE amplitude (evoked with f2 at 16 kHz) was measured repeatedly before, during, and after a train of shocks to the efferent bundle; efferent suppression of DPOAE amplitude (the difference between the two dashed lines) was measured. Panel E shows average values of efferent suppression at different test frequencies obtained from a cohort of animals using the assay illustrated in panel D.
efferent effect (Fig. 7E), except at 32 kHz, where the reduced efferent effect in the GluR<sub>H9254</sub> mice is well explained by the OHC dysfunction in that region, as seen by the threshold elevation in both ABRs and DPOAEs (Fig. 3A and B). Since the efferent suppressive effect on cochlear sound-induced vibration arises by reducing the OHCs’ contribution to cochlear amplification, efferent effect size is always reduced in areas of OHC dysfunction. We conclude that the deletion of GluR<sub>H9254</sub> did not affect efferent synaptic transmission and that efferent dysfunction cannot account for the enhanced vulnerability to acoustic injury.

**Vestibular morphology and function.** The presence of GluR<sub>δ1</sub> in vestibular hair cells (both type I and type II) and vestibular ganglia (36) suggested a role in vestibular function. We first analyzed morphology of vestibular end organs in osmicated plastic sections and hematoxylin-and-eosin-stained paraffin sections (see Materials and Methods). There were no changes in morphology of the saccule, utricle, or semicircular canals or in their afferent innervation in the GluR<sub>δ1</sub> mice (Fig. 8A to D; data not shown). Vestibular function was measured at 2 months, both behaviorally (Rotarod and swim tests) and electrophysiologically (VsEP, the summed neural activity of vestibular afferents from the utricle and saccule, and the ascending vestibular pathway, evoked by linear acceleration stimuli). The loss of GluR<sub>δ1</sub> had no significant effect on the ability of mice to remain on a rotating rod as its speed of revolution increased (Fig. 8E) or on the time required to right themselves and begin swimming after being dropped into a water bath (data not shown). On average, VsEP thresholds were slightly higher in GluR<sub>δ1</sub> mice than in GluR<sub>δ1</sub> mice. However, differences among the groups were not statistically significant (ANOVA) (Fig. 8F). Similarly, P1 peak latency, P2 peak latency, P1-N1 amplitudes, and P2-N2 amplitudes were not significantly different among the three genotypes (multivariate ANOVA). All response parameters were similar to normative values in normal young and adult mice (18, 19). These data suggest that the absence of the GluR<sub>δ1</sub> does not significantly alter gravity receptor function or balance behavior.

![Image](image-url)
Hippocampal morphology and function. Because of the high level of GluR61 mRNA and protein in adult hippocampus (Fig. 2B) (25, 36), we compared hippocampal morphology and synaptic function in GluR61+/+ and GluR61−/− mice at 2 months of age. As shown in Fig. 9A, no gross morphological differences were detected among the genotypes. Because the deletion of GluR62 strongly affects long-term depression of synaptic transmission in the cerebellum, we examined the role of GluR61 in synaptic transmission and synaptic plasticity at excitatory synapses between CA3 and CA1 pyramidal neurons (CA3-CA1 synapses) in hippocampal slices (Fig. 9B and C). Recordings of the extracellular fEPSPs showed that the loss of GluR61 did not cause any significant changes in synaptic transmission (Fig. 9B). Thus, input-output curves recorded over a wide range of stimulus intensities were normal in GluR61−/− mice compared to their GluR61+/+ littermates. We next explored the effect of GluR61 deficiency on LTP at CA3-CA1 synapses. We chose a 200-Hz stimulation protocol that induced a compound LTP that consisted of both presynaptic and postsynaptic modules of LTP expression (47). We found no significant changes in compound LTP between GluR61+/+ and GluR61−/− mice (Fig. 9C). Thus, the 200-Hz tetanic stimulation fEPSPs was increased to 159 ± 13% of initial levels in slices from GluR61−/− mice (Fig. 9C). Thus, the 200-Hz tetanic stimulation fEPSPs was increased to 159 ± 13% of initial levels in slices from GluR61−/− mice (mean ± SEM; n = 10) and 174 ± 17% (n = 15; P was >0.05) by the Kolmogorov-Smirnov test) of their initial levels, when measured 60 min after tetanization in slices from GluR61−/− and GluR61+/+ mice, respectively. (D) Performance in the place task of the water maze did not show significant differences among GluR61+/+, GluR61−/+ (−/+), and GluR61−/− mice at 2 to 3 months of age (n = 6, 10, 11, respectively). Mean latency to reach the hidden platform is shown on each of the 10 test days. All groups performed similarly in this task. The vertical bar indicates one standard error of the difference (SED) in group means.

FIG. 9. Hippocampal morphology and function appear normal in GluR61−/− mice. (A) Hematoxylin and eosin staining of hippocampi in GluR61+/+ (+/+) and GluR61−/− (−/−) mice at 2 months old. No obvious differences in neuronal location, number, and position were detected between the hippocampuses of GluR61+/+ and GluR61−/− mice. Synaptic transmission appeared normal in hippocampal slices of GluR61+/+ and GluR61−/− mice at 2 months old (B and C). (B) Recordings of the extracellular fEPSPs showed that the loss of GluR61 did not cause any significant changes in synaptic transmission over a wide range of stimulus intensities. (C) A 200-Hz tetanic stimulation enhanced the fEPSPs to 159 ± 13% (mean ± SEM; n = 10; P was >0.05 by the Kolmogorov-Smirnov test) of their initial levels, when measured 60 min after tetanization in slices from GluR61−/− and GluR61+/+ mice, respectively. (D) Performance in the place task of the water maze did not show significant differences among GluR61+/+, GluR61−/+ (−/+), and GluR61−/− mice at 2 to 3 months of age (n = 6, 10, 11, respectively). Mean latency to reach the hidden platform is shown on each of the 10 test days. All groups performed similarly in this task. The vertical bar indicates one standard error of the difference (SED) in group means.
acquisition of this task (ANOVA; group \( \times \) day; \( F = 1.33, \text{df} = 18,216; P > 0.05 \)).

**DISCUSSION**

Targeted disruption of GluR\( \delta \)1 causes significant hearing loss at high frequencies, associated with reductions of both OHC function and EP, the resting potential of the lumen of the cochlear duct that helps drive receptor currents into sensory cells. These findings provide the first in vivo evidence of a functional role for this largely uncharacterized orphan glutamate receptor, which is consistent with its prominent inner ear expression. Given the prevalence of congenital or acquired high-frequency hearing loss in human ears, the locus encoding GluR\( \delta \)1 represents a candidate disease gene (39).

In contrast, the apparently normal function and morphology in vestibular sensory-end organs and hippocampus in GluR\( \delta \)1\(-/-\) mice suggest that functional redundancy exists for the GluR\( \delta \)1 expressed in these areas. Given the lack of other members of the GluR\( \delta \) family in human and mouse genome sequences, it is conceivable that other proteins with little sequence homology to GluR\( \delta \)1 may compensate for the lack of GluR\( \delta \)1 in vestibular end organs and hippocampus.

**Cochlear dysfunction and EP reduction.** The high-frequency threshold elevation observed in the GluR\( \delta \)1\(-/-\) mice was on the order of 20 to 45 dB as measured in the neural response (ABR) and roughly half that as measured in the preneural response (DPOAE). This hearing loss was associated with a reduction in the EP of 20 to 25 mV, as measured in the basal turn at roughly the 45-kHz place. A number of lines of evidence converge to suggest that both the pattern and degree of threshold elevations are well explained qualitatively and quantitatively by the EP reduction (29), i.e., there is no reason to assume dysfunction in the sensory cells and nerve fibers per se.

Decreasing the EP reduces the driving force for sound-elicited transduction currents into both IHCs and OHCs. This reduction seen by the OHCs reduces their somatic electro-illumotility and thereby decreases mechanical motions of the cochlear partition and elevates thresholds as seen by both DPOAEs and ABRs (35). Threshold elevation in cochlear neurons is further increased by effects at the IHCs, since these IHCs provide exclusive synaptic drive to 95% of these nerve fibers. The EP reduction seen by IHCs reduces the receptor potentials which drive synaptic transmission, even without a change in cochlear vibration. Thus, cochlear dysfunction from EP reduction results in larger changes in ABR (neural) thresholds than in DPOAE thresholds (which require only normal OHC function). Indeed a recent empirical comparison of ABR shifts and DPOAE shifts in furosemide-treated gerbils showed a ratio very similar to that seen here (29).

According to studies of click-evoked neural potentials in cats, there is roughly a 1-dB threshold shift in ABR for each 1-mV decrement in EP (37). Given that click-evoked thresholds in cat are dominated by midfrequency (5 to 10 kHz) neurons (1) and that the OHC contribution to the cochlear amplifier increases with frequency, it is not unlikely that, in the 16- to 45-kHz region of the mouse, the relationship between neural thresholds and EP reduction is greater than 1 dB/mV. Thus, the ABR and DPOAE shifts seen in the present study are well explained by the magnitude of the EP shift.

**EP reduction and loss of spiral ligament cells.** The EP is generated by coordinated ion pumping activity of numerous cell types in the spiral ligament and the stria vascularis. Numerous other deafness mutations appear to affect hearing via their effects on EP and cochlear ion homeostasis. Mutations in Cx26, Claudin-11, Pendrin, Claudin-14, Nkcc1, Kcc4, and various channels (e.g., Kir4.1 and Isk) can cause EP reduction and corresponding elevation of cochlear thresholds (2–4, 7, 11, 12, 22, 28, 41). Mice lacking Pou3f4, a transcription factor expressed in spiral ligament fibrocytes, showed a 50-mV EP reduction associated with a 70- to 80-dB elevation of ABR thresholds. Interestingly, such a profound cochlear dysfunction (much more dramatic than that seen here with the loss of GluR\( \delta \)1) was associated with very subtle morphological changes. Hair cells and the rest of the organ of Corti were normal in Pou3f4 mice, with ultrastructural changes noted only in spiral ligament fibrocytes (type I and type II) (30). Mice lacking otospiralin, a protein of unknown function produced by spiral ligament fibrocytes, displayed modest threshold elevation (20 dB by ABR) associated with subtle changes in morphology of type II and type IV fibrocytes, visible only at the ultrastructural level. EP was not measured in this mutant line (6).

Type IV fibrocytes in GluR\( \delta \)1\(-/-\) mice were eliminated throughout the basal turn. However, this fibrocyte loss was probably not the cause of the EP reduction. Acoustic overstimulation experiments in mice have shown that type IV fibrocytes, among the most vulnerable cells in the ear, can be eliminated after moderate noise exposures, yet ABR thresholds and EP values can completely recover (17, 42). Similarly, in a mouse (C57BL/6) with progressive, high-frequency age-related hearing loss, type IV fibrocytes are also among the first cells to disappear from the basal turn (13) and, although high-frequency thresholds are elevated, the EP is not reduced (23, 31) (K. Hirose and M. C. Liberman, unpublished data).

Given that GluR\( \delta \)1 was expressed in IHCs, OHCs, and spiral ganglion neurons but not in the spiral ligament, possible explanations for the EP shifts based on cellular changes outside the stria and ligament must be considered. One possible link is that the generation of a normal EP must depend on appropriate recycling of K\(^+\) from the hair cells to the stria via the spiral ligament (43), and the loss of GluR\( \delta \)1 may disrupt that recycling in either the IHC or the OHC areas. The organ of Corti is both mechanically labile and “leaky” to ion flux, as a result of the effects of the loss of GluR\( \delta \)1 on one or more supporting cells. Direct measurement of the input impedance of scala media may address this issue, although such an approach is tedious and artifact prone.

**Glutamatergic transmission and loss of OHC function.** Although there is a rich efferent innervation of hair cells and cochlear neurons, there is no evidence for glutamatergic synapses in the efferent system; correspondingly, our findings showed no effects of GluR\( \delta \)1 deletion on efferent innervation or the strength of efferent-evoked effects on cochlear response (i.e., DPOAEs). However, the striking increase in vulnerability of the ears of GluR\( \delta \)1\(-/-\) mice to temporary acoustic injury seen in this study was consistent with a role of GluR\( \delta \)1 in OHCs. In particular, the similarity in noise-induced DPOAE shifts and ABR shifts suggests that increased vulnerability is occurring presynaptically, e.g., involving OHCs and their role as cochlear amplifiers. The presence of GluR\( \delta \)1 in OHCs in
our study and others (36) is consistent with such a notion. The afferent synapse between OHCs and type II cochlear nerve fibers is poorly understood. It is not clear whether afferent transmission there is glutamatergic (33, 34); glutamate excitotoxicity is not seen in the OHC area after acoustic overstimulation (33, 34), and the efferent synapses on OHCs are clearly cholinergic in nature with end effects mediated via the α9/10 nicotinic acetylcholine receptors (9, 40). Thus, it is difficult to propose a compelling argument as to why GluRδ1 loss should enhance damage to OHCs per se. An alternate hypothesis is that the heightened vulnerability arises via increased fragility of the EP generation mechanisms such that the increased demands on the system imposed during acoustic overstimulation lead to further EP reductions not seen in mice with more robust ion homeostasis.

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