# Divergent roles for thyroid hormone receptor β isoforms in the endocrine axis and auditory system

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Thyroid hormone receptors (TRs) modulate various physiological functions in many organ systems. The TRα and TRβ isoforms are products of 2 distinct genes, and the β1 and β2 isoforms are splice variants of the same gene. Whereas TRα1 and TRβ1 are widely expressed, expression of the TRβ2 isoform is mainly limited to the pituitary, triiodothyronine-responsive TRH neurons, the developing inner ear, and the retina. Mice with targeted disruption of the entire TRβ locus (TRβ-null) exhibit elevated thyroid hormone levels as a result of abnormal central regulation of thyrotropin, and also develop profound hearing loss. To clarify the contribution of the TRβ2 isoform to the function of the endocrine and auditory systems in vivo, we have generated mice with targeted disruption of the TRβ2 isoform. TRβ2-null mice have preserved expression of the TRα and TRβ1 isoforms. They develop a similar degree of central resistance to thyroid hormone as TRβ-null mice, indicating the important role of TRβ2 in the regulation of the hypothalamic-pituitary-thyroid axis. Growth hormone gene expression is marginally reduced. In contrast, TRβ2-null mice exhibit no evidence of hearing impairment, indicating that TRβ1 and TRβ2 subserve divergent roles in the regulation of auditory function.

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## Introduction

Thyroid hormone (triiodothyronine [$T_3$]) mediates its pleiotropic effects by binding to thyroid hormone receptors (TRs), which are nuclear transcription factors regulating gene expression by binding to specific thyroid hormone response elements (TREs) on the promoters of thyroid hormone–responsive genes (1). There are 3 $T_3$-binding isoforms of the TR: TRα1, TRβ1, and TRβ2 (1–4). The TRα and TRβ isoforms are the products of distinct genes residing on separate chromosomes (1). The 2 isoforms of TRβ are derived by alternative exon use within the TRβ locus (5–7). The receptors are entirely homologous, with the exception of distinct $NH_2$-termini. The TRβ1 isoform is widely expressed and is particularly abundant in the liver and kidney; but it is also present in the pituitary, the hypothalamus, and the developing nervous system (1).

In contrast, the expression of TRβ2 is relatively restricted and is most highly expressed in the pituitary (4). Significant expression has also been described in the hypothalamus (8, 9) and the developing ear (10). Very low level expression of TRβ2 has been described in peripheral tissues (11), but it is unknown whether these low levels subserve significant functions in those tissues. TRβ2 expression is highly regulated by $T_3$ (4, 12) and thyrotropin-releasing hormone (TRH) (13). In the pituitary, TRβ2 mRNA is more abundant than TRβ1 mRNA (4). Furthermore, selective depletion of TRβ2 mRNA in a pituitary-derived cell line (GH3) was associated with marked inhibition of $T_3$ responsiveness of the growth hormone (GH) gene (14). Thus, the differential expression of the TRβ isoforms and the predominant expression in the pituitary and hypothalamus raise the possibility that TRβ2 may play an important role in the central regulation of the hypothalamic-pituitary-thyroid axis.

Transfection experiments have revealed differential properties of TRβ1 and TRβ2 in vitro. For example, TRβ2 causes ligand-independent activation of the GH promoter in the presence of retinoid X receptor (RXR),
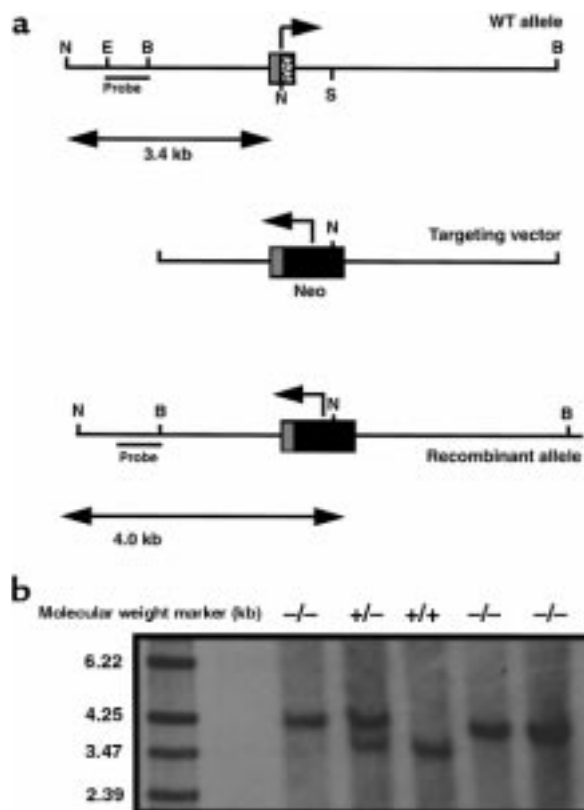
**Figure 1**

Targeted disruption of the TRβ2 exon. (**a**) Schematic representation of the TRβ2 exon and flanking DNA sequence. The targeting vector was generated by deleting a 1-kb *Nco*I-*Sac*I fragment encompassing the entire coding exon of the TRβ2 NH$_2$-terminus and the adjacent intron, and by inserting the neomycin resistance gene under the influence of the phosphoglycerate kinase promoter. Homologous integration of the targeting vector was confirmed by Southern blot of genomic DNA digested with *Nco*I and probed with an α-$^{32}$P–labeled, 700-bp *Eco*RI-*Bam*HI DNA fragment that is immediately upstream of the start of the targeting vector. The values 3.4 kb and 4.0 kb represent the expected sizes of the hybridized band in the WT and targeted alleles, respectively. B, *Bam*HI; E, *Eco*RI; N, *Nco*I; S, *Sac*I. (**b**) Representative Southern blot, demonstrating the targeted and WT alleles in KO (–/–), heterozygous (+/–), and WT (+/+) mice.

whereas TRβ1 mediates ligand-independent repression under these circumstances (15). On negative TREs, TRβ2 is a more potent mediator of ligand-independent activation than TRβ1 or TRα1 (16, 17). This property of TRβ2 has been mapped to amino acid residues 89–116 in its unique NH$_2$-terminus and can be transferred to TRα by exchanging the TRα NH$_2$-terminus with that of TRβ2 (18). RXR modulates the ability of TRβ1 and TRα1 to mediate negative regulation of the TRH gene in the presence or absence of its ligand, 9-*cis* retinoic acid. However RXR has no such effect on TRβ2 (19). Our laboratory has also demonstrated recently that whereas the transcriptional activity of TRβ1 and TRα1 can be modulated by interaction with the nuclear corepressor (N-CoR), TRβ2 does not possess this property (20). Mutations that cause selective pituitary resistance to thyroid hormone in humans selectively impair the function of TRβ2 but not TRβ1 (17). Hence, in tissues where both TR isoforms are expressed, there is ample in vitro evidence that these receptors may mediate distinct effects on gene transcription in the presence and absence of thyroid hormone.

Mice with targeted disruption of the TRβ locus (Trβ-null) (21), the TRα locus (22), and the TRα1 gene product (23) have been reported, and they have shed some light on the relative roles of each TR isoform in the regulation of the hypothalamic-pituitary-thyroid axis. TRβ-null mice exhibit elevated thyroid hormone concentrations and inappropriately elevated concentrations of thyrotropin (TSH) (21). Furthermore, in response to the administration of exogenous thyroid hormone, there is partial suppression of TSH, implying profound resistance to thyroid hormone (24). Hence, TRβ plays a key role in the central regulation of TSH. TRβ-null mice are also deaf, implying an important role for this isoform in the regulation of hearing (25). Given the coexistence of both TRβ isoforms in the pituitary and the developing cochlea, it is unclear if the hormonal abnormalities and the defective hearing are due to loss of TRβ1, TRβ2, or both. In contrast, selective ablation of the TRα isoform results in small declines in thyroid hormone concentrations, consistent with central hypothyroidism (22, 23).

To test the hypothesis that TRβ2 — by virtue of its restricted expression — is the key TR isoform responsible for the regulation of TSH, and to explore the possibility that TRβ1 and TRβ2 subserve distinct roles in the regulation of the auditory system, we generated transgenic mice with targeted disruption of the β2 isoform of the TR. These mice develop elevated concentrations of thyroid hormone and impaired suppression of thyroid hormone in response to the administration of exogenous T$_3$. Furthermore, the absence of TRβ2 profoundly impairs the activation of the TSHβ gene in vivo in the absence of thyroid hormone. Thus, the β2 isoform of the TR plays a critical role in the regulation of the hypothalamic-pituitary-thyroid axis. In contrast to mice that lack both TRβ isoforms, mice with an absence of TRβ2 do not exhibit hearing deficits.

## Methods

*Targeting vector.* PCR primers (5′-AGG TGC TAC TCT GAA GTG AG-3′ and 5′-ATA TGC TGC TAC TGG GCA CA-3′) that correspond to specific sequences within the TRβ2-specific NH$_2$-terminal coding exon were used to amplify the 129 embryonic stem (ES) cell genomic DNA coding a probe that was used to used to screen a 129 P1 genomic DNA library (Genome Systems Inc., St. Louis, Missouri, USA). A 70-kb fragment of the TRβ2 genomic locus was obtained, and an 8-kb *Bam*HI fragment containing the TRβ2-specific exon was subcloned as 2 contiguous *Bam*HI-*Eco*RI fragments (TRβ2 L and TRβ2 R, respectively). TRβ2 L contained the TRβ2-specific exon, the presence of which was confirmed by sequencing. A 1-kb fragment extending from an *Nco*I site 77 bp upstream of the transcription start
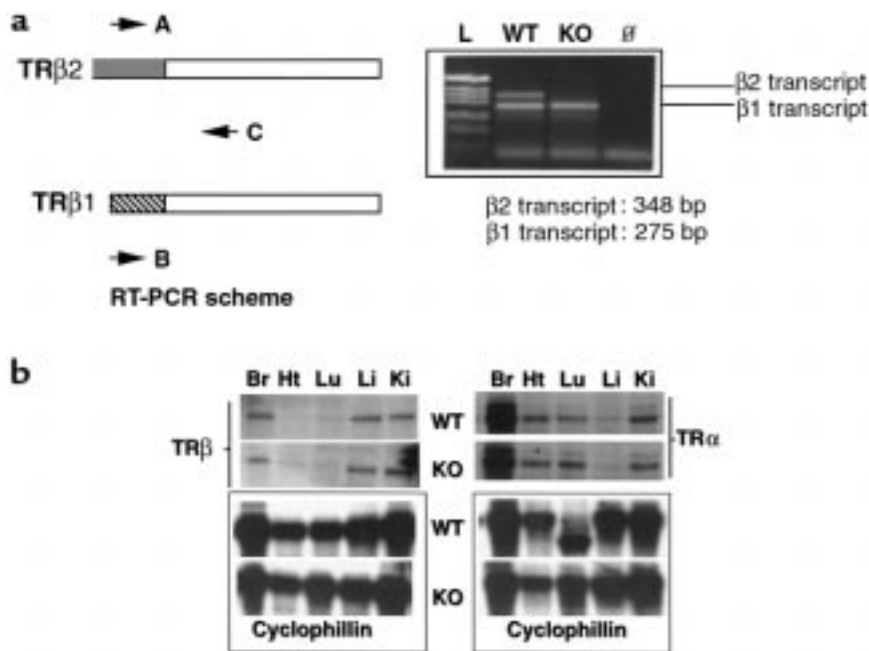
site to a *Sac*I site approximately 600 bp downstream of the end of the exon was excised, and a cDNA cassette containing the neomycin resistance gene under the influence of the phosphoglycerate kinase promoter was ligated into this space (Figure 1a). This maneuver thus eliminated the transcription start site, the entire TRβ2-specific exon, its splice donor acceptor site, and approximately 600 bp of the adjacent intron. TRβ2 L + Neo was then subcloned into a vector (pBluescript; Stratagene, La Jolla, California, USA) that contained TRβ2 R, thereby adding an additional 4.5 kb of homologous intronic DNA downstream of TRβ2 L + Neo. The vector was linearized with *Xho*I before electroporation into J1 ES cells (26, 27), and the cells were propagated in media containing G418.

*ES cell selection and generation of mutant mice.* A 700-bp *Eco*RI-*Bam*HI fragment immediately 5′ of the start of the targeting vector was obtained from a plasmid (gift of W.W. Wood, Health Sciences Center, University of Colorado, Denver, Colorado, USA) that contained 4.4 kb of the mouse TRβ2-specific locus as an *Eco*RI fragment. With this as a probe, an *Nco*I digest of genomic DNA reveals a 3.4-kb band for the endogenous allele and a 4.0-kb band for the targeted allele (Figure 1, a and b). Next, 211 G418-resistant colonies were screened. After expansion of these clones, genomic DNA was extracted using proteinase K digestion, high-salt precipitation, and DNA purification by ethanol precipitation. The DNA was digested with *Nco*I, and Southern blotting was performed after agarose gel electrophoresis. Homologous integration of the targeting construct was detected in 2 clones (76A and 107B), which were subsequently injected into 3-day-old blastocysts of the C57Bl 6 strain and then transferred into the uteri of pseudopregnant foster mothers. Chimeric offspring were successfully derived from clone 107B. Agouti off-spring from these chimera were genotyped by Southern blotting of genomic DNA isolated from tail clippings from 14- to 21-day-old animals. The DNA was extracted by overnight digestion with proteinase K and SDS, followed by high-salt precipitation, phenol chloroform extraction, and ethanol precipitation. DNA (10 μg) was digested with *Nco*I and then screened as described above for the ES cells. Offspring of the chimeric mice were either homozygous for the TRβ wild-type (WT) allele or heterozygous for the targeted allele. TRβ2-null (knockout [KO]) mice were then generated by crossing heterozygous mice. For all studies, the control mice used were either littermates of the TRβ2-null mice or offspring of WT littermates. Hence, all comparisons were made in mice of the same genetic background.

*RNA analysis.* Pituitary RNA was extracted from pooled pituitaries (5–8 animals) using guanidinium thiocyanate, selective precipitation, and isopycnic centrifugation with lithium chloride and cesium trifluoroacetate (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). For other tissues, RNA was extracted from tissue homogenates into a phenol and guanidinium thiocyanate solution (RNA Stat-60 reagent; Tel-Test Inc., Friendswood, Texas, USA) and then recovered by isopropanol precipitation. The presence of the TRβ1 transcript, and the presence or absence of the TRβ2 transcript, in pituitary RNA was established by performing RT-PCR on DNaseI-treated samples using the following 3 primers: TRβ2-specific 5′-ATG AAC TAC TGT ATG CCA GAG GTA-3′, denoted primer A; TRβ1-specific 5′-GCC TGG GAC AAG CAG AAG CCC CGT-3′, denoted primer B; and a 3′ primer common to TRβ1 and TRβ2: 5′-GTC TAA ATA GCT GGG CAT ATA CC-3′, denoted primer C (Figure 2a). PCR products were resolved on a 3% agarose gel. TRα and TRβ gene expression was assessed in the brain, heart, lung, liver, and
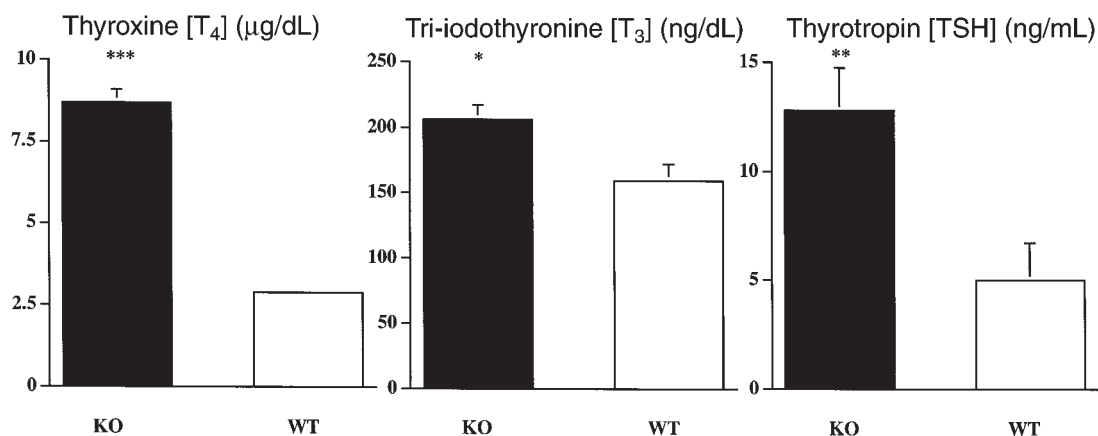
**Figure 3**
Basal serum concentrations of $T_4$, $T_3$, and TSH in KO and WT mice. Numbers of animals are 13 KO and 18 WT for $T_4$ assays, 18 KO and 14 WT for TSH assays, and 8 KO and 8 WT for $T_3$ assays. ***$P$ < 0.0001, **$P$ < 0.01, and *$P$ < 0.02 KO vs. WT.

kidney by RNase protection. Next, 20 µg of total RNA from each of these tissues was hybridized with α-$^{32}$P–labeled cRNA probes corresponding to sequences specific for the mouse TRα and TRβ, and processed using the Ribonuclease Protection Assay Kit (Ambion Inc., Austin, Texas, USA). The RNase-digested products were resolved on nondenaturing polyacrylamide gels. The TRα riboprobe was generated from a plasmid containing a 155-bp fragment of NH2-terminus of mouse TRα1. The fragment was obtained by RT-PCR of mouse brain total RNA using the primers 5′-GTG AAT GGA ACA GAA GCC AAG-3′ and 5′-CGT CTT TGT CCA GGT AAC TAG-3′, and was subcloned into the vector PGEM-T (Promega Corp., Madison, Wisconsin, USA). After verifying the sequence, the plasmid was linearized with *Nco*I, and the cRNA was transcribed with SP6 RNA polymerase. The TRβ riboprobe was derived from a 500-bp *Bam*HI-*Xba*I fragment of the TRβ genomic locus subcloned into PGEM-7 (Promega Corp.), which contained exon 6 and the flanking intronic sequence. The plasmid was linearized with *Bam*HI, and the cRNA was transcribed with T7 RNA polymerase. A cyclophillin riboprobe (Ambion Inc.) was used to control for loading. TSHβ and GH gene expression were assayed in pituitary total RNA by Northern blotting. In studies of TSHβ gene regulation, 5, 2.5, 1.25, and 0.625 µg of RNA obtained from hypothyroid mice were prepared by serial dilution. In studies of euthyroid and hyperthyroid mice, 15 µg of RNA was used. Samples were resolved on a 1.2% formaldehyde agarose gel, transferred to a nylon membrane, and hybridized to the entire cDNA of the β subunit of murine TSH (labeled with [α-$^{32}$P]dCTP) under high-stringency conditions. For the GH experiments, 5 µg of RNA was hybridized under high-stringency conditions to an α-$^{32}$P–labeled cDNA probe corresponding to 790 bp of the rat GH gene.

*$T_3$ suppression and hypothyroidism.* Blood was obtained for total thyroxine ($T_4$) and TSH from the tail vein. Experimental hypothyroidism was induced by admin-istering 150 µCi of $^{131}$I by intraperitoneal injection to mice who had been placed on a low-iodine diet (Harland Teklad Laboratory, Madison, Wisconsin, USA) for 8 days. Experiments on hypothyroid animals were performed 3 weeks after the administration of $^{131}$I, as all mice develop undetectable $T_4$ concentrations by the third week. Suppression of pituitary TSH production was attempted by daily intraperitoneal injections of $T_3$ (1 µg/mL) in buffered HEPES for 3 weeks at doses of 0.2 µg/100 g body weight during the first week, 0.5 µg/100 g body weight during the second week, and 1.0 µg/100 g body weight during the third week. Comparisons were made between KO mice and age-matched, WT controls of the same genetic background. Serum samples were obtained and pituitaries harvested on the day of the final $T_3$ dose. For studies of the regulation of GH gene expression by thyroid hormone, KO and control mice were treated for 3 days with 10 µg of $T_3$ per day by intraperitoneal injection, after which pituitaries were harvested for RNA. All aspects of animal care and experimentation performed in this study were approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Animals were maintained on a 12-hour light/12-hour dark schedule (light on at 0600 hours) and fed laboratory chow and water ad libitum.

*Hormone assays.* Total $T_4$ levels were measured in 10-µL serum samples in duplicate determinations by a mouse-specific RIA (ImmuChem-coated tube, $T_4$ $^{125}$I RIA Kit; ICN Pharmaceuticals, Costa Mesa, California, USA). Total $T_3$ was measured in 100-µL serum samples by RIA (ImmuChem-coated tube $T_3$ $^{125}$I RIA Kit; ICN Pharmaceuticals). TSH was measured in 50-µL serum samples in triplicate determinations by a specific mouse TSH RIA (Anilytics, Gaithersburg, Maryland, USA), reference preparation (AFP51718mp), a mouse TSH antiserum (AFP98991), and rat TSH antigen for radioiodination (NIDDK-rTSH-I-9) (all obtained from A.F. Parlow, Harbor University of California–Los Angeles Medical Center, Torrance, California, USA).

*Analysis of hearing.* To record auditory brainstem responses (ABRs), mice were anesthetized with xylazine (50 mg/kg intraperitoneally) and ketamine (100 mg/kg intraperitoneally). The external auditory canal was slit to provide a direct view of the eardrum, and a closed, calibrated acoustic system was positioned near the drum. Needle electrodes were inserted (vertex and pinna, with a ground near the tail), and responses were amplified (×10,000), filtered (0.3–3.0 kHz band pass), digitized, and averaged (1,024 responses, with equal numbers of opposite stimulus polarity) with custom software, including artifact rejection to eliminate large cardiac potentials. Stimuli were 5-millisecond tone pips (0.5 millisecond rise-fall, with $cos^2$ shaping) delivered at 40 per second. At each test frequency, sound pressure was varied in 5-dB steps from 5-dB sound pressure levels (SPLs) up to at least 20 dB above threshold, as defined by visual inspection of the response waveforms.

*Statistical analysis.* Differences were assessed by 1-tailed Student's *t* test (2-sample unequal variance).

## Results

*Generation of mice lacking TRβ2.* The targeting vector (Figure 1a), in which the TRβ2-specific exon was replaced by the neomycin resistance gene driven by the phosphoglycerate kinase promoter, was electroporated into J1 ES cells (26, 27). The targeted allele was readily detected by Southern blot analysis (Figure 1b). A total of 211 ES cell colonies were screened, and homologous recombination occurred in 2 clones (76A and 107B). After expansion, these clones were injected into

C57Bl6 blastocysts. Only ES cells from clone 107B successfully passed through the germ line of chimeric mice. Initial breeding yielded 115 mice. If the inheritance were mendelian, the expected frequency of WT, heterozygous, and homozygous (KO) mice would be 28, 57, and 27 respectively. We observed 24 WT, 62 heterozygous, and 29 homozygous mice, which is consistent with the expected mendelian frequency of inheritance. Hence, deletion of the TRβ2 exon was not associated with impaired embryonic survival. To demonstrate that our targeting strategy resulted in the selective loss of TRβ2 expression, we performed RT-PCR using isoform-specific 5′ primers and a common 3′ primer (Figure 2a). We were able to amplify the TRβ1, but not the TRβ2, transcript from pituitary RNA obtained from homozygous mice, indicating that our targeting strategy successfully inactivated TRβ2 expression. In WT mice, both transcripts were amplified. The RT-PCR fragments were subcloned, and sequence analysis verified that the 2 transcripts obtained in WT mice corresponded to TRβ1 and TRβ2, respectively, whereas the transcript in the KO mice was TRβ1. Although not quantitative, the RT-PCR analysis suggests that the levels of TRβ1 expression in the pituitaries of WT and KO mice were similar. TRα1-specific primers were also used to demonstrate that TRα expression was preserved in the pituitaries of TRβ2-null mice (data not shown).

*Impact on TRα and TRβ expression.* To assess more carefully the impact of our targeting strategy on TRβ and TRα gene expression, RNase protection assays were performed on total RNA obtained from the brains,
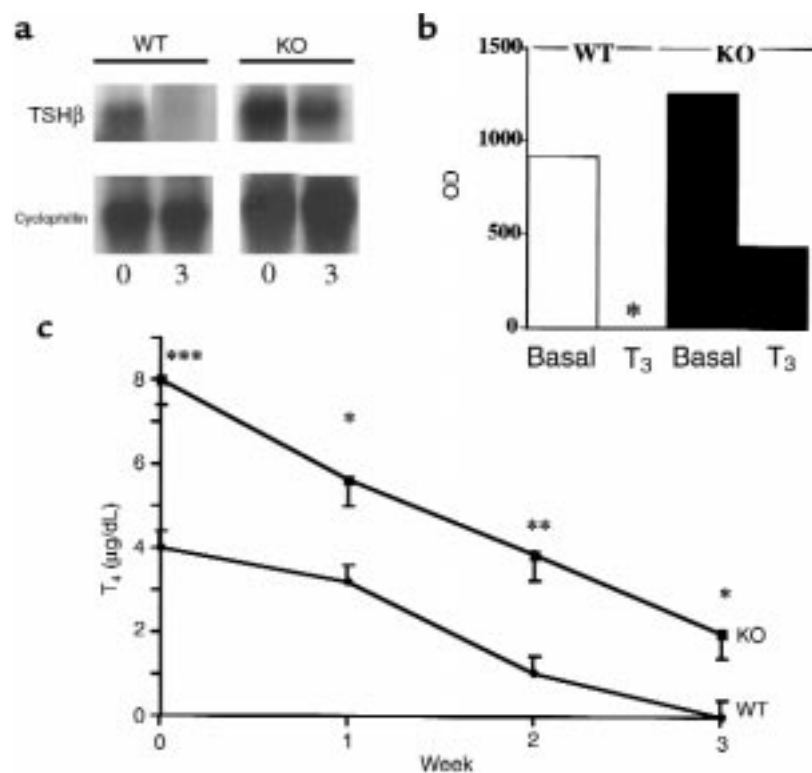


**Figure 4**
TSH and $T_4$ response to $T_3$ administration. (**a**) Northern blots of pituitary RNA from WT and KO mice at baseline (0) and after 3 weeks of $T_3$ administration (3). Blots were probed for TSHβ and cyclophillin. Note the complete suppression of TSHβ message by $T_3$ in WT mice, and the partial suppression in KO mice. (**b**) Densitometric analysis of TSHβ Northern blots corrected for loading with cyclophillin densities. *WT TSHβ mRNA after $T_3$ suppression was not detectable above background counts. (**c**) Serum $T_4$ measured at weekly intervals in KO ($n = 8$) and WT ($n = 6$) mice. Mice were injected daily with $T_3$ for the entire 3-week period. *$P < 0.02$, **$P < 0.01$, ***$P < 0.001$ vs. WT.
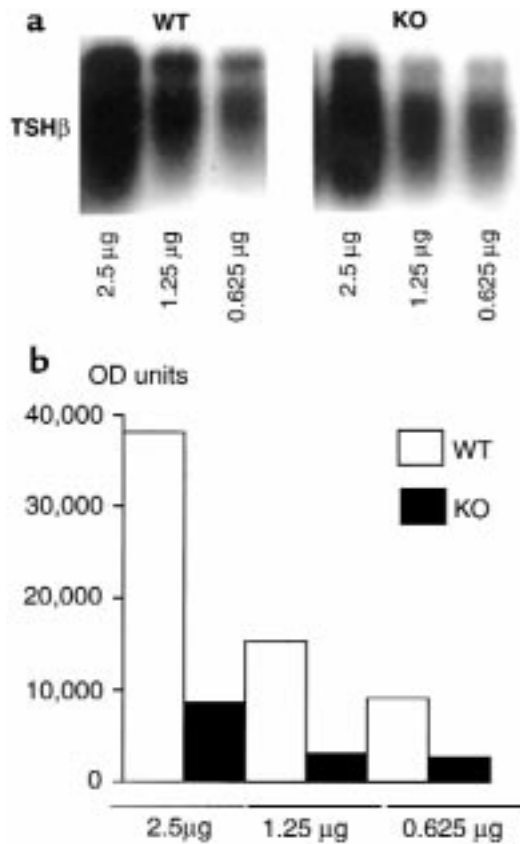
**Figure 5**
TSH response to hypothyroidism. (**a**) Northern blot of total RNA obtained from pooled pituitaries (*n* = 5 for WT and KO), demonstrating TSHβ mRNA responses in hypothyroid WT and KO mice. Amount of RNA loaded in each lane (in micrograms) is shown. (**b**) Densitometry of TSHβ mRNA abundance corrected for loading by hybridizing the same membranes with a cDNA for actin. Note the reduced response to hypothyroidism in KO vs. WT mice.

heart, lungs, livers, and kidneys of WT and KO mice. The TRα riboprobe was generated from a plasmid containing a 155-bp fragment of the $NH_2$-terminus of mouse TRα1. The TRβ riboprobe was derived from a 500-bp *Bam*HI-*Xba*I fragment of the TRβ genomic locus that contained exon 6 and the flanking intronic sequence. In both WT and KO mice, TRα expression was highest in brain, but also significant in the heart and kidney. In contrast, TRβ expression was most prominent in the liver, kidney, and brain. The pattern of TR expression was qualitatively similar between the WT and the KO mice (Figure 2b).

*Effect of loss of TRβ2 on basal thyroid hormone concentrations.* Absence of TRβ2 was associated with impairment in the regulation of TSH. Basal $T_4$ concentrations were 2- to 3-fold higher in KO mice compared with age-matched littermate controls (8.7 ± 0.4 μg/dL) versus WT mice (2.9 ± 0.1 μg/dL) (*P* < 0.0001). $T_3$ concentrations were increased by 40% (206.3 ± 11.0 ng/dL) versus WT mice (159.5 ± 12.9 ng/dL) (*P* < 0.02; Figure 3). The elevated thyroid hormone concentrations were associated with inappropriate TSH production. TSH concentra-

tions were 2.5-fold higher in KO mice (12.8 ± 1.9 ng/mL) versus WT mice (5.0 ± 1.7 ng/mL) (*P* < 0.005), which is compatible with resistance at the level of the pituitary (Figure 3). At the level of gene expression, TRβ2 KO mice exhibited increased levels of TSHβ mRNA (Figure 4a). There was no discernible effect, however, on α-subunit gene expression (data not shown).

*Impact on pituitary responsiveness to thyroid hormone.* To confirm the presence of central resistance to thyroid hormone, KO and control mice were treated for 3 weeks with pharmacological doses of $T_3$ to suppress pituitary TSH secretion. Serum $T_4$ concentrations were measured at weekly intervals, and TSHβ mRNA was measured in pituitaries obtained from WT and KO mice before and at the end of the 3-week period of $T_3$ administration. WT mice exhibited a progressive decline in $T_4$ concentrations throughout the treatment period; at the end of 3 weeks, the concentrations of $T_4$ were undetectable (Figure 4c). In a similar fashion, TSHβ gene expression was completely obliterated by $T_3$ administration in WT mice (Figure 4, a and b). In contrast, TRβ2 KO mice exhibited only partial suppression of $T_4$, with easily measurable concentrations (>2 μg/dL) after the 3-week period of $T_3$ treatment (Figure 4c). There was also resistance at the level of the TSHβ gene, with KO mice revealing persistent TSHβ gene expression despite receiving 3 weeks of pharmacological $T_3$ (Figure 4, a and b).

*Impact on ligand-independent activation of the TSHβ gene in vivo.* To assess the role of TRβ2 in mediating the activation of TSHβ-subunit gene expression in the absence of thyroid hormone, mice were rendered hypothyroid by administration of 150 μCi of [131]I. After 3 weeks, all mice exhibited $T_4$ concentrations below the limit of detection of the $T_4$ assay (<0.5 μg/dL). Pituitary RNA was obtained from hypothyroid WT and KO mice, and TSHβ expression was assessed by Northern blotting. TSHβ message was easily detected in 2.5, 1.25, and 0.625 μg of total pituitary RNA. It is very difficult to detect TSHβ message in these small concentrations of total RNA in euthyroid mice (data not shown). Hence, there was clear induction of TSHβ message by hypothyroidism in both sets of mice. However, the abundance of TSHβ mRNA was 4-fold greater in WT mice than in KO mice at all concentrations of RNA examined (Figure 5, a and b). Thus, in the absence of TRβ2, the degree of ligand-independent activation of the TSHβ gene is impaired in vivo.

*Impact on GH gene expression.* Given the expression of TRβ2 in the somatotroph (28), and the positive regulation of GH by $T_3$ (29), Northern blot analysis of GH gene expression was performed in control and KO mice at ambient $T_4$ levels and after 3 days of high-dose $T_3$ administration. TRβ2 KO mice exhibited a modest reduction (~30%) in GH gene expression. After $T_3$ administration, GH expression increased by 60% in WT mice and by 30% in TRβ2 KO mice, suggesting reduced $T_3$ responsiveness (Figure 6).

*Effect on hearing.* Given the profound impact of TRβ loss on hearing (25), we examined the ABRs in 8-week-old KO and WT mice. Surprisingly, TRβ2 KO mice did

not exhibit any deficit in hearing at any of the frequencies tested. In fact, the ABR thresholds between WT and KO mice were overlapping (Figure 7). The 1 elevated threshold value, seen at the highest test frequency in 1 WT mouse, presumably represents the onset of premature, age-related hearing loss, a progressive early onset hearing loss that has been well studied in the C57Bl6 strain (30).

## Discussion

We have successfully inactivated the $\beta2$ isoform of the TR. Our strategy was based on the fact that TR$\beta1$ and TR$\beta2$ differ only in their $NH_2$-termini and that these are encoded by distinct exons (5–7). The relative location of the exons encoding the TR$\beta1$ and TR$\beta2$ $NH_2$-termini are not known. It is also likely that in addition to alternate exon use, distinct promoters drive the expression of these 2 TR isoforms (6, 31, 32). It is clear from our data that disruption of the TR$\beta2$-specific exon does not interfere with the splicing of the TR$\beta1$-specific exons and the shared common exons. The selective targeting of TR$\beta2$ also did not affect the spatial expression of the TR$\beta1$ gene product. Although we did not examine the temporal expression of TR$\beta1$ in this study, our data strongly suggest that the regulatory elements governing TR$\beta1$ expression are not located within the TR$\beta2$-specific exon, nor in the first 600 bp of the adjacent intron that was also inactivated by our targeting strategy. This targeting strategy ensured that the entire coding sequence of the unique TR$\beta2$ $NH_2$-terminus was deleted. In fact, we were unable to detect by RT-PCR the existence of a truncated $NH_2$-terminal fragment that could potentially act as a dominant inhibitor. Therefore, these mice enabled us to define the physiological role of TR$\beta2$ in the regulation of the hypothalamic-pituitary-thyroid axis, and provided novel insight into differential roles of TR$\beta1$ and TR$\beta2$ in the regulation of auditory function.

The selective disruption of TR$\beta2$ confirms the important role that this isoform plays in the regulation of the hypothalamic-pituitary-thyroid axis. The 2- to 3-fold increase in thyroid hormone concentration seen in mice lacking TR$\beta2$ is similar to that reported in mice lacking the entire TR$\beta$ locus (21). The absolute hormone concentrations are not comparable, because our control values are lower than those reported by Forrest et al. (21). Nevertheless, it appears that the absence of TR$\beta2$ can account in large part for the hormonal abnormalities described in TR$\beta$-null mice. The residual TR$\beta1$ is therefore unable to compensate for the absence of TR$\beta2$. This may reflect qualitative differences in the ability of TR$\beta1$ to mediate the negative regulation of TSH by thyroid hormone. This possibility is supported by a large body of in vitro data that suggest that TR$\beta2$ is a more potent negative regulator of the TSH-subunit genes than is TR$\beta1$ (16, 17). However, the lack of TR$\beta2$ does not completely abrogate the responsiveness of the thyrotroph to thyroid hormone, as evidenced by the partial suppression of TSH$\beta$ mRNA after 3 weeks of $T_3$
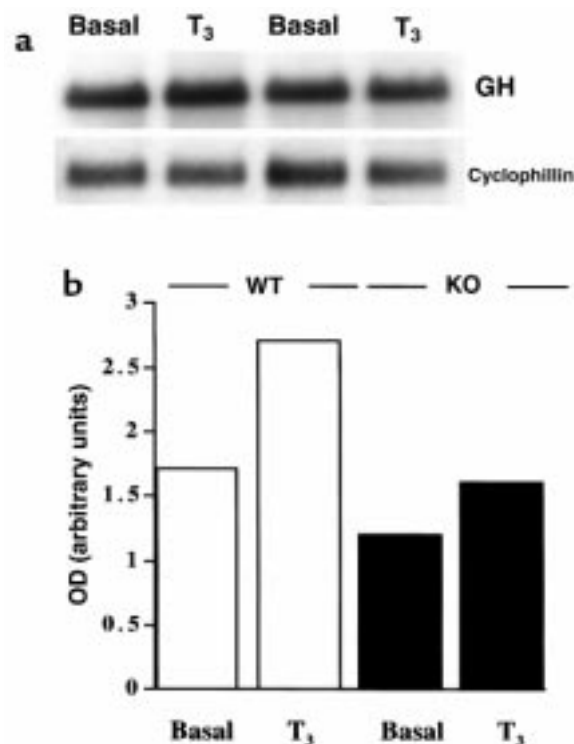


**Figure 6**
GH gene expression. (**a**) Representative Northern blot of total RNA obtained from pooled pituitaries for WT (first 2 lanes) and KO (last 2 lanes), demonstrating GH mRNA at ambient (basal) $T_4$ concentrations and after administration of high doses of $T_3$. Blots were also probed for cyclophillin. Five micrograms of RNA is loaded in each lane. Each lane represents RNA extracted from 3 pituitary glands. (**b**) Densitometry of GH mRNA abundance corrected for loading by hybridizing the same membranes with a cDNA for cyclophillin. Note the reduced basal GH expression and the blunted response to $T_3$.

treatment. The interpretation of the decline in $T_4$ levels is more complex. The fall in $T_4$ not only reflects impaired TSH suppression, but also reflects the ability of $T_3$ to increase the peripheral clearance of $T_4$ (33, 34). This is one possible explanation for the similarity in the slopes of the decline in $T_4$ in WT and TR$\beta2$-null mice. The important difference, though, is the presence of measurable $T_4$ in the TR$\beta2$-null animals at a time when $T_4$ levels are undetectable in WT mice. We believe that the persistence of $T_4$ in the TR$\beta2$-null mice after $T_3$ treatment is due to persistence of TSH.

The partial suppression of TSH in response to $T_3$ administration suggests that the residual TR$\beta1$ and TR$\alpha1$ are capable of mediating $T_3$-induced repression of the TSH-subunit genes. The recent reports of markedly elevated thyroid hormone concentrations in mice lacking both TR$\alpha$ and TR$\beta$ isoforms support this (35, 36). However, our data indicate that TR$\beta2$ is required to mediate complete suppression of the axis by thyroid hormone. Whether or not TR$\beta2$ alone can mediate all of the negative regulation of TSH production by thyroid hormone in vivo will only come from studies in mice with ablation of TR$\beta1$ and TR$\alpha$, but
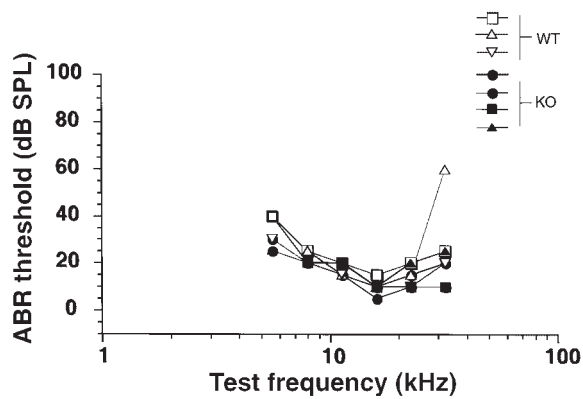
**Figure 7**
Evidence that cochlear function is normal in TRβ2 KO mice. ABRs in 8-week-old KO (*n* = 4) and WT (*n* = 3) mice. Threshold SPLs (0.0002 dyn/cm²) required for the detection of an ABR were obtained across a range of test-tone frequencies. Note that at all frequencies tested, TRβ2 KO mice exhibit no abnormality in ABR thresholds.

with preserved expression of TRβ2. The demonstration that the central resistance to thyroid hormone occurring in TRβ2 KO mice is due in part to impaired downregulation of TSHβ gene expression by $T_3$ does not preclude the possibility of impaired regulation at the level of the TRH neuron, given the expression of TRβ2 in the $T_3$-responsive TRH neurons of the paraventricular hypothalamus (PVN) (9). Our model now provides an important resource with which to analyze the role of TRβ2 in the regulation of TRH expression in the PVN over a wide range of thyroid hormone concentrations.

An important new finding from this study is the demonstration in vivo that TRβ2 is a critical mediator of the ligand-independent activation of TSHβ gene expression. In transfection experiments, we demonstrated previously that TRβ2 is a much more potent mediator of ligand-independent activation of the TSH-subunit and TRH genes than either TRβ1 or TRα1 (17, 18, 20). A potential mechanism for the differential activation capabilities of the TR isoforms on negatively regulated genes may reside in differential interactions with accessory proteins such as corepressors and coactivators. We have demonstrated previously that the ability of TRβ2 to mediate ligand-independent activation is independent of its interactions with N-CoR (20). In contrast, N-CoR binding limits the ligand-independent activation capability of TRβ1 and TRα1, and cotransfection of a potent dominant inhibitor of N-CoR converts these isoforms into strong mediators of ligand-independent activation (20). The molecular mediators of the ligand-independent activation of the TSHβ gene by TRs are currently unknown. It is likely that the enhanced potency of TRβ2 may reside in enhanced activation via its interaction with coactivator molecules.

Although our findings of impaired in vivo ligand-independent activation of the TSHβ gene strongly support earlier in vitro evidence that TRβ2 is the important mediator of ligand-independent activation, 2

additional possibilities warrant discussion. First, because TRβ2 is the most abundant TR isoform in the pituitary, loss of TRβ2 reduces the total number of pituitary TRs. Thus, the observed phenotype could simply represent the result of a net reduction in pituitary TR content. To ultimately prove that this defect is specific to the TRβ2 isoform, mice that selectively lack TRβ1 and TRα1 while retaining TRβ2 expression would need to be studied. If TRβ2 is the key mediator of ligand-independent activation of TSHβ in vivo, then such animals would be expected to exhibit no defects in the TSH response to hypothyroidism. Second, it could be argued that the impaired induction of TSHβ expression by hypothyroidism in the TRβ2-null mice may simply be due to the fact that basal TSHβ gene expression is already increased. We believe that this is unlikely because of our observations in transgenic mice with pituitary expression of a mutant TR transgene (37). Like the TRβ2-null mice, these animals exhibit pituitary resistance with a similar increase in basal TSH concentration. These animals increase their TSH concentrations 40-fold above baseline in response to hypothyroidism, indicating that despite higher basal TSH levels, thyrotroph responsiveness is retained. It is of interest that these transgenic mice with pituitary expression of a mutant TR express 40% less TSHβ mRNA than controls with similar degrees of hypothyroidism, which contrasts with the 75% decrease observed in the TRβ2 KO mice. Thus, the impact of the absence of the receptor on ligand-independent activation in vivo is greater than that observed in the presence of a powerful dominant-negative mutant TR. These 2 independent lines of evidence indicate that ligand-independent activation of the TSH-subunit genes can be modulated in vivo by altering TR expression and function, and they provide compelling evidence that TRs mediate ligand-independent activation of the TSHβ gene in vivo.

Thyroid hormone has long been known to be a potent inducer of GH gene expression (29, 38, 39). TRβ2 is expressed in the somatotroph (28), and there is evidence from studies in pituitary cell lines that TRβ2 is the major isoform mediating the stimulatory effect of thyroid hormone on GH gene expression (40). Furthermore, in the absence of thyroid hormone, TRβ2 exhibits potent ligand-independent activation of GH expression — in contrast to TRβ1, which mediates ligand-independent repression (15, 40). The TRβ2 KO mice therefore provided a unique model with which to evaluate the role of TRβ2 in the regulation of GH gene expression by thyroid hormone in vivo. Interestingly, TRβ2 KO mice exhibited decreased basal GH expression and a blunted response to $T_3$. The small decrease in basal GH expression is very similar to the decrease in GH expression recently reported in mice lacking both TRβ isoforms (36). This raises the possibility that the changes in GH expression in TRβ-null mice can be accounted for by loss of the TRβ2 isoform. It is important to note, however, that mice lacking both the TRα and TRβ isoforms exhibit profound

downregulation of GH expression (36), indicating that TRα and TRβ isoforms are both important in GH regulation by thyroid hormone.

Thyroid hormone plays an important role in auditory function. TRα and TRβ are expressed in the developing ear; however, the expression of TRβ is restricted to the cochlea (10). Congenital hypothyroidism in humans is associated in many cases with deafness. In rodent models of congenital hypothyroidism, this is associated with structural abnormalities of the cochlea (41–43). Genetic syndromes associated with loss of TRβ also result in deafness, which was a striking characteristic of the initial patient described with resistance to thyroid hormone; this patient had a homozygous deletion of TRβ (44). More recently, Forrest et al. (21) have demonstrated that genetic deletion of the entire TRβ locus in mice results in variable amounts of hearing loss. On average, ABR thresholds in TRβ-null mice were elevated by 20–40 dB. In individual animals, however, the threshold elevations ranged from a few decibels to complete loss of auditory responsiveness (25). There were no gross structural cochlear abnormalities seen, implying that TRβ plays an important role in the maturation and maintenance of normal cochlear function but not in morphogenesis. Recent evidence suggests that the basis for the impaired hearing in TRβ-null mice is a developmental delay in the expression of the fast-activating potassium conductance ($I_{K,f}$) in inner hair cells (45). Bradley et al. (10) demonstrated coexpression of TRβ1 and TRβ2 in the developing cochlea in rats. Our mice, with selective deletion of the TRβ2 isoform of the TR, reveal that TRβ1 is sufficient to mediate normal cochlear development and function. These observations highlight important isoform-specific differences in the regulation of hearing by TRβ. Therefore, it will be important to determine whether the developmental regulation of $I_{K,f}$ is normal in TRβ2-null mice.

The lack of an impact of TRβ2 deletion on hearing stands in contrast to its important role in the pituitary. The precise molecular mechanisms by which TRs regulate the development of hearing are unknown. As the target genes for the TR are identified in the cochlea, important insights into the factors governing the isoform-specific differences in gene regulation are likely to be obtained. A potential reason for the differential effects may lie in differences in the relative expression and relative roles of accessory cofactors in the ear versus the pituitary. It is known that retinoid receptors are expressed within the developing ear and are important heterodimer partners with the TRs on positively regulated genes (46). We have previously shown that TRβ1 and TRβ2 exhibit distinct functional interaction with RXRs and N-CoR (19, 20). On negative TREs, the transcriptional activity of TRβ1 can be modified by liganded RXR, whereas that of TRβ2 cannot (19). We have also shown that ligand-independent activation by TRβ1 and TRα1 is masked by corepressors, whereas the ligand-independent activation by TRβ2 is not (20). Hence, tissue-specific differences in the expression and

distribution of accessory cofactors may partly explain the differential roles of these TR isoforms in tissues in which they are coexpressed.

In summary, these studies have demonstrated that in keeping with its restricted expression, TRβ2 plays a central role in the regulation of the hypothalamic-pituitary-thyroid axis, and some role in the regulation of GH. In the thyrotroph, the function of TRβ2 cannot be replaced by the other TR isoforms. In the auditory system, however, normal hearing develops in the absence of TRβ2.

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