Deficient forward transduction and enhanced reverse transduction in the alpha tectorin C1509G human hearing loss mutation

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SUMMARY

Most forms of hearing loss are associated with loss of cochlear outer hair cells (OHCs). OHCs require the tectorial membrane (TM) for stereociliary bundle stimulation (forward transduction) and active feedback (reverse transduction). Alpha tectorin is a protein constituent of the TM and the C1509G mutation in alpha tectorin in humans results in autosomal dominant hearing loss. We engineered and validated this mutation in mice and found that the TM was shortened in heterozygous TectaC1509G/+ mice, reaching only the first row of OHCs. Thus, deficient forward transduction renders OHCs within the second and third rows non-functional, producing partial hearing loss. Surprisingly, both TectaC1509G/+ and TectaC1509G/C1509G mice were found to have increased reverse transduction as assessed by sound- and electrically-evoked otoacoustic emissions. We show that an increase in prestin, a protein necessary for electromotility, in all three rows of OHCs underlies this phenomenon. This mouse model demonstrates a human hearing loss mutation in which OHC function is altered through a non-cell-autonomous variation in prestin.

INTRODUCTION

Out of every 1000 children born, one is deaf and 17 are afflicted with sensorineural hearing loss before reaching the age of 18 years (Haggard and Pullan, 1989; Bhasin et al., 2006). Single-gene defects are responsible for over half of these cases and most are thought to affect the cochlea (Steel and Brown, 1996). A common clinical scenario is the identification of a child with a partial hearing loss who then slowly progresses to deafness over a period of years. Although certain gene mutations are known to affect inner ear-specific proteins and can be linked to progressive hair cell degeneration, the mechanisms behind most common causes of progressive hearing loss in childhood are essentially unknown (Cristobal and Oghalai, 2008).

The TECTA gene encodes alpha (α) tectorin, an extracellular protein constituent of the tectorial membrane (TM) and the otolithic membrane in the cochlea and vestibular system, respectively (Goodyear and Richardson, 2002). α-Tectorin contains several protein-protein interaction domains: an N-terminal entactin G1-like domain, three full and two partial von Willebrand factor (vWF) type D repeats, and a C-terminal zona pellucida (ZP) domain (Legan et al., 1997). Despite this understanding, however, the structural and functional roles of α-tectorin in the tectorial membrane are unclear. Mutations in TECTA (DFNA8/12) can cause either stable or progressive hearing loss depending on their location within the gene (Pfister et al., 2004). Mutations in the ZP domains are commonly associated with stable hearing loss, whereas mutations within the vWF domains tend to manifest as progressive hearing loss (Plantinga et al., 2006). Tecta null mice are deaf because the TM is detached completely from the organ of Corti; consequently, vibrations of the basilar membrane associated with the traveling wave do not lead to deflection of outer hair cell (OHC) or inner hair cell (IHC) stereocilia (Legan et al., 2000). Mice carrying a mutation in the ZP domain have congenital hearing loss because they have a misshapen TM that stimulates OHCs normally, but under-stimulates IHCs (Legan et al., 2005). Neither transgenic mouse has progressive hearing loss.

An autosomal dominant mutation in the human TECTA gene that presents clinically with partial hearing loss at birth followed by a steady rate of progressive hearing loss has been reported (Pfister et al., 2004). We hypothesized that altered biomechanical interactions between the TM and the OHCs would underlie the pathophysiology of this disorder. To study this possibility, we created this C1509G (cysteine-to-glycine) point mutation in the mouse Tecta gene. Here, we report that this human TECTA mutation causes reduced OHC forward transduction, as might be expected with a mutation that impacts the TM, but also increased reverse transduction. We further demonstrate that this increase is mediated through an elevation of OHC prestin, a protein that is essential for electromotility and cochlear amplification (Liberman et al., 2002; Dallos et al., 2008).

RESULTS

Generation of the TectaC1509G knock-in mouse

The TectaC1509G knock-in mouse was created using standard homologous recombination procedures (supplementary material Fig. S1A and see Methods). The C1509G mutation was re-created within the fourth vWF type D repeat domain (supplementary material Fig. S1B). Two embryonic stem (ES) cell lines were established using standard positive and negative selection...
techniques (see Methods). Mice were made from both ES cell lines and the presence of the C1509G mutation was confirmed in each line; sequencing from one of them (2G11) is shown (supplementary material Fig. S1C). Both knock-in lines showed similar histological and audiometrical results, although only data from the 2G11 line are presented in this report. Functionally, heterozygous $\text{Tecta}^{C1509G/+}$ and homozygous $\text{Tecta}^{C1509G/C1509G}$ mice did not demonstrate any obvious vestibular deficits, such as circling behavior, and were indistinguishable from wild-type $\text{Tecta}^{+/+}$ mice by gross examination.

**Histological studies**

$\alpha$-Tectorin mRNA is normally detectable in the mouse between embryonic day (E)12.5 and postnatal day (P)8, during the development of the TM (Rau et al., 1999). In situ hybridization at P0 confirmed that there were no differences in the expression patterns of $\alpha$-tectorin mRNA between wild-type, heterozygous and homozygous mice (Fig. 1A-C). Additional in situ hybridization studies at P3, P5 and P7 further verified that no differences in $\alpha$-tectorin expression were found during postnatal development (data not shown). Immunolabeling studies from the mid-basal turn of the cochlea (~0.75 turns from the round window) were performed at P0 (Fig. 1D-L). Qualitative assessment suggested that there were no gross differences in the patterns of expression of $\alpha$-tectorin, $\beta$-tectorin or otogelin between the genotypes. Altogether, these data demonstrate that the mutant $\alpha$-tectorin transcript and protein were produced during postnatal cochlear development, and that the mutant protein was incorporated into the TM.

Although the anatomy of the TM is notoriously sensitive to fixation and dehydration artifacts (Edge et al., 1998), we did perform histological studies using frozen sections at multiple developmental ages to provide a general assessment of TM morphology (Fig. 2). All images were taken from the mid-basal turn of the cochlea. The cross-sections revealed that there were anatomic differences between the genotypes, and that these differences became more pronounced during postnatal cochlear development. Qualitatively, the TM in heterozygous mice was thicker, but shorter, than the TM in wild-type mice. The TM in homozygous mice was even thicker and appeared to be detached from the sensory epithelium.

We also evaluated adult cochlear morphology using plastic sections of three wild-type, five heterozygous and five homozygous mice (Fig. 3A-C). As expected, differences in TM anatomy were clearly evident between the genotypes. The TM in heterozygous mice was shorter and did not appear to cover all three rows of OHCs, as seen in the wild-type mice. The TM in homozygous mice was loosely connected to the spiral limbus and was elevated off of the organ of Corti. Although the staining density within the center of the TM (the body) appeared qualitatively similar between the genotypes, the staining density at the distal end of the TM (the marginal band) appeared to be reduced in heterozygous and homozygous mice. Importantly, there were no visible differences in the anatomy of the rest of the cochlea, including the otic capsule bone, the spiral ligament, stria vascularis, basilar membrane, inner and outer hair cells, modiolus and spiral ganglion cells. We also imaged phalloidin-labeled whole-mount preparations of the adult cochlear epithelium in the mid-basal turn to assess the hair cells. In all three genotypes, there was no obvious loss of hair cells, and the organization and arrangement of the stereociliary bundles was qualitatively normal (Fig. 3D-F).

Transmission electron microscopy was performed to assess the ultrastructure of the TM within the mid-basal turn of the cochlea (Fig. 4). Qualitatively, there was a normal organization of the collagen fibrils and the striated sheet matrix within the body of the TM (Fig. 4A-C, top). At higher magnification, the dark and light filaments within the striated sheet matrix (Hasko and Richardson,
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1988) were visible in all three genotypes (Fig. 4A–C, bottom). However, there was partial disruption of the tightly packed fibrils surrounding the edges of the TM in heterozygous mice and severe disruption in homozygous mice (Fig. 4D–I). Together, these data suggest that the C1509G mutation in α-tectorin is particularly important in organizing the tightly packed fibrils along the rim of the tectorial membrane, which are presumably composed of collagen (Slepecky et al., 1992a; Slepecky et al., 1992b).

**Assessment of forward transduction**

Based on our histological studies, it appeared as though the heterozygous TM was malformed and may not contact all rows of OHCs. To directly assess this possibility, we studied the ability of sound to deflect OHC stereociliary bundles in freshly excised cochleae. Deflections of the hair cell stereociliary bundle permit cations to enter through mechanoelectrical transduction channels (Corey and Hudspeth, 1979; Dallos et al., 1982); this generates a receptor potential within the hair cell and this process is termed ‘forward transduction’. The Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) within hair cell stereocilia, as well as the soma, has been shown to vary during the process of forward transduction and can be measured by fluorescence imaging (Lumpkin and Hudspeth, 1995; Lumpkin et al., 1997; Lumpkin and Hudspeth, 1998; Yamoah et al., 1998; Beurg et al., 2009). Thus, we measured changes in [Ca\(^{2+}\)] within OHCs from each row in response to vibration of the stapes at acoustic frequencies (Yuan et al., 2010). We studied an easily accessible region of the cochlea that is ~1.25 turns up from the round window. An external solution containing 4 mM calcium was used in order to highlight variations in calcium channel conductance; this has been used previously to assess calcium conductance through hair cell mechanoelectrical transduction channels (Denk et al., 1995; Lumpkin and Hudspeth, 1995; Yamoah et al., 1998). We studied the [Ca\(^{2+}\)] within the soma rather than the stereocilia because it was easier to identify and served as an indirect marker of stereociliary stimulation.

First, we viewed the TM in cochleae from wild-type and heterozygous mice (n>20 mice for each group) using an external solution with a normal perilymphatic [Ca\(^{2+}\)] of 2 mM (Fig. 5A). Transmitted light imaging demonstrated that the wild-type TM covered all three rows of OHCs, whereas those from heterozygous mice appeared shorter. The TM from homozygous mice appeared to be elevated off the epithelium. Wild type, +/+; heterozygous, +/Gly; homozygous, Gly/Gly. Bar, 50 μm.

![Fig. 2. Development of the organ of Corti.](image)

Fluorescence imaging (Fig. 5B, top) revealed that the OHC soma from all three rows were clearly visible and of a similar intensity in wild-type mice. This indicates that the static [Ca\(^{2+}\)] was similar in OHCs from different rows. By contrast, differences were found between the rows of OHCs in heterozygous mice. OHCs within the second and third rows demonstrated lower fluorescence intensities than those in the first row. Homozygous mice demonstrated lower fluorescence intensity levels in all rows. Z-stack reconstructions (Fig. 5B, bottom) confirmed that the fluorescence intensity of the IHC and OHCs extended along their length and was not tightly localized to their cuticular plate regions. Again, there was little fluorescence in the surrounding supporting cells.

During acoustic stimulation of the stapes, we assessed for dynamic changes in the fluorescence intensity (ΔF/F) in a plane below the cuticular plate region of the OHCs. We found increases within OHCs from each row in wild-type mice, within OHCs from only the first row in heterozygous mice, and not within any OHCs.
in homozygous mice (Fig. 5C,D). These ΔF/F responses were statistically significant, although quite small and not visible by eye. Indeed the responses were only ~1%, similar to that seen at the basal end of the stereocilia by others (Lumpkin and Hudspeth, 1995; Lumpkin and Hudspeth, 1998), but much lower than the typical ΔF/F response for an action potential in a hippocampal neuron of ~50-100% (Reddy et al., 2008). This low signal reflects the relatively small current through the transduction channels and the resultant depolarization expected in this excised cochlear preparation, which does not have an endocochlear potential or a high [K+] surrounding the stereociliary bundles (for a review, see Geisler, 1998). Nevertheless, these findings support the concept that forward transduction only occurs within the first row of OHCs during sound transduction in heterozygous mice.

Assessment of cochlear function in vivo
In order to assess the ability of the TM to stimulate the OHCs in vivo, we measured the cochlear microphonic (CM), a field potential thought to emanate predominantly from the receptor potential within OHCs of the basal turn (Patuzzi et al., 1989). We used a stimulus frequency of 6 kHz in order to minimize the impact of the cochlear amplifier on the response, and varied the stimulus intensity to assess forward transduction. Wild-type mice demonstrated a normal response in which the CM was in phase with the stimulus and saturated at high stimulus intensities, whereas homozygous mice had a response that was reduced in amplitude and was a quarter of a cycle ahead of the stimulus trace (i.e. it led the stimulus by 90°) (Fig. 6A, left). In addition, at the onset of the stimulus, the CM was symmetrical in wild-type mice but asymmetrical in homozygous mice (Fig. 6A, right). These findings in the homozygous mice are identical to the responses of the Tecta null mouse, in which no tectorial membrane is present and stimulation of OHC stereocilia is hypothesized to occur secondary to viscous drag of the surrounding endolymphatic fluid (Legan et al., 2000). Importantly, the CM was above the noise floor and was larger than that found in situations when there is no endocochlear potential (Xia et al., 2007).

Interestingly, the phase of the CM from heterozygous mice varied with the stimulus intensity. At low levels, the CM was in phase with the stimulus. As the stimulus intensity increased, the CM had an increasing phase lead (Fig. 6A, left). We suspected this pattern represented the vector sum of field potentials generated by some OHCs that were stimulated in phase with the stimulus and by some OHCs that were stimulated out of phase with the stimulus. In order to assess how many OHC rows were stimulated by the heterozygous TM, we created a simple model (Fig. 6B,C). For example, if the heterozygous TM stimulated only the first row of OHCs, the CM from one row would be in phase and the CM from two rows would be out of phase. This was modeled by vector summing a third of the wild-type CM plus two-thirds of the homozygous CM (Row 1, blue line). Similarly, if the heterozygous TM stimulated the first two rows of OHCs, only the third row would be out of phase. This was modeled by vector summing two-thirds of the wild-type CM plus a third of the homozygous CM (Row 1 & 2, pink line). For both magnitude and phase, the data collected from heterozygous mice more closely matched the model where the TM only stimulates the first row of OHCs. Thus, the in vivo data are consistent with the results of the histological analyses and the calcium imaging studies.

Auditory brainstem responses (ABR) were used to assess auditory thresholds in young adult mice aged P28-P32. Over the measured frequency spectrum, heterozygous mice had 25-40 dB and homozygous mice had 30-50 dB ABR threshold elevations relative to wild-type littermates (Fig. 6D). These data indicate that heterozygous mice have partial hearing loss, similar to the partial congenital hearing loss found in humans with the same mutation (Pfister et al., 2004). By contrast, a more severe deficit was present in homozygous mice.

Assessment of reverse transduction
The main function of OHCs is to provide active feedback that amplifies and sharpens the tuning of the traveling wave (Dallos and Corey, 1991; Robles and Ruggero, 2001). This process involves ‘reverse transduction’, or force production by OHC electromotility in response to membrane potential changes (Brownell et al., 1985).
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We also measured detailed DPOAE amplitude versus stimulus intensity curves in wild-type and heterozygous mice using an F2=17.5 kHz (see Methods). This is predicted to assess the region of the cochlea that is approximately one turn up from the round window (i.e. between where our histological and calcium studies were performed) (Fig. 7A) (Muller et al., 2005). The overall DPOAE amplitudes in heterozygous mice were reduced, as might be expected with only one row of OHCs functionally attached to the TM. Both wild-type and heterozygous mice had the typical notch in their responses at higher stimulus levels, which is thought to reflect a non-linear cochlear amplifier with saturating input-output characteristics (Lukashkin et al., 2002; Lukashkin and Russell, 2002). However, after analyzing the slopes of DPOAE growth curves between threshold and the notch with linear fits, we found that heterozygous mice had higher slopes than wild-type mice (0.924±0.040 dB/dB vs 1.207±0.068 dB/dB in wild-type vs heterozygous mice, respectively; P=0.010). The larger growth slope suggests the possibility that, at stimulus levels capable of producing a DPOAE, one or more OHC processes responsible for DPOAE generation might be increased by the heterozygous mutation state. These could include forward transduction, conversion of transduction currents into receptor potentials, reverse transduction, or OHC-TM coupling.

Although DPOAEs are commonly used as an indirect method of assessing OHC electromotility, they require normal forward transduction. Since only one row of OHCs is stimulated in heterozygous mice, a comparison of DPOAE amplitudes does not reflect the ability of all OHCs to produce force. To selectively study OHC reverse transduction, we measured electrically evoked otoacoustic emissions (EEOAEs) in vivo. By applying an AC electric field across the cochlea, voltage drops occur within the OHCs, stimulating electromotility and a resultant EEOAE. This process has been shown to be essentially unaffected by the absence of a TM (Drexl et al., 2008). However, the prestin protein is crucial to OHC reverse transduction (Zheng et al., 2000; Dallos and Fakler, 2002; Dallos et al., 2006). For these experiments, we studied mice from crosses of Tecta<sup>C1509G</sup> mice with prestin (also known as Slc26a5) null mice, to generate all three genotypes of Tecta<sup>C1509G</sup> mice either with or without prestin in their OHCs (see Methods for details). Littermates aged 21-28 days were studied because it has been shown that no significant hair cell loss occurs before postnatal day 28 in prestin null mice (Wu et al., 2004).

In the prestin wild-type background, Tecta<sup>C1509G/+</sup> mice were found to have higher EEOAE amplitudes, which were statistically significant over most of the frequency spectrum, than wild-type mice (for every frequency between 4-60 kHz) (Fig. 7B). Tecta<sup>C1509G/C1509G</sup> mice had EEOAE amplitudes that were statistically different from wild-type mice at four frequencies (15, 17.5, 28.5 and 33.5 kHz) and that were not statistically different from heterozygous mice at any frequency. In the prestin null background, Tecta<sup>C1509G/+</sup> and Tecta<sup>C1509G/C1509G</sup> mice demonstrated near-complete loss of EEOAEs above 12 kHz, as was previously found to occur in prestin null mice (Drexl et al., 2008). Thus, there were statistically significant differences from prestin wild-type mice at all but two frequencies between 9-65 kHz. Importantly, however, there were no differences between the Tecta genotypes in the prestin null background, indicating that passive or active movement of charged moieties within the mutant TM.

One common way to assess OHC function in vivo is to measure distortion product otoacoustic emissions (DPOAEs). We found that heterozygous mice had a 10-30 dB DPOAE threshold elevation relative to wild-type mice (Fig. 6E). Homozygous mice had no reliable DPOAEs to the equipment limits, consistent with a complete detachment of the TM from the OHCs.
was not responsible for the differences in EEOAE amplitudes in the prestin wild-type background. This was expected because neither cysteine nor glycine is a charged amino acid at physiological pH. Taken together, these data demonstrate that there is more reverse transduction in Tecta heterozygous mice than in wild-type mice, and that this phenomenon involves prestin.

**Assessment of prestin expression**

We then assessed prestin expression within the three Tecta genotypes. Immunolabeling within whole-mount preparations of the cochlea revealed the typical prestin labeling pattern, demonstrating localization to the OHC lateral wall plasma membrane (Fig. 8A). We quantified the prestin fluorescence intensities and found that they were 1.9 times higher in heterozygous OHCs and 2.5 times higher in homozygous OHCs compared with wild-type OHCs (P<0.001, Fig. 8B; Table 1). In addition, the homozygous prestin intensity level was higher compared with that found in heterozygous OHCs (P=0.04). Within each genotype, the prestin labeling intensity was similar between each of the three rows of OHCs (P=0.05, Fig. 8C).

We also measured the levels of prestin by western blot analyses of whole cochleae from each genotype. After normalization, quantification of the band densities revealed higher levels of prestin in heterozygous and homozygous mice than in wild-type mice (P=0.01 for each comparison) (Table 1). No difference was found between heterozygous and homozygous mice (P=0.20). In one experiment, we also quantified the density of myosin VIIa, a hair cell-specific protein whose expression is found in both OHCs and in IHCs (Fig. 8D; supplementary material Fig. S2). The ratio of prestin:myosin VIIa was 1.7 times higher in heterozygous cochleae.
and 2.5 times higher in homozygous cochleae than in wild-type cochleae. These values are comparable to the findings of the immunofluorescence studies.

Lastly, we performed quantitative real time (RT)-PCR from whole cochleae to measure the relative ratio of prestin mRNA to that of the hair cell marker myosin VIIa (Fig. 8E; Table 1). Compared with wild-type mice, prestin levels were 1.6 times higher in heterozygous mutant mice and 1.5 times higher in homozygous mutant mice ($P<0.001$ and $P=0.005$, respectively). The difference between prestin mRNA levels in heterozygous and homozygous mice was not statistically significant ($P=0.37$).

**DISCUSSION**

Herein, we describe the impact of the human hearing loss C1509G point mutation in $\alpha$-tectorin on TM anatomy and cochlear physiology in a mouse knock-in model. This model demonstrates that $\alpha$-tectorin is crucial to the formation of the dense band of fibrils that surrounds the TM and is a key component of the attachment mechanism of OHC stereocilia to the TM.

**Fig. 6. In vivo electrophysiology from adult mice.** (A) Representative 6-kHz cochlear microphonic (CM) tracings for the three genotypes. Left: the stimulus intensity was increased from 10 to 100 dB and the CM tracings were plotted in an overlapping fashion. The 6-kHz acoustic stimulus waveform is shown at the bottom. The phase of the CM was in phase with the stimulus in wild-type mice and led the stimulus by 90° in homozygous mice. In heterozygous mice, the CM started in phase with the stimulus and as the intensity was increased, the phase progressively led the stimulus. Right: CM recordings at the stimulus onset (i.e. before the high-pass characteristics of the pre-amplifier filtered out the DC component of the signal) suggest differences in stereociliary bias points. Wild-type and heterozygous mice had symmetrical responses. By contrast, homozygous mice had asymmetrical responses. The stimulus intensities used to collect these data were 40, 55 and 85 dB SPL (sound pressure level) for wild-type, heterozygous and homozygous mice, respectively. (B,C) The amplitude and phase of the CM versus stimulus intensity are shown, along with models showing the effect of stimulating one row of OHCs (blue line) and two rows of OHCs (pink line). The heterozygous data were best fit by the one-row model. (D,E) Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) thresholds were moderately elevated in heterozygous mice and severely elevated in homozygous mice.

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attach to or stimulate any row of OHCs (Fig. 9). Thus, the partial congenital hearing loss found in humans with one copy of this mutation (Pfister et al., 2004) can be explained by the anatomic malformation of the TM. Essentially, OHCs from rows two and three are functionally neglected. Although they are present, they do not participate in normal forward transduction. This lack of appropriate stimulation prevents them from producing reverse transduction forces during normal hearing. All of our anatomic and physiologic studies involved the region of the cochlea that demonstrated the largest degree of hearing loss, as measured by ABR and DPOAE threshold shifts, the mid-basal to mid-apical regions (from ~0.75 to 1.25 turns up from the round window). Thus, there may be differences in the effect of this mutation at the extreme base or apex of the cochlea that were not identified by this assessment.

**Fig. 8. Increased prestin expression in OHCs from mutant mice.** (A) Prestin immunolabeling demonstrated higher fluorescence intensity within the lateral wall of OHCs in heterozygous and homozygous mice compared with wild-type mice. Each OHC row is labeled (1,2,3). All images were taken from the mid-basal turn. Bar, 8 μm. (B) Quantification of prestin immunofluorescence intensity within the OHC lateral wall demonstrated statistically significant increases in heterozygous and homozygous OHCs relative to wild-type OHCs. (C) Comparison of the fluorescence intensity between OHCs of different rows did not reveal any differences (ANOVA, P>0.05 for each genotype). (D) Western blot demonstrated an increase in prestin in cochleae from heterozygous and homozygous mice compared with cochleae from wild-type mice (+/+, 710 AU; +/Gly, 1172 AU; Gly/Gly, 1598 AU). Myosin VIIa levels from the same blot were roughly similar (+/+, 5811 AU; +/Gly, 5677 AU; Gly/Gly, 5214 AU). The prestin:myosin VIIa ratio, normalized to that of wild-type mice, was increased in heterozygous and homozygous mice (+/+, 1; +/Gly, 1.7; Gly/Gly, 2.5). (E) Quantitative RT-PCR analyses demonstrated elevated prestin transcript levels in both heterozygous and homozygous mice. Wild-type, +/+; heterozygous, +/Gly; homozygous, Gly/Gly. AU=arbitrary units.

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<th>Table 1. Comparison of prestin levels quantified by immunofluorescence, western blot and qRT-PCR among the α-tectorin C1905G knock-in mice genotypes</th>
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All values are normalized relative to those of +/+ mice. There is no variability for the western blot data in wild-type mice because normalization was performed separately for each experiment. By contrast, the immunofluorescence and qRT-PCR data normalization was performed as a grand average of all experiments. All values presented are mean ± S.E.M. P values come from non-paired t-test comparisons with wild-type mice.

**Tecta<sup>C1509G</sup>** transgenic mice have similarities and differences from the previously published mice with TM mutations. Because only one row of OHCs is involved in cochlear amplification, Tecta<sup>C1509G</sup> heterozygotes have distinct physiology from other previously published mice strains with an altered TM (Legan et al., 2000; Simmler et al., 2000; Legan et al., 2005; Russell et al., 2007). However, because neither Tecta<sup>C1509G</sup> homozygotes nor Tecta null mice (Legan et al., 2000) have a TM that is attached to any OHCs, both strains are physiologically identical. Anatomically, there are differences between these strains, however, because the TM in Tecta<sup>C1509G</sup> homozygous mice still maintains a thin attachment to the spiral limbus, whereas the TM in null mice is completely detached.

Ultrastructurally, the Tecta<sup>C1509G</sup> mutation produced no obvious alterations in the striated sheet matrix within the body of the TM.
which is similar to the otogelin null mouse (Simmler et al., 2000) and the Tecta Y1879C/+ mouse (Legan et al., 2005). This is quite different from Tecta null and Tectb null mice (Russell et al., 2007), both of which have disruption of the striated sheet matrix. The marginal band, covernet, bundles and Kimura’s membrane were clearly affected by the Tecta C1509G mutation; similar findings were present in the Tecta Y1879C/+ and Tecta null TM. However, only a portion of Kimura’s membrane in proximity to IHC stereocilia was altered in the Tectb null mouse, and these structures were unaffected in the otogelin null mouse. Thus, although it is evident that the non-collagenous glycoproteins α-tectorin, β-tectorin and otogelin are important to support the interactions between fibrils within the TM, the specific roles of each glycoprotein are not necessarily localized to specific domains of the TM. Nevertheless, our results and the previous descriptions of other Tecta mutant mice (Legan et al., 2000; Legan et al., 2005) demonstrate the importance of α-tectorin in supporting the rim of highly organized fibrils around the edge of the TM.

It might be argued that the calcium fluorescence assessments of forward transduction were altered by the elevated calcium concentration that we used in our external solution. Previous work in the chick has shown that changing the ionic concentration of the fluid surrounding the TM can cause it to swell or shrink by up to 300% (Freeman et al., 1994). However, in the mouse, the changes in the length and thickness of the TM when changing from endolymph to perilymph (i.e. from high K+ to high Na+, and from 20 μM Ca2+ to 2 mM Ca2+) are only 1-2% (Shah et al., 1995). In addition, the mouse TM does not change in cross-sectional area when the [Ca2+] in the perilymph is changed from 2 mM to 4 mM. Thus, although we cannot rule out subtle changes in TM anatomy as a result of our external solution, its ionic composition was not the reason why the heterozygous TM did not reach the second and third OHC rows. Furthermore, it is important to note that separate lines of evidence also support the concept that the heterozygous TM only contacts the first row of OHCs: the histology demonstrating a shorter TM and the in vivo CM magnitude and phase measurements.

OHC stereocilia connected to the TM are known to be biased so that about half of their transduction channels are open and have a symmetric CM response (Russell et al., 1986b). By contrast, this is not found in OHCs grown in culture without an overlying TM (Russell et al., 1986a) or in Tecta null mice that do not have a TM (Legan et al., 2000), which have an asymmetric CM response. This concept is confirmed in the Tecta C1509G mutation by the asymmetrical CM responses in homozygous mice that were noted in our experiment at the stimulus onset. Wild-type mice and heterozygous mice (where the CM response is dominated by the stereociliary bias that is greater than that of OHCs within the second and third rows, and that this is associated with differences in the somatic calcium level at rest.

The dynamic changes in OHC intracellular [Ca2+] in response to sound stimulation were above the noise floor in OHCs contacting the TM, but still demonstrated variability in peak magnitude (see differences between OHCs rows in the wild-type mice in Fig. 5D). This variation may be related to spatial variations of the dynamic changes in calcium along the length of the OHCs. For this study, we simply selected a cross-section to include all three rows of OHCs as close to the cuticular plate region as possible, as shown in Fig. 5B (bottom). This approach might be responsible for some of the variability between rows in the wild type, but was clearly adequate to demonstrate the difference between OHCs that were stimulated by, or not stimulated by, the TM in the heterozygous and
homozygous mice. Movement artifact is unlikely to have significantly contaminated our recordings of calcium changes during acoustic stimulation because the maximal displacements that occur with this technique are ~200 nm (Xia et al., 2007), whereas the depth resolution of the objective lens is ~1 μm.

A surprising finding of this study is that heterozygous and homozygous mice have an increased ability for reverse transduction by their OHCs (Fig. 9). Three lines of evidence (immunofluorescence, western blot and quantitative RT-PCR) show that one mechanism underlying this property is increased prestin levels in these genotypes relative to wild-type mice, which would be expected to produce increased OHC electromotility. We found that homozygous mice did not have larger prestin mRNA levels or EEOAE amplitudes than heterozygous mice, even though semi-quantitative immunolabeling and the western blot prestin:myosin VIIa ratio analysis suggest that they have more prestin per hair cell. One explanation is that the additional prestin in the homozygous OHCs compared with heterozygous OHCs may have variable functionality within the plasma membrane (Santos-Sacchi and Navarrete, 2002; Santos-Sacchi and Wu, 2004; Rajagopalan et al., 2007; Sturm et al., 2007). Indeed, previous work has suggested that prestin mRNA levels do not necessarily correlate with the level of functional prestin protein within OHCs (Xia et al., 2008). Lastly, EEOAEs are likely to be altered by the passive biomechanical properties and by variations in the voltage drop generated within the OHCs because of the malformed TM. Nevertheless, previously published data from the Tecta null mouse demonstrated a trend towards increased EEOAE amplitudes over wild-type mice (Drexel et al., 2008), supporting our finding in the TectaC1509G homozygous mouse.

Since prestin increases are found in all rows of OHCs in heterozygous and homozygous mice, the prestin increase is not a response that occurs within individual OHCs based on whether or not they are attached to the TM. Indeed since α-tectorin is not expressed by hair cells, this mutation should not directly affect OHCs at all and suggests a role for a non-cell-autonomous mechanism in prestin regulation. For example, it is possible that altered TM anatomy or biomechanics may misguide the normal process of OHC development and elongation. Alternatively, it is intriguing to consider the possibility that the partial hearing loss associated with this mutation may stimulate an unknown feedback mechanism designed to attempt to compensate for the hearing loss by enhancing the cochlear amplifier through an increase in prestin. This is not a unique concept, as other states of hearing loss have been demonstrated to increase prestin levels, including chronic salicylate administration (Yu et al., 2008) and noise exposure (Chen, 2006; Mazurek et al., 2007). This could be mediated through the efferent pathways, where each nerve non-specifically innervates many OHCs, or through reciprocal synapses between OHCs and their afferent terminals (Thiers et al., 2008).

TectaC1509G/+ mice represent a simplistic model of the most important pathophysiological aspects that are common to many forms of human hearing loss -- the loss of some OHCs owing to noise, aging, ototoxicity, inflammation, trauma, etc. (Spoendlin, 1985). In these situations, less than a full complement of OHCs is involved in amplifying the traveling wave and there is partial hearing loss. It is unknown whether the elevated prestin levels in TectaC1509G/+ mice are found in humans heterozygous for this mutation and in humans with other forms of hearing loss. However, it is conceivable that upregulation of prestin may be one mechanism that could predispose a patient to an increased rate of progressive sensorineural hearing loss. Potentially, this may occur if the excess prestin causes the OHCs to produce force at levels that are unsafe for the cell membrane; if it over-stiffens the OHC and puts the stereociliary bundles at a higher risk of trauma (Liberman and Beil, 1979); or if it increases the chance of separating the connections between the OHC stereocilia tips and the TM.

METHODS

Animals

The Baylor College of Medicine Institutional Animal Care and Use Committee approved the study protocol. We created the TectaC1509G/+ mouse using a standard homologous recombination replacement (Qiu et al., 1997) knock-in strategy (supplementary material Fig. S1A). Briefly, the right and left arm Tecta genomic DNA fragments flanking exons 13-14 were amplified by long-range PCR. The right arm (4333 bps) was subcloned into pBluescript II SK (+) before site-directed mutagenesis was used to create a single nucleotide change from thymine to guanine, which resulted in an amino acid change from cysteine to glycine at codon 1509 of exon 14. To facilitate genotyping of targeted loci, a second single nucleotide conserved change was made that created a unique BsmI restriction site at the adjacent codon 1510 without affecting the alanine codon (supplementary material Fig. S1B). The right arm containing the point mutation was inserted at XhoI and BamHI of a pFRt vector downstream of a PGKneo positive selection marker, which was flanked by two flipase recognition target (FRT) sites. The vector also contained a herpes simplex virus thymidine kinase (HSVTK) gene downstream of the BamHI site for negative selection. The left arm (2636 bps) was inserted upstream of PGKneo using PacI and Ascl. The targeting vector was linearized with PacI and electroporated into AB12 (129/Sv/Ev) embryonic stem (ES) cells. Homologous recombinants were verified by screening ES colonies by Southern blotting and PCR analyses (supplementary material Fig. S3). The right arm was confirmed by Southern blotting with a probe crossing exon 15 and by AvrII restriction. The PGKneo site was verified with a probe for part of the Neo sequence (data not shown). The left arm was confirmed by PCR (5’-TGACCCACGACCTCTGTTATTCTCTAT-3’ and 5’-GCACCATTTCATTAAATGGCCGGATA-3’). Overall, we found that two of 196 ES cell clones had undergone appropriate homologous recombination with the point mutation, which was confirmed by PCR and sequencing (5’-CGCATACGGGATGGTGGTCTG-3’ and 5’-CCTGCTTCATCTCTCTGCTGCAGGAGG-3’) (supplementary material Fig. S1C). Both clones were microinjected into blastocysts derived from C57BL/6 albino mice and chimeras were obtained. Germ line transmission (F1 generation) was obtained after chimeras were crossed with 129/SvEvSvEv wild-type mice. F1 heterozygotes were next crossed with a flipase1 line (FLP1, 129S4/SvJaeSor-Gr(ROSA)26Soim1(FLP1)Dym/H11032), Stock #003946, The Jackson Laboratory, Bar Harbor, Maine) to delete the PGKneo cassette, generating F2 heterozygotes. We confirmed the presence of the point mutation and the absence of the PGKneo site in these mice by sequencing and Southern blotting, as described above (data not shown). In order to reduce age-related hearing loss genes associated with the 129/SvEv background (Liberman et al., 2002), heterozygous...
F2 mice were crossed with wild-type CBA mice for three generations. All Tecta^{C1509G} mice studied were littermates of the F5-F6 generations in this background (supplementary material Fig. S4) and were between 21-28 days old, unless stated otherwise. This mouse strain can be obtained through The Jackson Laboratories (Stock #JR10826).

In situ hybridization
Detection of Tecta expression was performed using a non-radioactive, dual amplification, digoxigenin-tagged riboprobe system, as described previously for the robotic GenePaint platform (Visel et al., 2004). A 602 bp probe was amplified from the 3’ untranslated region of the Tecta gene using the following primer sequences: T3, 5’- CGAACTCAGGGTTTCTTC-3’ and T7, 5’-ACTTGAACACAAAAGTTATTTAAGG-3’. The specificity and sensitivity of the probe were confirmed using wild-type P0 and E14.5 cochlea.

Generation of the anti α-tectorin and β-tectorin antibodies
Rabbits were immunized (Charles River Breeding Laboratories, Kিফেল, Germany) with synthetic peptides corresponding to regions conserved between the human and the mouse tectorins (α-tectorin: amino acids 803-820, SGRLEHRKNSTTVESK, GenBank accession no. NM_009347; β-tectorin: amino acids 176-198, ETSEIGSDLFAVEAKGLSVRF, GenBank accession no. NM_009348). The antibodies were purified by protein A chromatography, and screened by immunohistochemistry, immunoblotting and ELISA against α-tectorin- and β-tectorin-specific peptides with standard negative controls. To exclude cross-reactivity, anti α-tectorin antibodies were tested against β-tectorin peptides and vice versa. Antibodies were also tested for tissue specificity by western blot using different tissue lysates (negative controls: heart, cortex; positive control: cochlea).

Immunolabeling of frozen sections
Cochleae were isolated from mice and fixed in 4% paraformaldehyde at 4°C overnight before cryoprotection in a sucrose gradient and embedding in OCT for frozen sectioning. Serial sections (10-12 μm) were then blocked for 1 hour in normal serum at room temperature and incubated with the primary antibody in phosphate buffered saline containing 0.1% Triton-X100 (PBST) overnight at 4°C in a humidified chamber. Sections were washed three times with PBST and then incubated with the secondary antibody at room temperature for 1 hour. The primary antibodies were rabbit anti α-tectorin (1:1000) (Winter et al., 2009), rabbit anti β-tectorin (1:1000) (Winter et al., 2009), rabbit anti otogelin (1:2000; provided by Christine Petit, France) (Cohen-Salmon et al., 1997), and rabbit anti-myosin VIIa (1:200; Affinity Bioreagents, Golden, CO). The secondary antibodies were Alexa Fluor 488 or Alexa Fluor 594 donkey anti-rabbit (1:500; Invitrogen). After washing with PBST again, the sections were embedded with antifade fluorescence mounting medium and the coverslips sealed with nail polish (Biomedia gel/mount, Foster City, CA). Images were acquired using an epi-fluorescence microscope (Axioplan 2, Zeiss, Germany). All presented images were taken ~0.75 turns up from the round window.

Plastic embedding for light and transmission electron microscopy
Anesthetized mice at P30 underwent cardiac perfusion with fixative (2.5% glutaraldehyde in 150 mM sodium cacodylate buffer, pH 7.2). The cochleae were further dissected and fixed by perilymphatic perfusion with fixative, followed by immersion in the same fixative for 2 hours at room temperature. Following three washes in 150 mM sodium cacodylate buffer, the cochleae were post-fixed with 1% osmium tetroxide in 150 mM sodium cacodylate buffer for 1 hour and the washed three times in 150 mM sodium cacodylate buffer. The cochleae were then decalcified in 500 mM EDTA for 3-5 days at 4°C. Following dehydration in a gradient ethanol series, the cochleae were embedded in Epon resin.

For light microscopy, 1 μm sections were cut and stained in 1% Toluidine Blue. Images were captured on an upright microscope using differential interference contrast optics (Axioplan 2). For transmission electron microscopy studies, 100 nm sections were cut, counterstained, and then viewed on a microscope (H-7500, Hitachi, Japan). Images were captured using a charge-coupled device (CCD) digital camera (Gatan, Pleasanton, CA) at a resolution of 2048×2048 pixels. All presented images were taken ~0.75 turns up from the round window.

Whole-mount cochlear epithelium preparations
Cochleae were isolated from mice at P32 and fixed in 4% paraformaldehyde at room temperature for 1 hour. The otic capsule was micro-dissected to reveal the organ of Corti and the cochleae were rinsed three times with PBST (10 minutes each time). Immunolabeling was performed by first blocking the cochleae with 4% donkey serum (017-000-121, Jackson ImmunoResearch Laboratories, West Grove, PA) in PBST for 1 hour at room temperature, before incubating with the primary antibody overnight at 4°C. The cochleae were washed four times with PBST, incubated with the secondary antibody at room temperature for 1 hour, and rinsed with PBS before imaging. The primary antibody was goat anti-prestin N-20 (1:200; SC-22692, Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibody was Texas Red donkey anti-goat (1:200; 705-076-1470, Jackson ImmunoResearch), both diluted in PBST. Stereocilia were labeled for 1 hour at room temperature by immersing the cochleae in Alexa Fluor 546-phalloidin (1:200; A22283, Invitrogen) diluted in PBS. The cochleae were rinsed with PBS before imaging.

After labeling, the cochleae were glued upright in a chamber and most were imaged using a custom-built upright microscope. The core of the microscope consisted of a movable objective microscope (MOM) (Sutter) fitted with a 20× objective (NA0.95, XLUMPlanFl, Olympus America, Center Valley, PA). We used a femtosecond Ti:sapphire laser (Chameleon, Coherent, Santa Clara, CA) to provide two-photon excitation, and fluorescence was detected by a photomultiplier tube after appropriate optical filtering. Lateral scanning of the laser beam was obtained by two galvanometer-actuated mirrors, and axial scanning was controlled by a separate actuator moving the objective lens. All of the hardware was controlled by ScanImage open-source software (Pologruto et al., 2003), which was modified for each experiment. For quantification of prestin, the immunofluorescence intensity along the lateral wall of the OHCs was quantified from images collected on a confocal system (LSM 510, Zeiss). All presented images were taken ~0.75 turns up from the cochlear round window.

Calcium imaging
Cochleae were harvested from mice age P21-P28 and studies were performed in a standard extracellular solution, similar in
composition to perilymph with the exception of an elevated calcium level. It contained, in mM: 142 NaCl, 4 KCl, 4 CaCl$_2$, 10 HEPES, 10 glucose, and had a pH of 7.3 and osmolality of 305 mOsm/kg. The extracellular solution was continuously bubbled with oxygen throughout all experimental procedures. The cochlea were glued upright in a chamber, and the otic capsule bone and Reissner’s membrane dissected open to reveal the organ of Corti, ~1.25 turns up from the round window. Oregon Green BAPTA-1 AM (O-6807; Invitrogen), at a concentration of 10 μM, diluted in extracellular solution was applied for 45 minutes. Excess dye was rinsed away and the cochlear epithelium was imaged using our custom-built upright microscope. Sequential images of the epithelium were saved every 500 milliseconds (ms) while the stapes was stimulated at acoustic frequencies using a piezoelectric probe. A piezoelectric probe mechanically drove the stapes at 8 kHz at an intensity approximating 80 dB SPL, as described previously (Xia et al., 2007).

Fluorescence images were collected and stored for off-line analyses. Image J (NIH) was used to quantify pixel intensity within regions of interest. Matlab (version 13, Mathworks) was used to fit photobleaching background curves with a single exponential, and regions of interest. Matlab (version 13, Mathworks) was used to fit magnitude of the response at 6 kHz was determined by FFT. However, the bioamplifier system does not pass DC signals (high-pass corner frequency of ~20 Hz) and it has a flat frequency response until its innate low-pass corner frequency of ~22 kHz. Thus, in order to describe the asymmetry of the CM (Fig. 6A, left), we only analyzed the response at the onset of the stimulus, before the asymmetry became filtered out.

The stimulus was a 30 ms 6-kHz tone, repeated every second, with an intensity range from 10-100 dB SPL. By measuring the speaker output with the probe tip microphone in the ear bar, fast Fourier transform (FFT) analysis demonstrated that all stimulus harmonics and noise at all other frequencies were at least 50 dB below the primary signal at all stimulus intensities. The CM signal measured by the bioamplifier was digitized at 200 kHz and the magnitude of the response at 6 kHz was determined by FFT.

In vivo experiments

Procedures performed on anesthetized mice included the measurement of the CM, auditory brainstem responses (ABR), distortion product otoacoustic emissions (DPOAEs), and electrically evoked otoacoustic emissions (EEOAEs). Mice of either sex were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg). Supplemental doses of anesthesia were administered to maintain areflexia to paw pinch.

Sine wave stimuli were generated digitally using Matlab (Release 13, The Mathworks, Natick, MA), converted to analog signals using a digital-to-analog converter running at 200 kHz, and then attenuated to the appropriate intensity according to our experimental design (RP2 and PA5, Tucker-Davis Technologies, Alachua, FL) (Oghalai, 2004). To generate the acoustic stimuli, two different speaker systems were used: high-frequency piezoelectric speakers for the DPOAE measurements (EC1, Tucker-Davis Technologies) and a super tweeter (Radio Shack) for the ABR and CM measurements. The speakers were connected to an ear bar inserted into the ear canal and calibrated from 4 to 95 kHz by a probe-tip microphone (type 8192, NEXUS conditioning amplifier, Bruel and Kjar, Denmark) inserted through the ear bar. The tip of the microphone was within 3 mm of the tympanic membrane.

Cochlear microphonic (CM) measurements

The CM is a field potential that reflects the summation of hair cell transduction currents, primarily from OHCs, at the basal turn of the cochlea (Dallos, 1975; Cheatham and Dallos, 1982; Patuzzi et al., 1989; Cheatham and Dallos, 1997; Patuzzi and Moleirinho, 1998). After rigidly securing the mouse in a head holder, the pinna was surgically resected. The bulla was carefully opened medial to the tympanic annulus to expose the round window. The stapled artery was preserved. The ear bar was then inserted into the ear canal and secured. The CM was measured from the ball-ended tip of a Teflon-coated silver wire (0.003 inch diameter, A-M Systems, Carlsborg, WA) advanced onto the round window membrane with a micromanipulator. The signal was referenced to a silver wire inserted under the skin near the vertex of the skull. The ground electrode was placed in the hind leg. A bioamplifier was used (DB4, Tucker Davis Technologies) to amplify the signals 100 times and no filtering was used. However, the bioamplifier system does not pass DC signals (high-pass corner frequency of ~20 Hz) and it has a flat frequency response until its innate low-pass corner frequency of ~22 kHz. Thus, in order to describe the asymmetry of the CM (Fig. 6A, left), we only analyzed the response at the onset of the stimulus, before the asymmetry became filtered out.

The stimulus was a 30 ms 6-kHz tone, repeated every second, with an intensity range from 10-100 dB SPL. By measuring the speaker output with the probe tip microphone in the ear bar, fast Fourier transform (FFT) analysis demonstrated that all stimulus harmonics and noise at all other frequencies were at least 50 dB below the primary signal at all stimulus intensities. The CM signal measured by the bioamplifier was digitized at 200 kHz and the magnitude of the response at 6 kHz was determined by FFT.

Auditory brainstem response (ABR) measurements

The ABR signal was measured with a bioamplifier (DB4, Tucker Davis Technologies) from a needle electrode positioned at the ventral surface of the tympanic bulla referenced to an electrode placed at the vertex of the skull, as described previously (Wenzel et al., 2007a; Wenzel et al., 2007b). A ground electrode was placed in the hind leg. The stimulus was a 5-ms sine wave tone pip of alternating polarity, with cos$^2$ envelope rise and fall times of 0.5 ms and a repetition time of 50 ms. The stimulus intensity ranged from 10 to 90 dB SPL in 10 dB steps. The frequency range studied was 4–90 kHz. Two hundred and fifty ABR responses were sampled at 25 kHz over the 50-ms repetition time and averaged. Thresholds were calculated off-line. At each frequency, the peak-to-peak voltage of the ABR waveform was measured and the data interpolated over the range of stimulus intensities. The ABR threshold was determined at four standard deviations above the noise floor. If no ABR response was detected, even at our equipment limits of 90 dB SPL, we arbitrarily defined the threshold to be 90 dB SPL.

Distortion product otoacoustic emission (DPOAE) measurements

DPOAE thresholds were measured, as described previously (Xia et al., 2007). Briefly, the stimuli for eliciting DPOAEs were two sine wave tones of differing frequencies (F2=1.2*F1) of 1-second duration, with F2 ranging from 4 to 90 kHz. The two tones were presented at identical intensities, which ranged from 20 to 80 dB SPL in 10 dB increments. The acoustic signal detected by the microphone in the ear bar was digitized at 200 kHz and the magnitude of the 2F1-F2 distortion product determined by FFT. The surrounding noise floor was also calculated by averaging 20 adjacent frequency bins around the distortion product frequency. DPOAE thresholds were calculated off-line by interpolating the data and identifying when the signal was greater than ~5 dB SPL and greater than two standard deviations above the noise floor. If no DPOAE response was detected, even at our equipment limits of 80 dB SPL, we arbitrarily defined the threshold to be 80 dB SPL.
**Electrarily evoked otoacoustic emissions (EOOAE)**

We studied Tecta^C1509G/+ mice that were bred into the prestin null background (generously provided by Jian Zuo) (Zheng et al., 2000). Double-heterozygous offspring were then bred for another generation and we studied the following six genotypes – prestin wild-type: Tecta wild type, heterozygote and homozygote; and prestin homozygous (null): Tecta wild-type, heterozygote and homozygote. We did not study mice with one copy of the prestin gene. EOOAEs were assessed by passing an alternating current through the cochlea, which generates a voltage drop across all OHCs and causes them to produce force. The resultant movement of the cochlear partition produces a sound emission that can be measured by the microphone in the ear (Ren and Nuttall, 2000; Reyes et al., 2001). Full details of our EEOAE protocol have been published previously (Xia et al., 2007).

**Western blotting**

For each experiment, fresh cochleae were dissected from two animals of each Tecta genotype (a total of four cochleae per genotype) in cold Hanks’ balanced salt solution (HBSS). The cochleae were homogenized in ice-cold lysis buffer containing protease inhibitors (Tris-HCl 50 mM, EDTA 2 mM, phenylmethylsulfonyl fluoride 1 mM and leupeptin 1 µg/ml) and placed on ice for 5 minutes to allow the bony capsule pieces to fall to the bottom of tube. The supernatants were transferred to a new tube and centrifuged at 20,000 x g at 4°C for 30 minutes. The new supernatants were removed, the pellet was solubilized in SDS-PAGE sample buffer at 100°C for 5 minutes, and the samples were then run on 7.5% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Blocking was performed with 4% donkey or goat serum, 5% milk (made from 4% non-fat powdered milk dissolved in PBS) and 0.1% Tween 20 in PBS. The membrane then was probed with anti-prestin (1:200, N-20, Santa Cruz) or anti-myosin VIIa (1:200; Affinity Bioreagents, Golden, CO) as the primary antibody, and an HRP-conjugated donkey anti-goat or an HRP-conjugated goat anti-rabbit (1:2000, Vector Labs, Burlingame, CA) antibody, respectively, as the secondary antibody. The membrane was washed and incubated with an appropriate enhanced chemiluminescence (ECL) (Pierce) substrate. Signals were detected by film imaging.

**Quantitative RT-PCR**

Total RNA was extracted from the cochleae of P21 wild-type, heterozygous and homozygous mutant mice using TRIzol reagent (Invitrogen). Samples were treated with DNaseI and purified using the RNeasy mini kit, according to the manufacturer’s protocol (Qiagen, Valencia, CA). cDNA was synthesized from 1 µg of RNA using the RT^2 First Strand Kit (SuperArray Bioscience Corporation, Frederick, MD). Quantitative real-time PCR reactions were performed on 10 ng of cDNA using the TaqMan master mix and plotted with SigmaPlot (11.0, Systat Software, San Jose, CA). Statistical analysis was assessed by the Student’s non-paired or paired two-tailed t-test, as needed (for three or more groups), or the Student’s non-paired or paired two-tailed t-test (for two groups). P values <0.05 were considered statistically significant. All values presented are mean±standard error of the mean (S.E.M.).

**TRANSLATIONAL IMPACT**

**Clinical issue**

Deafness, the most common sensory disorder, often strikes in childhood. In many cases, a child initially identified with partial deafness develops profound hearing loss over a period of months to years, often because of a gradual degeneration of the outer hair cells, which are required to amplify sound vibrations, and are also responsible for the exquisite sensitivity and frequency selectivity of mammalian hearing. Over half the cases of childhood deafness are due to single-gene defects, but the specific mechanisms by which many of these mutations cause progressive sensorineural hearing loss are not clearly defined. The ease of genetic manipulation in mice enables the creation of mutant alleles and the detailed dissection of the degenerative process.

**Results**

This manuscript uses a mouse model to study the pathophysiological mechanisms underlying deafness caused by an autosomal dominant mutation in the alpha tectorin gene (Tecta C1509G). Tecta protein localizes to the tectorial membrane, an acellular gelatinous structure that interacts with the sensory hair cell stereociliary bundles and transduces sound within the mammalian cochlea. Children with the TECTA C1509G mutation are born with partial hearing loss, which progressively worsens with age.

Tecta C1509G mice have hearing loss because the tectorial membrane is congenitally malformed, such that sound stimulates only the first row of outer hair cells, instead of all three rows. Thus, forward transduction (the process of converting sound pressure waves into voltage signals) is defective. Outer hair cells are also crucially important for amplifying and sharpening the tuning of the traveling soundwave, by a process termed reverse transduction. Surprisingly, reverse transduction was increased in Tecta C1509G mice, owing to upregulation of the outer hair cell electromotility motor protein prestin. Since Tecta is not expressed within hair cells, a non-cell-autonomous mechanism is responsible, which partially compensates for the hearing loss caused by defective forward transduction.

**Implications and future directions**

The Tecta C1509G mouse provides a unique opportunity to study how the targeted loss of outer hair cell stimulation alters cochlear physiology and cellular gene expression. Intriguingly, the increase in reverse transduction might also cause the progressive hearing loss associated with this mutation, perhaps by putting the outer hair cells at greater risk of damage from noise exposure or aging.

The findings from this study have general applicability to all the many forms of human hearing loss caused by deficient outer hair cell stimulation. Tecta C1509G mice may also be of use as a model system for the development of therapies to slow the rate of hearing loss. Such a therapy would be of great importance, allowing children with progressive hearing loss to lengthen their window of hearing while they are in the crucial process of learning speech and language, and reducing a major cause of societal isolation and depression in the elderly.

**Statistical analysis**

Data were analyzed with Microsoft Excel (Microsoft Office 2003) and plotted with SigmaPlot (11.0, Systat Software, San Jose, CA). Statistical significance was assessed using the one-way ANOVA followed by the non-paired Student’s t-test, as needed (for three or more groups), or the Student’s non-paired or paired two-tailed t-test (for two groups). P values <0.05 were considered statistically significant. All values presented are mean±standard error of the mean (S.E.M.).

**CTTGGT-3’; Myo7a probe: 6FAM-CAAACCTCAAGAG-AGG-BHQ1.**
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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS


SUPPLEMENTARY MATERIAL

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Deficient forward transduction and enhanced reverse transduction


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