Original Article

MOLECULAR METABOLISM

Leptin counteracts hypothermia in hypothyroidism through its pyrexic effects and by stabilizing serum thyroid hormone levels

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ABSTRACT

Objective: Thyroid hormones (TH) are essential for the homeostatic control of energy metabolism and the regulation of body temperature. The hypothalamic–pituitary–thyroid (HPT) axis is regulated by negative feedback mechanisms, ensuring that TH levels are maintained at a constant level. However, the feedback mechanisms underlying the resetting of the HPT axis regulation in the control of body temperature are still not fully understood. Here, we aimed to determine the thermoregulatory response in hypothyroid mice to different environmental temperatures and the underlying mechanisms.

Methods: Distinct thermogenic challenges were induced in hypothyroid female C57BL/6N and leptin-deficient *ob/ob* mice through housing at either room temperature or thermoneutrality. The thermogenic and metabolic effects were analyzed through metabolic chambers, 18F-FDG-PET/MRI, infrared thermography, metabolic profiling, histology, gene expression and Western blot analysis.

Results: In hypothyroid mice maintained at room temperature, high leptin serum levels induce a pyrexic effect leading to the stabilization of body temperature through brown adipose tissue thermogenesis and white adipose tissue browning. Housing at thermoneutrality leads to the normalization of leptin levels and a reduction of the central temperature set point, resulting in decreased thermogenesis in brown and white adipose tissue and skeletal muscle and a significant decline in body temperature. Furthermore, anapyrexia in hypothyroid leptin-deficient *ob/ob* mice indicates that besides its pyrexic actions, leptin exerts a stimulatory effect on the HPT axis to stabilize the remaining TH serum levels in hypothyroid mice.

Conclusion: This study led to the identification of a previously unknown endocrine loop in which leptin acts in concert with the HPT axis to stabilize body temperature in hypothyroid mice.

Keywords Thyroid hormone; Thermogenesis; Brown adipose tissue; White adipose tissue browning; Beige adipose tissue; Leptin

1. INTRODUCTION

Circulating thyroid hormone (TH) levels are tightly regulated by the hypothalamic–pituitary–thyroid (HPT) axis. Hypothalamic thyrotropin-releasing hormone (TRH) secretion leads to the release of thyroid-stimulating hormone (TSH) from the pituitary, which stimulates thyroid hormone production and secretion from the thyroid glands. Both at the level of the hypothalamus as well as pituitary, TH can exert negative feedback inhibition of TRH or TSH release, respectively, to curb its own secretion. This tight control of the HPT axis and TH serum levels is moreover affected by multiple other environmental challenges or endogenous hormonal cues such as shortage of food, elevated leptin levels or low environmental temperatures [1].

Temperature homeostasis itself largely depends on TH-driven thermogenesis. Exposure to cold stimulates an adaptive thermogenic response of brown adipose tissue (BAT) by the increase in sympathetic outflow to BAT and by increasing the expression and activity of deiodinase 2 (*Dio2*), resulting in enhanced local conversation of thyroxine (T4) to 3,3',5-triiodothyronine (T3) and thyroid hormone receptor (TR) saturation [2]. Local T3 and the catecholamine norepinephrine (NE)

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Abbreviations: TH, thyroid hormone; HPT, hypothalamic–pituitary–thyroid (HPT) axis; TRH, thyrotropin-releasing hormone; TR, thyroid hormone receptor; 18F-FDG, 18F-fluoro-deoxy-glucose; PET/MRI, positron emission tomography/magnetic resonance imaging

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released from the sympathetic nerve endings act synergistically to stimulate UCP1 expression, mitochondrial uncoupling and heat generation in BAT [3]. Furthermore, TRH itself affects thermogenesis in BAT by the activation of BAT innervating neurons [4,5]. TH also sustain obligatory thermogenesis by direct effects on target genes and tissues, e.g skeletal muscle [2]. Recent studies indicate that TH control peripheral heat loss through the tail surface, which induces compensatory BAT thermogenesis in TRα1 mutant mice [6]. TH induces browning of the white adipose tissue (WAT) by central effects or through direct activation of TRβ [7–10]. Mice with deletion of all TR isoforms display competent BAT recruitment and UCP1 expression but depressed thermogenesis [11]. Similarly, TRα knockout mice suffer from defective thermogenesis despite normal UCP1 levels [12]. Last, hyperthyroid mice had a higher body temperature that was independent of brown or beige fat thermogenesis but rather driven by skeletal muscle thermogenesis and an elevation of the central body temperature set-point [13]. Together, these reports indicate a complex interplay between the central and peripheral TH-regulated pathways to govern body temperature regulation. Overall, a balanced HPT axis appears to be of utmost importance for the stabilization of body temperature.

Human and rodent studies report an inverse relationship between leptin production within adipose tissue and thyroid activity [14–16]. The application of leptin to patients with leptin deficiency leads to the normalization of serum TH and TSH serum levels [17]. Furthermore, processing of leptin is compromised under hypothyroid conditions in mice, which suggests the existence of a regulatory loop by which the TH affects energy homeostasis [18]. The fasting-induced suppression of the HPT axis is an adaptive response to decreased energy expenditure during starvation. Leptin has been proposed as the critical signal to initiate the neuroendocrine response to fasting [19]. In mice with diet-induced obesity, augmentation of the HPT axis is controlled through central leptin signalling [20]. Interestingly, the administration of leptin to gradually cold-exposed double-mutant *ob/ob* $\times$ UCP1$^{-/-}$ mice protects body temperature, whereas vehicle-treated controls develop hypothermia. This was partly through the enhanced production of T3 together with the stimulation of sarcoendoplasmic reticulum Ca(2+) ATPase 2a (SERCA) expression in skeletal muscle [21]. This important role of leptin in thermoregulation was confirmed in *ob/ob* mice subjected to leptin treatment, which reacted with elevated body temperature due to a reduction of heat dissipation through the tail [22,23]. Interestingly, thermogenesis was unaffected in these leptin-treated *ob/ob* mice [22,23]. A number of studies have demonstrated that, in addition to its central actions, leptin may have peripheral actions, such as endothelium-mediated vasodilation, that might inhibit sympathetically induced vasoconstriction [24]. However, the precise mechanisms linking TH and leptin signalling in the regulation of body temperature remain incompletely understood.

In this study, we aimed to close this gap in knowledge by analyzing the thermoregulatory response in hypothyroid mice to different environmental temperatures. Overall, our studies led to the identification of a previously unknown endocrine loop in which leptin acts in concert with the HPT axis to stabilize body temperature.

2. MATERIAL AND METHODS

2.1. Animals, housing and treatment

Female C57BL/6NTac mice were purchased from Taconic Europe (Lille Skensved, Denmark) and female *ob/ob* mice (B6.V-Lep ob/obJRj) were purchased from Janvier Labs (Saint Berthevin Cedex, France). In all experimental cohorts, hypothyroidism was induced in 10-week-old mice by feeding an iodine-free chow diet supplemented with 0.15% 6-*n*-propyl-2-thiouracil (PTU, catalog TD 97061; Harlan Teklan, Madison, WI, USA) for 4 weeks. Housing at room temperature (20–22 °C) represents a moderate but significant cold stress [25]. Therefore, hypothyroidism was induced while mice were housed at room temperature (21 °C) or thermoneutrality (30 °C). Euthyroid mice fed standard chow diet (Altromin GmbH, Lage, Germany) served as controls and were housed under identical conditions ($n = 5$ mice per experimental condition). For leptin treatment, C57BL/6NTac mice were subjected to the same study design but recombinant murine leptin (2 μg/g BW/day, i.p.; Peprotech, Rocky Hill, New Jersey, USA) was administered in their final three days. Following perfusion with PBS and decapitation of the mice, samples were collected from intrascapular brown (BAT), gonadal white (gWAT) and inguinal white (ingWAT) adipose tissue and skeletal muscle (quadriceps), snap frozen in liquid nitrogen and stored at −80 °C until further use. All experiments with mice were carried out according to the guidelines approved by the local authorities of the State of Saxony, Germany, as recommended by the responsible local animal ethics review board (Regierungspräsidium Leipzig, Germany (TVV13/15 and TVV18/16).

2.2. Body composition

Whole body composition (fat mass, lean mass and total body water) was determined in conscious mice by nuclear magnetic resonance technology with an EchoMRI700™ instrument (Echo Medical Systems, Houston, TX, USA). Five animals per experimental group were measured.

2.3. Metabolic chambers

Oxygen consumption, carbon dioxide production, energy expenditure, substrate utilization (respiratory exchange ratio, RER) and home-cage activity were measured in temporally single-house mice using a climate-controlled indirect calorimetry system (TSE System, Bad Homburg, Germany). Analyses were carried out using ANCOVA and the R-based CalR package with body weight as the covariate, as reported previously [26].

2.4. Determination of serum parameters

Serum TT4 and fT3 concentrations were determined using commercial ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Germany). Serum leptin was determined using the mouse leptin ELISA (CrystalChem, Elk Grove Village, USA). Values below the limit of quantification (25 nmol/l for T4 and 1.4 pg/ml for T3) were set to 12.5 nmol/l or 0.7 pg/ml, respectively).

2.5. Type I iodothyronine deiodinase (DIO1) activity

Livers (approximately 50 mg) were homogenized on ice in 0.5 ml PED50 buffer (0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 50 mM dithiothreitol (DTT)). Protein concentrations were measured with the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard following the manufacturer's instructions (Bio-Rad Laboratories, Veenendaal, The Netherlands). Using 7 5 μl of 100–500-fold diluted homogenate incubated for 30 min at 37 °C in a final volume of 0.15 ml with 0.1 μM rT3 and with the addition of approximately 1 $\times$ 10^5 cpm [3,3′5′-125I]rT3 in PED10 (0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 10 mM DTT), we were able to determine DIO1 activity by duplicate measurements on a Shimadzu HPLC system in combination with a Waters Symmetry C18 column (4.6 $\times$ 250 mm, 5 μm) coupled to a Perkin Elmer Radiomatic scintillation analyzer (150 TR Flow). One sample of each group was incubated in the presence of 500 μM PTU in order to inhibit D1

activity representing a tissue blank. DIO1 activity was calculated by subtracting the activity measured in the tissue blank from the activity measured without PTU and expressed as pmol 3,3′T2 generated per minute per mg protein [27].

2.6. Quantitative real-time-PCR (qPCR)

For the quantification of gene expression, qPCR was performed using the LightCycler System LC480 and LightCycler-DNA Master SYBR Green I Kit (Roche, Mannheim, Germany) as described previously [9]. Primer sequences are listed in Supplementary table 1. Gene expression was calculated by the delta-delta Ct method using *Rplp0* as a reference gene [28]. The relative gene expression was calculated by setting the mean of the euthyroid control group to 1 and then calculating each individual value of the groups of mice studied.

2.7. Histomorphology

Inguinal and gonadal WAT (iWAT and gWAT, respectively) as well as intrascapular BAT (iBAT) were collected and fixed in 4% paraformaldehyde (pH 7.4) for 24 h at 4 °C. After paraffin embedding and sectioning, tissues were stained with hematoxylin and eosin. Microscopic examination was performed using an Axio Observer microscope (Carl Zeiss, Jena, Germany). Images were obtained using ZEN2012 software (Carl Zeiss, Jena, Germany).

2.8. ^{18}F-FDG PET/MRI of BAT activation

Small animal PET/magnetic resonance (MR) imaging studies were performed using a dedicated high-resolution scanner (nanoScan, Mediso Medical Imaging Systems, Hungary). Anaesthetized (induction at 4%, maintenance at 1.8% isoflurane in 60%/40% oxygen/air) mice were injected intraperitoneally with 14.5 ± 1.3 MBq [^{18}F]-FDG followed by a list-mode scan between 30 and 60 min after injection. Data reconstruction was performed as described previously [9]. Standardized uptake values (SUVs) of [^{18}F]FDG were determined in manually drawn PET/MR-based volumes of interest (VOIs) in the iBAT, iWAT and liver. To exclude possible unspecific alterations in the [^{18}F]-FDG distribution associated with the hypothyroid state, SUV ratios (SUVR) were calculated to normalize the iBAT/iWAT uptake to the liver uptake [29].

2.9. Brown fat, tail and rectal temperature measurements

For the measurement of surface BAT and tail temperature, infrared thermography was performed at the end of the study during the light phase and at room temperature (VarioCAM® hr; Infratec, Dresden, Germany). Three images of each mouse were taken while the animal was moving freely on the bottom of the cage. Since infrared images already showed that in wild-type mice, heat is dissipated throughout the tail with comparable tail root temperatures, we determined tail temperature at 2.5 cm from the tail root in these mice and at 0.5 cm from the tail root in *ob/ob* mice (see Figures 2C+H).

2.10. Statistical analyses

Data are shown as means ± SEM. As indicated in the figure legends, analysis of variance (ANOVA) or repeated measures ANOVA was used to compare more than two groups, followed by Bonferroni's or Sidak post-hoc test. When more than one variable influenced the variable being measured, two-way ANOVA was performed to test for a significant effect of each variable as well as an interaction between variables followed by Sidak post-hoc test. Statistics were performed using GraphPad Prism software (GraphPad 9.0.2(161), San Diego, CA, USA). Data for energy expenditure were analyzed using ANCOVA with body weight as the covariate as reported previously [30]. The statistical significance was defined as $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

3. RESULTS

3.1. Temperature-dependent regulation of the HPT axis in response to antithyroid treatment

In mice with T3 and T4 levels in the euthyroid (normal) range, housing at thermoneutrality (30 °C) did not influence TH levels in comparison with mice housed at room temperature (21 °C; Figure 1A+B). When mice were subjected to antithyroid PTU treatment to induce hypothyroidism, housing temperature significantly affected TH serum levels. While four weeks of PTU treatment was sufficient to induce systemic hypothyroidism in mice housed at 30 °C, it was not sufficient for littermates housed at 21 °C. In 30°C-housed mice, hypothyroidism was confirmed by a 36% and 95% reduction in serum T3 and T4 levels, respectively, together with a 90% reduction in hepatic *Dio1* mRNA expression and DIO1 activity, a very sensitive marker of TH action [31] (Figure 1C). In mice housed at 21 °C, PTU treatment significantly reduced T4 levels but not fT3 levels nor hepatic *Dio1* gene expression or DIO1 activity (Figure 1D). In summary, the data indicate that thermoneutrality makes mice more susceptible to the induction of hypothyroidism by PTU.

3.2. Inverted thermoregulatory profile in hypothyroid mice in response to ambient temperature

Since TH is important for the regulation of body temperature [2], we next aimed to investigate whether the degree of hypothyroidism is reflected by a change in the body temperature in relation to differing housing temperatures. As depicted in Figure 1E, the decline in TH levels at 30 °C in hypothyroid mice was accompanied by a significant reduction in body temperature. After three weeks of PTU treatment, the body temperatures of hypothyroid mice housed at thermoneutrality dropped significantly compared with corresponding euthyroid controls. In contrast, body temperatures remained stable in hypothyroid mice when housed at 21 °C (Figure 1E). At the end of the study, body temperatures in hypothyroid mice housed at 30 °C were significantly decreased compared with euthyroid littermates ($p < 0.001$) and ~2 °C lower than in hypothyroid littermates housed at 21 °C ($p_{temp} < 0.05$; $p_{interact} < 0.005$; Figure 1F). This is surprising since thermoneutrality is defined as the ambient temperature range where basal metabolism is sufficient to maintain body temperature. Instead, our data suggest an aberrant regulation of body temperature in hypothyroid animals at thermoneutrality but not at room temperature.

3.3. Tail heat loss in PTU-treated mice is dependent on ambient temperature

So far, our data indicate that housing temperature significantly affects the decline of TH levels in hypothyroid mice. In contrast, we also observe the stabilization of body temperature in hypothyroid mice at room temperature but not at thermoneutrality. It is known that adipokine leptin, apart from its regulatory function on the HPT axis, also has a thermoregulatory function [18,32,33]. Therefore, we next aimed to investigate whether leptin signalling is changed in hypothyroid mice at different housing temperatures. As shown in Figure 2A, development of weight gain was similar in hypothyroid mice housed at 21 °C and 30 °C, resulting in comparable body weight at the end of treatment (Figure 2A–B). Independent of the thyroid state, mice housed at thermoneutrality exhibited significantly lower fat mass accompanied by higher lean mass (Figure 2C). Despite comparable fat mass, we

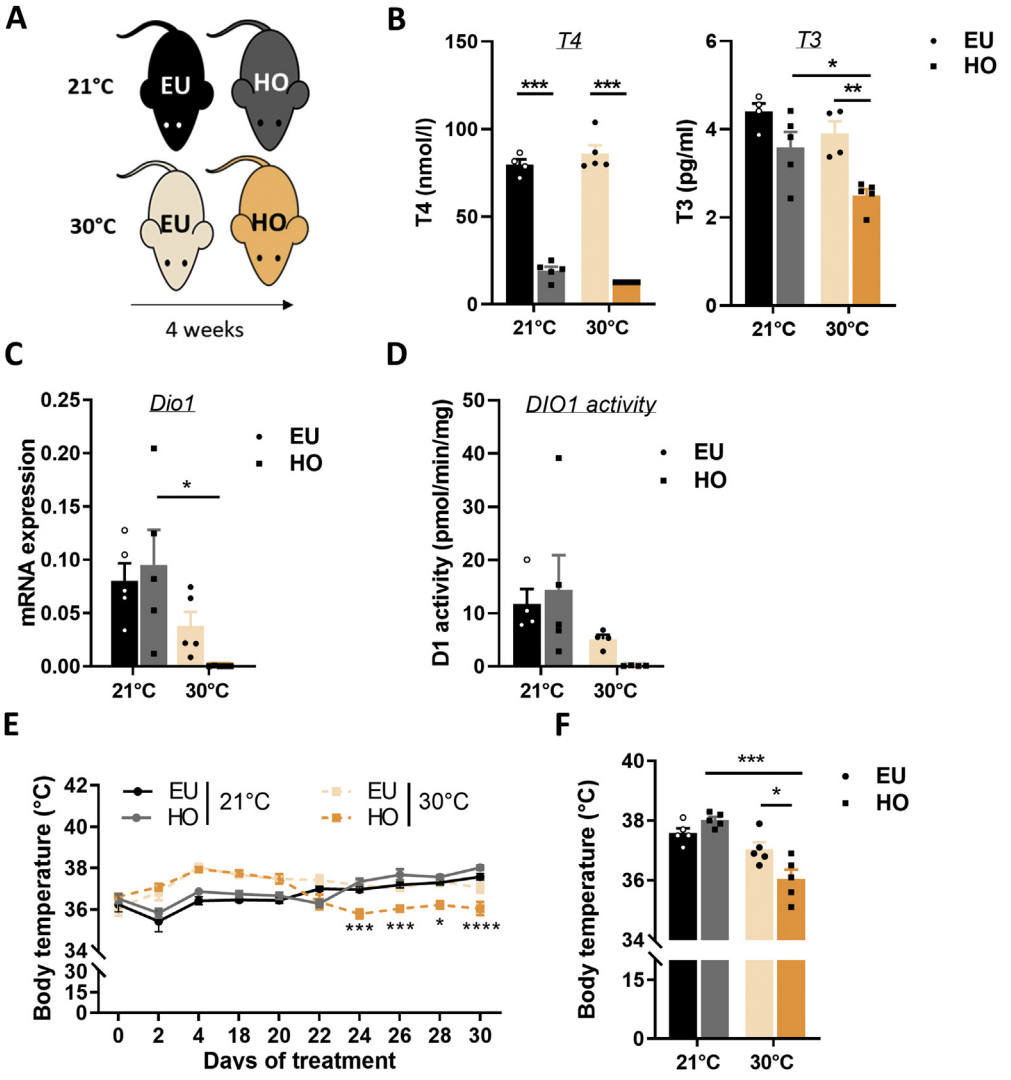

Figure 1: Effect of housing temperature on thyroid hormone serum levels and the regulation of body temperature in euthyroid control (EU) and hypothyroid mice (HO). (A) Schematic representation of housing temperature and thyroid status during experiment. (B) Serum T4 (thyroxine) and T3 (3,3′,5-triiodothyronine) levels as well as (C) hepatic *dio1* (Deiodinase 1) gene expression and (D) DIO1 activity in euthyroid controls and hypothyroid mice after 4 weeks of PTU (6-*n*-propyl-2-thiouracil) treatment and housing at 30 °C or 21 °C. (E) Regulation of body temperature in response to PTU treatment at different housing temperatures over 4 weeks. (F) Final body temperature of euthyroid controls and hypothyroid mice after 4 weeks of PTU treatment. All parameters were measured in euthyroid controls and PTU-treated hypothyroid mice housed at 21 °C or 30 °C ($n = 4$–5/group). Data are represented as mean ± SEM. The statistical significance was determined using two-way ANOVA with Sidak's post-hoc multiple comparisons test, with $p^* < 0.05$, $^{**}p < 0.01$and $^{***}p < 0.001$.

observed elevated leptin serum levels in hypothyroid mice housed at 21 °C compared with those observed in euthyroid controls. Leptin levels were also ∼2.5-fold higher in 21 °C than in 30°C-housed hypothyroid littermates (p_{temp} <0.01; Figure 2D). In leptin-deficient *ob/ob* mice, leptin acts as a pyrexic agent and increases body temperature through the reduction of tail heat loss [22]. Since increased thermal conductance (e.g. heat loss over the tail surface) results from impaired TH signaling [6], we next assessed whether leptin signalling is involved in heat dissipation over the tail surface and thereby contributes to the regulation of body temperature. Therefore, tail surface temperatures were measured by infrared imaging, which indicates vasoconstriction or vasodilation as a surrogate parameter for heat loss [34]. Figure 2E clearly demonstrates distinctive regulation of heat dissipation from tail in hypothyroid mice but not in euthyroid mice in response to housing temperature. Intriguingly, high leptin levels in hypothyroid mice at 21 °C were associated with a massive heat loss over the tail surface, since tail temperatures were ∼3 °C higher than in euthyroid controls (Figure 2E–F). Most remarkably, the tail temperatures of hypothyroid mice at 21 °C housing were ∼2 °C higher than those of 30°C-housed littermates (Figure 2F), potentially indicating an important thermoregulatory role of leptin in hypothyroid mice. Thus, our data propose a model whereby elevated leptin serum levels in hypothyroid mice housed at 21 °C may trigger heat loss over the tail surface while a restoration of leptin levels at 30 °C augments tail vasoconstriction, thereby preventing such a heat loss. To test this hypothesis, we treated leptin-deficient *ob/ob* mice with PTU following the treatment regime of wild-type mice (Figure 2G). Remarkably, the thermoregulatory profile of hypothyroid *ob/ob* mice was diametrically opposed to that observed before in hypothyroid wild-type mice. Similar to wild-type mice, housing temperatures did not affect the regulation of body temperature

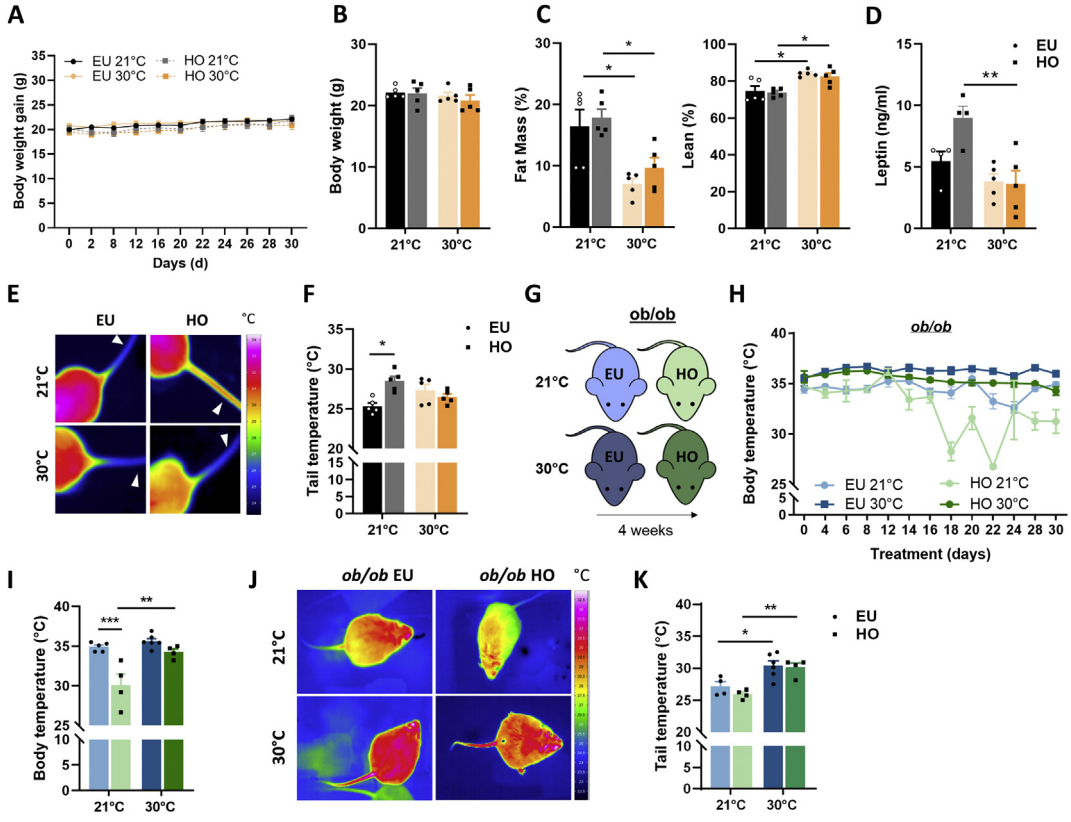

Figure 2: Leptin regulates the tail surface temperature in hypothyroid mice in response to the housing temperature. (A) Body weight gain and (B) mean body weight, (C) fat and lean mass and (D) leptin serum levels of euthyroid control (EU) and 6-*n*-propyl-2-thiouracil (PTU)-treated hypothyroid mice (HO) housed at 21 °C or 30 °C for 4 weeks. ($n = 4–5$/group). (E) Representative images from infrared thermography measuring the tail surface temperature of euthyroid controls and hypothyroid mice kept at 21 °C or 30 °C. (F) Quantification of the tail temperature measured 1.5 cm from the tail base. ($n = 4–5$/group). (G) Schematic representation of the treatment regimen in *ob/ob* mice. (H) Body temperature development and (I) body end-point temperature of euthyroid and hypothyroid *ob/ob* mice kept at 21 °C or 30 °C ($n = 3–4$/group). (J) Representative infrared images reflecting tail surface temperature and (K) quantification of tail temperature in euthyroid and hypothyroid *ob/ob* mice housed at 21 °C or 30 °C, measured 0.5 cm from the tail base. Data are represented as mean ± SEM. The statistical significance between euthyroid and hypothyroid mice housed at 21 °C and 30 °C was determined using two-way ANOVA with Sidak's post-hoc multiple comparisons test, with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

in euthyroid *ob/ob* mice (Figure 2H–I). Hypothyroid *ob/ob* mice failed to stabilize their body temperature at 21 °C and body temperature significantly dropped after 14 days of PTU diet (Figure 2H). At the end of PTU treatment, 21 °C housed hypothyroid *ob/ob* mice were hypothermic and torpid (31.2 ± 1.2 °C; Figure 2I). Tail surface temperatures were significantly lower than in 30 °C housed hypothyroid *ob/ob* littermates ($p < 0.01$; Figure 2J–K). Taken together, these data suggest that although the high leptin levels at 21 °C in hypothyroid wild-type mice may contribute to enhanced tail heat loss, this effect seems negligible compared with their far more important role in the stabilization of the body temperature.

3.4. Leptin stimulates the HPT axis to defend serum T4 levels

Next, we investigated the potential interplay of leptin and temperature on the control of the HPT axis. Previous reports have demonstrated that leptin regulates the HPT axis by direct and indirect stimulation of the TRH neurons in the PVN [32,35]. First, we explored whether leptin deficiency affects the stabilization of T3 and T4 serum levels in euthyroid vs hypothyroid *ob/ob* mice housed at 21 °C or 30 °C (Figure 3A). Overall, housing temperature did not affect TH levels in euthyroid *ob/ob* mice (Figure 3B+C). In hypothyroid *ob/ob* mice housed at 21 °C or 30 °C, T4 levels were reduced to levels below the limit of detection, compared with euthyroid *ob/ob* controls (Figure 3B) Similarly, we found reduced circulating T3 levels in hypothyroid vs. euthyroid *ob/ob* mice at both housing temperatures (Figure 3C). When comparing the TH serum levels between wild-type and *ob/ob* mice, the decline in T3 and T4 serum levels in response to PTU treatment at 21 °C appeared to be more pronounced in *ob/ob* mice than in hypothyroid WT mice (Table 1). The T4 levels of hypothyroid *ob/ob* mice housed at 30 °C were comparable to those of hypothyroid WT mice housed at 30 °C (Table 1), indicating that leptin is required to stabilize the T4 levels in hypothyroid wild-type mice at 21 °C. Nonetheless, it is still unclear why T3 serum levels were reduced after 4 weeks of PTU administration at 30 °C housing but not at 21 °C. In this respect, it was interesting to observe that PTU treatment significantly inhibited hepatic *Dio1* mRNA expression and DIO1 activity at 30 °C but not at 21 °C (Figure 1C). This was not due to a higher uptake of PTU through diet at 30 °C, which otherwise could explain hepatic liver DIO1 activity (Figure 1D).

Several human and rodent data indicate that leptin directly induces hepatic *Dio1* expression and activity [36–38]. We hypothesized that the high DIO1 activity observed in 21°C-housed mice but not in 30°C-housed hypothyroid mice is due to high leptin levels. To address this question, hypothyroid WT mice at both housing temperatures were

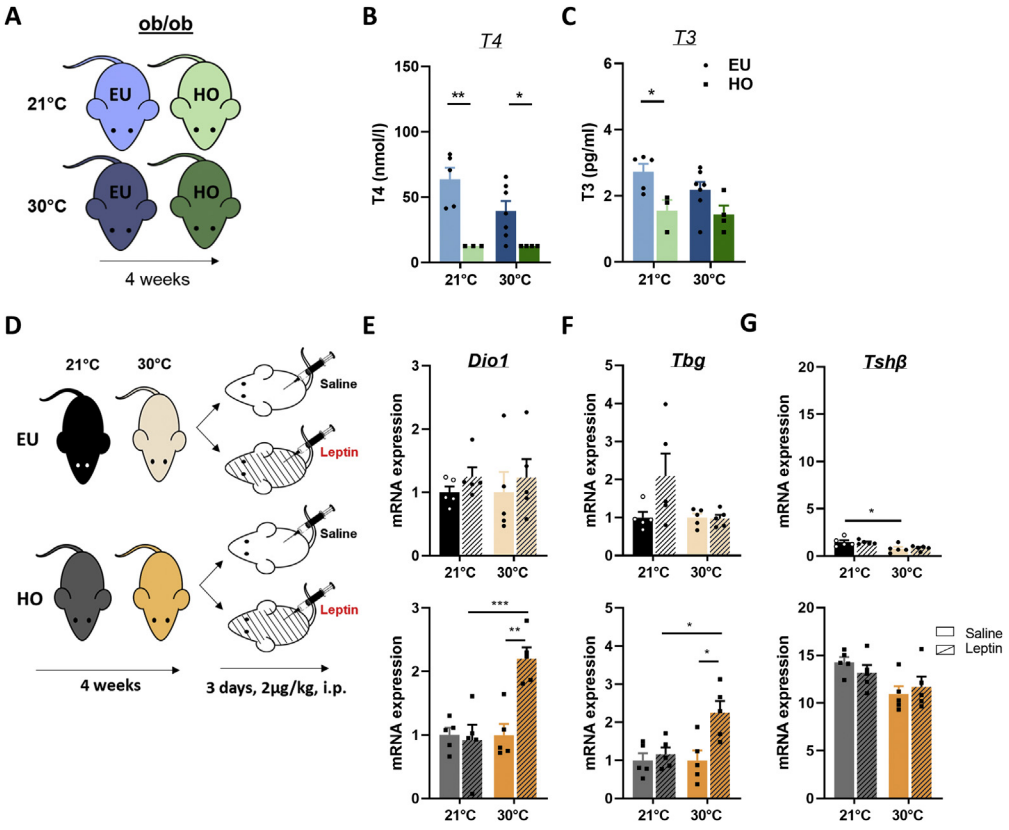

Figure 3: Leptin stimulates the HPT axis to defend serum T4 levels. (A) Representation of housing temperature and thyroid status in *ob/ob* mice. (B) T4 (thyroxine) serum levels and (C) T3 (3,3′,5-triiodothyronine) serum levels in hypothyroid (HO) and euthyroid (EU) *ob/ob* mice. (D) Schematic representation of the housing temperature and treatment regimen of hypothyroid mice treated with leptin. (E) Hepatic gene expression of *Dio1*, (F) *Tbg* and (G) pituitary *Tshβ* in euthyroid and hypothyroid mice housed at 21 °C or 30 °C treated with leptin (2 µg/g BW) or vehicle for 3 days ($n = 4–5$/group). Data are represented as mean ± SEM. The statistical significance between euthyroid and hypothyroid mice housed at 21 °C and 30 °C was determined using two-way ANOVA with Sidak's post-hoc multiple comparisons test, with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Table 1 — Serum levels of fT3, TT4 and TSH in euthyroid and hypothyroid WT and *ob/ob* mice.

		Euthyroid			Hypothyroid		
		T3 (pg/ml)	T4 (nmol/ml)	Leptin	T3 (pg/ml)	T4 (nmol/ml)	Leptin
WT	22 °C	4.40 ± 0.19	79.75 ± 3.02	5.4 ± 0.8	3.58 ± 0.35	21.88 ± 3.40	9.0 ± 0.9
WT	30 °C	3.90 ± 0.28	86.06 ± 4.66	3.8 ± 0.6	2.50 ± 0.15	12.5 ± 0.00[a]	3.6 ± 1.1
ob/ob	20 °C	2.73 ± .24	63.59 ± 8.9	n.d.	1.55 ± 0.33	12.5 ± 0.00[a]	n.d.
ob/ob	30 °C	2.39 ± 0.15	43.83 ± 7.37	n.d.	1.44 ± 0.27	12.5 ± 0.00[a]	n.d.

All values are presented as mean ± SEM.
n.d. — not determined.
[a] Below detection limit. Values were calculated as mean of the lowest standard (25 nmol/l) and blank (0 nmol/l).

treated daily with leptin (2 µg/g BW day; i.p.) during the last three days of the 4-week PTU treatment regime (Figure 3D). Leptin treatment had no effects on hepatic *Dio1* or thyroxine-binding globulin (*Tbg*) expression in euthyroid mice regardless of the housing temperatures (Figure 3E+F). In hypothyroid mice, leptin induced a highly significant increase in *Dio1* and *Tbg* expression at 30 °C but not at 21 °C, compared with the saline-treated controls. Higher hepatic expression levels of *Dio1* and *Tbg* in leptin-treated mice were associated neither with increased T3 or T4 serum levels nor with pituitary TSH mRNA levels (supplementary table 2 and Figure 3G). Together, these results indicate that high leptin serum levels may contribute to stabilized serum TH levels in hypothyroid wild-type mice at 21 °C, possibly through direct activation of peripheral TH metabolizing enzymes.

3.5. SNS-driven BAT thermogenesis compensates for heat loss in PTU-treated mice

So far, our data suggest the following scenario: In hypothyroid mice at 21 °C, higher leptin serum levels help stabilize the body temperature and TH serum levels, with simultaneous increase in heat loss *via* the tail surface. The absence of sympathetic stimulation at 30 °C augments tail vasoconstriction through the normalization of leptin serum levels but fails to stabilize body temperature. The significant decline in T3 and T4 levels below the required threshold may further explain why thermogenesis remains impaired despite the observed tail vasoconstriction. To evaluate the role of leptin in TH-driven regulation of body temperature, we next performed a metabolic characterization of hypothyroid and euthyroid mice at both housing temperatures.

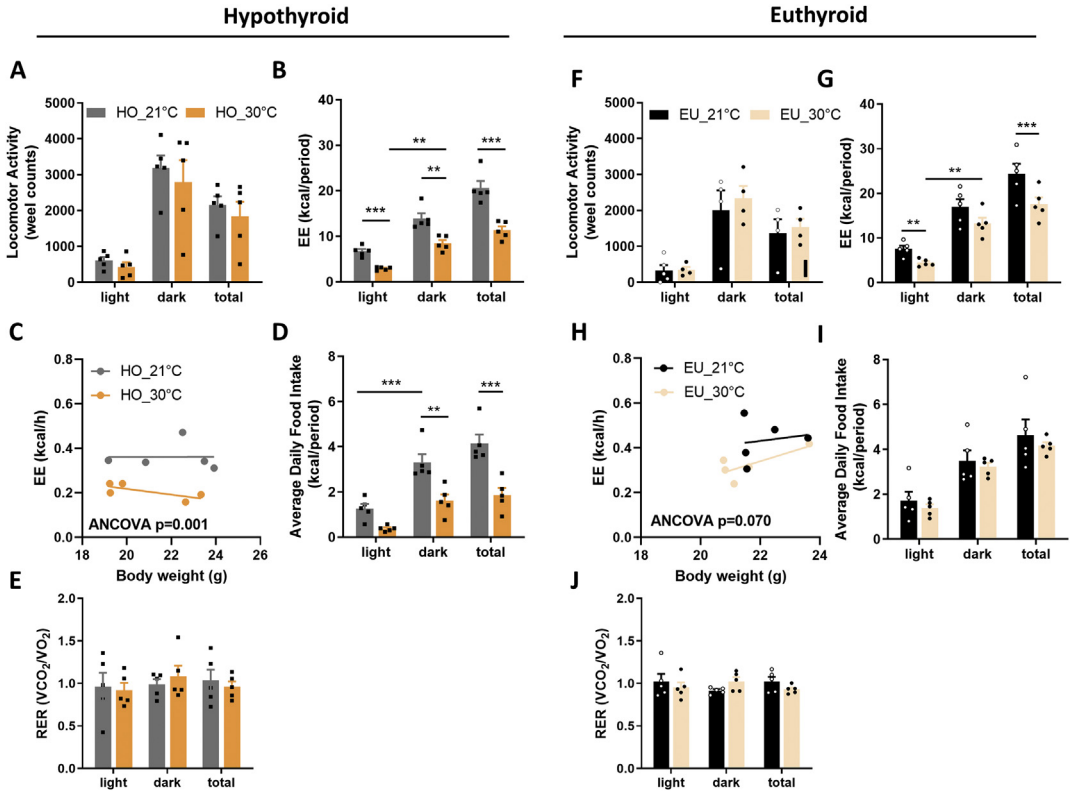

Figure 4: Effects of housing temperature on energy expenditure in hypothyroid mice. Locomotor activity and energy expenditure (EE), food intake and respiratory exchange ratio (RER) were calculated in total and during the light and dark phase in hypothyroid mice (A, B, D, E) and euthyroid animals (F, G, I, J) while housed at 21 °C or 30 °C for 48 h. (C) Regression plots of EE against body weight with the ANCOVA test using body weight as the covariate, with $p = 0.001$ for hypothyroid mice housed at 21 °C or 30 °C. (H) Regression plots of EE against body weight with ANCOVA test using body weight as the covariate; $p = 0.070$ for euthyroid mice housed at 21 °C or 30 °C. The statistical significance was determined using two-way ANOVA with Sidak's post-hoc multiple comparisons test and the Student's *t*-test, with $^{**}p < 0.01$ and $^{***}p < 0.001$.

Regardless of thyroid status, housing temperature did not affect locomotor activity (Figure 4A+F). Thermoneutral housing led to a marked reduction in energy expenditure (EE) in hypothyroid mice (Figure 4B). Following ANCOVA to adjust for differences in body weight, energy expenditure of hypothyroid mice was higher at 21 °C compared with 30 °C housing temperature ($F(1,7) = 25.694$, $P = 0.001$, partial $\eta^2 = 0.730$; Figure 4C). Food intake was significantly higher in hypothyroid mice maintained at 21 °C with an unaltered respiratory exchange ratio (RER), indicating no differences in preference for carbohydrates and lipids as fuels to fit the increased energy demand (Figure 4D–E). Euthyroid mice showed no phenotypic changes in food intake or RER between housing temperature (Figure 4I–J). EE was reduced in euthyroid mice housed at 30 °C, but this was not significant after adjustment in body weight ($F(1,7) = 4.533$, $P = 0.070$, partial $\eta^2 = 0.393$; Figure 4G–H)). Altogether, these findings suggest that housing temperature is a significant variate for EE in hypothyroid mice. Since BAT is a major thermogenic target of TH, we next quantified heat radiation from the scapular region by thermal imaging as a marker for iBAT thermogenesis. When housed at 30 °C, the iBAT temperatures of hypothyroid mice were 1.7 °C lower than those of hypothyroid mice housed at 21 °C ($p < 0.001$; Figure 5A). Hypothyroid mice housed at 21 °C further displayed the typical brown multilocular lipid droplet phenotype in their iBAT compared with the unilocular cell phenotype of iBAT from littermates housed at 30 °C (Figure 5B). Consistent with this, we found an elevated expression of thermogenic genes (*Ucp1, Dio2, Cidea and Elovl3*) in the iBAT of 21°C-housed hypothyroid mice compared with 30°C-housed littermates (Figure 5C). Last, small animal PET/MRI demonstrated decreased uptake of [^{18}F]FDG in the iBAT of hypothyroid mice at 30 °C vs. 21°C-housed littermates ($p < 0.01$; Figure 5D). Prompted by the altered BAT morphology, elevated expression of thermogenic genes and increased glucose uptake, we next assessed whether chronically increased sympathetic nervous system (SNS) activity could explain this hyperactive BAT state. The first evidence for such an elevated SNS activity was the decrease in the iBAT expression of the beta-3 adrenergic receptor (*Adrb3*) in hypothyroid mice at 21 °C housing temperature (Figure 5C). A*drb3* expression is known as the inversely correlated marker for SNS activity, and its decreased expression points to a desensitizing mechanism in response to increased basal BAT NE turnover [39]. Lipolysis was decreased in BAT at 21 °C, as demonstrated by the lack of phosphorylated hormone-sensitive lipase (pHSL) in Western blot analysis (Figure 5E). Consistent with this, the amount of UCP1 protein levels was higher in hypothyroid mice housed at 21 °C (Figure 5F). Overall, these data suggest a critical role for the SNS in the thermoregulatory activation of iBAT in hypothyroid mice exposed to mild cold stress.

3.6. PTU treatment induces browning of iWAT and SERCA activation in skeletal muscle

Next to BAT, thermogenesis is mostly driven by skeletal muscle and, possibly, beige inguinal WAT (iWAT). Here, prompted by our findings in BAT, we first assessed the browning of the iWAT in hypothyroid mice housed at 21 °C or 30 °C. Multilocular "beige" adipocytes in the iWAT of hypothyroid mice were predominantly detected in mice housed at

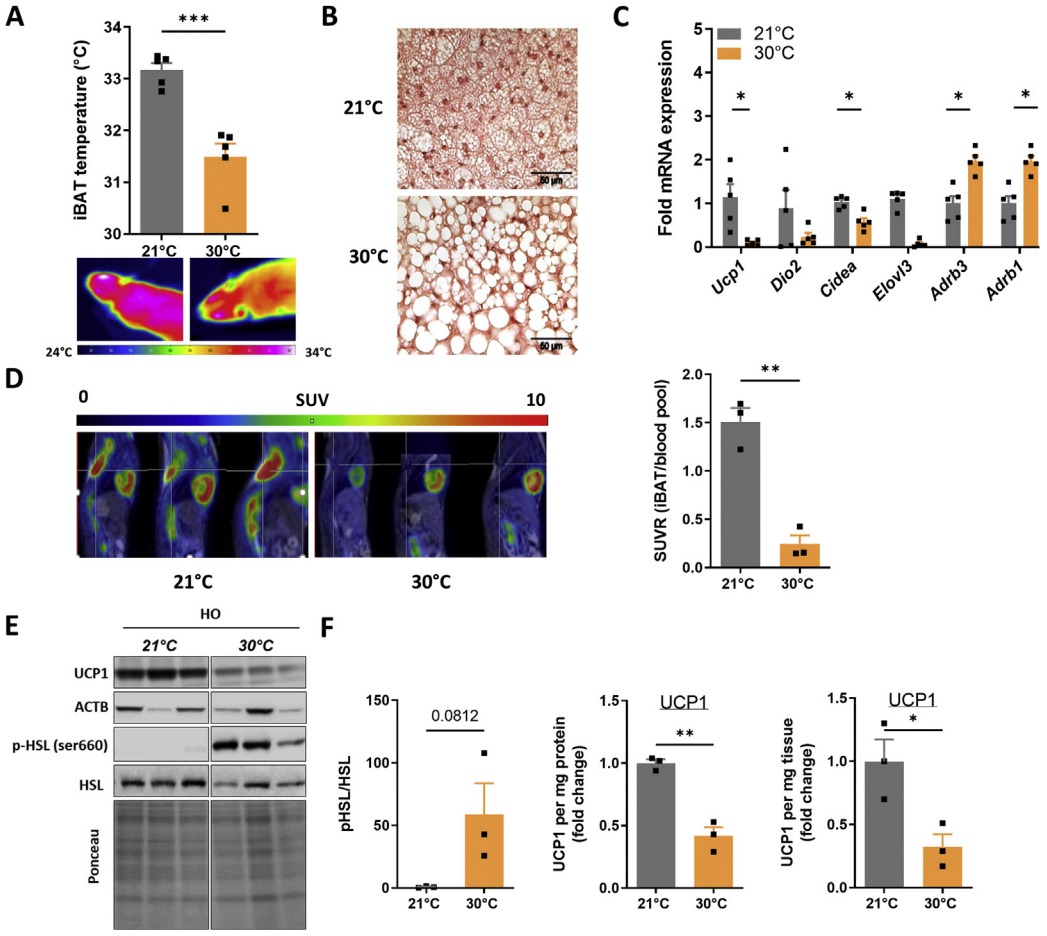

Figure 5: BAT thermogenesis of hypothyroid mice at 21°C or 30°C housing temperature. (A) Representative images reflecting heat dissipation from BAT and quantification of BAT temperature in hypothyroid mice housed at 21 °C or 30 °C ($n = 4–5$/group). (B) Representative images of hematoxylin and eosin (H&E) staining of BAT and (C) mRNA levels of thermogenic genes expressed in the BAT of hypothyroid mice at 21 °C and 30 °C ($n = 5$/group). (D) [^{18}F]FDG PET/MR fused images and SUVR of iBAT of hypothyroid mice housed at 21 °C or 30 °C ($n = 3$/group). (E) Immunoblots of UCP1, phosphorylated HSL (Ser660) and total HSL in the biBAT of hypothyroid mice kept at 21 °C or 30 °C. ($n = 3$/group). (F) Relative UCP1 content per mg of protein and mg of tissue ($n = 3$/group). The statistical significance between euthyroid and hypothyroid mice housed at 21 °C and 30 °C was determined using the Student's *t*-test. Data are represented as mean ± SEM, with $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

21 °C but also occurred to a lesser extent at 30 °C housing temperature (Figure 6A). As in BAT, the expression of thermogenic genes was upregulated in the iWAT of hypothyroid mice at 21 °C vs. 30 °C (Figure 6B). This corresponded with higher UCP-1 protein levels and increased uptake of [^{18}F]]FDG in the iWAT of mice housed at 21 °C compared with hypothyroid mice housed at 30 °C (Figure 6C+D).

To evaluate the contribution of muscle activity to the regulation of body temperature, we quantified the expression of genes that have previously been described in the context of TH-induced thermogenesis in the skeletal muscle [13], and found them, except for *Sln*, to be collectively downregulated in hypothyroid mice at 30 °C versus littermates housed at 21 °C (Figure 6E). Notably, sarcolipin (*Sln*) expression was increased >18 fold in hypothyroid mice housed at 30 °C, suggesting a compensatory mechanism in response to impaired TH signaling in muscle and in BAT [21,40].

Taken together, these data indicate that elevated leptin levels in hypothyroid mice at 21 °C exert a pyrexic effect and stabilize TH levels, leading to increased thermogenesis in iBAT and iWAT, which compensates for the augmented tail heat loss. In contrast, 30 °C housing prevents the increase in leptin levels and tail heat loss, but the robust decline in T3 and T4 levels strongly impairs iBAT, skeletal muscle and iWAT thermogenesis, which explains the decrease in the body temperature (Figure 7).

4. DISCUSSION

In this work, we observed an inverted thermoregulatory profile in mice with systemic hypothyroidism in response to housing temperature, which is due to a complex interaction between leptin signaling and the HPT axis. Leptin levels are increased in hypothyroid mice in response to ambient housing temperature, while thermoneutrality prevents leptin upregulation. High leptin levels appear to be required for stabilizing the body temperature in hypothyroid wild-type mice, as leptin-deficient *ob/ob* mice housed at the ambient temperature became hypothermic and torpid in response to PTU treatment (Figure 2G). Our data further show that the mechanisms by which leptin reverses the decline in body temperature are likely based on a concerted pyrexic action of leptin on central thermoregulation and on peripheral TH levels. The concept of TH-induced thermoregulation through peripheral vasoconstriction was recently exemplified in TRα1 mutant mice, in which lower body temperature despite elevated BAT thermogenesis is due to excessive heat loss through the tail surface [6]. The TRα1

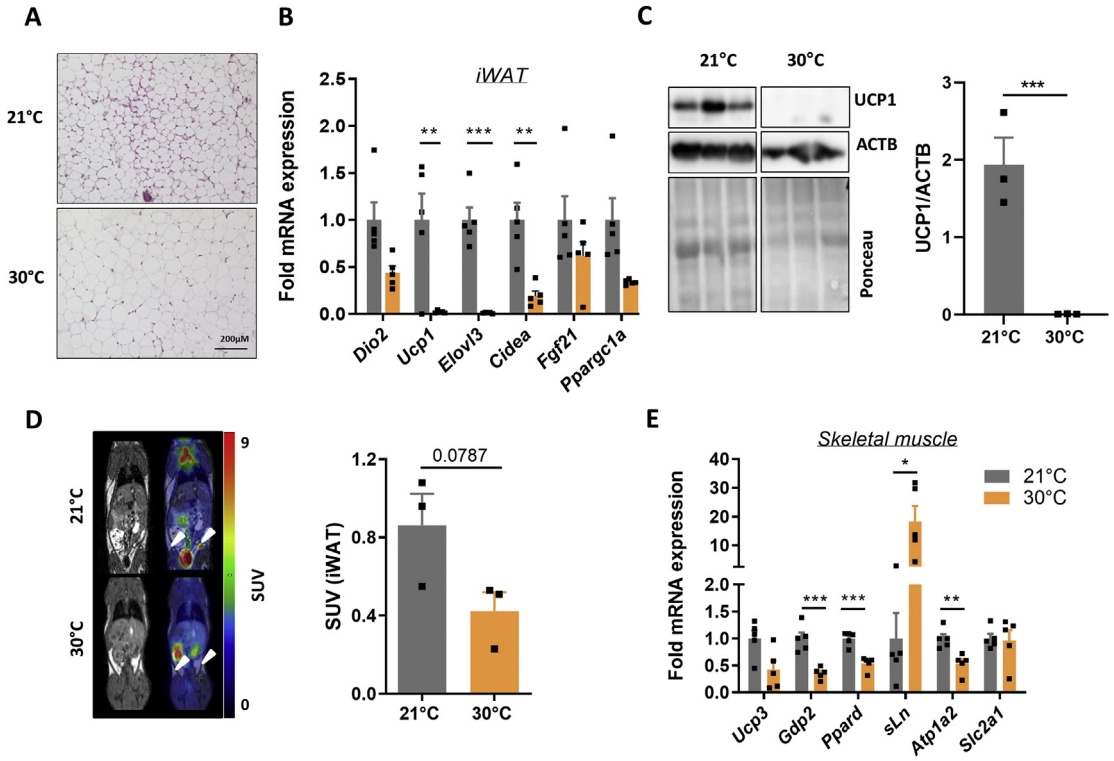

Figure 6: Thermogenic capacity of the iWAT and skeletal muscle of hypothyroid mice housed at 21°C or 30°C. (A) Representative images of hematoxylin and eosin (H&E) staining of iWAT from PTU-treated mice kept at 21 °C or 30 °C. (Scale bar, 200 µm) (B) Expression of thermogenic genes and (C) UCP1 protein in inguinal white adipose tissue of hypothyroid mice (HO) ($n = 4–5$/group). (D) Representative [^{18}F]FDG/MRI images and the corresponding ratios of the standard uptake value (SUVR) in inguinal adipose tissue (arrows) in PTU-treated and untreated control mice kept at 21 °C or 30 °C taken 45 min after [^{18}F]FDG injection ($n = 3$/group). (E) Expression of TH target genes in the skeletal muscle of hypothyroid mice housed at 21 °C and 30 °C ($n = 4–5$/group). qPCR data are expressed as fold variation relative to hypothyroid mice housed at 21 °C ($n = 4–5$/group). The statistical significance was determined using a multiple t-test with correction for multiple comparison using the Holm–Sidak method, with $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

mutation causes a 10-fold reduced T3 binding affinity and significantly impairs tail artery contractions upon adrenergic stimulation [6]. Our data suggest that, depending on the housing temperature, TH and leptin signaling may converge to increase body temperature. At 21 °C and in a hypothyroid state, high leptin levels appear to alter the central set-point controlling body temperature [6,41,42], resulting in the activation of thermogenesis in BAT, WAT and skeletal muscle. This phenomenon appears to be independent of the vasoconstriction in the tail and driven by the impairment of adrenergic sensitivity [6,41,42]. At thermoneutrality, the body temperature of hypothyroid mice declines rapidly due to the absence of pyrexic effects, normalized leptin levels and intact tail artery vasoconstriction.

Our data suggest the possibility that leptin is a causal factor for the regulation of body temperature in hypothyroid mice in response to housing temperature. Indeed, body temperature in hypothyroid *ob/ob* mice is reciprocally regulated in response to ambient vs. thermoneutral housing compared with hypothyroid wild-type mice; i.e., in hypothyroid *ob/ob* mice housed at 21 °C, we observed vasoconstriction accompanied by hypothermia but vasodilation with increased heat dissipation at 30 °C (Figure 2H). The absence of leptin in *ob/ob* mice was further associated with a strong decline in TH levels in response to PTU treatment, which was independent of the housing temperature. The direct comparison of TH levels from hypothyroid wild-type mice with that of hypothyroid *ob/ob* mice indicates the ability of leptin to stabilize TH levels in hypothyroid mice at 21 °C (Table 1). Thus, high leptin levels are required to maintain TH levels in hypothyroid mice at room temperature. This phenomenon has been observed before in mice with diet-induced obesity, in which leptin directly regulates TRH neurons, which enables the maintenance of TH levels [20]. Another study by Vella et al. indicated that reduced leptin levels during fasting are associated with the suppression of *Trh* expression in the PVN and the activation of hepatic pathways that metabolized T4 in order to reduce T4 levels during nutritional stress [43]. However, changes in gene expression involved in hepatic TH metabolism that would explain the stabilized TH levels, e.g. *Cyp2b10*, *Sulta1d1*, *Sulta2a1*, *Ugt1a1* or *Cyp7b1*, were not observed in our cohort (data not shown). Other studies suggest the direct modulation of DIO1 activity by leptin [37,44]. In this respect, it is worth noting that the acute administration of leptin to hypothyroid mice induced a strong increase in *Dio1* and *Tbg* mRNA expression in liver at 30 °C but not at 21 °C (Figure 3D). Chronically elevated leptin levels may stabilize TH levels in hypothyroidism through altered local TH action. However, in our hands, acute leptin challenges did not translate into higher TH levels (supplementary table 2).

In summary, our findings reveal that a balanced TH state appears to be a prerequisite for the regulation of body temperature upon environmental challenges. By controlling circulating TH levels and local TH action, our body can fine-tune heat production in thermogenic tissues as well as heat dissipation and conservation. Our data are further

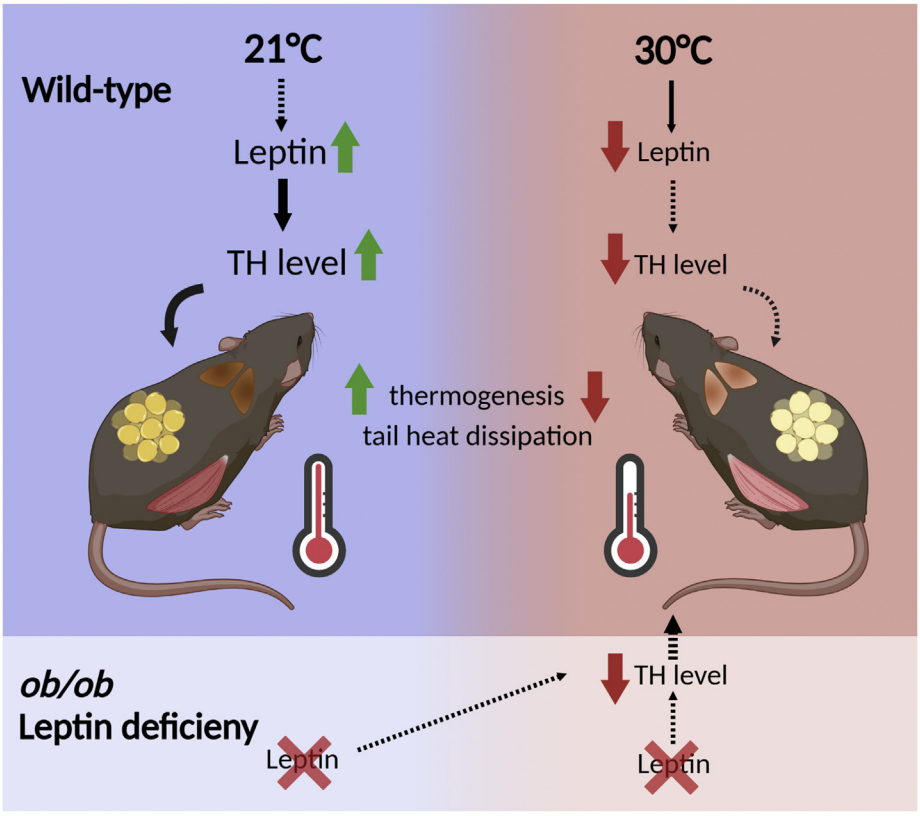

Figure 7: The proposed model showing that high leptin levels in hypothyroid mice at 21°C induce a pyrexic effect and, through stabilization of the TH levels, lead to thermogenesis in BAT, WAT and muscle. At 30 °C, thyroid hormone (TH) levels in hypothyroid mice drop through normalized leptin serum levels. Consequently, the absence of the pyrexic effect together with intact tail vasoconstriction and low TH levels impair thermogenesis in BAT, iWAT and muscle. This phenomenon is reflected in leptin-deficient *ob/ob* mice, wherein hypothyroidism is associated with a rapid drop in the TH levels and significant reduction in the body temperature regardless of the housing temperature. The graphic was created with BioRender.com.

consistent with the recently established role of central TH signaling as a rheostat for the CNS control of the body temperature set point, governed *via* a complex interplay of systemic TH action, SNS signalling and BAT thermogenesis [13,45–47]. Thermoregulatory pathways induced by TH are highly dynamic, and subtle dose- or time-dependent changes may provoke robust changes in thermoregulatory organs. In our studies, we may have reached a "sweet spot" in the transition from euthyroidism to hypothyroidism, where T3 was still sufficient to allow thermogenesis in BAT, iWAT and muscle. Leptin appears to play a fundamental role in this triangular control of TH action, adrenergic sensitivity and adipocyte thermogenesis at this transitional TH state. Identifying the exact mechanisms for this leptin–TH interplay is thus urgently warranted, as they will greatly contribute to our understanding of the central and peripheral effects of TH.

AUTHOR CONTRIBUTIONS

JW: experimental work, animal studies, data analysis and interpretation; LR: experiments and data interpretation; MK and PB: [^{18}F]]FDG PET/MRI and data analysis; AB: DIO1 activity measurements and data analysis; PTP: data analysis and interpretation; NK and JTH: experimental work and data interpretation; MB, AT and MS: data interpretation; JM: conceptualization, data interpretation and writing of the manuscript; KK: conceptualization, supervision, experimental work, validation, data analysis and interpretation and writing of the manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101348.

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