Role of Thyroid Hormone Receptor Subtypes $\alpha$ and $\beta$ on Gene Expression in the Cerebral Cortex and Striatum of Postnatal Mice

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The effects of thyroid hormones (THs) on brain development and function are largely mediated by the control of gene expression. This is achieved by the binding of the genomically active $T_3$ to transcriptionally active nuclear TH receptors (TRs). $T_3$ and the TRs can either induce or repress transcription. In hypothyroidism, the reduction of $T_3$ lowers the expression of a set of genes, the positively regulated genes, and increases the expression of negatively regulated genes. Two mechanisms may account for the effect of hypothyroidism on genes regulated directly by $T_3$: first, the loss of $T_3$ signaling and TR transactivation, and second, an intrinsic activity of the unliganded TRs directly responsible for repression of positive genes and enhancement of negative genes. To analyze the contribution of the TR subtypes $\alpha$ and $\beta$, we have measured by RT-PCR the expression of a set of positive and negative genes in the cerebral cortex and the striatum of TR-knockout male and female mice. The results indicate that TR$\alpha_1$ exerts a predominant but not exclusive role in the regulation of positive and negative genes. However, a fraction of the genes analyzed are not or only mildly affected by the total absence of TRs. Furthermore, hypothyroidism has a mild effect on these genes in the absence of TR$\alpha_1$, in agreement with a role of unliganded TR$\alpha_1$ in the effects of hypothyroidism. (Endocrinology 154: 1940–1947, 2013)

The effects of thyroid hormones (THs) on brain development and function are mediated primarily by genomic actions involving the control of the expression of many genes (1–3). This is achieved by binding to nuclear TH receptors (TRs), which are ligand-regulated transcription factors (4). In mammals TRs are encoded by two genes, $THRA$ and $THRB$. The $THRA$ gene produces several proteins of which only TR$\alpha_1$ binds the active hormone $T_3$ and therefore functions as a hormone receptor. The $THRB$ gene produces two $T_3$-binding isoforms, TR$\beta_1$ and TR$\beta_2$. The TRs regulate transcription by binding to specific sequences present in regulatory regions of the target genes (5, 6). The TRs can also regulate transcription by direct interactions with other transcription factors (7, 8). TH can exert positive or negative regulation (1). The positively regulated genes are repressed in the absence of $T_3$ and stimulated in its presence. The negatively regulated genes are stimulated in the absence of $T_3$ and inhibited in its presence. In the present paper, we use the term positive genes to mean genes with decreased expression in hypothyroid animals and negative genes those with increased expression in hypothyroid animals, independently of the mechanism of regulation, which is known for some of the genes that we have studied (9–11).

Despite the role of TRs in mediating the physiological effects of TH, it is known that the absence of TRs is not equivalent to TH deprivation, ie, hypothyroidism (12). For example, the morphological changes induced by hypothyroidism in the cerebellum of wild-type (WT) mice, are not observed in mice devoid of TR$\alpha_1$ (13), indicating that the effects of hypothyroidism are partly due to unliganded receptor activity. In agreement with this, a hypo-
thyroid phenotype is produced by expression of mutant TRα1 with dominant-negative activity due to intact DNA binding but deficient in hormone binding (14).

Differences in physiological roles between the TR subtypes α and β, or the TRβ isofoms, TRβ1 and TRβ2, depend mainly on their patterns of expression in different tissues. Hepatic gene expression profiles in vivo were very similar in TRα or TRβ knockout mice (15), indicating that in liver, TRα and TRβ regulate a similar set of genes. This agrees with recent findings demonstrating that when individually expressed in the same cell types, the TR subtypes regulate largely overlapping sets of genes, with differences mostly in the kinetics of regulation (16, 17). However, the TRs also display some selectivity on gene regulation in some experimental settings (18–20). For example, Klf9 is induced by T3 in N2a neuroblastoma cells expressing TRβ1, but not in N2a cells expressing TRα1 (21).

Based on the concentrations of TR protein in brain, predominantly in the form of TRα1 (22), it is thought that the effects of TH on the brain are mostly mediated through TRα1, but a detailed study on the relative contributions of TRα and TRβ on brain gene expression has not been made. In the present work, we have studied the role of the TRs in the mouse cerebral cortex and striatum. To this end, we analyzed the expression of a set of positively and negatively regulated genes (2, 3) in these regions from WT mice and from mice devoid of TRα1, TRβ, or both. The results indicate a primary, but not exclusive, role of TRα1 in positive and in negative regulation. We also studied whether the combined absence of TRα1 and TRβ had similar effects as the absence of the hormone. The results indicated that hypothyroidism induced a more profound effect on brain gene expression than complete absence of TRs, in agreement with a role of unliganded TR in hypothyroidism. The role of unliganded TRα1 was demonstrated on some genes by showing that hypothyroidism had no effect, or the effect was milder, on mice devoid of TRα1 than on WT mice.

**Materials and Methods**

**Handling of animals**

Protocols for animal handling were approved by the local institutional Animal Care Committee, following the rules of the European Union. Animals were housed in temperature-controlled (22°C ± 2°C) and light-controlled (12-hour light, 12-hour dark cycle; lights on at 7:00 am) conditions and had free access to food and water. Mice of a hybrid genetic background of 129/Ola-x 129/Sv 1 BALB/c-x C57BL/6 (23–26) were used. We started by crossing TRα1+/+ TRβ−/− male mice with female of the same genotype (F0) to generate all possible genotypic combinations (F1). The mice used in the experiments were obtained by appropriate crossings of F1 to generate WT, TRα1−/− TRβ+/+, TRα1+/− TRβ−/−, and TRα1+/+ TRβ+/− mice (F2) and were indistinctly male or female of age postnatal day 21 (P21). The TRα1 deletion was detected by using the following combination of primers: forward 5'-cagatccagagatccagaa-3', reverse 5'-gtatgggagctgcatctatccaag-3' and the TRα1+/−-specific reverse primer 5'-cactgccatatggtgtg-3'. The TRβ deletion was detected by using the following combination of primers: forward 5'-cagacagacagagactcttgct-3', reverse 5'-ccctgagcacaagcataaatg-3' and the TRβ+/−-specific reverse primer 5'-gtgcagcagcctgctg-3'. Hypothyroidism was induced in pregnant and lactating dams by administering a drinking solution containing 0.02% 1-methyl-2-mercapto-imidazole (MMI; Sigma Chemical Co, St Louis, Missouri) plus 1% KCIO4 ad libitum. These antithyroid compounds were given from gestational day 9 and throughout the lactating period until the end of the experiment on P21. These compounds cross the placenta and are present in the milk, so that the pups derived from the treated dams were also hypothyroid.

**RNA preparation and quantification by real-time RT-PCR**

The pups were killed by decapitation on P21. The cerebral cortex and the striatum were rapidly dissected out, frozen on dry ice, and kept at −80°C until RNA isolation. RNA was isolated using the Trizol procedure (Invitrogen, Carlsbad, California) with an additional step of chloroform extraction for RNA isolation. The quality of RNA was analyzed using a BioAnalyzer (Agilent, Santa Clara, California). cDNA was prepared from 250 ng of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California). Quantitative PCR assays were performed on microfluidic cards or single-tube PCR. For the microfluidic cards, we used TaqMan low-density arrays (Applied Biosystems), format 48a (P/N 4342253). cDNA aliquots corresponding to 10 ng of starting RNA from individual mice were used, with TaqMan Universal PCR Master Mix, No Amp Erase UNG (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The PCR program consisted in a hot start of 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. For analysis, we used the 2-Ct method. As an internal control we included 18S RNA and PpiA (peptidylprolyl cis/trans isomerase A or cyclophilin A). The use of either reference RNA gave similar results, so that the data were all normalized to the 18S RNA. Data are expressed relative to the values obtained for the control WT, which was given a value of 1.0 after correction for 18S RNA. For single-tube PCR, a CDNA aliquot corresponding to 5 ng of the starting RNA was used, with TaqMan Assay-on-Demand primers and the TaqMan Universal PCR Master Mix, No Amp Erase UNG (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The PCR program consisted in a hot start of 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. For analysis, we used the 2-Ct method. As an internal control we included 18S RNA and PpiA (peptidylprolyl cis/trans isomerase A or cyclophilin A). The use of either reference RNA gave similar results, so that the data were all normalized to the 18S RNA. Data are expressed relative to the values obtained for the control WT, which was given a value of 1.0 after correction for 18S RNA. For single-tube PCR, a CDNA aliquot corresponding to 5 ng of the starting RNA was used, with TaqMan Assay-on-Demand primers and the TaqMan Universal PCR Master Mix, No Amp Erase UNG (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The PCR program consisted in a hot start of 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. PCs were performed in triplicates, using the 18S gene as internal standard and the 2-Ct method for analysis.

**Statistical analysis**

Differences between means were obtained by 1- or 2-way ANOVA, depending on the experiment, and the Tukey or Bonferroni’s post hoc tests, respectively. Calculations were done us-
ing the GraphPad Prism software (http://www.graphpad.com/prism/). The experimental groups were formed with about the same number of male and female pups, and sex of the animals was not considered a factor in statistical analysis.

Results

The goal of this work was to analyze the relative roles of TRα1 and TRβ on the expression of TH-dependent genes in the cerebral cortex and the striatum of P21 mice. We analyzed 27 genes regulated positively and 14 genes regulated negatively by TH (Supplemental Data 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). These genes were identified in previous microarray analysis of the cerebral cortex (3) and the striatum (manuscript in preparation). The criterion to define these genes as positively or negatively regulated by TH was that hypothyroidism induced a decreased or increased expression, respectively, in at least 1 brain region. The expression of each gene was measured by quantitative PCR in RNA samples of individual mice from four genotypes: WT, TRα1/−/−, TRβ/−/−, and TRα1/−/−TRβ/−/−. As a reference for the TH dependence of each gene, we also included a group of WT mice rendered hypothyroid from prenatal stages. The effects of hypothyroidism on the cortex expression of most genes were as previously described (2), with the exception of Col6a2. This gene was included in the study as a negative gene based on previous findings. However, in the present study, the higher mean expression value in hypothyroidism was not statistically significant (Supplemental Data 1).

Figure 1 shows the expression patterns in cortex and striatum of 4 selected genes, Kcnj10, Cbr2, Cirbp, and Angptl4. Individual results for all 41 genes are shown in Supplemental Figure 1. The 4 genes selected in Figure 1 are examples illustrating the observed patterns of regulation (see also Figure 7). When hypothyroidism was performed in the WT mice, expression of Kcnj10 and Cbr2 decreased, whereas expression of Cirbp and Angptl4 increased, classifying these genes among the positive and negative genes, respectively. The effect of hypothyroidism may be compared with the effect of the combined absence of TRα1 and TRβ (abbreviated as αβ). In the cortex and the striatum, the absence of αβ increases the expression of the negatively regulated gene Cirbp to the same level as in hypothyroidism. For Kcnj10, the effect of αβ deficiency was in the same direction but not as strong as the effect of hypothyroidism. In contrast to the strong effects of hypothyroidism, αβ deficiency was without effect on Cbr2 in the cortex and the striatum and on Angptl4 in the striatum. The effect of αβ deficiency on cortex Angptl4 was less clear. Single inactivation of TRα1 or TRβ had variable effects, with a decreased expression of Kcnj10 and Cirbp in the striatum and increased expression of Angptl4 in the cortex of TRα1-deficient mice.

Figures 2 through 5 show the relative changes of gene expression in reference to the expression in the WT, given a value of 1.0 and represented as a dotted line in the figures. The genes were ordered in the figures in relation to the value obtained for the hypothyroid WT mice. For the positive genes (Figures 2 and 3), the strongest effect of hypothyroidism was on Agxt2l1 in the cortex and Cd72 in the striatum with more than 90% reduction. There was no quantitative correlation between the effects of hypothyroidism in the cortex and striatum (r = 0.13, P = .52, not shown). As extreme examples, Cd72 and Vdr were among the most strongly affected genes in the striatum, whereas in the cortex, they were little or not affected. Neither the lack of TRα1 nor the lack of TRβ induced consistent changes, although the mean expression was below the WT value for the TRα1−/− and above the WT for the TRβ−/−. The absence of αβ had in general the strongest effect and in the same direction as hypothyroidism. The effect of αβ deficiency on some genes approached that of hypothyroidism. This was the case for Hr, Pvalb, Kcnj10, Nefm, Nefb, or Sema7a in the cortex and for Cd72, Vdr, Pvalb, Aldh1a1, Nefm, Hr, or Nefb in the striatum. Other genes remained at or near WT levels (Flywch2, ler5, Ith3, Nrtn,
and Pqnr6 in the cortex and Agxt2l1, Cbr2, Flywch2, Ier5, Itih3, Klf9, and Pdp1 in the striatum) (Figures 2 and 3 and Supplemental Figure 1). The results partially agree with a predominant role of TRα1 in TH-mediated brain gene expression. The absence of TRβ was associated with normal or increased gene expression, probably due to the increased TH levels in these mice (24). In the absence of TRα1, TRβ was able to sustain gene expression to near WT euthyroid levels.

For the negative genes (Figure 4 for the cortex and Figure 5 for the striatum), the effects of hypothyroidism showed a positive correlation between the cortex and the striatum (Figures 2 and 3 and Supplemental Figure 1). The number of animals was as indicated in the legend for Figure 1.
 striatum ($r = 0.76$, $P = .0014$, not shown). Compared with the positive genes, a strongest effect of the absence of TRα1 was observed as a whole. Hypothyroidism increased the expression of 13 genes in the cortex from 1.5- to 4-fold and 14 genes in the striatum from 1.3- to 5.5-fold. The absence of TRα1 increased the expression of several genes in the cortex and in the striatum at least 1.5-fold. In contrast, the absence of TRβ induced minimal changes. The absence of αβ was similar to the absence of TRα1, with 9 genes increasing at least 2-fold in the cortex and 6 genes increasing at least 2-fold in the striatum. We may conclude that also for the negative genes, TRα1 was more relevant for gene expression than TRβ. As for the positive genes, the effects of hypothyroidism were in general stronger than the absence of TRs.

The effects of hypothyroidism on gene expression could be due to 2 factors. First, the reduction of T3 signaling directly related to the reduction of TR occupancy and transactivation. In this case, the absence of receptors should be similar to the effects of TH deprivation. Second, the unliganded TRs might have intrinsic activity and directly inhibit or stimulate the expression of positive or negative genes, respectively. The correlations between the effects of the lack of TRs and hypothyroidism for the positive and negative genes are shown in Figure 6. In all cases, the correlations were significant, but the slopes of the regression lines were lower than 1, indicating that the effect of hypothyroidism was stronger than the effect of TR deficiency. From the values of the y-intercepts it may be calculated that the effect of TR deprivation, ie, the loss of T3 signaling accounted for about 70%–80% of the effect of hypothyroidism on the positive genes and 60% for the negative genes on average.

To confirm that the effects of hypothyroidism on some genes were due at least in part by the activity of unliganded receptors, especially TRα1, we analyzed the effect of hypothyroidism on gene expression in TRα1-deficient mice. Figure 7 shows the response of the genes described in Figure 1 (Kcnj10, Cbr2, Cirbp, and Angptl4). In WT hypothyroid mice, the expression of Kcnj10 and Cirbp decreased and the expression of Cbr2 and Angptl4 increased. On the other hand, whereas hypothyroidism had a similar effect on the expression of Kcnj10 and Cirbp in the presence or absence of TRα1, it was without effect on Cbr2 and Angptl4 in the absence of TRα1, indicating that the effects of hypothyroidism on these 2 genes was due to the repressing (Cbr2) or inducing (Angptl4) activity of unliganded TRα1. The results obtained using 12 positive

![Figure 6](https://academic.oup.com/endo/article-abstract/154/5/1940/2423679)
genes and 7 negative genes in the cerebral cortex and/or the striatum are shown in Supplemental Figure 2. Compared with the effect of hypothyroidism on the WT mice, in the absence of TRα1, hypothyroidism had a much lower effect on \( \text{Agxt2l1}, \text{Gls2}, \text{Cbr2}, \text{Flywch2}, \text{Ithb3}, \text{Angptl4}, \text{Gpc3}, \text{Mmdc2}, \text{and} \text{Ly75} \) in at least 1 region. In addition, the test for interaction in the 2-way ANOVA comparing the response of WT and TRα1-deficient mice to hypothyroidism was highly significant \((P < .001)\) for most genes, indicating an influence of genotype in the response.

**Discussion**

In the present work, we have analyzed the role of TRα and TRβ in the control of brain gene expression. To this end, we have measured the expression of genes that we have previously identified as TH-dependent after microarray analysis of the cerebral cortex and striatum from normal and hypothyroid P21 mice. The genes analyzed cover a wide range of physiological and biochemical processes, reflecting the complexity of TH action in the brain and the pleiotropic effects of hypothyroidism (Supplemental Data 1). Although the goal of this paper was not to discuss each brain processes regulated by THs, it is pertinent to briefly summarize the main functions of the genes analyzed. They include genes involved in different aspects of metabolism, such as \( \text{Hmgcs2} \) (ketogenesis), \( \text{Aldh1a1} \) (retinol metabolism), \( \text{Cbr2} \) (nicotinamide adenine dinucleotide phosphate-dependent oxidoreductase), \( \text{Gls2} \) (glutamine metabolism), \( \text{Pla2g5} \) (membrane phospholipids), and \( \text{Angptl4} \) (glucose metabolism); genes involved in cell proliferation (\( \text{Ly75} \)), differentiation (\( \text{Cd72} \)), and survival (\( \text{Cirbp} \)); neurofilaments, such as \( \text{Nefb} \) and \( \text{Nefm} \); or related to tubulin processing (\( \text{Agbl3} \)); neurofilaments, such as \( \text{Itih3}, \text{Col6a1}, \text{Mamdc2}, \) and \( \text{Ctnn2} \); development (\( \text{Sema7a} \)); transcription (\( \text{Dbc1}, \text{Hr}, \text{Klf9} \)); \( \text{Ca}^{2+}/\text{calmodulin signaling in the dendritic spines (Nrtn)}, \text{K}^{+} \) channel (\( \text{Kcnj10} \)), and \( \text{Ca}^{2+} \)-binding proteins (\( \text{Pvalb} \) and \( \text{Calb} \)). We have previously studied the expression of many of these genes in other contexts, such as inactivation of the monocarboxylate transporter 8, and types 2 and 3 deiodinases (2, 3).

By comparing their expression in \( \text{TRa1}^{-/-} \) and \( \text{TRβ}^{-/-} \) mice relative to the WT, we conclude that both receptor subtypes are involved in the regulation of gene expression in brain. TRα1 appears to have a primary role, but the lack of this receptor affects only a subset of the genes. Absence of both receptor types increases the number of genes affected, and in many cases, the effect approaches quantitatively the effect attained by hypothyroidism, indicating that in the absence of TRα1, TRβ maintains gene expression near normal levels. On the other hand, the absence of TRβ results in little change, with increased expression of a few positive genes and decreased expression of a few negative genes.

In these effects of receptor deficiency, we have to take into account possible changes of TH concentrations that might have contributed to the observed changes. The effects of TRα1 deficiency are not probably due to lower \( \text{T}_3 \) concentration, because cerebral cortex concentrations of \( \text{T}_4 \) and \( \text{T}_3 \) are not modified in the \( \text{TRa1}^{-/-} \) mice (13). However, the increased or decreased expression of some genes observed in the absence of TRβ is most likely due to the known enhancement of TH production in the \( \text{TRβ}^{-/-} \) mice (24). This would result in increased \( \text{T}_3 \) action through the remaining TRα1, and increased or decreased expression of positive and negative genes, respectively. More difficult is to analyze the contribution of the highly increased TH concentrations in the double \( \alpha \beta \) deficiency (25). We cannot discard that overactivation of non-genomic pathways (1) might play a role in these mice. However, we think that this possibility is unlikely given that \( \alpha \beta \) deficiency either has no effect on gene expres-
sion or the effect goes in the same direction as in hypothyroidism.

Another important concern is how the cellular heterogeneity of the brain regions might have influenced the gene expression changes. Indeed, genetic studies have revealed a cellular complexity that goes well beyond the classical cell type subdivisions of the brain based on morphology and neurotransmitter production (27, 28). Different cell groups might respond differently to THs in the regulation of expression of individual genes. Also, the responsive cells might be a minor component of the total cellular repertoire of the region under study. It is well known that some individual genes may be sensitive to THs in some cell populations and not in others despite expressing TRs in adequate amounts (29). As an example, Nrgn, a gene regulated directly by T3 at the transcriptional level (30), is very sensitive to TH in the striatum, dentate gyrus, and layer 6 of cerebral cortex, whereas other layers of the cortex and hippocampus are not sensitive (31). This is the reason for the lower effect of hypothyroidism on Nrgn expression and on other genes such as Vdr and Cd72 in the whole cortex compared with the striatum. Only quantitative in situ hybridization techniques, with a detailed account of the gene expression responses by individual cell groups, should be able to provide a complete picture.

A full interpretation of the data would also require a previous understanding of the mechanisms of regulation by T3 on each individual gene, specifically whether they are direct or indirect responses. Several lines of evidence indicate that many of the genes studied in this work are most probably direct targets of TH. Some of them have been specifically studied in this regard, with confirmation of direct regulation at the cellular level and identification of thyroid-responsive elements. These include Nrgn (9, 30), Hr (10), Klf9 (11, 21), Gbp3 (32), and Angptl4 (33). Others respond to the administration of a single T3 dose to adult rats (Aldh1a1, Bcar3, Hr, Itih3, and Klf9) (34) or to primary mouse cerebrocortical neurons (Aldh1a1, Bcar3, Calb, Flywch2, Gbp3, Gpc3, Hr, Kcnj10, Klf9, Nefh, Nefm, Paqr6, Sema7a, and Sbh) (35). Studies are in progress to analyze gene responses in primary cells derived from TR-deficient mice.

With the above limitations in mind, we found little evidence for receptor subtype specificity among the positive genes. Some of the genes analyzed in this work have also been examined in other cellular contexts. In HepG2 cells, Kcnj10 was positively regulated by T3 through TRα1 and Gpc3 was negatively regulated, in general agreement with our findings (16). In contrast, Angptl4, which behaves as a negative gene in cortex and striatum, in HepG2 cells is a TRβ-specific positive gene (33). Concerning the negative genes, the absence of TRα1 was in general more effective than for the positive genes, indicating that TRα1 was more involved in negative regulation of brain genes than TRβ. These results contrast with the predominant effect of TRβ on negative regulation of the Tshb gene (20).

Compared with the effects of hypothyroidism, the absence of TRα1 and TRβ, and therefore complete lack of T3 signaling through the TR, led either to no changes in gene expression or to changes that were in the same direction as in hypothyroidism but generally of less severity. Taking together all genes, the effects of hypothyroidism and of TRα1 plus TRβ inactivation were correlated, but the regression line indicated a stronger effect of hypothyroidism. The results are compatible with the idea that the effects of hypothyroidism on the expression of some genes is due to the activity of the unliganded TR, consisting of repression of positive genes and activation of negative genes. It is likely that the effects of unliganded TR in brain hypothyroidism are primarily due to TRα1. This was demonstrated by showing that on some genes, hypothyroidism had no effect on the TRα1−/− mice. A clear example was Cbr2 in cortex and striatum. On others, there was a significant effect of hypothyroidism on the TRα1−/− mice but milder than the effect on the WT, for example, Kcnj10 also in both regions. This may indicate that unliganded TRβ might also play a role in hypothyroidism in agreement with the effects of mutant TRβ1 (36, 37).

A final conclusion of these studies is that TRα1 exerts a predominant but not exclusive role in the regulation of gene expression in the cerebral cortex and the striatum. This may be a direct consequence of the higher abundance of TRα1 relative to TRβ in brain or may reflect differences in the action kinetics between the two receptor subtypes, as pointed out recently for nonneural cell lines in culture (16, 17).

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