Thyroid hormone receptor $\beta 2$ is strongly up-regulated at all levels of the hypothalamo–pituitary–thyroidal axis during late embryogenesis in chicken

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Abstract

In this study, we tried to elucidate the changes in thyroid hormone (TH) receptor $\beta 2$ (TR $\beta 2$) expression at the different levels of the hypothalamo–pituitary–thyroidal (HPT) axis during the last week of chicken embryonic development and hatching, a period characterized by an augmented activity of the HPT axis. We quantified TR $\beta 2$ mRNA in retina, pineal gland, and the major control levels of the HPT axis – brain, pituitary, and thyroid gland – at day 18 of incubation, and found the most abundant mRNA content in retina and pituitary. Thyroidal TR $\beta 2$ mRNA content increased dramatically between embryonic day 14 and 1 day post-hatch. In pituitary and hypothalamus, TR $\beta 2$ mRNA expression rose gradually, in parallel with increases in plasma thyroxine concentrations. Using *in situ* hybridization, we have

demonstrated the presence of TR $\beta 2$ mRNA throughout the diencephalon and confirmed the elevation in TR $\beta 2$ mRNA expression in the hypophyseal thyrotropes. *In vitro* incubation with THs caused a down-regulation of TR $\beta 2$ mRNA levels in embryonic but not in post-hatch pituitaries. The observed expression patterns in pituitary and diencephalon may point to substantial changes in TR $\beta 2$ -mediated TH feedback active during the perinatal period. The strong rise in thyroidal TR $\beta 2$ mRNA content could be indicative of an augmented modulation of thyroid development and/or function by THs toward and after hatching. Finally, THs proved to exert an age-dependent effect on pituitary TR $\beta 2$ mRNA expression. *Journal of Endocrinology* (2008) **196**, 519–528

Introduction

Late embryogenesis and the perinatal period in the chicken embryo are associated with substantial physiological changes, such as yolk sac retraction, transition from chorioallantoic to lung respiration, and the onset of endothermy (Decuypere *et al.* 1990). During this important phase of development, plasma thyroid hormone (TH) concentrations change markedly – with a profound increase toward the end of embryogenesis – and contribute to the critical events taking place around hatching (Decuypere *et al.* 1990). THs are known to play an important role in controlling their own secretion through feedback effects acting on the different levels of the hypothalamo–pituitary–thyroidal (HPT) axis. In birds, information on TH feedback is scarce. Most studies dealing with TH feedback have been performed at the hypophyseal level in embryos as well as in post-hatch chicks. Gregory *et al.* (1998) demonstrated a suppressive effect of THs on pituitary thyrotropin (TSH)- β mRNA levels in cultured anterior pituitary cells of 19-day-old embryos (E19). An increase in TSH β mRNA expression was observed in 19-day-old

embryos and 1-day-old (C1) chicks treated with methimazole, indicating that TH feedback is functional at this stage of development (Muchow *et al.* 2005). The THs are also able to inhibit *in vitro* corticotropin-releasing hormone (CRH)-induced TSH release in E19 and C8 animals (Geris *et al.* 1999).

THs exert their effects, including their feedback regulation, by binding to TH receptors (TRs) in the target cells, stimulating or inhibiting gene transcription. In chicken, three functional TR variants have been cloned: TR α , TR $\beta 0$, and TR $\beta 2$ (Sap *et al.* 1986, Forrest *et al.* 1990, Sjöberg *et al.* 1992). As TR $\beta 2$ is strongly expressed in chicken pituitary (Grommen *et al.* 2005) – it is even the most abundant isoform in rodent pituitary gland (Bradley *et al.* 1989, Hodin *et al.* 1989) – and given its role in mammalian TRH and TSH gene expression (Langlois *et al.* 1997, Abel *et al.* 1999, 2001, Nakano *et al.* 2004), our study focused on this receptor type. Our primary goal was to increase our knowledge of the TH feedback active during late embryogenesis and hatching and to examine whether a role is reserved for TR $\beta 2$ herein. We first determined TR $\beta 2$ mRNA levels in several brain parts, pituitary, thyroid gland, pineal gland, and retina.

Subsequently, TRβ2 mRNA expression was quantified by real-time PCR in diencephalon, pituitary, and thyroid gland – the major control levels of the HPT axis – during the last week of embryonic development. Using *in situ* hybridization (ISH), we verified whether the density of the hypophyseal TRβ2 mRNA signal changes in the thyrotropic cells at E16 compared with E20 and investigated its distribution in diencephalon. As TH concentrations increase substantially during the perinatal period, we determined whether THs are able to alter TRβ2 mRNA expression levels in the embryonic and post-hatch pituitary *in vitro*.

Materials and Methods

All experiments were approved by the ethical committee for animal experiments of the K U Leuven.

Distribution of TRβ2 mRNA in embryonic tissues

Tissue sampling Fertilized eggs from Cobb broiler chickens were purchased from Poel-Houben (Lummen, Belgium) and incubated in a laboratory incubator as described previously (Darras *et al.* 1992). The start of incubation was called day 1 (E1). At E18 animals were decapitated and the different brain parts, pituitary glands (pooled per 4), pineal glands (pooled per 4), thyroid glands (pooled per 3 pairs), and retinal tissue (pooled per 3 pairs) were collected, immediately frozen in liquid nitrogen, and stored at –80 °C.

RNA isolation and real-time PCR Total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer’s instructions. To rule out any genomic DNA contamination of the RNA, the samples were treated with DNase I using the DNA-free kit (Ambion, Austin, TX,

USA). For each tissue, three individual samples or pools were used for analysis. One microgram of total RNA was reverse transcribed using 0.375 U/μl AMV reverse transcriptase (Invitrogen). Five microliter of cDNA, corresponding to ~50 ng total RNA, were used for real-time PCR. Primers for TRβ2 – located in the unique amino-terminal region of the receptor (Sjöberg *et al.* 1992) – and the active reference β-actin were chosen using the Primer Express software v.2.0 (Applied Biosystems, Warrington, UK) and are listed in Table 1. The real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System thermal cycler (Applied Biosystems) in a total volume of 25 μl with the following components: 1×Platinum SYBR Green (Invitrogen), 300 nM forward primer, and 300 nM reverse primer. The thermal cycle parameters used were 2 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. The amplification program was followed by a dissociation protocol to detect any non-specific amplification. For each gene, a non-template control and a sample that was not reverse transcribed were added as negative controls. A 1:5 dilution series of pituitary cDNA was added as a standard. Each experimental sample was analyzed *in triplo*, and standard samples were measured *in duplo*.

TRβ2 mRNA expression at the different levels of the HPT axis during the last week of embryonic development

Tissue sampling Fertilized eggs from Ross (pituitary) or Cobb (diencephalon, thyroid gland) broiler chickens were purchased from Avibel (Halle-Zoersel, Belgium) and Poel-Houben respectively and incubated in a laboratory incubator for 21 days as described earlier. At day 20 (E20) of embryonic development, we made a distinction between animals that had already switched from allantois to lung respiration, which we

Table 1 Primers and probes used to amplify chicken TRβ2, β-actin, and GAPDH with real-time PCR

	Sequence (5'–3')	Amplicon
TRβ2 (X62642)		
Forward primer	CCCAGCTGCTGGTAGCAATT	bp 55–74
Reverse primer	CCTGAGCATCACAACTGCTGTA	bp 125–146
β-actin (NM_205518)		
Forward primer	ATGGCTCCGGTATGTGCAA	bp 103–122
Reverse primer	TGTCTTTCTGGCCCATACCAA	bp 202–222
GAPDH (NM_204305)		
Forward primer	GAAGCTTACTGGAATGGCTTTCC	bp 729–751
Reverse primer	GATATCATCATCTTGGCTGGTTTCTC	bp 825–799
	Sequence (5'–3')	Bp numbering
Taqman probes		
TRβ2	FAM-AGTCCACTGATTATTGC-NFQ-MGB	bp 85–100
β-actin	FAM-CCCCAGACATCAGGGT-NFQ-MGB	bp 179–194

The EMBL accession number is indicated in parentheses beside each gene. TRβ2, thyroid hormone receptor β2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FAM, 6-carboxy fluorescein; NFQ, non-fluorescent quencher; MGB, minor groove binder; bp, base pair.

called the internal pipping stage (E20IP), and animals that had not perforated the membrane of the air chamber, which was termed the non-pipping stage (E20NP). Blood and tissue samples were taken every day starting from E14 until 1-day-old chicks (C1). After collecting blood samples by heart puncture (embryos) or by decapitation (post-hatch chicks), pituitary glands (pooled per 5), thyroid glands (pooled per 3 pairs), and hypothalami were collected, frozen in liquid nitrogen, and stored at -80°C . To examine the changes in TRβ2 mRNA expression in the thyrotropes and non-thyrotropic cell regions with ISH, E16 and E20IP pituitaries were collected and kept in 4% (w/v) paraformaldehyde (PFA) in PBS (pH 7.4) at 4°C . After 24 h, tissues were cryoprotected overnight in the same solution with 30% (w/v) sucrose. The pituitaries were subsequently stored at -80°C until sectioning. Twenty micrometer sections were cut with a cryostat and kept in a cryoprotectant solution containing 30% (v/v) ethylene glycol and 30% (v/v) glycerol in 0.1 M phosphate buffer at -80°C .

RNA isolation and real-time PCR In a first experiment, pituitary total RNA was isolated using the RNeasy Total RNA Isolation System (Promega), while in a second experiment, diencephalic and thyroidal total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's guidelines. Three pools of pituitary glands and thyroid glands and four diencephali of each developmental stage were used for RNA isolation. The samples were subsequently treated with DNase I using the DNA-free kit (Ambion). Reverse transcription was performed with the Reverse Transcription Reagents (Applied Biosystems) or using 0.375 U/μl AMV reverse transcriptase (Invitrogen). Five microliter of cDNA, corresponding to ~ 50 ng or 100 ng total RNA, were used for real-time PCR. For the measurement of TRβ2 and β-actin expression in the pituitary gland, the primers were combined with a Taqman probe (Table 1). The real-time PCR was performed as described in Grommen *et al.* (2005). When measuring the diencephalic and thyroidal TRβ2 expression, the same primers were applied with SYBR Green using the real-time PCR protocol described in the tissue distribution experiment. In pituitary gland, β-actin mRNA was expressed at a constant level during late embryogenesis as measured with real-time PCR, while GAPDH was a more stable active reference in diencephalon and thyroid gland (own observation). The primers for GAPDH are listed in Table 1. For each gene, a non-template control and a sample that was not reverse transcribed were added as negative controls. For the TRβ2 mRNA expression in pituitary, a 1:2 dilution series of pituitary cDNA was added as a standard on every 96-well optical reaction plate. The diencephalic and thyroidal TRβ2 mRNA values were calculated using the $\Delta\Delta\text{C}_t$ method after performing a validation experiment as described in Applied Biosystems User Bulletin No.2 (P/N 4303859). Each experimental sample was analyzed *in triplo*, and standard samples were measured *in duplo*.

RIA Plasma 3,5,3',5'-tetraiodothyronine (T_4) concentrations were measured as described previously (Van der Geyten *et al.* 2001).

Combined ISH-immunohistochemistry (IHC) Riboprobes were transcribed from 1 μg linearized plasmid containing a cDNA insert of chicken TRβ2 in the presence of 50 μCi [^{35}S]UTP (Perkin-Elmer, Wellesly, MA, USA) and 20 U RNA polymerase (SP6 for antisense and T7 for sense probes; Roche Diagnostics). The TRβ2 insert was chosen in the unique amino-terminal region of the receptor and is located from base pairs 74–338 (EMBL accession number X62642). *In situ* hybridization was performed on free-floating cryosections of E16 and E20IP pituitaries and followed by immunohistochemical staining of the thyrotropic cells with a homologous anti-TSHβ antibody as described by De Groef *et al.* (2003).

Quantification of TRβ2 ISH signal Sections were analyzed with a Leitz DM RBE microscope equipped with a color video camera (Optronics Engineering, Goleta, CA, USA) and connected to a computer-aided analysis system (Bioquant, R and M Biometrics, Nashville, TN, USA) as described in Arckens *et al.* (1998). The thyrotropic cells were identified in light microscopy mode based on anti-TSH immunoreactivity. The areal surface of each immunoreactive cell was outlined using the computer mouse. Upon switching to dark-field mode, we then automatically counted the number of overlaying pixels, with a brightness exceeding a pre-determined threshold, per μm^2 inside these TSH cell outlines. The number of silver grain pixels/ μm^2 was determined for ~ 100 thyrotropic cells per age group. In addition, we measured the number of silver grain pixels/ μm^2 for ~ 80 regions of comparable size but positioned in between the thyrotropes for both age groups (Table 2).

Localization of TRβ2 mRNA in diencephalon

Tissue sampling and preparation Cobb broiler chicks (Poel-Houben) were kept in warmed cages and provided with food and water *ad libitum*. Brains from C5 chicks were fixed by transcardiac perfusion with 1% PFA in PBS for 1 min and PBS

Table 2 Quantification of the number of silver grain pixels (TRβ2 mRNA *in situ* hybridization signal) overlaying the thyrotropic cells and areas covered by non-thyrotropic cells in the pituitary of 16-day-old chicken embryos (E16) and 20-day-old embryos at the internal pipping stage (E20IP). Values are means $\pm$ S.E.M. with $n = \sim 100$ for the thyrotropic cells and $n = \sim 80$ for the non-thyrotropic cell regions.

	E16	E20IP
Thyrotropes	12.41 $\pm$ 1.49	30.59 $\pm$ 1.66***
Non-thyrotropic cells	12.74 $\pm$ 0.76	34.29 $\pm$ 1.61***

Data are expressed as pixels/100 μm^2 . A significant difference between both age groups is indicated with *** (student's *t*-test, $P < 0.001$). TRβ2, thyroid hormone receptor β2.

with 4% PFA for 10 min. After removal, brains were kept in PBS containing 4% PFA for 24 h at 4 °C, in PBS for another 24 h at 4 °C and cryoprotected overnight in PBS with 10% sucrose at 4 °C. Finally, the tissue was embedded in 10% gelatin (w/v) and 10% sucrose in PBS and stored at -80 °C. Cryosections of 30 μ m were cut and stored at -80 °C.

In situ hybridization Sections were fixed with 4% PFA in PBS for 10 min. After washing in PBS for 3 $\times$ 5 min, the slides were treated for 10 min with 0.2 M HCl, 10 min with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine, 30 min with PBS with 1% (v/v) Triton X-100 (TX), and washed 3 $\times$ 5 min in PBS. Next, the sections were pre-hybridized for 2 h at room temperature in hybridization buffer (50% (v/v) formamide, 10% (w/v) dextran sulfate, 5 $\times$ Denhardt's, 0.62 M NaCl, 20 mM sodium piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 6.8), 10 mM EDTA, and 0.2% (w/v) SDS, adjusted to 50 ml with diethylpyrocarbonate-treated water), containing 50 mM dithiothreitol, 250 μ g/ml herring sperm DNA, and 250 μ g/ml yeast RNA. Finally, the sections were incubated overnight at 80 °C in hybridization buffer containing 400 ng/ml antisense or sense digoxigenine (DIG)-labeled cRNA probe. Riboprobes were transcribed from 1 μ g linearized pCRII-TOPO plasmid (Invitrogen) containing an insert of the chicken TR β 2 (base pairs 74–338, base pair numbering based on EMBL accession number X62642) in the presence of DIG RNA labeling mix and 40 U RNA polymerase, SP6 for antisense, and T7 for sense probes (Roche Diagnostics). A spotting test was performed to check the efficiency of the labeling. Coverslips were removed by soaking the sections in 5 $\times$ saline sodium citrate (SSC) at 72 °C. After washing for 45 min in 2 $\times$ SSC at 72 °C, 2 $\times$ 5 min in 2 $\times$ SSC at room temperature and 3 $\times$ 5 min in PBS with 0.1% TX, slides were treated for 1 h at room temperature with PBS containing 4% (w/v) BSA, 0.1% TX, and 0.1 M lysine. They were subsequently incubated overnight at 4 °C in alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) diluted 1/5000 in PBS with 4% BSA and 0.1% TX. The alkaline phosphatase signal was detected using the nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT/BCIP) chromagen system (Roche Diagnostics) according to the manufacturer's guidelines.

In vitro incubation of pituitaries with 3,5,3'-tri-iodothyronine (T₃) or T₄

Sampling, incubation, and analysis of TR β 2 mRNA expression Studies were performed on pituitaries of embryonic (E18) and post-hatch (C10) Ross broiler chickens (Avibél). Animals were killed by decapitation and the pituitaries were immediately transferred to 24-well plates with ice-cold M199 medium (Gibco). The medium was replaced with pre-warmed medium, and the pituitaries were incubated individually at 37 °C for 1 h of pre-equilibration.

The medium was then substituted with new pre-warmed medium (control) or medium containing 10 or 100 nM T₃ or T₄. The pituitaries were collected after 30 min of incubation. For each treatment, three pools of eight pituitaries were frozen on dry ice and stored at -80 °C until analysis. Total RNA was isolated using the RNeasy Total RNA Isolation System (Promega). The real-time PCR analysis was performed as described for the pituitary TR β 2 expression during embryonic development. Results were calculated using the $\Delta\Delta C_t$ method. For each age group, the different treatments were analyzed on separate 96-well plates, and on each plate one pool was chosen as a calibrator.

Statistical analysis

Statistical analysis was performed using the SAS program (SAS Institute, Cary, NC, USA). Real-time PCR data of the TR β 2 mRNA expression in diencephalon, pituitary gland, and thyroid gland were analyzed using Scheffé's lines on ranked

Figure 1 Distribution of TR β 2 mRNA in various tissues of 18-day-old embryos measured with real-time PCR. Results were calculated using the standard curve method and expressed as mean relative values $\pm$ S.E.M. ($n=3$ tissues or 3 pools of tissues).

Figure 2 Thyroid hormone receptor β 2 mRNA expression in (A) thyroid gland and diencephalon and (B) pituitary gland during the last week of embryonic development, expressed as mean relative values $\pm$ S.E.M. (thyroid gland, $n=3$ pools; diencephalon, $n=4$; pituitary, $n=3$ pools). Plasma T₄ concentrations corresponding to each experiment are shown in the upper panels as means (pmol/ml) $\pm$ S.E.M. (A, $n=20$; B, $n=13-17$). Samples with a common letter are not significantly different (TR β 2: Scheffé's test on ranked values, $P<0.05$; T₄: Scheffé's test, $P<0.05$).

values. Statistical differences in silver grain pixel counts between E16 and E20IP pituitaries were calculated using a student's t -test. The non-parametric Wilcoxon (Rank Sum) Test was used for the statistical analysis of the *in vitro* incubation data.

Results

Distribution of TR β 2 mRNA in embryonic tissues

TR β 2 mRNA expression was determined in different parts of the brain, pituitary gland, thyroid gland, pineal gland, and retinal tissue of 18-day-old embryos using real-time PCR (Fig. 1). At this embryonic stage, TR β 2 mRNA could be detected in all the examined tissues, but the most abundant expression was observed in retina and pituitary gland, with

respectively a ten- and threefold higher TR β 2 mRNA amount compared with diencephalon.

TR β 2 mRNA expression at the different levels of the HPT axis during the last week of embryonic development

TR β 2 mRNA expression was measured in thyroid gland and diencephalon (Fig. 2A) as well as in pituitary gland (Fig. 2B) from E14 till C0/C1 with real-time PCR. In thyroid tissue, we observed a dramatic rise in TR β 2 mRNA values during this developmental period as TR β 2 mRNA expression had increased 20-fold at E18 and 60-fold at C1 in comparison with E14. In both pituitary gland and diencephalon, mRNA levels rose gradually during the last week of embryogenesis with peak values at embryonic day 20. At E20IP, pituitary and diencephalic mRNA expression was significantly higher

Figure 3 Combined *in situ* hybridization (ISH)–immunohistochemistry (IHC) for the detection of TR β 2 mRNA (black dots) and thyrotropic cells (brown) in the pituitary gland of 16-day-old (E16) and 20-day-old embryos at the internal pipping stage (E20IP). (A and B) ISH–IHC using the antisense probe. (C) ISH–IHC using the sense probe (negative control). Bar=10 μ m.

compared with E14 and E15, and in case of the pituitary also E16. The developmental pattern of TR β 2 mRNA expression in diencephalon and pituitary closely resembled concurring plasma T₄ profiles (Fig. 2A and B).

The substantial change in pituitary TR β 2 mRNA expression was confirmed by ISH combined with IHC detection of the thyrotropic cells (Fig. 3A and B). At both incubation stages TR β 2 mRNA was present in the thyrotropic cells, but substantial ISH signal was also visible on other hypophyseal cell types. The grain density, expressed as pixels/100 μ m², was approximately three times higher in the thyrotropes – as well as in the area covered by non-thyrotropic cells – of E20IP pituitary sections in comparison

with E16 (Table 2); when performing an ISH with the sense probe, no signal was observed (Fig. 3C).

Localization of TR β 2 mRNA in diencephalon

We have also detected TR β 2 mRNA expression in the hypothalamic region of a 5-day-old chick using ISH with DIG-labeled riboprobes. The staining signal indicated that this receptor was widely expressed throughout the diencephalon including the peri- and paraventricular nuclei (Fig. 4A and B). When using the sense probe, no signal was observed (Fig. 4C).

Effect of THs on TR β 2 mRNA expression in E18 and C10 pituitaries

Pituitaries of E18 and C10 chicks were treated *in vitro* with two doses (10 and 100 nM) of T₃ or T₄ for 30 min (Fig. 5). A twofold decline in TR β 2 mRNA expression occurred after treating E18 pituitaries with 10 nM T₃ as well as with 100 nM T₃ or T₄. TR β 2 mRNA expression in C10 pituitaries was unaffected regardless of the treatment dose.

Discussion

In this study, we demonstrated the presence of TR β 2 mRNA in chicken thyroid gland, pituitary gland (including the thyrotropic cells), and diencephalon – together constituting the HPT axis. Guissouma *et al.* (1998) found that chicken TR β 2 – in contrast with chicken TR α – is able to induce a T₃-dependent regulation of rat TRH transcription when co-expressed with a rat TRH promoter-luciferase construct in murine hypothalamus. The amino terminus of the chicken TR β 2 is 87% identical to the human isoform, and amino acids important for the negative regulation of TRH by T₃ in humans are completely conserved in chicken (Langlois *et al.* 1997). In combination with our present findings, these observations suggest a role for TR β 2 in the negative feedback regulation of TSH and TSH-regulating hormones in the chicken. Next to TR β 2, TR α and TR β 0 have also been detected in the pituitary gland of the domestic duck (Bishop *et al.* 2000) and the hypothalamus of the chick (Forrest *et al.* 1991). Therefore, these receptor isoforms could also contribute to the TH feedback effects in the HPT axis. However, TR β isoforms seem to play a more important role than TR α in the TH-regulated inhibition of TRH gene expression, since only TR β 0- and not TR α -dependent TRH transcription was modulated by T₃ in chicken hypothalamic cells (Lezoualc'h *et al.* 1992). Likewise, several studies in mammals point to an important role for the β -isoforms in mediating TH-induced TSH and TRH down-regulation (Weiss *et al.* 1997, Abel *et al.* 1999, 2001). Moreover, TR β 2 exhibited the strongest suppression of the TSH β promoter in the presence of T₃ in CV1 cells (Nakano *et al.* 2004).

At the level of the pituitary gland, we observed that TR β 2 mRNA levels rose gradually during the last week of chicken embryogenesis. THs are known to decrease TSH β mRNA

Figure 4 Localization of TR β 2 mRNA in the hypothalamic region of a 5-day-old chick with *in situ* hybridization (ISH). (A and B) ISH using the antisense probe. (C) ISH performed with the sense probe (negative control). Bar=200 μ m (A) or 50 μ m (B and C) (PVN, paraventricular nucleus; PHN, periventricular nucleus; III, third brain ventricle).

levels in chicken anterior pituitary cells (Gregory *et al.* 1998), and a negative TH responsive element has been found in the promoter region of the TSH β gene in the Japanese crested ibis (Kawasaki *et al.* 2003). Therefore, the strong increase in TR β 2 mRNA levels before hatching – which can also be observed in the thyrotropes specifically (present study) – could account for the lowered activity of the HPT axis early post-hatch. Besides suppressing TSH β gene transcription directly, THs are able to up-regulate the expression of hypophyseal somatostatin (SS) receptor subtypes 2 and 5 (SSTR2, SSTR5) – which mediate the inhibition of TRH- and CRH-induced TSH release by SS (Geris *et al.* 2003, De Groef *et al.* 2007). The respective decrease in TSH β (De Groef *et al.* 2006) and increase in SSTR2 and SSTR5 mRNA levels between E19 and hatching could be the result of the coinciding rise in TR β 2 mRNA expression. It is interesting to note that TR β 2 is not confined to the thyrotropic cells and that the increase in TR β 2 mRNA expression in the pituitary during the last week of embryonic development occurs in thyrotropic as well as non-thyrotropic hypophyseal cells. Hence, changes in receptor expression in the pituitary likely also reflect an altered TH effect on other hormonal axes.

TR β 2 mRNA was widely expressed in chicken diencephalon and was also readily detected in all brain parts with real-time PCR. Together with the widespread distribution of immunoreactive TR β 2 in the central nervous system of the rat (Lechan *et al.* 1993), this implicates a significant role for TR β 2 in mediating TH action in vertebrate brain. As we

measured ontogenic changes of TR β 2 mRNA levels in the entire diencephalon, the observed elevated receptor expression could either represent a global change in TR β 2 mRNA levels or an augmented TR β 2 mRNA content in certain nuclei. In the paraventricular nucleus (PVN) and periventricular nucleus (PHN), TR β 2 may function to regulate the expression of TSH-releasing and release-inhibiting hormones as in mammals. THs are able to reduce TRH expression in the rodent PVN (Koller *et al.* 1987, Segerson *et al.* 1987, Yamada *et al.* 1989, Kakucska *et al.* 1992) and TR β 2 plays an important role in modulating mammalian TRH gene expression (Dupré *et al.* 2004).

The marked elevation in TR β 2 mRNA amount in the thyroid gland suggests a significant rise in the sensitivity of the thyroidal follicular cells for TH modulation. In contrast with the hypophyseal and diencephalic TR β 2 mRNA expression, the increase in mRNA values in the thyroid gland during late embryogenesis is much more pronounced and does not parallel the plasma T₄ pattern toward and after hatching. At this point, it is not clear whether the rise in thyroidal TR β 2 mRNA signifies an elevated regulation of thyroid gland development by THs, or an increased ultra-short feedback regulation of thyroid function. The THs are able to induce cell proliferation in FRTL-5 cells (Akiguchi *et al.* 1992) and bovine thyroid cells depleted of endogenous T₃ (Di Fulvio *et al.* 2000), but studies using transgenic mice proposed an important function for mainly TR α in the growth of the thyroid gland (Gauthier *et al.* 1999). Various studies suggest a suppressive action of THs on their production organ as THs

Figure 5 Expression of TR β 2 mRNA in pituitaries of 18-day-old embryos (E18) and 10-day-old chicks (C10) incubated with 10 or 100 nM T₃, T₄, or control medium for 30 min. Relative differences were measured with real-time PCR and calculated using the $\Delta\Delta C_t$ method. Results were expressed as mean relative values $\pm$ S.E.M. ($n=3$ pools of tissues). Significantly different results compared with corresponding control values are indicated with * (Wilcoxon rank sum test, $P<0.05$).

evoke inhibitory effects mainly – both *in vivo* and *in vitro* – on several TSH-stimulated processes in the mammalian thyroid gland such as iodine uptake and sodium iodide symporter mRNA expression (Yu *et al.* 1976, Akiguchi *et al.* 1992, Spitzweg *et al.* 1999). The elevated TR β 2 mRNA content could therefore include an augmented negative action of THs on their production organ before and after hatching, which perhaps signifies that local fine-tuning becomes more and more important once TH concentrations have started to increase substantially.

As plasma TH concentrations change considerably during the second half of embryogenesis, we investigated the effect of TH treatment on the TR β 2 mRNA expression in E18 and C10 pituitaries *in vitro*. The THs reduced TR β 2 expression levels only in embryos and not in post-hatch chicks. A similar repression of TR β 2 mRNA levels was observed when GH3 cells were incubated with T₃ (Hodin *et al.* 1989, Jones *et al.* 1993), while hypothyroidism leads to an up-regulation of TR β 2 mRNA in adult rat pituitary gland (Li & Boyages 1997). The different response of embryonic versus post-hatch pituitaries to a short-term TH challenge, as observed in this study, could be the result of an age-specific difference in regulatory factors participating in the control of TR β 2 gene transcription. Several co-activators and co-repressors involved

in TR-mediated effects (e.g. steroid receptor coactivator (SRC)-1–3, SYT, NCoR) are expressed in an age-dependent way in mammals (Martinez de Arrieta *et al.* 2000, Iwasaki *et al.* 2005, Schmidt *et al.* 2007), but virtually no data concerning these regulatory molecules in chicken are available. The down-regulation of chicken TR β 2 mRNA content in embryonic pituitary following an acute TH treatment *in vitro* may seem contradictory to the increasing expression of TR β 2 coinciding with the rising T₄ concentrations during late embryogenesis. It is not unlikely though, that an increase in plasma T₄ concentrations for a longer period of time – in contrast with the short-term TH administration in our experiment – has an opposite effect on TR β 2 mRNA expression.

In conclusion, we have investigated whether the TR β 2 mRNA expression at the major control levels of the HPT axis alters during late embryogenesis and the perinatal period in the chicken. The presence of TR β 2 mRNA in these tissues may imply a role for this TR isoform in TH-mediated feedback effects. We detected a significant rise in pituitary and diencephalon TR β 2 mRNA levels toward hatching, and although expression was not restricted to the pituitary thyrotropes and the hypothalamic PVN and PHN, this significant increase could translate into an augmented

regulation of the production of TSH and its regulatory hormones. In thyroid tissue, an impressive rise in receptor expression was noticeable during late embryogenesis till C1, indicative of an increasing modulation of thyroid development and/or function by THs. Furthermore, the regulation of TR β 2 expression by THs seems to be age dependent as T₃ and T₄ down-regulate receptor levels only in embryonic and not in post-hatch pituitaries *in vitro*.

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