Autonomous functions of murine thyroid hormone receptor TRα and TRβ in cochlear hair cells

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A B S T R A C T
Thyroid hormone acts on gene transcription by binding to its nuclear receptors TRα1 and TRβ1. Whereas global deletion of TRβ1 causes deafness, global TRα1-deficient mice have normal hearing thresholds. Since the individual roles of the two receptors in cochlear hair cells are still unclear, we generated mice with a hair cell-specific mutation of TRα1 or deletion of TRβ1 using the Cre-loxP system. Hair cell-specific TRβ1 mutant mice showed normal hearing thresholds but delayed BK channel expression in inner hair cells, slightly stronger outer hair cell function, and slightly reduced amplitudes of auditory brainstem responses. In contrast, hair cell-specific TRα1 mutant mice showed normal timing of BK channel expression, slightly reduced outer hair cell function, and slightly enhanced amplitudes of auditory brainstem responses. Our data demonstrate that TRβ1-related deafness originates outside of hair cells and that TRα1 and TRβ1 play opposing, non-redundant roles in hair cells. A role for thyroid hormone receptors in controlling key regulators that shape signal transduction during development is discussed. Thyroid hormone may act through different thyroid hormone receptor activities to permanently alter the sensitivity of auditory neurotransmission.

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1. Introduction

Thyroid hormone (TH) deficiency in congenital hypothyroidism leads to disturbance of the normal development of the organ of Corti and deafness (Deol, 1973; Knipper et al., 2001; Uziel, 1986). Indeed, any disturbance of TH homeostasis through hereditary congenital thyroid disorders or iodine deficiency can cause deafness (Forrest, 1996; Ritter, 1967). TH exerts its function by binding to thyroid hormone receptors (TRs), which are ligand-dependent transcription factors encoded by the related Thra and Thrb genes (Sap et al., 1986; Weinberger et al., 1986). It is generally assumed that TRα1 knockout mice have a milder phenotype than mice with a dominant negative TRα1 mutation (Bernal et al., 2003; Flamant et al., 2002; Hashimoto et al., 2001) because mutated TRα1 can still recruit co-repressors, while in a TRα1 knockout, TRβ1 may extend its function to other target genes, compensating for the loss of TRα1. Indeed, it has been a matter of debate to what extent TRα1 and TRβ1 exhibit specific functions that cannot be replaced by the other TH receptor isoform. In the inner ear, a local effect of TH on cochlear targets is likely, as the deletion of deiodinase 2 and the subsequent failure to convert cochlear thyroxine (T4) into active tri-iodothyronine (T3) lead to deafness (Ng et al., 2004). This makes it unlikely that deafness is due to indirect neuronal or morphological effects from other organs (reviewed in Forrest et al., 2002; Ng et al., 2013)). Both TH receptors, TRα1 and TRβ1, are expressed in hair cells during the critical developmental period before the onset of hearing function, although the peak of TRα1 precedes that of TRβ1 (Knipper et al., 1989; Lautermann and ten Cate, 1997; Ng et al., 2013). This critical period is when TH is essential for development of normal hearing (Knipper et al., 2001). Deafness in humans and mice related to TR dysfunction has been associated with a deficiency of TRβ (Forrest et al., 1996a;
Refetto et al., 1993), whereas TRα1 knockout mice do not show hearing loss (Forrest et al., 1996a, 1996b; Rüsch et al., 1998).

However, auditory defects have been reported recently in mice with a TRα1 point mutation, which were proposed to result from bone ossification defects of the ossicles (Cordas et al., 2012). An exacerbation of the cochlear phenotype in TRα1/TRβ double knockout mice has been reported (Rüsch et al., 2001). Ng and colleagues found that TRα1 overexpression improves hearing defects in TRβ1 knockout animals (Ng et al., 2001).

Therefore, TRα and TRβ functions might have merely redundant effects in the auditory system. However, we have described specific effects such as a lack of KCNQ4 expression in TRα1m™ mutant mice (Winter et al., 2006), in which a point mutation reduces the affinity of TRα1 for TH (Tinknikov et al., 2002). Furthermore, delayed prestin expression and immature prestin distribution was found in hypothyroid animals (Cimerman et al., 2013; Weber et al., 2002) as well as in TRβ1 knockout mice (Winter et al., 2006), suggesting that the two receptor functions do not fully overlap. Furthermore, deafness in TRβ1 knockout mice (Forrest et al., 1996a, 1996b), which do not express any functional TRβ protein, neither TRβ1 nor TRβ2, was proposed to result from the delayed expression of the large conductance voltage and Ca2+-activated potassium (BK) channel in cochlear inner hair cells (Rüsch et al., 1998), for which TRβ obviously cannot compensate. These results all point to individual functions of TRα and TRβ in the cochlea.

Previous studies showed that mice with hair cell-specific deletion of TRβ1 after postnatal day 11 (P11) have normal hearing (Winter et al., 2005). This indicates that the origin of hearing loss in TRβ1−/− mutant mice is manifested before P11 and that TRβ1 displays a negligible role in hair cells after onset of hearing. The role of TRβ1 deletion in hair cells before hearing onset for the development of hearing deficits is still elusive. To specifically address the question of specific roles for TRα and TRβ in hair cells and hearing, we generated hair cell-specific, tamoxifen-inducible TRα1- and TRβ1 mutant mice with inactivation of TRα1 and TRβ1 gene activity before hearing onset, respectively, before hearing onset. This was achieved by using the Cre-loxP system and a transgenic Math1-CreERTM mouse strain (Chow et al., 2006).

In these mice, the expression of Cre recombinase is under the control of the Math1 enhancer and recombinase activity is induced upon systemic tamoxifen administration (Chow et al., 2006). In the cochlea, Math1 is exclusively expressed in inner hair cells (IHCs) and outer hair cells (OHCs) as early as embryonic day (E) 13. Math1 is downregulated in these cells after postnatal day (P) 4, appearing at P7 (Birmingham et al., 1999; Woods et al., 2004). As displayed in Fig. 1, in rodents, the critical developmental time period of the inner ear is between the onset of fetal thyroid gland function at approximately embryonal day E15–17 (Deol, 1968; Ritter, 1967) and the onset of hearing function around P12. TRα and TRβ gene expression starts early during the auditory ontogenesis (Bradley et al., 1994). From P2 onward, the multicellular layer of the greater epithelial ridge disappears, the inner sulcus is formed and the detachment of the tectorial membrane occurs. Morphogenetic maturation in rodents is completed around P12. Prestin is expressed from approximately P2 onwards, KCNQ4 from approximately P4 onwards, and BK expression in rodents starts around P10. With the onset of hearing, ABR responses can be recorded and reach mature characteristics at around P21 (Christ et al., 2004).

As TRα and TRβ are both expressed in hair cells (Lautermann and ten Cate, 1997), a comparison between these two mouse mutants, one heterozygous for a dominant negative mutation, the other one homozygous for a null allele, would reveal possible autonomous functions of the respective receptors in hair cells.

Using the tamoxifen-inducible Cre-loxP system and the transgenic Math1-CreERTM mouse as tools to introduce early developmental mutations of TRs restricted to hair cells in the cochlea, we demonstrate that the deafness upon deletion of TRβ function has its origin outside of hair cells. Further, TRα and TRβ in hair cells exert subtle but specific effects on systemic hearing function. Finally, both receptors alter the sensitivity of sound transmission in an opposing way, possible through targetting a common key regulator.

2.Materials and methods

2.1. Generation of hair cell-specific TRα1 and TRβ1 mutant mice

For the generation of hair cell-specific TRα1 and TRβ1 mutant mice, we used two “floxed” alleles, one for THRα (thyroid hormone receptor α-activating function-2 mutation inducible (TRα2M™), in which Cre/loxP recombination permits the expression of a dominant-negative TRα1-AMM receptor (Quignod et al., 2007), and one for THRβ, TRβ1/2, in which Cre/loxP recombination eliminates coding exon 5, encoding the end of the DNA-binding (Winter et al., 2009). We also used a TRβ1-deleted allele (TRβ1−/−) (Forrest et al., 1996b). These were combined with Math1-CreERTM mouse line (Chow et al., 2006). The Math1-CreERTM is a fusion protein consisting of Cre recombinase and an altered ligand-binding domain of the estrogen receptor (CreERTM), which allows activation of recombinase activity only upon binding of tamoxifen, rather than endogenous estrogen (Chow et al., 2006). Math1, a transcription factor is involved in the development of the cerebellum (Ben-Arie et al., 1997) and hair cells of the cochlear and vestibular organs (Birmingham et al., 1999) is expressed in inner ear hair cells, cerebellum granule cells, but not in other inner ear cell types. We produced by repeated crosses double heterozygous TRα2M™/−/Math1-CreERTM, TRβ1−/−/Math1-CreERTM (subsequently named TRα2M™Math1 and TRβ1−/−Math1 for simplicity) and TRβ1−/− knockout mice (Fig. 2A–F).

Math1-CreERTM mouse line were also crossed to ROSA26 reporter (R26) mouse strain (Soriano, 1999) to verify recombination patterns. To demonstrate that tamoxifen specifically induced Cre in hair cells, X-gal staining of P3 ROSA26Math1CreERTM cochleae was performed. For this, heterozygous R26 mice were scheduled bred with heterozygous Math1-CreERTM mice (Fig. 2G). Four different genotypes were expected in the offspring (Fig. 2G), but only one genotype was expected to show a blue precipitate in IHCs and OHCs after X-gal staining (Fig. 2G. ROSA26CreERTMMath1CreERTM, written in blue). At P3, pups were sacrificed and cochleae were dissected and stained for X-gal. Positive X-gal staining was expected only from those pups where ROSA PCR products of 603 bp (ROSA wild-type locus) and 297 bp (ROSA knock-in locus) and also a Cre PCR product of 358 bp for the Cre locus could be amplified (Fig. 2H, lane 1). Genotypes lacking either the Cre locus (Fig. 2H, lane 2) or the R26 locus (Fig. 2H, lane 3) or both (Fig. 2H, lane 4) would not be expected to delete the floxed gene locus in hair cells. Whole-mount preparation of two cochleae from mice with confirmed R26 knock-in locus and Cre locus were stained with X-gal (Fig. 2I). All hair cells of all other cochlear turns were stained blue (Fig. 2I, shown for the apical and basal cochlear turns). The X-gal staining of one representative apical cochlear turn is shown in higher magnification, to better visualize the blue staining of the three rows of OHCs (solid arrows) and the one row of IHCs (open arrow). This observation is in accordance with Math1 expression in the cochlea and shows that the mode of tamoxifen application and the concentration chosen induces Cre activity and causes deletion of the floxed locus.

It is important to note in this context that Chow et al., 2006, upon analyzing the expression pattern of the Math1CreERTM construct using X-gal staining, showed lacZ-labeling exclusively in inner ear hair cells (cochlear and vestibular) and cerebellar granule cells (Chow et al., 2006) but not, for example, in the cochlear nucleus, the superior olivary complex (SOC) or the lateral lemniscus. Thus, a presumptive TRα1 function on medial olivocochlear (MOC) neurons would be intact in TRα2M™Math1 mice but deleted in global
TRα<sub>AMI</sub> mice (Quignodon et al., 2007). In all experiments, littersmates without Math1-CreERT<sup>TM</sup> transgene were used as controls.

2.1.1. Tamoxifen application
Maternal treatment with tamoxifen at E14 allowed to trigger Cre/loxP recombination in TRα<sub>AMI</sub>/Math1 and TRβ<sub>L2/C176</sub>-Math1 and R26R-Math1-CreERT<sup>TM</sup>embryos. For this purpose, 24 mg tamoxifen (Sigma–Aldrich, Taufkirchen, Germany) were dissolved in 400 µl corn oil (Sigma–Aldrich) at 37 °C using an ultrasonic water bath for instant use. Pregnant females were injected intraperitoneally with a volume of 100 µl. The females were killed by cervical dislocation at E19 and the pups were given to a foster mother (NMRI outbred strain; Charles River, Sulzfeld, Germany).

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.

2.2. Mouse genotyping
DNA was prepared from earmarks (QIamp DNA mini kit, Qiagen, Hilden, Germany) and PCR used for genotyping. Presence of the Math1-CreERT<sup>TM</sup> transgene was performed using 5′-ACGACCAAGTGACAGCAATG-3′ and 5′-CCATGCCTCAGACGATTAG-3′ as primers (Li et al., 2004). The TRα<sub>AMI</sub> and wild-type alleles were distinguished after PCR amplification (5′-GAAGTG ACTCAAATTGGG-3′, 5′-GAGGAAGGAGAGAAGATG-3′) was used to identify the floxed TRβ locus, two forward primers (5′-CATCTATGTTGCATGGCAACAGACT-3′, 5′-CAGCCACCTGGAGCAGAGCAGA-3′) and one reverse primer (5′-ACGCTACTTGTTGTGTAACAG-3′) producing PCR products with the expected size of 1111 bp (floxed locus) and 931 bp (wild-type locus). PCR using three oligonucleotides (5′-AAATGCGCTCTGATGTATAT-3′, 5′-GGGAGAGGTTGTCCCTCAACC-3′, and 5′-GGAGGCGAGAAATGCAATG-3′) was used to detect the presence of a 297 bp fragment specific for the R26R transgene, as described previously (Soriano, 1999).

2.3. Tissue preparation, histological staining, and immunohistochemistry
Cochleae of immature and mature offspring were isolated and dissected as previously described (Knipper et al., 2000). β-galactosidase activity was analyzed in immature (P3) R26RMath1CreERT<sup>TM</sup> mice through X-gal staining. Cochleae of R26RMath1CreERT<sup>TM</sup> mice were fixed and stained as previously described (Winter et al., 2009). Staining of cochlear whole-mount preparations was performed in staining solution containing 0.1% X-gal (Sigma–Aldrich). In case of a successful deletion of the floxed R26R locus, the presence of β-galactosidase protein was easily visualized by tissue incubation with the enzyme’s substrate (X-gal), resulting in a blue precipitate only in cells expressing Cre recombinase. For immunohistochemistry, sections were thawed and either permeabilized with 0.5% Triton X-100 (Sigma–Aldrich) for 10 min at room temperature, blocked with 4% normal goat serum in PBS, and incubated with primary antibody in 1% normal goat serum in PBS containing 0.1% Triton X-100 overnight at +4 °C or permeabilized with 0.1% Triton X-100 for 3 min at room temperature and preblocked with 1% bovine serum albumin in PBS and incubated overnight at +4 °C with primary antibodies. For double labeling studies, specimens were simultaneously incubated with both antibodies for identical time periods. Primary antibodies were detected with Cy3- (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and Alexa488- (Invitrogen, Life Technologies GmbH, Darmstadt, Germany) conjugated secondary antibodies. Sections were embedded with Vectashield mounting medium containing nuclear marker DAPI (Vector Laboratories, Burlingame, CA, USA). The following primary antibodies were used: rabbit polyclonal anti-BKα (Alomone Labs, Jerusalem, Israel), rabbit polyclonal anti-KCNQ4 (Rüttiger et al., 2004; Winter et al., 2006), and goat polyclonal anti-prestin (Santa Cruz Biotechnology, Dallas, TX, USA).

Sections and cochlear whole-mount preparations for X-gal staining were viewed using either an Olympus AX70 microscope or Olympus BX61 microscope both equipped with epifluorescence. Images were acquired using a CCD camera and analyzed with
Fig. 2. Inactivation of TRα or TRβ and recombination pattern in the inner ear hair cells. Cre/loxP recombination was induced by tamoxifen treatment at E14 in either TRα<sup>AMi</sup>Math1 or TRβ<sup>L2/C0</sup>Math1 embryos. Littermates without Math1-CreERT<sup>TM</sup> were used as controls. Cre/loxP recombination either activates the expression of a dominant-negative TRα<sup>L400R</sup> mutant receptor in heterozygous hair cells, or deletes the coding exon 5 of the remaining TRβ. (A) Crosses for the production of tamoxifen-treated TRα<sup>AMi</sup>Math1 embryos. (B) Crosses for the production of tamoxifen-treated TRβ<sup>L2/C0</sup>Math1 embryos. (C) Structure of the floxed Thra allele. (D) Structure of the floxed Thrb allele. (E, F) PCR for genotyping. (G) Heterozygous R26<sup>ki/wt</sup> mice were bred with heterozygous Math1-CreERCre/wt mice. Tamoxifen treatment was performed at E14. Only one genotype (ROSA26<sup>ki/wt</sup>Math1-CreERCre/wt, highlighted in blue) should show a blue staining of IHCs and OHCs. (H) Genotyping of R26R<sup>Math1-CreER</sup> mice using R26R- and Cre-specific oligonucleotides. LacZ expression following a blue staining of IHCs and OHCs was expected in mice where two PCR products for the R26R locus with size of 603 bp (R26R, wild-type locus) and 297 bp (R26R knock-in locus) and additionally a PCR product of 358 bp (Cre locus) could be detected (lane 1). Lanes 2 to 4 present genotypes of control animals where no blue stained hair cells are expected. (I) Whole-mount-X-gal staining on 3-day-old (P3) cochlear whole-mount preparation showed blue staining of OHCs and IHCs, demonstrated for the apical and basal cochlear turn. Solid arrows mark the three rows of OHCs; the open arrow marks the row of IHCs. Scale bars, 200 μm (overview images), 20 μm (magnification, right panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
A

TRβ-Ctr

B

TRβl/Math1

C

TRβ

D

Fig. 3. Morphology of the tectorial membrane in 6 to 7-month-old hair cell-specific TRβl/Math1 knockout mice compared with age-matched TRβ-Ctr and constitutive TRβ-/- knockout mice. Cross sections of epoxy-resin-embedded cochleae reveal a similar morphology of the tectorial membrane (TM) for TRβ-Ctr (A) and hair cell-specific TRβl/Math1 knockout mice (B) that is different to the tectorial membrane of constitutive TRβ-/- knockout mice (C). Scale bars, 50 μm. (D) Boxplots for dimensional measurements of TM (width, thickness, area). TRβ-Ctr: n = 49 sections, 1 animal; TRβl/Math1: n = 53 sections, 2 animals; TRβ-/-: n = 44 sections, 1 animal.

cell^F or cellSens software (Olympus Soft Imaging Solutions – OSIS, Hamburg, Germany). Images were processed with Photoshop.

For anatomical studies of the tectorial membrane, cochleae were epoxy-resin embedded and analyzed according to (Winter et al., 2009).

2.4. Hearing measurements

Auditory brainstem response (ABR) and distortion products of otoacoustic emission (DPOAE) measurements were performed as previously described (Engel et al., 2006; Knipper et al., 2000; Schimmang et al., 2003).

2.5. Statistical analyses

Unless otherwise noted, data were represented as mean ± standard deviation (s.d.); differences of the mean were tested for statistical significance either by Student’s t-test, one-way ANOVA, or two-way ANOVA for repeated measurements (two-way ANOVA) with α = 0.05. Greenhouse-Geisser adjustment was applied for the F-tests involving the repeated measure variable. Particularly, statistics for the repeated measure variables in the hearing measurement data focused on the main effect of genotype (genotype) and the genotype × frequency or genotype × intensity interaction. All ANOVA tests were followed by Tukey’s HSD post hoc tests or multiple t-tests with Bonferroni-Holm’s adjustment of α-level. The resulting p values are reported in the legends. ’p < 0.05; ‘’p < 0.01; ‘’’p < 0.001; n.s., not significant.

To visualize data of the tectorial membrane, boxplots were used (median, quartiles).

3. Results

3.1. Hair cell-specific Thrb knockout mice (TRβl/Math1) develop a normally shaped tectorial membrane

Deafness as a result of congenital hypothyroidism (Deol, 1973; Uziel, 1986; Uziel et al., 1985) and loss of TRβ in TRβ-/- mice (Winter et al., 2009) has been associated with tectorial membrane malformation. Using light microscopy, we analyzed the width, thickness, and area of the tectorial membrane of 6 to 7-month-old TRβ controls (Fig. 3A and D), TRβl/Math1 mice with hair cell-specific deletion of TRβ (Fig. 3B and D), and constitutive TRβ-/- mice (Fig. 3C and D) as described previously (Winter et al., 2009). Using histological examination, we did not observe any malformation of the tectorial membrane in TRβl/Math1 mice (Fig. 3B), whereas tectorial membranes in TRβ-/- mice were clearly malformed (Fig. 3C) compared with TRβ control animals (Fig. 3A). Boxplots for the parameters width, thickness, and area revealed that tectorial membrane morphology of TRβl/Math1 mice was not different from controls (TRβ-Ctr) (Fig. 3D). Malformation of the tectorial membrane was only observed in TRβ-/- mice (Fig. 3C and D). We therefore conclude that the tectorial membrane malformation observed under conditions of T3 deprivation or in TRβ-/- mice (Rusch et al., 1998) is caused by lack of TRβ activity outside rather than inside the hair cells.

3.2. Hair cell-specific Thrb knockout mice (TRβl/Math1) show delayed expression of BK channels in inner hair cells

The constitutive deletion of TRβ (Rusch et al., 1998), which includes the TRβ1 and TRβ2 isoforms, and the conditional deletion of TRβ1/2 around P11 both result in delayed expression of BK channels and onset of BK currents in IHCs (≥P12) (Winter et al., 2009). In contrast, the normal timing of expression of BK protein in OHCs has been suggested to be controlled by rescue from TRα suppression (Winter et al., 2007). In line with this hypothesis, using immunolabeling on cochlear cryosections, we found that the tamoxifen-induced deletion of the Thrb locus in TRβl/Math1 mice revealed no difference in the expression of BK protein in OHCs when compared with TRβ controls at P15 (Suppl. Fig. 1A and B) and P30 (Suppl. Fig. 1C and D). In contrast, in IHCs of TRβl/Math1 mice an obvious difference in the expression of BK channels was observed, particularly in the large clusters of expression at the apical segment of the lateral membrane, with lesser differences at the basal pole of the cell. The pre- or postsynaptic identity of the basal BK clusters has to be clarified in future studies. BK protein was absent...
from the apical IHC segment at P12 (Fig. 4B) and P15 (Fig. 4D) of TRβ<sup>L1/C0</sup>-Math1 mice when compared with age-matched TRβ-Ctr mice (Fig. 4A and C). At P30, BK expression in the apico-lateral IHC membrane still appeared weaker in TRβ<sup>L1/C0</sup>-Math1 mice compared with controls (Fig. 4E and F). The disturbance in the timing of BK upregulation in IHCs affects the higher precision of temporal coding in the ascending auditory pathway (Kurt et al., 2012), a process that therefore may be hampered in TRβ<sup>L1/C0</sup>-Math1 mutants.

3.3. Hair cell-specific Thrb knockout mice show subtly reduced auditory brain stem responses and slightly stronger growth functions of distortion products of otoacoustic emissions

The delayed BK expression in IHCs in the absence of TH was accompanied by a delayed developmental downregulation of Ca<sup>2+</sup> currents and an immature-like less efficient exocytosis with a higher dependence on Ca<sup>2+</sup> (Brandt et al., 2007) (J.E., unpublished results). To investigate the contribution of TRβ to sound coding by hair cells, we measured auditory brainstem responses (ABRs). To assess OHC function, we measured distortion product otoacoustic emissions (DPOAEs).

ABR signals represent the summed activity of neurons in the ascending auditory pathway and are measured by averaging the sound-evoked electrical response recorded by subcutaneous electrodes. Sound-evoked brainstem responses elicited by either click or tone-burst stimuli are commonly used to objectively analyze auditory processing. No significant differences in ABR hearing thresholds were found between 3-month-old TRβ<sup>L1/C0</sup>-Math1 and control mice as measured by click- (Fig. 5A, TRβ-Ctr: 18.2 ± 3.22 dB SPL, n = 10 ears from 6 mice; TRβ<sup>L1/C0</sup>-Math1: 17.1 ± 6.08 dB SPL, n = 34 ears from 21 mice; Tukey’s HSD test, p = 0.905) and tone-burst-evoked ABR (f-ABR) measurements (Fig. 5C, TRβ-Ctr: n = 9 ears from 6 mice; TRβ<sup>L1/C0</sup>-Math1: n = 25 ears from 21 mice; Tukey’s HSD test, p = 0.854). Click-ABR thresholds of 1-7 months-old mice with constitutive deletion of TRβ (TRβ<sup>–/–</sup>) were significantly higher than the thresholds of TRβ-Ctr and the TRβ<sup>L1/C0</sup>-Math1 mice (TRβ<sup>–/–</sup>: 71.7 ± 10.49 dB SPL, n = 14 ears from 7 mice; one-way ANOVA, p < 0.001, post hoc Tukey’s HSD test between TRβ<sup>–/–</sup> and TRβ-Ctr mice, p < 0.001, and between TRβ<sup>–/–</sup> and TRβ<sup>L1/C0</sup>-Math1 mice, p < 0.001; Fig. 5A). Similarly, f-ABR thresholds of TRβ<sup>–/–</sup> mice were significantly elevated compared with the thresholds of the control and TRβ<sup>L1/C0</sup>-Math1 mice (TRβ<sup>–/–</sup>: n = 8 ears from 7 mice; two-way ANOVA, p < 0.001 for genotype, post hoc Tukey’s HSD test between TRβ<sup>–/–</sup> and TRβ-Ctr mice, p < 0.001, and between TRβ<sup>–/–</sup> and TRβ<sup>L1/C0</sup>-Math1 mice, p < 0.001, Fig. 5C). ABR waveforms reflect synchronous discharge of elements of the auditory pathway (Johnson and Kiang, 1976), from the cochlear nerve (wave I) through the cochlear nucleus and superior olivary complex (wave II and III), lateral lemniscus and inferior colliculus (wave IV), manifested as distinct temporally synchronized peaks (Fig. 5B). Mean click-ABR waveforms revealed no significant difference between TRβ<sup>L1/C0</sup>-Math1 and TRβ-Ctr mice (Fig. 5B). We observed a subtle, though not significant, reduction of ABR peak-to-peak amplitude of wave I (Fig. 5D, left panel, TRβ-Ctr: n = 7 ears from 4 mice; TRβ<sup>L1/C0</sup>-Math1: n = 9 ears from 7 mice; two-way ANOVA, p = 0.693 for genotype and p = 0.419 for genotype × intensity interaction) and wave IV (Fig. 5D, right panel, TRβ-Ctr: n = 6 ears from 5 mice; TRβ<sup>L1/C0</sup>-Math1: n = 14 ears from 10 mice; two-way ANOVA, p = 0.563 for genotype and p = 0.220 for genotype × intensity interaction), which suggests that summed activity tends to be reduced in higher brainstem nuclei in TRβ<sup>L1/C0</sup>-Math1 mice.

OHC function was assessed by measuring DPOAEs, acoustic signals that are caused by the nonlinearity of the cochlear amplifier when two tones are presented simultaneously. The electromechanical force generated by the OHCs makes a crucial contribution to this nonlinearity and thus to the generation of DPOAEs. Distortion products can be detected with a microphone placed in the ear canal (reviewed in Janssen et al., 2006)). DPOAE amplitudes (Fig. 6A) and thresholds (Fig. 6B) of 3-month-old TRβ<sup>L1/C0</sup>-Math1 and control mice were analyzed. Maximum DPOAE amplitudes at stimulation level (11) of 50 dB SPL were not significantly different between TRβ<sup>L1/C0</sup>-Math1 and control mice (Fig. 6A, TRβ-Ctr: 14.6 ± 7.43 dB SPL, n = 12 ears from 6 mice; TRβ<sup>L1/C0</sup>-Math1: 18.2 ± 5.54 dB SPL, n = 37 ears from 20 mice; Tukey’s HSD test, p = 0.184). DPOAE thresholds at seven pairs of stimulation frequencies (Fig. 6B) were also not significantly different (TRβ-Ctr; n = 12 ears from 6 mice; TRβ<sup>L1/C0</sup>-Math1: n = 37 ears from 20 mice; Tukey’s HSD test, p = 0.644). However, we found a higher number of frequencies where the mean threshold values of the TRβ<sup>L1/C0</sup>-Math1 mice were smaller than in the TRβ-Ctr mice (inset table in Fig. 6B), indicating a tendency of lower DPOAE thresholds in the TRβ<sup>L1/C0</sup>-Math1 mice than the TRβ-Ctr mice. DPOAE maximum
amplitudes of TRβ−/− mice were significantly lower than the amplitudes of TRβ-Ctr and TRβL1/Math1 mice. Numbers in bars represent the group mean DPOAE amplitudes in dB SPL. (B) Analysis of frequency-specific DPOAE thresholds did not show significant differences between the TRβL1/Math1 and TRβ-Ctr mice although the mean threshold values of the TRβL1/Math1 mice were more often lower than the mean threshold values of TRβ-Ctr mice (5 out of 7 frequencies, inset table), showing a tendency of lower DPOAE thresholds in the TRβL1/Math1 mice compared with the TRβ-Ctr mice. TRβ−/− mice had significantly elevated DPOAE thresholds at all stimulation frequencies. (C) The input–output (I/O) growth function of the DPOAE amplitudes at stimulation frequency (f2) of 11.3 kHz showed significantly larger DPOAE amplitudes for TRβL1/Math1 mice when compared to TRβ-Ctr mice.

Amplitudes of TRβ−/− mice were significantly lower than the amplitudes of TRβ-Ctr and TRβL1/Math1 mice (TRβ−/−: −0.2 ± 6.1 dB SPL, n = 5 ears from 5 mice; one-way ANOVA, p < 0.001, post hoc Tukey’s HSD test between TRβ−/− and TRβ-Ctr mice, p < 0.001, and between TRβ−/− and TRβL1/Math1 mice, p < 0.001; Fig. 6A). DPOAE thresholds of TRβ−/− mice were significantly higher than the thresholds of TRβ-Ctr and TRβL1/Math1 mice (TRβ−/−: n = 5 ears from 5 mice; two-way ANOVA, p < 0.001 for genotype, post hoc Tukey’s HSD test between TRβ−/− and TRβ-Ctr mice, p < 0.001, and between TRβ−/− and TRβL1/Math1 mice, p < 0.001; Fig. 6B). Input-output (I/O) growth function of DPOAEs was measured at the frequency of best hearing (11.3 kHz) and a significantly larger I/O growth function was found for TRβL1/Math1 mice (Fig. 6C, TRβ-Ctr: n = 8 ears from 6 mice; TRβL1/Math1:
n = 38 ears from 20 mice; two-way ANOVA, p = 0.022 for genotype and p = 0.727 for genotype × intensity interaction). This indicates that deletion of TRβ in hair cells enhances the output function of OHCs but markedly reduces the output function of IHCs.

3.4. Loss of TRX1-mediated TH transcriptional regulation in hair cells does not affect BK expression

The failure to terminate repression of TH-dependent genes through TRX despite the presence of TH, as expected in TRXAMIMath1 hair cells, did not alter the expression of BK channels in IHCs (Fig. 7) and OHCs (Suppl. Fig. 2). Neither BK expression at the apico-lateral (neck) membrane, at the basal pole of the IHC (Fig. 7), nor at the base of the OHCs (Suppl. Fig. 2) was different along the tonotopic axis, as shown for P14 and P30, for example. This finding is the first clear phenotypic difference between TRXAMIMath1 and TRXAMIMath1 mutants.

3.5. Loss of TRX1-mediated TH regulation of transcription in hair cells subtly enhances afferent auditory signal propagation

In TRXAMIMutant mutants, the basal sensitivity (threshold) of sound processing, as measured by similar click-evoked ABR thresholds, was not affected (Fig. 8A, TRXAMIM-Ctr: 13.4 ± 3.86 dB SPL, n = 6 ears from 4 mice; TRXAMIMMath1: 15.1 ± 2.66 dB SPL, n = 8 ears from 6 mice; two-sided t-test, p = 0.358); nor were tone-burst-evoked ABR thresholds (Fig. 8C, TRXAMIM-Ctr: n = 6 ears from 4 mice; TRXAMIMMath1: n = 8 ears from 6 mice; two-way ANOVA, p = 0.711 for genotype and p = 0.579 for genotype × frequency interaction).

However, analysis of the fine structure of ABR waveforms, especially of waves I and IV (Fig. 8B), revealed significantly larger peak-to-peak amplitudes for medium to high stimulus intensities, reflected by a significant genotype × intensity interaction for the growth functions of both waves (Fig. 8D, left panel, wave I: TRXAMIM, Ctr: n = 7 ears from 5 mice; TRXAMIMMath1: n = 6 ears from 4 mice; two-way ANOVA, p = 0.344 for genotype and p = 0.033 for genotype × intensity interaction; Fig. 8D, right panel, wave IV: TRXAMIM-Ctr: n = 6 ears from 4 mice; TRXAMIMMath1: n = 6 ears from 5 mice; two-way ANOVA, p < 0.001 for genotype and p = 0.017 for genotype × intensity interaction). In conclusion, in contrast to TRB1L1/−Math1 mutant mice, TRX−Math1 mutants have enhanced IHC output activity.

3.6. Loss of TRX1-mediated target gene transcription in hair cells leads to reduced DPOAE amplitudes, despite normal KCNQ4 and prestin expression

We next examined whether OHC transduction driving DPOAEs was affected by the persistent TRX1-mediated target gene suppression. In contrast to TRB1L1/−Math1 mice (Fig. 6A and B), DPOAE measurements performed in 3-month-old TRXAMIMath1 mice revealed significantly lower maximum DPOAE amplitudes at a stimulation level (11) of 50 dB SPL than in age-matched controls (Fig. 9A, TRXAMIM-Ctr: 22.2 ± 5.08 dB SPL, n = 6 ears from 6 mice; TRXAMIMMath1: 15.1 ± 5.23 dB SPL, n = 9 ears from 6 mice; two-sided t-test, p = 0.022). Although stimulation at threshold levels at different frequencies did not result in changed DPOAE amplitudes (Fig. 9B, TRXAMIM-Ctr: n = 5 ears from 4 mice; TRXAMIMMath1: n = 9 ears from 6 mice; two-way ANOVA, p = 0.386 for genotype and p = 0.052 for genotype × frequency interaction), we found that the number of frequencies at which the mean threshold values of TRXAMIMath1 mice were higher than in TRXAMIM-Ctr mice was larger (inset table in Fig. 9B), showing that TRXAMIMMath1 mutants tend to have higher DPOAE thresholds. The i/o growth function of DPOAEs at best stimulation frequency (11.3 kHz) revealed smaller amplitudes in TRXAMIMMath1 over almost all super-threshold stimulation levels, although the main effect of genotype did not reach statistical significance by a small margin (Fig. 9C, TRXAMIM-Ctr: n = 6 ears from 4 mice; TRXAMIMMath1: n = 9 ears from 6 mice; two-way ANOVA, p = 0.054 for genotype and p = 0.118 for genotype × intensity interaction). These reduced DPOAE amplitudes were observed despite the fact that there were no alterations of the basic OHC phenotype. Consistent with these results, the expression and distribution of KCNQ4 and prestin, as analyzed by immunolabeling in TRXAMIMath1 and control mice, did not reveal any obvious differences along different cochlear turns (Fig. 9D and E).

In conclusion, while TRB1L1/−Math1 and TRXAMIMath1 effects on ABR wave amplitudes and growth functions of DPOAEs, when considered individually, are small, the opposing effects of the two receptors on IHC and OHC output function are striking. These results reveal a subtle and as yet unappreciated role of TH receptors α and β in hair cells in defining the sensitivity of auditory transmission.

4. Discussion

The present study emphasizes that deafness reported in hypothyroidism and upon TRβ dysfunction does not result from a lack of TRβ activity inside hair cells. Rather, the autonomous and specific roles of TRX and TRβ neglected to date, likely act in the critical developmental period before or during hearing onset and may permanently alter inner and outer hair cell output activity. This topic is still a subject of controversy (Chatonnet et al., 2011; Picou et al., 2012).
4.1. Pathological shape of the tectorial membrane is not caused by loss of TRβ-mediated transcriptional regulation in hair cells

Our study addresses the consequences of a cell-specific deletion of TRβ (TRβ1/2) in hair cells driven by the Math1 enhancer expressed before hearing onset (E13-P7), leading to dysfunction of TRα and deletion of TRβ during the TH-dependent period of the developing organ of Corti in the inner ear. (Knipper et al., 2000).

As a first conclusion of the present study, the TH- and TRβ-dependent genes controlling proper formation of the tectorial membrane under normal conditions are active outside of hair cells: malformation of the tectorial membrane is found in the absence of TH (Deol, 1973; Knipper et al., 2000; Uziel, 1986), in the absence of all TRs (TRα and TRβ) (Rüsch et al., 2001), in the absence of only TRβ (Winter et al., 2009), but not in the hair cell-specific deletion of TRβ. We postulate that coordinated TRβ expression in supporting cells of the immature Kölliker’s organ (Knipper et al., 1999) is essential to form a normal tectorial membrane. In the absence of TRβ apoptosis of the greater epithelial ridge of Kölliker’s organ is delayed, causing delayed formation of the inner sulcus (Deol, 1973; Knipper et al., 2000; Uziel, 1986). This leads to a longer period of β-tectorin secretion as previously suggested (Knipper et al., 2001) and, as shown in TRβ-/- mice, causes a thicker tectorial membrane with increased β-tectorin levels (Knipper et al., 2001). Such a pathological increase in tectorial membrane mass is deleterious for hearing (Legan et al., 2000).

4.2. TRα- and TRβ-mediated TH effects differ in outer hair cells

Data in the present study show that TRα and TRβ have long-lasting and opposing effects on the growth functions of DPOAEs. This observation may be explained by the cochlear amplification process of OHCs, which is mostly based on voltage-dependent electromotile responses of OHCs carried by the motor protein prestin (Slc26a5) (Zheng et al., 2000), which feeds energy back into the cochlear partition. Electromotile responses of OHCs can be measured as nonlinear capacitance changes. OHC motility is not affected when TH was omitted after birth (He et al., 2003). When hypothyroidism is induced before birth, expression of the motor protein prestin is delayed (Weber et al., 2002). These findings indicate that TH controls the timing of gene expression during a very short time window. Also, in OHCs of TRβ-/- and Tshrhyt mutant mice, expression patterns of prestin, although delayed, finally mature (Winter et al., 2007), as do nonlinear capacitance and electromotility in these mice (Rüsch et al., 1998; Walsh et al., 2003). This strongly suggests that the permanent effects of TRα and TRβ malfunction on active cochlear mechanics, shown in the mature system in the present study, is not caused by altered prestin function. We therefore conclude that TRα or TRβ in hair cells is unlikely to permanently alter the DPOAEs amplitudes by directly targeting the motor protein prestin.

The opposing actions of TRα and TRβ may also be explained by an indirect effect on DPOAEs by affecting OHC membrane conductances, such as through potassium channels. Complete absence or delay of KCNQ4 expression in hypothyroidism has been shown in rats (Winter et al., 2006, 2007), Tshrhyt mutant mice, expression patterns of prestin, although delayed, finally mature (Winter et al., 2007), as do nonlinear capacitance and electromotility in these mice (Rüsch et al., 1998; Walsh et al., 2003). This strongly suggests that the permanent effects of TRα and TRβ malfunction on active cochlear mechanics, shown in the mature system in the present study, is not caused by altered prestin function. We therefore conclude that TRα or TRβ in hair cells is unlikely to permanently alter the DPOAEs amplitudes by directly targeting the motor protein prestin.

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discussed to potentially causing OHC cell death that under hypothyroidism occurs over time as observed in Pax8−/− mice (Christ et al., 2004) or when KCNQ4 is pharmacologically blocked (Nouvian et al., 2003). Our data do not show an absence or delay of KCNQ4 surface expression in OHCs of hair cell-specific TRα3/AMI mice (TRα3/AMI Math1), in contrast to global TRα3/AMI mutants (Quignodon et al., 2007). This indicates that in global TRα3/AMI mutants, altered TRα activity in non-hair cells causes the previously observed ongoing suppression of KCNQ4 (Quignodon et al., 2007; Winter et al., 2006, 2007), possibly through a missing maturation step of efferent innervation which coincides with MOC efferent function (reviewed in (Simmons et al., 2002)). However, how maturation of efferent innervation and KCNQ4 expression may be coupled is elusive. Since KCNQ4 expression does not seem to be controlled by TRα in hair cells, an altered KCNQ4 expression level therefore cannot be responsible for altered DPOAE function in TRα3/AMI Math1.

Finally, key regulators that set the sensitivity of OHCs during the TH-sensitive period might cause permanently altered OHC function. The degree of increasing cochlear mechanical responses to low-level sound (Dolan and Nuttall, 1988; Murugasu and Russell, 1996) and lowering them to high-level sound in the mature OHC (Wiederhold and Kiang, 1970), may thus critically depend on the sensitivity of the cochlear amplifier, which is set during the critical developmental period prior to hearing function. Two different possibilities may explain how TRs influence the setting of OHC output. First, the efferent input modulating OHC function is inhibited by α9/α10 nicotinic acetylcholine receptors (nAChRs) due to a functional coupling of the α9/α10 complex to KCa channels. Thus, ligand-gated Ca2+ entry through nAChRs (Fuchs and Murrow, 1992a,b) is coupled to Ca2+-dependent K+ efflux carried by the BK and SK2 currents, which leads to hyperpolarization of OHCs (Housley and Ashmore, 1991; Maison et al., 2013). In turn, this hyperpolarization can inhibit OHC electromotile responses (Dallos, 1992). No obvious changes in the BK expression pattern were apparent in OHCs, in neither TRα3/AMI Math1 nor TRα3/AMI Math1 mice, demonstrating no direct effect of TRs on BK surface expression. Future studies, however, should consider α9/α10 nAChRs or SK2 as TR targets. So far, however, there is no evidence that hypothyroidism affects nAChR currents or protein density, in either the cochlea or brain (Alzoubi et al., 2007). Second, it has been shown only recently that a disruption of the OHC Ca2± homeostasis, normally mediated by the canonical transient receptor potential channel subtype 3 (TRPC3), significantly increases DPOAE growth functions (Wong et al., 2013). It was concluded that through altered Ca2± homeostasis, the acquisition of proper OHC conductances during the critical period prior to onset of hearing was permanently altered, thereby changing the sensitivity of the OHCs.
4.3. TRα- and TRβ-mediated TH effects differ in inner hair cells

Initially, deafness of constitutive TRβ-deficient mice was not attributed to a pathological tectorial membrane but rather to delayed BK channel expression in IHCs (Rutsch et al., 1998). Normal hearing of BKα−/− mice in the first months of life (Rütiger et al., 2004) excluded an essential role of IHC BK channels for hearing. Absence or delay of BK expression would, however, lead to deficits in the precision of IHC temporal and amplitude coding, which has been recently demonstrated in mice with hair cell-specific deletion of BK (Kurt et al., 2012).

Due to the delayed expression of BK channels in TRβ1/1-Math1 mice (until P30), repolarization of IHCs in response to sound-evoked depolarization may be transiently altered. In the absence of TH, depolarization-induced exocytosis from the secondary releasable pool (SRP) is reduced, which likely reflects disturbed re-supply of vesicles to the active zones (Johnson et al., 2010). This phenomenon was ascribed to the absence of coupling exocytosis with endocytosis, a mechanism that typically starts with the onset of hearing (Duncker et al., 2013). A decline of exocytosis from a SRP was also observed, interestingly, in the pachanga mouse (OtofPga/Pga) (PangrisPf et al., 2010), which consequently developed reduced ABR wave I. The tendency of reduced (although not significantly) ABR wave I in TRβ1/1-Math1 mice therefore might result from disturbed key regulators for coupling exocytosis with endocytosis.

In contrast to the observations in TRβ1/1-Math1, BK expression is normal in IHCs of TRβ4/4-Math1 mice. Preliminary data indicate global TRαAMT mutants have a higher developmental peak Ca2+ current amplitude and higher Ca2+ current amplitudes than wild-types in the third postnatal week in IHCs (J.E., unpublished results). While more detailed studies are required, it is likely that unliganded TRαx persistently suppresses a gene that controls the developmental regulation of Ca2+ currents. Indeed, it is tempting to speculate that TRα and TRβ differentially control the expression of IHC ion channels. If we consider that TRβx exerts an opposing effect on cellular output also on the OHC level, it is also tempting to hypothesize that these different TR functions occur through targets that control Ca2+ homeostasis in both hair cell types. Altered Ca2+ homeostasis prior to hearing onset caused by TRPC3 deletion has been shown to change the input-output function of sound processing (Wong et al., 2013). These types of TR-mediated changes in membrane conductances during a critical time period would have the potential to permanently adjust the output of IHCs and OHCs and thereby the sensitivity of auditory transmission.

In conclusion, the opposing effects of TRαx and TRβ in inner and outer hair cells on both ABR and DPOAE amplitudes may suggest that the two receptors differentially regulate key steps in hair cell maturation. Depending on the timing of maximal TR isoform expression, the sound transduction input-output function and auditory neurotransmission could thereby be permanently shaped.

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Appendix A. Supplementary Material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2013.08.025.

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