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Essential role of UCP1 modulating the central effects of thyroid hormones on energy balance

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ABSTRACT

Objective: Classically, metabolic effects of thyroid hormones (THs) have been considered to be peripherally mediated, i.e. different tissues in the body respond directly to thyroid hormones with an increased metabolism. An alternative view is that the metabolic effects are centrally regulated. We have examined here the degree to which prolonged, centrally infused triiodothyronine (T3) could in itself induce total body metabolic effects and the degree to which brown adipose tissue (BAT) thermogenesis was essential for such effects, by examining uncoupling protein 1 (UCP1) KO mice.
Methods: Wildtype and UPC1 KO mice were centrally-treated with T3 by using minipumps. Metabolic measurements were analyzed by indirect calorimetry and expression analysis by RT-PCR or western blot. BAT morphology and histology were studied by immunohistochemistry.
Results: We found that central T3-treatment led to reduced levels of hypothalamic AMP-activated protein kinase (AMPK) and elevated body temperature (0.7 °C). UCP1 was essential for the T3-induced increased rate of energy expenditure, which was only observable at thermoneutrality and notably only during the active phase, for the increased body weight loss, for the increased hypothalamic levels of neuropeptide Y (NPY) and agouti-related peptide (AgRP) and for the increased food intake induced by central T3-treatment. Prolonged central T3-treatment also led to recruitment of BAT and britening/beiging ("browning") of inguinal white adipose tissue (iWAT).
Conclusions: We conclude that UCP1 is essential for mediation of the central effects of thyroid hormones on energy balance, and we suggest that similar UCP1-dependent effects may underlie central energy balance effects of other agents.

1. INTRODUCTION

Thyroid hormones (THs) 3,3′,5,5′ tetraiodothyronine (T4) and 3,3′,5-triiodothyronine (T3) control important biological processes, including metabolism and energy balance [1—3]. Hyperthyroidism is associated with an increase in metabolic rate. There are basically two models for how thyroid hormones induce this hypermetabolism. According to the classical view, the effect is (mainly) peripheral, i.e. thyroid hormones affect peripheral tissues directly and increase their metabolism through mechanisms not presently fully understood. In contrast, according to a novel view, the effect is (mainly) central, in that thyroid hormones may affect centers in the brain that stimulate the metabolism of certain peripheral organs via the (sympathetic) nervous system (SNS) [4—6]. Indeed, both types of thyroid hormone action may occur.

An organ that is likely to be responsible for an increase in metabolism is brown adipose tissue (BAT). Due to the presence of the uncoupling protein 1 (UCP1) in the mitochondria of BAT, the tissue has a special ability and high efficiency for converting food or stored energy (body fat) into heat [7]. The possibility of central effects of thyroid hormones in the regulation of BAT thermogenesis was proposed a long time ago [4], but this was mainly based on a central effect of thyroid hormones on body temperature control and, through this, a secondary effect on BAT. We later presented evidence for a more specific link between the central effects of thyroid hormones on BAT [5]. Particularly, central T3 affects cells in the ventromedial nucleus of the hypothalamus (VMH). This decreases the phosphorylation (and thus the activity) of AMP-activated protein kinase (AMPK) in the tissue, and this leads to increased tone in the sympathetic nerves innervating BAT. However, an actual increase in metabolic rate signaled through this pathway has not as yet been demonstrated, and it is therefore also unknown to what extent the stimulation of BAT is responsible for the metabolic thermogenic response and for the other metabolic effects of central T3-treatment. The aim of the present study, therefore, was to

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investigate the role of BAT and UCP1 on the thermogenic effects of central T3-treatment. We found that the entire thermogenic effect of central T3-treatment was dependent upon the presence of UCP1. Additionally, further metabolic effects, such as T3-induced hyperphagia, were also entirely UCP1-dependent. The possible significance of these observations on the understanding of T3 effects in model animals, as well as in humans, is evident, and this pathway may additionally be involved in the mediation of broad metabolic effects of other centrally acting agents.

2. EXPERIMENTAL PROCEDURES

2.1. Animals

Ucp1(−/−) (UCP1 KO) male mice (8-10 weeks-old; initially generated by replacing exon 2 and part of exon 3 with a neomycin resistance gene by homologous recombination [8]) and their corresponding wildtype (*C57BL/6*), to which the UCP1 KO mice had been backcrossed for >10 generations, were obtained from our own breeding of inbred lines in the *Wenner-Gren Institute*.

2.2. Experimental layout

The mice were acclimated for one month to 18 °C to recruit BAT. They had *ad libitum* access to standard chow and tap water and were exposed to a 12 h light/12 h dark cycle. During the third week at 18 °C, they were separated into single cages. Throughout the actual experiment, the mice were moved to a thermoneutral environment (30 °C with relative humidity of 45−52%) in order to acutely eliminate the extra-metabolism needed to defend the body temperature at lower temperatures. Body temperature, body weight and quantity of chow consumed by each mouse were measured every day at 10.30−11.30 AM (i.e. in the middle of the light phase). The general layout of the experiment was as follows: on day 0, after measuring body temperature and body weight, the mice underwent surgery to position the osmotic minipumps delivering vehicle or T3 directly into the brain. For the anesthesia, we added 1 ml of ketamine and 250 μl of xylazine to 6 ml of saline and the volume injected was 100 μl per 10 g of body weight. Mice were then moved to 30 °C for recovery. On day 1, mice were kept undisturbed at 30 °C. On day 2, the mice were placed in indirect calorimetry metabolic chambers (the temperature in the chambers was set to 30 °C). On days 3 and 4, calorimetric measurements were performed; mice were taken out of the boxes for a short period of time each day to measure different parameters. On day 5, mice were removed from the metabolic chambers and placed in a room at 30 °C. On day 6, body composition was measured, and mice were anesthetized with CO_2 and then decapitated. Several tissues were dissected out. We used 10 animals per experimental group. Since there is a limitation in the number of metabolic cages, mice were divided in 5 cohorts each containing 2 animals for each treatment (2 WT/vehicle + 2 WT/T3 + 2 KO/vehicle + 2 KO/T3). One wildtype mouse from the vehicle-treated group was removed from all compilations because it behaved totally differently from the rest of the mice in the same group (including this mouse yields a P value of 0.06 for the T3 effect on the mean metabolic rate). The study was approved by the *Animal Ethics Committee of the Northern Stockholm Region*.

2.3. Central T3 administration

Central T3 was administered by osmotic minipumps (micro-osmotic pump, model 1007D, *Alzet;* Cupertino, CA, USA) that were placed subcutaneously in the scapular region of mice. The minipumps were directly connected to an intracerebroventricular (ICV) cannula targeting the lateral right ventricle in the brain, as previously described [9—11].

T3 (8 ng/day; *Sigma*; St Louis, MO, USA) or its vehicle (saline + 1 mM NaOH) was continuously delivered during the experiment. The dose of T3 was selected on the basis of our former studies [5,12], with consideration of the higher metabolism of mice as compared to rats. (This dose is infinitely small compared to the doses that are used to induce T3 effects peripherally and found not to affect circulating levels of T4 and T3, demonstrating lack of leaking from the cerebrospinal fluid to the circulation [5,12,13]). Immediately after the surgery, mice were placed at 30 °C.

2.4. Metabolic measurements

Every day (from day 0 to day 6) between 10:30−11:00 AM, mice were taken out of the chambers (if relevant), and body temperature was determined with a rectal probe for mice (BAT-12 microprobe thermometer, *Physitemp Instruments, Inc*; Clifton, NJ, USA). Body weight and the quantity of food consumed were also measured at this time. Food was weighed when supplied, and remaining food was subtracted to calculate the food eaten per day.

2.5. Indirect calorimetry

Indirect calorimetry was analyzed with the *INCA System* (*Somedic*; Hörby, Sweden) [14]. Two days after the placement of the osmotic minipump, mice in their home cages were placed in a temperature-controlled (30 °C) box through which air was pumped. After calibrating the system with the reference gases (18% and 25% O_2 in N_2), the metabolic rate was measured for 3 nights and the corresponding days. O_2 consumption (VO_2) and respiratory quotient (RQ) were recorded every second minute.

2.6. Tissue dissection

At the end of the treatment (day 6), mice were sedated with CO_2 before decapitation and dissected to collect brain, interscapular brown adipose tissue (iBAT) and inguinal white adipose tissue (iWAT). Tissues were weighed, quickly frozen in liquid nitrogen and stored at −80 °C until use.

2.7. Western blotting

Mediobasal hypothalamic dissections (removed from the frozen brains) and iBAT total samples were homogenized in lysis buffer (consisting of a mix of 5 ml Tris−HCl, 0.5 ml 0.2 M EGTA, 0.5 ml 0.2 M EDTA, 1 ml Triton-X 100, 1 ml 0.1 M sodium orthovanadate, 0.21 g sodium fluoride, 0.22 g sodium pyrophosphate and 9.2 g of 0.27 M sucrose, made up with distilled water to 100 ml and adjusted to 7.5 pH) and freshly added protease inhibitor cocktail tablets (*Roche Diagnostics*; Indianapolis, IN, USA). The protein concentration was determined by the *Bradford Method* and the total protein content of the tissues was calculated. The protein lysates were subjected to SDS-PAGE, electrotransferred to polyvinylidene difluoride membranes (PVDF; *Millipore*; Billerica, MA, USA) with a semidry blotter and probed with antibodies against UCP1 and AgRP (*Abcam*; Cambridge, UK), pAMPKα-Thr172 (*Cell Signaling*; Danvers; MA, USA), NPY (*Santa Cruz Biotechnology*; Dallas, TX, USA), α-tubulin and β-actin (*Sigma*; St Louis, MO, USA), as previously described [5,10,11,15—17]. Each membrane was then incubated with the corresponding secondary antibody: anti-mouse, anti-rabbit or anti-goat (all of them from *DAKO*, Glostrup, Denmark). The membranes were exposed to an X-ray film and developed using developer (*Developer G150, AGFA HealthCare*: Mortsel, Belgium) and Fixator (*Manual Fixing G354; AGFA HealthCare*; Mortsel, Belgium). Quantification was performed with the *ImageJ 1.33* software (*NIH*, Bethesda, MD, USA). Values were expressed relative to β-actin levels (hypothalamic samples) or α-tubulin (iBAT samples). No significant

changes were found in the protein levels of the loading controls (see below; Supplemental Figure 3).

2.8. Real-time quantitative PCR

Real-time PCR was performed to analyze UCP1 mRNA levels in iWAT. Specific sets of primers and probes for UCP1 (*TaqMan® Gene Expression Assays, Ref Mm00494070_m1*; *Life Technologies*; Carlsbad, CA, US) were used, as previously described [5,10]. All reactions were carried out using the following cycling parameters: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Values were expressed relative to HPRT (hypoxanthine-guanine phosphoribosyl-transferase) levels (Probe: FAM-5′- CGACCCTCAGTCCCAGCGTCGT-GAT-3′-TAMRA, Fw Primer: 5′-AGCCGACCGGTTCTGTCAT-3′, Rv Primer: 5′-GGTCATAACCTGGTTCATCATCAC -3′; *Eurofins Genomics*; Les Ulis, France).

2.9. Hematoxylin-eosin staining and UCP1 immunohistochemistry

Adipose tissue depots were fixed in 10% buffered formaldehyde and subsequently treated for histological study by dehydration (increasing alcohol concentrations), mounting in xylene and immersion in paraffin. The paraffin blocks were sliced into 3 μm sections that were processed, deparaffinized in xylene, rehydrated and rinsed in distilled water and then stained either for hematoxylin-eosin or UCP1 immunohistochemistry. In the hematoxylin-eosin study, slices were first stained with hematoxylin for 5 min, washed and stained again with eosin for 1 min. For UCP1 immunohistochemistry, slices were incubated overnight with the primary antibody (UCP1, *Abcam;* Cambridge, UK), washed and incubated with the secondary antibody (*ENVISION* (a mixture of anti-mouse and anti-rabbit from *DAKO*; Glostrup, Denmark)). Images were taken in an optical microscope with a digital camera Olympus XC50 (*Olympus Corporation*; Tokyo, Japan) at 40X. Adipocyte area was quantified using *ImageJ 1.33* software (*NIH*, Bethesda, MD, USA).

2.10. Statistical analysis

All data are presented as mean $\pm$ SEM. For statistical analysis, SPSS was used and statistical significance was determined by Student's *t*-test or 2-way ANOVA in the case of the body weight, food intake and temperature measurements (Figure 2). $P < 0.05$ was considered significant.

3. RESULTS

3.1. Central T3 infusion induces oxygen consumption in mice

To establish the existence of thermogenesis induced by central T3-infusion, we used mice that were chronically thyroid hormone-stimulated centrally through minipumps connected to a cannula targeting the lateral ventricle of the brain. To ensure that the mice had available a sufficiently high BAT thermogenic capacity to make T3-induced thermogenesis from BAT observable, mice were acclimated to 18 °C for about a month before the experiment [7]. Further, to ensure that any extra heat contribution from thyroid-hormone-induced thermogenesis would be observable, the acute analysis of thermogenesis was performed at thermoneutrality. As seen in Figure 1A (left, blue line), as expected, marked circadian alterations in metabolism were observed in the untreated wildtype mice. In the T3-treated mice (red line), oxygen consumption (VO$_2$) was clearly elevated. Thus, this is the first functional confirmation that the increases earlier observed, e.g. SNS activity in BAT in centrally T3-infused animals [5], are really associated with increased VO$_2$. Additionally, this constitutes evidence that the central model for (certain) T3 effects, so far only demonstrated

in rats, is also valid in mice. In similar experiments, where the mice were also acclimated to 18 °C but where the indirect calorimetry experiments were also performed at 18 °C, we failed to observe any effect of central T3 infusion on VO$_2$ (Supplemental Figure 1, upper curves).

To accurately define the VO$_2$ specifically induced by central T3 infusion, we subtracted the running means of the vehicle-infused mice from those of the T3-treated mice (Figure 1B, left, black line). It becomes very evident that the increased VO$_2$ was only observed during the dark phase; this was unexpected. The central T3 infusion system is continuously active and the increases in sympathetic activity to BAT reported earlier were observed during the light phase (in rats) [5]. It is not presently possible to ascribe the strong diurnal effect of the T3-infusion to interaction with any other circadian events, although feeding and muscular activity would, of course, be strong candidates. Also, in the former experiments, when sympathetic activation was detected, T3 was given as a single bolus [5], but here a chronic minipump treatment was utilized.

3.2. UCP1 is essential for T3-induced oxygen consumption

To examine the significance of BAT-derived heat production for the T3-induced VO$_2$, we followed VO$_2$ in mice lacking UCP1. These mice are devoid of adrenergically induced VO$_2$ in their brown adipocytes [18], as well as in their brite/beige adipocytes [19,20]. As seen in Figure 1A, right, these mice also showed marked circadian patterns in their metabolism in the untreated state. There was, however, no obvious effect on VO$_2$ of central T3 infusion in these UCP1-ablated mice. Indeed, when the specific metabolic effect of central T3 infusion was calculated (Figure 1B, left, red line), the line did not deviate positively from zero. Formally, the UCP1-dependence of the T3-effect can be plotted (Figure 1B, right) as the difference between the two curves (Figure 1B, left). As thus seen from Figure 1B, the entire effect of central T3 infusion on VO$_2$ is mediated via UCP1, and no alternative mechanism for increased metabolism is called into action in the absence of UCP1. This is thus the first demonstration that not only is BAT involved in the increased energy expenditure induced by central T3 infusion but that the increase is totally dependent on the presence of UCP1.

The data above are quantitatively evaluated in Figure 1C–E. Due to the marked qualitative differences between the light and dark phases evident from Figure 1A–B, these phases have been separately analyzed. In the wildtype mice, there was no statistically significant effect of central T3 infusion during the light phase (Figure 1C, left), but central T3 infusion significantly increased the VO$_2$ by 22% during the dark period (an increase of 17% when analyzed for the light and dark phases together; $P = 0.03$). No significant effects of central T3 infusion were seen in the UCP1-ablated mice, either during the light phase or during the dark phase (Figure 1C, right). In Figure 1D, the effect of T3 during the light phase on the wildtype mice in comparison with the effect in UCP1-ablated mice is shown. Whereas no differences were observed during the light phase, the entire T3 effect seen in the wildtype mice depends on the presence of UCP1 (Figure 1E).

Next, we evaluated the effect of central T3 on the RQ of wildtype and UCP1 KO mice. Our data showed that central T3 promoted a tendency to increase the RQ that was lost in UCP1 null animals (Supplemental Figure 2A–E).

3.3. UCP1 is essential for the pyrexia ("fever") caused by central T3 infusion

In Figure 2, we display the development of the body temperature observed in the middle of the light phase during the entire experiment.

In the wildtype mice (Figure 2A, left), there was an indication of a higher body temperature in the T3-treated mice by the second day after the application of the T3-delivering system, and a clear sustained difference was observed from day 3 and onwards. This increase in

body temperature induced by central T3 infusion amounts to about 0.7 °C (Figure 2B). Most notably, the T3 treatment counteracted the decrease in body temperature seen over the experimental period. For the UCP1-ablated mice, there was no consistent increase in body
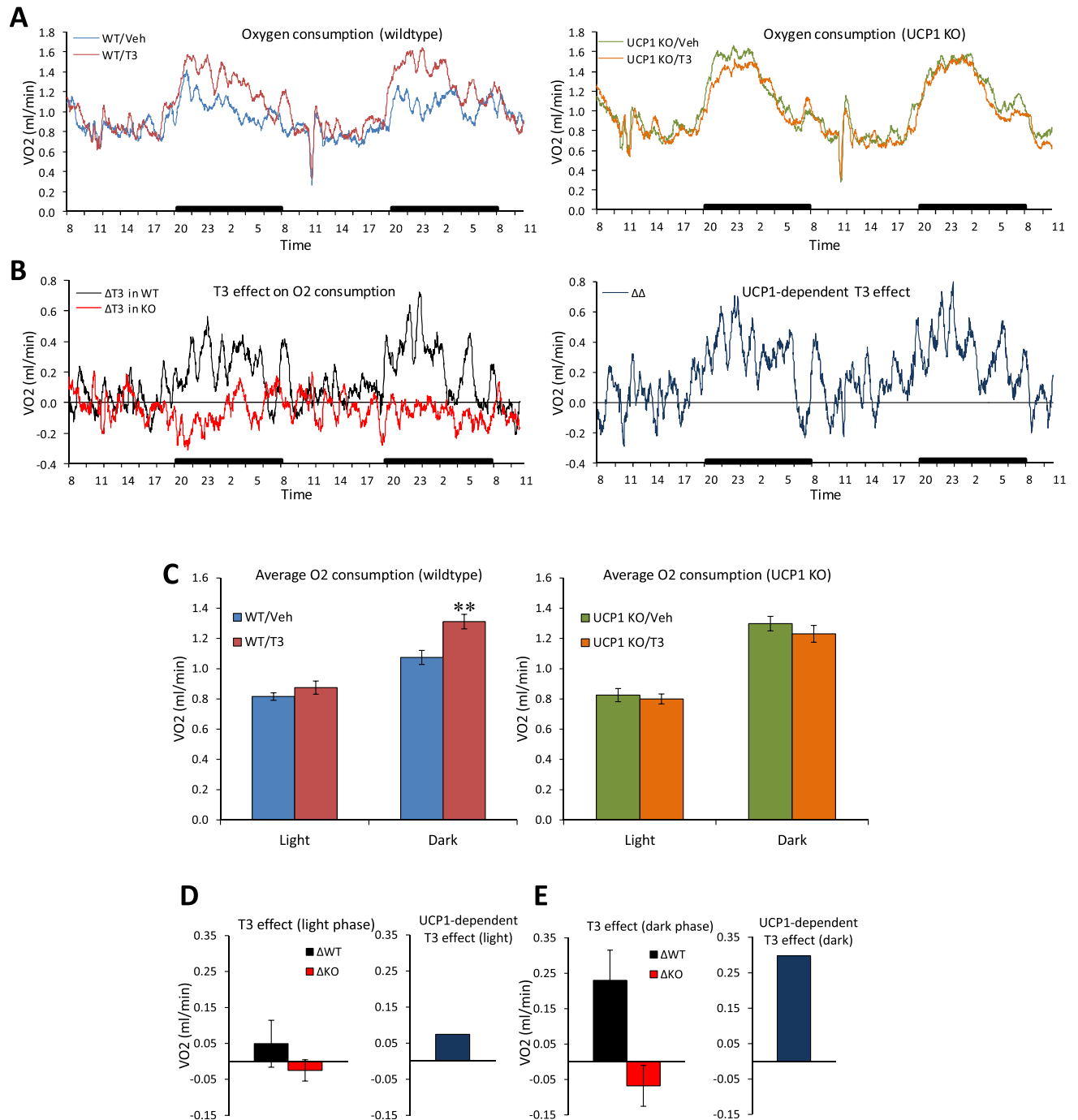
Figure 1: Central T3 infusion induces extra oxygen consumption in a UCP-dependent process. Mice were preacclimated to 18 °C, implanted with T3-delivering minipumps and, two days later, transfered to metabolic chambers where their rate of oxygen consumption (VO$_2$) was followed. The first day in the chambers is not shown. **A)** Two days VO$_2$ in wildtype (left) and UCP1 KO mice (right) centrally infused with T3 or vehicle. Mean data from 9 to 10 mice. **B)** Left: The effect of T3 on metabolic rate (from A) calculated as Δ = T3 values minus vehicle values; wildtype black line, UCP1 KO red line. Right: The UCP1-dependent T3 effect calculated from B left as $\Delta\Delta$ = Δwildtype values minus ΔKO values. **C)** Mean metabolic rate represented as the average O$_2$ consumption during the light and during the dark phases in wildtype (left) and UCP1 KO mice (right), centrally treated or not with T3. Values were calculated for each mouse by adding the two last days divided by 2 and then calculating the average for all mice in each group (n = 9–10). **D)** T3 effect on the light phase metabolic rate represented as Δ = T3 values minus vehicle values (left), and as the UCP1-dependent T3 effect (right). **E)** As D but during the dark phase. Data are means (A, B) or means $\pm$ SEM (C, D, E) of 9 (wildtype vehicle) or 10 (all other) mice; **P $<$ 0.01 T3-treated *vs.* vehicle as analyzed by unpaired t-test.
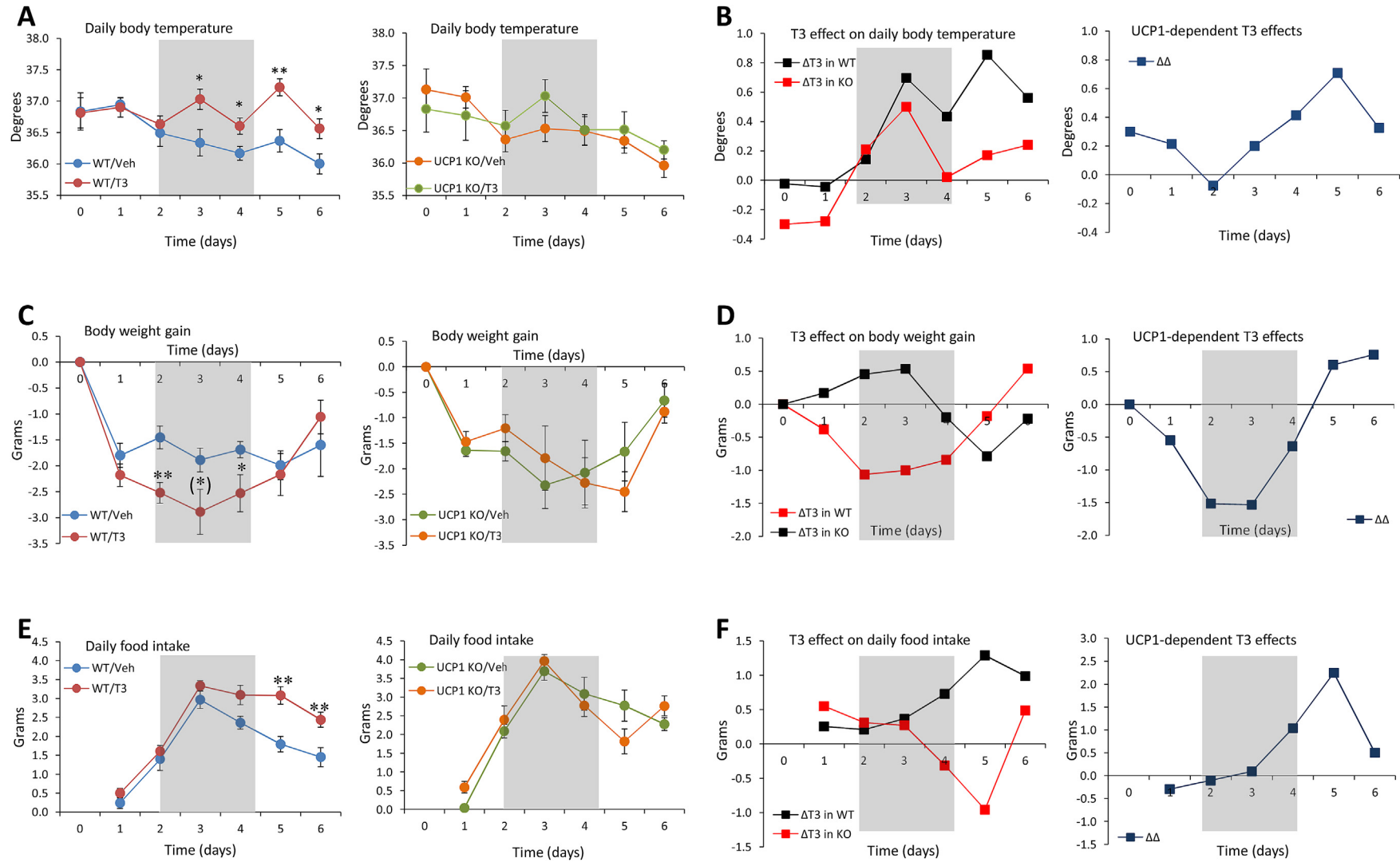
Figure 2: UCP1-dependent effects of central T3 infusion on body temperature, body weight and food intake. The mice depicted in Figure 1 were followed during the entire T3-treatment period. **A)** Body temperature from day 0 in wildtype (left) and UCP1 KO mice (right) centrally treated with T3. **B)** T3 effect on body temperature represented as Δ = T3 values minus vehicle values (left) and UCP1-dependent T3 effect represented as $\Delta\Delta$ = Δwildtype values − ΔKO values (right). **C)** Body weight gain in wildtype (left) and UCP1 KO mice (right) centrally treated with T3. The initial average body weight of each group was: WT/Veh 30.7 ± 0.4 g, WT/T3 30.1 ± 0.7 g, KO/Veh 27.0 ± 0.9 g and KO/T3 27.3 ± 0.9 g. Note that the mice were pre-acclimated to 18 °C, and at this subthermoneutral temperature, UCP1-ablated mice are not expected to become more obese than wildtype, rather, the reverse. **D)** Effect of T3 on body weight gain, represented as Δ = T3 values minus vehicle values (left), and UCP1-dependent T3 effect represented as $\Delta\Delta$ = Δwildtype values minus ΔKO values (right). **E)** Daily food intake in wildtype (left) or UCP1 KO mice (right) centrally treated with T3. **F)** Effect of T3 on daily food intake, represented as Δ = T3 values minus vehicle values (left) and UCP1-dependent T3 effect represented as $\Delta\Delta$ = Δwildtype values minus ΔKO values (right). The time when the mice were inside the metabolic cages is represented by the light grey rectangle. Data are mean ± SEM as in Figure 1; *, ** and (*) P < 0.05, <0.01 and = 0.06 T3-treatment *vs.* vehicle as analyzed by unpaired t-test at single time points. 2-way ANOVA was also applied to analyze the dependence of time and treatment. Figure 2A, **left**: effect of treatment P = 0.000; effect of time: P = 0.001; Figure 2A, **right**: effect of treatment P = 0.286; effect of time: P = 0.015; Figure 2C, **left**: effect of treatment P = 0.006; effect of time: P = 0.014; Figure 2C, **right**: effect of treatment P = 0.969; effect of time: P = 0.007; Figure 2E, **left**: effect of treatment P = 0.000; effect of time: P = 0.000; Figure 2E, **right**: effect of treatment P = 0.774; effect of time: P = 0.000.

temperature, and for both treated and untreated animals, the body temperature decreased over time (Figure 2A, right).

The increase in body temperature observed in the hyperthyroid state is often interpreted as hyperthermia, i.e. the animal produces more heat than it can dispose of. The data presented here do not support the concept that the higher body temperature in the T3-treated mice is a hyperthermia caused by the increase in heat production, because this is observed during the light phase when no increase in VO_2 is evident (Figures 1 and 2). The higher body temperature in the T3-treated mice should therefore be considered as pyrexia ("fever"), i.e. a defended new body temperature. This conclusion is principally in agreement with certain earlier studies on hyperthyroid rats indicating that they maintain a defended increase in body temperature [21,22].

3.4. UCP1 is essential for the effect of central T3 infusion on body weight and food intake

As seen in Figure 2C, left, the surgery and the implantation of the infusion system led to a direct decrease in body weight of about 2 g. In the non-treated wildtype mice, body weight was thereafter maintained at this level. However, in the T3-treated mice, a statistically significant transient decrease of about 1 g was seen. This T3-induced body weight loss was similar to that observed in rats [5,12]. In the UCP1-ablated mice, the initial body weight after the operation was generally maintained, and no consistent effect of T3-treatment was observed (Figure 2C, right). Thus, the extra body weight loss was fully UCP1-dependent (Figure 2D) and may be associated directly with the increased metabolism induced during eating time (dark phase) (Figure 1).

Probably as a consequence of the anesthesia and the ICV surgery, during the day after the implantation of the infusion system, the mice did not eat (Figure 2E), explaining the initial loss of body weight (Figure 2C). In the following days, feeding was restored in the non-treated wildtype mice (Figure 2E, left) but then the intake gradually declined. Food intake in T3-treated wildtype mice remained high, making them hyperphagic relative to non-treated mice. This relative hyperphagia clearly allowed for the recovery in weight gain observed in the T3-treated mice from day 3 (Figure 2C—D). There was no effect of T3 treatment on food intake in the UCP1 KO mice (Figure 2E, right), implying that the positive effect of T3 on food intake was dependent on the presence of UCP1 (Figure 2F).

3.5. UCP1 is essential for the central T3 infusion-induced expression of orexigenic neuropeptides

At the end of the experiment, the mice were killed and their mediobasal hypothalami examined for the expression of the feeding-promoting neuropeptides neuropeptide Y (NPY) and agouti-related peptide (AgRP). No significant changes were found either in the protein levels of the loading control (β-actin) or in the basal levels of NPY and AgRP between wildtype and UPC1 KO mice (Supplemental Figure 3A—B). As previously shown [5,12], and in keeping with its orexigenic effect (Figure 2E—F), central administration of T3 increased the protein expression of these neuropeptides in the hypothalamus of wildtype mice (Figure 3A—B, left). This may be a direct effect of T3 on the relevant hypothalamic nuclei [12]. However, strikingly, central T3 infusion failed to induce the expression of the neuropeptides in the UCP1-ablated mice (Figure 3A—B, right) in which, correspondingly, no increase in food intake was seen (Figure 2E—F). This is an indication that activation of BAT leads to an increased utilization of stored energy (Figure 2C—E), signaling to the brain that its energy stores are diminished.

3.6. Absence of effect of central T3 infusion in UCP1-ablated mice is not due to impaired AMPK signaling (or defective AMPK response)

The effects of central T3 infusion on BAT are mediated via decreased phosphorylation of hypothalamic AMPK [5]. To examine the possibility
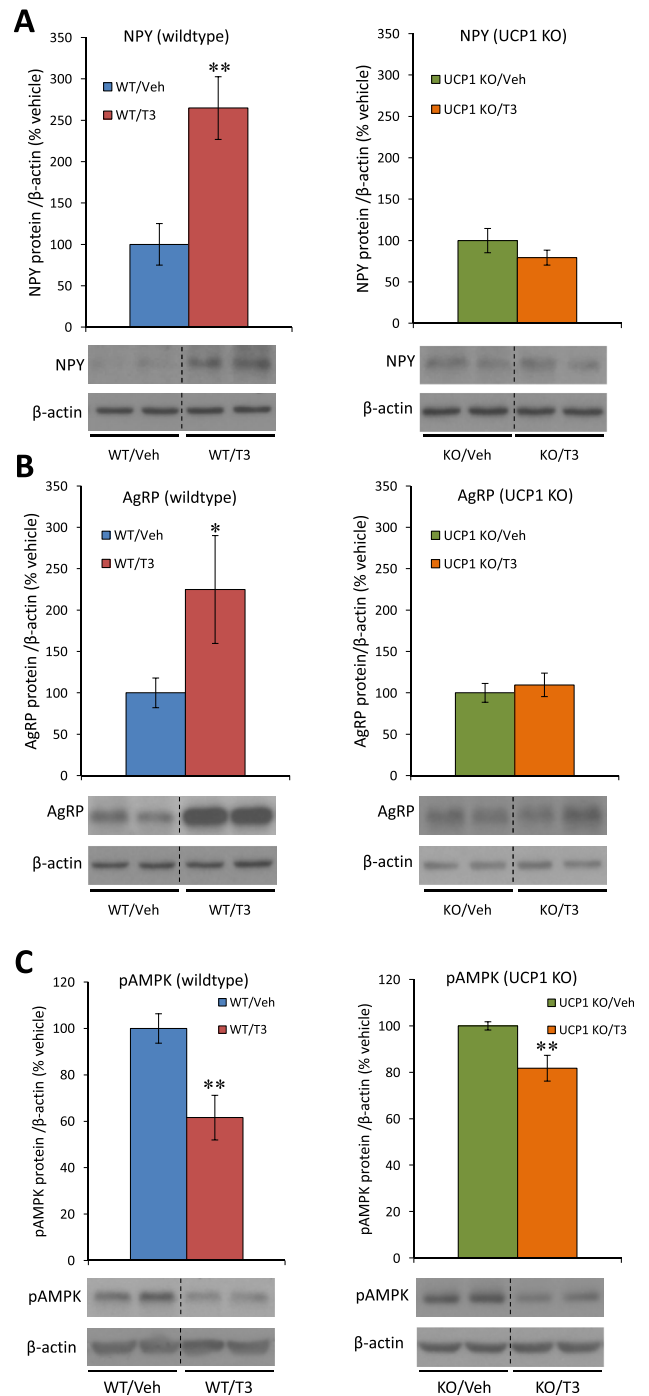

Figure 3: UCP1 dependence of the effect of central T3 on hypothalamic neuro-peptide and pAMPK levels. After the end of the T3-treatment, mice were killed and hypothalami dissected out. The graphs show hypothalamic protein levels (upper panels) and representative western blot images (lower panels) of NPY **(A)**, AgRP **(B)** and pAMPK **(C)** in wildtype (left) and UCP1 KO mice (right) centrally treated with T3. Note that all samples from each genotype were run in parallel in the same gel. Data are means ± SEM for 7 mice. * and **P < 0.05 and 0.01 T3-treatment *vs.* vehicle. Vehicle levels were for each genotype set to 100%.

that the absence of T3 effects in UCP1-ablated mice could be due to an unsuccessful response, we examined pAMPK levels in the mediobasal hypothalamus of wildtype and UCP1 KO. However, T3 reduced the hypothalamic levels of pAMPK in both groups of animals (Figure 3C); no significant changes were found either in the protein levels of the loading control (β-actin) or in the basal levels of pAMPK between wildtype and UPC1 KO mice (Supplemental Figure 3C). Thus, the central T3 signaling is intact in the UCP1-ablated mice, and the absence of effects of UCP1 in these mice must be explained at the peripheral level.

3.7. Central T3 infusion increases BAT UCP1 levels

In rats, the T3-induced hypothalamic AMPK inactivation leads to increased sympathetic tone in the nerves innervating BAT [5]. As chronic sympathetic stimulation is the physiological recruitment mechanism for BAT [7], we examined the effect of chronic T3 infusion on BAT recruitment state. Wildtype T3-treated mice showed enlarged interscapular BAT, as estimated by wet weight (Figure 4A); but an increase in BAT wet weight is not necessarily associated with a recruitment of tissue and could instead indicate BAT inactivity (due to lipid accumulation). However, as total protein amount also tended to be increased in the T3-treated mice (Figure 4B), these data are compatible with BAT recruitment.

We further measured the level of UCP1 protein in BAT. We found that in wildtype mice, relative (Figure 4C) and total (Figure 4D) levels of UCP1 protein (per mg protein) were increased after T3-treatment; no significant changes were found in the protein levels of the loading control (α-tubulin) (Supplemental Figure 3D). Of course, in the UCP1-ablated mice no UCP1 was detected (Figure 4C). Overall, in keeping with our former observations on mRNA levels in rats [5], these data indicate that

central T3 treatment induces UCP1 protein levels in wildtype mice and thus probably an increase in thermogenic capacity with time.

3.8. Central T3 infusion induces britening of WAT

After the end of the chronic infusion of T3 into the brain, the body composition was analyzed by MRI before the mice were decapitated. As seen in Figure 5A, the total amount of fat in the body was modestly higher in the T3-treated wildtype mice than in the vehicle-treated group (P = 0.09), probably reflecting the higher food intake. No differences were found in the UCP1-ablated mice (Figure 5A—B).

The morphology of the inguinal white adipose (iWAT) depot was analyzed by hematoxylin-eosin staining. The size of adipocytes was reduced after central T3 infusion, thus changing the morphology towards a more BAT-like morphology; no differences were found in UCP1-ablated mice (Figure 5C—E). When the sections were stained for UCP1, no staining was observed in untreated wildtype mice, but positive immunoreactivity was seen in wildtype mice treated with T3 (Figure 5D). As expected, no UCP1 immunoreactivity was observed in UCP1-ablated mice (Figure 5D). We also examined the effect of central T3 infusion on UCP1 mRNA levels in the inguinal adipose tissue, as expressed versus the reference gene HPRT. T3-treatment did not alter the expression of the reference gene (Supplemental Figure 3E), but the expression of UCP1 was significantly increased (Figure 5F).

4. DISCUSSION

4.1. Central T3 induces oxygen consumption through a UCP1-dependent circadian mechanism

Thermogenic effects of thyroid hormones have generally been considered to be peripheral, in that certain (or most) tissues would
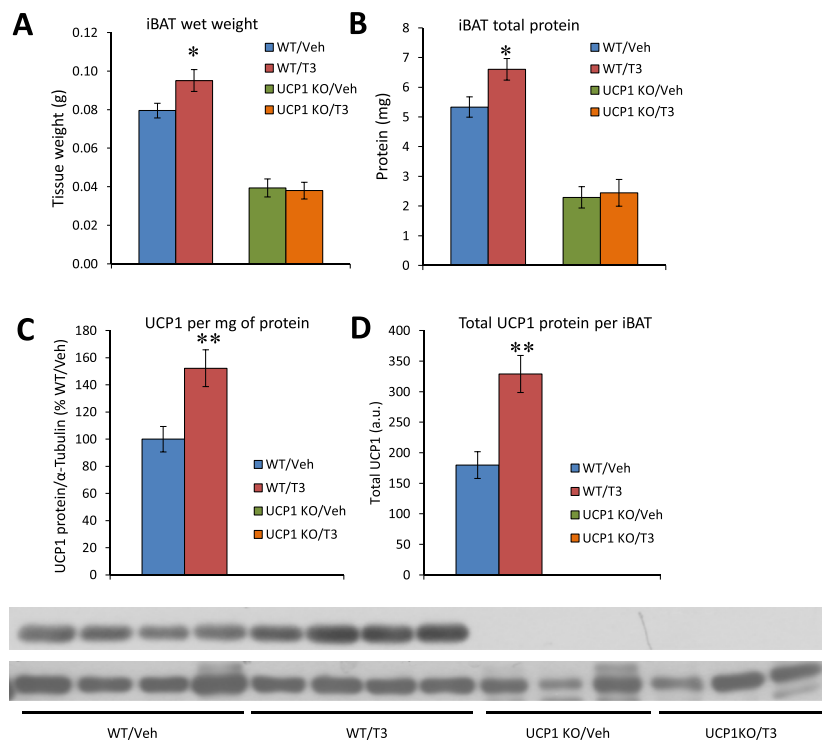

Figure 4: Central T3 infusion recruits brown adipose tissue. At the end of the T3-treatment, interscapular brown adipose tissue (iBAT) was dissected out. **A)** BAT wet weight and **B)** total protein levels in iBAT in wildtype and UCP1 KO mice centrally treated with T3. Data are means ± SEM from n = 9 (WT/Veh group) or n = 10 (all other groups). **C)** Relative UCP1 protein levels per mg of protein (upper panel, left) and representative western blot images (lower panel). Note that all the samples were run in parallel in the same gel. **D)** Estimated total UCP1 protein content in iBAT was obtained by multiplying the data in **B** with the data in **C** for each mouse. n = 7 animals per experimental group.
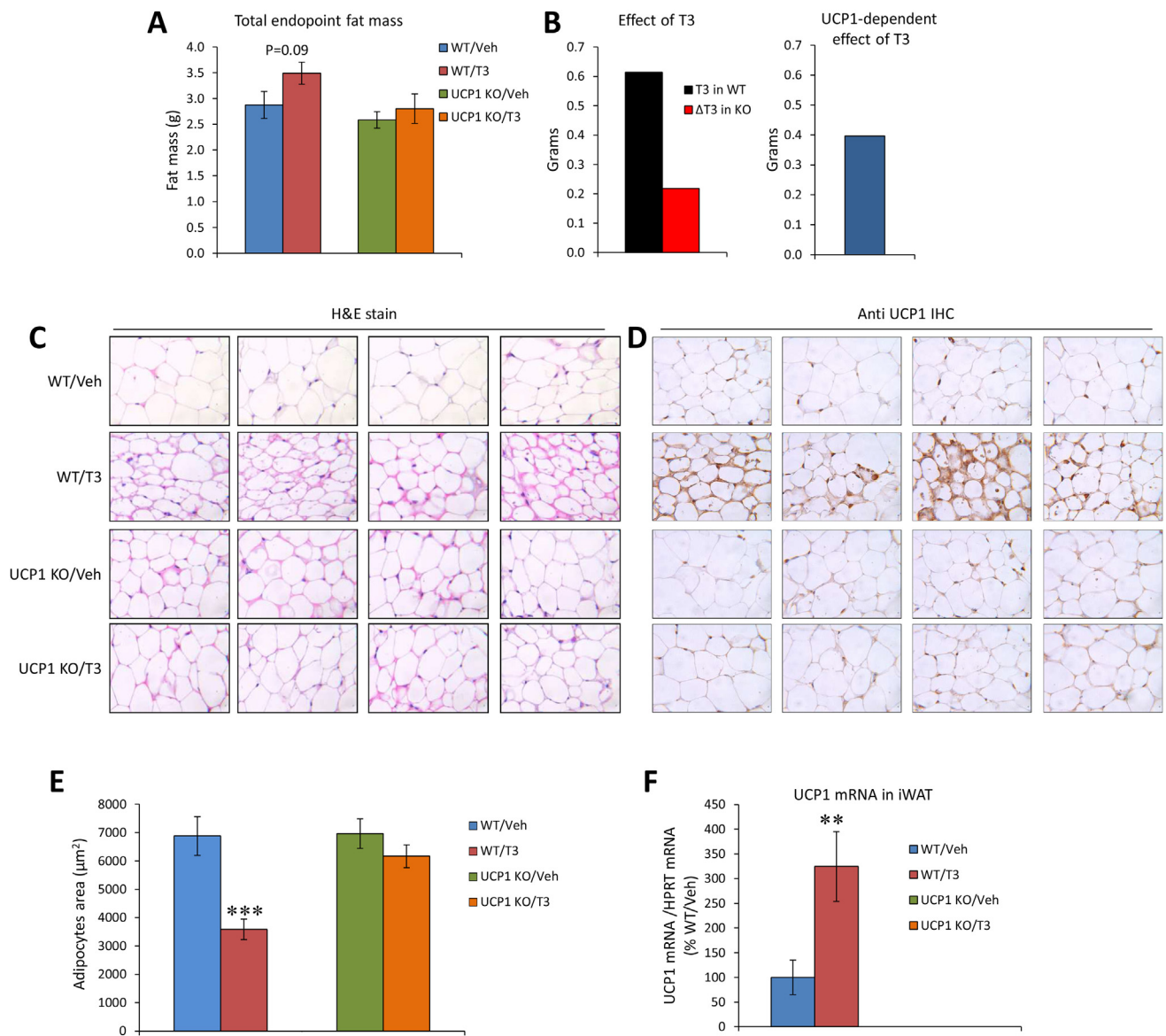
Figure 5: Central T3 infusion recruits brite adipose tissue. At the end of the T3-treatment, inguinal white adipose tissue (iWAT) was dissected out. **A)** Total body fat mass measured by MRI at the endpoint of the experiment, in wildtype and UCP1 KO mice centrally treated with T3. **B)** Effect of T3 on the total body fat mass, represented as Δ = T3 values minus vehicle values (left); UCP1-dependent T3 effect represented as $\Delta\Delta$ = Δwildtype values minus ΔKO values (right). **C)** Hematoxylin-eosin staining and D) UCP1 immunohistochemistry, showing UCP1 in sections from iWAT depots in wildtype and UCP1 KO mice centrally treated with T3. The histographs are from 4 different mice but representative for all 39 mice examined (9 in the WT/Veh group and 10 in the other 3 groups). **E)** Adipocyte area in wildtype and UCP1 KO mice centrally treated with T3. n = 9. ***P < 0.001 *vs.* WT vehicle. **F)** Effect on UCP1 mRNA levels in iWAT in wildtype and UCP1 KO mice centrally treated with T3. UCP1 mRNA levels were normalized to HPRT levels, and the mean level in vehicle-treated wildtype mice set to 100%. n = 9–10. Ct values were WT/Veh: 25.81 ± 0.73 and WT/T3: 23.25 ± 0.51, P < 0.01; undetected in the UCP1 KO. Data are mean ± SEM, **P < 0.01, T3-treatment *vs.* vehicle.

respond thermogenically to a thyroid hormone signal, and the metabolism of the total animal would through this be increased [1–3]. Presently, the possibility that (some of) the metabolic effects of thyroid hormones instead are centrally induced is discussed [5]. In the present study, we have for the first time demonstrated that T3, solely through a brain-mediated process, can induce thermogenesis mediated by UCP1 and thus by brown, and perhaps brite/beige adipose tissues. The effects of centrally administered T3 on body temperature are independent of stimulated VO₂. The effects on body weight and food intake (and on orexigenic neuropeptide levels in the brain) are indirect, peripherally mediated and result from the activation of BAT caused by

central T3. Chronic activation of BAT through central T3 results in enhanced recruitment of the tissue, and britening/beiging of inguinal WAT is also observed. Thus, central mediation may explain many if not all of the metabolic and thermogenic effects of thyroid hormones (Figure 6).

The metabolic effects of thyroid hormones on BAT are centrally mediated [2,5]. Specifically, T3 inhibits AMPK in the VMH, promoting increased expression of UCP1 through activation of the SNS [5]. However, the essentiality of BAT for centrally induced thyroid thermogenesis has not been demonstrated as yet. We observed here that T3 infusion in wildtype mice significantly enhanced VO₂. No effect of
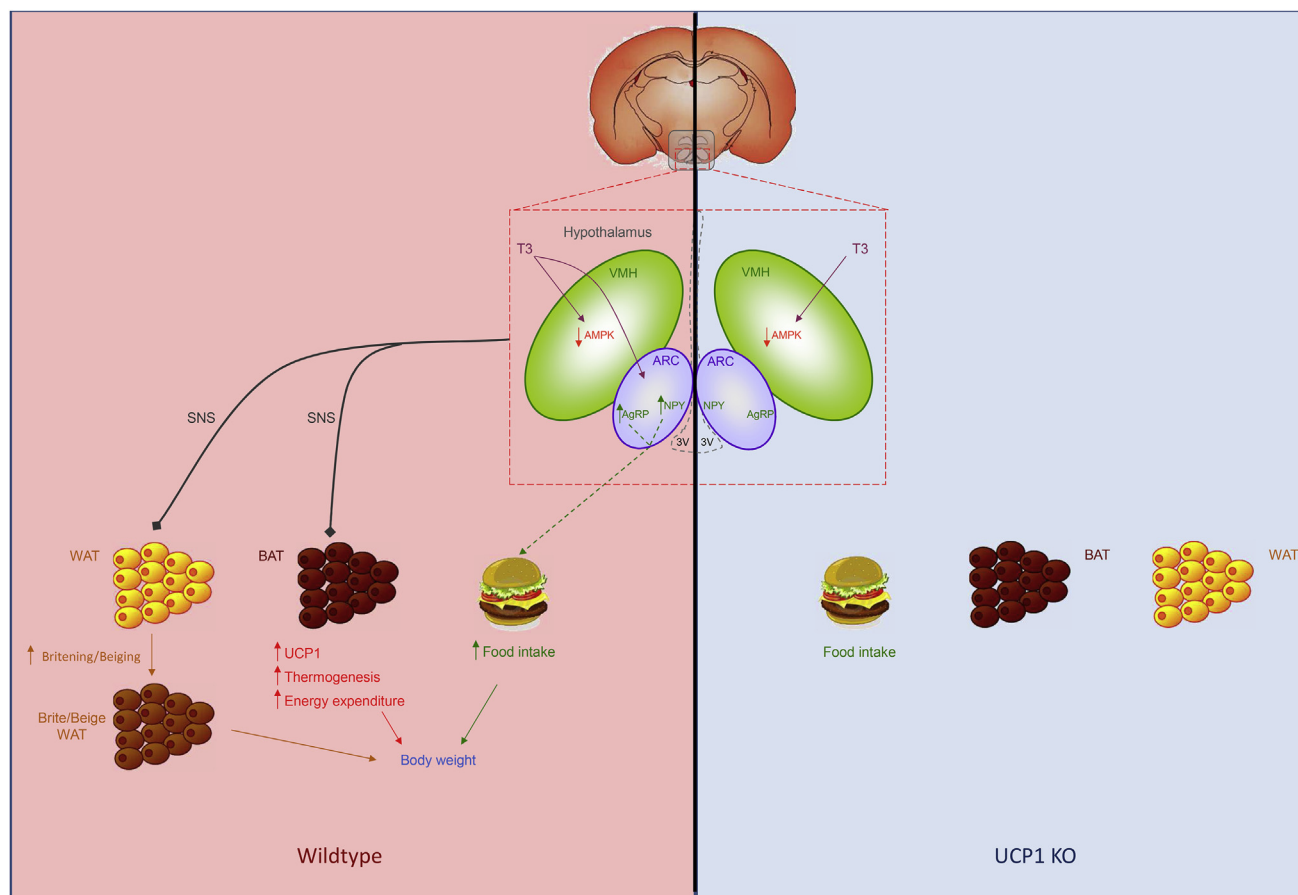
Figure 6: Role of UCP1 role in modulating the central effects of T3 on energy balance. In wildtype mice (left), central T3 regulates feeding through agouti-related peptide (AgRP) and neuroptide Y (NPY) in the arcuate nucleus of the hypothalamus (ARC). In addition, central T3 modulates the thermogenic program in the brown adipose tissue (BAT) and the britening/beiging ("browning") of white adipose tissue (WAT); this effect is mediated via inhibition of AMP-activated protein kinase (AMPK) in the ventromedial nucleus of the hypothalamus (VMH). This leads to increased thermogenesis, energy expenditure and weight loss, despite hyperphagia. In UCP1 KO mice (right), although central T3 inhibits hypothalamic AMPK, it fails to induce any change in feeding, BAT thermogenesis, energy expenditure and browning of WAT. This data demonstrate that UCP1 is essential for the central actions of T3 on energy balance. 3V: third ventricle; SNS: sympathetic nervous system.

central T3-treatment was found in mice lacking UCP1. This thus demonstrates the essential role of UCP1 for central T3-induced thermogenesis. However, the enhanced VO_2 was only observable during the night, despite the chronic release of T3 centrally. This must mean that the heat production is not due to a direct effect of T3 on nerves controlling sympathetic stimulation of BAT - but rather seems to facilitate/enhance the innate nightly BAT stimulation that is most likely associated with feeding. The mechanism for the selective effect during the dark phase is unknown. Additionally, as illustrated in Supplemental Figure 1, the effect is not observable in mice studied at normal animal house temperatures. This is understandable, as any "extra" stimulation of BAT would be compensated by a thermoregulatory decrease in the stimulation caused by the cold experienced by the mice at this temperature [23]. Additionally, in keeping with this idea, we have found that performing the experiment at thermoneutrality (as here) in mice pre-acclimated to thermoneutrality resulted in no observable T3-induced thermogenesis (data not shown). This is understandable in that the BAT in such animals has a low thermogenic capacity, and the effect would not be measurable. It may be extrapolated that in humans, who generally live under thermoneutral conditions, there would be no brown-fat-derived thermogenesis of the type described here. However, as discussed below, chronic central T3-treatment, a situation that

mimics central hyperthyroidism [5], leads to BAT recruitment, so an enhanced thermogenic capacity would develop with time, even in humans at thermoneutrality.

Our observations also demonstrate that no mechanism other than UCP1 can mediate central T3-induced thermogenesis. Therefore, it can be concluded that there are no alternative sources of increased metabolism induced by central T3-treatment, mediated by any mechanism in any tissue.

4.2. Central T3-induced elevation of body temperature is independent of UCP1-mediated thermogenesis

In contrast to the clear UCP1-dependence of the effect of T3 on VO_2, an elevation of body temperature caused by central T3-treatment was visible in wildtype mice during the light phase when no elevated thermogenesis was evident in T3-treated mice. This is in itself a direct demonstration that the T3-induced increase in body temperature is not hyperthermia (as sometimes implied concerning thyroid hormone-induced increased body temperatures). This means it is not due to an overproduction of heat that the mice cannot dispose of. Rather, T3-treatment alters the body temperature "set-point", i.e. T3 induces a fever-like state. Although there are several indications that enhanced body temperature caused by thyroid

hormone is a fever [21,22], the present study is the first to indicate that it can be directly caused by central thyroid hormone interacting in the brain. It may also be noted that these data underline the tenet that body temperature increases cannot be used as a proxy for VO$_2$. Indeed, the higher body temperatures here are achieved during the light phase without increased VO$_2$. As they are at thermoneutrality, the mice likely can adjust their heat loss in order to maintain their preferred body temperature, e.g. by altering vasoconstriction/dilation in their tails [24]. Another important question is whether the fever-like state induced by central T3 is cytokine-mediated. Preliminary analysis of circulating interleukin-1 (IL-1) and interleukin-6 (IL-6) showed no changes after ICV T3 (data not shown), which may suggest that they are not involved in the pyrexic actions of T3. In any case, these data should be taken with care and further work will be necessary, for example using IL-1 and IL-6 deficient mice to address this question.

4.3. Central T3-treatment effects on hypothalamic neuropeptides are dependent on brown-fat activation

Our observations here indicate that the positive effects of T3-treatment on food intake earlier observed [5] - and the corresponding increases in the levels of food-intake-stimulatory peptides in the hypothalamus [12] - do not occur in mice that lack UCP1. The molecular details of this mechanism are unclear but one possibility may be that lack of thermogenic machinery in the UPC1 KO mice would account for less fuel demands and a lack of necessity for increased feeding. Whatever the case, these data suggest that UCP1 contributes to the control of energy balance not only through the regulation of energy expenditure but also through appetite control by modulating T3 action. An alternative might be the existence of humoral feedback mechanisms between the brain and BAT; for example recent data from our group have demonstrated that bone morphogenetic protein 8B (BMP8B), a batokine, exerts a deep impact on hypothalamic networks [16]. Changes in adiposity may also promote alterations in leptin levels that could affect neuropeptide expression. In this regard, it has been already reported that the anorexic effect of leptin is reduced in UCP1 KO and that UCP1 enhances the leptin action at the hypothalamus [25]. Further work will be necessary to address the exact mechanism by which UCP1 deficiency in BAT leads to changes in neuropeptide expression. In this sense, it is also important to consider that a limitation of our study was the analysis of neuropeptide expression by using western blot from mediobasal hypothalamic samples, which does not allow conclude nuclei-specific regulations. Therefore, an *in situ* hybridization approach would be more adequate. Those things said, it is also important to keep in mind that we observed changes in the expression of AgRP and NPY. AgRP is specifically expressed in the medial part of the arcuate nucleus (ARC) [26], suggesting that the protein changes are specific for that neuronal population. In the case of NPY, it is expressed in both the ARC and the dorsomedial nucleus of the hypothalamus (DMH), where its levels are constitutively lower. Moreover, NPY in the DMH has been demonstrated to be highly expressed only in very specific situations, such as pregnancy and lactation [27—29] as well as in melanocortin deficiency [30]; thus, it is unlikely that it can significantly contribute to the protein levels that we detected by western blot.

4.4. Chronic central T3-stimulation induces BAT recruitment

Chronic adrenergic stimulation of BAT leads to BAT recruitment [7]. As expected, BAT in T3-treated mice shows clear signs of recruitment, such as increased total protein in the tissue, increased concentration of UCP1 in total tissue protein and, most importantly, in the total amount of UCP1 in the tissue (the biochemical parameter that most accurately reflects the total thermogenic capacity of the tissue [7,31]). The recruitment process induced by T3-treatment indicates that even if mice (or humans) would be acclimated to thermoneutrality, and thus possess very little BAT with a very limited thermogenic response to T3-treatment, central hyperthyroidism would with time lead to substantial recruitment of BAT and thus to acquiring significant thermogenic capacity.

4.5. Central T3-induced britening/beiging in inguinal WAT is UCP1 dependent

Also in certain WAT depots, chronic adrenergic stimulation leads to expression of UCP1 (see [23]). Here, we show for the first time that central T3 administration also increases UCP1 mRNA in the iWAT. The signaling pathway for this effect is not self-evident. A suggestion could be the observations (see [23]) that the brain regions innervating BAT and WAT are in similar anatomical locations. Thus, it could simply be that the nerves innervating WAT are activated when the nerves for the BAT are activated, and the effect of central T3-treatment would therefore be similar in the two tissues.

4.6. The relationship between central T3-induced thermogenesis and peripherally T3-induced thermogenesis

The present study is limited to investigating the significance of UCP1 for the actions of centrally infused T3. Although it is likely that this pathway is important for the general effect of thyroid hormones in the body, the conclusions from the present study cannot be extrapolated to explain all the effects of peripheral administration of thyroid hormones. The central pathway studied here may or may not be the only way T3 can activate thermogenesis. Thus, UCP1-dependent or UCP1-independent metabolic effects of peripheral thyroid hormone may exist, only to be revealed through future studies.

We have here studied the results of central administration of T3 on metabolic rate, body weight and food intake. There are probably more effects of central T3-induced sympathetic activation [32,33]. To which degree such effects are also dependent on BAT activation has not been examined here. However, as central T3-treatment leads to substantial increases in metabolic rate and in food intake, and as these effects are totally dependent on BAT activity, it may be that additional effects are BAT dependent: increased metabolism, food intake and food handling in the body will clearly affect many other systems in the body (heart rate, liver metabolism) that may or may not be secondary to the phenomena described here. Analysis of liver and cardiovascular function in centrally T3-treated UCP1 KO mice will examine that possibility.

4.7. Perspectives

The central mechanism (the VMH AMPK-SNS-BAT axis) leading to BAT activation was initially described for thyroid hormones [5]. However, there are now data indicating that this pathway is shared by other agents that are experimentally administered centrally, such as leptin [34], BMP8B [16], estradiol [10], glucagon like peptide-1 (GLP-1) [35] and nicotine [17,36]. It is tempting to speculate that the central thermogenic effect of those factors may also be impaired in UCP1 KO mice. This hypothesis will require further investigation and will help to understand the physiological mechanism modulating energy homeostasis. All data investigating these central effects of diverse agents stimulating and perhaps being mediated through BAT are obtained in rodents. The present realization that adult humans possess active BAT [37] evidently implies that similar mechanisms may occur, at least in a subpopulation of humans.

APPENDIX A.   SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.01.008.

CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

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