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Maternal thyroid hormones are transcriptionally active during embryo–foetal development: results from a novel transgenic mouse model

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Abstract

Even though several studies highlighted the role of maternal thyroid hormones (THs) during embryo–foetal development, direct evidence of their interaction with embryonic thyroid receptors (TRs) is still lacking. We generated a transgenic mouse model ubiquitously expressing a reporter gene tracing TH action during development. We engineered a construct (TRE2×) containing two TH-responsive elements controlling the expression of the LacZ reporter gene, which encodes β-galactosidase (β-gal). The specificity of the TRE2× activation by TH was evaluated in NIH3T3 cells by cotransfecting TRE2× along with TRs, retinoic or oestrogen receptors in the presence of their specific ligands. TRE2× transgene was microinjected into the zygotes, implanted in pseudopregnant BDF1 (a first-generation (F1) hybrid from a cross of C57BL/6 female and a DBA/2 male) mice and transgenic mouse models were developed. β-gal expression was assayed in tissue sections of transgenic mouse embryos at different stages of development. *In vitro*, TRE2× transactivation was observed only following physiological T3 stimulation, mediated exclusively by TRs. *In vivo*, β-gal staining, absent until embryonic day 9.5–10.5 (E9.5–E10.5), was observed as early as E11.5–E12.5 in different primordia (*i.e.* central nervous system, sense organs, intestine, etc.) of the TRE2× transgenic embryos, while the foetal thyroid function (FTF) was still inactive. Immunohistochemistry for TRs essentially colocalized with β-gal staining. No β-gal staining was detected in embryos of hypothyroid transgenic mice. Importantly, treatment with T3 in hypothyroid TRE2× transgenic mice rescued β-gal expression. Our results provide *in vivo* direct evidence that during embryonic life and before the onset of FTF, maternal THs are transcriptionally active through the action of embryonic TRs. This model may have clinical relevance and may be employed to design end-point assays for new molecules affecting THs action.

Keywords: maternal thyroid hormone • thyroid receptors • thyroid responsive element • transgenic mouse • embryonic development • central nervous system • reporter gene

Introduction

Tetraiodothyronine (T4) is secreted by the thyroid gland as a prohormone that is converted in the tissues to triiodothyronine (T3), the active hormone that binds to the nuclear receptors and initiates thyroid hormone (TH) action [1]. In extrathyroidal tissues,

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T3 concentration in the intracellular and nuclear compartments is dependent on (*i*) the circulating levels of THs, (*ii*) their rates of entry and exit in and out of the cell and the nucleus, (*iii*) the rate of T4 to T3 conversion and (*iv*) T3 degradation in the cell [2]. The formation and degradation of T3 in tissues are dependent primarily on the activities of three selenodeiodinases (type 1, type 2 and type 3) that catalyse the selective removal of iodine from iodothyronines and convert the precursor T4 into the active hormone T3, and the inactive hormones reverse T3 and T2 [3]. Importantly, the cellular uptake and the release of THs are mediated by transporters. Among these, monocarboxylate transporter 8 (MCT8) is particularly important in the transport of T3 into the brain [4, 5].

THs are necessary for differentiation, growth and metabolism in mammals as well as lower organisms [6]. Its biological action is mediated by thyroid hormone receptors (TRs) binding to TH-responsive elements (TREs) in the regulatory regions of target genes that regulate gene transcription by chromatin remodelling [7, 8]. In the absence of TH, TRs bind a complex of inhibitory proteins that promote deacetylation and inhibit gene transcription [9]. Recently another mechanism by which the nuclear corepressor NCoR modulates expression of positive TRs targets and regulates the response to set levels of T3 was demonstrated [10].

Both T4 and T3 have been importantly detected in the rat embryo and foetal brain before the onset of foetal thyroid function (FTF) [11, 12]. T4 has been found in human embryonic cavities in the first trimester of pregnancy, thus suggesting that maternal T4 could cross the placenta. From the coelomic fluid it may reach the embryo *via* the yolk sac, and may be functionally important for the developing embryo, while its own thyroid is not yet functioning [13]. In addition, significant levels of total T4 were found in umbilical cord sera of term neonates affected by congenital hypothyroidism, unable to produce any T4 due to total iodide organification defects. After birth, T4 serum levels gradually decreased, and became undetectable within 2 weeks, thus indicating that substantial amounts of T4 are transferred from mother to foetus during late gestation [14]. Nuclear T3 receptor has been interestingly found in human foetal brain, liver, heart and lung at early and mid gestation [15, 16].

Indeed, maternal THs have been consistently described to play a crucial biological role in brain development, affecting dendritic elongation and branching of Purkinje cells, synaptogenesis, proliferation and migration of granule cells, and myelination [7, 17]. Both maternal hypothyroidism and hyperthyroidism have deleterious effects on the outcome of human pregnancy [18]. High maternal THs levels (not associated with autoimmune thyroid disease), by itself, produce foetal thyrotoxicosis, impair embryogenesis and cause higher miscarriage rates through a direct toxic effect of excess THs on the human foetus [18].

Furthermore, maternal thyroid failure even in terms of hypothyroxinemia alone (reduced FT4 and normal TSH levels), or subclinical hypothyroidism may be harmful to the human foetus. In fact, several rodent models demonstrated that maternal hypothyroidism may have different effects in the offspring: (*i*) severe defects in the cerebral and cerebellar cortex, and in visual and auditory development [7, 19–21]; (*ii*) inappropriate gene expression in foetal rat brain [22, 23] and (*iii*) alterations in cell migration in the cerebral cortex and hippocampus [19, 24, 25].

Maternal hypothyroxinemia is 150–200 times more prevalent than congenital hypothyroidism and results in lack of TH in embryo–foetal primordia during early pregnancy, before the onset of FTF [26, 27]. Severe iodine deficiency during pregnancy may induce maternal and foetal hypothyroxinemia, and cause neurological dysfunctions in the progeny [28–32]. Importantly, permanent alterations in the cytoarchitecture of the cerebral cortex appear in the progeny of hypothyroxinemic but not in hypothyroid dams [33].

The hypothesis that maternal THs can interact with embryonic TRs is supported by two facts: (*i*) maternal T4, which is essential for the development of the brain and other foetal organs was found in coelomic and amniotic fluid in human beings and other species before FTF became active [25, 34]; (*ii*) TRs are expressed in the cerebral cortex of first trimester human foetuses [15, 35], as well as in rodent embryonic and adult neural tissues [36–38].

However, direct evidence to support a physiological interaction between maternal TH and embryonic TRs during the early and late stages of embryogenesis is still lacking [25].

To address this question, transgenic mouse models expressing the LacZ reporter gene (encoding β-galactosidase [β-gal]) under a TRE, have been generated to specifically trace maternal TH transcription activity during early and late embryo–foetal development.

Materials and methods

Engineering the transgenic vector TRE2×

To generate TRE2× we used a truncated enhancer region (MBP-TRE-18) of the native myelin basic protein (MBP-TRE-33) gene promoter [39–41]. We chose this sequence (MBP-TRE-18) and configuration because it provides a selective and robust response to TRs ($TR\beta_1 >> TR\alpha_1$) [39]. TRE2× containing two MBP-TRE-18 [39, 40] spaced by two nucleotides (gc) was cloned into the pNASSβ vector (generously provided by Dr. G. Piaggio, Regina Elena Cancer Institute, Roma, Italy) expressing LacZ reporter gene in order to create pTRE2×. The structure of pTRE2× is shown in Fig. 1A. The following forward and reverse oligonucleotides were used for vector generation: 5′-CCTCGAGAAGCTTACCTCGGCTGAGGACACGgcACCTCGGCTGAGGACACGG-3′ and 5′-GATCCCGTGTCCTCAGCCGAGGTgcCGTGTCCTCAGCCGAGGTAAGCTTCTCGAGGCATG-3′, respectively. These comprised (from 5′ to 3′): four restriction sites, SphI, XhoI, HindIII and BamHI, and two TRE-18 spaced by two base pairs (bp) (gc). The resulting double-stranded oligo was digested and subcloned into the SphI site downstream of TK minimal promoter of the pBLCAT2 vector [39]. A TK construct (named pNASSβ-TK), deleted of the MBP-TRE-18 sequences, was used as a negative control. All plasmids were checked by automated DNA sequence analysis.

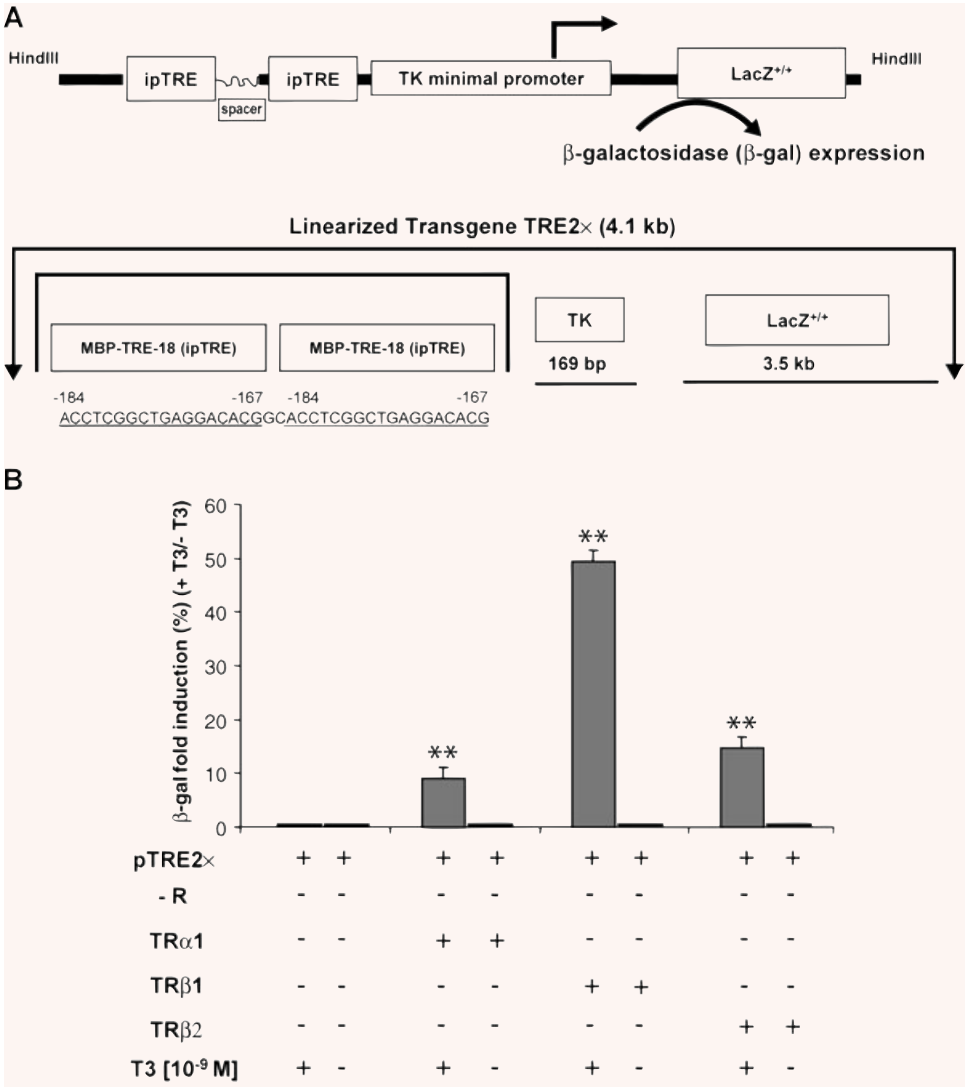

Fig. 1 Effects of T3 binding to TRs on the transactivation of TRE2×. (**A**) The structure of the transgene TRE2× (4.1 kb) is as follows: (*i*) two truncated enhancer regions (thyroid response elements: myelin basic protein (MBP-TRE-18) gene promoter, –184/–167) of the native MBP-TRE-33, each one composed of 18 nucleotides with four half-sites arranged as an imperfect inverted palindrome (ipTRE), which are required for the T3 thyroid receptors in order to bind the DNA; (*ii*) a thymidine kinase minimal promoter (TK) originating from herpes simplex virus upstream at the 5′-flanking region of the LacZ reporter gene. (**B**) TRE2× transactivation in the presence or absence of TRs and T3 (10^{-9} M). After 72 hrs NIH3T3 cells treated or untreated with T3 (10^{-9} M) were harvested and β-gal activity (an indicator of the interaction between T3 and TRs on TRE2×) was quantified. The fold induction of normalized β-gal activity (β-gal/luciferase/total protein) is calculated by the ratio between the effects in the presence and in the absence of T3. The maximum effect on TRE2× transactivation was provided by TRβ1>>>TRβ2>TRα$_1$ (**$P < 0.01$). These results represent the average ± S.D. of at least three independent experiments each performed in duplicate.

Cell transfections

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (FCS) (Sigma, St. Louis, MO, USA). Cell transfections were performed by electroporation according to Desvergne *et al.* [42], in the presence of hormone-free FCS-supplemented medium. Briefly, 6 μg of pTRE2× or pTK along with 12 μg of each of the following nuclear receptor plasmid were electroporated using the Bio-Rad GenePulser at 960 μfarads and 250 V: rat TRα$_1$, rat TRβ$_1$, mouse oestrogen receptor (ER)α and ER-β (generously provided by Dr. A. Farsetti), mouse TRβ$_2$, retinoic X receptors (RXR)α and RXR-γ (generously provided by Dr. P. Yen, Johns Hopkins Bayview Medical Center, Baltimore, USA), mouse RXR-β (generously provided by Dr. C. Gaetano, Istituto Dermopatico dell'Immacolata, Roma, Italy), or mouse retinoic acid receptor (RAR)α and RAR-β (generously provided by Dr. M. Cippitelli, Regina Elena Cancer Institute, Roma, Italy).

Transgenic mouse models

Animal care

Animal care procedures were conducted in accordance with the guidelines of the European Community Council Directives (86/609/EEC). Animals

were kept under controlled temperature (22 ± 2°C) and light (12 hrs light: 12 hrs dark cycle; lights on at 07:00 am) conditions, and had free access to food and water.

Transient transgenic embryos (transient transgenic model)

Linearized pTRE2× (named TRE2×) was injected into (C57Bl/6xDBA/2)F2 zygotes as described by Nagy *et al.* [43]. After zygote implantation, foster mothers were killed at embryonic day 9.5 (E9.5), E10.5, E11.5, E12.5, E13.5 (early embryo development) and E15.5 and E17.5 (late embryo development) in order to analyse TRE2× genome integration and β-gal expression.

Stable transgenic mice

To generate stable transgenic lines, linearized TRE2× transgene was injected into (C57Bl/6xDBA/2) F2 zygotes as described by Nagy *et al.* [43]. One founder (out of 60 pups) was identified by PCR and homozygous mice were analysed by quantitative real-time PCR.

Transgenic embryos derived from homozygous mice matings were collected at E9.5, E10.5, E11.5, E12.5, E13.5, E15.5 and E17.5, and analysed for β-gal and nuclear receptors expression.

Screening of transgenic mice by PCR

Genomic DNA was extracted from mouse placenta or mouse tail biopsies by the addition of 1% SDS, 50 mM Tris-HCl (pH 8.0) and 10 mg/ml of proteinase-K (Invitrogen, San Giuliano Milanese, Italy), and incubated overnight at 55°C. DNA was purified by phenol-chloroform extraction and ethanol precipitation. TRE2× and TK transgenic embryos were screened by PCR using the following primers, respectively: (*i*) TRE2×-Forward-5′-ACCTCGGCTGAGGACACGGCACC-3′ and LacZ-Reverse-5′-GGCCGTAACCGACCCAGCGCCCG-3′ that amplify the DNA region comprised between the TRE-TK (enhancer-promoter region) and LacZ sequences and (*ii*) TK-Forward-5′-GGATCCGGCCCCGAGCGTCT-3′ and LacZ-Reverse-5′-GGCCGTAACCGACCCAGCGCCCG-3′ that amplify the region between the TK promoter and LacZ sequences. PCR analysis was carried out on 200 ng genomic DNA by TAQ polymerase (Invitrogen). After 40 cycles (1 min. at 95°C, 1 min. at 67°C and 1 min. at 72°C) the PCR products of TRE2× and TK were analysed on a 0.8% agarose gel. As positive controls for TRE2× and TK transgene amplicons, we used the pNASSβ and pBLCAT2 vectors, respectively. As negative control, we used genomic DNA without LacZ gene sequences.

Genotyping assay by real-time PCR

Homozygous mice were identified by quantitative real-time PCR and confirmed by breeding test. The breeding test was performed by mating between homozygous mice and wild-type mice. Quantitative real-time PCR was performed according to Shish *et al.* [44] on genome DNA of different founders in order to quantify TRE2× copy number. LacZ primers (forward: ACGCGCGAATTGAATTATGG and reverse: GTTGACTGTAGCGGCTGATGTT) were designed using the Primer Express oligo design software (Applied BioSystems, Foster City, CA, USA) and synthesized by Integrated DNA Technologies (Coralville, IA, USA). SYBR Green I assay and the ABI 7500 Fast Real-time PCR System were used for detecting real-time PCR products using the master template standard curve approach according to Shih *et al.* [44].

Hypothyroidism

Hypothyroidism was induced in TRE2× transgenic female mice according to Schneider *et al.* [45] by treatment with 0.1% 2-mercapto-1-methylimidazole (MMI) (Sigma) and 1% potassium perchlorate ($KClO_4$) (Sigma) dissolved in deionized water and administered by oral gavage (200 μl per day) for approximately 4–6 weeks. In addition, low iodine diet (LID) was used. TRE2× transgenic female mice were continuously treated with 0.1% MMI and 1% $KClO_4$ beginning approximately 4–6 weeks before pregnancy induction and with 0.1% MMI alone combined with LID from E11.5 until the end of pregnancy they were treated.

Serum total T4 and T3 concentration were measured by coated tube radioimmunoassay (RIA)s (Diagnostic Products, Los Angeles, CA, USA) adapted for mouse serum using 25 and 50 μl serum, respectively [46]. TSH was measured in 50 μl serum using a sensitive, heterologous disequilibrium double-antibody precipitation RIA [46]. The minimal detectable concentrations in the assay were 10 mU/l for TSH, 3.21 nmol/l for total T4 and 0.15 nmol/l for total T3. All mouse sera in this study were assayed for TSH activity by a blind tester.

This animal study was approved by Institutional Review Board (Regina Elena Cancer Institute, Rome, Italy).

Treatment with T3

We treated 14 hypothyroid transgenic mice (TRE2×$^{+/+}$) with L-T3 (Sigma-Aldrich, Milan, Italy) (1.5 μg/100 g body weight per day) by intraperitoneal injections for 4 days (from E9.5 to E12.5). As control group, 14 hypothyroid transgenic mice (TRE2×$^{+/+}$) received the vehicle alone. The animals were killed at E12.5; transgenic embryos were collected and stained for β-gal activity (β-gal expression). Blood samples were collected 6 hrs after the last T3 dose from the tail or cava veins. The sera were tested for TSH, total T3 and T4 levels according to Pohlenz *et al.* [46].

β-gal enzymatic assay in embryos

β-gal activity assay (β-gal expression) was carried out on E9.5, E10.5, E11.5, E12.5 and E13.5 transient and stable embryos as described by Vernet *et al.* [47]. Briefly, embryos were fixed using 1% paraformaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA and 0.02% NP40 in PBS (pH 7.3) for 30–90 min. at 4°C. After several washes in 0.02% NP40 in PBS, they were stained at 37°C overnight in the dark using 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, 0.02% NP40, 20 mM Tris-HCl (pH 7.3) and 1 mg/ml X-gal dissolved in PBS. β-gal protein was stable overnight at 37°C according to Nagy *et al.* [43]. Embryos were post-fixed in 4% paraformaldehyde/PBS for 12–24 hrs, dehydrated in an ascending series of alcohols, embedded in paraffin and sectioned on the coronal or sagittal planes at 10 μm. Embryos at E15.5 and E17.5 were embedded in OCT compound (Bioptica, Milan, Italy) and frozen in cold isopentane, and β-gal histochemistry was performed on frozen tissue

sections as described by Signoretti *et al.* [48]. Finally, selected β-gal stained sections of E9.5, E10.5, E11.5, E12.5, E13.5, E15.5 and E17.5 embryos were processed for immunohistochemical analysis.

Immunohistochemistry

Ten-micrometre-thick sagittal or coronal sections of E9.5, E10.5, E11.5, E12.5, E13.5, E15.5 and E17.5 transient and stable embryos were obtained with a Leica sliding microtome and mounted in series on superfrost slides. One series was counterstained with 1% neutral red, cover slipped in Eukitt mounting medium and photographed to describe the distribution of β-gal^{+} areas. The others series were used for immunohistochemistry (IHC) using antibodies against TRs, RXRs and RAR. To localize TRs, sections were processed at room temperature (r.t.). They were incubated in 0.3% H_2O_2 in 0.1 M PB-saline (PBS) for 30 min. in order to inhibit endogenous peroxidises and then washed in PBS. Sections were transferred for 1 hr into PBS containing 0.3% Triton X-100, 10% normal goat serum and 3% bovine serum albumin. Sections were incubated overnight (TRs) or for 48 hrs (RXRs and RAR) at 4°C in a solution containing a rabbit polyclonal antibody against TRα$_1$, TRβ$_1$, TRβ$_2$, RXR-α, RXR-β, RXR-γ (generously provided by Dr. P. Yen, Johns Hopkins University, USA) or RAR-α (sc-551, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Binding of the primary antibody was visualized by incubating the tissue with biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA; diluted 1:200 in PBS-T) for 4 hrs at r.t., followed by an avidin-biotin-peroxidase complex (Vector Laboratories, Elite ABC Kit, Burlingame, CA, USA) for 2 hrs at r.t.; the peroxidase was detected using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) or 3-Amino-9-ethylcarbazole (AEC, Dako, Glostrup, Denmark) as chromogen. After cover slipping in Mowiol, sections were observed using a Nikon Eclipse E-800 light microscope and photographed by a Coolpix 990 digital camera (Nikon, Celentano Firenze, Italy).

Paraffin-embedded sections of rat pituitary were used as positive controls for TRs. For RXR and RAR families rat pituitary and liver paraffin-embedded sections were used. The specificity of the antisera, certified by the manufacturer, was validated by omitting the antisera from the incubation medium. In addition, as a further negative control for TRs we used pituitary paraffin-embedded tissues from mice TRα$_1^{-/-}$ and PAX8$^{+/-}$ (female number 268 and male number 280) and TRβ$^{-/-}$ and PAX8$^{+/-}$ (female number 166 and male number 183), kindly provided by Dr. Heike Heuer ('Neuroendocrinology' Leibniz Institute, Jena). The colocalization between β-gal activity (blue staining) and nuclear receptors expression was evaluated by immunoreacting E9.5, E10.5, E11.5, E12.5 E13.5, E15.5 and E17.5 embryos, previously reacted for β-gal in whole mount, on 10-μm-thick sagittal or coronal serial sections. Because DAB precipitates could have masked the light blue β-gal staining, β-gal^{+} adjacent sections were considered for comparison. Similarly, some embryos were reacted for IHC without β-gal staining. Immunohistochemical staining (immunoreactivity) was evaluated semi-quantitatively as weakly (+), moderately (++) and strongly (+++) positive depending on the intensity; negative (−) when the staining was clearly absent, and uncertain (+/−) when the staining was not clearly positive.

Statistical analysis

P-values below 0.05 were considered significant and calculated by Student's t-test and ANOVA test. The results represent the average ± S.D. Statistical analysis was carried out using Excel Software version 1997–2003.

Results

β-gal expression as a marker of TH action

We cloned TRE into a vector expressing β-gal (LacZ gene reporter). This gene reporter is widely used due to its sensitivity for *in vivo* labelling of different cell populations by catalysing a colorimetric (blue) chemical reaction [49]. We therefore used this molecular system to trace TH action during embryo–foetal development.

First, we assessed TRE2× specificity (Fig. 1A) by transient cotransfections performed in NIH3T3 cells using TRs, RXRs, RARs or ERs expression vectors in the presence or absence of the following ligands: all-trans retinoic acid, 9-*cis*-retinoic acid and oestradiol, used at a final concentration of 5 μM, 10^{-7} M and 10^{-8} M, respectively. TRE2× transactivation was not detected upon treatment with any of these ligands (data not shown).

Conversely, upon T3 treatment (10^{-9} M), transgene TRE2× transactivation was significantly induced in terms of β-gal expression only in the presence of TRα$_1$ (fold induction = 9.1 ± 0.2), TRβ$_1$ (fold induction = 49.36 ± 1.2), or TRβ$_2$ (fold induction = 14.7 ± 1.2) ($P < 0.01$) (Fig. 1B).

The empty vector TK (without TRE sequences) was used as a negative control and did not elicit any LacZ transcriptional activity (data not shown).

Transgene expression in euthyroid transient embryos derived from TRE2× microinjection (transient model)

Upon TRE2× injection into (C57Bl/6xDBA/2)F2 zygotes followed by implantation (implanted zygotes = 921) into 72 euthyroid female mice (body weight = 24 g ± 0.3). Euthyroid mice showed values of TSH less than 10 mU/l, T4 51.60 nmol/l and T3 1.14 ± 0.09 nmol/l. TRE2× transactivation (β-gal expression) was analysed in 175 embryos (E9.5, E10.5, E11.5, E12.5, E13.5 and E17.5) (Table 1). Examples of β-gal^{+} transient transgenic embryos are shown in Fig. 2. TK^{+} transient transgenic embryos, used as negative controls, did not show any β-gal expression (data not shown).

Maternal TH action during early stages of development in euthyroid transient transgenic embryos

At E9.5 no β-gal staining was detected (Fig. 3A). TRs IHC at E9.5 showed weak expression of TRα$_1$, TRβ$_1$ and TRβ$_2$ in the midbrain and medulla oblongata (Fig. 3B–D). On the contrary, retinoic receptors (both RXR and RAR) were highly expressed (data not shown).

Transgenic embryos from euthyroid mice showed consistent β-gal expression in the same anatomical sites, as early as E11.5–E12.5. At E11.5–E12.5, the staining was localized in different

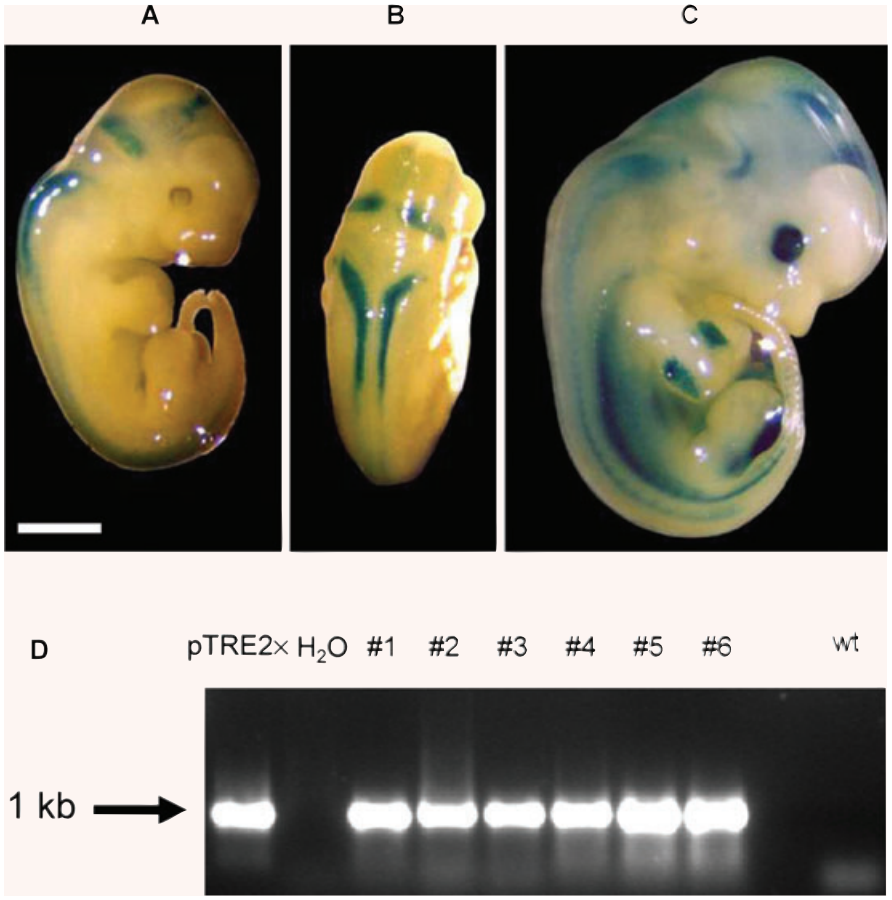

Fig. 2 Overall β-gal expression at E11.5 and E12.5. (**A–C**): Euthyroid transient transgenic embryos at E11.5 (**A**, lateral view; **B**, dorsal view) and E12.5 (**C**, lateral view). β-gal expression (blue staining) is localized in the metencephalic and diencephalic vesicles and in the ganglia of the cranial nerves (E11.5); in addition, in the E12.5 the staining is localized also in the eye and in the ear regions. Scale bar = 1 mm. In (**D**), E11.5 and E12.5 transient transgenic embryos (from #1 to #6) TRE2× positive (1 kb), screened by PCR analysis; wild-type (wt) DNA represents a sample without nucleotide sequences of LacZ reporter gene, used as a negative control.

Table 1 β-gal expression in TRE2× positive transgenic embryos during early (E9.5, E10.5, E11.5, E12.5 and E13.5) and late (E15.5 and E17.5) stages of development

Number of mice	Development	No. of transient transgenic embryos [c]β-gal expression	No. of embryos from stable transgenic mice [c]β-gal expression
12	[a]E9.5	0/30	0/20
12	[a]E10.5	0/30	0/20
12	[a]E11.5	7/20	15/15
12	[a]E12.5	33/75	20/20
12	[a]E13.5	4/10	12/12
12	[b]E15.5	-	8/8
12	[b,*]E17.5	4/10	13/13

E = embryonic day; a = early development; b = late development. [c]β-gal expression was consistently detected in the same anatomical sites of transgenic embryos derived from TRE2× positive transgenic mice (see text). No ectopic sites of β-gal expression were observed. *Thyroid foetal function is active.

primordial anatomical areas (Fig. 4), *i.e.* cerebellum, mesencephalon and myelencephalon. In particular, β-gal was highly expressed in the diencephalic vesicle (epithalamus, dorsal wall of the III ventricle) and in the mantle layer of alar plate of the lateral wall of the metencephalon, in the medulla oblongata, the marginal and mantle layers of the lateral wall of the midbrain, the mantle layer of the lateral wall of the spinal cord, the ocular annexa and the retina, the vestibular primordia (semicircular canal and endolymphatic sac) and in the trigeminal ganglion. Interestingly, the presumptive trigeminal ganglion, facial ganglion, vestibulococlear ganglion complex, inferior ganglion of the vagal nerve, the hypoglossal nerve, the sympathetic chain and the roots of the spinal nerves expressed high levels of β-gal. In contrast, β-gal protein expression was rarely detected in the ventricular layer of the telencephalon. In the skeleton, β-gal protein expression was found in the mesenchymal head of the palatine process, the maxillary process, the bones of the upper limb and in the myotomal muscle mass. In the circulatory system, β-gal was detected in the internal carotid, the sixth arch and the mesenteric arteries. A line of positive cells was also observed surrounding the abdominal aorta towards the gut. Moreover, β-gal expression was weakly detected in the liver, the stomach, the duodenum, the dorsal mesentery of the hindgut, the mesenchymal tissue surrounding

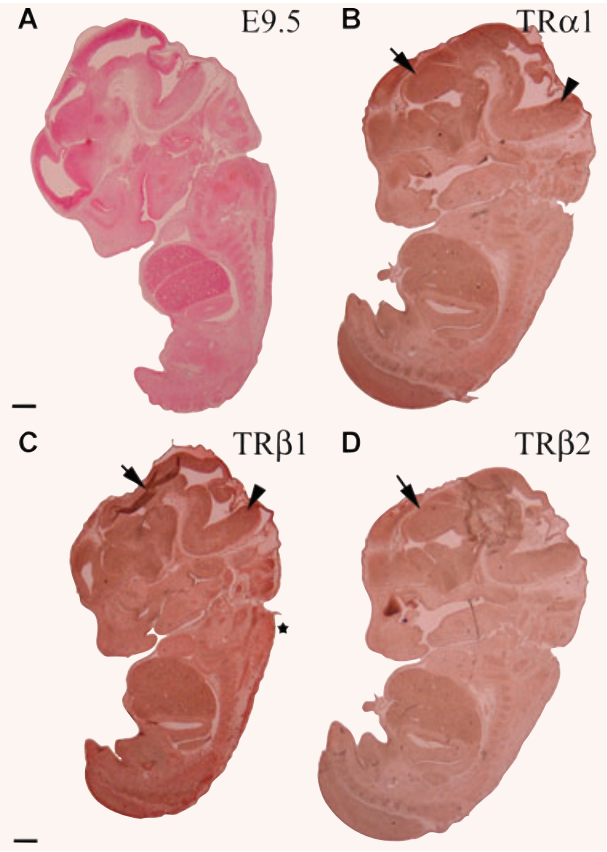

Fig. 3 β-gal expression and TRs IHC at E9.5. Euthyroid transient transgenic embryos at E9.5 did not show β-gal expression (**A**). (**B**) TRα$_1$, (**C**) TRβ$_1$ and (**D**) TRβ$_2$ show very weak IHC positivity in the CNS. Arrows point to the midbrain and arrowheads to the medulla oblongata. Parasagittal sections. Scale bar = 100 μm.

the trachea and oesophagus, the atria and in the umbilical cord. Skin primordia (dermatomes) also showed β-gal expression (data not shown).

TRs IHC in β-gal$^+$ anatomical areas showed intense labelling for TRβ$_1$ and TRβ$_2$, and weak labelling for TRα$_1$, RXR-α, RXR-β, RXR-γ and RAR-α (Fig. 5). RXRs and RAR-α protein expression was unchanged compared to E9.5–E10.5 (data not shown).

Nuclear receptors expression in the early stages of development

At E11.5–E12.5 TRα$_1$ IHC shows an intense labelling in the central nervous system (CNS) primordia and colocalizes with β-gal staining (Fig. 5). This was not seen in the ocular and vestibular annexes, nor in the peripheral nervous system, which were negative for TRα$_1$ IHC. Telencephalic vesicles were instead labelled by TRα$_1$ IHC but not by β-gal. TRβ$_1$ IHC displayed a similar intense labelling of cell bodies in the CNS (Fig. 5), with the exception of the spinal cord. In addition, we observed TRβ$_1$ colocalization with the β-gal staining around blood vessels and the airways. Similarly, TRβ$_2$ IHC gave an intense staining in the CNS primordia, even in the spinal cord (Fig. 5), and in the sense organs primordia (data not shown). On the contrary, RXR-α always gave the lightest labelling, except for some labelling in the spinal cord (motoneurons) and in the spinal nerve roots, without colocalization with β-gal staining (data not shown). RXR-β IHC stained the CNS with less intensity than TRα$_1$ and TRβ$_1$ (Fig. 5). In this case, labelling was colocalized with β-gal staining only in the optic nerve, whereas colocalization was almost absent in the brain and in the spinal cord. RXR-γ IHC gave a weak staining, with a low degree of colocalization with β-gal staining in the brain with no staining outside the brain (Fig. 5).

TH action during late stages of development in euthyroid transient transgenic embryos

At late stages of development (E15.5–E17.5), when FTF (E17.5) is active and the embryo is at the trilaminar disc stage, β-gal expression was exclusively detected: (*i*) in the small intestine primordia (Fig. 6); (*ii*) in the vibrissal follicular nerves (maxillary and mandibular divisions of the trigeminal cranial nerve) (Fig. 6); (*iii*) in the scapula and rib cartilage primordia (ossification centres) (data not shown) and (*iv*) in the Meckel's cartilage (data not shown).

Nuclear receptors expression in the late stages of development

At E17.5 TRs IHC staining was intense and colocalized with β-gal staining in the small intestine primordia (*i.e.* TRα$_1$ immunoexpression), the nerves of the whiskerpad, the follicles of the vibrissae (Fig. 6) and in the scapula and cartilage primordia (data not shown). In the cerebral cortex, TRα$_1$ was detected (Fig. 6), in the cortical plate and in the subventricular zone, whereas it was less intense in the subplate. An intense immunoreactivity was also detected in the hippocampus and in the striatum, and some positivity was also observed in the developing ear and spinal cord. TRβ$_1$ and TRβ$_2$ IHC gave similar results (Fig. 6). TRs immunoreactivity was also evident in β-gal$^-$ areas (*i.e.* in the telencephalon). RXR-β (Fig. 6) and RXR-γ (data not shown) immunoreactivities were very weak, whereas RAR-α expression was intense (Fig. 6). RXRs expression was unmodified at E17.5 compared to the early stages examined, whereas RAR-α expression was strongly increased (Fig. 6).

β-gal expression in euthyroid embryos generated from a transgenic line (stable model)

We generated a transgenic line responsive to TH action, which was able to stably express β-gal protein in the early and late

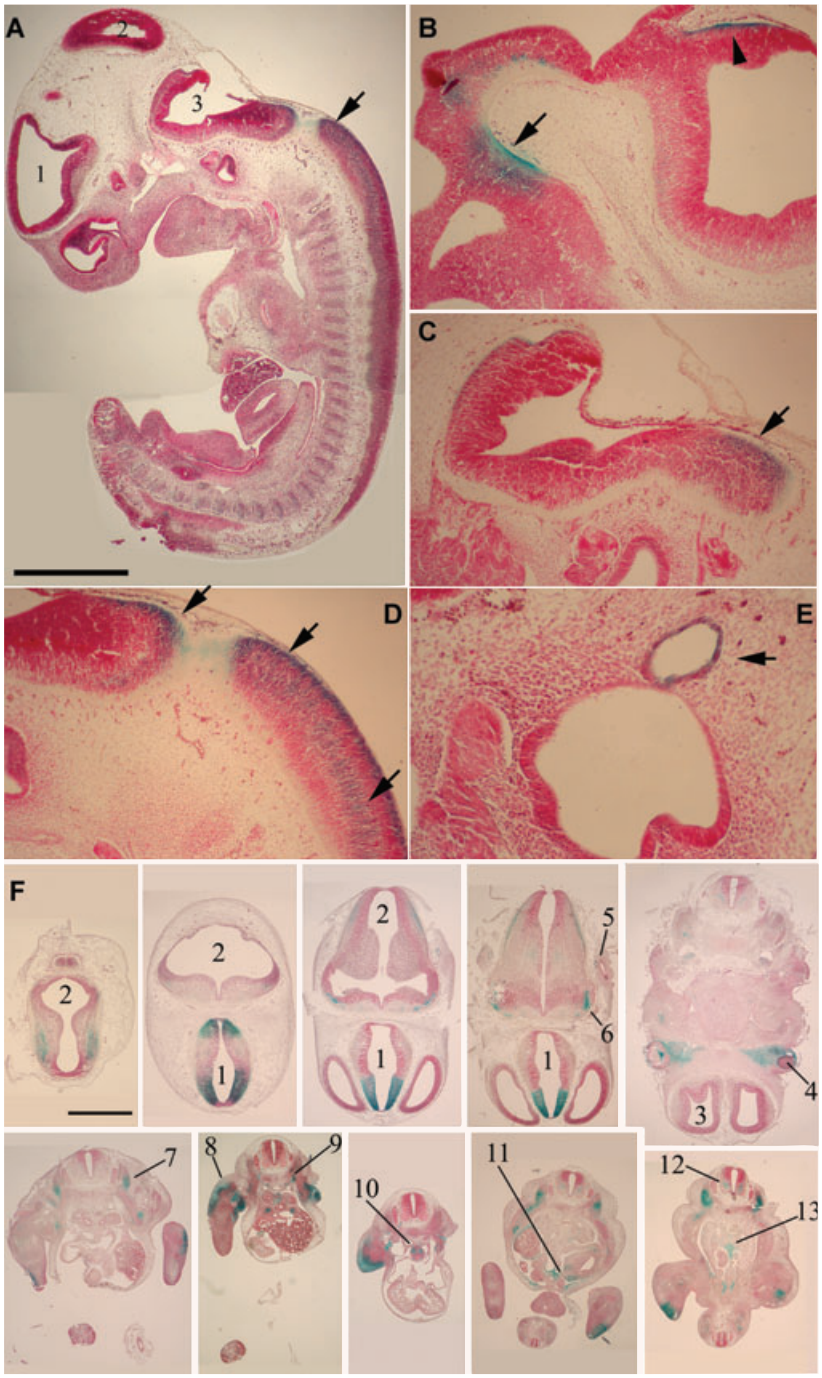

Fig. 4 β-gal expression at E11.5 and E12.5 of euthyroid transient transgenic embryos. (**A–E**): TRE2× transgenic embryo at E11.5. β-gal-reacted embryos (blue) counterstained with neutral red. In (**A**), parasagittal section: 1) lateral ventricle; 2) third ventricle; 3) fourth ventricle. Arrow points the medulla oblongata. In (**B**), higher magnification of the diencephalic vesicle: the arrow points the hypothalamic area, whereas the arrowhead points to (**C**) ponto-mielencephalic area, with the arrow pointing to the medulla oblongata. In (**D**) particular of (**A**), with arrows pointing to β-gal expression in the medulla oblongata and in the cranial spinal cord. In (**E**), arrow points to a semicircular canal in the developing ear. Scale bar = 1 mm in (**A**), and 400 μm in (**B–E**). In (**F**), TRE2× transgenic embryo at E12.5. Craniocaudal coronal sections through the head, the thorax and abdomen of the embryo. 1 – third ventricle; 2 – fourth ventricle; 3 – lateral ventricles; 4 – optic stalk; 5 – endolymphatic sac; 6 – pons; 7 – premuscle mesodermal condensation; 8 – cartilage of the forelimb bud; 9 – neural crest; 10 – oesophagus; 11 – small intestine; 12 – basal plate of the spinal cord; 13 – vagal trunk. Scale bar: f = 1mm

stages of development. TRE2× copy number in homozygous transgenic lines is reported in Fig. 7. β-gal was consistently expressed in 108 homozygous transgenic embryos both at early (E11.5, E12.5, E13.5) and late stages of development (E15.5 and E17.5) (Table 1). No β-gal expression, however, was observed at E9.5–E10.5.

Maternal TH action during early stages of development in euthyroid stable transgenic embryos

β-gal was highly expressed at early stages of development as early as E11.5–E12.5. In particular, we detected β-gal expression

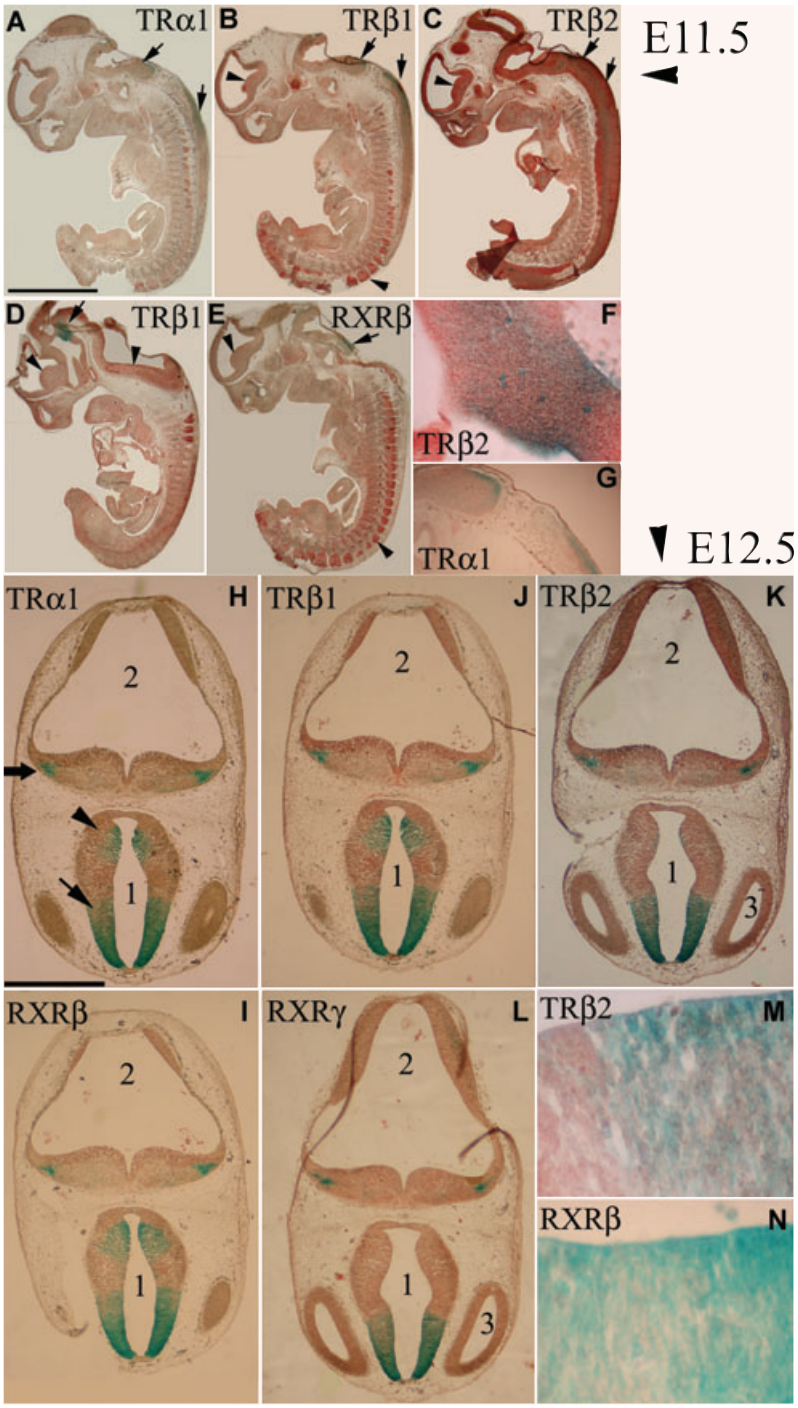

Fig. 5 Double labelling with β-gal and nuclear receptors IHC at E11.5 and E12.5 of euthyroid transient transgenic embryos. (**A**–**G**): IHC against thyroid (TRs) and retinoid (RXRs) receptors in midsagittal sections of β-gal$^+$ E11.5: (**A**) anti-TRα_1; (**B**) anti-TRβ_1 (diencephalon, medulla oblongata); (**C**) anti-TRβ_2 (diencephalon, medulla oblongata); (**D**) anti-TRβ_1 (diencephalon, mesencephalic vesicle); (**E**) anti-RXR-β (diencephalon); (**F**) anti-TRβ_2, detail in the area of the diencephalic vesicle; (**G**) anti-TRα_1, detail of the medulla oblongata and cranial spinal cord. Scale bar = 1 mm in (**A**–**E**), 100 μm in (**F**) and 400 μm in (**G**). Arrows point to areas of colocalization between β-gal positivity and TR-immunoreactivity (diencephalon, medulla oblongata and spinal cord), whereas arrowheads point to areas of TRs-immunoreactivity devoid of β-gal staining (pons and dorsal root ganglia). (**H**–**N**): IHC with antibodies against TRs and RXRs in coronal sections of the brains of β-gal$^+$ at E12.5: (**H**) anti-TRα_1; (**J**) anti-TRβ_1; (**K**) anti-TRβ_2; (**I**) anti-RXR-β; (**L**) anti-RXR-γ; (**M**) anti-TRβ_2, detail in the area of the developing hypothalamus; (**N**) anti-RXR-β, detail of the cranial spinal cord (as in Fig. 4D). Scale bar = 1 mm in (**H**–**L**) and 100 μm in (**M**, **N**). 1: third ventricle; 2: fourth ventricle; 3: lateral ventricle. Thin arrow points to the colocalization of β-gal and TR-immunoreactivity in the diencephalon (thalamus), the arrowhead in the diencephalon (hypothalamus) and the thick arrow in the midbrain.

at E11.5 in the otic vescicle (wall of the endolymphatic diverticular appendage) and in the diencephalon. β-gal expression was also observed at E12.5 in the diencephalon (thalamus, hypothalamus, epithalamus, dorsal wall of the III ventricle) (Fig. 8A–C), and in the area of the future neurohypophysis, known as the infundibular recess of the third ventricle, indicated by the ectodermal primordium of the adenohypophysis (Rathke's pouch). At E13.5 β-gal expression was marked in the medulla oblongata, rostral extremity of the endolymphatic duct, origin of choroid plexus differentiating from roof of fourth ventricle, choroid plexus

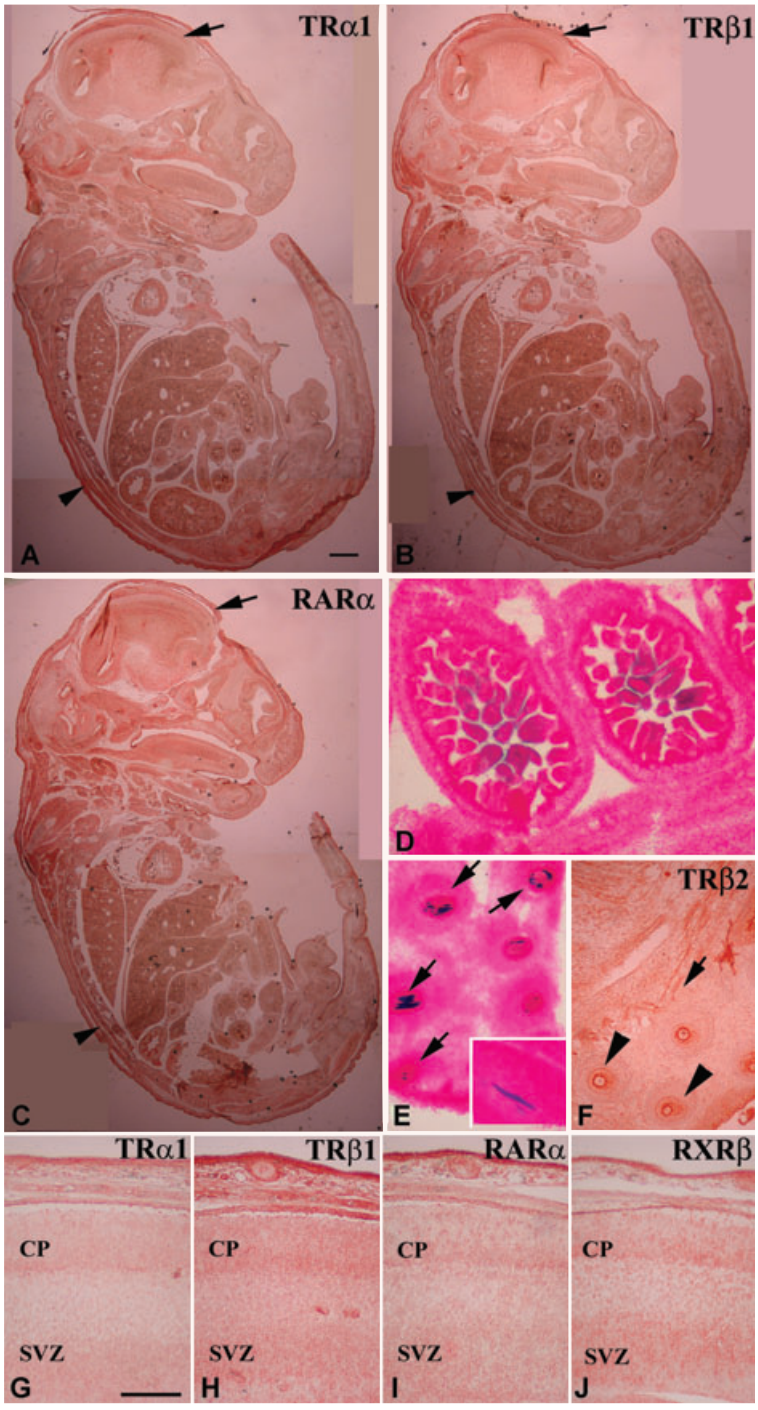

Fig. 6 Double labelling with β-gal and nuclear receptors IHC at a late stage (E17.5) of development of euthyroid transient transgenic embryos. **(A–C)** IHC with antibodies against thyroid (TRs) and retinoic (RARs) receptors: $TR\alpha_1$ in (**A**), $TR\beta_1$ in (**B**), RAR-α in (**C**) and β-gal expression **(D–F)** in transgenic embryos at E17.5. Scale bar corresponds to 800 μm in (**A–C**), 80 μm in (**D**) and 200 μm in (**E–F**). In (**A–C**) the arrows point to the developing cerebral cortex and the arrowheads to the spinal cord, respectively. In (**D**), β-gal expression in the developing small intestine, and in (**E**), β-gal expression (and in (**F**), $TR\beta_2$ immunostaining) in the nerves (arrows) innervating the whiskerpad (**E,** tangential section and **F,** transverse section). Higher magnification of $TR\alpha_1$ **(G)**, $TR\beta_1$ **(H)**, RAR-α **(I)**, and retinoid receptor RXR-β **(J)** IHC of the developing cerebral cortex: CP = cortical plate, SVZ = subventricular zone. Scale bar = 160 μm. In (**A–D**), the small intestine primordia are labelled by both IHC and β-gal histochemistry. The peripheral nerves innervating the whiskerpad (inset in **E** and arrow in **F**) and the follicles of the vibrissae (arrows in **E** and arrowheads in **F**) are labelled by both β-gal and $TR\beta_2$ IHC.

within central part of lumen of fourth ventricle and the area above the olfactory epithelium. At stages E11.5, E12.5 and E13.5, similar to our findings in the transient transgenic embryos, we detected TRs and β-gal colocalization in the aforementioned anatomical primordia.

TH action during late stages of development in euthyroid stable transgenic embryos

At late stages of development diencephalic differentiation is almost complete and various anatomical sites can be distinguished. The

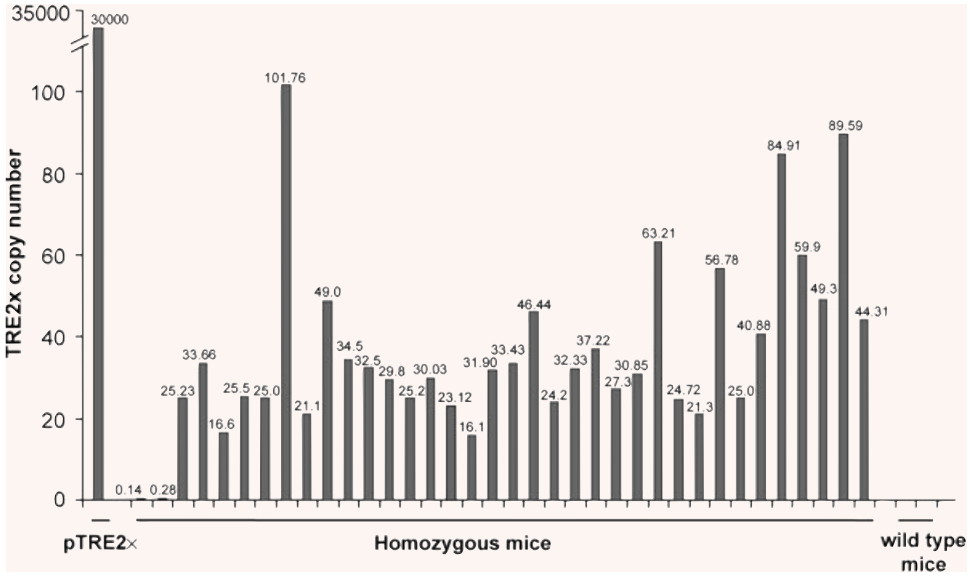

Fig. 7 Transgene TRE2× copy number in stable transgenic mice. Quantitative real time PCR shows TRE2× copy number integrated into the genome of homozygous transgenic mice. pTRE2× is the non linearized vector (positive control) and DNA from wild-type mice was used as a negative control.

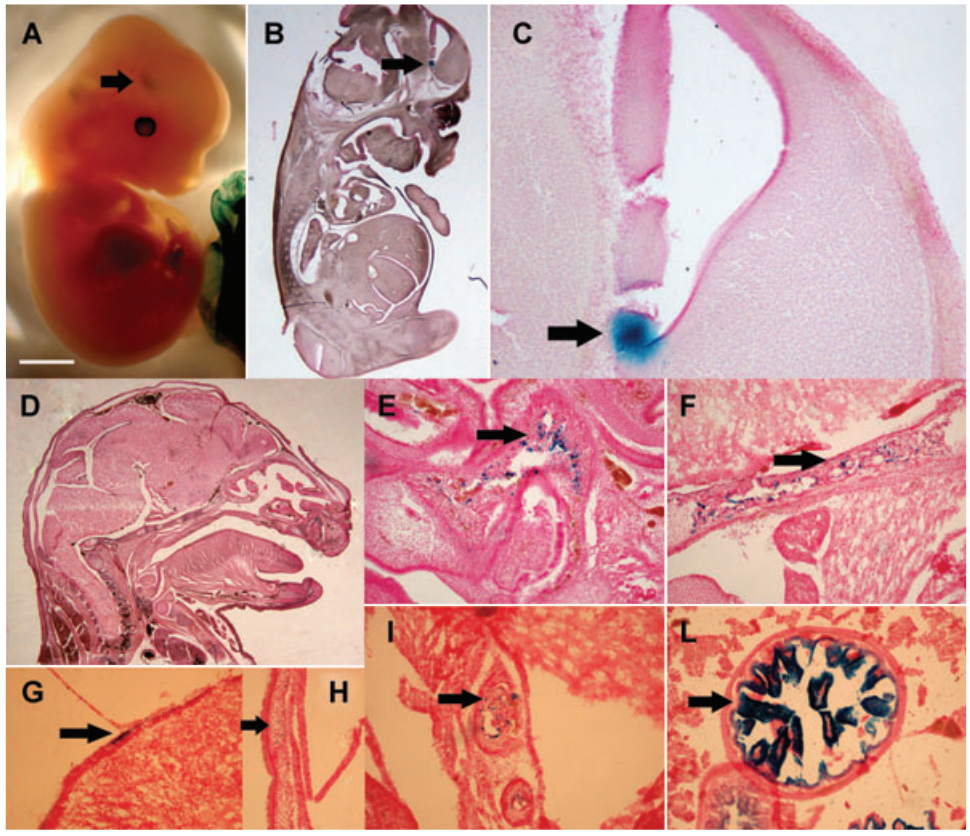

Fig. 8 β-gal expression in TRE2× positive stable transgenic embryos. **(A-C)** β-gal expression in the diencephalon (arrows) in E12.5 embryo *in toto* **(A)** and in neutral red stained sagittal sections at low **(B)** and high **(C)** magnification. **(D–L)** Sagittal sections of β-gal expression at E17.5 by neutral red stains: low **(D)** and high **(E–L)** magnification in the whiskerpad (arrow) **(E)**, occipital bone primordium (arrow) **(F)**, medulla oblongata (arrow) **(G)**, skin (arrow) **(H)**, vertebrae (arrow) **(I)** and small intestine (arrow) **(L)**. Scale bar: A, B = 1 mm; C = 100 μm; D = 2 mm; E–L = 200 μm.

neurohypophysis and the epiphysis are distinct as evaginations of the diencephalic wall in the hypothalamus and epithalamus, respectively. At E15.5 no β-gal expression was found in the diencephalic primordium, whereas it was detected in the primordia of follicles of vibrissae associated with lower lip, the olfactory epithelium, the zone of cartilage primordium of nasal septum and in the ossification centres of the mandible. At E17.5, β-gal activity was detectable in the wall of the medulla oblongata, the whiskerpad, the

occipital bone primordium, the vertebrae and in the small intestine primordia (Fig. 8D–L). Consistent with the results obtained at E17.5 in transient transgenic embryos, we detected TRs (*i.e.* TRα_1 immunoexpression in the small intestine primordia) and β-gal colocalization in the aforementioned anatomical primordia.

Hypothyroidism in pregnant TRE2× transgenic mice treated with T3

As expected, β-gal staining was not detectable in the early stages (E12.5) in 36 embryos obtained from 14 hypothyroid transgenic females mice (TSH 4,749 ± 938 mU/l, T4 29.60 ± 0.16 nmol/l, T3 0.75 ± 0.16 nmol/l) (body weight = 27 g ± 0.24). Goitres were observed in the hypothyroid mice.

Further, IHC against TRs (Fig. 9) did not show significant differences in the expression of all receptors, both at localization and intensity levels, when hypothyroid mouse embryos were compared to euthyroid mouse embryos.

In order to further validate the specificity of the TRE× transactivation by TH, we used two groups of 14 hypothyroid transgenic female mice. One group of mice was treated with T3 (1.5 μg/100 g body weight per day) from E9.5 to E12.5 while treatment with 0.1% MMI and low iodine diet were maintained. The control group was treated with the vehicle only. T3 in a dose of 1.5 μg was effective in decreasing the serum TSH levels in the hypothyroid transgenic mice to the physiological baseline levels (TSH < 10 mU/l) as well as increasing T3 levels (14.2 ± 2.71 nmol/l) and T4 levels (28.31 ± 0.08 nmol/l). The control mice on the other hand maintained a low T4 (11.06 ± 0.15 nmol/l) and low T3 (1.10 ± 0.3 nmol/l) and a high TSH (4,481.66 ± 778.2 mU/l) ($P < 0.001$). No β-gal expression was detected in the E12.5 transgenic embryos from the control group (Fig. 10A). Importantly, treatment with T3 in the hypothyroid mice rescued β-gal expression in the diencephalon primordium of E12.5 transgenic embryos (Fig. 10B), producing a similar pattern to that observed in the euthyroid transgenic mouse embryos.

In summary, our results show that in euthyroid condition, maternal THs are able to cross placental barrier and to be transcriptionally active through embryonic TRs as early as E11.5 (early stage) of embryo–foetal development (Fig. 11). On the contrary, transplacental transfer of maternal THs is inhibited with maternal hypothyroidism (low T4 and T3 and high TSH) (Fig. 11). Treatment with T3 in hypothyroid pregnant mice (from approximately 4–6 weeks before pregnancy until E12.5) was able to switch on the TRE2× transactivation and β-gal expression at the early stages (E12.5) of development when FTF is still not active.

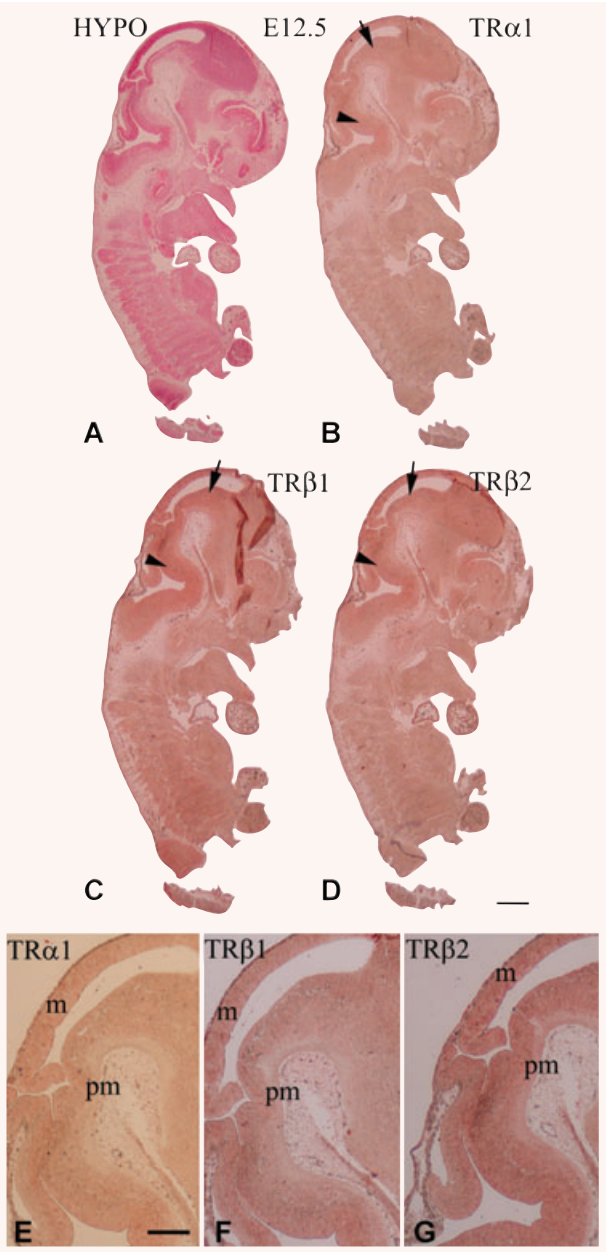

Fig. 9 β-gal expression and TRs IHC in hypothyroid (hypo) E12.5 transgenic embryos. At E12.5, β-gal expression was absent **(A)**, whereas TRα_1 **(B)**, TRβ_1 **(C)** and TRβ_2 **(D)** IHC were comparable to that shown in Fig. 5. Arrows point to the midbrain and arrowheads to the medulla oblongata. Parasagittal sections. Scale bar = 400 μm. Higher magnification of TRα_1 **(E)**, TRβ_1 **(F)** and TRβ_2 **(G)** IHC. m = roof of midbrain; pm = region of pons-midbrain junction.

Discussion

The simultaneous presence of maternal THs and nuclear thyroid receptors (TRs) during embryo–foetal development does not necessarily indicate that maternal THs are biologically active [25]. However, recent evidence shows that maternal T4 is necessary for early physiological neurogenesis [33, 50].

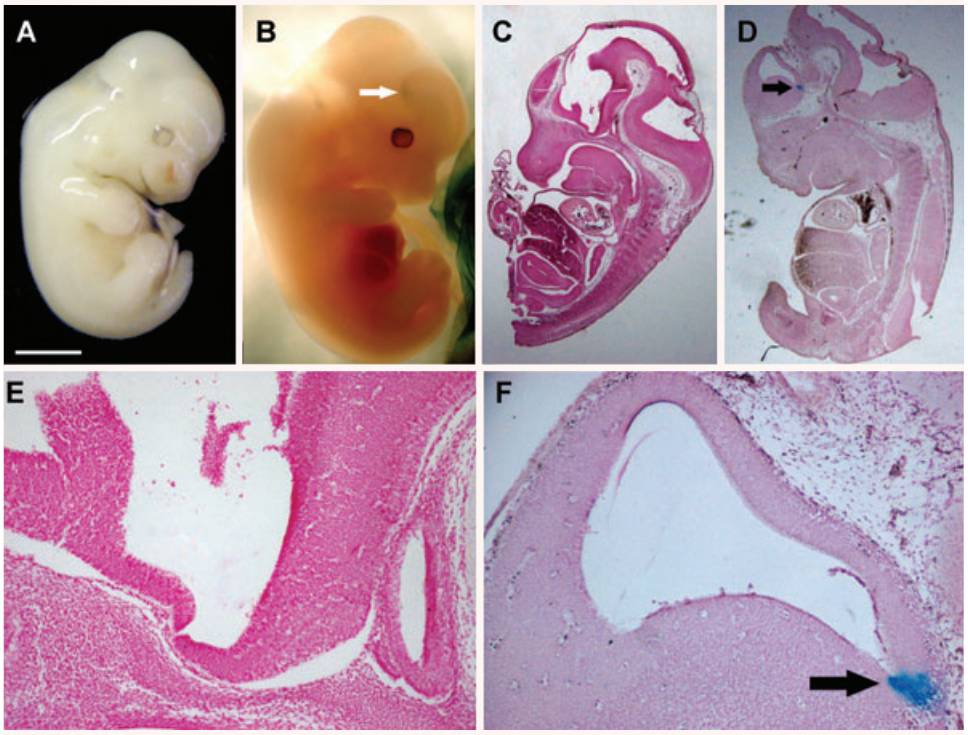

Fig. 10 β-gal expression in hypothyroid stable transgenic mice treated with T3. **(A)** Hypothyroid transgenic mouse embryo at E12.5 treated with vehicle (negative control) did not show β-gal expression (no blue staining). **(B)** Hypothyroid transgenic mouse embryo at E12.5 treated with T3 (1.5 μg/100 g body weight per day) from E9.5 to E12.5 shows β-gal expression (blue staining, arrow) in the brain (diencephalon primordium). (**C**, low magnification and **E**, high magnification): neutral red staining shows absence of β-gal expression in the diencephalon primordium. (**D**, low magnification and **F**, high magnification): neutral red staining shows β-gal expression (blue staining) in the diencephalon primordium (arrow). Scale bar: (**A**, **B**) = 1 mm; (**C**, **D**) = 1 mm; (**E**, **F**) = 100 μm.

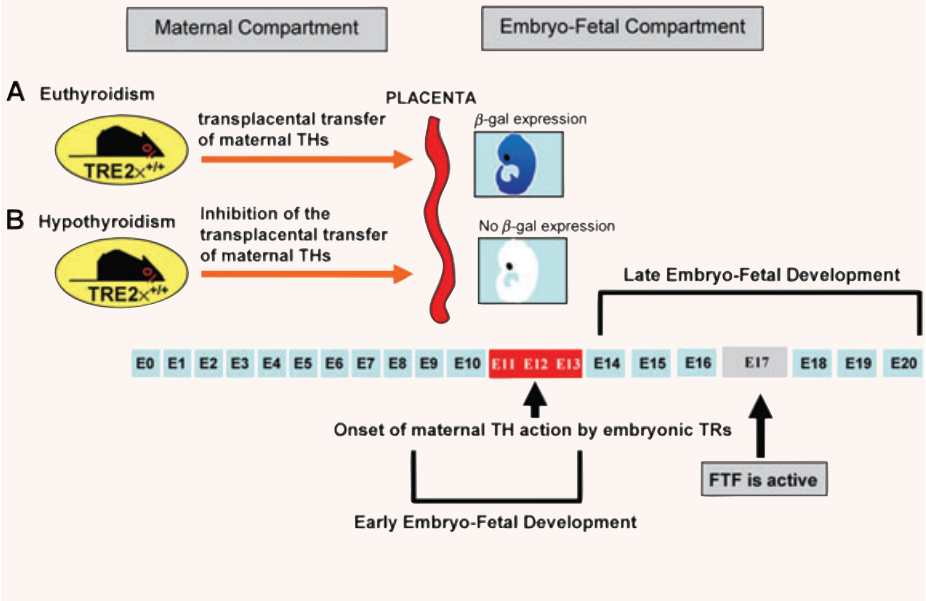

Fig. 11 TRE2× transgenic mouse model. **(A)** Maternal THs cross the placental barrier as early as E11.5, before FTF is active, and transactivate the TRE2× transgene through embryonic TRs (see Fig. 1A, B). TRE2× transactivation permits β-gal expression. β-gal expression (blue staining) is used as a molecular marker to trace THs action during early (E11.5, E12.5, E13.5) and late (E15.5 and E17.5) stages of embryo–foetal development. **(B)** Conversely, when the maternal thyroid gland is functionally blocked (hypothyroidism: low T3 and T4 and high TSH), transplacental transfer of THs is inhibited, and TRE2× is not transactivated in the early stages of development by TRs; no β-gal expression (no blue staining) is therefore detected in the TRE2× positive transgenic embryos.

Here, we sought to investigate whether maternal TH transcriptional activity during early and late foetal development is TRs mediated. We generated a novel transgenic mouse model using both transient and stable genetic engineering techniques [51] in order to corroborate an active role of maternal THs in the embryo.

Our model was able to trace TH action (TH-indicator) during early and late development by the expression of β-gal (that is encoded by LacZ), generated through the transcriptional activity of a TH-dependent murine thyroid hormone response element (TRE2×), arising from the myelin basic protein gene-TRE. Our *in vitro* validations show that TRE2× transactivation is strongly responsive to T3 action by $TR\beta_1$, $TR\beta_2$ or $TR\alpha_1$, but not to other nuclear receptors and ligands such as oestrogen or retinoic acid. Previous studies also demonstrated a selective transcriptional activity on the myelin basic protein gene-TRE only by TR [39, 40, 52].

Using transgenic embryos, we show that maternal THs cross the placental barrier and are transcriptionally active during embryo–foetal development in different primordia, before the onset of FTF (E15.5–E17.5). Importantly, we found β-gal expression as early as E11.5, a crucial early stage of CNS development. Previous studies detected T4 and T3 by RIA in E10–E12 rat trophoblasts and E13–E20 embryos and placentas, as well as in amniotic fluid [11]. On the contrary, Quignodon *et al.* demonstrated T3 signalling at E15.5 (late stage of CNS development) in the midbrain roof by the use of a chimeric yeast Gal4 system [53].

Similar to previous results [11], our study shows that THs cross the placental barrier at an early stage (E11.5) of embryonic development; at this stage the FTF is still inactive and the embryo–foetal primordia are sensitive to maternal TH action.

Here, TRE2× transactivation resulted in β-gal expression in different embryo–foetal primordia, particularly during the early and late development. Importantly, we observed β-gal expression in the same anatomical sites of several euthyroid transgenic embryos, whereas it was completely absent in embryos of transgenic hypothyroid mice. Thus, these results suggest that maternal THs are present and active during embryo–foetal development, and that TRE2× transactivation is specifically driven by TH.

Interestingly, we did not detect β-gal expression before E11.5. This result may suggest that THs are not involved in the neurulation mechanisms, in spite of the presence of TRs at these stages. Early CNS differentiation comprised two phases: (*i*) neurulation and segmentation (E8–E9.5–E10.5) consisting of the formation of the three primary brain vesicles and (*ii*) regionalization of the neural tube (*i.e.* diencephalon primordium, E11.5–E12.5) [54]. The lack of TRE2× transactivation before E11.5 may be related to different expression levels of TH transporters or type 2 and 3 deiodinases (D2 and D3) in the placenta during development [55–59]. Importantly, the main specific TH transporter, MCT8, has been recently identified by Friesema *et al.* [60], and found to be expressed in human normal placenta at 6 weeks of gestation, with evidence for increasing expression during advanced gestation [55]. In addition, human placenta expresses high levels of D3 that appears to be important in maintaining circulating and tissue levels of foetal THs exceedingly low relative to maternal or adult levels. This mechanism has led to the concept that TH levels during development are tightly regulated within narrow limits to avoid either an excess or a deficiency of TH and that the D3, along with the D2, is widely responsible for this specific regulation [58]. Furthermore, Chan *et al.* have been reported that D2 and D3 mRNA levels and enzyme activities in human placenta were higher at early gestation than at late gestation [59]. Koopdonk-Kool *et al.* [61] have been reported significantly higher overall placental D3 than D2 activities at all gestations.

However, further studies are needed to better understand the role of TH before E11.5 during mouse development.

In our study, the detection of β-gal expression at E11.5 strengthens previous studies that demonstrated that maternal THs play a critical role in cell migration and formation of neuronal layers, as well as in neuronal and glial cell differentiation and synaptogenesis [62, 63]. The staining of β-gal in the trigeminal ganglion (represents one of the first primordia of the neural crest to differentiate in the mesencephalic region), as detected in our study, highlights the role of THs on neuronal and glial cell differentiation. Further, because β-gal was expressed in different areas of the brain (*i.e.* diencephalon) or in the sense organs primordia at an early stage, our data show that maternal THs are transcriptionally active in the development of these anatomical structures. At E15.5–E17.5 (late stage) when the FTF is finally settled, β-gal expression was detected in different primordia compared to its expression in the early stages, such as small intestine, follicular nerves of the vibrissae, bones, skin (dermatomes), muscle, blood vessels, etc. We hypothesize that at these late stages, most THs available to embryo–foetal primordia are probably still of maternal origin, because foetal thyroid becomes active only at E17.5.

Similar to the results found in the transient transgenic embryos, β-gal expression was detected in the embryos obtained from the stable transgenic line, both at early and late stages of development. However, the pattern of β-gal expression in the stable embryos was less diffuse compared to the transient transgenic embryos. This may be due to the transgene transmission through germ line. In fact, a proportion of transgenes undergo germ-line heritable epigenetic modifications, including DNA methylation or histone modifications due to the chromatin packaging, which could affect the transgene expression levels by transcriptional repression [64, 65]. Less frequently, the expression levels of the transgene may be influenced by endogenous silencers or enhancers surrounding the regions of insertion [66].

Our stable transgenic line consistently expressed β-gal at the early stages, *i.e.* in the brain (*i.e.* diencephalic primordia, medulla oblongata) and sense organs primordia (otic vescicle, olfactory epithelium, retina), whereas at late stages (E15.5–E17.5) this pattern no longer appeared, and the expression was specifically localized in other primordia, *i.e.* in the bones, follicular nerves of the vibrissae, as well as in the small intestine primordia, but also in the medulla oblongata.

Our results from the stable, as well as from the transient, transgenic embryos highlight the essential role of TH for normal brain development during embryo–foetal life, as previously reported [19, 25, 67, 68]. Of relevance, in the rat, changes in

maternal TH availability during early stages of development – equivalent to the end of the first and beginning of the second trimester in human beings – irreversibly affect neurogenesis and radial migration of neurons into the developing cerebral cortex and hippocampus [19].

To the best of our knowledge, this is the first transgenic mouse model that traces THs action during diencephalon differentiation (prosencephalic regionalization) – the brain region that includes the thalamus, hypothalamus and epithalamus, which is involved in the physiological regulation of several biological processes. Moreover, our data reveal β-gal expression in the small intestine primordia at E17.5. This result importantly confirms the fact that THs are involved in mammalian intestinal epithelial development, and may affect its fundamental processes of growth and differentiation. Interestingly, hypothyroid animals display marked cryptvillus hypoplasia and thyroidectomy in adult rats leads to a decrease in jejunal crypt mitotic rate, whereas T3 administration induces mucosal hyperplasia [69–72]. T3 has two major influences on the adult small intestine, including a trophic on crypt cells and alteration in the pattern of brush-border enzyme expression in the villus. In addition, Malo *et al.* identified a novel response element that appears to mediate T3-induced activation of the enterocyte differentiation marker (*i.e.* intestinal alkaline phosphatase) [73]. T3 mediates its effects through TRs, and TRs knockout mice were studied to determine the effects of T3 on intestine development and differentiation. Although TRβ knockout mice appear to have no intestinal abnormalities, TRα knockout mice display marked hypoplasia in the crypts and villi and decreased levels of digestive enzymes (*i.e.* lactase, sucrase, aminopeptidase) [74–77]. $TR\alpha_1$ therefore regulates intestinal development and homeostasis by controlling epithelial proliferation in the crypts. This process involves positive control of the Wnt/β-catenin pathway [78–79].

Other transgenic mouse models were used to study TH action during embryo–foetal development. Quignodon *et al.* used the yeast UAShsp68/Gal4/ $TR\alpha_1$/LacZ system and found β-gal expression in the roof of the midbrain at late stages (E15.5) [53]. Nagasawa *et al.* used $TR\beta_1$/LacZ system and described β-gal expression in the midbrain, auditory vesicles, limbs and face at early stages (E9.5–E12.5), and in the root of whisker follicles and the intestine primordia at E17.5 [80]. The differences in embryonic sites and stages of transgene expression between our model and the previous ones described may be explained by the different types of transgenes used [81].

In our study T3 treatment from E9.5 to E12.5 in hypothyroid pregnant transgenic mice was able to rescue β-gal expression in the diencephalon primordium of E12.5 transgenic embryos, reproducing a similar pattern to that observed in the euthyroid transgenic mouse embryos. This result highlights that TRE2× transactivation is specifically driven by TH. Furthermore, we showed that embryo–foetal primordia expressing β-gal by TRE2× transactivation through TH action are also positive for TRs both at early and late stages, thus suggesting that TH action may be mediated by embryonic TRs. However, not all embryonic TR^+ areas were $\beta\text{-gal}^+$; the absence of β-gal expression may suggest that in those tissues either there were no maternal TH or TH were transcriptionally inactive despite the presence of TRs. The biological activity of TH is determined by the availability of the active hormone T3 for binding to nuclear receptors in target cells. This depends on different factors: (*i*) the circulating concentrations of T3 and its precursor T4; (*ii*) the activity of the selenoprotein iodothyronine deiodinases (D1, D2 and D3) that catalyse the production and degradation of T3 and (*iii*) the activity of TH transporters (*i.e.* MCT8, MCT10, OATP1C1) that facilitate cellular uptake and/or efflux of the iodothyronines [3, 5, 55, 58, 60, 82–87]. Importantly, an absence of TH transporters (*i.e.* MCT8) or deiodinases expression might be critical to inhibit TH action [3, 5, 58, 82, 83]. Interestingly, D2 and D3 are widely expressed in different foetal tissues during development and are important in regulating the circulating and tissue TH levels during this period [58]. Furthermore, nuclear TH and TRs action is dependent on the recruitment of coregulators such as coactivators and corepressors, both crucial for the transcriptional regulation of their target genes [10, 88, 89]. Indeed, TRs are therefore involved in many biological processes through the regulation of gene transcription by binding to nuclear transacting factors [90]. TRs mRNA has a distinct ontogenetic expression during rat embryo–foetal development: $TR\beta_1$ and $TR\beta_2$ contribute to the regulation of neuroblast proliferation, whereas $TR\alpha_1$ is critical for neuronal differentiation [36]. In human beings, $TR\alpha_1$ and $TR\beta_1$ mRNAs were found in the first trimester in foetal brain [91]. In addition, different TR-knockout mouse models showed that some embryonic genes such as those involved in auditory and visual development are regulated by TRs [92–98], thus suggesting that TRs are relevant for auditory and visual function. Interestingly, mice lacking both $TR\alpha_1$ and TRβ display that these T3-receptors can affect different functions, including female fertility, control of the pituitary–thyroid axis and bone development [99].

Herein, we investigated $TR\alpha_1$, $TR\beta_1$ and $TR\beta_2$ expression, and consistent with other studies [100] we found their expression to be present during early stages (E9.5) of development when maternal TH action is still not active, *i.e.* β-gal expression is lacking. This result may suggest a repressor role by unliganded TRs [101] in the early stages of embryo–foetal development before THs cross the placental barrier. In corroboration with this finding, the unliganded TRα (apo-receptor) binds DNA and may repress the transcription of genes involved in cardiac contractile activity between foetal and postnatal life of the mouse [102]. Intriguingly, hypothyroid mice deficient for $TR\alpha_1$ did not show cerebellar alterations such as delayed granule cell migration and arrested Purkinje cell growth [103]. These results supported the idea that repression by the unliganded $TR\alpha_1$, and not the lack of TH, is responsible for the hypothyroid phenotype; THs therefore have a permissive effect on cerebellar granule cell migration through derepression by the $TR\alpha_1$ isoform [103]. In addition, $TR\alpha_2$ is unable to bind T3 and has been shown to exert dominant inhibitory action on T3/TRs-dependent transactivation of target genes [104–106]. In our study, we did not analyse $TR\alpha_2$ expression. However, others described a temporal expression pattern of $TR\alpha_2$ coincident with that of $TR\alpha_1$ during early and

late stages of the foetal rat nervous system development; nevertheless, TRα_2 levels were markedly higher [36], thus suggesting that TRα_2 might be a critical regulator of TH action by interfering with T3 effect on the expression of brain-specific genes.

In summary, our study shows a tissue-specific ontogenetic expression of TRs as well as a synergic action between maternal TH and embryonic TRs, as early as E11.5 by the regulation of TRE2$\times$ transcriptional activity (β-gal expression) in the transgenic embryos. TH/TR action presumably regulates the transcription of target genes involved in the development of CNS (*i.e.* diencephalon differentiation), intestine and other embryo–foetal primordia before FTF is active. These results are reinforced by the finding that when the maternal thyroid gland is functionally blocked (hypothyroidism: low T3 and T4 and high TSH), β-gal expression is completely absent despite the presence of TRs, and is rescued with T3 treatment.

In conclusion, our transgenic mouse model may be a reliable tool to investigate molecular mechanisms underlying maternal TH action during early and late embryo–foetal development. It may also represent a useful model to analyse maternal hypothyroidism, transient gestational hypothyroxinemia or hyperthyroidism effects during embryo–foetal development. This model may have clinical relevance and may be employed to design end-point assays whereby new molecules affecting or mimicking TH action in early and late embryo–foetal development could be tested.

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