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Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor β: evidence for tissue-specific modulation of receptor function

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The diverse functions of thyroid hormone (T3) are presumed to be mediated by two genes encoding the related receptors, TRα and TRβ. However, the *in vivo* functions of TRα and TRβ are undefined. Here, we report that targeted inactivation of the mouse TRβ gene results in goitre and elevated levels of thyroid hormone. Also, thyroid-stimulating hormone (TSH), which is released by pituitary thyrotropes and which is normally suppressed by increased levels of thyroid hormone, was present at elevated levels in homozygous mutant (*Thrb*$^{-/-}$) mice. These findings suggest a unique role for TRβ that cannot be substituted by TRα in the T3-dependent feedback regulation of TSH transcription. *Thrb*$^{-/-}$ mice provide a recessive model for the human syndrome of resistance to thyroid hormone (RTH) that exhibits a similar endocrine disorder but which is typically caused by dominant TRβ mutants that are transcriptional inhibitors. It is unknown whether TRα, TRβ or other receptors are targets for inhibition in dominant RTH; however, the analysis of *Thrb*$^{-/-}$ mice suggests that antagonism of TRβ-mediated pathways underlies the disorder of the pituitary–thyroid axis. Interestingly, in the brain, the absence of TRβ may not mimic the defects often associated with dominant RTH, since no overt behavioural or neuroanatomical abnormalities were detected in *Thrb*$^{-/-}$ mice. These data define *in vivo* functions for TRβ and indicate that specificity in T3 signalling is conferred by distinct receptor genes.

Keywords: c-*erb*A/genetic disease/thyroid hormone receptor/transcription

Introduction

The thyroid gland produces thyroid hormone at the appropriate levels required for development and for homeostatic maintenance in adults (Oppenheimer and Samuels, 1983; Tata, 1993). Congenital thyroid disorders resulting in either insufficient or excessive levels of thyroid hormone can lead to serious defects, for example in development of the brain and auditory system. Thus, precise regulation of the function of the thyroid gland is essential. The thyroid gland is under the control of thyroid-stimulating hormone (TSH) which is released by the pituitary thyrotropes. Fluctuations in circulating levels of thyroid hormone are strictly regulated by a feedback loop in which increased thyroid hormone levels suppress TSH expression, which in turn reduces thyroid activity. The importance of this control is evident in the syndrome of resistance to thyroid hormone (RTH), a genetic disease characterized by a complex range of symptoms including goitre, pituitary dysfunction and neurological disorders (Refetoff *et al.*, 1967). A salient feature of RTH is the malfunction of the pituitary–thyroid axis, which results in elevated levels of both thyroid hormone and TSH (Refetoff *et al.*, 1993).

The identification of two genes encoding the related thyroid hormone receptors, TRα and TRβ (Sap *et al.*, 1986; Weinberger *et al.*, 1986), suggested that the expression of distinct TR genes may determine the specificity of the cellular response to thyroid hormone (Forrest, 1994). TRα and TRβ are nuclear hormone receptors that consist of a central DNA binding region and a C-terminal domain that binds the thyroid hormone triiodothyronine (T3). TRα and TRβ act as T3-dependent transcription factors, either inducing or repressing gene expression in response to T3, although they also display a form of T3-independent repression of transcription (Damm *et al.*, 1989). Both can bind to target DNA sequences either as homodimers or as heterodimers with retinoid X and other nuclear receptors (Stunnenberg, 1993; Chambon, 1994). The TRα gene is widely expressed from early developmental stages, whereas the TRβ gene is highly restricted until later in embryogenesis when it is induced in brain, pituitary and other tissues (Forrest *et al.*, 1990; Strait *et al.*, 1990; Bradley *et al.*, 1992). Two N-terminal receptor variants, TRβ1 and TRβ2, are generated by differential promoter utilization and alternative splicing (Hodin *et al.*, 1989; Wood *et al.*, 1991; Sjöberg *et al.*, 1992). The differential expression of the TR genes suggests that they mediate distinct functions, with TRβ playing a specific role in tissues that include the nervous system and pituitary. However, since the TRα gene is also expressed in these tissues, the extent to which the individual genes provide unique or overlapping *in vivo* functions is not clear.

The analysis of naturally occurring mutant TR genes in disease states has yielded some clues about the *in vivo* functions of TRα and TRβ. RTH is typically an autosomal dominant disease caused by mutations in the C-terminus of TRβ that impair its response to T3 (Usala *et al.*, 1988; Refetoff *et al.*, 1993). The mutant receptors are capable of forming dimers that bind to DNA but they are defective

in T3-dependent transcriptional regulation. Furthermore, they inhibit transactivation by wild-type TRβ, TRα and retinoic acid receptors and they inhibit T3-dependent transcriptional repression mediated by wild-type TRs in co-transfection assays (Sakurai *et al.*, 1990; Chatterjee *et al.*, 1991; Baniahmad *et al.*, 1992; Nagaya and Jameson, 1993; Ng *et al.*, 1995). These observations have led to the hypothesis that RTH arises from the antagonism of one or more of the normal receptor gene products. In one unusual kindred, with a particularly complex array of symptoms, RTH was found to be recessive (Refetoff *et al.*, 1967). This kindred carried a genomic rearrangement that probably deleted several genes including the TRβ gene (Takeda *et al.*, 1992). However, since the rearrangement was extensive and undefined, no phenotype could be attributed to one particular missing gene.

Therefore, to analyse the *in vivo* function of TRβ and its role in RTH, we generated mice with a targeted disruption of the TRβ gene. The phenotype of these mice identifies a key role for TRβ, that cannot be substituted by TRα, in regulation of the pituitary–thyroid axis. This mutant mouse also represents a recessive model for RTH, which demonstrates that the loss of TRβ results in a similar endocrine disorder to that associated with dominant RTH. In contrast, in the nervous and auditory systems, the loss of TRβ has consequences different from those associated with dominant TRβ mutations in human RTH. These findings suggest the existence of tissue-specific mechanisms that modulate the action of TRβ both in normal situations and in RTH.

Results

Targeted disruption of thyroid hormone receptor β gene

To inactivate the mouse TRβ gene (*Thrb*), a 3.0 kb deletion was introduced into the exon encoding the first zinc finger of the DNA binding domain (exon 3; Figure 1A). This was predicted to preclude expression of any functional protein since the mutation disrupted the essential DNA binding and T3 binding domains. Embryonic stem cells were electroporated with the targeting vector and resistant colonies were screened by Southern blot analysis to identify clones that had undergone correct targeting by homologous recombination. These were used to generate chimeric mice that transmitted the mutation through the germline.

Heterozygous ($Thrb^{-/+}$) mice derived from the chimeras were intercrossed to generate litters containing homozygous progeny. These litters contained $Thrb^{+/+}$, $Thrb^{-/+}$ and $Thrb^{-/-}$ progeny segregating with Mendelian frequencies (n = 139, 269 and 121 progeny or 26, 51 and 23%, respectively), demonstrating that the mutation was not deleterious to survival. Figure 1B shows the genotypes recorded using the 3′ probe (see Figure 1A) for Southern blot analysis of DNA digested with *Bam*HI and *Eag*I. The 19.0 kb wild-type band was absent in homozygotes, which carried instead a single mutant fragment of 10.0 kb. The structure of the mutant allele was confirmed by analysis with several independent probes. Figure 1C shows a representative analysis of *Kpn*I-digested DNA. The 5′ probe (see Figure 1A) detected a wild-type band of 3.8 kb and a mutant band of 6.5 kb, both of which were present in heterozygotes, whereas the *neo* probe detected the 6.5 kb band only in homo- and heterozygotes. This demonstrated that a single *neo* integration was linked to the mutant allele as predicted.

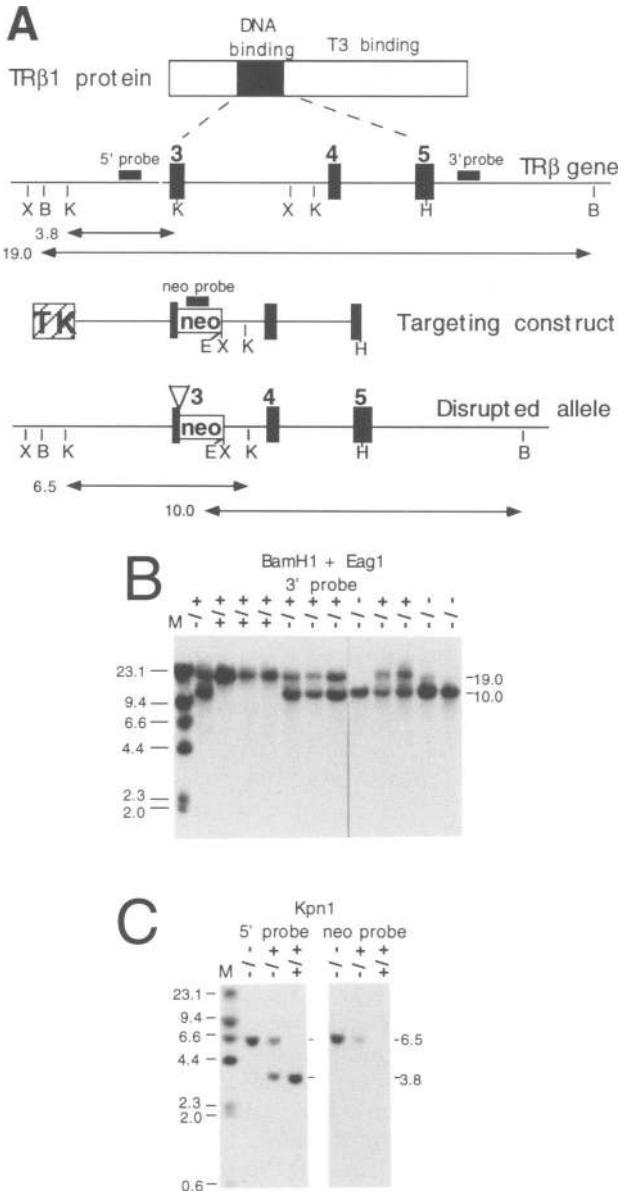

Fig. 1. Disruption of the TRβ gene by homologous recombination. (**A**) Diagram of the TRβ1 protein showing the DNA binding domain (black) and T3 binding domain and, below this, the part of the gene encoding the DNA binding region (exons 3, 4 and 5). The targeting construct contained a 3.0 kb deletion including part of exon 3 and adjacent intron sequences. Both the *neo* and *tk* selectable markers were in the same transcriptional orientation as the TRβ gene. The structure of the mutant allele is shown on the bottom line. The 5′, 3′ and *neo* probes used for Southern blot analyses and the fragment sizes detected with the 3′ probe upon digestion with *Bam*HI and *Eag*I and with the 5′ and *neo* probes upon digestion with *Kpn*I are indicated. Relevant restriction enzyme sites: X, *Xba*I; B, *Bam*HI; K, *Kpn*I; H, *Hin*dIII; E, *Eag*I. (**B**) Genotypes of progeny of $Thrb^{-/+}$ intercrosses. DNA samples digested with *Bam*HI and *Eag*I were analysed with the 3′ probe to identify three $Thrb^{+/+}$, six $Thrb^{-/+}$ and three $Thrb^{-/-}$ mice. (**C**) Analyses with 5′ and *neo* probes on *Kpn*I-digested DNA from representative $Thrb^{-/-}$, $Thrb^{-/+}$ and $Thrb^{+/+}$ individuals. The predicted wild-type 3.8 kb and mutant 6.5 kb bands were detected with the 5′ probe. The single mutant band and absence of bands in $Thrb^{+/+}$ DNA detected with the *neo* probe demonstrated the absence of additional, random insertions of the targeting vector.

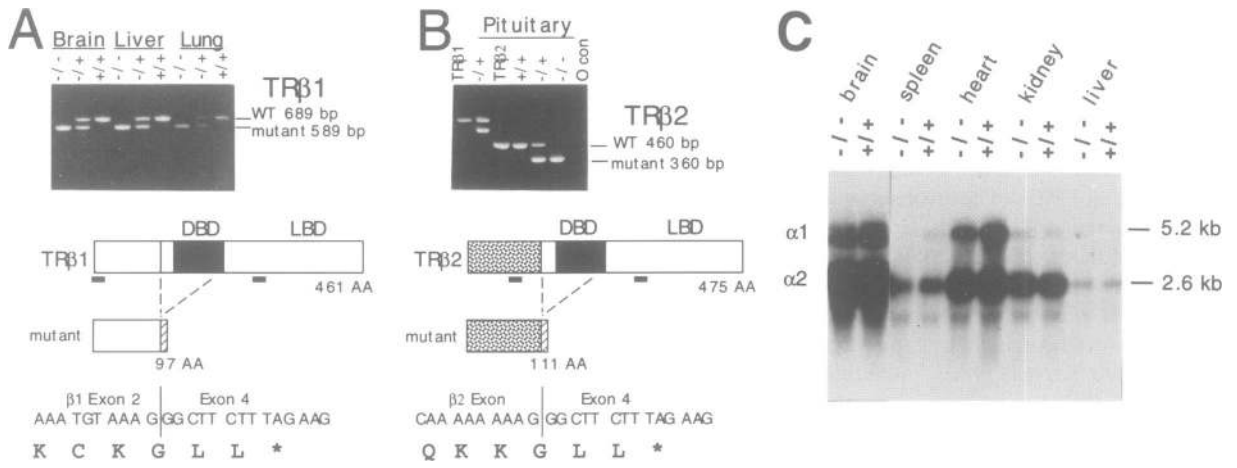

Fig. 2. Expression of the mutant and wild-type TR genes. (A and B) RNA from different tissues of *Thrb*$^{+/+}$, *Thrb*$^{-/+}$ and *Thrb*$^{-/-}$ mice was analysed by RT–PCR with primers specific for TRβ1 (A) and TRβ2 (B). (**A**) A wild-type (+/+) band of 689 bp and a mutant (–/–) band of 589 bp were detected. The deletion included the exon 3 splice donor and resulted in the residual portion of the exon not being recognized as an exon, such that the *Thrb*$^{-/-}$ mRNA entirely lacked exon 3. The deleted mRNA encoded a putative peptide that terminated after eight out-of-frame bases in exon 4 at a TAG stop codon (asterisk in the partial sequence of the junction, at bottom). (**B**) In pituitary RNA, a TRβ2 wild-type (+/+) band of 461 bp and a mutant (–/–) band of 361 bp were detected. *Thrb*$^{-/+}$ pituitary RNA contained wild-type and mutant forms of both TRβ2 and TRβ1. The *Thrb*$^{-/-}$ mRNA lacked exon 3 and terminated after eight bases in exon 4. Lanes (left to right): TRβ1, TRβ1 plasmid as control for TRβ1 wild-type band; –/+, *Thrb*$^{-/+}$ pituitary sample; TRβ2, TRβ2 plasmid as control for TRβ2 wild-type band; +/+, –/+, –/–, pituitary samples from *Thrb*$^{+/+}$, *Thrb*$^{-/+}$ and *Thrb*$^{-/-}$ mice. 0 con, negative control reaction containing no sample template. The predicted TRβ1 protein sizes in *Thrb*$^{+/+}$ and *Thrb*$^{-/-}$ mice were 461 and 97 amino acids, respectively and for TRβ2, 475 and 111 amino acids, respectively. (**C**) Northern blot analysis of TRα mRNA in different tissues of *Thrb*$^{+/+}$ or *Thrb*$^{-/-}$ mice. Tissues were pooled from adult male mice of each genotype and analysed with a probe that detected both TRα1 and TRα2 splice variants. Results were confirmed with independent probes specific for either TRα1 or TRα2.

Expression of mutant and wild-type TR genes

To demonstrate that the mutation inactivated the TRβ gene, RNA from different tissues was analysed. Northern blot analysis showed that an ~6.5 kb TRβ mRNA was expressed at similar levels in *Thrb*$^{+/+}$ and *Thrb*$^{-/-}$ littermates and that *neo* sequences were not incorporated into this mRNA in *Thrb*$^{-/-}$ mice (data not shown). This ruled out the possible expression of a fused *neo*–TRβ mRNA. To investigate the nature of the mRNA in *Thrb*$^{-/-}$ mice, cDNA was generated by RT–PCR. Using TRβ1-specific primers, a product that was ~100 bp shorter than the wild-type product was identified (Figure 2A), indicating that the *Thrb*$^{-/-}$ mRNA carried an internal deletion. Sequence analysis of the *Thrb*$^{+/+}$ and *Thrb*$^{-/-}$ cDNAs revealed that the mutation deleted exon 3 sequences and fused TRβ1 exon 2 to exon 4, resulting in an aberrant open reading frame that terminated after 8 bp in exon 4. Thus, even if the mutant mRNA could be translated, the protein product would lack the DNA binding and T3 binding domains of TRβ. The same deletion was confirmed in TRβ1 mRNA from a range of tissues including brain, liver, lung and pituitary.

The structure of the 5′ region of the TRβ gene is complex and it is capable of tissue-specific splicing to generate the TRβ1 and TRβ2 variants that diverge in their N-termini at the junction with the deleted exon 3. Therefore, to demonstrate that the mutation also disrupted TRβ2, pituitary mRNA was analysed with TRβ2-specific primers (Figure 2B). This revealed an mRNA that could only encode a truncated N-terminal peptide of TRβ2. Thus, in all tissues examined, the mutation precluded expression of functional TRβ1 or TRβ2 and demonstrated that the gene was essentially inactivated.

To investigate if expression of the TRα gene was altered as a consequence of the TRβ mutation, TRα mRNA was examined by Northern blot analysis. Figure 2C shows that, in a range of tissues, no obvious differences were detected between *Thrb*$^{+/+}$ and *Thrb*$^{-/-}$ mice for mRNA encoding either the TRα1 or TRα2 C-terminal splice variants. Thus, although no functional TRβ was expressed in *Thrb*$^{-/-}$ mice, there was no indication of a gross compensatory alteration in TRα expression.

Thyroid pathology in homozygous mutants

Thrb$^{-/-}$ mice were viable, they displayed normal growth rates and weight gain and they were fertile. Necropsy failed to reveal gross abnormalities in most organs, with the exception of the thyroid gland which was variably enlarged in *Thrb*$^{-/-}$ mice (Figure 3A–D). Quantitative image analysis of histological sections indicated that thyroid areas were 1.5- to 2.0-fold increased ($P<0.05$) in overall size in homozygotes (mean ± SEM in mm^2, 0.58 ± 0.09, $n = 10$) compared with heterozygous (0.35 ± 0.04, $n = 9$) and wild-type (0.39 ± 0.04, $n = 8$) mice at 5 weeks of age. There was no significant difference between *Thrb*$^{-/+}$ and *Thrb*$^{+/+}$ mice. Higher magnification revealed a diffuse enlargement of *Thrb*$^{-/-}$ thyroid glands resulting from an increase in both the number and size of follicles (Figure 3E and F). The colloid of follicles from *Thrb*$^{-/-}$ mice frequently contained large phagocytic-like cells that were often multinucleated and other cellular debris that was probably derived from degenerating epithelial cells (Figure 3G).

This pathology suggested that the *Thrb*$^{-/-}$ thyroid glands were in a hyperactive state with increased epithelial cell turnover, indicating that the mutation caused a recessive hyperthyroid-like condition. No difference was detected between the sexes, and the enlargement persisted in mice

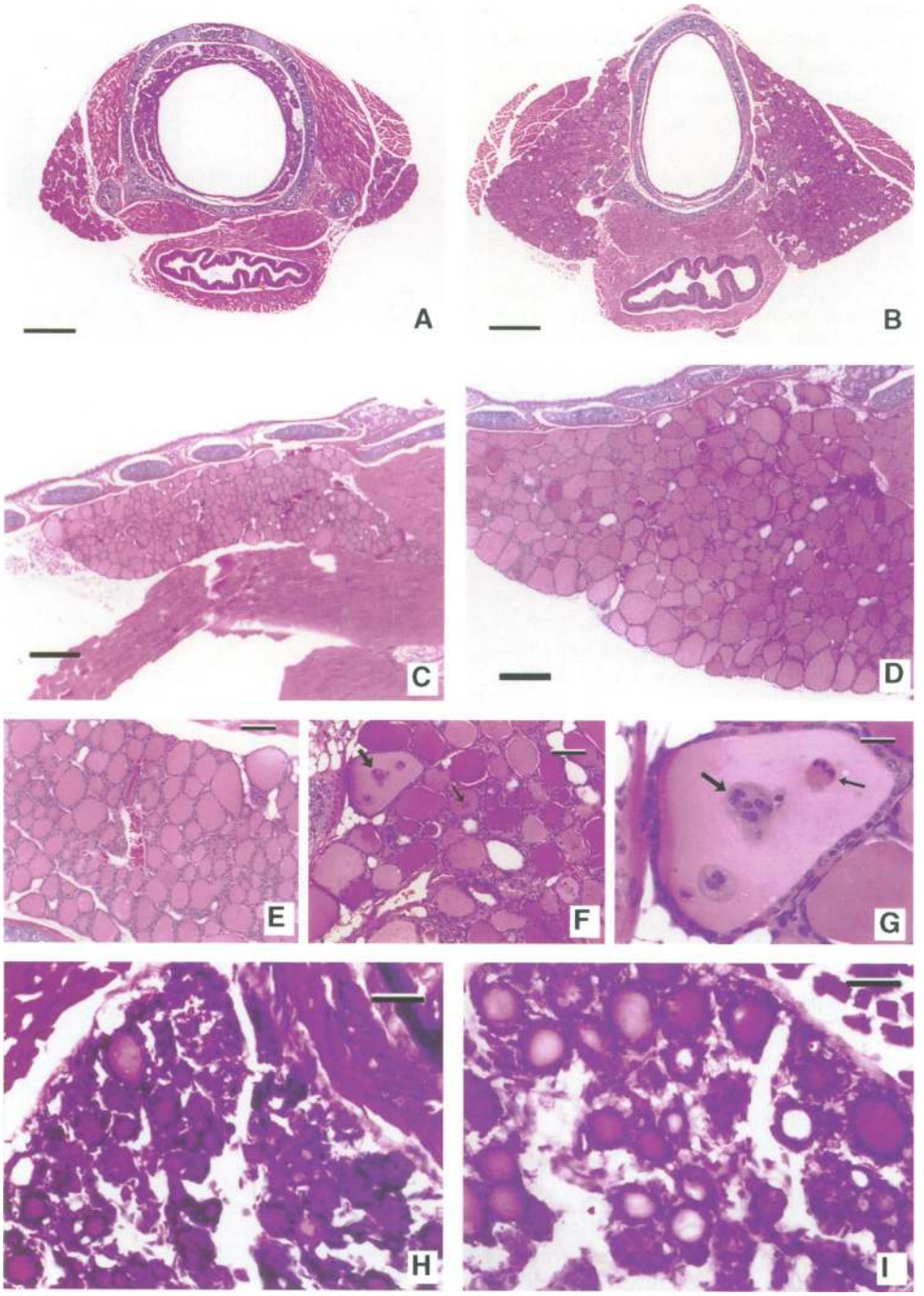

Fig. 3. Thyroid enlargement in *Thrb*$^{-/-}$ mice. (**A** and **B**) Transverse sections through the thyroids, trachea and oesophagus of 5-week-old *Thrb*$^{+/+}$ (A) and *Thrb*$^{-/-}$ (B) mice. The *Thrb*$^{-/-}$ sample showed bilateral thyroid enlargement. Scale bars = 380 μm. (**C** and **D**) Longitudinal sections through the thyroids of 18-week-old *Thrb*$^{+/+}$ (C) and *Thrb*$^{-/-}$ (D) mice. The *Thrb*$^{-/-}$ sample was enlarged due to increased numbers and size of follicles. Scale bars = 270 μm. (**E, F** and **G**) Higher magnification of thyroid tissue from 18-week-old *Thrb*$^{+/+}$ (E) and *Thrb*$^{-/-}$ (F) mice. The *Thrb*$^{-/-}$ tissue (F) had more large follicles than the *Thrb*$^{+/+}$ tissue (E) and also displayed a high incidence of intra-follicular debris, consisting of large, sometimes multinucleated phagocyte-like cells (large arrow) and degenerating tissue that may derive from the follicular epithelium (small arrow); scale bars = 70 μm; also shown at higher magnification in (G); scale bar = 20 μm. (**H** and **I**) Thyroid pathology in mice at post-natal day 7. *Thrb*$^{-/-}$ thyroid tissue (I) contained a greater proportion of enlarged follicles than did the *Thrb*$^{+/+}$ sample (H); scale bar = 20 μm.

analysed at 5, 18 and 40 weeks of age. The condition was not rapidly progressive since the pathology was not more pronounced, with no evidence of hyperplasia, in 40-week-old mice. Image analysis of thyroid sections demonstrated an approximately constant ratio of areas of colloid:epithelium in *Thrb*$^{-/-}$ (mean ± SEM, 1.02 ± 0.08, n = 10), *Thrb*$^{-/+}$ (0.86 ± 0.1, n = 9) and *Thrb*$^{+/+}$ (0.91 ± 0.1, n = 8) mice. Thyroid size increased in all genotypes with age, but there was no significant difference in the ratio of colloid:epithelium between *Thrb*$^{-/-}$ and normal mice. The thyroid glands of *Thrb*$^{-/-}$ mice at post-natal day 7 also displayed an increase in the numbers and size of colloid-containing follicles, indicating that the condition arose at an early age (Figure 3H and I).

Hormonal disorder

The observed thyroid pathology of the *Thrb*$^{-/-}$ mice suggested that there could be abnormalities in thyroid hormone levels. Analysis of serum thyroid hormones revealed that the levels of total thyroxine (T4), the major product of the thyroid gland, were significantly elevated in *Thrb*$^{-/-}$ mice at 5–40 weeks of age, irrespective of gender. Figure 4A shows that mean T4 levels were elevated ~2.5-fold in a representative analysis of 10-week-old mice (means ± SEM for *Thrb*$^{-/-}$, *Thrb*$^{-/+}$, *Thrb*$^{+/+}$ were 11.5 ± 1.07, 4.6 ± 0.3, 4.1 ± 0.3 μg/dl, respectively). Parallel increases in free T4 were observed in *Thrb*$^{-/-}$ mice (1.7 ± 0.18 ng/dl) compared with *Thrb*$^{-/+}$ (0.6 ± 0.05) and *Thrb*$^{+/+}$ (0.5 ± 0.06) mice. This confirmed the predicted thyroid hyperactivity and excluded abnormal serum binding or transport of T4 as the cause of the elevated serum hormone levels. Preliminary data indicated that there was a general decrease of T4 levels in older *Thrb*$^{-/-}$ mice (~1.5 years of age), suggesting that the hyperactivity was ameliorated with age. The levels of total and free T3, the main biologically active form of thyroid hormone, were also elevated in *Thrb*$^{-/-}$ mice. The levels of total T3 were somewhat variable regardless of the genotype, perhaps indicating variability in the peripheral conversion of T4 to T3 in this mouse strain. However, free T3 levels were consistently elevated.

Failure to regulate thyroid-stimulating hormone

Elevation of thyroid hormone levels normally suppresses TSH production by the pituitary thyrotropes. However, the mean serum levels of TSH were significantly elevated in *Thrb*$^{-/-}$ compared with *Thrb*$^{-/+}$ or *Thrb*$^{+/+}$ mice at 5–40 weeks of age, irrespective of gender (Figure 4B). Thus, despite the high levels of thyroid hormones, TSH was paradoxically elevated in *Thrb*$^{-/-}$ mutants. Northern blot analysis of pituitary RNA showed that levels of mRNA encoding TSHα and TSHβ subunits were elevated 2.5- and 3.3-fold respectively compared with *Thrb*$^{+/+}$ mice (Figure 4C), suggesting that the increased TSH levels in mice lacking TRβ reflected abnormal regulation of TSH gene transcription. Histological examination of pituitary glands from *Thrb*$^{-/-}$ mice revealed no abnormalities, and immunohistochemical analysis showed no abnormal pattern of cells staining positively for the TSH subunits (Figure 4D–G). Thus, the over-production of TSH detected in *Thrb*$^{-/-}$ mice resulted from defective thyrotrope function rather than from hyperplasia or malformation of the pituitary gland.

Central nervous system (CNS) function and anatomy

The absence of, or excessive exposure to, T3 during a critical embryonic and neonatal period can impair brain development (Legrand, 1984). To investigate if the absence of TRβ and/or the associated increase in thyroid hormone levels caused neurological defects, the function of the nervous system in *Thrb*$^{-/-}$ mice was assessed using a range of behavioural tests (Figure 5). These analyses were valid since mice, like humans or rats, are susceptible to behavioural defects associated with congenital thyroid disorders, and similar tests have demonstrated learning disabilities in the hypothyroid (*hyt*) mutant mouse (Anthony *et al.*, 1993). In a stringent version of the Morris

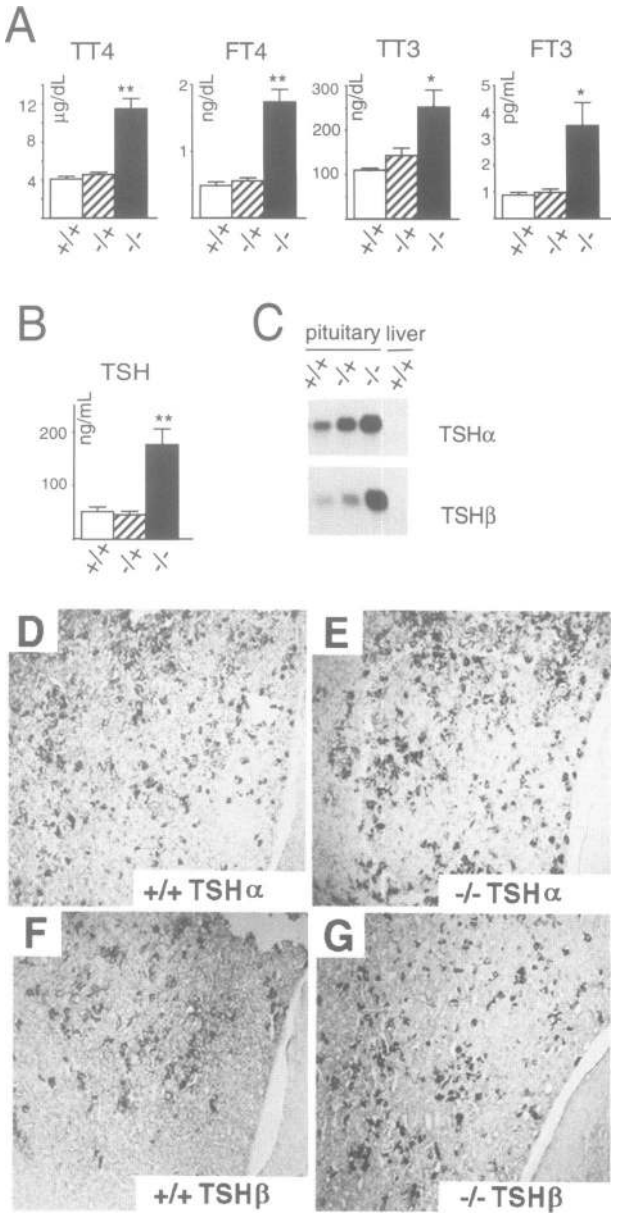

Fig. 4. Elevated levels of thyroid hormones and thyroid-stimulating hormone in *Thrb*$^{-/-}$ mice. (**A**) Thyroid hormone levels (means ± SEM) in serum from mice at 10 weeks of age determined by radioimmunoassay. *Thrb*$^{-/-}$, *Thrb*$^{-/+}$ and *Thrb*$^{+/+}$ groups contained respectively, n = 7, 9 and 5 (total T4; TT4); n = 7, 9 and 5 (free T4; FT4); n = 5, 9 and 5 (total T3; TT3); n = 5, 7 and 4 (free T3; FT3). (**B**) Levels of TSH in 5-week-old mice (means ± SEM). *Thrb*$^{-/-}$, *Thrb*$^{-/+}$ and *Thrb*$^{+/+}$ groups contained, respectively, n = 8, 10 and 7. TT4, FT4 and TSH levels in *Thrb*$^{-/-}$ mice were significantly elevated over levels in *Thrb*$^{-/+}$ and *Thrb*$^{+/+}$ mice at $P<0.01$(**). TT3 and FT3 were significantly elevated at $P<0.05$ (*). (**C**) Northern blot hybridization analysis of pituitary mRNA from *Thrb*$^{-/-}$, *Thrb*$^{-/+}$ and *Thrb*$^{+/+}$ mice using probes specific for the TSHα and β subunits; mRNA sizes were ~0.7 kb (TSHα) and 0.6 kb (TSHβ). As a control for specificity, neither probe detected a band in liver. (**D–G**) Immunohistochemical analysis of TSH subunits in coronal sections of the anterior lobe of the pituitary of *Thrb*$^{+/+}$ (D and F) and *Thrb*$^{-/-}$ (E and G) mice immunostained for TSHα (D and E) or TSHβ (F and G).

water task, requiring the mice to locate a hidden platform to escape, *Thrb*$^{-/-}$ and *Thrb*$^{+/+}$ mice learned to escape equally well with repeated trials over 9 days. When the platform was removed, mice of both genotypes spent

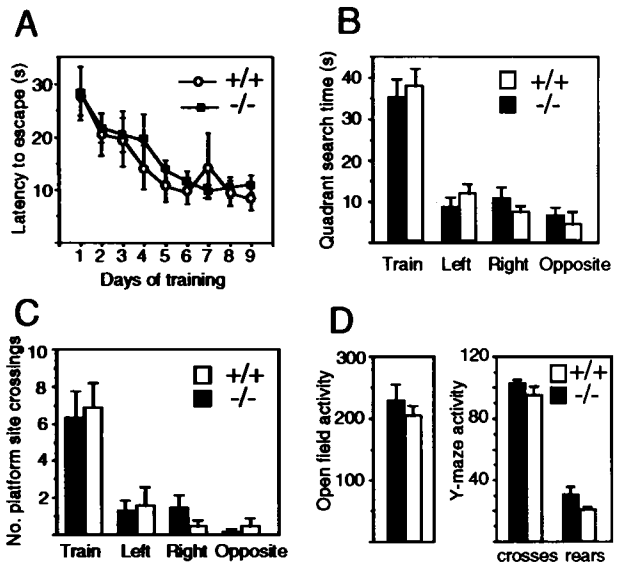

Fig. 5. Behaviour of *Thrb*$^{+/+}$ and *Thrb*$^{-/-}$ mutant mice. (**A**) Spatial learning in the Morris water maze task. Mice were tested for their ability to learn to escape using a hidden platform in four trials per day for 9 days. (**B** and **C**) The water maze was considered to consist of four quadrants. After 9 days of training, the platform was removed and the mice were examined for (B) time spent in the trained quadrant (train) where the platform had been located and (C) number of crossings of the platform site. (**D**) Using photocells, open field activity was measured as the number of times the mouse crossed an infrared beam in 5 min. Y-maze activity was measured as the number of crossings within the maze and the number of times the mouse reared. Test groups contained $n = 7$ mice (both genders) at ~12 weeks of age. However, results shown in (A) were based on groups of $n = 6$ mice, which omitted data for one mouse of each genotype that failed to learn.

equivalent time and activity in the quadrant where the platform had been located, indicating that both genotypes performed well in a spatial learning task. Contextual fear conditioned learning and responses in the pre-pulse inhibition task that may indicate attention deficits were not significantly different in *Thrb*$^{-/-}$ mice (data not shown). However, these studies may not be conclusive as they employ an acoustic stimulus to which the mutants could not respond reliably due to defective auditory function (Forrest *et al.*, 1996). In other tests, such as activity in an open field and Y-maze, *Thrb*$^{-/-}$ and *Thrb*$^{+/+}$ mice also behaved similarly. Histological and histochemical analysis of the CNS of *Thrb*$^{-/-}$ mice revealed no obvious abnormalities in brain anatomy, including structures known to be sensitive to T3, such as the cerebellum and hippocampus. Furthermore, analysis of hippocampal field potentials did not indicate defects in long-term potentiation (G.Golarai, J.Connor, T.Curran and D.Forrest, unpublished data). In conclusion, while developmental delays and attention deficits were not excluded, no overt neurological defects were detected in adult *Thrb*$^{-/-}$ mutants, suggesting that TRβ has subtle rather than major functions in neurodevelopment.

Discussion

Dysfunction of the pituitary–thyroid axis

Thrb$^{-/-}$ mice display a chronic disorder of the endocrine system evidenced by a hyperactive goitre and elevated thyroid hormone levels from early post-natal stages. These mice also displayed elevated serum levels of TSH that, through persistent stimulation of the thyroid, are probably the immediate cause of the observed thyroid pathology. Furthermore, the paradoxical elevation of TSH, despite the increased thyroid hormone levels, indicates that the pituitary is no longer sensitive to suppression by thyroid hormone. This unregulated production of TSH is likely to be due to impaired thyrotrope function rather than to malformation of the pituitary, since there were no obvious abnormalities in the pattern of TSH immunoreactive cells in *Thrb*$^{-/-}$ pituitary glands. These findings demonstrate a critical function for TRβ in the regulation of the pituitary–thyroid axis.

Previous studies indicated that the negative control of TSH by T3 is exerted at the level of transcription, since the TSHα and β subunit genes possess regions that confer T3-dependent suppression in co-transfection assays and in transgenic mice (Chatterjee *et al.*, 1989; Wood *et al.*, 1989; Kendall *et al.*, 1994). Our analyses suggest a specific role for TRβ in mediating this negative regulation since the mRNA levels of the TSH subunits were elevated in *Thrb*$^{-/-}$ pituitaries. This is in accord with the expression of TRβ mRNA in the anterior pituitary (Hodin *et al.*, 1989; Bradley *et al.*, 1992) and in thyrotropes in pituitary primary cultures and cell lines (Childs *et al.*, 1991; Wood *et al.*, 1991). This proposed role for TRβ need not exclude other functions that could contribute to the T3-sensitive regulation of thyroid activity. For example, the TRβ gene is also expressed in the paraventricular nucleus of the hypothalamus that produces thyrotropin-releasing hormone (Bradley *et al.*, 1992; Lechan *et al.*, 1994) which stimulates thyrotrope activity. TRβ and TRα mRNA has also been identified in the thyroid (Brönnegård *et al.*, 1994), suggesting that this gland itself may respond directly to T3. However, given the major role of the pituitary in the control of thyroid activity, our results suggest that the loss of TRβ in thyrotropes is the principal cause of the unregulated TSH release and, consequently, of the thyroid pathology. It is likely that TRβ directly regulates transcription of the TSH genes, although confirmation awaits biochemical characterization of the physiologically relevant protein complexes.

Our results indicate that the TRα gene, which is also expressed in the anterior pituitary and in thyrotrope lines (Wood *et al.*, 1991; Bradley *et al.*, 1992), is unable to substitute for TRβ in the control of thyrotrope function. Despite considerable *in vitro* analysis, very few studies have uncovered differences in the DNA binding and transactivation functions of TRα and TRβ (Lezoualc'h *et al.*, 1992; Strait *et al.*, 1992). It has been proposed previously that the TRα and TRβ genes have distinct developmental and tissue-specific functions based on their differential expression (Forrest, 1994). The present results support the view that the two receptors are also functionally distinct even when co-expressed in the same cell type, which could reflect subtle differences in their preferences for dimerization, DNA binding or in their interaction with co-factors. These findings also suggest that caution should be exercised in the interpretation of *in vitro* studies on the DNA binding and transactivation properties of any one form of TR, to avoid unwarranted generalization about the functions of both TRα and TRβ.

Table I. Comparison of the phenotype of *Thrb*$^{-/-}$ mice with the recessive form of RTH syndrome

	Human[a]		Mouse	
	Control	RTH patient	Control	*Thrb*$^{-/-}$ mutant
Thyroids	Normal	2–3× enlarged	Normal	1.5–2× enlarged
T4 (μg/dl)	9.0–9.9	20.0–26.7	3.3–4.8	8.9–27.9
FT4 (%)[b]	−18/−21	+88/+125	−50/0	+183/+400
T3 (ng/dl)	117	294–830	93–125	155–387
FT3 (pg/ml)	NA	NA	0.6–1.1	1.7–5.6
TSH (%)[c]	−58/0	−51/+187	−40/0	+113/+391
Hearing	Normal	Impaired	Normal	Impaired

[a]Human data are from a single family [kindred G; (Refetoff *et al.*, 1967; Takeda *et al.*, 1992)] and represent the range of values recorded in three recessive cases of RTH and their parents as controls.
[b]Free T4 levels are shown as percentages above (+) or below (–) the upper limit of normal; for mice, percentages are relative to the upper limit recorded in wild-type mice in this study.
[c]TSH levels are shown as percentages above (+) or below (–) the upper limit of normal defined here as the highest control value recorded in the human family or in *Thrb*$^{+/+}$ mice.
NA, data not available.

Model for RTH syndrome: transcriptional mechanisms

The endocrine disorder of the *Thrb*$^{-/-}$ mouse closely resembles the characteristic symptoms of human RTH syndrome and, thus, establishes the *Thrb*$^{-/-}$ mouse as a recessive model for this disease. Indeed, abnormal production of TSH is considered to be the major cause of the thyroid hyperactivity in RTH (Refetoff *et al.*, 1980). Significantly, our results also demonstrate that loss of TRβ results in a hormonal disorder similar to that typically associated with dominant RTH mutations. This raises the intriguing question, in terms of the mechanisms of transcription underlying RTH, of how loss of TRβ can be functionally equivalent to the mixed presence of mutant and wild-type TRβ that occurs with a heterozygous dominant mutation.

Little is understood of the mechanisms by which the dominant mutant receptors produce RTH, except that they act as poorly defined, pleiotropic transcription inhibitors. This has led to proposals that RTH results from interference with the residual wild-type TRβ, TRα, retinoid receptors or other nuclear factors, such as AP1 (Ono *et al.*, 1991; Baniahmad *et al.*, 1992; Nagaya and Jameson, 1993; Ways *et al.*, 1993). However, the simplest interpretation of our results is that antagonism of only TRβ is required in the relevant tissues of the pituitary–thyroid axis. By implication, in dominant RTH, the mutant receptor may inhibit the remaining wild-type TRβ to produce an effective 'knock-out' of TRβ, equivalent to the homozygous null mutation. In this model, both recessive and dominant mutations could abolish the activity of TRβ, and the alternative proposal of antagonism of TRα, retinoid receptors or other factors, need not be invoked, since it is impossible for this to occur in *Thrb*$^{-/-}$ mice. Although more complex, indirect models are possible, these require additional assumptions that are unsupported by the presently available data. In dominant RTH, specific antagonism of TRβ could be achieved, for example, by formation of inactive dimers between the wild-type and mutant TRβ, which would imply that there are normal *in vivo* functions for wild-type TRβ homodimers. While *in vitro* evidence suggests that TRs function as heterodimers with RXRs (Stunnenberg, 1993; Chambon, 1994), TRβ has also been shown to form relatively stable homodimers on certain response elements (Ng *et al.*, 1995). The present data suggest that there may be distinct physiological functions for both TRβ homodimers and heterodimers with RXRs or other nuclear receptors.

Studies on the human kindred with recessive RTH did not indicate whether loss of TRβ caused any or all of the associated array of symptoms, since the genomic rearrangement was too extensive to allow any phenotype to be ascribed to a specific gene (Refetoff *et al.*, 1967; Takeda *et al.*, 1992). The phenotype of *Thrb*$^{-/-}$ mice establishes that loss of TRβ alone is sufficient to produce an RTH-like endocrine phenotype (Table I). Indeed, it is informative to compare the phenotype of the *Thrb*$^{-/-}$ mouse with the phenotype of human recessive RTH since this delineates aspects of the human kindred, including the endocrine disorder and deafness (Forrest *et al.*, 1996), that can be attributed to loss of TRβ as opposed to any other genetic change.

Neurodevelopment and implications for tissue specificity of TR function

Surprisingly, given the critical role of T3 in brain development (Legrand, 1984), *Thrb*$^{-/-}$ mice displayed no overt abnormality in neuroanatomy, behaviour or in hippocampal long-term potentiation. These data suggest that TRβ plays a subtle rather than a major role in brain development, despite the fact that the TRβ gene is highly induced in the neonatal CNS during the critical period of T3 sensitivity (Forrest *et al.*, 1991; Mellström *et al.*, 1991). However, this does not exclude the possibility of a subtle delay rather than a permanent defect with respect to some features of brain development, as occurs, for example, with myelination in hypothyroidism (Walters and Morell, 1980; Rodriguez-Pena *et al.*, 1993). Alternatively, secondary changes could arise that mask the absence of TRβ. For example, the elevated levels of thyroid hormones in *Thrb*$^{-/-}$ mice could cause greater saturation of TRα to stimulate an enhanced activity that fortuitously compensates for the loss of TRβ. Comparative studies with mice lacking TRα will be required to address directly the relationship between the different TR genes and CNS development.

It is interesting that the lack of overt defects in CNS function in the *Thrb*$^{-/-}$ mice and in the human case of

recessive RTH (Refetoff *et al.*, 1967) contrasts with reports that mental retardation and attention deficit–hyperactivity disorders are present in >70% of dominant RTH cases (Hauser *et al.*, 1993; Weiss *et al.*, 1994). Thus, whereas the loss of TRβ may have only relatively mild or undetected consequences for brain development, a dominant TRβ mutant may be more potent in disrupting critical neurological functions. It is possible, therefore, that pathways not mediated by TRβ are targets for the dominant mutant receptors in the CNS. Thus, in contrast to the pituitary–thyroid axis described above, where our results indicate that loss of TRβ causes the defect, CNS deficiencies in dominant RTH may result from the antagonism of other pathways, perhaps mediated by TRα or other receptors. This implies that in some tissues, such as in the pituitary–thyroid axis, TRβ has certain autonomous functions that may not involve interaction with other receptors, whereas in the CNS, the action of TRβ may involve other receptor-mediated pathways.

Genetic studies also reveal that while recessive and dominant TRβ mutations cause a similar endocrine disorder, dominant mutant receptors fail to recapitulate the phenotype caused by loss of TRβ in other systems. In addition to the hormonal disorder, we have shown that the *Thrb*$^{-/-}$ mice have impaired auditory function (Forrest *et al.*, 1996). In contrast, hearing defects are generally not present in dominant RTH (Refetoff *et al.*, 1993), suggesting that, in the auditory system, a dominant mutant TRβ may retain partial activity. A possible explanation is provided by reports that a wild-type TR can mediate both T3-dependent and T3-independent regulation of transcription (Damm *et al.*, 1989; Fondell *et al.*, 1993). Thus, a T3-insensitive mutant of TRβ might still mediate essential T3-independent functions in certain cells, perhaps by interacting with tissue-specific co-factors (Chen and Evans, 1995; Hörlein *et al.*, 1995; Lee *et al.*, 1995). In summary, dominant and recessive TRβ mutations may only be equivalent in restricted situations, such as in the pituitary–thyroid axis, where TRβ may function independently of other receptors. In other situations, such as in the brain and auditory system, a dominant mutant of TRβ may result in more and less severe phenotypes, respectively, than the loss of TRβ. These variations probably reflect tissue-specific differences in the function of TRβ with respect to its interaction with other factors and possibly its T3-dependent and independent functions.

Materials and methods

Targeting vector

A chick TRβ cDNA insert (Forrest *et al.*, 1990) was used to isolate and map genomic clones encompassing the TRβ gene from a bacteriophage λ library of DNA from a 129/sv mouse (Stratagene). The genomic clones were used to construct the targeting vector (pB21; Figure 1A) that contained a neomycin-resistance selection marker (*neo*) and a thymidine kinase gene (*tk*) for negative selection against random integration, using established procedures (Forrest *et al.*, 1994). The vector contained from 5′ to 3′: a herpes simplex virus *tk* gene from pMC1-HSVTK, a 3 kb fragment of TRβ genomic DNA extending to a *Kpn* I site in coding exon number 3, a *neo* gene from pgkneobpA and a 4 kb *Xba*I–*Hin*dIII genomic fragment containing TRβ exon 4 and part of exon 5. The construct was linearized at the 5′ end of *tk* with *Bam*HI.

ES cell selection and generation of mutant mice

W9.5 ES cells were electroporated with targeting vector, then selected with G418 (Gibco) and ganciclovir (a gift of Syntex Corp) and after 8 days, resistant colonies were picked for screening as described (Forrest *et al.*, 1994). One clone out of 240 was identified as positive for homologous recombination; additional clones were obtained at a similar frequency in further experiments. The correct chromosome content of positive clones was determined, then ES cells were injected into C57BL/6J blastocysts (Stewart, 1993) to generate male chimeric offspring. These were bred with C57BL/6J females to generate progeny with germline transmission of the mutation, carried on a hybrid genetic background of 129/sv and C57BL/6J strains. Analyses utilized progeny derived from a single targeted ES cell clone. Mice were maintained in light:dark cycles of 12:12 h (changes 6.00 a.m./6 p.m.) and were fed with PicoLab mouse diet 20 (Purina Mills, Inc.). All animal experiments were performed according to approved protocols.

Southern blot hybridization analysis and genotype determination

ES cell or mouse tail DNA was prepared, digested with *Bam*HI and *Eag*I then analysed on 0.7% TAE agarose gels for Southern blot analysis as described (Forrest *et al.*, 1994) with the 3′ (*Avr*II/1.7 kb fragment) and 5′ (*Sal*I–*Pml*I/2.1 kb fragment) probes shown in Figure 1A. Membranes were washed twice in 0.1× SSC/0.2% SDS at 62°C, then once at 65°C.

RNA analyses

Total cellular RNA was prepared from individual tissues or from the pooled pituitaries of 10 male mice of each genotype. Samples of RNA (10 μg) were subjected to Northern blot analysis using antisense oligonucleotide probes that were labelled at their 5′ ends with ^{32}P as described (Forrest *et al.*, 1991). Probes were derived from mouse sequences: TSHα positions 222–268 (Chin *et al.*, 1981); TSHβ positions 176–221 (Gurr *et al.*, 1983); TRα positions 427–476 (Prost *et al.*, 1988). A PhosphorImager (Molecular Dynamics) was used to quantitate bands. For RT–PCR, 10 μg samples of RNA were used to make first strand cDNA enriched for TRβ sequences by using an antisense primer derived from positions 1527–1570 of the 3′-terminal coding exon TRβ (Wood *et al.*, 1991). PCR analyses utilized pairs of primers specific for products encoding either the TRβ1 or TRβ2 N-terminal variants [Figure 2; common 3′ antisense primer, positions 696–720; TRβ1-specific sense primer, positions 30–74; TRβ2-specific sense primer, positions 351–373 (Wood *et al.*, 1991)]. Reactions involved denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 45 s for 35 cycles using AmpliTaq in standard buffer (Cetus). Products were analysed on 0.8% TBE agarose gels, then purified for DNA sequence determination.

Histology and immunohistochemistry

Organs were fixed in 10% neutral-buffered formalin, embedded in paraffin and 4–6 μm thick sections prepared and stained with haematoxylin and eosin. Thyroids were analysed in transverse sections from mice at 5 weeks (n = 10 for each genotype) and at 40 weeks (n = 4/group) of age. Thyroid areas and ratios of colloid:epithelium were determined on comparable sections by computer imaging using NIH Image software. Similar analyses were performed on longitudinal sections of mice at 18 weeks of age (n = 6/group). For histology of adult brains and pituitaries, mice were anaesthetized then perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). 14 μm thick sections of brain in sagittal and coronal planes from wild-type (n = 3) and *Thrb*$^{-/-}$ mice (n = 3) were stained with cresyl violet. Additional sections were stained for acetyl cholinesterase activity and for mossy fibre synaptic organization in the dentate gyrus of the hippocampus using Timm histochemistry. Pituitary glands were post-fixed in fresh fixative overnight then embedded in paraplast-plus™, for sectioning at 5 μm and mounting onto polyionic-treated slides (Superfrost-plus, Fisher Scientific). For immunostaining, paraffin was removed with three rinses in xylene followed by rehydration through a series of graded alchohols, then rinsed three times for 10 min in 1× PBS. Sections were treated with 4% normal goat serum (NGS), 0.4% Triton X-100, 1% bovine serum albumin (BSA) for 20 min to block non-specific binding, then exposed to the primary antibody at 4°C overnight. Rabbit antisera against rat TSHα (AFP-66P9986) and TSHβ (AFP-1274789; both gifts from the National Hormone and Pituitary Program, NIDDK, NICHHD and USDA) were used at a dilution of 1:1000 in PBS, 4% NGS, 0.4% Triton X-100, 1% BSA. Sections were rinsed five times for 10 min in 1× PBS, 0.02% Triton X-100, 1% BSA and exposed to secondary antibody for 1 h (goat anti-rabbit, 1:1000 in PBS, 0.02% Triton X-100, 1% BSA). Sections were rinsed three times for 10 min in PBS, 0.25% BSA and exposed to an avidin–biotin sandwich procedure for 30 min (ABC Elite, Vectastain). Sections were rinsed twice for 10 min in PBS and antigen

was visualized using DAB, followed by rinses in PBS and dehydration in alchohol. Analyses were performed on adult female *Thrb*$^{-/-}$ (n = 4) and *Thrb*$^{+/+}$ (n = 4) mice. Male mice yielded similar results.

Hormone assays

Mice were immobilized with CO_2 and bled from the retro-orbital sinus. Sampling for all experiments was performed at the same time of day (9.00–11.00 a.m.) to avoid day–night cycle variations. Serum levels of free and total T4 and T3 were determined with Coat-A-Count radioimmunoassay kits (Diagnostic Products Corp). TSH levels were determined by Hazleton Laboratories (Vienna, VA) using a radioimmunoassay that had been calibrated using a mouse TSH reference preparation (LH/TSH AFP-51718 MP) obtained from Dr A.F.Parlow, Harbor-UCLA Medical Center. Statistical comparisons employed the t-test.

Learning and behaviour

Open field activity (Pauly et al., 1993). Mouse movement in a circular open field 60 cm in diameter was tracked by infrared photocells and the number of times a beam was broken was counted by computer. Each mouse was tested for 5 min and all mice were tested within 1 h of each other.

Y-Maze activity (Marks et al., 1989). The maze contained three symmetrical arms 26 cm long, 6.1 cm wide and 10.2 cm high. The floor and ceiling were red translucent acrylic plastic; the sides were black acrylic plastic. Mice were placed in the maze for 3 min and crossings within sections and number of rears made were counted.

The Morris water task. This task used a hidden platform and a distributed training procedure. The apparatus and room were described by Paylor *et al.* (1993). Mice were given four trials per day for 9 days. On day one, mice were placed on the platform and allowed to swim for 10 s. This was repeated twice for the mice to become accustomed to the apparatus. On each set of four trials, the starting position was randomized and mice were allowed to swim for 60 s or until they located the platform. Mice that did not locate the platform in 60 s were given a score of 60 s and placed on the platform. The time to reach the platform was recorded for each of the total 36 trials. After completing training on day nine, mice were given a 60 s probe trial in which the platform was removed and their behaviour recorded for the number of crossings of the training site and the time spent in each of the quadrants. Contextual fear conditioning was performed as described (Paylor *et al.*, 1994). Prepulse inhibition was tested as described (Geyer *et al.*, 1993) with modification for the mouse.

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