International Journal of Obesity (2015) **39,** 105–113

www.nature.com/ijo

ORIGINAL ARTICLE

CREB/TRH pathway in the central nervous system regulates energy expenditure in response to deprivation of an essential amino acid

T Xia[1], Q Zhang[1], Y Xiao, C Wang, J Yu, H Liu, B Liu, Y Zhang, S Chen, Y Liu, Y Chen and F Guo

BACKGROUND: In the central nervous system (CNS), thyrotropin-releasing hormone (TRH) has an important role in regulating energy balance. We previously showed that dietary deprivation of leucine in mice increases energy expenditure through CNS-dependent regulation. However, the involvement of central TRH in this regulation has not been reported.
METHODS: Male C57J/B6 mice were maintained on a control or leucine-deficient diet for 7 days. Leucine-deprived mice were either third intracerebroventricular (i.c.v.) injected with a TRH antibody followed by intraperitoneal (i.p.) injection of triiodothyronine (T3) or i.c.v. administrated with an adenovirus of shCREB (cAMP-response element binding protein) followed by i.c.v. injection of TRH. Food intake and body weight were monitored daily. Oxygen consumption, physical activity and rectal temperature were assessed after the treatment. After being killed, the hypothalamus and the brown adipose tissue were collected and the expression of related genes and proteins related was analyzed. In other experiments, control or leucine-deficient medium incubated primary cultured neurons were either infected with adenovirus-mediated short hairpin RNA targeting extracellular signal-regulated kinases 1 and 2 (Ad-shERK1/2) or transfected with plasmid-overexpressing protein phosphatase 1 regulatory subunit 3C (PPP1R3C).
RESULTS: I.c.v. administration of anti-TRH antibodies significantly reduced leucine deprivation-stimulated energy expenditure. Furthermore, the effects of i.c.v. TRH antibodies were reversed by i.p. injection of T3 during leucine deprivation. Moreover, i.c.v. injection of Ad-shCREB (adenovirus-mediated short hairpin RNA targeting CREB) significantly suppressed leucine deprivation-stimulated energy expenditure via modulation of TRH expression. Lastly, TRH expression was regulated by CREB, which was phosphorylated by ERK1/2 and dephosphorylated by PPP1R3C-containing protein Ser/Thr phosphatase type 1 (PP1) under leucine deprivation *in vitro*.
CONCLUSIONS: Our data indicate a novel role for TRH in regulating energy expenditure via T3 during leucine deprivation. Furthermore, our findings reveal that TRH expression is activated by CREB, which is phosphorylated by ERK1/2 and dephosphorylated by PPP1R3C-containing PP1. Collectively, our studies provide novel insights into the regulation of energy homeostasis by the CNS in response to an essential amino-acid deprivation.

International Journal of Obesity (2015) **39,** 105–113; doi:10.1038/ijo.2014.65

Keywords: leucine deprivation; TRH; CREB; hypothalamus; PPP1R3C; ERK1/2

INTRODUCTION

Leucine is an essential amino acid (EAA), and altered leucine homeostasis is associated with numerous metabolic manifestations including glucose use, lipid metabolism and energy expenditure.[1,2] Notably, leucine deprivation serves as a research model that is essential for exploring whole-body metabolism.[3–6] Mammals exhibit multiple adaptive mechanisms that sense and respond to fluctuations in AA starvation.[7] The response to AA deprivation in mammals is considered to be characterized by repression of protein synthesis and upregulation of the AA biosynthesis and their transporters.[8–10] When rats were fed a diet with a balanced EAA profile but moderately low in protein ($< 50\%$ of protein restriction), they became hyperphagic because the increased food intake could provide adequate protein for the maintenance of many important physiological activities in the body.[11,12] Despite the increased energy intake, body weight was not increased, suggesting an altered energy partition.[12] Similar experiments have been performed in human subjects. For example, human subjects who ate low-protein omelettes consumed nearly two times as much energy as participants who ate high-protein omelettes.[13] A 9-week study on elderly women found that energy intake is increased under a low-protein diet, but they do not gain weight, suggesting elevated energy expenditure.[14] Thus, low-protein diet leads to compensatory hyperphagia to meet nutritional requirements in animals and humans, as well as compensatory increases in energy expenditure, possibly to fight for the extra energy intake. However, in the case when energy expenditure is not induced, they might gain weight.[15] Therefore, it is very important to study the underlying mechanisms.

In the central nervous system (CNS), numerous hormones including thyrotropin-releasing hormone (TRH) have important roles in the regulation of energy homeostasis.[16] TRH-expressing

Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, The Graduate School of the Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai, China. Correspondence: Professor F Guo, Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, The Graduate School of the Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China.
E-mail: ffguo@sibs.ac.cn
[1]These authors contributed equally to this work.
Received 27 November 2013; revised 18 March 2014; accepted 6 April 2014; accepted article preview online 15 April 2014; advance online publication, 20 May 2014

neurons are located in the paraventricular nucleus of hypothalamus (PVN).[17] TRH increases biosynthesis and secretion of thyroid-stimulating hormone from the pituitary,[18,19] which in turn stimulates biosynthesis of the thyroid hormones thyroxine (T4) and triiodothyronine (T3). Conversely, TRH itself responds to alterations in metabolism and external environment, including thyroid hormones, glucocorticoids, leptin, cold and various nutritional conditions.[20–22]

TRH is regulated by several transcription factors[23,24] including cAMP-response element binding protein (CREB). Phosphorylated-CREB binds to the consensus cAMP-response element (CRE) site in TRH promoter and activates gene transcription.[25–27] CREB can be phosphorylated by an array of kinases including extracellular signal-regulated kinases 1 and 2 (ERK1/2)[28] and dephosphorylated by protein phosphatases including protein Ser/Thr phosphatase type 1 (PP1) and protein Ser/Thr phosphatase type 2A.[29,30] These observations raise the possibility that CREB/TRH-dependent signaling regulates energy homeostasis.

We previously showed that leucine deprivation-increased energy expenditure in mice, which was controlled by a CNS-dependent pathway.[4,6] Our current study showed that CREB/TRH-dependent pathway is crucial for regulating energy expenditure under leucine deprivation.

MATERIALS AND METHODS

Animals and diets

Wild-type C57BL/6J mice were obtained from the Shanghai Laboratory Animals Co. Ltd (Shanghai, China). Male, 8–10-week-old mice were maintained on a 12-h light/dark cycle at 24 °C and provided free access to commercial rodent chow and tap water before the experiments. Control (nutritionally complete AA) and leucine-deficient ((−) leu) diets, which are isocaloric and compositionally the same in terms of carbohydrate and lipid component, were obtained from Research Diets Inc. (New Brunswick, NJ, USA). As described previously,[3,5] at the start of the feeding experiment, mice were acclimated to a control diet for 7–10 days and then randomly assigned to either control or (−) leu diet groups with free access to control or (−) leu diet, respectively, for 7 days. These experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of INS, SIBS and CAS.

Construction of plasmids and adenovirus

pRC-CMV-PPP1R3C was described previously.[31] Adenovirus-mediated short hairpin RNA targeting CREB (Ad-shCREB) or adenovirus-expressing ERK1/2-specific short hairpin RNA (Ad-shERK1/2) was generated with the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequence designed for the knockdown of CREB is: 5′-CCTGCAGACATTAACCATGA-3′ and for ERK1/2 is: 5′-GCAATGACCACATCTGCTACT-3′.

Primary hypothalamic neuron isolation and treatments

Primary cultures of hypothalamic neurons were prepared as described previously.[4] On day 10, primary cultured hypothalamic neurons were infected with Ad-shERK1/2 (10^8 pore-forming unit per 60 cm^2 cells), or scramble control shRNA, followed by treatment with (−) leu medium or complete medium, prepared as described previously.[4]

Intracerebroventricular administration experiments

Intracerebroventricular (i.c.v.) administration experiments were conducted as described previously.[4,6] A sterile stainless-steel cannula was stereotaxically implanted into the right lateral brain ventricle (−0.5 mm anterior and 1.0 mm lateral relative to bregma and 2.5 mm below the surface of the skull). After 7 days recovery, 1 μl of anti-TRH antibodies (0.5 μg in 1 μl phosphate-buffered saline (PBS);[32] PROGEN Biotechnic GmbH, Heidelberg, Germany) or PBS, 1 μl of TRH (P1319-50MG; Sigma, St Louis, MO, USA) (0.1 mM in artificial cerebrospinal fluid[33]) or artificial cerebrospinal fluid was administered once daily for 7 days. A measure of 1 μl 5×10^8 pore-forming unit per mice of Ad-shCREB or control adenovirus was injected into the third ventricle (at the midline coordinates of 1.6 mm posterior to the bregma and 5.0 mm below the bregma), followed by 5 days recovery before experimental diets were applied. Our preliminary experiments showed that the best knockdown efficiency of Ad-shCREB expression in the hypothalamus occurred on the twelveth day after injection.

Intraperitoneal injection of T3

A measure of 100 μl T3 (0.1 mg ml^{-1}; Sigma)[34] or 10 mM NaOH as a control was administered once daily for 7 days.

T3 measurements

Thyroid hormone T3 in serum is determined using Elisa Kits from R&D Systems (Minneapolis, MN, USA).

Metabolic parameters measurements

Indirect calorimetry was measured in a comprehensive lab animal monitoring system (CLAMS; Columbus Instruments, Columbus, OH, USA), as described previously.[6] Regarding mice receiving i.c.v. injection or cannula implanted, mice were put in CLAMS on the fifth day following initiation of experimental diets. Rectal temperatures of mice were measured at 1500 hours (basal metabolic state) using a rectal probe attached to a digital thermometer (Physitemp Instruments, Clifton, NJ, USA).

RNA isolation and relative quantitative RT-PCR

RNA isolation and reverse transcription-polymerase chain reaction was performed as described previously.[3,4,6] The sequences of primers used in this study are available upon request.

Western blot analysis

Western blot was performed with primary antibodies (anti-p-ERK1/2 (Ser235/236), anti-ERK1/2, anti-p-CREB (Ser133) and anti-CREB antibodies (Cell Signaling Technology, Beverly, MA, USA), anti-UCP1, anti-PTG antibodies (above from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-actin antibody (Sigma) as described previously.[4,6]

Immunohistochemistry staining

Immunohistochemistry (IHC) staining was performed with primary antibodies anti-p-CREB (Ser133) antibody (Cell Signaling Technology) and anti-TRH antibody (PROGEN Biotechnic GmbH), as described previously.[4,6]

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed according to the manufacturer's protocol (Millipore, Bedford, MA, USA). Immunoprecipitation was performed with anti-CREB antibodies (Cell Signaling Technology) or normal rabbit immunoglobulin G (Santa Cruz Biotechnology) for negative control at 4 °C overnight. Immunoprecipitated TRH promoter was quantified using polymerase chain reaction with primers designed to amplify the region encompassing the 180 bp containing the CRE site in mouse TRH (forward, 5′-ATGAGGACTGTGAGGTATAAC-3′ and reverse, 5′-GAGGGGTGGTCCTGTGATA-3′) or an upstream region encompassing 180 bp (NC, from −2000 to −1820) that is not involved in CREB response (forward, 5′- TTGGCTTAGATCCCAAG -3′ and reverse, 5′-TTAATTGGTGGG GGAA-3′).

Phosphatase activity assay

Phosphatase activity was determined by Phosphatase Assay Kit (Invitrogen) according to the manufacturer's instruction. Phosphatase activity was measured in a fluorescence microplate reader.

Statistical analysis

All values are presented as mean ± s.e.m. Differences between groups were analyzed either by the Student's *t*-test or one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls (SNK) test, differences in which $P < 0.05$ were considered statistically significant.

RESULTS

Intracerebroventricular administration of anti-TRH antibodies significantly reduces leucine deprivation-stimulated energy expenditure

To investigate the possible involvement of TRH in leucine deprivation-stimulated energy expenditure, we measured TRH levels in the hypothalamus. The levels of the *Trh* mRNA and protein were increased in the hypothalamus of leucine-deprived mice compared with control diet-fed male C57J/B6 mice (Figures 1a and b). TRH is produced almost exclusively in the PVN neurons.[17] Here, we found that the levels of TRH were higher in PVN of leucine-deprived mice compared with mice fed a control diet, as shown by IHC staining (Figure 1c). Furthermore, serum thyroid-stimulating hormone levels were also increased in the leucine-deprived mice (Supplementary Figure S1), which indicated the presence of elevated levels of mature TRH.[35,36]

The method of i.c.v. antibodies to TRH can selectively neutralize the activities of endogenous TRH, which has also been used in many other published studies.[32,37,38] The effects of neutralizing the central effects of TRH were determined by the decreased levels of T3, which is a downstream factor of TRH.[20] To access the contribution of hypothalamic TRH in leucine deprivation-induced energy expenditure, we blocked TRH function by i.c.v. administration of anti-TRH antibodies[32,37,38] once daily while maintaining the mice on a control or (−) leu diet for 7 days. I.c.v. injection of TRH-Ab did not affect food intake, but attenuated the reduction in body weight caused by leucine deprivation compared with control mice (Supplementary Figure S2). Next, we assessed energy expenditure by performing indirect calorimetry, and measuring physical activity and rectal temperature of the mice. Consistent with previous results,[3] the 24-h O_2 consumption was markedly increased and the respiratory exchange ratio (RER, VCO_2:VO_2) was lower during both dark and light phases in PBS-treated mice maintained on a (−) leu diet, as compared with mice maintained on a control diet (Figures 1d and e). By contrast, the effects of leucine deprivation on oxygen consumption and RER were abolished in mice i.c.v. injected with anti-TRH antibodies (Figures 1d and e). However, locomotor activities were not affected by i.c.v. administration of anti-TRH antibodies in mice under either diet (Figure 1f). I.c.v. administration of anti-TRH antibodies also significantly blocked leucine deprivation-increased rectal temperature (Figure 1g) and UCP1 expression in brown

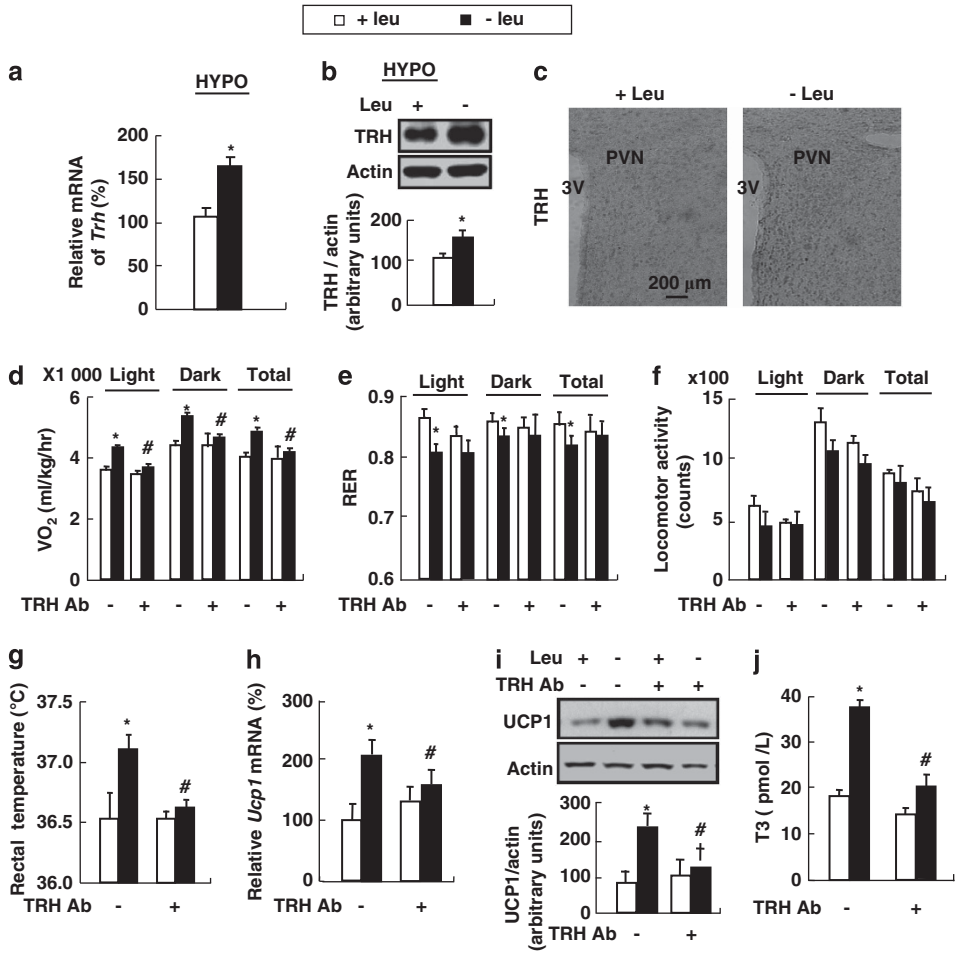

Figure 1. I.c.v. administration of anti-TRH antibodies significantly reduces leucine deprivation-stimulated energy expenditure. Mice received i.c.v. administration of anti-TRH antibodies (+ TRH-Ab) or PBS (− TRH-Ab) once a day for 7 days under a control (+ leu) or − leu diet for 7 days. Energy expenditure was measured by indirect calorimetry. Data are means ± s.e.m. of at least two independent experiments ($n = 6$–10 for each group). Statistical significance was determined by one-way ANOVA followed by the SNK test: $^{*}P < 0.05$ (for the effect of (−) leu versus control diet within the same i.c.v. group), $^{\#}P < 0.05$ (for the effect of with versus without anti-TRH antibodies under (−) leu diet). (**a**) *Trh* mRNA; (**b**) TRH proteins (upper, western blot; lower, quantitative measurements of TRH relative to actin); (**c**) IHC staining for TRH in the hypothalamus: images of TRH staining in the PVN. 3 V, third ventricle. Scale bar, 200 μm. Images shown are representative of several animals for each group. (**d**) Twenty-four- h oxygen consumption (VO_2); (**e**) RER; (**f**) physical activity; (**g**) rectal temperature; (**h**) *Ucp*1 mRNA in BAT; (**i**) UCP1 protein in BAT (upper, western blot; lower, quantitative measurements of UCP1 protein relative to actin); (**j**) T3 levels in the serum.

adipose tissue (BAT) compared with PBS administration (Figures 1h and i). Notably, baseline conditions showed no significant differences in body weight and food intake among the groups when the anti-TRH antibodies were administered. This could be because of the different sensitivity of the hypothalamus to TRH antibodies because of the different secretory volumes of TRH between control and leucine-deprived mice.

TRH stimulates energy expenditure via altering serum T3 levels under leucine deprivation

Increased TRH expression has been shown to promote T3 production,[20] suggesting that the effects of hypothalamic TRH are possibly mediated by alteration of serum T3 levels under leucine deprivation. Supporting this possibility, we found that serum T3 levels were increased under leucine deprivation and this increase was blocked by i.c.v. administration of anti-TRH antibodies (Figure 1j). To assess the role for T3 in mediating effects of TRH, we i.c.v. injected anti-TRH antibodies and examined whether subsequent intraperitoneal (i.p.) injection of T3 could reverse the suppressive effect of anti-TRH antibodies on leucine deprivation-induced energy expenditure in mice.

I.p. injection of T3 promoted food intake, but reversed the blocking effect of anti-TRH antibodies on leucine deprivation-decreased body weight (Supplementary Figure S3), which agrees with the well-known effect of T3 on increasing energy expenditure with a compensatory increase in food intake.[39] As predicted, T3 injection reversed the suppressive effect of anti-TRH antibodies on leucine deprivation-induced oxygen consumption and RER (Figures 2a and b), and on rectal temperature and UCP1 expression in BAT (Figures 2d–f). T3 injection, however, had no effect on physical activity in any group (Figure 2c). As expected, T3 injection increased serum T3 levels (Figure 2g).

Intracerebroventricular injection of Ad-shCREB significantly blocks leucine deprivation-stimulated energy expenditure via modulation of TRH expression

We previously showed that hypothalamic phosphorylated-CREB was increased during leucine deprivation.[4,6] In this study, IHC staining showed that the levels of phosphorylated-CREB were mainly increased in the PVN (Figure 3a) and elevated slightly in other regions of the hypothalamus (data not shown) in leucine-deprived mice, when compared with mice fed a control diet. To determine whether CREB binds to the CRE site of TRH promoter under leucine deprivation, we performed chromatin immunoprecipitation assays. Consistent with a *Trh* expression in the hypothalamus under leucine deprivation, the amounts of TRH promoter were higher in immunoprecipitates obtained using anti-CREB antibodies in leucine-deprived mice compared with the control group (Figure 3b). By contrast, in the absence of the CREB-specific antibody, the CRE-containing promoter was not precipitated in any of the cases (Figure 3b). To ensure that the CRE-containing promoter was being specifically precipitated, we also amplified, from the same immunoprecipitates, the upstream region that is not involved in the CREB response. No band was detected in the hypothalamus of mice under any treatments when either anti-CREB antibody or control immunoglobulin G was used (Figure 3b). To test the possibility that CREB mediates the effects of leucine deprivation-induced energy expenditure, we inhibited the expression of hypothalamic CREB by i.c.v. administration of Ad-shCREB. First, we validated the effects of i.c.v. Ad-shCREB by examining mRNA levels of *Creb* in the hypothalamus. As predicted, *Creb* gene expression was significantly lower in the Ad-shCREB group than in the control group (Figure 3c). Furthermore, down-regulation of CREB also blocked leucine deprivation-induced *Trh* expression compared with Ad-scramble mice (Figure 3c).

We found that i.c.v. administration of Ad-shCREB did not affect food intake, but it attenuated leucine deprivation-dependent reduction in body weight relative to control (Supplementary

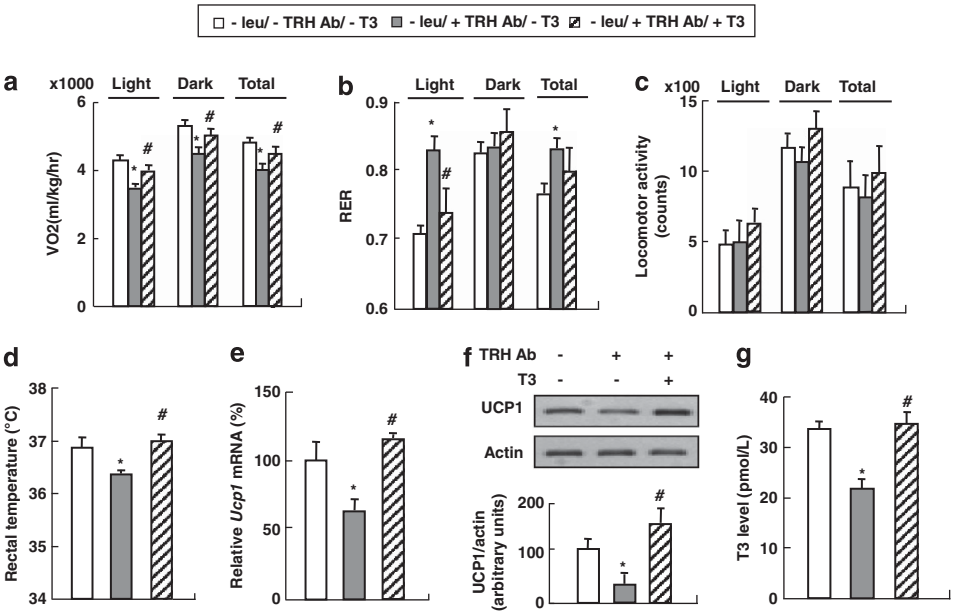

Figure 2. TRH regulates energy expenditure via alteration of serum T3 levels under leucine deprivation. Mice received i.c.v. injection of anti-TRH antibodies (+TRH-Ab) or PBS (−TRH-Ab), followed by i.p. injection of thiiodothyronine (+T3) or NaOH (−T3) once daily and maintenance on a (−) leu diet for 7 days. Data are means ± s.e.m. of at least two independent experiments ($n = 6$ for each group). Statistical significance was determined by the Student's *t*-test: $^{*}P < 0.05$ (for the effect of with versus without anti-TRH antibodies in the absence of T3), $^{\#}P < 0.05$ (for the effect of with versus without T3 in mice receiving anti-TRH antibodies administration). (**a**) Twenty-four-h oxygen consumption (VO_2); (**b**) RER; (**c**) physical activity; (**d**) rectal temperature; (**e**) *Ucp*1 mRNA in BAT; (**f**) UCP1 protein in BAT (upper, western blot; lower, quantitative measurements of UCP1 protein relative to actin); (**g**) T3 levels in the serum.

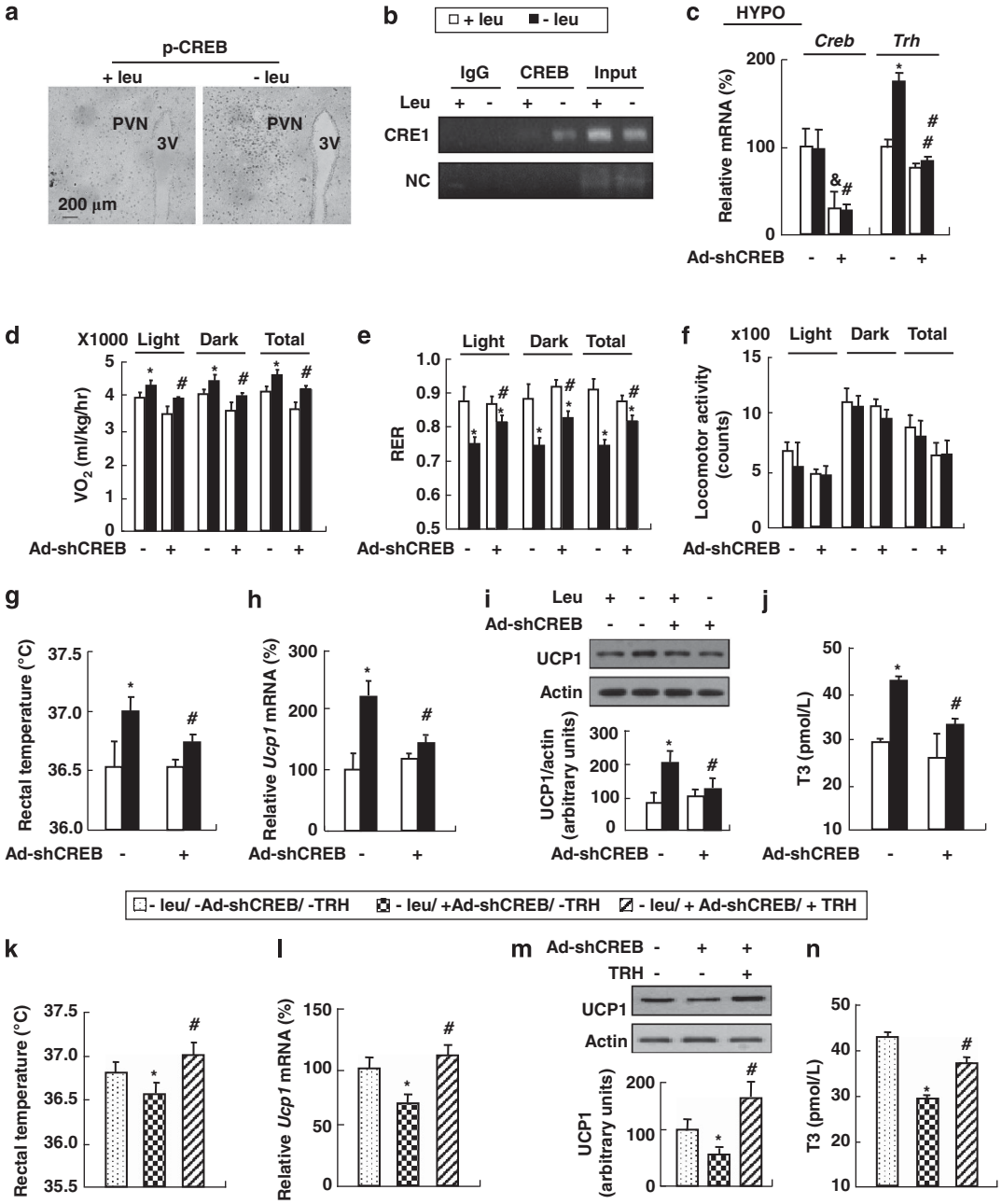

Figure 3. I.c.v. injection of Ad-shCREB significantly blocks leucine deprivation-stimulated energy expenditure via modulation of TRH expression under leucine deprivation. (**a** and **b**) Mice were fed a control (+leu) or (−) leu diet for 7 days. (**c**–**j**) Mice received i.c.v. injection of Ad-shCREB (+Ad-shCREB) or scrambled RNA (−Ad-shCREB) and then fed a control (+leu) or (−) leu diet for 7 days. Energy expenditure was measured by indirect calorimetry. Data are means ± s.e.m. of at least two independent experiments ($n = 6$ for each group). Statistical significance was determined by one-way ANOVA followed by the SNK test: $^{*}P < 0.05$ (for the effect of (−) leu versus control diet within the same i.c.v. group), $^{\#}P < 0.05$ (for the effect of with versus without Ad-shCREB under (−) leu diet), $^{\&}P < 0.05$ (for the effect of with versus without Ad-shCREB under control diet). (**k**–**n**) Mice received i.c.v. injection of +Ad-shCREB or −Ad-shCREB, followed by injection of +TRH or artificial cerebral spinal fluid (−TRH) once daily and maintenance on a (−) leu diet for 7 days. Data are means ± s.e.m. of at least two independent experiments ($n = 6$–7 for each group). Statistical significance was determined by the Student's *t*-test: $^{*}P < 0.05$ (for the effect of with versus without Ad-shCREB in the absence of TRH), $^{\#}P < 0.05$ (for the effect of with versus without TRH in mice receiving Ad-shCREB). (**a**) IHC staining for phosphorylated (p)-CREB in the hypothalamus: images of TRH staining in the PVN. 3 V, third ventricle. Scale bar, 200 μm. Images shown are representative of several animals for each group; (**b**) chromatin immunoprecipitation (ChIP) assay in the hypothalamus; (**c**) *Trh* and *Creb* mRNA; (**d**) 24-h oxygen consumption (VO_2); (**e**) RER; (**f**) physical activity; (**g**) rectal temperature; (**h**) *Ucp*1 mRNA in BAT; (**i**) UCP1 protein in BAT (upper, western blot; lower, quantitative measurements of UCP1 protein relative to actin); (**j**) T3 levels in serum; (**k**) rectal temperature; (**l**) *Ucp*1 mRNA in BAT; (**m**) UCP1 protein in BAT (upper, western blot; lower, quantitative measurements of UCP1 protein relative to actin); (**n**) T3 levels in the serum.

Figure S4). Ad-shCREB exposure also significantly blocked leucine deprivation-increased total energy expenditure (24-h O_2 consumption). The RER in the Ad-shCREB-treated mice showed a slight but significant reversal compared with Ad-scramble-treated mice under leucine deprivation (Figures 3d and e). However, we did not detect significant differences in physical activity among the groups (Figure 3f). Consistent with changes in energy expenditure, the increased body temperature and UCP1 expression in BAT caused by leucine deprivation were also significantly attenuated in Ad-shCREB mice (Figures 3g–i). Moreover, similar blocking effects on leucine deprivation-induced increase in serum T3 levels were also observed following i.c.v. administration of Ad-shCREB (Figure 3j).

To test whether hypothalamic CREB regulates leucine deprivation-induced energy expenditure by modulating TRH, we i.c.v. injected Ad-shCREB (which lowered *Trh* expression; see Figure 3c), and then examined whether subsequent i.c.v. administration of TRH could reverse the suppressive effect of Ad-shCREB on leucine deprivation-induced increase in rectal temperature and UCP1 expression in BAT in mice. Food intake was not affected in Ad-shCREB-treated leucine-deprived mice following injection with TRH, but the suppressive effects of Ad-shCREB on leucine deprivation-decreased body weight was significantly reversed by TRH injection (Supplementary Figure S5). In accord with these results, i.c.v. injection of TRH also largely reversed the suppressive effects of i.c.v.-injected Ad-shCREB on leucine deprivation-induced increase in rectal temperature, UCP1 expression in BAT and serum T3 levels (Figures 3k–n).

Trh expression is regulated by CREB that is phosphorylated by ERK1/2 and dephosphorylated by PPP1R3C-containing PP1 under leucine deprivation *in vitro*

CREB is known to be phosphorylated by a collection of kinases, including ERK1/2.[28] We found that ERK1/2 phosphorylation was increased significantly in the hypothalamus of leucine-deprived mice compared with control mice (Figure 4a). To confirm a role for ERK1/2 in the regulation of CREB phosphorylation and *Trh* expression, we infected primary cultured neurons with Ad-shERK1/2 or control Ad-scramble and then maintained these cells in a medium with or without leucine. Knocking down of ERK1/2 blocked leucine deprivation-induced CREB phosphorylation partially, which is to be expected because CREB can also be phosphorylated by numerous other kinases.[40] However, leucine deprivation-induced *Trh* expression was completely arrested compared with control adenovirus-infected cells maintained on a (−) leu medium (Figures 4b and c).

CREB can be dephosphorylated by several protein phosphatases, including PP1.[29,30] In this study, we found that total phosphatase activity was lower in the hypothalamus of leucine-deprived mice compared with control mice (Figure 4d). By analyzing mRNA levels of different subunits of PP1, we found that mRNA levels of protein phosphatase 1 regulatory subunit 3C (*Ppp1r3c*),[41] but not other subunits, were markedly lower in the hypothalamus of leucine-deprived mice compared with control mice (Figure 4e). To investigate the possibility that PPP1R3C has a key role in PP1-dependent attenuation of CREB phosphorylation, we transfected a PPP1R3C-overexpressing plasmid or a control vector into primary cultured hypothalamic neurons and maintained these cells in a (−) leu medium or in a control medium. Overexpression of PPP1R3C attenuated leucine deprivation-induced CREB phosphorylation and *Trh* expression compared with control cells maintained on a (−) leu medium (Figures 4f and g); in parallel, phosphatase activity assays demonstrated that the overexpression of PPP1R3C was accompanied by a significant increase in phosphatase activity in the cells (Figure 4h).

DISCUSSION

The CNS, particularly the hypothalamus, has a crucial role in maintaining energy homeostasis by integrating signals from nutrients and hormones.[6,16] TRH has an important role in the regulation of energy balance[20] and is considered as a metabolic sensor.[21] An alteration in central TRH expression results in corresponding changes in serum T3 levels and sympathetic nervous system activity, which are also key players in mediating the effects of TRH on metabolic changes in peripheral tissues.[20,42] Here, we demonstrate a key role for central TRH in the stimulation of energy expenditure during leucine deprivation, which extends the understanding of TRH function under different nutritional status. Furthermore, we found that the effects of TRH are mediated via modulation of serum T3 levels, as T3 injection largely reversed the suppressive effects of i.c.v. anti-TRH antibodies on UCP1 expression in BAT during leucine deprivation. We previously showed that physical activity was not altered under leucine deprivation.[3] Here we found that physical activity was also not affected by i.c.v. TRH-Ab. These results suggest that physical activity and thermogenesis are possibly controlled by disparate mechanisms under leucine deprivation. Supporting this notion, other studies have also shown that physical activity and thermogenesis are not necessarily changed in the same manner.[43,44]

TRH biosynthesis has been studied in various cell lines that were co-transfected with the TRH promoter and expression vectors of thyroid hormone receptors or CREB.[24,27,45] Several studies have indicated that phosphorylated-CREB can bind to the CRE site in the TRH promoter, the one overlapping the thyroid hormone response element, named Site-4 (TGACCTCA; at −59/−52 of rat TRH promoter),[21,24,46] to activate the gene expression.[25,26] However, the regulation of *Trh* by CREB *in vivo* and the physiological manifestations are still poorly understood.

Many studies, including those conducted on brain-specific CREB knockout mice,[47] have demonstrated that CREB is vital for many functions in the CNS, including memory formation, survival and growth of multiple neuronal subtypes.[48,49] It has also been shown that deletion of CREB in the PVN in mice results in the development of obesity due to the impaired BAT activation and body temperature.[50] Consistent with these results, our current study also indicates a key role for CREB in the regulation of energy expenditure under leucine deprivation. Moreover, we have shown that the effects of CREB are mediated by modulation of TRH expression, which agrees with an *in vitro* study showing that CREB can regulate TRH expression.[23] In contrast to our observations, Franck Chiappini *et al.*[51] reported that deletion of CREB in the PVN stimulates *Trh* expression. This discrepancy, however, might be explained by the upregulation of another protein named CREM, which is responsible for increased *Trh* expression in the animal models used by Chiappini and co-workers.[51]

Protein phosphorylation and dephosphorylation are both posttranslational modifications that turn many protein enzymes on and off, and thereby alter their function and activity.[52] CREB can be phosphorylated by an array of kinases,[40] including ERKs. Our results show that ERK activation is required for CREB phosphorylation and induction of TRH during leucine deprivation. Consistent with our results, another study showed that ERK can regulate TRH expression *in vitro*.[23] Conversely, CREB can also be dephosphorylated by phosphatases such as PP1 and protein Ser/Thr phosphatase type 2A.[29,30] Our data suggest that PP1 is the protein phosphatase responsible for dephosphorylation of CREB. PP1 contains a catalytic subunit and a regulatory subunit.[53] PPP1R3C is a regulatory subunit of PP1, which can modulate its substrate specificity and activity. Our studies revealed that PPP1R3C expression was decreased in the hypothalamus of leucine-deprived mice, and overexpression of PPP1R3C largely restored protein phosphatase activity and inhibited leucine

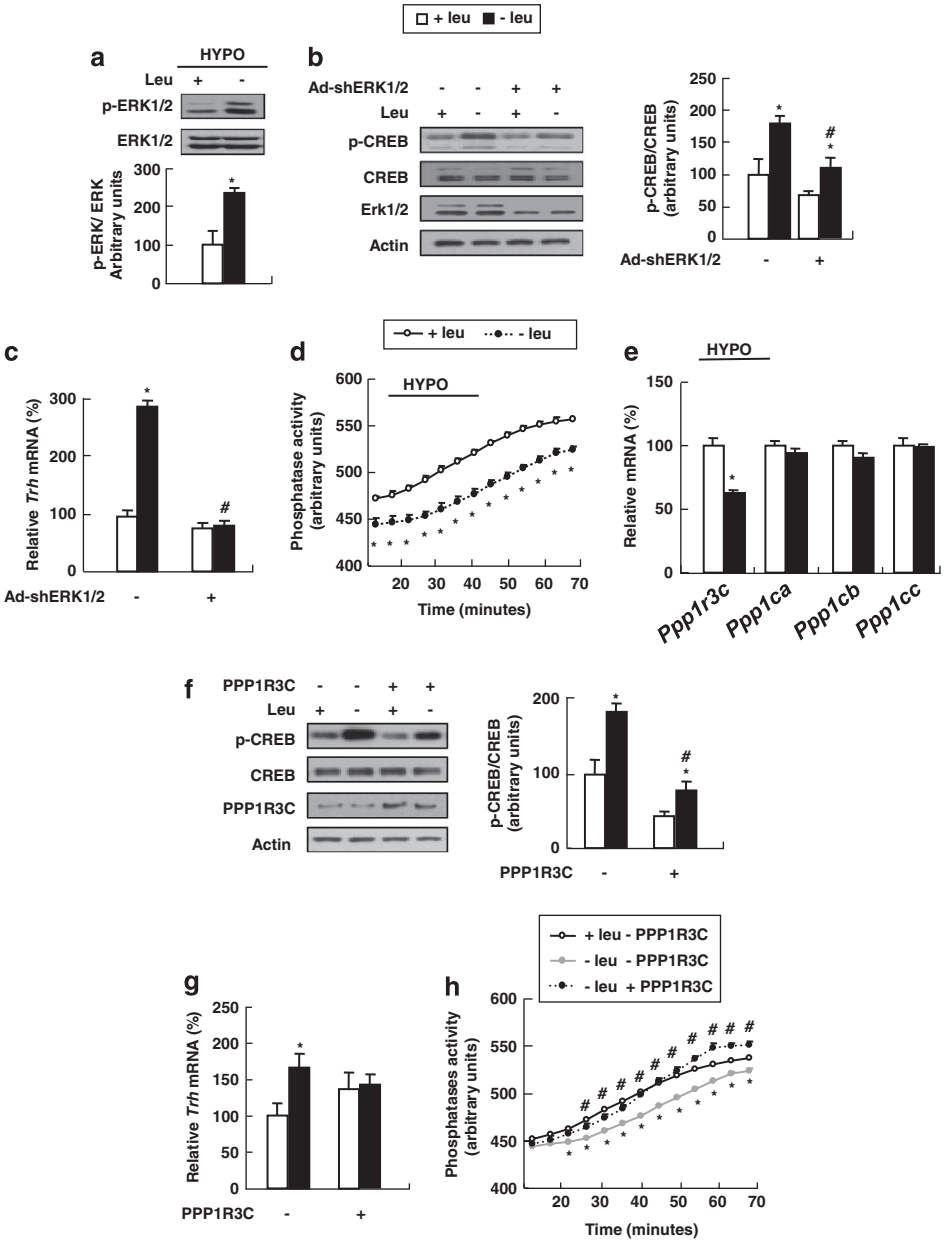

Figure 4. *Trh* gene expression is regulated by CREB that is phosphorylated by ERK1/2 and dephosphorylated by PPP1R3C-containing PP1 under leucine deprivation *in vitro*. (**a**, **d** and **e**) Mice were fed a control (+leu) or (−) leu diet for 7 days. Data are mean ± s.e.m. for at least two independent experiments with mice maintained on each diet for each experiment ($n = 6$–9 for each group). Statistical significance was determined by one-way ANOVA followed by the SNK test: $^{*}P < 0.01$ (for the effect of (−) leu versus control diet). (**b**, **c**, **f** and **g**) Primary cultured hypothalamic neurons were infected with adenovirus-expressing ERK-specific short hairpin RNA (+Ad-shERK) or control scramble shRNA (−Ad-shERK) in (b and **c**), or transfected with plasmid pRC-CMV-PPP1R3C (+PPP1R3C) or control plasmid pRC-CMV (−PPP1R3C) in (**f** and **g**), followed by incubation in medium with (+leu) or without (−leu) for another 48 h. Data are means ± s.e.m. of at least two independent experiments ($n = 6$ for each group). Statistical significance was determined by one-way ANOVA followed by the SNK test: $^{*}P < 0.05$ (for the effect of (−) leu versus (+) leu within the same adenovirus or plasmid group), $^{\#}P < 0.05$ (for the effect of with versus without Ad-shERK1/2 (or pRC-CMV-PPP1R3C) under (−) leu diet). (**a**) ERK1/2 proteins (upper, western blot; lower, quantitative measurements of p-ERK1/2 protein relative to total ERK1/2); (**b**) CREB and ERK1/2 proteins (left, western blot; right, quantitative measurements of phosphorylated (p)-CREB and p-ERK1/2 protein relative to total CREB and total ERK1/2, respectively); (**c**) *Trh* mRNA; (**d**) total phosphatase activity; (**e**) gene expression of phosphatase subunits; (**f**) CREB and PPP1R3C proteins (left, western blot; right, quantitative measurements of p-CREB relative to total CREB); (**g**) *Trh* mRNA; (**h**) total phosphatase activity.

deprivation-increased CREB phosphorylation and TRH induction in primary cultured hypothalamic neurons. This key observation demonstrates that the regulatory subunit PPP1R3C modulates PP1 activity. In fact, similar effects have been observed in the regulation of other enzyme activities by regulatory subunits.[54]

Given the importance of CREB and TRH in the regulation of energy expenditure under leucine deprivation, we speculate that ERK/CREB-dependent pathway is crucial for the regulation of energy expenditure during leucine deprivation. Supporting this possibility, a recent study has shown that inhibition of hypothalamic ERK activity by i.c.v. injection of an ERK inhibitor significantly attenuates leptin activation of thermogenesis.[55] Furthermore, whereas most of previous studies focused on the effects of AA deprivation on protein synthesis mediated by translational control,

our current study provide evidence demonstrating that protein modification also occurs and has an important role in energy expenditure during leucine deprivation.

The RER is the ratio of CO_2 produced to O_2 consumed by the body (VCO_2:VO_2). In case of carbohydrate-only oxidation, RER is equal to 1.0, whereas with fat-only oxidation, RER equals to 0.7.[56] Therefore, the value of RER reflects the source of fuel under conditions examined. Leucine deprivation decreases RER compared with control diet-fed mice,[3] suggesting that fuel source has switched to lipid. The fuel source is also changed following different treatment in this study. I.c.v. injection of TRH-Ab or Ad-shCREB attenuated the suppression of RER by leucine deprivation, suggesting that more carbohydrate was used as fuel source in these treatments. Furthermore, i.p. injection of T3 reversed the upregulation of RER by i.c.v. TRH-Ab, reflecting that T3 elicits increased fat oxidation. The fuel source change induced by leucine deprivation is very likely mediated via adipose sympathetic nervous system, as levels of serum norepinephine and 3-adrenoceptor expression in white adipose tissue and BAT were increased in leucine-deprived mice.[3]

Mouse TRH is produced as a prepro-mRNA that encodes for five copies of mature TRH, which is cleaved from basic amino acids by prohormone convertases 1 and 2, and carboxypeptidase E.[20,35,36] We assumed that both pro- and mature TRH should be induced under leucine deprivation, based on our western blot results showing increased hypothalamic pro-TRH expression and serum T3 levels, as a reflection of mature TRH levels, in leucine-deprived mice.[3] High-performance liquid chromatography fractionation and radioimmunoassay analysis[35] would be required to clarify this issue more precisely in the future.

The effects of an imbalanced AA diet, such as a diet lacking a single EAA, have not been tested in humans. However, the effects of single EAA deprivation have been investigated in animal models extensively. For example, a (−) leu diet has been shown to lead to anorexia, but not hyperphagia, in mice and rats.[5,57] We found the increased energy expenditure was caused by increased thermogenesis, due to increased UCP1 expression in BAT of leucine-deprived mice.[3] These results also indicate that the decreased food intake should not be the primary factor driving to increased energy expenditure in leucine-deprived mice. Based on the results obtained from mouse, we consider leucine deprivation may influence fat mass in humans, which needs further study and is a long-term goal of our laboratory.

In conclusion, our data indicate a novel role for TRH in regulating energy expenditure via T3 during leucine deprivation (Figure 5). We have further shown that TRH expression is activated by CREB, which is phosphorylated by ERK1/2 and dephosphorylated by PPP1R3C-containing PP1. Taken together, our studies provide novel insights into the regulation of energy expenditure by the CNS. Our results also suggest that the enhancement of posttranslational modification of proteins is another important adaptive and beneficial response to the condition of an EAA deprivation and more investigations on the molecular mechanisms should be carried on.

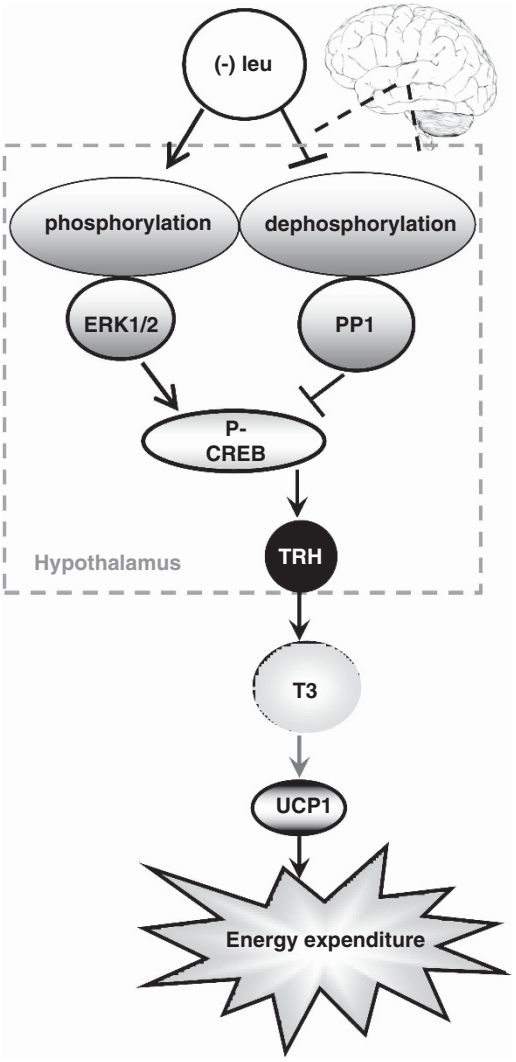

Figure 5. Model of leucine deprivation regulation of energy expenditure. Leucine deprivation activates hypothalamic CREB via phosphorylation and dephosphorylation synchronously, which increases UCP1 in BAT through TRH signaling.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Science and Technology of China (973 Program 2010CB912502); National Natural Science Foundation (81130076, 31271269, 81100615, 81300659, 81390350 and 81325005); the Key Program of Shanghai Scientific and Technological Innovation Action Plan (13JC1409000); International S&T Cooperation Program of China (Singapore 2014DFG32470). FG was also supported by the One Hundred Talents Program of the Chinese Academy of Sciences.

REFERENCES

1 Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF *et al.* A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 2009; **9**: 311–326.
2 Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E *et al.* Metabolite profiles and the risk of developing diabetes. *Nat Med* 2011; **17**: 448–453.
3 Cheng Y, Meng Q, Wang C, Li H, Huang Z, Chen S *et al.* Leucine deprivation decreases fat mass by stimulation of lipolysis in white adipose tissue and upregulation of uncoupling protein 1 (UCP1) in brown adipose tissue. *Diabetes* 2010; **59**: 17–25.
4 Cheng Y, Zhang Q, Meng Q, Xia T, Huang Z, Wang C *et al.* Leucine deprivation stimulates fat loss via increasing CRH expression in the hypothalamus and activating the sympathetic nervous system. *Mol Endocrinol (Baltimore, MD)* 2012; **25**: 1624–1635.
5 Guo F, Cavener DR. The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. *Cell Metab* 2007; **5**: 103–114.
6 Xia T, Cheng Y, Zhang Q, Xiao F, Liu B, Chen S *et al.* S6K1 in the central nervous system regulates energy expenditure via MC4R/CRH pathways in response to deprivation of an essential amino acid. *Diabetes* 2012; **61**: 2461–2471.
7 Kilberg MS, Balasubramanian M, Fu L, Shan J. The transcription factor network associated with the amino acid response in mammalian cells. *Adv Nutr (Bethesda, MD)* 2012; **3**: 295–306.

8 Hinnebusch AG. The eIF-2 alpha kinases: regulators of protein synthesis in starvation and stress. *Sem Cell Biol* 1994; **5**: 417–426.
9 Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. *Annu Rev Nutr* 2005; **25**: 59–85.
10 Wek RC. eIF-2 kinases: regulators of general and gene-specific translation initiation. *Trends Biochem Sci* 1994; **19**: 491–496.
11 White BD, He B, Dean RG, Martin RJ. Low protein diets increase neuropeptide Y gene expression in the basomedial hypothalamus of rats. *J Nutr* 1994; **124**: 1152–1160.
12 Du F, Higginbotham DA, White BD. Food intake, energy balance and serum leptin concentrations in rats fed low-protein diets. *J Nutr* 2000; **130**: 514–521.
13 Porrini M, Santangelo A, Crovetti R, Riso P, Testolin G, Blundell JE. Weight, protein, fat, and timing of preloads affect food intake. *Physiol Behav* 1997; **62**: 563–570.
14 Castaneda C, Charnley JM, Evans WJ, Crim MC. Elderly women accommodate to a low-protein diet with losses of body cell mass, muscle function, and immune response. *Am J Clin Nutr* 1995; **62**: 30–39.
15 Hill JO, Melanson EL, Wyatt HT. Dietary fat intake and regulation of energy balance: implications for obesity. *J Nutr* 2000; **130**: 284S–288S.
16 Blouet C, Schwartz GJ. Hypothalamic nutrient sensing in the control of energy homeostasis. *Behav Brain Res* 2010; **209**: 1–12.
17 Lechan RM, Wu P, Jackson IM. Immunolocalization of the thyrotropin-releasing hormone prohormone in the rat central nervous system. *Endocrinology* 1986; **119**: 1210–1216.
18 Hall R, Amos J, Garry R, Buxton RL. Thyroid-stimulating hormone response to synthetic thyrotrophin releasing hormone in man. *BMJ* 1970; **2**: 274–277.
19 Harris AR, Christianson D, Smith MS, Fang SL, Braverman LE, Vagenakis AG. The physiological role of thyrotropin-releasing hormone in the regulation of thyroid-stimulating hormone and prolactin secretion in the rat. *J Clin Invest* 1978; **61**: 441–448.
20 Nillni EA. Regulation of the hypothalamic thyrotropin releasing hormone (TRH) neuron by neuronal and peripheral inputs. *Front Neuroendocrinol* 2010; **31**: 134–156.
21 Hollenberg AN. The role of the thyrotropin-releasing hormone (TRH) neuron as a metabolic sensor. *Thyroid* 2008; **18**: 131–139.
22 Lechan RM, Fekete C. The TRH neuron: a hypothalamic integrator of energy metabolism. *Progr Brain Res* 2006; **153**: 209–235.
23 Cote-Velez A, Perez-Martinez L, Charli JL, Joseph-Bravo P. The PKC and ERK/MAPK pathways regulate glucocorticoid action on TRH transcription. *Neurochem Res* 2008; **33**: 1582–1591.
24 Harris M, Aschkenasi C, Elias CF, Chandrankunnel A, Nillni EA, Bjoorbaek C *et al.* Transcriptional regulation of the thyrotropin-releasing hormone gene by leptin and melanocortin signaling. *J Clin Invest* 2001; **107**: 111–120.
25 Bianco AC, Kim BW. Deiodinases: implications of the local control of thyroid hormone action. *J Clin Invest* 2006; **116**: 2571–2579.
26 Sarkar S, Legradi G, Lechan RM. Intracerebroventricular administration of alpha-melanocyte stimulating hormone increases phosphorylation of CREB in TRH- and CRH-producing neurons of the hypothalamic paraventricular nucleus. *Brain Res* 2002; **945**: 50–59.
27 Cote-Vélez A, Pérez-Maldonado A, Osunab J, Barreraa B, Charlia J-L, Creb Joseph-Bravo P. and Sp/Krüppel response elements cooperate to control rat TRH gene transcription in response to cAMP. *Biochim Biophys Acta (BBA)* 2011; **1809**: 191–199.
28 Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science (New York, NY)* 1996; **273**: 959–963.
29 Shima H, Tohda H, Aonuma S, Nakayasu M, DePaoli-Roach AA, Sugimura T *et al.* Characterization of the PP2A alpha gene mutation in okadaic acid-resistant variants of CHO-K1 cells. *Proc Natl Acad Sci USA* 1994; **91**: 9267–9271.
30 Wadzinski BE, Wheat WH, Jaspers S, Peruski Jr LF, Lickteig RL, Johnson GL *et al.* Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation. *Mol Cell Biol* 1993; **13**: 2822–2834.
31 Luo X, Zhang Y, Ruan X, Jiang X, Zhu L, Wang X *et al.* Fasting-induced protein phosphatase 1 regulatory subunit contributes to postprandial blood glucose homeostasis via regulation of hepatic glycogenesis. *Diabetes* 2011; **60**: 1435–1445.
32 Tamura Y, Shintani M, Nakamura A, Monden M, Shiomi H. Phase-specific central regulatory systems of hibernation in Syrian hamsters. *Brain Res* 2005; **1045**: 88–96.
33 Diop L, Pascaud X, Junien JL, Bueno L. CRF triggers the CNS release of TRH in stress-induced changes in gastric emptying. *Am J Physiol* 1991; **260**: G39–G44.
34 Crupi R, Paterniti I, Campolo M, Di Paola R, Cuzzocrea S, Esposito E. Exogenous T3 administration provides neuroprotection in a murine model of traumatic brain injury. *Pharmacol Res* 2013; **70**: 80–89.
35 Sanchez VC, Goldstein J, Stuart RC, Hovanesian V, Huo L, Munzberg H *et al.* Regulation of hypothalamic prohormone convertases 1 and 2 and effects on processing of prothyrotropin-releasing hormone. *J Clin Invest* 2004; **114**: 357–369.
36 Harper ME, Seifert EL. Thyroid hormone effects on mitochondrial energetics. *Thyroid* 2008; **18**: 145–156.
37 Hernandez DE, Arredondo ME, Xue BG, Jennes L. Evidence for a role of brain thyrotropin-releasing hormone (TRH) on stress gastric lesion formation in rats. *Brain Res Bull* 1990; **24**: 693–695.
38 Garcia SI, Dabsys SM, Martinez VN, Delorenzi A, Santajuliana D, Nahmod VE *et al.* Thyrotropin-releasing hormone hyperactivity in the preoptic area of spontaneously hypertensive rats. *Hypertension* 1995; **26**: 1105–1110.
39 Coll AP, Farooqi IS, O'Rahilly S. The hormonal control of food intake. *Cell* 2007; **129**: 251–262.
40 Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* 1999; **68**: 821–861.
41 Doherty MJ, Young PR, Cohen PT. Amino acid sequence of a novel protein phosphatase 1 binding protein (R5) which is related to the liver- and muscle-specific glycogen binding subunits of protein phosphatase 1. *FEBS Lett* 1996; **399**: 339–343.
42 Alkemade A. Central and peripheral effects of thyroid hormone signalling in the control of energy metabolism. *J Neuroendocrinol* 2009; **22**: 56–63.
43 Bachman ES, Dhillon H, Zhang CY, Cinti S, Bianco AC, Kobilka BK *et al.* betaAR signaling required for diet-induced thermogenesis and obesity resistance. *Science (New York, NY)* 2002; **297**: 843–845.
44 Ohki-Hamazaki H, Watase K, Yamamoto K, Ogura H, Yamano M, Yamada K *et al.* Mice lacking bombesin receptor subtype-3 develop metabolic defects and obesity. *Nature* 1997; **390**: 165–169.
45 Guo F, Bakal K, Minokoshi Y, Hollenberg AN. Leptin signaling targets the thyrotropin-releasing hormone gene promoter *in vivo*. *Endocrinology* 2004; **145**: 2221–2227.
46 Lee SL, Stewart K, Goodman RH. Structure of the gene encoding rat thyrotropin releasing hormone. *J Biol Chem* 1988; **263**: 16604–16609.
47 Valverde O, Mantamadiotis T, Torrecilla M, Ugedo L, Pineda J, Bleckmann S *et al.* Modulation of anxiety-like behavior and morphine dependence in CREB-deficient mice. *Neuropsychopharmacology* 2004; **29**: 1122–1133.
48 Silva AJ, Kogan JH, Frankland PW, Kida S. CREB and memory. *Annu Rev Neurosci* 1998; **21**: 127–148.
49 Mantamadiotis T, Lemberger T, Bleckmann SC, Kern H, Kretz O, Martin Villalba A *et al.* Disruption of CREB function in brain leads to neurodegeneration. *Nat Genet* 2002; **31**: 47–54.
50 Chiappini F, Cunha LL, Harris JC, Hollenberg AN. Lack of cAMP-response element-binding protein 1 in the hypothalamus causes obesity. *J Biol Chem* 2011; **286**: 8094–8105.
51 Chiappini F, Ramadoss P, Vella KR, Cunha LL, Ye FD, Stuart RC *et al.* Family members CREB and CREM control thyrotropin-releasing hormone (TRH) expression in the hypothalamus. *Mol Cell Endocrinol* 2013; **365**: 84–94.
52 Barford D, Das AK, Egloff MP. The structure and mechanism of protein phosphatases: insights into catalysis and regulation. *Annu Rev Biophys Biomol Struct* 1998; **27**: 133–164.
53 Goldberg J, Huang HB, Kwon YG, Greengard P, Nairn AC, Kuriyan J. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* 1995; **376**: 745–753.
54 Price NE, Mumby MC. Effects of regulatory subunits on the kinetics of protein phosphatase 2A. *Biochemistry* 2000; **39**: 11312–11318.
55 Rahmouni K, Sigmund CD, Haynes WG, Mark AL. Hypothalamic ERK mediates the anorectic and thermogenic sympathetic effects of leptin. *Diabetes* 2009; **58**: 536–542.
56 Schrauwen P, Westerterp KR. The role of high-fat diets and physical activity in the regulation of body weight. *Br J Nutr* 2000; **84**: 417–427.
57 Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, Wek RC *et al.* Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science (New York, NY)* 2005; **307**: 1776–1778.

Supplementary Information accompanies this paper on International Journal of Obesity website (http://www.nature.com/ijo)