Thyroid hormone exerts negative feedback on hypothalamic type 4 melanocortin receptor expression

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The type 4 melanocortin receptor MC4R, a key relay in leptin signaling, links central energy control to peripheral reserve status. MC4R activation in different brain areas reduces food intake and increases energy expenditure. Mice lacking *Mc4r* are obese. *Mc4r* is expressed by hypothalamic paraventricular Thyrotropin-releasing hormone (TRH) neurons and increases energy usage through activation of *Trh* and production of the thyroid hormone tri-iodothyronine (T_3). These facts led us to test the hypothesis that energy homeostasis should require negative feedback by T_3 on *Mc4r* expression. Quantitative PCR and in situ hybridization showed hyperthyroidism reduces *Mc4r* mRNA levels in the paraventricular nucleus. Comparative *in silico* analysis of *Mc4r* regulatory regions revealed two evolutionarily conserved potential negative thyroid hormone-response elements (nTREs). In vivo ChIP assays on mouse hypothalamus demonstrated association of thyroid hormone receptors (TRs) with a region spanning one nTRE. Further, in vivo gene reporter assays revealed dose-dependent T_3 repression of transcription from the *Mc4r* promoter in mouse hypothalamus, in parallel with T_3-dependent *Trh* repression. Mutagenesis of the nTREs in the *Mc4r* promoter demonstrated direct regulation by T_3, consolidating the ChIP results. In vivo shRNA knockdown, TR overexpression approaches and use of mutant mice lacking specific TRs showed that both TRα and TRβ contribute to *Mc4r* regulation. T_3 repression of *Mc4r* transcription ensures that the energy-saving effects of T_3 feedback on *Trh* are not overridden by MC4R activation of *Trh*. Thus parallel repression by T_3 on hypothalamic *Mc4r* and *Trh* contributes to energy homeostasis.

energy homeostasis | gene transcription | leptin pathway | ligand-dependent repression | thyrotropin-releasing hormone

Reversing the increasing incidence of obesity requires knowledge of physiological regulations controlling energy homeostasis. Understanding how central genes involved in endocrine and metabolic axes are regulated is crucial to these problems.

Thyroid hormones (THs) regulate metabolism and appetite (1, 2). THs, particularly the biologically active form tri-iodothyronine, T_3, stimulate the basal metabolic rate. Hyperthyroidism leads to increased catabolism and weight loss; hypothyroidism causes weight gain (3). TH levels are kept within physiological ranges through hypothalamo-pituitary neuroendocrine feedback loops. Increased T_3 represses transcription of hypothalamic *Thyrotropin-releasing hormone*, *Trh* (4), the master regulator of the hypothalamo-pituitary-thyroid (HPT) axis (5). In turn, decreased T_3 output reduces metabolism and energy usage (6).

Hypothalamic TRH neurons integrate numerous metabolic, endocrine, and neuronal signals (7). T_3-responsive TRH neurons in the paraventricular nucleus (PVN) express all the functional TH nuclear receptors (TRs) and a key membrane receptor involved in energy homeostasis, namely the type 4 melanocortin receptor (MC4R). In situ hybridization studies show that nearly all TRH neurons in the caudal-medial parvocellular PVN express MC4R (8).

MC4R, a membrane α-melanocyte stimulating hormone (αMSH) receptor, is integral to central leptin/melanocortin signaling (9). Leptin, a major satiety hormone, regulates energy homeostasis through food intake, energy partition, and thermogenesis (9). Centrally, leptin signaling is relayed mainly through the hypothalamic melanocortin system and MC4R-expressing neurons. *Mc4r* expression is widespread in the brain (10, 11), with high levels in the hypothalamus and brainstem, areas involved in energy homeostasis (12). The hypothalamus governs metabolism through complex neuroendocrine regulations. The brainstem also integrates metabolic signaling, notably regulating thermogenesis via the autonomic nervous system (13).

In the PVN, leptin stimulates TRH production by coordinating pathways that culminate in MC4R activation and increased intracellular cAMP levels. A cAMP response binding (CREB)-responsive element in the *Trh* promoter (8) drives *Trh* transcription, stimulating T_3 production and energy expenditure. Further, hypothalamic T_3 stimulates orexigenic pathways (14, 15) and dampens anorexigenic signals (16, 17). Despite these roles, no studies have addressed how brain TH levels contribute to central metabolic control through *Mc4r* expression.

Because MC4R activation stimulates the HPT axis, we examined whether homeostasis involved negative feedback regulation by T_3 on *Mc4r* expression. We found that thyroid status modulated endogenous *Mc4r* mRNA levels in brain areas relevant to metabolism. *In silico* examination of human and mouse *Mc4r* proximal regulatory regions showed two conserved potential negative thyroid hormone responsive element (nTREs). To assess if TRs were associated with nTREs, we used TR antibodies for ChIP on regulatory regions from *Mc4r* and *Trh*. Differential patterns of TR binding to hypothalamic *Mc4r* and *Trh* nTREs were found as a function of thyroid status. Three different in vivo gene transfer (iGT) approaches in wild-type and TR-mutant mice were used to analyze further T_3 regulation of *Mc4r* transcription and to define the roles of different TRs. iGT was used first for reporter studies with control and mutated *Mc4r* promoter constructs in wild-type and $TR\alpha^{o/o}$ and $TR\beta^{-/-}$ mice (18, 19), then to introduce shRNA against TR isoforms, and finally, to overexpress TRs. The results confirmed that T_3 exerts direct regulation on *Mc4r* and that different TR isoforms have distinct transcriptional regulatory roles.

The data provide insight into ligand-dependent repression mechanisms and reveal an integrative function of the hypothal-

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amus in metabolic homeostasis through coordinated, T_3-dependent feedback on hypothalamic *Mc4r* gene, together with that of *Trh*.

Results

Thyroid Status Regulates Endogenous *Mc4r* Gene Expression. MC4R activates PVN *Trh* transcription (8). Thus we hypothesized that, as for *Trh* in the PVN, *Mc4r* could undergo feedback by T_3. To test this possibility, hypo- and hyperthyroidism were induced in 2-month-old mice by 6-*n*-propyl-2-thiouracil (PTU) and thyroxine (T_4) treatment, respectively (Fig. S1*A*). The physiological consequences of modified thyroid status during this late growth phase were confirmed by the differential weight gain of treated mice (Fig. S1*B*). Thyroid effects on endogenous *Mc4r* and *Trh* mRNA expression were assessed by quantitative PCR (qPCR) and normalized with *Gapdh* mRNA (*SI Materials and Methods*). Levels were analyzed in the PVN, where *Mc4r* is expressed in hypophysiotropic TRH neurons (8), and in the arcuate nucleus (ARC) and the brainstem, areas also involved in metabolism. The frontal cortex served as a control because it expresses *Mc4r* but is not linked to energy homeostasis.

In the PVN, hypothyroidism significantly increased both *Mc4r* ($P < 0.01$) and *Trh* ($P < 0.001$) mRNA levels versus levels in euthyroid controls (Fig. 1*A*). However, hyperthyroidism modified neither *Trh* ($P = 0.07$) nor *Mc4r* expression in the PVN within the time frame used (13 days). In situ hybridization confirmed that PTU-induced hypothyroidism strongly increased *Mc4r* mRNA expression in the PVN compared with T_4-treated animals (Fig. 1*B*). In the ARC, hypothyroidism increased *Mc4r* ($P < 0.05$) but, as expected (20), did not modify *Trh* (Fig. S2*A*). Conversely, hyperthyroidism decreased *Mc4r* expression in the ARC ($P < 0.05$), whereas, somewhat surprisingly, it increased *Trh* levels ($P < 0.05$) (Fig. S2*A*). As predicted (17), thyroid status modified *Neuropeptide Y* (*Npy*) *and Pro-opiomelanocortin* (*Pomc*) expression in the ARC (Fig. S2*D*). In the brainstem, hypothyroidism slightly, but significantly, reduced *Mc4r* mRNA, and hyperthyroidism induced a dramatic, almost 10-fold, decrease ($P < 0.001$) (Fig. S2*B*). In the cortex, *Mc4r* was not altered (Fig. S2*C*). As expected (21), neither in brainstem nor in cortex was *Trh* transcription affected by thyroid status (Fig. S2 *B* and *C*).

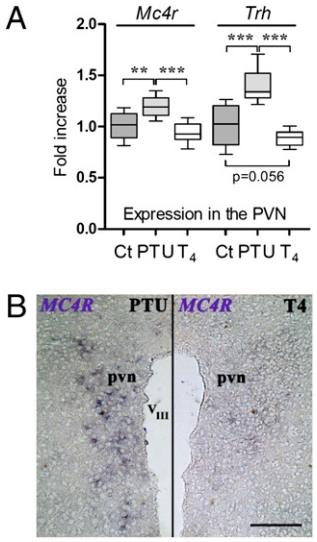

Fig. 1. Thyroid status alters *Mc4r* expression in the hypothalamic PVN. (A) Real-time PCR quantification of *Mc4r* and *Trh* mRNA from PVN of adult male mice treated for 13 days by PTU (hypothyroid) or T_4 (hyperthyroid), or euthyroid controls (Ct). Gene expression was normalized with *Gapdh*. Shown are pooled results of two independent experiments (n = 7 or 8 per group). Nonparametric ANOVA followed by permutation test was used to assess statistical significance. **, $P < 0.01$; ***, $P < 0.001$. (B) In situ hybridization shows a decrease in *Mc4r* mRNA expression in the PVN following T_4 treatment, compared with PTU-treated mice. pvn, paraventricular nuclei; V_{III}, third ventricle. (Scale bar, 1 mm.)

T_3-Dependent *Mc4r* and *Trh* Repression Requires Gene-Specific TR/DNA Interactions. To determine if TH-induced regulatory responses involve direct interactions of TR, ChIP assays were used. Human and mouse *Mc4r* proximal regulatory regions share two putative monomeric TRE half-sites (Fig. S3*A*): TRE1 and TRE2. TRE1, at −92 bp before the transcription start site (TSS) in h*MC4R*, is conserved in mouse, rat, pig, and human (22). TRE2, at +374 bp after the TSS, resembles the nTRE conserved in mouse and human *Trh* (23).

TR binding was analyzed on the most conserved nTREs identified in *Mc4r* (TRE1) and *Trh* (site 4) regulatory regions. ChIP using anti-TRβ antibodies (Upstate Biotechnologies) was carried out on hypothalami from hypothyroid newborn mice. In these conditions, TRβ was absent from the *Mc4r* TRE1 (Fig. 2*A*), whereas TRβ was present on the *Trh* TRE site 4 (Fig. 2*B*). Following T_3 treatment, TRβ was recruited to the *Mc4r* TRE1 region (Fig. 2*A*), but it decreased significantly on the *Trh* regulatory region (Fig. 2*B*). In no case, with or without T_3, was any binding found on the irrelevant control regions of *Trh* or *Mc4r* promoters (Fig. 2 *A Right* and *B Right*). A second set of anti-TRβ antibodies (provided by R. Denver, University of Michigan, Ann Arbor, MI) gave similar TR/DNA binding patterns for both genes (Fig. S4 *A* and *B*).

Given the association of TRs with the nTREs shown by in vivo ChIP assays, the functionality of the putative nTREs in mediating T_3 signaling was tested in a physiological context using iGT (24). Transcription from *Mc4r* reporter plasmids bearing the nTREs driving *Firefly* (f.) or *Renilla* (r.) luciferase (luc) expression (Fig. S2 *E–G*) was followed in hypothalami of euthyroid or hypothyroid pups. Hypothyroidism increased T_3-independent MC4R-luc transcription 2-fold versus euthyroid mice (Fig. S2*E*). T_3 treatment of hypothyroid pups reduced MC4R-luc transcription dose dependently (Fig. 2*C*), with repression reaching more than 65% at the maximal T_3 dose used ($P < 0.001$) or 50% with 3,5,3-triiodothyroacetic acid, a T_3 analogue (Fig. S2*F*). The T_3 repression of MC4R-luc paralleled that of TRH-luc (Fig. S2*G*).

When either the MC4R-luc or TRH-luc was cotransfected with an appropriate control (cytomegalovirus-luciferase, CMV-luc), only the physiologically relevant promoters, and not the CMV promoter, were repressed (Fig. S5 *A* and *B*). Given the parallel regulation of *Mc4r* and *Trh* transcription, we used a cotransfection approach, mixing an *Mc4r* reporter construct coupled to the *Renilla* luciferase sequence (MC4R-r.luc) with TRH-f.luc. In this cotransfection paradigm, T_3 repressed transcription by 30% and 52% for *Mc4r* and *Trh* reporter plasmids, respectively (Fig. S5*C*).

To test the roles of the putative *Mc4r* TREs, site-directed mutations of either or both of the *Mc4r* promoter nTREs was done, producing three constructs (Fig. S3). Mutation of TRE1 (TRE1m) decreased T_3-independent expression ($P < 0.001$) and abolished T_3-dependent repression of *Mc4r* transcription (Fig. 2*D*). In contrast, mutating TRE2 (TRE2m) had no effect (Fig. 2*E*). When both TREs were mutated (DBm), a similar profile to the TRE1m construct was observed (Fig. S4*C*). In all these experiments, the non-mutated *Trh* construct was cotransfected as a positive control for T_3-dependent regulation (Fig. S4*C*).

TRs Exhibit Isoform-Specific Contributions to *Mc4r* Expression. Given that site-directed mutagenesis of *Mc4r* promoter and ChIP analyses argued for direct regulation of *Mc4r* by T_3 and TRs, the roles of TR isoforms were tested using expression vectors encoding TRα1 (Fig. 3*A*), TRβ1 (Fig. 3*B*), or TRβ2 (Fig. 3*C*). TRα1 reduced T_3-independent transcription by 30% ($P < 0.05$). The TRβ isoforms did not modify T_3-independent transcription. However, overexpression of TRβ1 or TRβ2 enhanced the repressive effects of T_3 on *Mc4r* transcription. T_3 repressed *Mc4r-r.luc* transcription by 20% ($P < 0.05$) in the presence of a control empty vector,

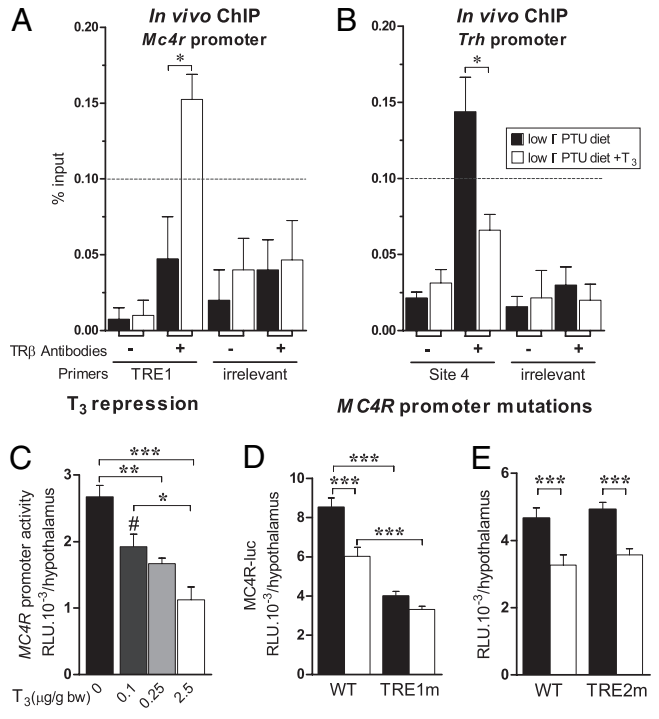

Fig. 2. T_3 directly regulates *Mc4r* expression. (*A* and *B*) PCR quantification of ChIP assays carried out on hypothalami from hypothyroid newborn mice treated with T_3 (2.5 µg/g bw) 20 h before sacrifice. Samples were immunoprecipitated with TRβ-specific antibodies and amplified with either MC4R-TRE1 or TRH site 4 primers or their respective irrelevant control primers. For negative controls, samples were processed through immunoprecipitation without antibody (–, background). Results represent the occupancy of TRβ isoforms at the TRE1 site in the *Mc4r* promoter and at the TRE-site 4 in the *Trh* promoter. Data are presented as percentage of input (starting sonicated DNA used for ChIP). The threshold value for a positive signal was set at 0.1% of input (dashed line). Graphs represent means of four independent experiments. Student's *t* test assessed difference between the groups. (*A*) TRβ isoforms are present at the TRE1 site in *Mc4r* promoter only after T_3 treatment. (*B*) TRβ isoforms are present at the TRE site 4 in the *Trh* promoter in hypothyroid animals but are absent after T_3 treatment. Negative controls included primers spanning irrelevant sequences in both gene sequences (right half of each graph). (*C*) Dose-dependent repression of *Mc4r* promoter activity by T_3 in hypothyroid mice. (*D*) Mutation of the putative TRE1 half-site abrogates T_3-independent *Mc4r* promoter activity and its T_3-dependent repression. (*E*) Mutation of the TRE2 putative half site is without effect. One-day-old pups were transfected in the hypothalamus with 2 µL of a 250 ng/µL solution of PEI-complexed MC4R-f.luc and then were injected s.c. with T_3 [2.5, 0.25, or 0.1 µg/g body weight (bw)] or vehicle (NaCl 0.9%). Luciferase was measured 24 h later. RLU, relative light units. Means ± SEM are shown. One-way ANOVA statistical analysis was followed by Bonferroni's multiple comparisons to assess statistical differences. In T_3 treatment at 2.5 µg/g bw, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.05$ for control group vs. group treated with T_3 at 0.1 µg/g bw.

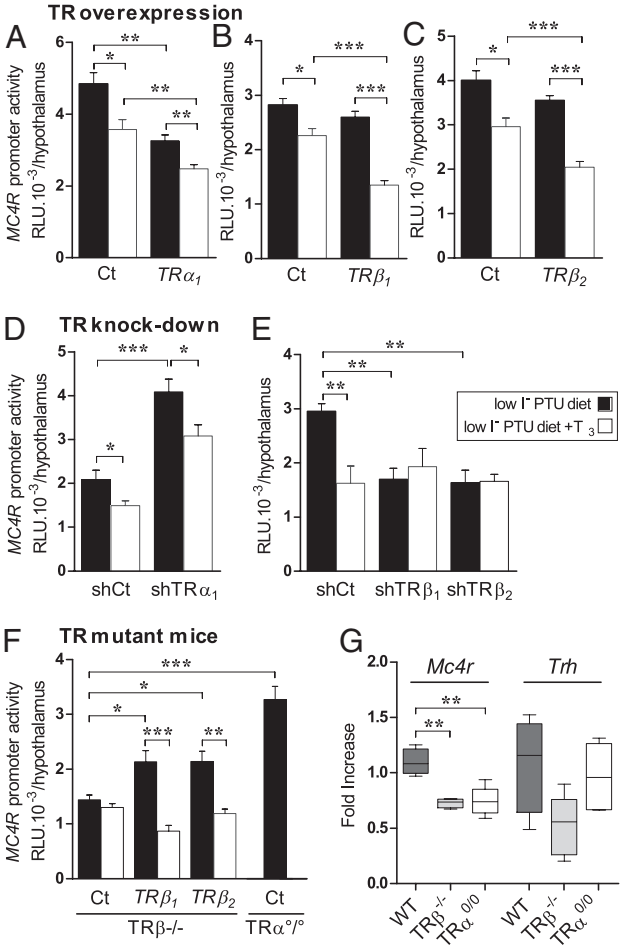

Fig. 3. TRs display isoform-specific roles on *Mc4r* promoter. (*A*) TRα1 overexpression diminishes T_3-independent *Mc4r* promoter activity. (*B* and *C*) TRβ1 or TRβ2 overexpression reinforces T_3-dependent repression of *Mc4r*-promoter activity. Pups were transfected in the hypothalamic region of the brain with 2 µL of a 250 ng/µL solution of MC4R-f.luc with pSG5-TRα1, pSG5-TRβ1, pSG5-TRβ2, or empty pSG5 vector (25 ng/µL). (*D*) shTRα1 activates T_3-independent MC4R-f.luc expression and abrogates T_3-dependent repression. (*E*) shTRβ1 or shTRβ2 represses T_3-independent *Mc4r* promoter activity and abrogates T_3-induced repression. Hypothalamic transfections used MC4R-f.luc (250 ng/µL) and either pCMV-H1-shTRα1, pCMV-H1-shTRβ1, pCMV-H1-shTRβ2, or the empty vector pCMV-H1 (400 ng/pup). (*F*) $TR\alpha^{°/°}$ and $TR\beta^{-/-}$ animals were transfected with MC4R-f.luc with or without TRβ1- or TRβ2-expressing plasmid. T_3 treatment, 2.5 µg/g bw. (*G*) *Mc4r* and *Trh* mRNA expression levels in adult TRβ ($TR\beta^{-/-}$) and TRα ($TR\alpha^{°/°}$) knockout mice. Real-time PCR quantification of *Mc4r* and *Trh* mRNA from the PVN of adult male wild-type (WT), $TR\beta^{-/-}$, or $TR\alpha^{°/°}$ mice. Gene expression was normalized with *Gapdh*. Results are pooled from two independent experiments ($n = 7$ or 8 per group). One-way ANOVA statistical analysis was performed, followed by Bonferroni's multiple comparisons to assess statistical differences.

whereas repression reached 48% with TRβ1 ($P < 0.001$) and 42% with TRβ2 ($P < 0.001$) (Fig. 3 *B* and *C*).

To examine TR-specific effects further, knockdown with plasmids containing the hybrid cytomegalovirus-polymerase III histone H1-RNA promoter (pCMV-H1) expressing shRNAs was applied. Because neither the pCMV-H1 empty vector nor the pCMV-H1 vectors coding for irrelevant shRNA sequences (shGFP) or a scrambled sequence (shSCR) affected TRH-, MC4R- or control CMV-driven transgenes (Figs. S6 and S7), pCMV-H1 was used as a control. Two shRNAs against each TR isoform (shTRs, see Table S2) were designed from published TRβ1 and TRβ2 siRNAs (25).

The shRNA method was validated against the well-documented (24, 26, 27) specificity of TRα1, TRβ1, and TRβ2 isoforms on hypothalamic *Trh* regulation (Fig. S6) and then was applied to the *Mc4r* promoter. ShTRα1 significantly (55%) increased T_3-independent *Mc4r* transcription in hypothyroid newborn mice and maintained T_3-dependent repression (Fig. 3*D*). Conversely, expression of shRNA against TRβ1 or TRβ2 significantly decreased T_3-independent transcription (by 42% and 44%, respectively) (Fig. 3*E*) and abolished T_3 repression. Knockdown of TRβ1 and TRβ2 thus confirmed the lack of differential effect between these two isoforms on *Mc4r* promoter activity seen with overexpression. These results were reproduced using another set of TRβ1 or TRβ2 shRNA (Fig. S7 *B* and *C*).

TR-specific contributions to *Mc4r* regulation also were investigated in mice lacking TRβ or TRα gene products ($TR\beta^{-/-}$ or

TRα$^{o/o}$ mice). In TRβ$^{-/-}$ mice, MC4R-luc was insensitive to T_3 (Fig. 3*F*, *Left*). Overexpression of TRβ1 or TRβ2 restored T_3-dependent *Mc4r* promoter repression and increased T_3-independent *Mc4r* promoter expression ($P < 0.001$ in each case), confirming the roles of both TRβ isoforms seen with overexpression or shTR experiments. Moreover, T_3-independent MC4R-luc expression was increased significantly in TRα$^{o/o}$ pups ($P < 0.001$) compared with TRβ$^{-/-}$ pups (Fig. 3*F*), confirming the repressive role of TRα in T_3-independent *Mc4r* transcription. Analysis of endogenous *Mc4r* levels using qPCR on mutant mice showed that loss of either TR significantly modified *Mc4r* expression in the PVN (Fig. 3*G*) but not in the ARC (Fig. S7*D*).

Discussion

Metabolic homeostasis requires coordination of activity in specific hypothalamic nuclei, notably those governing food intake and energy expenditure. One key actor in these energy-regulating networks is the αMSH receptor MC4R (18). *MC4R* mutations in humans are associated with obesity and ≈5% rate of associated morbidity (28). Mice lacking the *Mc4r* gene are severely obese, and heterozygous mice show intermediate phenotypes, demonstrating a gene–dosage effect for *Mc4r* in energy balance (29). Another key player is T_3, which determines metabolic rate (3). Here, using multiple in vivo approaches, we show that these two signaling pathways are linked centrally by T_3 repression of *Mc4r*.

Thyroid Status Modifies *Mc4r* Expression in Brain Areas Relevant to Metabolic Regulation. *Mc4r* is widely expressed in the brain, with high levels in brainstem and hypothalamus, notably the PVN where *Mc4r* is found in *Trh* neurons (10, 11). qPCR and in situ hybridization showed hypothyroidism to increase endogenous *Mc4r* expression in the PVN. This increase parallels the well-known rise in PVN *Trh* mRNA seen in hypothyroidism (21, 30). The increase in *Mc4r* mRNA was modest, but small changes in regulatory genes can significantly impact targets in terms of physiology, development, and evolution (31). Hypothyroidism also substantially raised, and hyperthyroidism repressed, *Mc4r* expression in the ARC. In the brainstem, also targeted by the melanocortin pathway, *Mc4r* again was repressed by hyperthyroidism, but to a much larger extent. As expected, neither in brainstem nor in cortex was *Trh* expression affected by thyroid status. *Mc4r* expression also was unchanged in the cortex. Thus thyroid status specifically alters *Mc4r* expression in brain areas relevant to metabolic regulation.

Evidence for Direct T_3-Dependent Regulation of *Mc4r* Expression. The putative nTRE, TRE1, identified in the *Mc4r* 5′ region, provides a molecular basis for T_3 action on *Mc4r* expression. TRE1 mutation dramatically decreases *Mc4r* promoter activity and leads to loss of T_3-dependent repression. TRE1, a nonclassical sequence conserved in mammals, is thought to bind monomeric nuclear receptors. TRE1 is situated in the core-promoter region and next to a CAAT box (22), suggesting a role for TRs in transcriptional machinery recruitment and direct transcriptional regulation of *Mc4r* by T_3. This hypothesis is supported by the in vivo ChIP results showing TRβ recruitment on the putative TRE1 sequence of *Mc4r* promoter and on site 4 in the *Trh* promoter. Data from many in vitro studies on positively regulated genes suggest a model wherein TRs bind to TREs with or without ligand, T_3 (32). In vivo, the TREs studied from the *Mc4r* and *Trh* promoters showed distinct TRβ recruitment patterns as a function of T_3 presence or absence. *Mc4r* TRE1 recruited only low levels of TRβ in the absence of T_3, and T_3 induced a large increase in TRβ binding. In contrast, TRβ was found on *Trh* TRE site 4 without hormone, as previously shown (33), and T_3 dissociated TRβ from *Trh* TRE site 4. These differences may contribute to the different sensitivities of the two genes to T_3-dependent repression. Indeed, *Mc4r* TRE1 and *Trh* site 4 display very different sequences (Fig. S8 *B* and *C*), and the neighboring chromatin contexts differ for each TRE. The *Mc4r* TRE1 sequence could bind nuclear receptors as monomers (22), allowing TR to exert its own distinct transcriptional properties. The *Trh* site 4, on the contrary, preferentially binds TR/retinoid X receptor heterodimers (23). This TRE also overlaps with a canonical CREB-response element (8) that mediates rapid responses to cAMP/protein kinase A pathways. Thus *Trh* site 4 could recruit different sets of transcriptional coregulators and induce T_3 response kinetics different from that of the *Mc4r* TRE1.

The data thus provide in vivo data on the molecular mechanisms of T_3-dependent gene repression by TRβ in a physiological context. It should be borne in mind, however, that other TR response elements also could participate in the regulations studied, and that the contribution of TRα remains to be clarified.

Specific Contributions of TRα and TRβ Isoforms to *Mc4r* Regulation. To analyze further the transcriptional regulations induced by TH, we used an iGT paradigm in the newborn mouse hypothalamus. With iGT, hypothalamic neurons are transfected with plasmid DNA complexed with polyethylenimine (PEI). PEI is one of the most exploited nonviral gene transfer agents and can be used in the same manner as its viral counterparts (e.g., adeno-associated virus or lentivirus). Previous results using this technique established that it provides region- and neuron-specific regulations (24). Somatic iGT transgenesis in pups confirmed that hypothyroidism upregulates transcription from the *Mc4r* promoter, whereas T_3 represses hypothalamic MC4R-luc. These changes are synchronous with T_3 effects on *Trh* transcription (Fig. 4).

Functional TRs (TRα1, TRβ1, and TRβ2) colocalize in many hypothalamic nuclei (5, 21). Each isoform exerts specific transcriptional activities on the *Trh* promoter (4, 24, 26, 27), differences which are caused by variations in N-terminal sequence (34). Overexpression and knockdown studies in newborn mice revealed TR isoform-specific and age-dependent effects on *Mc4r* transcription. For TRα, transient, hypothalamic knockdown of TRα activated *Mc4r* transcription in the newborn, but its long-term, generalized knockout resulted in repression in the adult. These stage-dependent differences could result from developmental compensatory effects in the mutant mouse. For TRβ, similar effects on T_3-dependent regulation of *Mc4r* were seen in newborn and adult mice. Comparing the roles of each TRβ isoform in *Mc4r* and *Trh* transcription, that of TRβ1 appears common to *Mc4r* and *Trh* promoters, and that of TRβ2 seems to be gene specific. TRβ2 activates T_3-independent *Mc4r* basal expression, but for *Trh* regulation, it mediates T_3-dependent repression (26). As discussed earlier, the mechanisms underlying such gene-specific regulations might be related to differences in the TRE sequences seen in *Mc4r* and *Trh* promoters, leading to gene-specific recruitment of transcription factors.

Physiological Relevance of T_3-Dependent Repression of *Mc4r* Expression. The leptin/melanocortin pathway impinges on the HPT axis, adapting energy expenditure to metabolic reserves (8). Our results reveal a feedback loop targeting the melanocortin relay, with T_3 repressing

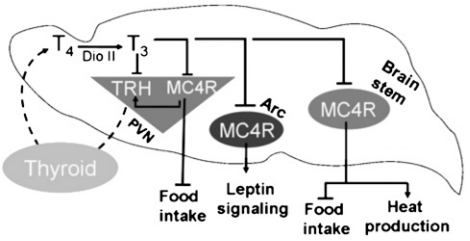

Fig. 4. Schema of T_3 repression of *Mc4r* expression in brain. The physiological consequences of modified thyroid status on *Trh* and *Mc4r* gene regulation and metabolic output are played out through modulation of *Mc4r* expression in key brain areas affecting metabolism, in parallel with *Trh* repression. Coordinated T_3 repression of *Mc4r* has energy-saving consequences. See *Discussion* for commentary.

Mc4r expression not only via hypothalamic *Trh* but also in other key energy-related brain regions. TH-dependent repression of *Mc4r* will have multiple physiological consequences. A first short-term consequence is orexigenia and increased weight gain, as seen in the T_4-treated mice (Fig. S1*B*). Indeed, hypothalamic T_3-dependent repression of *Mc4r* expression should reduce the sensitivity of TRH neurons to leptin/melanocortin inputs. Moreover, increased T_3 also would reduce hypothalamic sensitivity to Agouti-related protein (AgRP) signaling and other MC4R ligands. More than 50% of hypophysiotropic TRH neurons receive AgRP fibers, and αMSH fibers represent 38% of the innervation to PVN TRH neurons. Thus T_3-dependent repression of *Mc4r* would dampen brain global responsiveness to melanocortin anorectic signaling while stimulating orexigenic pathways through NPY signaling. Again, our findings are consistent with this prediction. T_3 modulated the leptin-sensitive neuropeptides *Pomc* and *Npy* in the ARC, downregulating the *Pomc* precursor for αMSH and increasing *Npy* mRNA, in line with published data (15, 17). In contrast, because hypothyroidism increases *Mc4r* expression in the PVN and ARC, low T_3 would sensitize TRH neurons and their ARC counterparts to MC4R ligands (35), enhancing central metabolic responses (Fig. S8*A*). Future work should address whether the effects of TH on *Mc4r* expression are uniform across different hypothalamic cell populations or are limited to given neuronal categories. For instance, corticotropin-releasing hormone neurons, as well as TRH neurons, express MC4R (36). Thus T_3 regulation of *Mc4r* could affect other neuroendocrine circuits. The largest repressive effect of T_3 on *Mc4r* expression was in brainstem, which controls thermogenesis and meal size (37–39). Here, T_3 repression of *Mc4r* could modulate autonomic outputs governing peripheral energy homeostasis and food intake.

The fact that *Mc4r* is regulated by thyroid status in different brain areas having distinct effects on metabolism has major physiological significance. Overall increased MC4R signaling will decrease food intake, increase thermogenesis, and restrict meal size. Each of these effects will reduce energy reserves and will, in parallel, be amplified by high T_3. To avoid excess catabolism, T_3 feeds back on *Trh* (thereby reducing T_3 production), providing a first line of defense against energy dissipation. However, if this process occurs without putting a brake on MC4R signaling, the energy-saving effects of T_3 feedback on *Trh* would be neutralized or overridden: MC4R stimulates *Trh* transcription through cAMP and CREB (8), potentially overriding T_3 feedback on *Trh*. Thus, coordination of leptin/melanocortin signaling and HPT axis pathways is achieved by a double T_3-dependent feedback loop, reinforcing maintenance of metabolic homeostasis (Fig. 4 and Fig. S8*A*).

During evolution, the selection of a syn-repression control by T_3 on these two central genes could have provided a selective advantage through optimal regulation of energy conservation and homeostasis. The presence of the nTRE we identified in all mammals examined (mouse, rat, human, and pig) is a strong argument for the significance of this endocrine regulation in homeotherms.

In conclusion, this study demonstrates that thyroid status regulates *Mc4r* expression in major brain areas relevant to metabolic homeostasis. T_3 directly mediates the regulation of *Mc4r* expression. Taken together, the data reinforce the concept that T_3 has a major role in integrating endocrine and metabolic signaling at the central level.

Materials and Methods

Animals. Animal care and experimentation were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee, Veterinary Services Direction, Paris, France. Swiss wild-type mice were from Janvier. Mice lacking all TRβ or all TRα gene products [$TR\beta^{-/-}$ (19) or $TR\alpha^{0/0}$ (18)] were provided by J. Samarut (Ecole Normale Supérieure, Lyon, France). For investigations of hypothyroidism, 8-week-old male mice were given iodine-deficient food containing 0.15% PTU (Harlan) and drinking water with 0.5 g/L PTU (Sigma-Aldrich) for 13 days before they were killed. To induce fetal and neonatal hypothyroidism, dams were given the iodine-deficient PTU diet from day 14 of gestation through lactation. For investigations of hyperthyroidism, adult mice were treated with 1.2 μg/mL of T_4 in drinking water for 13 days before they were killed.

Assessment of T_4 Levels. Total T_4 blood concentrations were quantified using ELISA kits (AbCys, Paris, France).

In Situ Hybridization. In situ hybridization protocol was adapted from (40) (see *SI Materials and Methods*).

Plasmids. TRH-f.luc and TRα1, TRβ1, and TRβ2 plasmids were as described (27). MC4R-f.luc plasmid, provided by H. Krude (Charity Medical University, Berlin, Germany), contains −653 to +448 bp of the *MC4R* promoter cloned upstream of the *Firefly* luciferase-coding sequence of the pGL3 basic Luciferase reporter vector (Promega, Charbonniéres les Bains, France). MC4R-*Renilla* luciferase (MC4R-r.luc) was designed in the laboratory (see *SI Materials and Methods*).

Two putative TREs in the h*MC4R* promoter region were mutated using the QuikChange II XL Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) per the manufacturer's protocol. Primers for mutagenesis (Table S1) were from Invitrogen. Three MC4R-r.luc transgenes were generated and sequenced: TRE1m, TRE2m, and DBm (mutations in both TRE1 and TRE2).

The plasmid encoding shRNA against TRα1 (shTRα1) has been published (41). The two sets of shRNA against TRβ isoforms (25), shGFP and shSCR, bear the same backbone as shTRα1. Design (Table S2 and S3) and cloning are described in *SI Materials and Methods*.

In Vivo Transfection and Luciferase Assays. DNA/PEI complexes and iGT were adapted from (24). For single and cotransfection iGT protocols and T_3 treatments see *SI Materials and Methods*.

In Vivo Chromatin Immunoprecipitation. Pups were treated with T_3 or vehicle and killed 20 h later. Dorsal hypothalamic regions including the PVN were dissected. Samples were fixed in 1% formaldehyde solution and sonicated. Control and T_3-treated samples were used for ChIP with a mix of anti-TRβ1 and anti-TRβ2 antibodies (Millipore) or without antibody (negative control). Precipitated DNA fragments were purified. Primers spanning the TRE sequences in *Mc4r* and *Trh* promoter were used in qPCR to measure enrichment of DNA samples. Negative controls comprised primers spanning irrelevant sequences in both gene sequences. The detailed ChIP protocol is provided in *SI Materials and Methods*.

qPCR. qPCR procedures used for *Mc4r, Trh, Pomc*, and *Npy* mRNA detection are described in *SI Materials and Methods*. Primers for specific TR mRNA detection were as described (21).

Statistical Analysis. qPCR results: boxes represent the fifth to ninety-fifth percentiles around the median with whiskers for minimum and maximum values. Statistical analysis for qPCR data compared the means of fold increase values ± SD, using nonparametric ANOVA, followed by a permutation test (Cytel Studio software, Cambridge, MA) to compare the control and treated groups. Independent experiments ($5 \leq n \leq 8$) were repeated three times, providing similar results and data were pooled.

iGT results are presented as means ± SEM per group. ANOVA was used to compare groups followed by multiple-comparisons Bonferroni's post-test. Each experiment was carried out with $n \geq 10$ and was repeated at least three times providing the same results. In all cases, typical experiments are shown, differences were considered significant at $P < 0.05$ with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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