Thyroid status co-regulates thyroid hormone receptor and co-modulator genes specifically in the hypothalamus

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Abstract Regulation of Thyrotropin Releasing Hormone (*TRH*) transcription in the hypothalamus represents the central control point of thyroid function. To examine the expression of potential *TRH* regulatory components, we simultaneously amplified, by semi-quantitative multiplex PCR system, nine key genes from ≤100 ng total RNA from two brain areas (hypothalamus and cortex) under different thyroid states. Expression of TR_1 and TR_2 isoforms, key elements in *TRH* regulation, was modified by thyroid status in the hypothalamus but not in the cortex. Similarly, hypothyroidism increased specifically hypothalamic levels of three co-modulator genes. This study provides the first demonstration of tissue specific co-regulation of a set of genes by thyroid status within a defined brain area.

Keywords: Thyroid hormones; *TRH* transcription; Gene synexpression; Semi-quantitative multiplex PCR; Tissue specific gene regulation; Paraventricular nucleus of the hypothalamus

1. Introduction

Differential gene activity largely accounts for the regulation of cell differentiation underlying development and for tissue specific contributions to homeostasis. Many physiological regulations require strict temporal and spatial regulation of gene activity. One paradigm for differential gene regulation in a defined neuronal context is that of hypothalamic specific transcriptional regulation of Thyrotropin Releasing Hormone (*TRH*), specifically regulated by thyroid hormones in the paraventricular nuclei (PVN) of the hypothalamus [1,2]. This regulation represents the central control point of thyroid function. Tri-iodothyronine (T_3)-dependent repression of *TRH* transcription occurs through thyroid receptors (TR), members of the nuclear receptor superfamily.

As for other nuclear receptors, TR action is modulated by interaction with co-regulator proteins (for review, see [3,4]). Little is known about TR and co-modulator regulation in the hypothalamus and whether tissue specific regulation of the genes encoding proteins potentially involved in *TRH* regulation occurs.

To address these questions, we set up a semi-quantitative multiplex PCR system to analyze, among the genes potentially involved in T_3-dependent *TRH* gene regulation, which ones were specifically regulated by thyroid status in the PVN. To verify the PVN-specific nature of the regulation, we analyzed the expression of the same genes in another brain region, i.e., the motor and cingulate cortex, where TRH is not expressed. The results bolster the hypothesis that TRβ receptors and both co-repressors and co-activators can act synergistically to play modulatory roles in *TRH* regulation and that their regulation by thyroid status is PVN specific.

2. Materials and methods

2.1. Animal care and treatment

Animal studies were approved by the Direction des Services Veterinaires de Paris and conducted in accordance with the principles and procedures of the NIH Guidelines for Care and Use of Experimental Animals.

Hypo- or hyperthyroidism (T3 levels: 16.42 ± 3.51 and 981 ± 111 ng/dl, respectively, vs. 69.30 ± 1.96 ng/dl for euthyroidism) was induced in male OF1 six weeks old mice (Janvier, Le Genest St Isle, France) as described by Dupre et al. [5] for 13 days.

2.2. Tissue isolation

Animals were decapitated after light anesthesia with diethyl ether. To respect circadian rhythms, animals were always sampled between 10 a.m. and 12 a.m. Plasma samples were taken for verification of thyroid status (as in Dupre et al. [5]). Brains were removed, and the PVN and the area corresponding to the motor and cingulated cortex were micro-dissected and kept in RNA*later* (Ambion Inc., Austin, TX, USA).

2.3. RNA extraction and cDNA synthesis

PVN from three mice were pooled and total RNAs from PVN or cortex were extracted using RNAble (Eurobio, Les Ulis, France) following the manufacturer's protocol. From 100 ng to 1 μg of total RNA were reverse-transcribed using Superscript II RNase H^- reverse-transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Control reactions were done without reverse-transcriptase. Final cDNA reaction products were diluted with H_2O to concentrations corresponding to 20 ng of starting RNA (20 ng RNA equivalents) per microliter and stored at −80 °C.

2.4. Primers

Multiplex primer design was optimized according to Henegariu et al. [6] and Zangerberg et al. [7], using Oligo 4.0 S software (National Biosciences Inc., Plymouth, USA). Briefly, primers fulfilled the following conditions: 20 to 26 mer, with TT or CC at the 3′ terminus to avoid primer dimerization, with a GC content between 40% and 60% and an annealing temperature of 60 ± 1 °C. The amplified sequences were between 102 and 357 bp long and were chosen in regions of least

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Table 1
Sequence of oligonucleotide primers used in the semi-quantitative multiplex RT-PCR experiments: oligonucleotide positions are indicated as subscript

Gene name	Size (bp)	5′ Oligonucleotide (5′–3′)	3′ Oligonucleotide (5′–3′)	GenBank™	Primer (nM)
TRα_1	168	$_{1251}$CAG AGG GTG GGA GCT GGT	CCT GTC CAA GGG CTG GAG GGT	X51983	800
TRα_2	102	$_{1215}$GCA TGT TGT TCA GGG TCC GCA GGT	GGG CTC TTC GGG CTC TGG TGC T	X07751.1	50
TRβ_1	155	$_{-41}$GAA TGC CAG TAC AGA AGA TGA CCC	GTG CAG GCA GGC TTC AGA CAT T	S62756.1	50
TRβ_2	143	$_{97}$AGT CCG GCT CTC AGT GGT CGT	GCC ATG TCC AAG TCA GAG TCC	NM-009380.1	250
TRH	253	$_{14}$GGC TGA TGA TGG CTC TGG CTT T	ACG TCT TCC TCC TTC TCC TCC CTT T	NM-009426	50
NcoR	321	$_{3506}$TTT CAT CGT TGC GGG GCT CTA TT	TCC TTC CAC TGC CTC ATA GCT TCT TT	NM-011308	100
SMRT	284	$_{359}$CAT CAC CCC TGC TGG CCA CT	CCA GGC TTC GGT GCT TTG ATT	AP113001	200
CBP	357	$_{1270}$ATG ACT GTC CTG TTT GCC TCC CTT T	TTG GTG GCT GTT GAT CTG TTG TTA TT	S66385	75
SRC1	233	$_{2102}$GCA GCC CCT CAG ACA TCA CCA CTT	CTT TAC GTC ATC CAG GCA CAG GTT	U64828	100

homology (Table 1). All primer sets were tested individually to optimize amplification conditions. We next amplified all targets in the same tube, starting with equimolar primer concentrations (200 nM of each primer). After adjustments, final concentrations of each primer set ranged from 50 to 800 nM (Table 1).

2.5. Multiplex reaction component optimization

Salt concentrations, dNTP concentration and *Taq* DNA polymerase availability affect multiplex performance. Each parameter was tested and optimized separately. The optimal multiplex PCR mix was found to be: 50–800 nM of each primer set (see Table 1), dATP, dTTP, dGTP: 400 μM, dCTP: 25 μM, 2 μCi of [α-^{32}P]dCTP, 1.5× of PCR buffer, and 2.5 units of *Taq* DNA polymerase per 50 μl of reaction (thermal profile: 94 °C, 5 min; 26 cycles of 94 °C, 1 min, 60 °C, 1 min, 72 °C, 2 min; final elongation: 8 min).

2.6. Quantitation

For quantifying and comparing physiological situations, an invariant endogenous standard must be included. We used Ambion's QuantumRNA™ 18S Internal Standard kit (using 18S/"competimer" ratio of 1/9) which was amplified simultaneously with 5 or 20 ng of RNA equivalents as described above. PCR products were separated in a 10% polyacrylamide minigel in 1× Tris–Borate–EDTA buffer (2 h/100 V). Fixed gels were dried before overnight exposition with a storage Phosphor screen (Molecular Dynamics, Amersham Pharmacia Biotech) and intensity was measured by PhosphorImager (Molecular Dynamics), then quantified with the ImageQuant software (Molecular Dynamics). Amounts of each specific product were expressed as a percent of internal standard.

2.7. Statistical analysis

Means ± S.E.M. are given for indicated sample numbers (each sample representing a pool of three animals). Statistical comparisons between controls and treated groups were done using unpaired Student's *t*-test. Differences were considered significant at $P < 0.05$. When appropriate, ANOVA analysis was also used, giving the same levels of significance.

3. Results

We first optimized the semi-quantitative multiplex method. For the primers, annealing temperatures between 52 and 60 °C were tested and the highest usable (60 °C) chosen. Individual primer concentrations required adjustment, as each target is expressed at different levels, a fact that induces variable competition for the amplification. Fig. 1A shows an example of such competition. When no TRβ_1 primers are present, TRα_1 amplifies well, whereas when TRβ_1 primers are introduced, TRα_1 amplification is less efficient. We determined optimal concentrations for each primer set to achieve equivalent yields of all PCR products and similar levels of amplification as when the primers were amplified individually. These conditions were

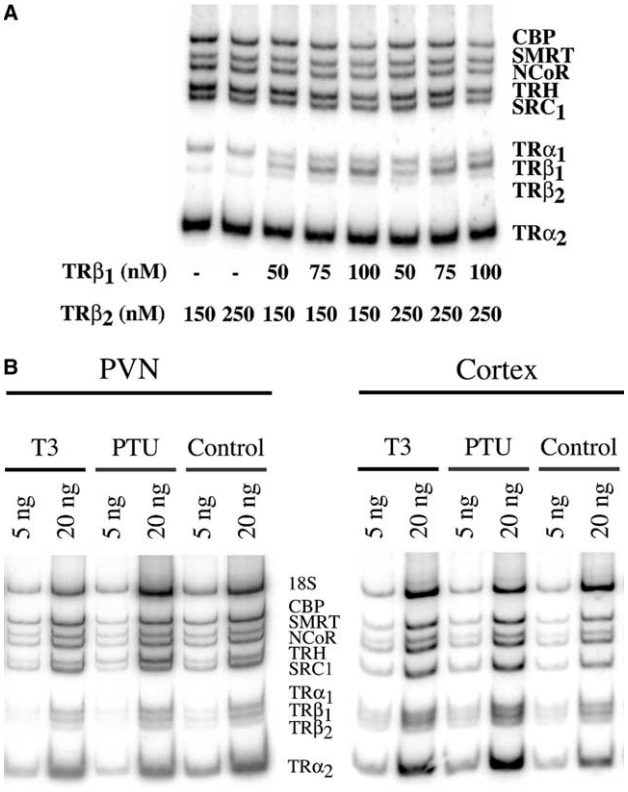

Fig. 1. Semi-quantitative multiplex PCR optimization and application to RNA from PVN and cortex. (A) 20 ng RNA equivalents were amplified as described in Section 2. Primer concentrations were fixed (see Table 1), except for TRβ_1 and TRβ_2 primers, which were varied as indicated. Optimal conditions were found to be 50 nM of TRβ_1 and 250 nM of TRβ_2. (B) Nine targets were simultaneously amplified with an internal control (18S) from 5 to 20 ng of RNA equivalents from PVN or cortex, with [α-^{32}P]dCTP for PhosphorImager semi-quantitation. Individual gene expression was compared across control and T_3-treated (T3) or PTU-treated (PTU) animals. Note that amplified product increases with the amount of RNA used, confirming PCR linearity.

critical for subsequent semi-quantification. We confirmed reaction linearity using 3 concentrations of RNA equivalents (5, 20 and 100 ng), using 10 cycles more than the number of cycles used in the final condition (26 cycles). We obtained dose-dependent amplifications (data not shown).

This optimized semi-quantitative multiplex RT-PCR method was used to compare, in two brain areas, the expression of several genes (*TRH*, four TRs isoforms and four co-modulators)

potentially implicated in *TRH* regulation. For relative quantification of targets, 18S rRNA was amplified as an internal standard. Target signals showed linear dependence on cDNA quantity (5 or 20 ng of RNA equivalents) introduced in the reaction (Fig. 1B). Thus, amplification was concentration-dependent ensuring that changes observed in the ratio of a given gene PCR product to 18S PCR product truly reflected a change in RNA abundance. The tissue specificity and physiological relevance of the system was confirmed by the observation that the 45% increase in *TRH* gene expression in the PVN of hypothyroid animals was in the same order of magnitude as previously reported [1], and that *TRH* expression was absent from the cortex of all groups (Fig. 1B). By simplex PCR (data not shown), we verified that this gene was not expressed in the cortex, confirming that the multiplex system was not responsible for non-amplification.

To study effects of hypo- and hyperthyroidism on gene expression, relative quantification was done, starting with two genes that are neither functional TRs nor co-modulator protein: *TRH* and $TR\alpha_2$. Hypothyroidism (6-*n*-propyl-2-thiouracil (PTU) treatment) significantly increased *TRH* expression in the PVN (+44%, $P < 0.0001$), whereas hyperthyroidism (T_3 treatment) decreased to a lesser, but significant, extent (−12%, $P < 0.05$) the expression of *TRH* in the same brain region (Fig. 2A). *TRH* was not expressed in the cortex (Fig. 1B), as expected. In hypothyroidism, $TR\alpha_2$ expression was significantly increased in both brain areas, whereas hyperthyroidism had no effect on $TR\alpha_2$ expression in either region (Fig. 2A and B).

We next quantified the expression of functional TR isoforms ($TR\alpha_1$, $TR\beta_1$ and $TR\beta_2$) in the PVN (Fig. 3A) and in the cortex (Fig. 3B). Clear, tissue specific, differences in the effects of thyroid status were seen. Hypothyroidism strongly increased the relative levels of both $TR\beta_1$ (+39%, $P < 0.001$) and $TR\beta_2$ (+63%, $P < 0.0001$) mRNAs in the PVN but not in the cortex (compare Fig. 3A and B), whereas no significant effect of hyperthyroidism or hypothyroidism versus controls was observed on $TR\alpha_1$ expression in the PVN. However, ANOVA applied to the $TR\alpha_1$ data showed the differences between hypothyroid and hyperthyroid samples to be significant ($P < 0.01$). In contrast, hyperthyroidism caused an extremely significant decrease of $TR\alpha_1$ gene expression in the cortex (−36%, $P < 0.0001$) but not in the PVN, where the decrease is not significant compared to

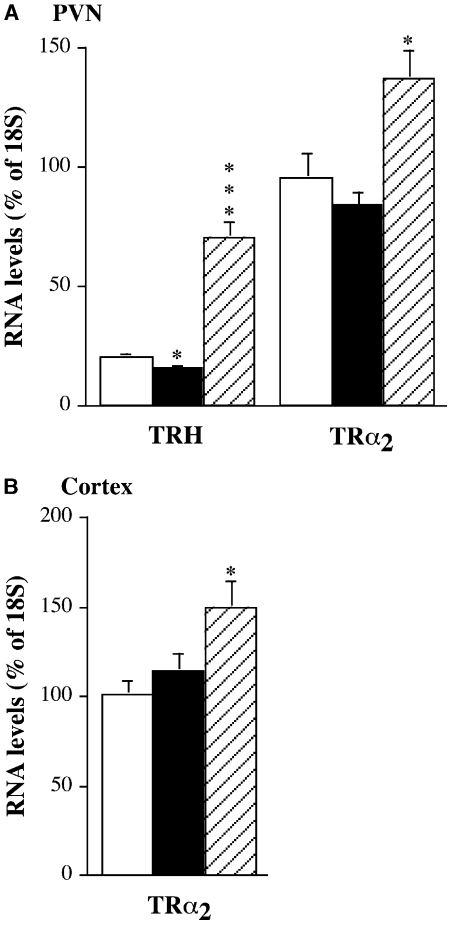

Fig. 2. Tissue specific regulation of TRH and $TR\alpha_2$ expression by thyroid status in PVN and cortex: following multiplex PCR, the amounts of TRH and $TR\alpha_2$ RNA are quantified and expressed as a percent of the internal standard. In this and the following figures, values are given as means ± S.E.M., $n = 8$–14. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ compared to control group. Control (□), T3-treated (■), PTU-treated (▨).

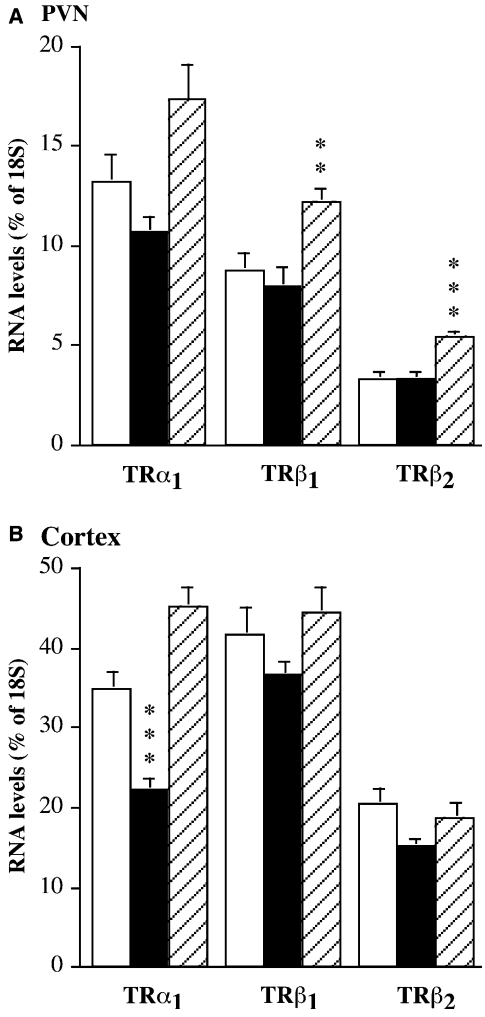

Fig. 3. Tissue specific regulation of $TR\alpha_1$, $TR\beta_1$ and $TR\beta_2$ expression by thyroid status in PVN and cortex: following multiplex PCR, the amounts of $TR\alpha_1$, $TR\beta_1$ and $TR\beta_2$ RNA are quantified and expressed as a percent of the internal standard. Control (□), T3-treated (■), PTU-treated (▨).

the control group. Hyperthyroidism had no significant effect on the expression of $TR\beta_1$ and $TR\beta_2$ isoforms neither in the PVN nor in the cortex.

Similarly, clear-cut tissue specificity was seen when examining the effects of thyroid status on co-modulator expression (Fig. 4). In the PVN, hypothyroidism increased the expression of both co-activators, *CREB-Binding Protein* (*CBP*) and *Steroid Receptor-Coactivator 1* (SRC_1) (+27%, $P < 0.001$ and +47%, $P < 0.0001$, respectively), and of both two co-repressors examined: *Silencing Mediator for Retinoid and Thyroid hormone receptor* (*SMRT*) and *Nuclear Receptor Co-Repressor* (*NCoR*) (+93% and +88%, respectively, $P < 0.0001$) (Fig. 4A). In contrast, in the cortex, the effect of hypothyroidism on co-modulator expression was limited to a slight (+28%), but significant ($P < 0.05$), increase in *SMRT* levels (Fig. 4B). The effects of hyperthyroidism were less marked in both the PVN and the cortex. In the PVN, only *CBP* levels were significantly repressed by T_3 treatment (−26%, $P < 0.0001$) (Fig. 4A), whereas in the cortex a small, but significant reduction in *SMRT* levels (−16%, $P < 0.05$) was observed (Fig. 4B).

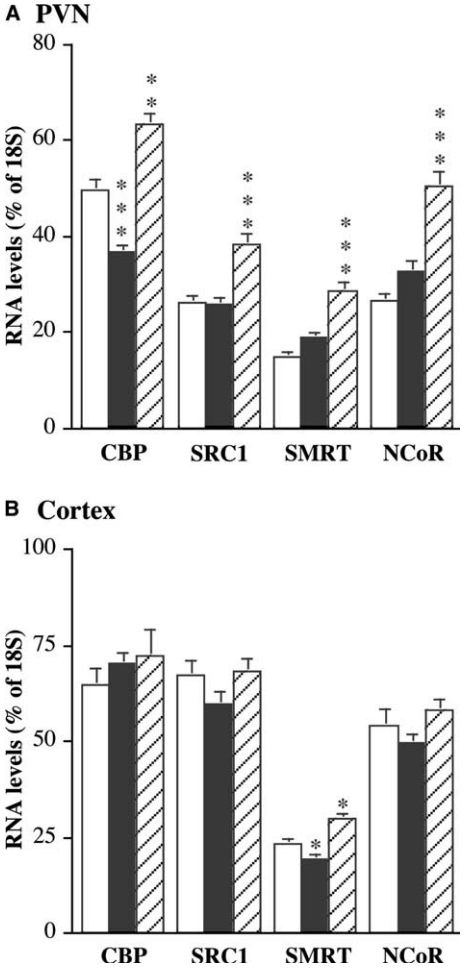

Fig. 4. Tissue specific regulation of the co-modulator CBP, SRC_1, SMRT and NCoR expression by thyroid status in PVN and cortex: following multiplex PCR, the amounts of CBP, SRC_1, SMRT and NCoR RNA are quantified and expressed as a percent of the amount of the internal standard. Control (□), T3-treated (■), PTU-treated (▨).

4. Discussion

We compared thyroid status effects on the expression of *TRH* itself, four *TR* isoforms ($TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$ and $TR\beta_2$) and four co-modulators potentially involved in *TRH* transcription (two co-activators, *CBP* and SRC_1 and two co-repressors, *NCoR* and *SMRT*). Our working hypothesis was that genes encoding proteins involved in transcriptional control of *TRH* might undergo coordinated, tissue-specific regulation by physiological signals, in a manner similar to that reported for coordinated developmental expression of genes involved in the given pathways [8]. First, we analyzed if these genes showed co-regulation under physiological variations, i.e., hypo- or hyperthyroidism. Second, we studied if this regulation was specific to the site of regulated *TRH* transcription, i.e., the PVN [1,2].

To this end, we set up and optimized a semi-quantitative multiplex RT-PCR method. This approach was dictated by the very low quantities of material available (each mouse PVN weighs <1 mg).

We found first significant physiological co-regulations of multiple genes in a restricted brain area and second, that PTU treatment induces larger regulatory responses than T_3 treatment. This latter finding suggests that the brain is more protected against hyperthyroidism than hypothyroidism. One of the eventual explanations is that despite the rapid entry of T_3 into the brain [9,10], hyperthyroidism would be actively buffered by inactivating deiodinases, in particular, Type 3 iodothyronine deiodinase (D3), the major T_3- and T_4-inactivating enzyme (for review, see [11]) levels of which increase in hyperthyroidism throughout the CNS.

4.1. Hypothyroidism increases TRβ but not $TR\alpha_1$ expression in the PVN

In the PVN, both TRβ mRNA isoforms were significantly increased by hypothyroidism, whereas for $TR\alpha_1$ mRNA, the regulation was not significant compared to the control group. Of the three main functional isoforms ($TR\alpha_1$, $TR\beta_1$ and $TR\beta_2$), $TR\alpha_1$ is most ubiquitously expressed [12], whilst $TR\beta_1$ has a more restricted yet still relatively wide distribution, showing high expression in the CNS and liver. $TR\beta_2$ expression is tightly restricted to the CNS, being strongly expressed in the pituitary [13] and the PVN [14]. Recently, an in vivo study with knock-out mice lacking the $TR\beta_2$ isoform [15] showed that the T_3-dependent repression of TRH in the PVN was completely abolished in $TR\beta_2$ null-mice, suggesting that this isoform plays a critical role in the mediation of T_3 negative feedback on the hypothalamic–pituitary–thyroid axis. Moreover, Dupre et al. [5] show that in new-born $TR\beta^{-/-}$ mice, TRH regulation is abolished, and is rescued by either $TR\beta_1$ or $TR\beta_2$ overexpression but not $TR\alpha_1$. The result reported here, showing specific regulation of the $TR\beta$ isoforms, consolidates the idea that $TR\beta_1$ and $TR\beta_2$ are the key isoforms involved in *TRH* regulation in the hypothalamus ([5,15–19]). Moreover, it is important to note that the regulation of TRβ mRNA is strictly tissue specific, and occurs only in the PVN, the brain region involved in the hypothalamic–pituitary–thyroid axis regulation, but not in the cortex.

As to $TR\alpha_1$, which according to most reports [5,16,17] is apparently not involved in the negative regulation of TRH, we find that its mRNA is not significantly modified by hypothyroidism compared to control in the PVN, but is strongly

repressed by hyperthyroidism in the cortex, bolstering the idea that this isoform may play a more general role on the control of the brain homeostasis and brain activity.

4.2. Brain region specific regulation of nuclear receptor co-modulators by hypothyroidism

Hypothyroidism increases the expression of all the co-modulators studied in the PVN. At first sight, it seems striking that co-repressors and co-activators are regulated in a similar manner, despite the fact that they can have opposite effects on transcription of many positively regulated genes. However, here we are dealing with a brain region where *TRH* gene is negatively regulated, and the roles and relative contributions of co-modulators in this regulation have yet to be elucidated.

Moreover, several studies have suggested that co-repressors and co-activators may reside in the same complex. For instance, Li et al. [20] have shown that NCoR and SMRT interact with the co-activator ACTR (Activator of Thyroid Receptor) and that interestingly, this interaction, at least for NCoR, enhanced TRβ-mediated activation, with the presence of NCoR raising the local concentration of co-activator at the target gene promoter. Likewise, NCoR and CBP have been shown to bind simultaneously to the homeobox heterodimer pbx-hox [21]. Thus, our findings of a co-regulation of co-activators and co-repressors are consistent with this hypothesis of their co-existence and possible interaction within a single-unit.

4.3. Exploitation of semi-quantitative multiplex RT-PCR

To our knowledge, it is the first time that semi-quantitative multiplex RT-PCR has been used for simultaneously following nine different target RNAs. Ideally, this method should be adapted to real time PCR to achieve better quantification. However, even using the most recent real-time PCR apparatus available, one cannot actually analyze more than three, sometimes four, targets (including the internal standard) in the same tube. Here, we were working on RNA extracted from the PVN of adult mice. The two PVN from a single brain weigh <1 mg and so the PVNs from three brains were pooled to ensure extraction of sufficient total RNA (about 5 μg) of good quality. Thus, we needed to analyze several genes simultaneously on limited starting material, which also excluded the use of cDNA microarrays, which would have required approximately 400 PVNs per condition tested. What is more, DNA microarray methodology is rather with insensitive a twofold difference between groups being necessary to be considered relevant. The multiplex approach revealed variations of at least 30–40%, which is more than relevant for physiological effects, especially as we are dealing with regulatory proteins.

The observed tissue specific co-regulation of a set of genes within the hypothalamus raises the question of the mechanisms involved. Clearly, one might expect conserved regulatory sequences such as TREs in the promoter regions of the target gene concerned. An in silico approach coupled with a functional assay to test putative conserved sequences would be the ideal method to verify such a hypothesis.

In conclusion, this is the first demonstration of brain region specific co-regulation by thyroid status of a given gene set. This co-regulation of genes by thyroid status within a defined area of the hypothalamus underscores the physiological requirement for coordinated gene expression that should facilitate the integrative functions of this brain region.

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