Cellular/Molecular

Deafness in TRβ Mutants Is Caused by Malformation of the Tectorial Membrane

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Thyroid hormone receptor β (TRβ) dysfunction leads to deafness in humans and mice. Deafness in $TR\beta^{-/-}$ mutant mice has been attributed to TRβ-mediated control of voltage- and Ca^{2+}-activated K^+ (BK) channel expression in inner hair cells (IHCs). However, normal hearing in young constitutive $BK\alpha^{-/-}$ mutants contradicts this hypothesis. Here, we show that mice with hair cell-specific deletion of TRβ after postnatal day 11 (P11) have a delay in BKα expression but normal hearing, indicating that the origin of hearing loss in $TR\beta^{-/-}$ mutant mice manifested before P11. Analyzing the phenotype of IHCs in constitutive $TR\beta^{-/-}$ mice, we found normal Ca^{2+} current amplitudes, exocytosis, and shape of compound action potential waveforms. In contrast, reduced distortion product otoacoustic emissions and cochlear microphonics associated with an abnormal structure of the tectorial membrane and enhanced tectorin levels suggest that disturbed mechanical performance is the primary cause of deafness resulting from TRβ deficiency.

Key words: hearing; TRβ; BK; conditional knock-out mice; tectorin; exocytosis

Introduction

Thyroid hormone (TH) and its receptors are essential for the development of hearing as congenital thyroid disorders or iodine deficiency can cause deafness (Forrest, 1996). TH receptors (TR) are ligand-dependent transcription factors encoded by the related TRα and TRβ genes (Sap et al., 1986; Weinberger et al., 1986). TRα1 and TRβ are expressed during embryonic and postnatal development of the cochlea (Bradley et al., 1994), indicating that the cochlea is a direct site of T3 action (Knipper et al., 2001).

Genetic ablation of TRβ in mice severely impairs auditory-evoked brainstem responses (Forrest et al., 1996), whereas mice with TRα deletion exhibit normal hearing (Ng et al., 2001). Moreover, the resistance to thyroid hormone (RTH) syndrome in humans is associated with TRβ mutations and can present with mild to profound hearing impairment (for review, see Weiss and Refetoff, 2000; Refetoff and Dumitrescu, 2007).

Since cochlear malformations were not reported in constitu-

tive $TR\beta^{-/-}$ mice (Forrest et al., 1996), their deafness was attributed to impaired functional rather than morphological maturation (Rüsch et al., 1998). A severe retardation of the expression of the inner hair cell (IHC) fast-activating potassium current termed $I_{K,f}$ (Dulon et al., 1995; Kros et al., 1998) was observed in $TR\beta^{-/-}$ mice (Rüsch et al., 1998). Consequently, inappropriate maturation of auditory brainstem neurons that extract timing information was suggested as a possible reason for deafness (Rüsch et al., 1998). In contrast to the suggested necessity of BK channels during IHC development (Kros et al., 1998), germline deletion of BKα, the pore-forming subunit (KCNMA1) of the BK channel, resulted in normal hearing (Rüttiger et al., 2004; Pyott et al., 2007).

We compared the cochlear and hearing phenotype of TRβ mutants with either germline deletion ($TR\beta^{-/-}$) or postnatal deletion of TRβ restricted to hair cells ($HC-TR\beta^{-/-}$). Analyses of both mouse lines exclude that delayed $I_{K,f}$ expression or retro-cochlear defects cause hearing loss in $TR\beta^{-/-}$ mice. Instead, our results suggest that malformation of the tectorial membrane (TM) results in reduced mechanical performance of the cochlea and deafness.

Materials and Methods

Generation of hair cell-specific TRβ-deficient mice. The $TR\beta^{lox}$ (TRβ L2) allele was constructed by homologous recombination, introducing two loxP sites 5′ and 3′ to exon 5. A FRTNeoFRT cassette was also introduced downstream of the 3′ LoxP sequence for *in vitro* drug selection and later eliminated by FLP (flippase)-mediated recombination, leaving only one FRT (flippase recombination target) sequence. We verified by PCR and

Southern blotting that Cre (cyclization recombination) recombinase-mediated recombination results in the deletion of exon 5, both in embryonic stem cells and in mice. Exon 5 deletion introduces a frame shift, so that the deleted locus has the potential to encode only truncated products, whose homology extend to the DNA binding domain (amino acid 178 of TRβ1). TRβ L2/L2 mice (mixed C57BL/6J/129/SV background) were crossed with constitutive TRβ$^{-/-}$ (C57BL/6J/SV129 background) (Forrest et al., 1996) mice to generate TRβ L2/-mutants. TRβ$^{-/-}$ mice were crossed with Prestin-Cre transgenic mice, termed PCre (SV129/FVB background) (Tian et al., 2004) to obtain heterozygous Prestin-Cre transgenic TRβ$^{+/-}$ mice (TRβ$^{+/-}$PCre). TRβ L2/-mutants were crossed with TRβ$^{+/-}$PCre mice to generate TRβ L2/+, used as controls (HC-TRβ-Ctr), TRβ$^{-/-}$, used as constitutive knock-out controls, and Prestin-Cre transgenic TRβ L2/-mutants, displaying hair cell-specific deletion of TRβ (HC-TRβ$^{-/-}$). Offspring was genotyped by RT-PCR, and animals of respective genotypes were repetitively tested for their hearing thresholds at the age of 1–4 months as indicated. As control animals for the TRβ$^{-/-}$ mice we used either wild-type (TRβ$^{+/+}$), heterozygous TRβ$^{+/-}$ (Forrest et al., 1996), or TRβ$^{+/-}$TR α1$^{+/-}$ mice (Rusch et al., 2001), all termed TRβ-Ctr.

Care and use of the animals and the experimental protocol were reviewed and approved by the animal welfare commissioner and the regional board for scientific animal experiments in Tübingen.

Immunohistochemistry and histological staining. Procedure of standard immunohistochemistry is described by Knipper et al. (2000). For anatomical studies cochleae were isolated, fixed in paraformaldehyde, decalcified in EDTA and postfixed in osmium tetroxide. After dehydration in solutions of ethanol at increasing concentrations cochleae were embedded in epoxy resin. Serial sections of 10 μm were counterstained with toluidine blue. Thickness (height), width, and cross-sectional area of the tectorial membrane were measured according to (Keiler and Richter, 2001). Measurements were done by using the software Cell^F from Soft-Imaging Systems (Olympus) and compared by paired two sample Student's *t* test.

Antibodies. The α-tectorin and β-tectorin antibodies were generated by immunizing one rabbit in each case (Charles River) with synthetic peptides corresponding to conserved regions of mouse α- and β-tectorin. For further information, see supplemental material, available at www.jneurosci.org. In addition, we used rabbit polyclonal anti-BKα (Alomone Laboratories) and rabbit polyclonal anti-KCNQ4 (Rüttiger et al., 2004; Winter et al., 2006).

Electrophysiological recordings. Whole-cell voltage-activated K$^+$ currents and voltage-activated capacitance changes were recorded in IHCs using acute explants of the organ of Corti (apical turn) as described previously (Brandt et al., 2007).

Hearing measurements. All physiological recordings were performed under anesthesia in a soundproof chamber (IAC) as described previously (Müller et al., 1997; Rüttiger et al., 2004). For further information, see supplemental material, available at www.jneurosci.org.

Western blotting. Western blot analysis of cochlear tissue of TRβ-Ctr and TRβ$^{-/-}$ mice was performed as described previously (Schug et al., 2006). For quantification of α-tectorin (Santa Cruz, s.c.-18035, 1:400) and β-tectorin (see above, 1:3000) levels in TRβ$^{-/-}$ mice, the α- respectively the β-tectorin Western blot was performed simultaneously with the ezrin antibody (Dianova, 1:750). Densitometric analysis was performed using the Soft Imaging System Cell^F from Olympus. The intensities were normalized to the level of the codetected ezrin as an internal control (Knipper et al., 2001). Control and knock-out mice were compared and data expressed as mean percentage of control (set at 100%) ± SD. Western blots were performed on three different sets of animals with one repetition.

Figure 1. *A*, *B*, ABR measurements in HC-TRβ-Ctr, HC-TRβ$^{-/-}$, and TRβ$^{-/-}$ mice. At 3–4 months, TRβ$^{-/-}$ mice show a significant elevation of click-evoked ABR thresholds (*A*). Frequency-specific ABR thresholds of HC-TRβ$^{-/-}$ mice show a significant threshold elevation at frequencies <8 kHz (*B*). *C–F*, BKα expression (red, arrowheads) in HC-TRβ-Ctr and HC-TRβ$^{-/-}$ mice at indicated ages. Note lack of BKα expression in IHCs and OHCs of HC-TRβ$^{-/-}$ mice at P15. Nuclei are stained in blue with DAPI. Scale bars, 20 μm. *G–J*, Typical IHC voltage-activated K$^+$ currents in HC-TRβ-Ctr mice at P15 (*G*) and P18 (*I*) compared with IHCs of HC-TRβ$^{-/-}$ mice (*H*, *J*).

Results

Hair cell-specific TRβ-deficient mice (HC-TRβ$^{-/-}$) have normal hearing despite retarded expression of BK channels

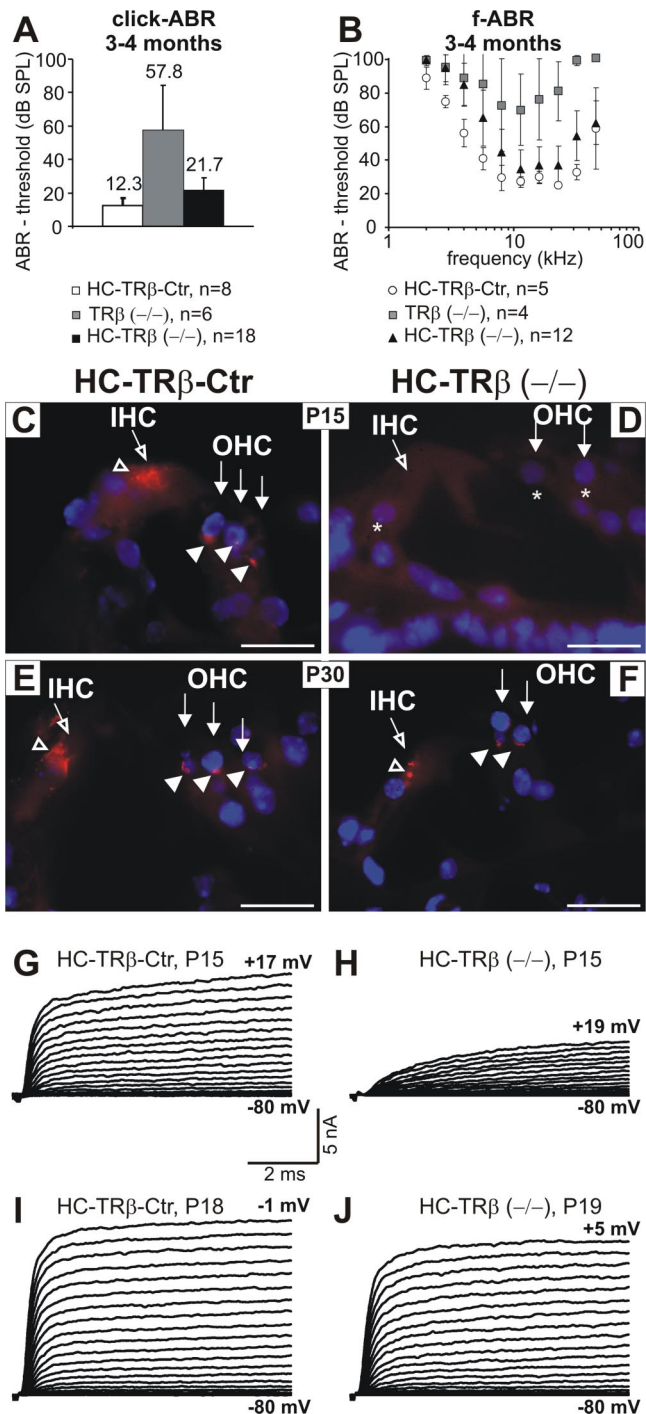First, we aimed to define the time period when deafness manifests in TRβ mutants by comparing hearing function and physiology of conditional and constitutive TRβ knock-out mice (Fig. 1). The Prestin-Cre transgenic mouse strain (Tian et al., 2004) was used to generate TRβ mutant mice carrying a hair cell-specific gene deletion (see Materials and Methods). Onset of Cre activity was demonstrated by LacZ expression after Cre-mediated recombination using X-gal staining. For
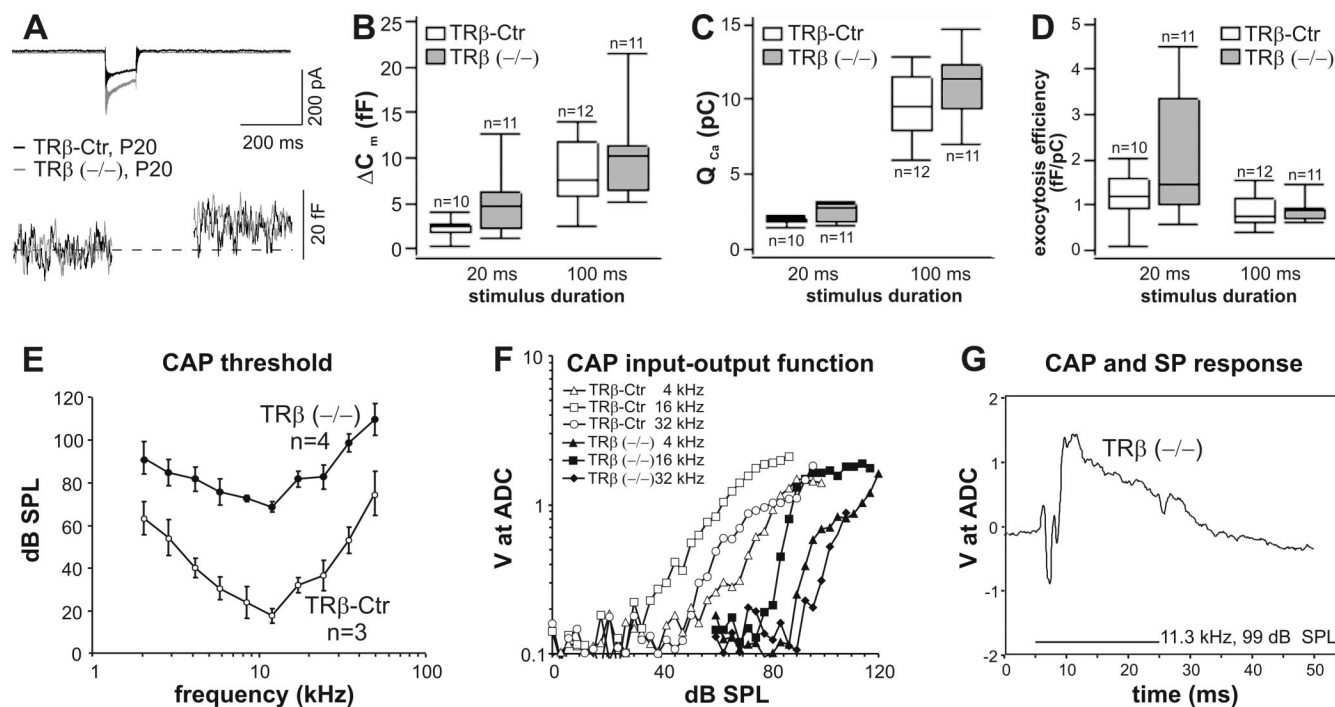
Figure 2. Exocytosis in IHCs (**A–D**) and CAP responses (**E–G**). **A**, Ca²⁺ currents of a P20 TRβ-Ctr (black trace) and a P20 TRβ⁻/⁻ IHC (gray trace) evoked by stepping from −80 to 0 mV for 100 ms (top) and capacitance increases indicating exocytosis (bottom). **B–D**, Box-and-whisker plots of capacitance change ΔC_m (**B**), Ca²⁺ charge (**C**) and resulting exocytosis efficiency (**D**). Horizontal lines at the median (bold), 25 and 75% quartiles (box), and extreme values (whiskers) for TRβ-Ctr and TRβ⁻/⁻ IHCs aged P17–P20. **E**, CAP threshold elevation was evident in the whole frequency range, especially in the frequency range of normal best sensitivity. **F**, CAP input–output functions in TRβ⁻/⁻ mice were shifted to higher SPLs and had steeper slopes than those for TRβ-Ctr mice; maximum CAP amplitudes were similar. **G**, Time course of CAP and SP responses in TRβ⁻/⁻ mice; the stimulus is indicated by the line above the time axis.

further information, see supplemental material and supplemental Figure 1, available at www.jneurosci.org as supplemental material. Auditory brainstem response (ABR) thresholds for click auditory stimuli (Fig. 1A) confirmed elevated hearing thresholds (Forrest et al., 1996) in 3- to 4-month-old TRβ⁻/⁻ mice [57.8 ± 26.6 dB sound pressure level (SPL), n = 6], whereas hair cell-specific HC-TRβ⁻/⁻ mice had hearing thresholds of 21.7 ± 11.2 dB SPL (n = 18) that were only slightly different from that of HC-TRβ-Ctr animals (12.3 ± 4.5 dB SPL, n = 8). Frequency-dependent ABR measurements (Fig. 1B) revealed a severe threshold shift in HC-TRβ⁻/⁻ mice, whereas hair cell-specific TRβ deletion in IHCs and outer hair cells (OHCs) from postnatal day 12 (P12) onwards may have affected hair cells only in apical cochlear turns (Fig. 1B). Nevertheless, similar to previous studies, BKα expression was delayed in IHCs of HC-TRβ⁻/⁻ mice (Fig. 1C–J). At P15, when BKα was expressed in IHCs and OHCs of HC-TRβ-Ctr mice (Fig. 1C), age-matched HC-TRβ⁻/⁻ mice did not show any BKα protein in IHCs and OHCs (Fig. 1D). At P30, however, there was no difference in the BKα expression of HC-TRβ-Ctr (Fig. 1E) and HC-TRβ⁻/⁻ mice (Fig. 1F). Recording voltage-activated K⁺ currents in IHCs from hearing onset onwards revealed a fast activating K⁺ current component in HC-TRβ-Ctr at P15 (Fig. 1G) indicative of BKα expression (Kros et al., 1998; Brandt et al., 2007), which was lacking in IHCs of HC-TRβ⁻/⁻ mice (Fig. 1H). Four days later, IHC K⁺ currents of a P19 HC-TRβ⁻/⁻ mouse (Fig. 1J) showed increased current amplitudes and accelerated activation kinetics that were comparable with those of the P18 control IHC (Fig. 1I). These findings indicate that the deafness of TRβ⁻/⁻ mutants is not caused by the lack of TRβ function in hair cells after the onset of hearing or by a TRβ-induced delay of BK

channel expression. Therefore, we analyzed the physiology of IHCs of constitutive TRβ⁻/⁻ mice in more detail.

To test if exocytosis is functional in TRβ⁻/⁻ IHCs, we performed capacitance measurements. Capacitance changes (ΔC_m) were recorded in response to voltage steps of 20 and 100 ms duration from the holding potential (−80 mV) to the potential of maximal Ca²⁺ influx (0 mV) for P20 TRβ-Ctr and TRβ⁻/⁻ mice (Fig. 2A–D). Figure 2A shows example current traces in response to a depolarizing step of 100 ms duration (top) and the corresponding capacitance changes (bottom) for TRβ-Ctr and TRβ⁻/⁻ mice. Average capacitance changes shown in a box-and-whisker plot (Fig. 2B) did not reveal significant differences of ΔC_m between IHCs of TRβ-Ctr and TRβ⁻/⁻ mice for both stimulus durations ($p > 0.05$) despite the larger variability of ΔC_m in TRβ⁻/⁻ mice. Neither the corresponding Ca²⁺ charge (Q_{Ca}) entering the cell during depolarization nor the efficiency of exocytosis, the ratio between ΔC_m and Q_{Ca}, showed significant differences (Fig. 2C,D) in TRβ⁻/⁻ IHCs compared with TRβ-Ctr IHCs. IHCs typically show an increase in exocytosis efficiency by the end of the third postnatal week (Beutner and Moser, 2001; Johnson et al., 2005), a developmental process controlled by TH (Brandt et al., 2007). Our results indicate that TH-dependent maturation of exocytosis is not mediated by the TRβ receptor (Fig. 2D).

To distinguish if deafness after deletion of TRβ is of cochlear and/or retrocochlear origin, we measured compound action potentials (CAP). Similar to ABR thresholds (Fig. 1) and findings of Forrest et al. (1996), CAP thresholds were clearly elevated in 3- to 4-month-old TRβ⁻/⁻ mice at all frequencies tested (Fig. 2E). TRβ-Ctr mice exhibited best thresholds of ~20 dB SPL at 11.3 kHz, whereas in age-matched TRβ⁻/⁻ mice, best thresholds of 70 dB SPL were found at 11.3
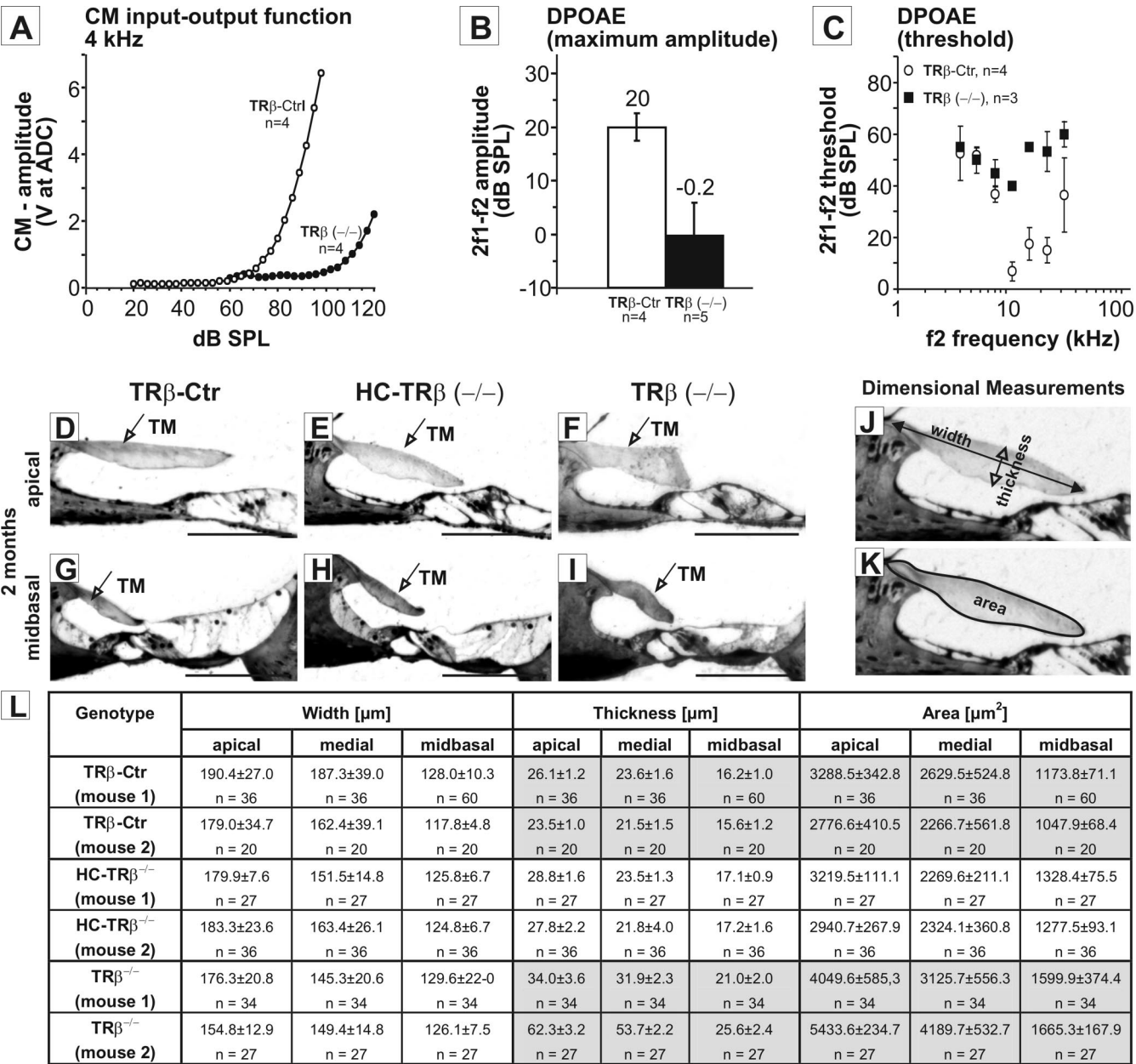
Figure 3. *A–C*, CMs and DPOAEs of TRβ-Ctr and TRβ $^{-/-}$ mice. CM are present in both TRβ-Ctr and TRβ $^{-/-}$ mice, although reduced in TRβ $^{-/-}$ mice (*A*). DPOAE amplitudes (*B*) and DPOAE thresholds (*C*) reveal a loss of active cochlear mechanics in TRβ $^{-/-}$ mice at frequencies above f1 = 8 kHz. *D–I*, TM structure of TRβ-Ctr, HC-TRβ $^{-/-}$, and TRβ $^{-/-}$ mice. Cross-sections of epoxy resin-embedded cochleae reveal morphological abnormalities of the TM in TRβ $^{-/-}$ mice (*F, I*) compared with TRβ-Ctr (*D, G*) and HC-TRβ $^{-/-}$ mice (*E, H*). Scale bars, 100 μm. *J–L*, Dimensional measurements (width, thickness, area) of the TM.

kHz (Fig. 2*E*). Input–output functions of the CAP responses were shifted to higher SPLs and tended to have steeper slopes than those from TRβ-Ctr mice, yet maximum amplitudes were similar (Fig. 2*F*). Despite a 50 dB elevation of threshold SPL in TRβ $^{-/-}$ mice, the waveform of the CAP and summating potential (SP) signal recorded at the round window for 11.3 kHz tone bursts were normal (Zidanic and Brownell, 1992) (Fig. 2*G*), indicating that action potentials were generated in the auditory nerve and transmitted to the brainstem. However, the increased slopes of the CAP input–output functions in TRβ $^{-/-}$ mice (Fig. 2*F*) point to recruitment and dysfunction of cochlear amplification.

Deafness of TRβ $^{-/-}$ mice is correlated with severe reduction in mechanical performance

To test outer hair cell performance, cochlear microphonics (CM) were analyzed from CM input–output functions in 2-month-old TRβ-Ctr and TRβ $^{-/-}$ mice. A severe reduction of CM was noted in TRβ $^{-/-}$ mice (Fig. 3*A*). CM input–output functions at 4 kHz indicated a threshold elevation from 60 to 90 dB SPL; at 100 dB SPL CM amplitudes were reduced by 20 dB (Fig. 3*A*). In addition, distortion product otoacoustic emission (DPOAE) thresholds, measured as a function of f2-frequency at various frequencies, were altered. In TRβ $^{-/-}$ mutants, no 2f1-f2 amplitudes baseline was detected using f2-frequency of 16 kHz

(Fig. 3B), and significantly elevated 2f1-f2 thresholds >8 kHz were observed (Fig. 3C), supporting the notion of impaired OHC function. This was surprising since TRβ mutation was hypothesized to affect inner rather than outer hair cell function as previous studies indicated normal endocochlear potentials, OHC mechanotransducer currents and nonlinear capacitances (Rüsch et al., 1998).

Because hypothyroidism has been shown to cause malformation of the TM (Uziel, 1986), we re-examined TM morphology using an epoxy resin embedding approach. Considerable structural abnormalities of the TM were observed in 2-month-old TRβ$^{-/-}$ mice (Fig. 3F,I) compared with TRβ-Ctr (Fig. 3D,G) and HC-TRβ$^{-/-}$ mice (Fig. 3E,H). Obvious thickening and enlargement of the TM (Fig. 3F,I,J–L, thickness, area, highlighted in gray) were verified by measuring 20–60 cross-sections of apical, medial, and midbasal cochlear turns from two TRβ-Ctr, HC-TRβ$^{-/-}$, and TRβ$^{-/-}$ mice, respectively. Both thickening and enlargement of the TM in TRβ$^{-/-}$ mice were most prominent in the apical turns. Furthermore, the enlarged TM showed an increased α- and β-tectorin immunolabeling. There was an obvious increase in α-tectorin (Fig. 4A–D) and β-tectorin immunoreactivity (Fig. 4E–H) along the entire tonotopic axis, which was found in 3 (of 3) different specimens of 4- to 5-month-old TRβ$^{-/-}$ mice. For quantification of α- and β-tectorin levels, Western blotting and densitometric analysis were performed using dissected cochlear tissue. The intensities of α- and β-tectorin were normalized to the level of the codetected house keeping protein ezrin (Fig. 4I,J), yielding an increase to 180 ± 27% for α-tectorin (Fig. 4I) and 147 ± 15% for β-tectorin (Fig. 4J) in TRβ$^{-/-}$ mice relative to TRβ-Ctr mice.

Discussion

In the present study, hair cell-specific deletion of the TRβ gene after P11 is shown to delay BK current expression in HC-TRβ$^{-/-}$ IHCs, similar as in constitutive TRβ$^{-/-}$ mutants (Rüsch et al., 1998). Nevertheless, HC-TRβ$^{-/-}$ mice developed normal hearing, indicating (1) a dispensable role of BK for basic hearing function, and (2) a negligible role of TRβ after onset of hearing. The finding that TRβ$^{-/-}$ IHCs show normal Ca^{2+} currents and normal maturation of exocytosis at P20 suggests that maturation of exocytosis efficiency, recently shown to require TH (Brandt et al., 2007), proceeds independently of TRβ. Indeed, evidence is increasing that in addition to TRβ, liganded TRα1 also plays a role for development of many organs (Flamant and Samarut, 2003; Quignodon et al., 2007) including the cochlea (Winter et al., 2006, 2007).

Normal shape of the CAP response waveform in TRβ$^{-/-}$ mice despite up to 50 dB threshold elevation implies normal exocytotic function of IHCs. The elevated thresholds most likely reflect a loss of sensitivity because of impaired mechanical performance of the organ of Corti. Normal exocytosis and shape of the CAP waveform show that action potentials are generated in the auditory nerve and transduced to the brainstem in TRβ$^{-/-}$ mice. However, we cannot exclude that lack of TRβ activity in central auditory nuclei before P11 contributes to hearing loss in TRβ$^{-/-}$ mice. Considering normal endocochlear potential and OHC nonlinear capacitance in TRβ$^{-/-}$ mutants (Rüsch et al., 1998), their reduced DPOAEs and CM shown here are expected to arise from improper mechanical excitation of OHCs. Malformation of the TM has been described in hypothyroidism (Deol, 1973; Uziel, 1986) and was observed in TRα1$^{-/-}$β$^{-/-}$ double mutants (Rusch et al., 2001). So far, abnormalities have not been

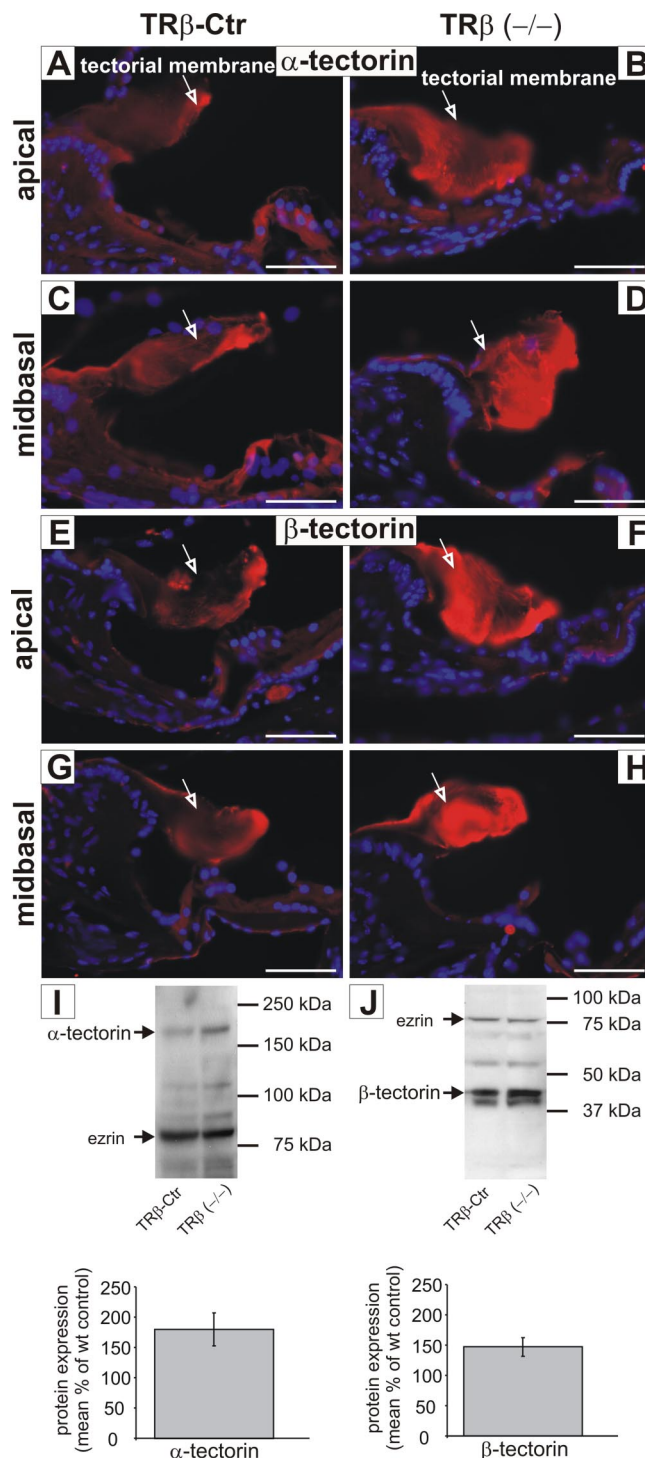**Figure 4.** α- and β-tectorin in TRβ-Ctr and TRβ$^{-/-}$ mice. **A–H**, Immunohistochemistry against α-tectorin (**A–D**, red) and β-tectorin (**E–H**, red) shows a more abundant expression in TRβ$^{-/-}$ compared with TRβ-Ctr mice. Nuclei are stained in blue with DAPI. Scale bars, 20 μm. **I, J**, Western blots and densitometric quantification of α-tectorin (**I**) and β-tectorin expression levels (**J**) in TRβ$^{-/-}$ mice compared with TRβ-Ctr animals. The house keeping protein ezrin was used for normalization. Data are expressed as mean percentage of control ± SD.

observed in light microscopic analyses of TRβ$^{-/-}$ animals (Forrest et al., 1996; Rusch et al., 2001) probably because specimens were analyzed at around P20.

In humans, a mild auditory phenotype is associated with heterozygosity for mutations in the THRB gene that reduce T3-

binding capacity and cause the autosomal dominant disorder RTH (Brucker-Davis et al., 1996). Analysis of a homologous knock-in mouse model for RTH that segregates the orthologue of a well characterized human RTH mutation revealed profound hearing impairment at all tested frequencies associated with normal structure of the basilar membrane and stria vascularis but severe abnormalities of the TM (Griffith et al., 2002). Similar to our present findings, Griffith et al. (2002) showed a significant thickening of the TM.

There is no doubt that an intact TM is required for proper deflection of OHC stereocilia (Gummer et al., 1996). Missense mutations of one of the major TM proteins, α-tectorin (Legan et al., 2000), leads to autosomal dominant nonsyndromic hearing impairment (Verhoeven et al., 1998; Mustapha et al., 1999). The mouse model Tecta$^{-/-}$ displays complete detachment of the TM and impaired hearing thresholds as OHCs cannot effectively respond to basilar membrane motion (Legan et al., 2000).

In TRβ$^{-/-}$ mutants, the detachment of the TM from the cells of the greater epithelial ridge (GER) is delayed for days, and in TRα1$^{-/-}$β$^{-/-}$ double mutants, it can be delayed for weeks (Rusch et al., 2001). In conclusion, we suggest that a delay in reshaping the GER in TRβ$^{-/-}$ mutants disturbs the correct timing of TM secretion (Rau et al., 1999) resulting in a likely prolonged secretion of tectorins and a pathological TM mass. According to recent findings for β-tectorin, changes in TM mass may directly alter cochlear microphonics (Russell et al., 2007).

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