Different functions for the thyroid hormone receptors TRα and TRβ in the control of thyroid hormone production and post-natal development

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Introduction

Thyroid hormones are known to induce metamorphosis in amphibians by mediating remodelling of specific tissues and organs (for review see Tata, 1993; Kaltenbach, 1996). Triiodothyronin (T3) is essential for post-natal development of mammals, as hypothyroidism leads to growth retardation and impaired neurogenesis (Legrand, 1986). T3 is also involved in many aspects of adult life. In humans, hypothyroidism affects the function of many organs and results in alterations of thermogenesis and behaviour (Legrand, 1986).

The functions of T3 are mediated by three nuclear thyroid receptors, TRα1, TRβ1 and TRβ2, encoded by two genes, TRα and TRβ, respectively (Sap et al., 1986; Weinberger et al., 1986). The TRβ locus generates the β1 and β2 receptors by using two different promoters and alternative splicing. The TRα locus generates the α1 receptor and three non-T3 binding proteins: TRt2, which results from alternative splicing of the TRα primary transcript (Koenig et al., 1989; Lazar et al., 1989); and TRαΔ1 and TRαΔ2 transcripts, which are generated from an internal promoter located in intron 7 of the TRα locus (Chassande et al., 1997). The thyroid hormone receptors belong to the family of nuclear receptors which includes retinoic acid receptors, 9-cis retinoic acid receptors, vitamin D3 receptors and peroxisome proliferator-activated receptors (Laubet et al., 1992). All these receptors contain a DNA-binding domain and a ligand-binding domain; they mediate ligand-dependent transcriptional control of target genes (Mangelsdorf et al., 1995). The β1 and β2 receptors only differ in their N-terminal region. The α and β receptors display remarkably conserved sequences in their DNA-binding, ligand-binding and ligand-dependent trans-activation domains, but their sequences differ completely in the N-terminal region. The consequences of these structural homologies is that all three receptors bind the same ligand and the same motifs on DNA. Despite these similarities, recent in vivo investigations of the respective functions of these receptors have suggested that each of them is likely to mediate a limited number of the physiological activities of T3.

The TRs not only mediate the action of T3 but also play a role in maintaining the concentration of ligand at a constant level. This is achieved by feedback regulation involving pituitary thyroid stimulating hormone (TSH). T3 negatively controls the synthesis and secretion of TSH and thyrotropin releasing hormone (TRH) at the level of pituitary and hypothalamus, respectively, through a negative feedback loop (Lezoualc’h et al., 1992; Scanlon and Toft, 1996). The disruption of the TRβ gene leads to hyperthyroxinemia (Forrest et al., 1996; Weiss et al., 1998). In contrast, mice with targeted disruption of the TRα1 and TRαΔ1 genes (TRα1−/−) exhibit a moderate hyperthyroxinemia and mild hypothyroidism (Wikstrom et al., 1998), and mice lacking both TRα1 and TRα2 display hyperthyroxinemia which increases upon aging and severe hypothyroidism (Fraichard et al., 1997). These data suggest that the products of both the α and β genes are involved, although in different ways, in the control of TSH production.

Other physiological functions are differentially affected by the deletion of either of the receptors. The inactivation of the TRβ gene results in impairment of the auditory
function, but no alteration in development, metabolism or neurological functions has been described in these animals (Forrest et al., 1996). \( TR\alpha^{-/-} \) mice show an abnormal heart rate and lower body temperature (Wikstrom et al., 1998). Mice which lack both the \( TR\alpha \) and the \( TR\beta \) isoforms (\( TR\alpha^{-/-} \)) exhibit growth retardation, a lower body temperature, a delayed maturation of bone and intestine and they die shortly after weaning (Fraichard et al., 1997). From these studies, it is clear that \( \alpha \) and \( \beta \) receptors are differentially involved in the control of developmental, endocrine and metabolic processes.

The difference in the pattern of expression of the three receptors may partly account for these functional differences. The pleiotropic effect of the mutation in \( TR\alpha \) is consistent with the wide distribution of \( TR\alpha \) transcripts, whereas the more limited alterations generated by the ablation of the \( TR\beta \) gene are in agreement with the more restricted pattern of expression observed for \( TR\beta \) transcripts. A few organs and tissues such as early chick embryos (Forrest et al., 1991; Flamant and Samarut, 1998) and rat cerebellum (Bradley et al., 1989) express only \( \alpha \) isoforms but most tissues express several isoforms with varying ratios. Redundancy may occur in tissues where both \( \alpha \) and \( \beta \) receptors are expressed.

To investigate the respective roles of \( TR\alpha \) and \( TR\beta \) genes, we have generated mice with a targeted disruption of the \( TR\beta \) gene and, using the previously described \( TR\alpha^{-/-} \) mice, produced double mutant mice. We have compared the phenotypes of wild-type, \( TR\alpha^{-/-}, TR\beta^{-/-} \) and \( TR\alpha^{-/-}TR\beta^{-/-} \) mice, and investigated the pituitary and thyroid functions of these mice in order to analyse the respective parts played by the \( \alpha \) and \( \beta \) receptors in the control of the production of thyroid hormones. We have also compared the effects of the single and double mutations on the maturation of bone and intestine, in order to analyse potential functional redundancies between \( \alpha \) and \( \beta \) receptors in these organs.

**Results**

**Production of mice with inactivated \( TR\beta \)**

To inactivate the \( TR\beta \) gene, a recombination cassette containing both a \( lacZ \) coding sequence and a \( Neo^R \) gene driven by the \( \beta\text{-}actin \) promoter was introduced downstream of exon 3 (Figure 1A). The presence of two polyadenylation sites downstream of the \( lacZ \) and \( Neo^R \) genes, respectively, was designed to prevent further transcription of both \( TR\beta1 \) and \( TR\beta2 \) mRNAs. Moreover, the region of the gene which encodes the DNA-binding domain of the receptors was removed in the replacement vector, to prevent the production of a chimeric protein that could potentially compete with other receptors for the binding to DNA. Two independent clones of recombinant embryonic stem (ES) cell clones were isolated and injected into blastocysts. Heterozygous mice were derived in an inbred 129SV background. Homozygous animals were obtained by intercrossing heterozygous animals (Figure 1B). As previously described (Forrest et al., 1996), homozygous animals were fertile and displayed hyperthyroxinemia.

**Production of mice with both inactivated \( TR\alpha \) and \( TR\beta \)**

\( TR\alpha^{+/+}, TR\beta^{-/-} \) mice were first crossed with \( TR\alpha^{+/+}, TR\beta^{+/+} \) mice to generate double heterozygous animals, which were fertile and intercrossed to yield viable and fertile \( TR\alpha^{+/+}, TR\beta^{-/-} \) mice. These latter mice were further intercrossed to generate double homozygous animals. \( TR\alpha^{-/-}, TR\beta^{-/-} \) mice were born alive with no obvious morphological or physiological alterations. Among 148 \( TR\beta^{-/-} \) pups, 45 (30.4%), 69 (46.6%) and 34 (23%) were \( TR\alpha^{-/-}, TR\beta^{-/-} \) and \( TR\alpha^{-/-}, TR\beta^{+/+} \), respectively. The newborn mice were able to move and suckle normally, compared with \( TR\beta^{-/-} \) or wild-type mice. These data show that the absence of both \( TR\alpha \) and \( TR\beta \) receptors does not impair the embryonic development of the mouse.

RT–PCR experiments were designed to monitor the expression of the transcripts produced from the \( TR\alpha \) and \( TR\beta \) loci. Figure 1C shows that no transcript containing sequences downstream of the \( lac\beta\text{-}actin \) cassette was detected in intestine or lung from \( TR\beta^{-/-} \) or \( TR\alpha^{-/-}, TR\beta^{-/-} \) mice.

**Altered growth rate and post-natal death in \( TR\alpha^{-/-}, TR\beta^{-/-} \) mice**

There was no obvious difference in the growth rates of wild-type and \( TR\beta^{-/-} \) animals, respectively (data not shown), in agreement with previous observations (Forrest et al., 1996). As previously described for the \( TR\alpha^{-/-} \) mice,
the newborn double mutant animals exhibited an altered growth rate. Until the age of 15 days, the difference in body weight between TRβ<sup>–/–</sup> and double mutant mice was moderate (Figure 2). From this age on, the growth of double mutants was stopped and after 3 weeks a decrease in body weight was observed, leading to death by the age of 5 weeks. Growth of the TRα<sup>–/–</sup>TRβ<sup>–/–</sup> mice was slower than that of TRβ<sup>–/–</sup> mice.

As for the TRα<sup>–/–</sup> mice, morphological examination of skeleton, liver, heart, lung and kidneys of 3-week-old TRα<sup>–/–</sup>TRβ<sup>–/–</sup> mice showed that these organs did not reveal overt abnormalities despite a significant reduction in size compared with wild-type or TRβ<sup>–/–</sup> animals (data not shown).

**Hyperproduction of TSH and thyroid hormones in TRα<sup>–/–</sup>TRβ<sup>–/–</sup> mice**

We showed previously that from the age of 3 weeks TRα<sup>–/–</sup> mice were progressively becoming hypothyroid. By the age of 5 weeks, plasma thyroid hormone concentrations reached values representing ~30% of hormone levels in normal mice (Fraichard et al., 1997). On the contrary, elevated plasma concentrations of TSH, T3 and thyroxin (T4) have been reported for TRβ<sup>–/–</sup> mice (Forrest et al., 1996). We measured the plasma concentrations of these hormones in 3-week-old wild-type, TRα<sup>–/–</sup>, TRβ<sup>–/–</sup> and TRα<sup>–/–</sup>TRβ<sup>–/–</sup> mice (Figure 3). In these experiments, comparison of the concentrations of TSH and thyroid hormones in TRα<sup>–/–</sup> mice versus wild-type mice did not have any statistical significance, as assessed by an unpaired t-test (p > 0.37 for the three parameters). This was due to the large variance and the low number of animals tested.

Comparisons of all parameters in TRβ<sup>–/–</sup> and TRα<sup>–/–</sup>TRβ<sup>–/–</sup> versus wild-type on the one hand, TRα<sup>–/–</sup> and TRβ<sup>–/–</sup> versus TRα<sup>–/–</sup>TRβ<sup>–/–</sup> on the other, were meaningful as assessed by p values < 0.001 (see Figure 3). Comparison of TRα<sup>–/–</sup> versus TRβ<sup>–/–</sup> was valid for T3 and T4 but not for TSH. In agreement with the observations from others (Forrest et al., 1996; Weiss et al., 1998), TSH, T4 and T3 concentrations in TRβ<sup>–/–</sup> mice were increased 3- to 6-fold compared with normal controls (Figure 3). In double mutant mice, both TSH and thyroid hormone concentration were surprisingly high. Plasma T4 and T3 levels were ~10-fold higher than those of normal mice, whereas plasma TSH concentration was increased by >100-fold (Figure 3).

**Histological features of the thyroid in TRα<sup>–/–</sup>, TRβ<sup>–/–</sup> and double mutants**

At the autopsy of 3-week-old mice, gross examination revealed an enlargement of the thyroid gland in TRβ<sup>–/–</sup> and TRα<sup>–/–</sup>TRβ<sup>–/–</sup> mice compared with wild-type mice, whereas in TRα<sup>–/–</sup> mice the thyroid gland was much smaller and could not be distinguished from the muscular and the adipose surrounding tissues. Histological comparison of the largest sections of the tracheal block confirmed the diffuse hyperplasia of the thyroid gland in the TRβ<sup>–/–</sup> (Figure 4, compare C1 with A1) and in the TRα<sup>–/–</sup>TRβ<sup>–/–</sup> mice (compare D1 with A1 in Figure 4), in contrast to the hypoplasia observed in the thyroid gland of the TRα<sup>–/–</sup> mice (Figure 4, compare B1 with A1). On the high magnification, the follicles in TRα<sup>–/–</sup> mice were small and disorganized (Figure 4, compare B2 with A2), TRβ<sup>–/–</sup> and double mutants exhibited an increase in the number of follicles but the thyroid glands of these animals differed in size and content of the follicles, aspects of colloid and development of vascularization (Figure 4, compare C2 and D2 with A2). In agreement with the observations of Forrest et al. (1996), Figure 4C2 clearly shows an increase in size of the follicles in TRβ<sup>–/–</sup> compared with wild-type

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**Fig. 2.** Growth curves of the seven pups’ progeny resulting from a TRα<sup>+</sup>/TRβ<sup>–/–</sup> mice intercross. Squares, TRβ<sup>–/–</sup> animals; triangles, TRβ<sup>–/–</sup>TRα<sup>+</sup> animals; circles, TRβ<sup>–/–</sup>TRα<sup>+</sup> animals.

**Fig. 3.** T3, T4 and TSH are markedly increased in mice lacking TRβ receptors. Thyroid hormones (total T4, total T3) and TSH concentrations in serum from 3-week-old wild-type (wt; black bars), TRα<sup>–/–</sup> (hatched bars), TRβ<sup>–/–</sup> (cross-hatched bars) or double mutant mice (white bars). The number of animals used for each measurement is indicated in parentheses on the top of each bar. Statistical analysis was performed using an unpaired t-test to assess the significance of the differences observed between the values obtained in the different genotypes.
Fig. 4. Histology of the thyroid gland in TR mutants. Transverse sections through the thyroid trachea block of a 3-week-old control mouse (A), and TRα−/− (B), TRβ−/− (C) and TRα−/−TRβ−/− (D) mutant mice. The TRβ−/− (C1) and TRα−/−TRβ−/− (D1) exhibited a bilateral thyroid enlargement and the TRα−/− (B1) exhibited a moderate but clear hypoplasia, compared with controls (A1). Scale bars in A1–D1, 200 μm. pt: parathyroid gland. Higher magnification showed that enlargement was due to increased number and size of follicles (f) in TRβ−/− mice (C2), compared with controls (A2). In TRα−/−TRβ−/− mutants (D2), small follicles exhibited signs of hyperactivity: colloid vacuoles (v), cuboid epithelium and hypervascularization (bv). Scale bars in A2–D2, 20 μm.
Knockouts of thyroid hormone receptors in mice

Fig. 5. Bone development is impaired in double mutant as in TRα−/− mice. Longitudinal bone sections (7 μm) of wild-type (A), TRβ−/− (B), TRα−/− (C) and TRα−/−TRβ−/− (D) mice. Bar, 200 μm. m, metaphysis; d, diaphysis. The horizontal arrow indicates the growth plate.

mice (Figure 4A2). In TRα+/−TRβ+/− mice (Figure 4D2), the follicles were smaller and contained a less abundant colloid with vacuoles near the epithelium. The capillaries filled with red cells seemed more numerous in TRα+/−TRβ+/− than in TRβ−/− mice. Active colloid resorption and increased vascularity observed in the thyroid gland of double mutant animals are consistent with hyperactivity.

Delayed maturation of bone and intestine in TRα+/−TRβ+/− mice

The overall morphology of the skeleton was not disturbed in TRβ−/− or in double mutant animals. Histological examination of the tibia of TRβ+/− mice did not reveal any difference compared with the wild-type animals (Figure 5A and B). In contrast, the tibia of TRα+/−TRβ+/− mice exhibited impaired development of epiphysial bone centres characterized by a hypertrophied cartilage associated with a low ossification (Figure 5D). This phenotype is identical to the one observed in TRα−/− mice (Figure 5C; Fraichard et al., 1997). This study was carried out with 2-week-old animals. At this age, TRα−/− and wild-type animals have similar thyroid hormone plasma concentrations (data not shown), avoiding any interference of hypothyroidy with the phenotype.

Morphological appearance of the small intestine in the four groups of 2-week-old animals was also studied. As previously described, TRα+/− animals showed reduced length of the intestine as well as a greater fragility compared with the wild-types. This was also observed in the double mutant mice. On the contrary, we did not observe any difference between wild-type and TRβ−/− animals. Histological analysis was then conducted at the proximal and distal levels of the small intestine and in the proximal colon. In accordance with previous observations, the colon was not affected by the receptor deficiency. Here we show histological staining at the distal ileum level (Figure 6). The intestinal size and the length of the crypt-villus unit was reduced in the TRα−/− compared with wild-type mice (Figure 6, C and D versus A and B). On the contrary, TRβ−/− animals did not show such intestinal alterations (Figure 6E and F). The intestines of the double knockout mice displayed a histological impairment similar to TRα−/− mice at the proximal jejunum level (data not shown; Fraichard et al., 1997). Strikingly, a much more dramatic alteration was observed in the distal ileum of double mutant mice compared with TRα−/− mice (Figure 6G–I). This severe phenotype was characterized by very few and short villi-lined, flat epithelial cells (Figure 6I). The thickness of the external muscle coats in double mutants was similar to TRα−/− DI (Figure 6H versus D).

Discussion

Unliganded TRβ is not responsible for the phenotype resulting from the disruption of the TRα gene

We have previously described a severe phenotype in mice lacking the TRα1 and TRα2 proteins (Fraichard et al., 1997).
Fig. 6. Small intestinal phenotypic maturation is more severely impaired in double mutant than in TRα–/– mice. Periodic acid–Schiff (PAS) staining of transverse section of wild-type (A and B), TRα–/– (C and D), TRβ–/– (E and F) and TRα–/–TRβ–/– (G and H) ileum. At low magnification (A, C, E and G), bar = 250 μm. v, villi; c, crypt. At higher magnification (B, D, F and H), bar = 100 μm. cml, circular muscle layer; lml, longitudinal muscle layer. At very high magnification (I), bar = 40 μm.

1997). TRα–/– animals displayed progressive hypothyroidism together with multiple disorders leading to death shortly after the weaning period. We postulated that this phenotype could be the result of hypothyroidism reinforced by the dominant negative activity of unliganded TRβ in these mice. The ablation of the TRβ gene does not correct the severe phenotype observed in TRα–/– animals, showing that the phenotype generated by the disruption of TRα is not the result of a dominant-negative activity of the TRβ aporeceptor.

TRα and TRβ cooperate to repress TSH production

The hyperthyroxinemia observed in TRβ–/– animals (our data; Forrest et al., 1996) indicates that, in normal mice, liganded TRβ receptors repress TSH synthesis, and this very probably mainly results from direct interaction of TRβ with negative response elements present in the TSHβ gene promoter (Wondisford et al., 1989; Wood et al., 1989), although TRH deregulation may also occur (Feng et al., 1994; Yamada et al., 1997). These data suggest that TRα alone is not able to fully control TSHβ expression. However, the comparison between TRβ–/– and TRβ–/– TRα–/– mice presenting extremely high circulating TSH levels, shows that TRα is also capable of repressing TSHβ expression. This confirms recent observations suggesting that TRα1 could regulate the production of TSH and thyroid hormones in the absence of TRβ receptors (Weiss et al., 1997). We propose that in normal mice, TRβ and
TRα cooperate to repress TSHβ transcription, TRβ being a more potent repressor.

In apparent contradiction with this model, we have previously shown that the absence of TRα receptors in wild-type mice leads to hypothyroxinemia (Fraichard et al., 1997), and others have described limited hypothyroxinemia in TRα1−/− mice (Wikstrom et al., 1998). These data suggested that the TRα proteins might balance the action of TRβ receptors and positively control the production of thyroid hormones. This contradiction is easily resolved if we assume that the absence of the weak repressor, TRα, eliminates the competition between the two types of receptors and enables the stronger repressor, TRβ, to fully exert its repression potential. This assumption is supported by data showing that TRβ proteins are more efficient than TRα1 in binding the negative thyroid hormone response element of the TSH promoter and repressing its activity (McCabe et al., 1998).

The TRαα and TRβ proteins have redundant functions in some but not all tissues

The extended pattern of expression of the TRα gene may account for the pleiotropic phenotype of TRα−/− mice. In contrast, TRβ−/− mice display a mild and tissue-restricted phenotype (Forrest et al., 1996; our data), consistent with their more restricted pattern of expression. In some tissues, however, the TRαα and TRβ genes are coexpressed (Bradley et al., 1989, 1992; Forrest et al., 1990, 1991; Macchia et al., 1990; Strait et al., 1990, 1991). This raises the possibility of a functional redundancy between TRα and TRβ proteins. Our data show that this assumption is verified in some, but not all tissues.

As bones from young TRα−/− and TRα+−/−TRβ−/− mice look identical, and are normal in TRβ−/− animals, it seems unlikely that TRβ plays any function in long bone formation although it is expressed in chondrocytes (Abu et al., 1997). We conclude that TRα, but not TRβ, is essential for the normal postnatal maturation of bone, and that TRβ cannot substitute for TRα in this process.

In contrast, histological analysis reveals clear differences between TRα−/−TRβ−/− and TRα+−/− mice in the distal ileum, but not in the jejunum or in the colon. In the ileum, both TRα and TRβ are coexpressed in the epithelium (Plateroti, M., Chassande, O., Fraichard, A., Gauthier, K., Freund, J.-N., Samarut, J. and Kedinger, M., submitted). Our data suggest that these genes are redundant in distal ileum, although TRβ is not able to fully compensate for the absence of TRα. They also suggest that TRβ is responsible for the partial recovery of the intestinal phenotype after treatment of TRα−/− mice by T3 injections (Plateroti, M., Chassande, O., Fraichard, A., Gauthier, K., Freund, J.-N., Samarut, J. and Kedinger, M., submitted). We assume that redundancy occurs for other functions and enables the rescue of TRα−/− mice by T3 (Fraichard et al., 1997).

The thyrocytes express TRβ and TRα isoforms (Selmin-Ruby and Roussel, 1996), but the functions of these proteins in the thyroid gland remain poorly understood. TSH exerts a tight control on the development and metabolism of the thyroid, as an increase in TSH concentration triggers hyperplasia and hyperplasia of the thyroid gland and results in enhanced secretion of thyroid hormones. Therefore, it is likely that the morphological and histological features of the thyroid gland in the TRβ−/− mice is the consequence of the increase in TSH concentration. Further increase in TSH concentration would be expected to result in enhanced hyperthyroso, as it is observed in pathological or experimental hypothyroidism or in TRβ−/−TRα1−/− mice (D. Forrest, personal communication). However, in our double mutant animals, which display a very high concentration of TSH, the hyperthyrosis of the gland is blunted. Since the disruption of the TRα gene in TRα−/− animals leads to hypothyrosis of the thyroid gland, it is possible that their absence in double mutant mice prevents a larger hyperthyrosis in response to TSH. From these data we assume that TRα plays an important role in the development of the thyroid gland. In conclusion, this work shows that the currently known T3 receptors are dispensable for the embryonic development of the mouse and that the products of the TRα and TRβ genes display tissue-specific functions with limited redundancy.

Further investigations using targeted disruption of the TRαα and TRβ genes should unravel the respective contribution of each receptor isofrom to peripheric functions, and the molecular basis of the tissue-specific functions.

Materials and methods

ES cell selection and generation of mutant mice

The TRβ targeting vector was constructed using pGNA6 as starting plasmid (gift from Dr P.Brulet). The 3′ arm homologous to TRβ was cloned from a λEMBL4 library (gift from Dr J.P.Magaud). The 5′ arm was amplified by PCR from mouse 129 DNA. The thymidine kinase gene was inserted as previously described (Fraichard et al., 1997). The targeting vector was digested with SpeI. ‘ENS’ ES cells were established from 129sv blastocysts in our laboratory by B.Pain and D.Aubert according to the technique described previously (Robertson, 1987). These cells were karyotyped as male and were proven to contribute with a very high efficiency to the germline when injected into blastocysts. We routinely maintain ‘ENS’ cells on MEF feeders in Glasgow minimal essential medium supplemented with 10% fetal bovine serum and 1000 U/ml LIF. ‘ENS’ ES cells were electroporated with 40 μg linearized plasmid, then selected with G418 (Gibco-BRL) and Gancyclovir and, after 10 days, clones were picked for screening. Positive clones were subsequently tested for correct karyotype and absence of mycoplasma and subsequently injected into blastocysts from C57B/6 recipient mice. The genotype of the progenies of the mice carrying a TRβ mutation was analysed using a PCR-based screening procedure. DNA (20–100 ng) extracted from tails was subjected to amplification using a mixture of four oligonucleotides: β3 (GGAGTCTCTACT-AGAGTAC) and lacZAS (CCTCTTCGCTATTACGCCAGCTGG) allowed the amplification of the mutant allele. β5 (GGTGCTGGATGACAGCAAGA) and β6 (CAGGAATTCGGCTTGTGCTT) allowed the amplification of the fragment from the wild-type allele. The amplification was carried out on a thermocycler (Perkin Elmer) using the following procedure: 94°C, 2 min, then (94°C, 15 s, 58°C, 15 s, 72°C, 60 s) for 35 cycles. PCR was performed using 0.5 μg of total RNA and 0.5 μg of random primers were mixed in 10 μl of water, heated to 68°C for 5 min and cooled to 37°C. Then, 10 μl of polymerization mix [100 mM Tris–HCl pH 8.3, 150 mM KCl, 20 mM dithiothreitol, 6 mM MgCl2, 0.5 mM each dNTP, 200 U reverse transcriptase (Promega)] were added and the mixture was incubated at 37°C for 30 min. PCR was performed using 0.8 units Eurobiotaq thermostable DNA polymerase (Eurobio, France) in a 50 μl reaction mix. One microlitre of reverse transcription mix was used in each reaction. The detection of TRβ transcripts was carried out using oligonucleotides E6 (GGTGCTGGATGACAGCAAGA) and E7A (GCATTACAGATGGGTCTGTG) were used as primers. Oligonucleotides VIS (GGAGATGATTCGCTCACTGAG) and 15A (CACGC-CTGCAGACAGACCATTCCGT) were used as primers to detect TRα transcripts. The amplification was performed using the following parameters: 94°C, 2 min, (94°C, 15 s, 58°C, 15 s, 72°C, 60 s) for 35 cycles.
**Intestine and bone analysis**

Intestine analysis. Jejunum and ileum were dissected from 2-week-old pups, fixed in Bouin’s reagent and embedded in paraffin for serial sections and morphological observations. Sections of 5 μm were stained according to the standard PAS protocol (Segal and Petras, 1992).

Bone analysis. Leg tibia were dissected out from 2-week-old pups, fixed in 10% alcohol and embedded in paraffin. Undecalcified sections (7 μm) were obtained and stained with Goldner’s trichrome, according to the bone histomorphometric protocol (Meunier, 1983). Longitudinal medial sections were analysed with a true colour image-processing workstation Visioblab 1000 (Biocon, France).

**Hormone assays and thyroid gland histology**

Three-week-old mice were anaesthetized with ketamine (Panpharma, France) + Hypnovel (Roche, France) and bled intracardially. Sampling for cell experiments was performed from 12 a.m. to 3 p.m. to minimize day–night variations. Serum concentrations of total T3 and total T4 were determined by specific radioimmunoassay, using sodium anilino-naphthalene sulfonate and acidic buffer, as binding inhibitors of serum proteins (Roussel et al., 1984). TSH was measured by specific radioimmunoassay, as described previously (Weiss et al., 1997). Tracheal blocks of mice were dissected out, immediately fixed in Bouin Holland, embedded in paraffin and cut in totality by serial sections of 5 μm. Sections were stained according to the standard Masson’s Trichrome protocol. Thyroid areas were compared on the largest sections.

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