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Family members CREB and CREM Control Thyrotropin-Releasing Hormone (TRH) Expression in the Hypothalamus

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Abstract

Thyrotropin-releasing hormone (TRH) in the paraventricular nucleus (PVN) of the hypothalamus is regulated by thyroid hormone (TH). cAMP response element binding protein (CREB) has also been postulated to regulate TRH expression but its interaction with TH signaling *in vivo* is not known. To evaluate the role of CREB in TRH regulation *in vivo*, we deleted CREB from PVN neurons to generate the *CREB1^{ΔSIM1}* mouse. As previously shown, loss of CREB was compensated for by an up-regulation of CREM in euthyroid *CREB1^{ΔSIM1}* mice but TSH, T₄ and T₃ levels were normal, even though *TRH* mRNA levels were elevated. Interestingly, *TRH* mRNA expression was also increased in the PVN of *CREB1^{ΔSIM1}* mice in the hypothyroid state but became normal when made hyperthyroid. Importantly, CREM levels were similar in *CREB1^{ΔSIM1}* mice regardless of thyroid status, demonstrating that the regulation of TRH by T₃ *in vivo* likely occurs independently of the CREB/CREM family.

Keywords

TRH expression; CREB; CREM; hypothalamic-pituitary thyroid axis; thyroid hormone

1. Introduction

Thyrotropin-releasing hormone (TRH) is a three amino acid (pyro-Glu-His-Pro-NH₂) peptide formed from the cleavage of prepro-TRH (Sato et al., 1992). *Prepro-TRH* mRNA is expressed in the central nervous system, the heart, the testis (Wilber and Xu, 1998) and also the pancreas and the gut (Morley, 1979). TRH has many different potential functions including the regulation of food intake (Diano et al., 1998, Fekete and Lechan, 2006, Fekete et al., 2002), adaptation to cold exposure (Ishikawa et al., 1984, Jaimes-Hoy et al., 2008,

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Conflict of Interest. The authors declare that they have no conflict of interest

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Sanchez et al., 2001, Uribe et al., 2011, Zoeller and Rudeen, 1992, Zoeller et al., 1995) and the regulation of core body temperature and energy expenditure (Lechan and Fekete, 2006, Nillni, 2010). However, the principal function of TRH is to regulate the set point of the hypothalamic-pituitary thyroid (HPT) axis. Indeed, loss of TRH function in mice or humans leads to central hypothyroidism (Bonomi et al., 2009, Carlson and Hershman, 1975, Rabeler et al., 2004, Winter and Signorino, 2001, Yamada et al., 1997).

TRH is synthesized and secreted by the paraventricular nucleus (PVN) of the hypothalamus, released at the median eminence (ME) and prompts secretion of thyroid-stimulating hormone (TSH) by the pituitary. In turn, TSH promotes the synthesis and release of the thyroid hormones (TH) T₃ and T₄ from the thyroid gland. Furthermore, negative feedback by T₃ represses TRH production (Carlson and Hershman, 1975, Dupont et al., 1976, Dyess et al., 1988, Grimm and Reichlin, 1973, Reichlin, 1975, Wenzel et al., 1975, Yamada and Wilber, 1990). While many regions of the brain express the TRH gene, TH regulates TRH production only in the PVN (Kakucska et al., 1992, Segerson et al., 1987). TRH neurons in the PVN also play an important role in the control of energy homeostasis by regulating TH production during nutritional deprivation (Fekete and Lechan, 2006, Fekete et al., 2002, Ishikawa et al., 1984, Jaimes-Hoy et al., 2008, Lechan and Fekete, 2006, Sanchez et al., 2001, Tuomisto et al., 1975, Uribe et al., 2011, Uribe et al., 2009, Zoeller and Rudeen, 1992, Zoeller et al., 1995). Indeed, TRH neurons receive input from other nuclei in the brain such as the arcuate nucleus that contains proopiomelanocortin (POMC) neurons and agouti related peptide (AgRP)/neuropeptide Y (NPY) neurons (Fekete et al., 2000, Harris et al., 2001, Perello et al., 2006, Toni et al., 1990, Vella et al., 2011), catecholaminergic neurons in the brainstem (Diano et al., 1998, Fuzesi et al., 2009, Sawchenko et al., 1985, Shioda et al., 1986, Tapia-Arancibia et al., 1985, Wittmann, 2008) and the neurons located in the dorsomedial nucleus of the hypothalamus (Lechan and Fekete, 2006, Mihaly et al., 2001, ter Horst and Luiten, 1986, Ulrich-Lai et al., 2011, Wittmann, 2008).

The production of TRH in the PVN is regulated at the level of transcription and through a number of post-transcriptional mechanisms. The TRH promoter contains multiple regulatory elements that recruit different transcription factors depending on the stimulus. During embryonic development, Krüppel-like factor 4 is important in controlling the biosynthesis of TRH in the hypothalamus, but this regulation disappears in the newborn (Perez-Monter et al., 2011). Negative feedback by T₃ is mediated in the PVN by the thyroid hormone receptor β 2 (TR β 2) isoform. The T₃/TR β 2 complex can bind to the TRH promoter via a proximal site termed site 4 (Hollenberg et al., 1995, Iwasaki et al., 1996, Satoh et al., 1996). It has been shown also that TRH promoter contains response elements for the glucocorticoid receptor (Cote-Velez et al., 2005, Diaz-Gallardo et al., 2010), STAT3 (Harris et al., 2001), c-Jun (Cote-Velez et al., 2005, Diaz-Gallardo et al., 2010), c-Fos (Diaz-Gallardo et al., 2010), Sp-1 (Diaz-Gallardo et al., 2010, Ren et al., 1998) and importantly cAMP response element binding protein (CREB) (Cote-Velez et al., 2011, Diaz-Gallardo et al., 2010, Harris et al., 2001, Hashimoto et al., 2000, Perez-Martinez et al., 1998, Wilber and Xu, 1998). Indeed, while CREB can bind to Site 4 it is likely that the principal CREB binding site is further upstream at -101/-94 (CRE2) in the TRH promoter (Cote-Velez et al., 2011, Diaz-Gallardo et al., 2010).

CREB1 has been postulated to play a key role in the regulation of TRH production, as it is a downstream target of both MC4R and NPY signaling in the PVN, which are pathways that play critical roles in the regulation of the thyroid axis by fasting. Furthermore, CREB1 may be the key to the activation of *TRH* mRNA expression in hypothyroidism but this has never been tested *in vivo*. CREB-signaling is complicated by the fact that a number of related transcription factors including cAMP response element modulator (CREM) and activating transcription factor 1 (ATF1) are also phosphorylated by PKA and can compensate for

CREB1 in its absence. Indeed, CREM is known to be up-regulated when CREB1 is deleted (Chiappini et al., 2011, Mantamadiotis et al., 2006, Mantamadiotis et al., 2002, Parlato et al., 2006) but it is also likely that both CREB1 and CREM have overlapping yet distinct roles in regulating target gene expression (Blendy et al., 1996, Mantamadiotis et al., 2002, Parlato et al., 2007, Parlato et al., 2006, Ruppert et al., 1992).

To test *in vivo* the role of CREB and CREM in the regulation of TRH expression in the PVN, we created a mouse model lacking CREB1 specifically in whole PVN including the hypophysiotropic TRH-neurons using *SIM1^{Cre}* mice. We recently showed that deletion of CREB1 in the PVN surprisingly induces an increase in *TRH* mRNA expression possibly due to the up-regulation of CREM (Chiappini et al., 2011). To better understand the role of CREB1 and CREM in the regulation of TRH and the HPT axis we further studied mice that lack CREB1 in the PVN by inducing both hypo and hyperthyroidism. Our data show that the absence of CREB1 in the PVN does not prevent the increase in *TRH* mRNA expression in hypothyroidism or its repression in hyperthyroidism in *CREB1^{ΔSIM1}* mice. While CREM is up-regulated in *CREB1^{ΔSIM1}* mice, its levels are similar in all thyroid states. Thus the TR must also play a critical role in the up-regulation of TRH in hypothyroidism and its repression in hyperthyroidism and the regulation of TRH by T₃ *in vivo* likely occurs independently of the CREB/CREM family.

2. Material and Methods

2.1. Animal Experiments

To generate specific CREB1 knockout (*CREB1^{ΔSIM1}*) animals, mice harboring the *CREB1^{loxP/+}* allele (a gift of Dr. G. Schutz) were crossed with *SIM1^{Cre}* transgenic mice that express *Cre-recombinase* in the PVN (Balthasar et al., 2005, Chiappini et al., 2011, Mantamadiotis et al., 2006, Mantamadiotis et al., 2002). The *CREB1^{loxP/loxP} × SIM1^{Cre}* mice obtained from this first breeding were crossed with the *CREB1^{loxP/loxP}* mice to obtain *CREB1^{loxP/loxP}* control littermates and *CREB1^{ΔSIM1}* mice. Mice were fed a chow or propylthiouracil (PTU/low iodine diet, Harlan Teklad) diet for 3 weeks and injected with saline (0.9%) or 2 μg/100g body weight of T₃ (triiodo-L-thyronine, Sigma) intraperitoneally for 3 days. All of the experiments were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

2.2. Immunohistochemistry

Brains were cut in 30 μm coronal sections. Sections containing the PVN were from bregma -0.58 to -1.22 mm. Immunohistochemistry methods have been previously described (Chiappini et al., 2011). Polyclonal anti-CREM antibody (1:50, Santa Cruz Biotechnology) recognizing most of the CREM isoforms including α and τ isoforms and polyclonal anti-CREB (1:100, Cell Signaling) were used followed by anti-rabbit IgG secondary antibody. Diaminobenzidine (DAB) kits were used (Vector Laboratories, Inc.) for detection. Immunohistochemistry analysis and quantification methods have been previously established (Chiappini et al., 2011, Itoi et al., 2004, Kiss et al., 1984, Kovacs and Sawchenko, 1996).

2.3. Thyrotropin Releasing Hormone (*TRH*) *In Situ* Hybridization

To determine the expression of *TRH* mRNA in the PVN, *in situ* hybridization was performed as previously described (Chiappini et al., 2011). Slides were counterstained with thionin to be able to match, compare and quantify the slices between the different conditions.

2.4. Quantification of the Images

The images were quantified using ImageJ (Public Domain, Developed at the National Institute of Mental Health, Bethesda, MD). The same threshold was used for comparison sets. The total number of positive pixels per unit area (pixel density) was calculated subtracting background from areas not expressing *TRH* mRNA. Pixel density in each region of the hypothalamus was averaged from a minimum of three matching sections of the PVN.

2.5. Real Time Quantitative (Q)-PCR

Total mRNA from male and female mice on a chow and PTU diet was extracted from the pituitary gland using RNA-STAT-60 according to the manufacturer's protocol and quantified with the NanoDrop®-ND1000 (Thermo Scientific). cDNA was obtained by using the Advantage® RT-for-PCR kit (Clontech Laboratories Inc.). To quantify *TRα*, *TRβ1*, *TRβ2*, *TSHβ*, *DIO2* and *PRL* gene expression, TaqMan® Assays were purchased from Applied Biosystems (Mm00617505_m1, Mm01316711_m1, Mn00437042_m1, Mm00437190_m1, Mm00515664_m1 and Mm00599949_m1). *Cyclophilin* was used as a control. Q-PCR was run using the Stratagene® MX3000P® (Agilent Technologies). The gene to *cyclophilin* ratio was calculated for each sample based on the arbitrary value of copies determined by the standard curve for each gene.

Paraventricular nucleus (PVN), median eminence (ME) and cortex were microdissected, mRNA extracted, transcribed into cDNA (see above) and *proconvertase 1 (PC1)* and *PC2* mRNA expression were determined by RT-Q-PCR using Applied Biosystems Sybercore® kit procedure and the following PC1, PC2 and Actin primers:

PC1:

Forward: 5'-GCA TCT GGT TGT CTG GAC CT-3';

Reverse: 5'-CCA GAG CTT TGG CAT TTA GC-3',

PC2

Forward: 5'-CTG ACT GTG CTC ACC TCC AA-3';

Reverse: 5'-CAA GGA CTC CGT AGC CAA AG-3')

These were performed on ABI 7500 Fast System SDS Software version 1.3.1 (Applied Biosystems), as published previously (Perello et al.).

2.6. TRH protein expression

PVN and ME samples were micro-dissected from mice brains using a rodent brain matrix for adult mouse maintained at 4°C on ice (ASI Instruments) and the mouse brain stereotaxic atlas (Franklin and Paxinos, 2007, Paxinos and Franklin, 2001) for the coordinates. PVN and ME samples were collected in 20 µL peptide extraction buffer (Sigma Protease Inhibitor-P8340-use 0.167ml/100ml 2N acetic acid) and denatured 15 min at 95°C. PVN and ME extracts were then subjected to SDS-PAGE followed by slicing and RIA to quantify TRH peptides as described previously (Nillni et al., 2001, Nillni et al., 1995) and total protein content was measured using the Bradford protocol.

2.7. Cell Culture and Transient Transfection

293T cells were transfected in six-well plates with 10 ng of a CMV-β-galactosidase expression vector and 50 ng of TRH promoter plasmid (pTRH-900) containing -900 to +55 of the TRH promoter upstream of the luciferase gene, which has been described previously (Harris et al., 2001, Hollenberg et al., 1995) or the empty luciferase vector pA3LUC to determine the response to cAMP (data not shown). To determine the response to CREB and

CREM, 293T cells were transfected with the above reporters and 50 ng of either pKCR2-CREB (Harris et al., 2001, Selkirk et al., 2006), pKCR2-CREMa or pCDNA3.1-CREMt2 (CREM plasmids are a generous gift from Dr P. Sassone-Corsi) as indicated. pTRH-900 was also co-transfected with pKCR2 and pCDNA3.1 alone (data not shown). All transfections were performed with Lipofectamine™ 2000 (Invitrogen) in serum-free OptiMem media (Invitrogen) as recommended by the manufacturer. Sixteen hours after transfection, the cells were treated with or without 250 μ M 8-Br-cAMP (Sigma) or 122 μ M forskolin (Invitrogen) for 8 hours. The cells were lysed and assayed for luciferase and β -galactosidase activity respectively. All transfections were performed in triplicate.

2.8. Statistical Methods

All data are presented as mean $\pm$ s.e.m. To test for differences between groups of mice an ANOVA-test was run followed by Bonferroni-Dunn *post-hoc* analysis. Data sets were analyzed using a two-tailed unpaired Student's *t*-test. Type one error α was set at 5% for all of our statistical analysis.

3. Results

3.1. *TRH* mRNA expression is significantly increased in *CREB1^{ΔSIM1}* mice but *TRH* protein expression is not altered

To determine the role of CREB1 in the regulation of *TRH* mRNA expression we generated new cohorts of *CREB1^{ΔSIM1}* mice which lack CREB1 in the PVN. As we demonstrated previously, the deletion of CREB1 in the PVN leads to an up-regulation of *TRH* mRNA expression in *CREB1^{ΔSIM1}* mice compared to control animals (Chiappini et al., 2011) (Fig. 1A). Despite this increase in *TRH* mRNA expression, *TRH* peptide levels were similar in *CREB1^{ΔSIM1}* mice and controls in both the PVN and the ME (Fig. 1B). To understand why *TRH* peptide levels were not increased despite increased mRNA expression we examined the expression of PC 1 and PC2 which process prepro-*TRH* to mature *TRH* (Hollenberg, 2008, Nillni, 2010). As shown in Figure 1C, expression of *PC1* and *PC2* mRNA in the PVN and ME of control and *CREB1^{ΔSIM1}* mice was not different (Fig. 1C). In this cohort we also examined *T₄* levels and found no difference between *CREB1^{ΔSIM1}* mice and controls of both males and females (Fig. 1D). We also confirmed in this new cohort of mice by immunohistochemistry that we deleted CREB1 in the hypothalamus where SIM1 is expressed (Chiappini et al., 2011) including the PVN, the supraoptic nucleus (SON) and the medial amygdala (MeAD) (Supplemental Fig. 1).

We next asked if CREB1 plays a role in the response of *TRH* to changes in TH levels. To do this we placed groups of control and *CREB1^{ΔSIM1}* mice on: 1) a chow diet; 2) a PTU/low iodine diet for 3 weeks or 3) a PTU/low iodine diet for 3 weeks with *T₃* administered intraperitoneally (I.P.) for the final 3 days. As shown in Fig. 2, hypothyroidism induces a significant 2-fold increase in *TRH* mRNA expression in both control and *CREB1^{ΔSIM1}* mice compared to the euthyroid state. However, *TRH* mRNA expression in hypothyroid *CREB1^{ΔSIM1}* mice was higher by at least 2-fold compared to both hypothyroid control mice and euthyroid *CREB1^{ΔSIM1}* mice. When *CREB1^{ΔSIM1}* and control mice were made hyperthyroid after PTU treatment *TRH* mRNA was repressed to a similar degree in *CREB1^{ΔSIM1}* and control mice (Fig. 2A and 2B).

3.2. Pituitary and thyroid gland function are not altered in *CREB1^{ΔSIM1}* mice

Since *TRH* mRNA levels were altered in the PVN of *CREB1^{ΔSIM1}* mice, we next wanted to determine if this impacted the pituitary or thyroid in *CREB1^{ΔSIM1}* mice. We measured serum levels of TSH, total *T₄* and *T₃* from the cohort of mice previously described in Fig 2. Despite the increased levels of *TRH* mRNA, serum TSH was unaffected in both the

euthyroid and hypothyroid states in control and *CREB1^{ΔSIM1}* mice (Fig. 3A) consistent with similar amounts of TRH reaching the median eminence. Similarly, total T₄ and total T₃ levels were not different (Fig. 3B and 3C). The PTU diet significantly decreased total T₄ and T₃ between the two groups of mice (control and *CREB1^{ΔSIM1}* mice) in both males and females (Fig. 3B and 3C). As expected T₃ administration for 3 days lowered the serum TSH to similar levels in control and *CREB1^{ΔSIM1}* mice (Fig. 3A). TSH levels in PTU mice treated with T₃ are higher than TSH levels of mice on a chow diet because 3 days of T₃ treatment is too short period to normalize TSH levels. Clearly circulating T₃ levels after exogenous injection are increased (Fig. 3C).

Next, we wanted to see if the expression of genes in the pituitary gland that are potentially involved in TSH production or known to respond to TRH were altered in *CREB1^{ΔSIM1}* mice. As shown in Fig. 4 there are no differences in *DIO2*, *TRα*, *TRβ1* and 2, *TSHβ* and *prolactin* mRNA levels between control and *CREB1^{ΔSIM1}* mice of both sexes regardless of thyroid status. These data are consistent with the TSH, total T₄ and T₃ levels observed.

3.3 CREM is significantly increased in absence of CREB1 *in vivo*, and can increase TRH expression *in vitro* but is not changed when the HPT axis is modified

To further understand why *TRH* mRNA expression is increased in the absence of CREB1, we further analyzed the role of the CREB1 family member CREM. CREM has been shown previously to be up-regulated in the absence of CREB1 in the PVN and in a variety of other neurons (Chiappini et al., 2011, Mantamadiotis et al., 2006, Mantamadiotis et al., 2002, Parlato et al., 2006). We thus performed immunohistochemistry for CREM using brain sections from the mice described in Fig. 2 and after normalizing for the background present quantified the expression of CREM. Similar to what we have shown previously (Chiappini et al., 2011), on a chow diet *CREB1^{ΔSIM1}* mice showed a significant increase in CREM protein expression as compared to control mice (Fig. 5A and 5B). Strikingly, CREM protein levels remain high in the PVN of *CREB1^{ΔSIM1}* mice regardless of the TH levels (hypothyroid or hyperthyroid state). Thus, the up-regulation of CREM appears to preserve and potentially enhance the basal expression of *TRH* mRNA in the PVN. However, changes in CREM levels play no role in determining the response of *TRH* mRNA to changing thyroid hormone levels.

Since expression of CREM leads to an increase in *TRH* mRNA expression in mice that lack CREB1 in the PVN, we next asked whether this could be due to enhanced action via the TRH promoter. Thus, we co-transfected a heterologous cell line (293T cells) with a construct containing 900 bp of the TRH promoter fused to luciferase (Hollenberg et al., 1995) and either CREB1 or two isoforms of CREM, CREMα or CREMτ2 that have been shown to increase the transcription of certain genes compared to other CREM isoforms (Kimmins et al., 2004, Ledo et al., 2000, Liu et al., 2005). The cells were then stimulated with either 8-Br-cAMP (250μM) or forskolin (122 μM), both potent activators of the CREB/CREM signaling pathways. Interestingly, CREM isoforms had no effect on basal TRH promoter activity but enhanced the forskolin and 8-Br-cAMP response compared to CREB1 (Fig. 6A and 6B).

4. Discussion

The hypophysiotropic TRH neurons in the PVN have the capacity to integrate a diverse number of signals in order to properly determine the set point of the HPT axis and ultimately determine circulating TH levels. The most potent regulator of *TRH* mRNA expression is T₃. Similarly, pathways important in energy expenditure also target hypophysiotropic TRH neurons as leptin receptors are present on a small proportion of PVN TRH neurons and both the MC4R and NPY receptors that are also present on hypophysiotropic TRH neurons (Vella

et al., 2011). Indeed, CREB1 has been postulated to be a key role in the regulation of TRH production *in vivo* as it is a downstream target of both MC4R and NPY signaling in the PVN and this pathway plays a critical role in the regulation of the thyroid axis by fasting. A role for CREB1 is further supported by the presence of a cAMP response element (CRE) in the proximal TRH promoter. While our earlier work indicated that Site-4, which binds the TR isoforms, can bind CREB1, a second site slightly upstream termed CRE-2 binds CREB with much higher avidity both *in vitro* and in ChIP assays (Cote-Velez et al., 2005, Diaz-Gallardo et al., 2010, Diaz-Gallardo et al., 2010, Harris et al., 2001, Iwasaki et al., 1996, Satoh et al., 1996). In addition, Sp-1/Krüppel-like pathways may also play a role in mediating CREB1 regulation of TRH (Cote-Velez et al., 2011). Thus, a central question has always been whether TRH-regulation of TRH requires CREB or its family members *in vivo*.

To answer this question we used mice that lack CREB1 in all PVN neurons (*CREB1^{ΔSIM}* mice). We have shown previously that despite the absence of CREB1, *CREB1^{ΔSIM}* mice have normal thyroid hormone levels and surprisingly higher levels of *TRH* mRNA in the PVN. Presumably, the compensatory up-regulation of CREM in these mice preserved thyroid function in this model and the up-regulation of *TRH* mRNA expression supports the notion that CREB family members directly regulate TRH gene regulation presumably through CREs present in the TRH promoter (Cote-Velez et al., 2011, Cote-Velez et al., 2005, Diaz-Gallardo et al., 2010, Diaz-Gallardo et al., 2010, Harris et al., 2001, Hollenberg et al., 1995, Iwasaki et al., 1996, Satoh et al., 1996). Importantly, the up-regulation of CREM in *CREB1^{ΔSIM}* mice allows us to now be able to quantify the input of CREB signaling in different thyroid states and determine whether T₃-signaling requires changes in CREB family members or whether it functions independently presumably through the TR isoforms.

As shown herein, CREM levels are remarkably stable in hypothyroid, euthyroid and hyperthyroid *CREB1^{ΔSIM}* mice. Since CREB1 is deleted and ATF1 is not apparently expressed in the rodent brain (Chiappini et al., 2011, Mantamadiotis et al., 2006, Mantamadiotis et al., 2002) the changes in *TRH* mRNA expression seen are independent of changes in the levels of CREB family members. These findings support the notion that T₃-signaling alters the ligand-independent and dependent functions of TR-isoforms to regulate TRH gene expression. However, because the CREB/CREM family recruits a number of co-activators including CBP/p300 and the CRTC's it remains possible that T₃-signaling somehow modifies the transcriptional activity of this pathway (Cardinaux et al., 2000, Carlezon et al., 2005). Further work will be required to determine this but since TRH levels are similarly elevated in both the euthyroid and hypothyroid states in *CREB1^{ΔSIM}* mice when compared to controls it would appear that CREM retains similar transcriptional activity.

Surprisingly, despite the up-regulation of *TRH* mRNA by CREM in the PVN there is no evidence of systemic hyperthyroidism in *CREB1^{ΔSIM}* mice. While this could be developmental as CREB1 is deleted during embryonic development (Alonso et al., 2007, Blendy et al., 1996, Mantamadiotis et al., 2002, Moeller et al., 2007, Moura et al., 2008, Parlato et al., 2007, Parlato et al., 2006, Ruppert et al., 1992) this is not likely to be the case. Indeed, in another mouse model where *TRH* mRNA levels are primarily elevated (Sabio et al., 2010) TH levels are elevated consistent with central hyperthyroidism. However, in *CREB1^{ΔSIM}* mice, TRH peptide levels in the PVN and median eminence are normal implying that despite elevated mRNA levels, post-translational pathways exist to keep peptide levels normal. Indeed, two pathways are described; 1. mature TRH is produced from a prohormone by the prohormone convertases PC1/3 and PC2; and 2. TRH peptide levels are regulated by the peptidase PP11, which is expressed in tanycytes that project to the median eminence (Sanchez et al., 2009, Vella and Hollenberg, 2009). Both of these

pathways are regulated by TH. Interestingly, *PC1* and *PC2* gene expression in the PVN and ME are not up-regulated in *CREB1^{ΔSIM1}* mice, which is consistent with the normal TSH and TH levels in *CREB1^{ΔSIM1}* mice. Thus it remains possible that PPII is mediating the effects seen or that the up-regulation of *TRH* mRNA by CREM is occurring in PVN neurons that do not project to the ME (Chiappini et al., 2011, Duplan et al., 2009, Saper et al., 1976, Sawchenko, 1998, Sawchenko and Swanson, 1982, Sawchenko et al., 1985, Swanson and Kuypers, 1980). However this latter point is unlikely, given the complete deletion of CREB in all PVN neurons.

In conclusion, the absence of CREB1 in the PVN leads to an increase of *TRH* mRNA expression in euthyroid and hypothyroid states. The increase of *TRH* mRNA expression is probably due to an increase of CREM expression that compensates for the absence of CREB1 (FIG. 7). However, the increase in *TRH* mRNA expression does not lead to an increase in TRH protein expression or in pituitary and thyroid function. In addition, since the levels of CREM do not change in *CREB1^{ΔSIM1}* mice (higher levels compared to the control) between euthyroid, hypothyroid and hyperthyroid states, the regulation of TRH by T₃ *in vivo* likely occurs independently of the CREB/CREM family (FIG. 7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Lack of CREB in PVN neurons leads to a significant increase of TRH expression
- Lack of CREB in PVN neurons leads to a significant increase of CREM expression
- Lack of CREB in PVN neurons does not affect regulation of TRH by thyroid hormones
- Increased of CREM *in vitro* regulates TRH expression but not *in vivo*
- regulation of TRH by T3 *in vivo* likely occurs independently of the CREB/CREM family

FIG. 1. TRH mRNA expression is significantly higher in *CREB1^{ΔSIM1}* mice compared to the *CREB1^{lox/lox}* mice but TRH protein level does not change
 (A) Images are from 3 different brains. ISH: *in situ* hybridization. n=5/group. *** p<0.01. The data are represented as mean ± s.e.m. Bar scale = 100 μm. (B) TRH peptide levels in the paraventricular nucleus of the hypothalamus (PVN) and median eminence (ME) in male and female *CREB1^{lox/lox}* and *CREB1^{ΔSIM1}* mice. (C) *Proconvertase 1 (PC1)* and *PC2* gene expression is unchanged in the PVN and ME of *CREB1^{ΔSIM1}* mice. *CREB1^{lox/lox}* n=8; *CREB1^{ΔSIM1}* mice n=7. (D) Total T₄ serum level from the same mice in (B). n=11 males n=5 females *CREB1^{lox/lox}* and n= 18 males and n=17 females *CREB1^{ΔSIM1}* mice on chow diet. The data are presented as mean ± s.e.m.

FIG. 2. *TRH* mRNA expression remains significantly higher in *CREB1*^{ΔSIM1} mice compared to the *CREB1*^{lox/lox} mice on PTU/low iodine diet but not in hyperthyroidism
(A) *TRH* mRNA *in situ* hybridization (ISH) with thionin staining (pictures on the bottom right) in *CREB1*^{ΔSIM1} and *CREB1*^{lox/lox} mice fed a chow diet, PTU/low iodine diet or PTU/low iodine diet injected with T₃ (intraperitoneal injection 2μg/100g body weight for 3 days). The figure shows 2 different brains from each group of mice per conditions. **(B)** Quantification of (A) with ImageJ. n=4/genotype/condition. The data are presented as mean ± s.e.m.

FIG. 3. The pituitary-thyroid axis is not affected in *CREB1^{ΔSIM1}* mice
(A) Thyroid-stimulating hormone (TSH), **(B)** total T₄ and **(C)** total T₃ levels in *CREB1^{ΔSIM1}* and *CREB1^{lox/lox}* mice fed a chow, PTU/low iodine or PTU/low iodine diet injected with T₃ (intraperitoneal injection 2μg/100g body weight for 3 days). n=4/genotype/condition. The data are presented as mean ± s.e.m. *p<0.05 *CREB1^{ΔSIM1}* and *CREB1^{lox/lox}* mice on a PTU/low iodine with or without T₃ are compared to the chow diet group. † p<0.05 *CREB1^{ΔSIM1}* and *CREB1^{lox/lox}* mice on a PTU/low iodine with T₃ are compared to *CREB1^{ΔSIM1}* and *CREB1^{lox/lox}* mice on a PTU/low iodine without T₃.

FIG. 4. No difference in gene expression in pituitary gland from *CREB1^{ΔSIM1}* mice compared to the *CREB1^{lox/lox}* mice on chow and PTU/low iodine diet

(A) Thyroid receptor beta 1 (*TRβ1*), (B) Thyroid receptor beta 2 (*TRβ2*), (C) Thyroid receptor alpha (*TRα*), (E) Thyroid stimulating hormone beta (*TSHβ*), (F) Deiodinase type 2 (*DIO2*), and (G) Prolactin (*PRL*) gene expression relative to *cyclophilin*. n=8 males n=7 females *CREB1^{lox/lox}* and n= 7 males and n=8 females *CREB1^{ΔSIM1}* mice on chow diet; n= 16 males and n=9 females *CREB1^{lox/lox}* and n= 12 males and n=12 females *CREB1^{ΔSIM1}* mice on PTU/low iodine diet. The data are presented as mean $\pm$ s.e.m.

FIG. 5. CREM protein expression is significantly higher in *CREB1^{ΔSIM1}* mice compared to the *CREB1^{lox/lox}* mice on chow and PTU/low iodine diet but not in hyperthyroidism (A) CREM immunohistochemistry in *CREB1^{lox/lox}* and *CREB1^{ΔSIM1}* mice fed a chow diet, PTU/low iodine diet or PTU/low iodine diet injected with T₃ (intraperitoneal injection 2μg/100g body weight for 3 days). The figure shows 2 different brains from each group of mice per condition. (B) Quantification of (A) with ImageJ. n=4/genotype/condition (same mice as shown in Figure 2). The data are represented as mean ± s.e.m. ANOVA-one way was run followed by *t*-test Bonferonni-Dunn *post-hoc* analysis 3v: Third ventricle; PVN: paraventricular nucleus of the hypothalamus. Bar scale = 100 μm.

FIG. 6. CREB1 and CREMα and CREMτ2 can regulate TRH expression

pTRH-900-Luc was co-transfected into 293T cells either with pKCR2-CREB1 or pKCR2-CREMα or pCDNA3.1-CREMτ2 and stimulated with (A) forskolin (122μM) (* $p < 0.05$ when compared their own control without forskolin; † $p < 0.05$ compared to wells transfected with pKCR2-CREB plasmid treated with forskolin) or (B) 8-Br-cAMP (8-Br-cAMP 250 μM) for 8 hours (* $p < 0.05$ when compared their own control without 8-Br-cAMP; † $p < 0.05$ compared to wells transfected with pKCR2-CREB plasmid treated with 8-Br-cAMP). The data are presented as mean $\pm$ s.e.m. Each experiment was performed with triplicate samples and each experiment was repeated 3 times.

FIG. 7. A model of TRH regulation by CREB/CREM and thyroid hormone

TR β 2 is bound to Site-4 either in the presence or absence of T₃ and determines the expression of TRH based on the recruitment of a still to be identified co-activator (CoA) or co-repressor (CoR) complex. In the absence of CREB1, CREM, which requires its phosphorylation at a unique regulatory site Ser117 (Monaco et al., 2004) to be active, regulates TRH expression presumably through the upstream CRE-2 (Cote-Velez et al., 2011). It is still not clear how TR 2 and CREB/CREM family members interact to regulate TRH expression (►).