Complete Activation of Thyroid Hormone Receptor β by T_3 is Essential for Normal Cochlear Function and Morphology in Mice

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Key Words

Thyroid hormone receptor • Hearing • Cochlea • *knock-in* mice

Abstract

Background/Aims: Thyroid hormones (THs) regulate many developmental processes, including the developmental onset of cochlear differentiation and function. TH action is mediated mostly by triiodothyronine (T_3) bound to thyroid hormone nuclear receptors (TRs). At positive regulated genes and in the absence of THs, nuclear co-repressors are bound to TRs and decrease basal transcription rate. Ligand (T_3) binding results in the dissociation of co-repressors and the recruitment of co-activators to the complex, which results in full transcriptional activation. **Methods:** We measured cochlear function in two *knock-in* mouse models: $TR\beta^{E457A/E457A}$, with the $TR\beta$ co-activator binding surface (AF-2) disrupted to prevent co-activator binding; and $TR\beta^{\Delta 337T/\Delta 337T}$, which is unable to bind T_3 . Cochlear morphology and function were analyzed in 10-week-old normal and mutated mice. Cochlear function was determined by measuring auditory brainstem responses, cochlear tuning and compound action potential (CAP)

thresholds. **Results:** All $TR\beta^{\Delta 337T/\Delta 337T}$ and 85% of the $TR\beta^{E457A/E457A}$ mice presented elevated CAP thresholds ($P < 0.05$ or less). Five percent of the $TR\beta^{E457A/E457A}$ mice presented normal CAP thresholds with broadened cochlear tuning. $TR\beta^{E457A/E457A}$ and $TR\beta^{\Delta 337T/\Delta 337T}$ presented developmental defects that led to a decreased width ($P < 0.01$) and an increased thickness ($P < 0.01$) of the tectorial membrane. In addition, $TR\beta^{\Delta 337T/\Delta 337T}$ animals showed an increased tectorial membrane area ($P < 0.01$). **Conclusion:** Both mutations were deleterious to tectorial membrane development and led to important alterations in cochlear morphology and loss of cochlear function.

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Introduction

Thyroid hormones (THs) and their receptors are required for the development of the cochlea. Hypothyroidism inhibits differentiation of the cochlea by unknown mechanisms. In humans, congenital hypothyroidism or iodine deficiency may impair hearing or cause profound deafness [1, 2]. In rodents, hypothyroidism causes deformities in several structures, such as the tectorial membrane, which normally contacts

the stereociliary bundles of the mechanosensitive hair cells [3-10]. Hearing loss has also been observed in the human syndrome of resistance to thyroid hormone (RTH, OMIM #188570) [11, 12].

Thyroid gland releases mainly thyroxine (T₄), however TH action is mediated mostly by triiodothyronine (T₃) bound to thyroid hormone nuclear receptors (TRs). TH action is mediated by different isoforms of thyroid hormone receptors (TRs), which are members of the nuclear receptor superfamily of ligand-modulated transcriptional factors [13]. Two genes encode all known ligand-binding TR isoforms: TR- α 1, TR β 1 and TR β 2, which mediate genomic T₃ effects. Their hormone response elements are DNA sequences that bind thyroid hormone receptors and cause specific nearby genes to respond transcriptionally to thyroid hormone. At positive regulated genes and in the absence of THs, nuclear co-repressors are bound to TRs and decrease the basal transcription rate. Ligand (T₃) binding results in the dissociation of co-repressors and the recruitment of co-activators to the complex, which results in full transcriptional activation.

The presence of TR β is essential for the normal development of the auditory system. Several transgenic animal models have been studied to determine how THs affect hearing function. TR β knockout mice have severe hearing impairment that has been associated with abnormal electrophysiological maturation of the inner hair cells in affected cochleae [4, 14-19]. Interestingly, morphologic abnormalities in TR β -knockout mice cochleae are not observed under light microscopy [6, 20]. However, when TR β was specifically deleted from hair cells, Winter et al. (2009) reported an abnormal tectorial membrane structure.

TR α 1 does not appear to be critical for hearing function because TR α - knockout mice have normal hearing function [4] while biallelic disruption of TR β generates deaf mice [4, 6, 17, 21].

The lack of T₃ action clearly affects cochlear function and development. Mutant animals carrying a point mutation that inactivates the thyrotropin receptor were insensitivity to sound, that were only partially reverse by T₄ treatment before they were born [22]. Furthermore, animals that have no thyroid due to the absence of Pax8 show abnormalities in the outer and middle ear structures, which are poorly developed, deformed or completely absent. These animals exhibit an auditory phenotype similar to that caused by systemic hypothyroidism or TR deletions [3, 22-24]. Little is known about the mechanisms that underlie T₃ action in the auditory system. However,

thyroid hormone and its receptors are required for the normal development of the auditory system.

In vitro studies have demonstrated that TR action is regulated by interactions with co-repressor (Co-R) and co-activator (Co-A) molecules [13]. Ligand binding to TRs results in the dissociation of Co-Rs and recruitment of Co-As to the TR AF-2 domain, which results in the activation of transcription. Given the importance of the presence of functional TRs for the development of the auditory system, we decided to compare two *knock-in* mouse models with different disruptions of TR β interactions with T₃ and co-factors. In one model, the AF-2 domain on TR- β where co-activators bind was disrupted, while Co-R and T₃-binding activity was maintained. In the second model, the T₃-binding domain was disrupted, and the Co-R-binding activity was maintained. Surprisingly, full activation of the TR- β by T₃ was essential for normal hearing function.

Materials and Methods

Ethical approval

All animal experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Chicago and by the Institutional Animal Care and Use Committee at Northwestern University.

Mice

Two different *knock-in* mice lines expressing mutant TR β were used: TR $\beta^{\Delta 337T/\Delta 337T}$ and TR $\beta^{E457A/E457A}$. The $\Delta 337T$ mutation is a natural mutation that has been observed in a family (Kindred S) with resistance to THs (for review see e.g. [12]). The E457A mutation is an artificial mutation that has been previously described [25]. Both mutations were introduced in the germ line of mice by homologous recombination [26, 27]. All mice used in these studies were from the same mixed genetic background (C57BL6/129Svj), and littermate wild-type (TR $\beta^{wt/wt}$) controls were used in all functional experiments. The genotypes of the animals were determined, as previously described [26-29].

Mice were maintained under 12-hour light/12-hour dark cycles (beginning at 6 am/6 pm). The animals had their cochlear function and morphology evaluated at 10 weeks of age. Blood samples were obtained from age- and gender-paired groups for total serum T₄ determination by radioimmunoassay.

Serum thyroxine measurement

A specific radioimmunoassay kit was used to measure the total serum thyroxine (T₄) levels (MP Biomedicals; New York, NY, USA). The detection limit and intra-assay variation were 1 μ g/dL and 1.4%, respectively. All samples were measured in duplicate within the same assay.

Animal surgery

Animal surgery was performed, as previously described [30]. In short, mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (80 mg/kg bodyweight). Maintenance doses of 17 mg/kg bodyweight were given throughout the experiment whenever the animal showed signs of increasing arousal. The depth of anesthesia was assessed every 30 minutes by the paw withdrawal reflex. After the animal was fully anesthetized, breathing was facilitated by performing a tracheotomy and securing a length of PE90 tubing into the opening of the trachea. The animal was positioned belly up on a heating pad, with the head mounted to a heated head holder. The bulla was surgically exposed and was opened to allow access to the cochlea. A silver electrode was hooked onto the bony rim of the round window of the cochlea, and a ground electrode was placed under the skin at the left jaw. After cutting the cartilaginous outer ear canal, a brass speculum (for the sound delivery system) was cemented with dental acrylic to the bony part of the outer ear canal. The surgical platform containing the animal was moved into a soundproof booth. Two chest electrodes were attached to monitor the heart rate, and a high-frequency tweeter (Beyer DT770-Pro, Beyerdynamic Inc., Farmingdale, NY) was coupled to the speculum at the ear canal.

Compound action potential (CAP) thresholds

CAP thresholds were determined using a modified tracking procedure and were defined as the sound level required for a 20- μ V N1/P1 amplitude at a given stimulus frequency [31]. For the measurements, tone bursts of 12 ms in duration, including a 1 ms rise and fall time, were presented at a rate of 4 Hz. To reduce the contribution of cochlear microphonics, responses to 32 consecutive tone-burst presentations delivered in opposite phases were averaged. Moreover, the overall noise of the recordings was reduced by bandpass filtering the response using a preamplifier and filter (ISO 80, World Precision Instruments, Sarasota, FL), with the highpass filter cutoff frequency set to 300 Hz and the lowpass filter cutoff frequency set to 3000 Hz. The amplifier gain was set to 60 dB. CAP thresholds were determined for a frequency range of five octaves with a resolution of three steps per octave. The highest frequency was 50 kHz.

Cochlear tuning

Tone-on-tone masking was used to measure the frequency selectivity of the cochlea. The probe tone (10 ms in duration, including a 1-ms rise/fall time) was overlapped by a masker (34 ms in duration, including a 5-ms rise/fall time). Stimuli were presented at a rate of 4 Hz. The initial step of the experiment was to adjust the probe's sound level to evoke a 40- μ V compound action potential. No masker was presented during the probe's adjustments. During subsequent measurements, the probe's frequencies (6 or 12 kHz) and sound levels were kept constant, while a masker was simultaneously presented at different frequencies and sound levels. For a given masker frequency, which began one octave above the probe's frequency, the masker's sound level was decreased until the CAP-amplitude was either 28 μ V, which was 3 dB below the

probe's response, or 20 μ V, which was 6 dB below. Sound levels obtained for the masker to reduce the probe's response by either 3 dB or 6 dB were plotted versus masker frequencies. A tuned response resulted in a minimum at the probe's frequency. To express the selectivity or sharpness of tuning (Q_{10dB}) of the response, the frequency in Hz at the minimum of the curve was divided by the width of the curve (in Hz) at masker levels 10 dB above the minimum.

Auditory brainstem responses (ABRs)

Auditory brainstem responses are differential responses from intradermal electrodes. The responses were obtained by subtracting the ipsilateral mastoid from vertex potentials that were measured relative to a ground electrode placed in the neck. The electrodes were connected to a differential amplifier (ISO-80, WPI; Sarasota, FL) with a high input impedance ($>10^{12}$ Ω) set at 10,000 times amplification. Further amplification (ten times) and filtering (300 to 3000 Hz) of the signal was obtained through a Frequency Device filter (IP90, Frequency Devices Inc., Ottawa, IL). The sampling rate was 200,000, and 512 trials were averaged. The ABR-threshold was defined as the sound level required for the first visual appearance of wave I or wave II in the recorded trace.

Sound System

Voltage commands for the acoustic stimuli were generated using custom-written software and a computer I/O board (KPCI 3110, Keithley, Cleveland, OH, USA) inserted into a PC. The voltage commands generated by the Keithley computer boards were fed into a buffer amplifier (Symetrix 303, Symetrix Inc, Lynnwood, WA, USA), and the signals were adjusted in amplitude using a programmable attenuator (8310 Series, Aeroflex Weinschel, Ann Arbor, MI, USA). The two channels were mixed using a signal mixer (SM5, Tucker-Davis Technologies, Alachua, FL, USA) before driving a Beyer DT770Pro headphone. The sound was calibrated using a real head coupler, as previously described [32]. In short, the fixed and dried head of an experimental animal was sliced along the medial plane. The soft tissue was removed, and the bulla was accessed. To approximate the *in vivo* experimental setup, the speaker was mounted to the outer ear canal while a tan, 1/8-inch Bruel and Kjaer microphone (Bruel & Kjaer North America Inc., Norcross, GA, USA) was placed at the tympanic ring. The membrane of the microphone was close to the natural position of the tympanic membrane. Maximum sound levels for the speaker were determined for a 1-V_{rms} driving voltage command to the speaker at different stimulus frequencies.

Morphology

To assess anatomical changes that may exist between animals, cochlear gross dimensions were measured as described before in fixed hemicochleae [33, 34]. Furthermore, cochlear anatomical changes were obtained from Araldite Resin-embedded cochleae.

Fixation of the cochleae. After cochlear function was determined, the mice were sacrificed with an intraperitoneal sodium pentobarbital injection (200 mg/kg body weight), and the animals were decapitated. The bullae were extracted and

trimmed in Hanks's Balanced Salt Solution (HBSS) to expose the cochleae. To allow better penetration of the fixative into the cochleae, a hole was made into the apex of the cochleae, and the stapes was dislodged before placing the specimens in 0.1 M phosphate buffer containing 4% paraformaldehyde. Cochleae were fixed for four hours at room temperature and were maintained in 0.1 M phosphate buffer at 4°C until slicing the hemicochlea or plastic embedding. For a few cochleae, a post-fixation with 1% osmium tetroxide for 10 minutes was performed.

Hemicochleae. The method of producing a hemicochlea has been previously published [34]. A hemicochlea is a cochlea cut in half along its mid-modiolar plane using a TPI vibratome with half of a double-edged razor blade. After the blade passed through the entire cochlea, the cochlea was gently cut from the block and was placed into a media-filled Petri dish for imaging that contained a dab of vacuum grease.

Embedding. Embedding in Araldite Resin plastic followed a standard protocol [e.g. 35]. In short, the specimens were rinsed in phosphate buffer three times for 15 minutes, and were dehydrated in a series of increasing concentration of Acetone (15 minutes in 25%, 15 minutes in 50%, 15 minutes in 75%, 15 minutes in 90%, 15 minutes in 100%, 20 minutes in 100%). After dehydration, the cochleae were embedded in Araldite-Epoxy Resin (7:1 Acetone:Resin, 1:1 Acetone:Resin, 1:7 Acetone:Resin, three times pure Resin). The plastic was cured for 12 hours in an oven at 65°C. Sections were cut perpendicular to the modiolus at 5 µm thickness on an ultramicrotome (LKB 8800 Ultratome III, Stockholm-Bromma, Sweden) and were placed on glass slides. The sections were stained with 1% toluidine blue (Sigma Aldrich, St Louis, MO) and 1% sodium tetraborate solution (1:20).

Capture images. The experimental setup consisted of an upright Leitz microscope (Medilux, Leitz, Grand Rapids, MI, USA) equipped with 10x, 20x and 40x Olympus (Olympus, Center Valley, PA, USA) water-immersion lenses and a trinocular viewing head. Pictures of the hemicochlea preparation were taken with a CCD camera (GP-MF602, Panasonic Corporation of North America, Rolling Meadows, IL, USA) attached to one port of the viewing head. Despite most of the organ of Corti tissues being light-transparent, different translucent structures could still be visualized by oblique illumination. In this experiment, the light source was a group of five red light-emitting diodes (LEDs) arranged below the microscope's condenser. The position of the LEDs was off the optical axis so that the preparation could be illuminated by oblique light [36].

The 2.25-2.5 turns of the mouse cochlea revealed four cut edges (Fig. 1). Measurements were taken at three cut edges because at the most apical edge no organ of Corti structures could be visualized. The distances of the cut edges used for measurements were approximately 1 mm, 2.1 mm, and 4.3 mm from the apical end of the basilar membrane. According to the frequency place map by Ehret [37] the basal cut edge would correspond to a best frequency of 20-30 kHz, at which age-related changes in hearing function can be seen at the age of the mice used [38-40].

Measuring the cochlear dimensions. Various dimensions of the hemicochlea were evaluated using the captured images.

Fig. 1. Image of a mouse hemicochlea preparation that shows the four cut edges, which are referred to as the (a) basal, (b) middle, and (c) apical cut edges, and (d) the helicotrema. Scale bar equals 100 µm.

Fig. 2. Sketch of the radial view of a cut edge of a hemicochlea. A: shows the structures that can be identified in the images, including the tectorial membrane [42], the outer and inner hair cells (OHC, IHC), the inner spiral sulcus (ISS), the tunnel of Corti [58], the basilar membrane pectinate and arcuate zone (BMP, BMA), the Deiter's cells (DC), the Boettcher cells, and the Claudius cells (CL). B: Linear measurements were performed as shown. Measurements that were determined were the widths of the basilar membrane pectinate zone and arcuate zone (a,b), the width of the tectorial membrane (c), the heights of the inner and outer pillar cells (e,d), the combined height of the basilar membrane hyaline matrix and the tympanic cover layer cells (g), the heights of the Claudius cells (j) and Boettcher cells (f), the height of the tectorial membrane (h) and the thickness of the tympanic cover layer cells (i). Heights of the outer hair cells and bodies of the Deiters cells were measured. C: Cross-sectional area measurements were obtained according to the shaded areas in the lower panel for the tectorial membrane (A), organ of Corti (B, see also text), and hyaline matrix of the basilar membrane (C).

Fig. 3. Serum T4 and auditory brainstem response thresholds obtained from $TR\beta^{wt/wt}$, $TR\beta^{\Delta 337T/\Delta 337T}$ and $TR\beta^{E457A/E457A}$ mice. (A) Classification of knock-in animals based on T_3 binding. (B) Total serum T4. (C) Triangles with an upward arrow indicate that no auditory response could be acoustically evoked up to the maximum sound level of the system. Five animals were used in each group. Data are presented as means $\pm$ SEM. * $TR\beta^{E457A/E457A}$, average of two animals.

The scale setting was converted from pixels to micrometers by determining the number of pixels between two lines of the image of a standard slide containing 10- μ m and 100- μ m divisions. The number of image pixels was converted into micrometers for linear measurements. Area measurements were obtained by tracing the structures of interest. The total number of pixels within the circumscribed area were counted and given a measurement for the cross-sectional area. Different dimensions of the cochlea were measured and are shown in Fig. 2, including the width of the basilar membrane (BM), BM arcuate and BM pectinate zones; the height of the basilar membrane, including separate heights for Boettcher cells (BC), Claudius cells [41] and hyaline matrix [41]. In addition, the height, width, and area of the tectorial membrane [42]; the heights of the outer hair cells and Deiters' cells (OHC, DC); the heights of the inner and outer pillar cells (IPC, OPC); and the area of the organ of Corti (OC) were measured. OC measurements did not include Hensen's cells, which are usually included because Hensen's cells and CL cannot be identified easily. Inner and outer pillar cell lengths were determined along the line shown in Fig. 2 starting at the junction between the outer pillar foot and marginal band of the basilar membrane to the head of the pillar cells.

Statistical analysis

Averages and standard errors were determined for each of the measurements. One-way ANOVA was employed to assess significance. The level at which the critical values for the F-statistics were evaluated was 0.05. When the analysis of variance indicated a significant difference between the obtained measurements for a selected structure or the CAP thresholds, a post-hoc honesty significant difference test (Tukey) was performed to make pairwise comparisons among the means. Differences were considered significant at $P < 0.05$.

Fig. 4. Compound action potential (CAP) thresholds obtained from $TR\beta^{wt/wt}$ mice. [24] Four $TR\beta^{wt/wt}$ mice from a mixed strain (129/C57BL/6). (Bottom) Averages of $TR\beta^{wt/wt}$ were compared with published CAP thresholds from the "gold standard" CBA/CaJ mouse strain [33].

Results

This study included 16 wild-type mice ($TR\beta^{wt/wt}$), 12 mice homozygous for the E457A mutation ($TR\beta^{E457A/E457A}$) and 5 mice homozygous for the $\Delta 337T$ mutation ($TR\beta^{\Delta 337T/\Delta 337T}$). The data in Fig. 3B shows significantly elevated total serum T4 in both mutant groups ($P < 0.001$).

Fig. 5. Compound action potential (CAP) thresholds obtained from $TR\beta^{E457A/E457A}$ mice. (A) Averages of $TR\beta^{wt/wt}$ are shown by filled circles. Averages of $TR\beta^{E457A/E457A}$ are shown by triangles. One $TR\beta^{E457A/E457A}$ mouse (ID M4422) is shown by diamonds. Five to seven animals were used in each group. Data are presented as the mean $\pm$ SEM. (B) Two representative cochlear tuning curves are shown. $TR\beta^{wt/wt}$ (circles) have narrow tuning curves, and the $TR\beta^{E457A/E457A}$ M4422 has a broad tuning curve (diamonds). Sharpness of tuning (Q_{10dB}) for all control animals was 4.1 ± 0.5 at 12 kHz and 2.0 ± 0.2 at 6 kHz. The tuning curves obtained from the $TR\beta^{E457A/E457A}$ M4422 (right panel) with normal CAP thresholds were broadened by 1.5 at 12 kHz and 1.7 at 6 kHz. Numbers are averages and corresponding standard errors.

$\Delta 337T$ mutation increases auditory brainstem response thresholds

Cochlear function in mutant mice and corresponding wild-type littermates was monitored by determining acoustically evoked ABR thresholds. Fig. 3C shows the ABR thresholds of the analyzed animals. The ABR thresholds for the $TR\beta^{\Delta 337T/\Delta 337T}$ mice were elevated by more than 60 dB SPL over the thresholds obtained from $TR\beta^{wt/wt}$ mice. Triangles with upward arrows indicated that no auditory response could be acoustically evoked up to the maximum sound level of the system. $TR\beta^{E457A/E457A}$ -ABR thresholds were moderately elevated by approximately 20 dB SPL. All frequencies were affected.

Compound action potential thresholds are elevated in $TR\beta^{E457A/E457A}$ mice

For the $TR\beta^{E457A/E457A}$ mutant, corresponding heterozygous (data not shown) and $TR\beta^{wt/wt}$ mice, cochlear function was quantified by measuring CAP thresholds (Fig. 4 and 5). At frequencies below 10 kHz, the $TR\beta^{wt/wt}$ littermates revealed CAP thresholds similar to CBA/CaJ mice, which are considered the “gold standard” (Fig. 4). Above 10 kHz, the CAP threshold was elevated when compared to the CBA/CaJ, and, above 30 kHz, sound levels of up to 90 dB SPL could not evoke auditory responses in $TR\beta^{wt/wt}$ mice (Fig. 4).

Differences in cochlear function above 20 kHz were expected because $TR\beta^{wt/wt}$ were bred from a 129/C57BL/6 background. The latter strains are known for age-related hearing loss, which manifests at 8–12 weeks of age (see Discussion). CAP thresholds obtained from heterozygous animals were similar to the CAP thresholds determined from control animals (data not shown). CAP thresholds were elevated for $TR\beta^{E457A/E457A}$ mice (Fig. 5A). Threshold elevation varied widely among the animals, and no response could be acoustically evoked from two animals. One $TR\beta^{E457A/E457A}$ mouse did not show any threshold elevation (ID M4422, Fig. 5A). However, the frequency selectivity of this animal was affected, which was shown by the tone-on-tone tuning curves (Fig. 5B, described later).

Cochlear compound action potential tuning is affected in $TR\beta^{E457A/E457A}$ mice

Cochlear CAP tuning was measured for all $TR\beta^{wt/wt}$ animals for which CAP thresholds were determined (Fig. 5B). Furthermore, cochlear CAP tuning was measured for the one $TR\beta^{E457A/E457A}$ (ID M4422) that displayed normal thresholds. The CAP tuning curve was measured at a few selected frequencies. Control animals had narrow tuning curves, while the $TR\beta^{E457A/E457A}$ mutant (ID M4422) had a broad CAP tuning curve. The sharpness of tuning (Q_{10dB}) for all control animals

	WIDTH (μm)			AREA (μm^2)			THICKNESS (μm)		
	Basal	Middle	Apical	Basal	Middle	Apical	Basal	Middle	Apical
CBA/CaJ	117 $\pm$ 5.5	170 $\pm$ 3.2	184 $\pm$ 4.4	2507 $\pm$ 340	5290 $\pm$ 279	6622 $\pm$ 413	30 $\pm$ 1.3	46 $\pm$ 1.6	54 $\pm$ 4.0
C57BL/6J	131 $\pm$ 1.5	175 $\pm$ 3.9	183 $\pm$ 6.2	2034 $\pm$ 118	4092 $\pm$ 89	6164 $\pm$ 322	27 $\pm$ 1.1	41 $\pm$ 0.8	50 $\pm$ 2.3
TR $\beta^{\text{E457A/E457A}}$	101 $\pm$ 2.8*	139 $\pm$ 3.6*	156 $\pm$ 4.1*	2258 $\pm$ 127	4073 $\pm$ 163	6155 $\pm$ 283	35 $\pm$ 1.5*	46 $\pm$ 1.4	59 $\pm$ 1.7*
TR $\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$	90 $\pm$ 15	126 $\pm$ 2.5	131 $\pm$ 11	2990 $\pm$ 687	4503 $\pm$ 613	13910 $\pm$ 868*	44 $\pm$ 4.1*	51 $\pm$ 4.3	74 $\pm$ 6.3*

Table 1. Basal, middle and apical measurements were taken at 4.3, 2.1 and 1 mm from the apical end of basilar membrane respectively. Five to 7 animals were used in each group. Data for the CBA/CaJ and the C57BL/6 were taken from a previously published manuscript [33]. Data are present as means $\pm$ SD. *P<0.01 (or less) compared to wild-type strains CBA/CaJ and C57BL/6J.

	CBA/CaJ	C57BL/6J	TR $\beta^{\text{E457A/E457A}}$	TR $\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$
Width				
BM (arcuate zone)	40.6 $\pm$ 1.9	40.6 $\pm$ 0.4	39.7 $\pm$ 2.2	33.8 $\pm$ 1.3
BM (pectinate zone)	95.7 $\pm$ 3.3	88.0 $\pm$ 1.3	81.6 $\pm$ 3.2	86.6 $\pm$ 10.2
Height				
Inner pillar cell	46.3 $\pm$ 2.5	45.1 $\pm$ 0.4	40.9 $\pm$ 3.0	32.9 $\pm$ 3.3
Outer pillar cell	45.6 $\pm$ 1.7	50.7 $\pm$ 1.3	46.1 $\pm$ 0.8	40.1 $\pm$ 1.2
Outer hair cell 1	14.7 $\pm$ 0.5	15.0 $\pm$ 0.4	14.5 $\pm$ 0.1	16.9 $\pm$ 0.8
Outer hair cell 2	13.9 $\pm$ 0.6	15.8 $\pm$ 0.5	14.1 $\pm$ 0.4	16.4 $\pm$ 0.9
Outer hair cell 3	13.8 $\pm$ 0.8	14.5 $\pm$ 0.8	13.8 $\pm$ 0.4	16.5 $\pm$ 1.1
Deiters' cell 1	22.4 $\pm$ 1.6	24.7 $\pm$ 2.1	25.9 $\pm$ 1.9	23.4 $\pm$ 2.2
Deiters' cell 2	21.9 $\pm$ 0.6	28.1 $\pm$ 2.3	25.2 $\pm$ 1.2	22.7 $\pm$ 2.8
Deiters' cell 3	25.1 $\pm$ 1.2	29.5 $\pm$ 1.7	24.5 $\pm$ 1.4	22.7 $\pm$ 3.4
Thickness				
Tympanic cover layer cells	6.5 $\pm$ 0.4	7.6 $\pm$ 0.5	6.7 $\pm$ 0.8	8.3 $\pm$ 1.1
Boettcher cells	18.3 $\pm$ 1.5	14.5 $\pm$ 1.2	13.6 $\pm$ 1.4	13.8 $\pm$ 2.0
Claudius cells	42.4 $\pm$ 5.3	46.9 $\pm$ 2.0	43.4 $\pm$ 4.1	29.8 $\pm$ 6.2
Hyaline matrix	13.3 $\pm$ 0.5	11.0 $\pm$ 0.4	15.4 $\pm$ 1.6	13.4 $\pm$ 0.2
Area				
Basilar membrane	1266 $\pm$ 51	1082 $\pm$ 90	1128.4 $\pm$ 243	923 $\pm$ 17.6
Organ of Corti	2832 $\pm$ 349	2612 $\pm$ 166	2174 $\pm$ 276	2590 $\pm$ 195

Table 2. Five to 7 animals were used in each group. Data for the CBA/CaJ and the C57BL/6 were taken from a previously published manuscript [33]. Data are present as means $\pm$ SD.

was 4.1 $\pm$ 0.5 at 12 kHz and 2.0 $\pm$ 0.2 at 6 kHz. Fig. 5B shows a representative example of the tuning curves obtained from the control animals and from TR $\beta^{\text{E457A/E457A}}$ mice with normal CAP thresholds. In particular, differences were apparent at 12 kHz. For the TR $\beta^{\text{E457A/E457A}}$ mice, the tuning was broadened by 1.5 at 12 kHz and 1.7 at 6 kHz.

For CAP threshold elevations of more than 60 dB SPL, no tuning curves could be determined in either the TR $\beta^{\text{E457A/E457A}}$ or TR $\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$ mice because a cochlear response could only be evoked in a small frequency range.

The tectorial membrane is affected in TR $\beta^{\text{E457A/E457A}}$ and TR $\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$ mice

The values obtained from the mutant mice (TR $\beta^{\text{E457A/E457A}}$ and TR $\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$) are shown in Table 1-4 and are compared to the wild-type animals from the CBA/CaJ and C57BL/6J strains [33].

The gross morphology of TR $\beta^{\text{E457A/E457A}}$ cochleae changed. Nuel's space was larger in TR $\beta^{\text{E457A/E457A}}$ mice compared to TR $\beta^{\text{wt/wt}}$ mice (Fig. 6). Moreover, along the entire cochlea, the width of the tectorial membrane was significantly shorter (Table 1, p<0.01); however, the differences in the cross-sectional area were not significant when compared with values obtained from CBA/CaJ or C57BL/6J mice [33].

Similar to the TR $\beta^{\text{E457A/E457A}}$ mutant, tectorial membrane width was significantly smaller in the TR $\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$ mice when compared with the CBA/CaJ or C57BL/6J mice (Fig.7, Table 1). Moreover, the TR $\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$ mutant had a significantly larger tectorial membrane cross-sectional area (Fig. 7, Table 1). These differences were significant when compared to the CBA/CaJ and C57BL/6J mouse strains.

To determine whether other tissue structures that can be observed in a cochlear cross-section were affected

Fig. 6. Cochlear cross-section at 3 mm from the basal cochlear end. The gross morphology was changed for the $\text{TR}\beta^{\text{E457A/E457A}}$ mouse. Nuel's space (*) was larger in the $\text{TR}\beta^{\text{E457A/E457A}}$ mice (upper panel) when compared to corresponding $\text{TR}\beta^{\text{wt/wt}}$ mice (lower panel). Scale bar equals 50 μm .

Fig. 7. Cochlear cross-section at 5 mm from the basal cochlear end. The upper image shows an enlarged tectorial membrane observed in the $\text{TR}\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$ mice when compared to corresponding $\text{TR}\beta^{\text{wt/wt}}$ mice (lower panel). Scale bar equals 50 μm .

	CBA/CaJ	C57BL/6J	$\text{TR}\beta^{\text{E457A/E457A}}$	$\text{TR}\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$
Width				
BM (arcuate zone)	49.1 $\pm$ 1.4	50.6 $\pm$ 2.5	46.5 $\pm$ 2.0	34.7 $\pm$ 0.7
BM (pectinate zone)	110.1 $\pm$ 3.4	105.8 $\pm$ 2.0	98.3 $\pm$ 2.0	81.0 $\pm$ 8.4
Height				
Inner pillar cell	52.6 $\pm$ 2.9	49.0 $\pm$ 0.7	45.1 $\pm$ 2.0	24.6 $\pm$ 2.3
Outer pillar cell	58.5 $\pm$ 1.6	58.1 $\pm$ 1.3	51.1 $\pm$ 1.6	50.3
Outer hair cell 1	18.8 $\pm$ 0.6	17.6 $\pm$ 1.0	20.4 $\pm$ 0.5	19.3 $\pm$ 5.9
Outer hair cell 2	18.5 $\pm$ 0.9	17.2 $\pm$ 0.5	19.4 $\pm$ 0.6	18.5 $\pm$ 1.6
Outer hair cell 3	16.4 $\pm$ 0.5	17.2 $\pm$ 0.6	17.9 $\pm$ 0.5	19.8 $\pm$ 0.4
Deiters' cell 1	32.5 $\pm$ 1.5	33.1 $\pm$ 1.3	34.5 $\pm$ 0.6	N/A
Deiters' cell 2	35.8 $\pm$ 2.0	33.9 $\pm$ 1.0	34.4 $\pm$ 0.8	N/A
Deiters' cell 3	46.2 $\pm$ 5.0	35.5 $\pm$ 1.3	34.4 $\pm$ 1.1	N/A
Thickness				
Tympanic cover layer cells	6.7 $\pm$ 0.4	6.4 $\pm$ 0.3	10.3 $\pm$ 0.5	7.9 $\pm$ 1.1
Boettcher cells	19.4 $\pm$ 1.1	16.0 $\pm$ 0.8	16.8 $\pm$ 2.0	12.0 $\pm$ 2.6
Claudius cells	60.1 $\pm$ 3.2	50.0 $\pm$ 2.9	51.1 $\pm$ 1.5	43
Hyaline matrix	12.9 $\pm$ 0.5	9.7 $\pm$ 0.3	18.8 $\pm$ 1.5	5.2 $\pm$ 4.3
Area				
Basilar membrane	1401 $\pm$ 58	1129 $\pm$ 47	709 $\pm$ 70	595 $\pm$ 65
Organ of Corti	3883 $\pm$ 357	3418 $\pm$ 146	3006 $\pm$ 67	3884 $\pm$ 39

Table 3. Five to 7 animals were used in each group. Data for the CBA/CaJ and the C57BL/6 were taken from a previously published manuscript [33]. Data are present as means $\pm$ SD.

	CBA/CaJ	C57BL/6J	$\text{TR}\beta^{\text{E457A/E457A}}$	$\text{TR}\beta^{\Delta 337\text{T}/\Delta 337\text{T}}$
Width				
BM (arcuate zone)	55.1 $\pm$ 2.6	54.7 $\pm$ 2.7	52.7 $\pm$ 3.6	39.1 $\pm$ 1.4
BM (pectinate zone)	108.5 $\pm$ 7.1	118.0 $\pm$ 8.1	104 $\pm$ 7.3	110.4 $\pm$ 5.3
Height				
Inner pillar cell	49.2 $\pm$ 1.9	50.5 $\pm$ 1.5	48.2 $\pm$ 3.4	44.7 $\pm$ 2.9
Outer pillar cell	53.4 $\pm$ 2.4	61.8 $\pm$ 1.8	57.2 $\pm$ 2.1	51.3 $\pm$ 1.8
Outer hair cell 1	22.2 $\pm$ 1.4	21.3 $\pm$ 0.5	24.0 $\pm$ 1.0	25.9 $\pm$ 1.2
Outer hair cell 2	20.6 $\pm$ 1.3	21.6 $\pm$ 0.8	22.4 $\pm$ 0.5	26.7 $\pm$ 3.4
Outer hair cell 3	19.9 $\pm$ 1.2	20.9 $\pm$ 0.9	19.9 $\pm$ 0.5	25.3 $\pm$ 2.1
Deiters' cell 1	35.1 $\pm$ 2.1	34.8 $\pm$ 1.3	36.6 $\pm$ 0.6	33.0 $\pm$ 0.8
Deiters' cell 2	35.0 $\pm$ 1.6	38.2 $\pm$ 1.7	37.6 $\pm$ 0.6	30.8 $\pm$ 3.1
Deiters' cell 3	39.2 $\pm$ 1.7	40.2 $\pm$ 0.9	40.2 $\pm$ 0.9	35.2 $\pm$ 3.8
Thickness				
Tympanic cover layer cells	8.7 $\pm$ 0.4	8.8 $\pm$ 1.2	10.4 $\pm$ 1.4	9.3 $\pm$ 0.9
Boettcher cells	N/A	N/A	14.3 $\pm$ 1.8	18.2 $\pm$ 0.1
Claudius cells	N/A	N/A	52.2 $\pm$ 9.2	42.1 $\pm$ 9.8
Hyaline matrix	12.0 $\pm$ 0.6	5.3 $\pm$ 0.4	11.1 $\pm$ 1.0	11.4 $\pm$ 4.0
Area				
Basilar membrane	1308 $\pm$ 110	1142 $\pm$ 112	762 $\pm$ 251	1529 $\pm$ 519
Organ of Corti	3448 $\pm$ 405	5065 $\pm$ 791	3461 $\pm$ 228	3870 $\pm$ 770

Table 4. Five to 7 animals were used in each group. Data for the CBA/CaJ and the C57BL/6 were taken from a previously published manuscript [33]. Data are present as means $\pm$ SD.

by both mutations, different cochlear structures were measured according to the sketch in Fig. 2. The lengths of the outer hair cells, Deiters' cells, and inner and outer pillar cells, as well as the heights of Claudius cells and Boettcher cells, and the thickness of the basilar membrane and hyaline matrix in mutants were not significantly

different from CBA/CaJ and C57BL/6 wild-type animals (Tables 2-4). Data for the CBA/CaJ and the C57BL/6 were taken from a previously published manuscript [33]. Except for the tectorial membrane (Table 1), no other values were significantly different among the mouse strains.

Fig. 8. Diagram illustrating wild-type and mutant thyroid hormone receptors bound to RXR, T_3 and co-factors. A correlation with hearing function is shown. RXR, retinoid X receptor; TR, wild-type thyroid hormone receptor β ; E457A, thyroid hormone receptor β carrying the E457A mutation; $\Delta 337T$, thyroid hormone receptor β carrying the $\Delta 337T$ mutation; Co-A, nuclear co-activator; and Co-R, nuclear co-repressor.

Discussion

In this study we aimed to determine the effects of two different TR β mutations on cochlear morphology and function. Deficits in cochlear function can result from TH deficiencies [4-6, 10, 15, 43]. In drug-induced hypothyroid animals, hearing thresholds were elevated to approximately 80 dB SLP at 8 kHz or to 95 dB SPL at 6 kHz [44]. The exact mechanisms and genes involved remain to be identified.

Decreased levels of THs and altered TH metabolism, an absence of TR β , or TR β dysfunction causes deafness in mice and human beings [3, 6]. TR β plays an important role in the normal development of the cochlea and in normal cochlear function in rodents [1, 45, 46].

In humans, a moderate auditory threshold elevation was observed with mutations in TR β that reduce T_3 binding capacity and cause RTH. Mild and mixed hearing loss has been reported in 15% of affected RTH individuals, and conductive hearing loss occurred in approximately 6% of affected individuals [47]. However, why individuals with identical TR β mutations should display different phenotypes even within the same family (and between littermates) is not known. One explanation is that the environment of cofactors in which TRs interact with TR-responsive genes regulates the ultimate pattern of gene expression. To investigate if interactions between TRs and co-factors affect hearing function, we studied two different TR β mutants.

The two mutant mice (TR $\beta^{\Delta 337T/\Delta 337T}$ and TR $\beta^{E457A/E457A}$) that we used mimic human RTH syndrome and exhibit altered TR β interactions with T_3 and co-factors (Fig. 3A). The TR $\beta^{\Delta 337T/\Delta 337T}$ mutation is a natural mutation that results from a deletion of threonine at position 337 and was originally observed in the S family with

RTH [41, 48-50]. TR $\beta^{\Delta 337T/\Delta 337T}$ mutant mice express a TR β that does not bind T_3 and is constitutively bound to Co-Rs, such as NCoR, and does not bind co-activators [26]. When a thyroid hormone receptor with this mutation binds to another TR isoform or to another nuclear receptor, it induces a dominant-negative effect. The second animal model, TR $\beta^{E457A/E457A}$, expresses a TR β receptor that normally binds to T_3 , binds and releases Co-R and cannot bind Co-activators (Co-A), due to an alteration in helix 12 [27]. Co-activators usually have histone acetyltransferase activity, and the lack of co-activator recruitment leads to a partial response to T_3 . Therefore, receptors bearing this mutation do not achieve a maximum response to T_3 [25, 27]. Down- and upregulation of different genes by T_3 are impaired in both models [26, 51]. In addition, both mutants have elevated levels of THs and are resistant to THs to different degrees [26, 51]. The TR $\beta^{\Delta 337T/\Delta 337T}$ mutation presents a more severe phenotype when compared to the TR $\beta^{E457A/E457A}$ mutation [26, 51]. The effects of the two mutations (TR $\beta^{\Delta 337T/\Delta 337T}$ and TR $\beta^{E457A/E457A}$) upon the pituitary-thyroid axis are consistent with the clinical phenotype of RTH. Moreover, data from both models have shown that retinal [52] and cerebellar functions are affected [26, 53].

Cochlear function was determined by measuring acoustically evoked ABRs to pure tones and click stimuli. Moreover, the ABR patterns for the animals with a remaining auditory response were similar to those evoked in normal-hearing animals. These results indicate that the functional deficits were caused by the cochlea and not by extra cochlear regions of the auditory pathway. Similar to the TR β knockout mice [17], the homozygous TR $\beta^{\Delta 337T/\Delta 337T}$ mutants had severe ABR threshold elevations of 60 dB SPL and more.

In contrast to the TR $\beta^{\Delta 337T/\Delta 337T}$ mutant mice, the

TR $\beta^{E457A/E457A}$ animals presented a variable phenotype. For some animals, cochlear function was almost unaffected, while other animals had severely elevated ABR thresholds (Fig. 1). On average, their cochlear function resembled mice with a P-box mutation in TR β [17]. TR β containing this mutation lacks the ability to bind to DNA; however, it is still able to bind to T $_3$. Those animals have ABR threshold elevations of 20–30 dB [17].

Further, to investigate hearing alterations in the TR $\beta^{E457A/E457A}$ animals, we evaluated their CAP thresholds. Only one animal was indistinguishable from the wild-type animals. Approximately 10% of the TR $\beta^{E457A/E457A}$ had elevated thresholds, and 85% were severely elevated (Fig. 8). These results are similar to the numbers reported for TR $\beta^{-/-}$ mice [6]. The heterozygous animals were similar to the wild-type animals, and the wild-type animals were similar to the “gold standard” CBA/CaJ mice [38]. Both knock-in strains used were derived from 129/C57BL/6 strains of mice that are known to be genetically susceptible to age-related hearing loss. Age-related hearing loss is manifested at 8–12 weeks of age [38], which may explain the CAP threshold elevations above 20 kHz. The heterozygous animals of both mutant strains revealed normal cochlear function.

To evaluate hearing loss in these animals, cochlear tuning was tested using tone-on-tone masking. Tone-on-tone masking curves are similar to single-fiber recordings obtained from the same animal. For wild-type animals, tuning was provided as a Q $_{10dB}$, which was between 1 and 5, and was similar to values reported in the literature [54]. For most of the homozygous animals, no masking curves could be recorded. The only masking curve we measured was for the one TR $\beta^{E457A/E457A}$ mouse with normal CAP thresholds. Interestingly, cochlear tuning was broadened for this animal, which indicated that cochlear function was ultimately affected.

The hearing loss observed in both models can be explained by alterations in cochlear morphology. At first, gross morphology of both knock-in strains appeared normal. The lengths of the outer hair cells, Deiters’ cells, and inner and outer pillar cells, the heights of Claudius cells and Boettcher cells, and the thickness of the basilar membrane and hyaline matrix in the mutants were not significantly different from other mouse strains, particularly the CBA/CaJ and C57BL/6J mice [33]. Furthermore, the cross-sectional areas of the organ of Corti were similar to previously published measurements for mice. The basilar membrane and cell sizes in the mutant mice were not significantly different when compared to controls or

the “gold standard” CBA/CaJ mice. The Nuel’s space in TR $\beta^{E457A/E457A}$ mice was greatly enlarged between the third row of outer hair cells and Hensen’s cells. The enlargement was observable in all homozygous mice and has not been observed in any other wild-type CBA/CaJ or C57BL/6J mouse. We can only speculate on the causes of these changes. TR β is essential for the expression of inner hair cell potassium channels [55]. These channels may also be involved in the “recycling” of potassium in the outer hair cells. Functional deficits from the potassium channels may result in decreased potassium uptake in the outer hair cells and the formation of a hydrops. Further experiments are required to test this hypothesis.

Defects in cochlear morphology include the abnormal persistence of afferent dendrites, an absence of efferent innervation [44], a lack of inner spiral sulcus formation [44], and decreased β -tectorin protein levels in the tectorial membrane [15]. Until recently, deafness in TR $\beta^{-/-}$ mice had been mostly attributed to a decreased expression of inner hair cell potassium channels [55] because cochlear malformations were not observed in TR $\beta^{-/-}$ mice [6]. A recent paper reinforced that deafness in TR β mutants was caused by a malformation of the tectorial membrane by delaying inner hair cell potassium channel expression [56]. An abnormally thick tectorial membrane was also reported in Pax8 $^{-/-}$ mice [57] and in the Thr β^{PV} mutation [14]. Similar to previous findings, the present mutant strains revealed altered tectorial membranes. The tectorial membrane was shorter in the radial direction; however, the membrane was thicker. Differences were significant for both mutants (TR $\beta^{E457A/E457A}$ and TR $\beta^{\Delta 337T/\Delta 337T}$) and were more prominent in the TR $\beta^{\Delta 337T/\Delta 337T}$ mutant mice; therefore, the alteration in function was proportional to tectorial membrane alteration (Fig. 8). Our data confirmed that with disrupted T $_3$ binding leading to abnormal development of the tectorial membrane and dramatically elevated thresholds, which was shown by Winter et al. [56]. Homozygous TR $\beta^{\Delta 337T/\Delta 337T}$ mutant mice had no functional TR β and were deaf. However, heterozygous mice presented normal hearing function, which suggests that one normal allele of the receptor is sufficient in mice. This finding is different from results in humans, as some degree of hearing loss is detected in more than 10% of heterozygous patients [12].

We showed that the lack of the ability to bind T $_3$ (TR $\beta^{\Delta 337T/\Delta 337T}$) and reduced amplitude of T $_3$ effect (TR $\beta^{E457A/E457A}$) are deleterious to tectorial membrane development and lead to important alterations in cochlear morphology and hearing impairment.

Abbreviations

ABR (auditory brainstem response); BC (Boettcher cells); BMp (basilar membrane pectinate); BMa (basilar membrane arcuate zone); CAP (compound action potential); CL (Claudius cells); Co-As (co-activators); Co-Rs (co-repressors); DC (Deiters' cells); HM (hyaline matrix); IPC (inner pillar cells); ISS (inner spiral sulcus); OC (organ of corty); OHC (outer hair cells); OPC (outer pillar cells); T3 (triiodothyronine); T4 (thyroxine); THs (thyroid hormones); TM (tectorial membrane); TR (thyroid hormone receptor).

Acknowledgements

Source of funding: CPR has been supported by a grant from the NSF (IBN-0415901). FEW has been supported by National Institutes of Health Grants DK49126 and DK53036. TMO has been supported by grants from the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Sociedade Brasileira de Endocrinologia e Metabologia (SBEM-Thyroid Grant).

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