SUPPLEMENTARY INFORMATION

Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance

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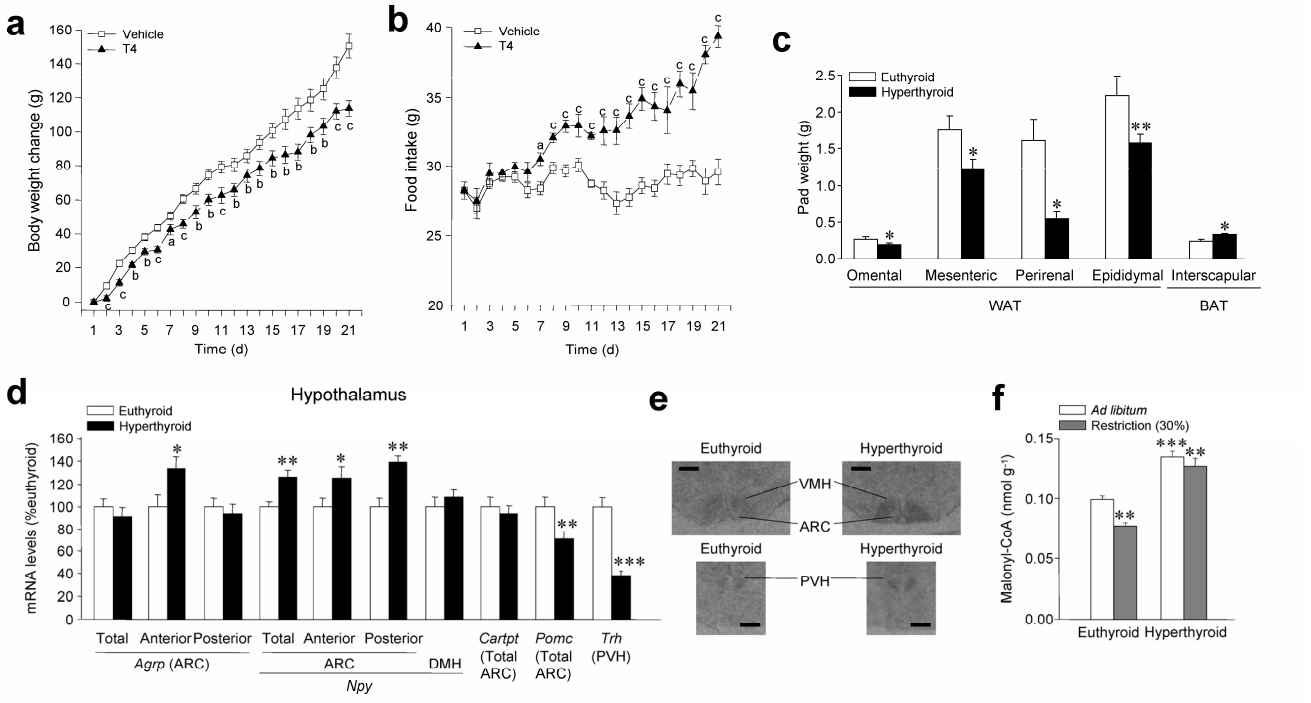
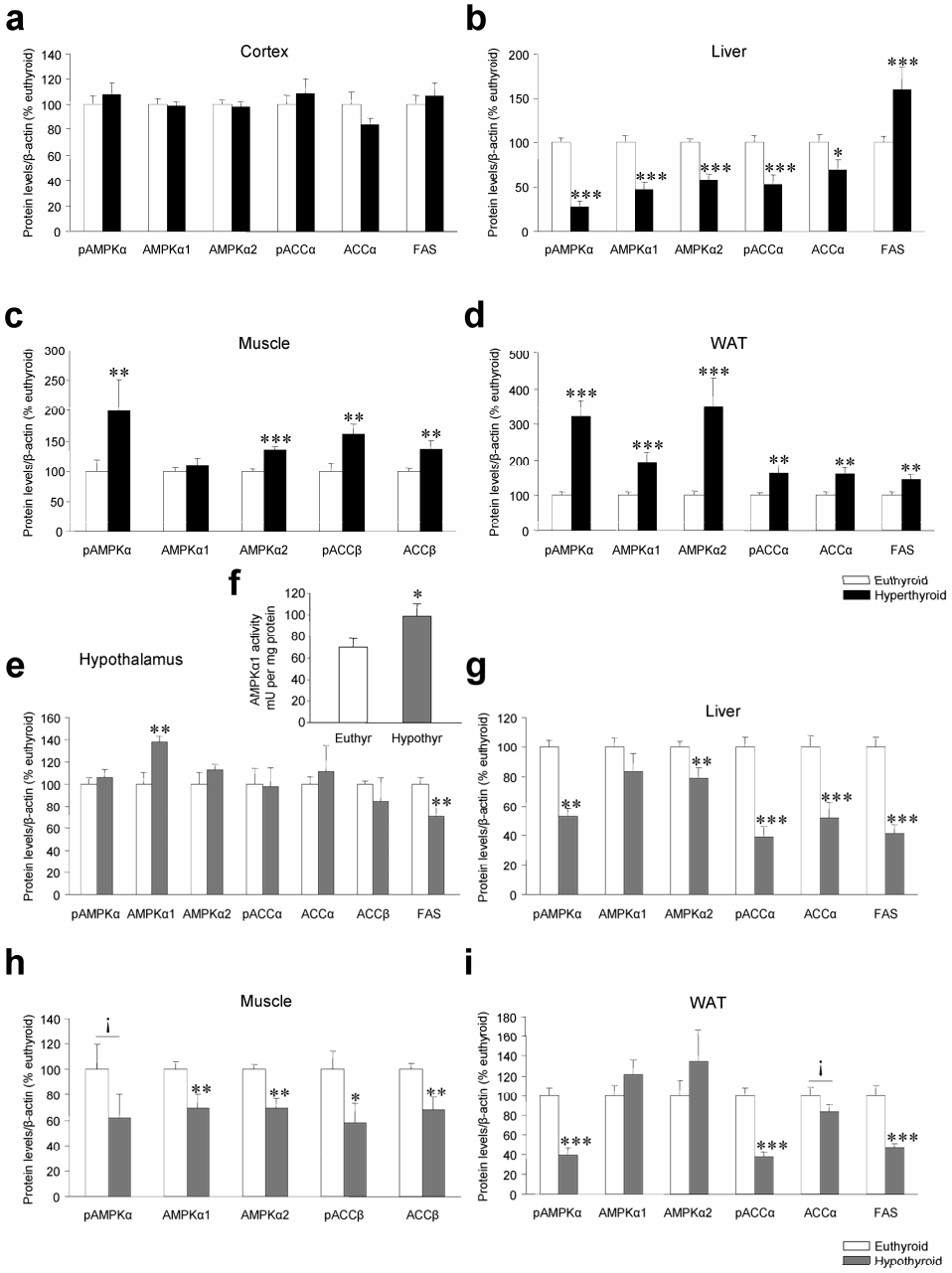
Supplementary Table 1: Body and plasma parameters and hypothalamic (PVH) TRH levels of euthyroid, hyperthyroid and hypothyroid rats

	Euthyroid	**Hyperthyroid**	**Hypothyroid**
Body length (cm, with tail)	44.44±0.18	44.25±0.25	42.63±0.46**
Body length (cm, without tail)	24.78±0.22	24.50±0.29	23.75±0.25**
Fat mass (g)	34.99±2.06	17.25±1.90 ***	-
Non-fat mass (g)	314.61 ±5.84	295.83±5.29**	-
Free water (g)	1.32±0.09	1.19±0.08	-
Temperature (ºC)	34.60±0.09	35.43±0.13***	-
Food efficiency (g $Kcal^{-1}$)	0.19±0.01	0.12±0.01***	-
TSH (ng ml^{-1})	3.47±0.20	0.42 ± 0.08***	27.17±1.71***
T4 (nmol l^{-1})	80.20±7.16	321.64±33.42***	25.0±7.67***
T3 (nmol l^{-1})	3.17±0.23	5.10±0.64***	1.28±0.19***
Glucose (mmol l^{-1})	7.36±0.15	7.38±0.35	6.12±0.23*
Insulin (ng ml^{-1})	2.11±0.34	2.48±0.41	1.50±0.55
Leptin (ng ml^{-1})	3.89±0.46	1.65±0.42***	3.40±1.05
Trh PVH (% euthyroid)	100.00±9.29	40.15±4.51***	189.68±14.45***

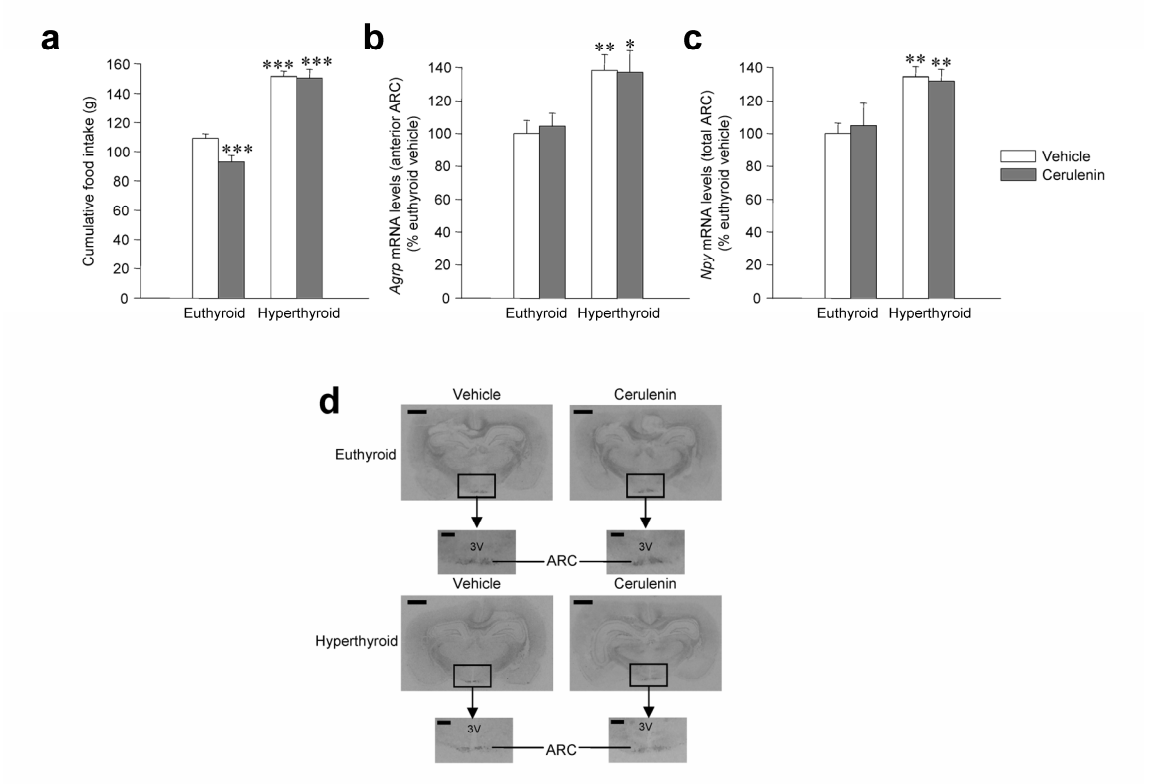
* : P<0.05 vs. Euthyroid
** : P<0.01 vs. Euthyroid
***: P<0.001 vs. Euthyroid



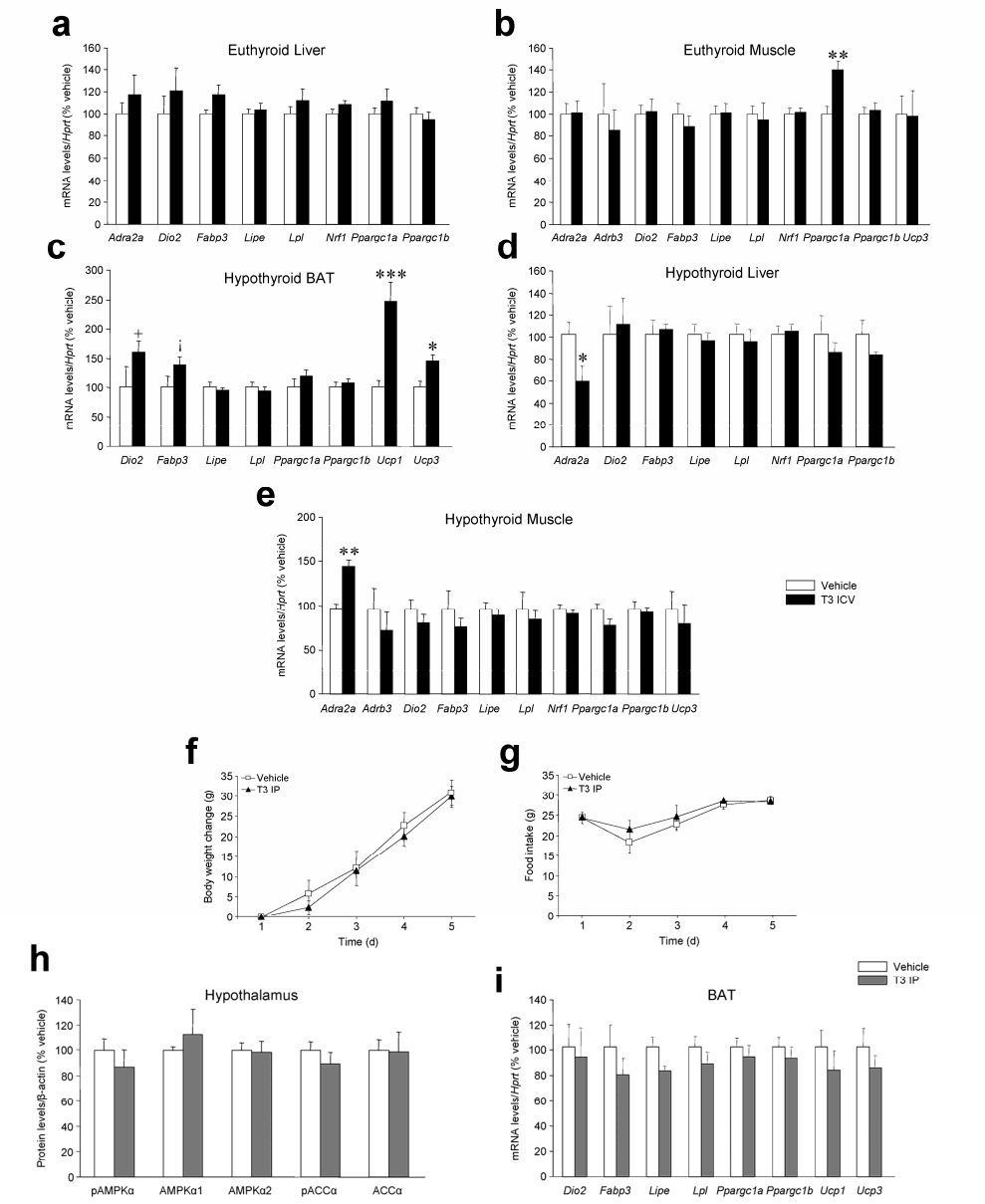
Supplementary Figure 1. Energy balance and hypothalamic parameters in hyperthyroid rats. (**a**) Body weight change, (**b**) daily food intake, (**c**) weights of fat pads, (**d**) hypothalamic neuropeptide mRNA expression, (**e**) in situ hybridization images (scale bar, 500 µm) showing *Fasn* mRNA expression and (**f**) hypothalamic malonyl-CoA response to food restriction of euthyroid and hyperthyroid rats. * or "a" $P < 0.05$, ** or "b" $P < 0.01$, *** or "c" : $P < 0.001$ *vs*. vehicle or euthyroid; all data are expressed as mean ± SEM. *Cartpt*: gene encoding CART (cocaine and amphetamine-regulated transcript).



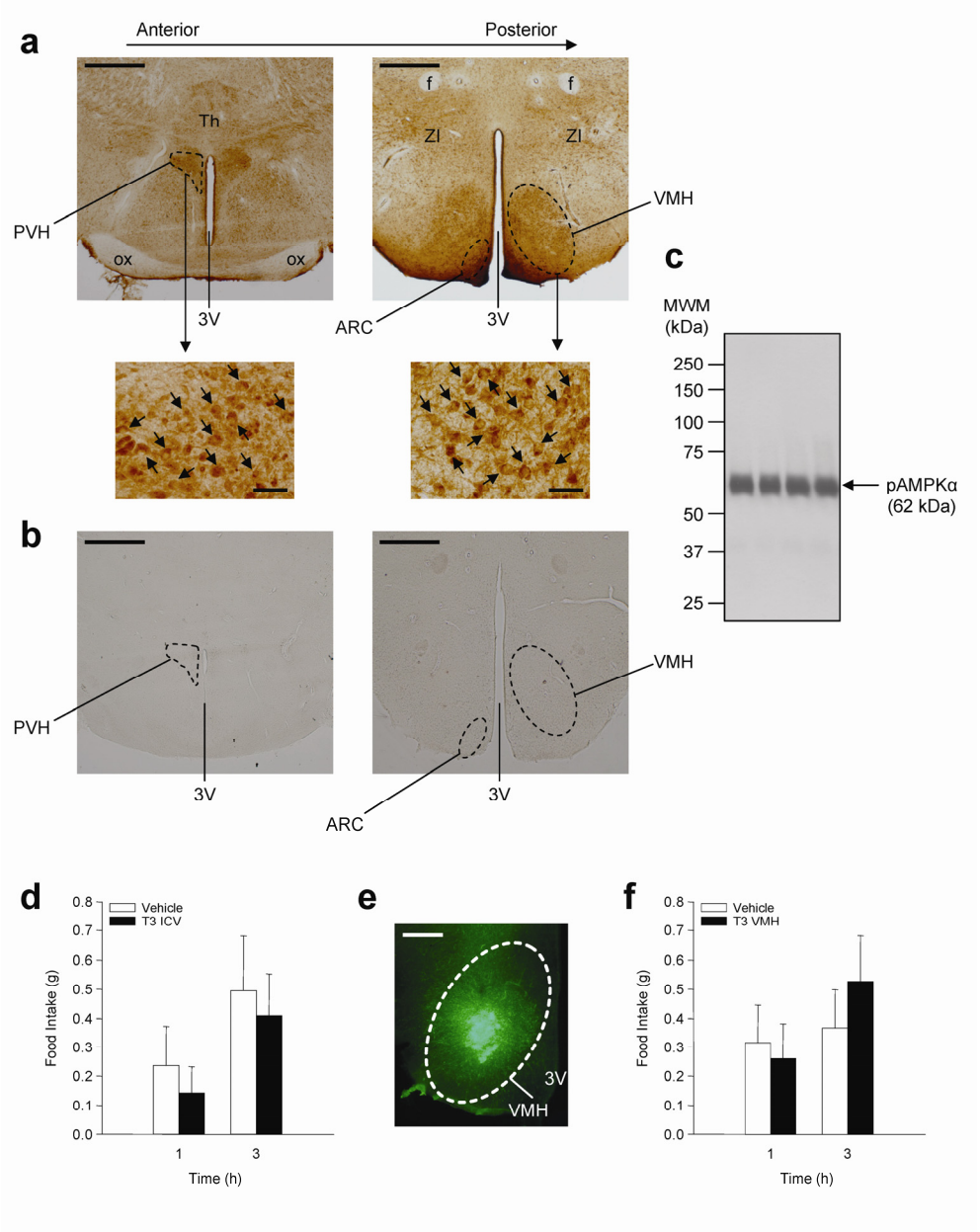
Supplementary Figure 2. Regulation of AMPK pathway in hyperthyroid and hypothyroid rats. (**a–d**) Protein levels of pAMPKα, AMPKα1, AMPKα2, pACCα, ACCα, and FAS in the brain cortex (**a**), liver (**b**), muscle (**c**) and WAT (**d**) of euthyroid and hyperthyroid rats. (**e–i**) Protein levels of pAMPKα, AMPKα1, AMPKα2, pACCα (or pACCβ in muscle), ACCα (or ACCβ in muscle) and FAS and AMPKα1 activity in the hypothalamus (**e, f**), liver (**g**), muscle (**h**) and WAT (**i**) of euthyroid and hypothyroid rats. ¡P = 0.08, *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* euthyroid; all data are expressed as mean ± SEM.



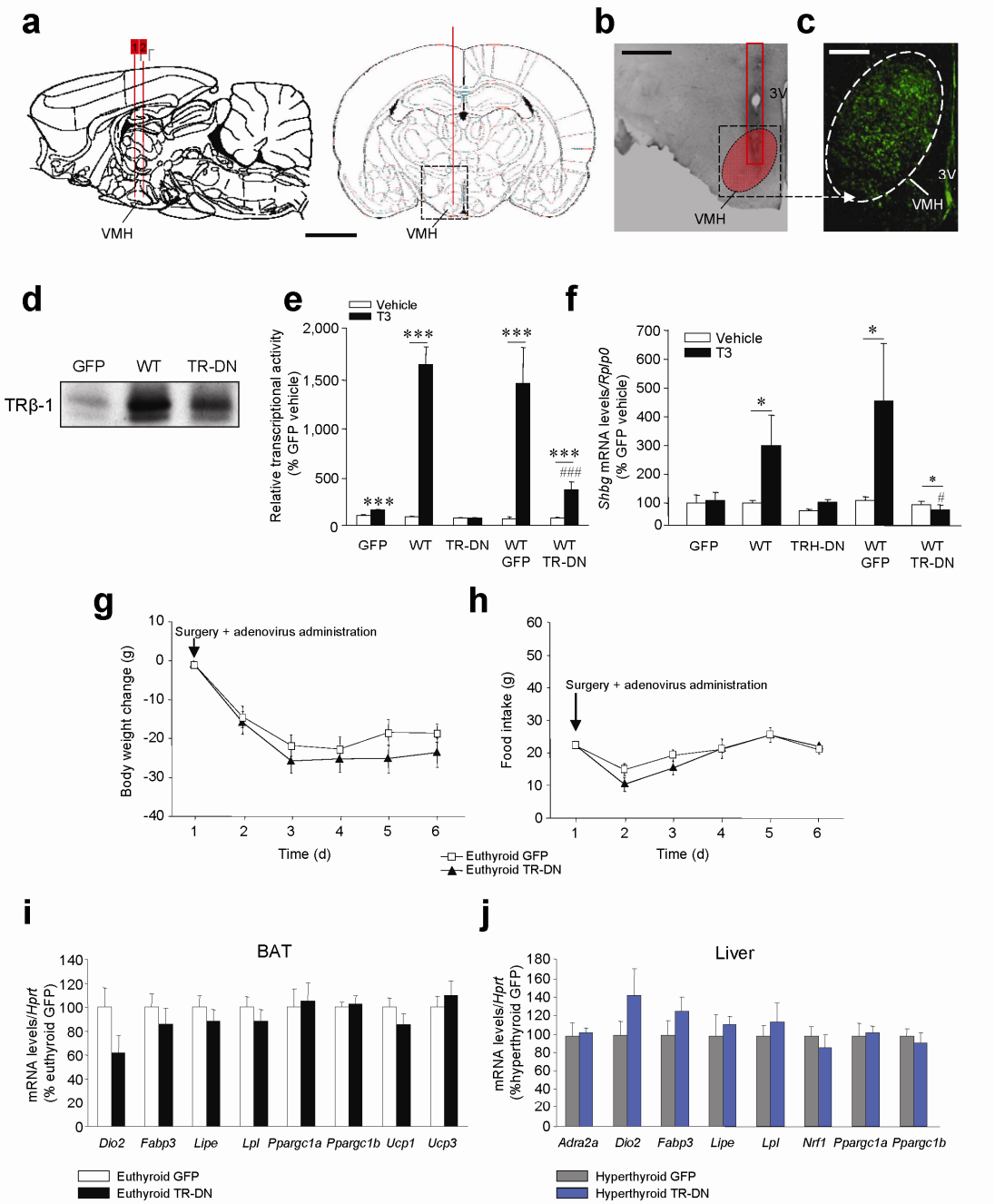
Supplementary Figure 3. Food intake and neuropeptide changes induced by central inhibition of FAS. (**a**) Cumulative food intake, (**b**) *Agrp* mRNA levels in the anterior ARC, (**c**) *Npy* levels in the total ARC and (**d**) in situ hybridization images (upper images: scale bar, 2 mm; lower images, scale bar, 500 µm) showing *Pomc* mRNA levels in the total ARC of euthyroid and hyperthyroid rats ICV-treated with cerulenin. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* euthyroid vehicle; all data are expressed as mean ± SEM.



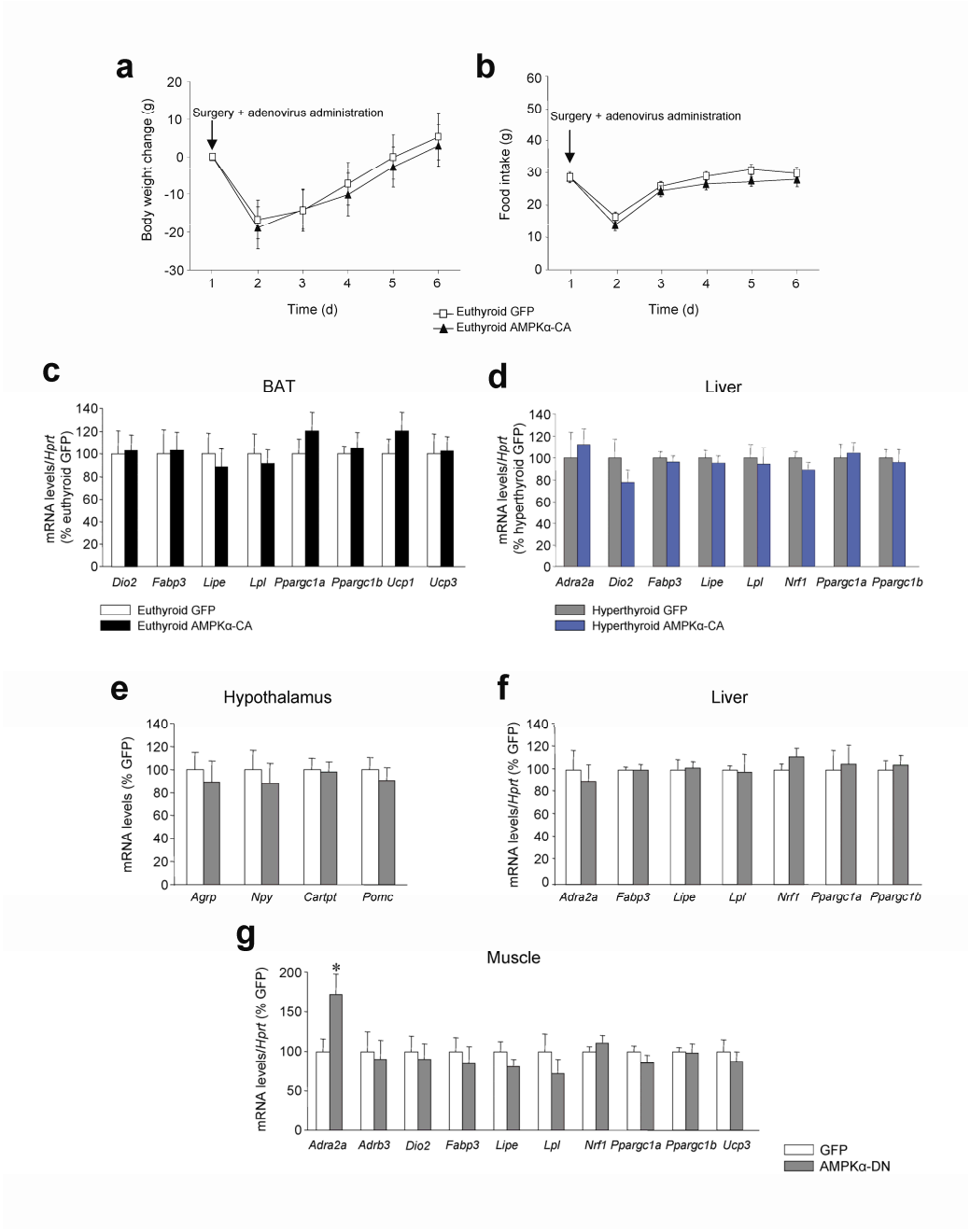
Supplementary Figure 4. Molecular and physiological effects of chronic central T3 administration. (**a–b**) mRNA expression profiles in the liver (**a**) and muscle (**b**) of euthyroid rats ICV-treated with T3 for 4 d. (**c–e**) mRNA expression profiles in the BAT (**c**), liver (**d**) and muscle (**e**) of hypothyroid rats ICV-treated with T3 for 4 d. (**f–i**) Body weight gain (**f**), daily food intake (**g**), hypothalamic pAMPKα, AMPKα1, AMPKα2, pACCα and ACCα protein levels (**h**) and mRNA expression profiles in the BAT (**i**) of euthyroid rats IP-treated with T3 for 4 d. ¡P = 0.08, +P = 0.06, , *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle; all data are expressed as mean ± SEM. *Adra2a:* gene encoding α2A-AR (adrenergic receptor alpha 2a); *Nrf1*: gene encoding NRF-1 (nuclear respiratory factor 1).



Supplementary Figure 5. Molecular and physiological effects of central T3 action in the hypothalamus. (**a–c**) Hypothalamic pAMPKα immunoreactivity (IR) (upper 40 ×, scale bar 500 μm; lower: 200 ×, scale bar, 20 μm), the dotted lines show the ARC, the PVH and the VMH (**a**). Pre-absorption with control peptide showing lack of positive pAMPKα-IR (40 ×, scale bar 500 μm) (**b**). Western blot showing the specificity of anti-pAMPKα-Thr172 antibody on hypothalamic protein extracts. The antibody just detected pAMPKα at the expected molecular weight (62 kDa) and no additional bands were observed (**c**). (**d**) Food intake of euthyroid rats treated with T3 ICV. (**e–f**) Immunofluorescence (40 ×, scale bar, 200 μm) showing the presence of the dye (flurorescein isothiocyanate dextran 7000) after co-microinjection with T3 into the VMH (**e**) and food intake of euthyroid rats treated with T3 stereotaxically into the VMH (**f**). All data are expressed as mean ± SEM. 3V: third ventricle; Th: thalamus; ox: optic chiasm; ZI: zona incerta (thalamus).



Supplementary Figure 6. Genetic ablation of thyroid hormone receptor in the VMH. (**a–c**) Localization studies (scale bar, 2 mm) showing the injection route for stereotaxic microinjections of adenoviral expression vectors into the VMH. Two injections (labeled as 1 and 2) were given in each VMH (scale bar, 2 mm) (**a**). The injection route enclosed in a red rectangle is precisely placed in the VMH (10 ×, scale bar, 500 μm) (**b**). Representative immunofluorescence (40 ×, scale bar, 200 μm) with anti-GFP antibody showing GFP expression in the VMH of rats treated with adenoviruses (**c**). (**d**) Western blot for protein levels of TRβ-1 in adenoviruses-infected HepG2 cells. (**e**) Transcriptional activity of a thyroid hormone response element containing reporter gene without or with 100 nM T3 in HepG2 cells, infected with control (GFP), wildtype (WT) or mutant thyroid hormone receptor dominant negative (TR-DN) expressing adenoviruses. (**f**) Expression of an endogenous target gene (*Shbg*) without or with 100 nM T3 in HepG2 cells, infected with control (GFP), wild type (WT) or mutant thyroid hormone receptor dominant negative (TR-DN) expressing adenoviruses. (**g–j**) Body weight change (**g**), daily food intake (**h**) and mRNA expression profiles in BAT of euthyroid rats (**i**) and liver of hyperthyroid rats (**j**) stereotaxically treated with a GFP-expressing adenoviruses or GFP plus TR-DN adenoviruses into the VMH. **P* < 0.05, ****P* < 0.001 *vs.* vehicle; #*P* < 0.05 WT TR-DN T3 *vs.* WT T3 and WT GFP T3; ###*P* < 0.001 WT TR-DN T3 *vs.* WT T3 and WT GFP T3; all data are expressed as mean ± SEM.



Supplementary Figure 7. Molecular and physiological effects of administration of AMPK-CA or AMPK-DN adenovirus in the VMH. (**a–d**) Body weight change (**a**), daily food intake (**b**) and mRNA expression profiles in BAT of euthyroid rats (**c**) and in liver of hyperthyroid rats (**d**) stereotaxically treated with a GFP-expressing adenoviruses or GFP plus AMPKα-CA adenoviruses into the VMH. (**e–g**) Hypothalamic neuropeptide mRNA expression (**e**), mRNA expression profiles in liver (**f**) and muscle (**g**) of euthyroid rats stereotaxically treated with adenoviruses expressing GPF or GFP plus AMPK-DN into the VMH. **P* < 0.05 *vs.* GFP. All data are expressed as mean ± SEM.

SUPPLEMENTARY METHODS

Animals and sample preparation. We used adult male Sprague-Dawley rats (9–11 weeks old, *Animalario General USC*; and *Harlan Sprague-Dawley*). USC and University of Iowa Animal Research Committees approved all procedures. All studies were conducted in accordance with the International Law on Animal Experimentation and National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. For all the experimental groups and analytic methods, we use 9–12 rats per group as a minimum. All the experiments were repeated at least twice. Rats were killed by cervical dislocation and quickly decapitated, in a separate room, and their tissues (brain, liver, BAT, WAT and muscle) removed. For hypothalamic studies, one set of brains animals per experimental group was removed intact for *in situ* hybridization analysis, when required. In another set of brains the hypothalamus was dissected, as previously described [1–5] for protein and activity or metabolite determination. All these samples were frozen immediately on dry ice and maintained at – 80 °C until processed. We collected trunk blood in heparinized tubes, centrifuged immediately, separated the plasma and kept frozen at – 20 °C until assayed. For immunohistochemical techniques rats were deeply anaesthetized with ketamine and xylaxine and then fixed by perfusion using formaline. Brains were removed and immersed for 2–4 h in the same fixative followed by a washing step in phosphate buffer 0.1M for 1 h. Fifty-μm vibratome sections were processed for immunohistochemical and double immunofluorescence free floating methods, as previously shown (see below) [2, 6].

Stereotaxic microinjection of adenoviral expression vectors and T3 microinjections. We placed rats in a stereotaxic frame (*David Kopf Instruments*) under ketamine-xylazine

anesthesia. The VMH was targeted bilaterally as previously reported [2]. Adenoviruses vectors (*Viraquest;* North Liberty) either *1)* GFP ($1x10^{12}$ pfu ml^{-1}, control adenoviruses) or *2)* GFP plus AMPKα dominant negative (AMPKα-DN; $1x10^{12}$ pfu ml^{-1}), or *3)* GFP plus AMPKα constitutively active (AMPKα-CA; $2x10^{10}$ pfu ml^{-1}), or *4)* GFP plus thyroid hormone receptor dominant negative (TR-DN; $3x10^{10}$ pfu ml^{-1}) were delivered at a rate of 200 nl min^{-1} for 5 min (1 µl per injection site) [2]. For the VMH microinjections, T3 (0.4 ng in 100 nl of DMSO) or vehicle (100 nl of DMSO) was given; the pipette was left in place for 5–10 min and then removed. We performed measurements after 1 h for the hypothalamic protein studies and during 6 h for the BAT SNA experiments. We mixed fluorescent dye (flurorescein isothiocyanate dextran 7000; *Sigma*) with T3 and vehicle for each injection to assess the accuracy of the injection site. Only animals that showed VMH-specific location of the injections (dye) were included (**Supplementary Fig. 5e**).

Sympathetic nerve activity recording. We anesthetized rats using intraperitoneal ketamine (91 mg kg^{-1}) and xylaxine (9.1 mg kg^{-1}) and maintained anesthesia with α-chloralose (initial dose: 25mg kg^{-1}, sustain dose: 50 mg kg^{-1} h^{-1}) via a catheter inserted in the femoral vein. The trachea was cannulated, and each rat was allowed to breathe spontaneously oxygen-enriched air. Rectal temperature was maintained at 37.5°C using a temperature-controlled surgical table and a lamp. We obtained multi-fiber recording of SNA from the nerve subserving BAT, as previously described [7, 8]. Using a dissecting microscope, a nerve fiber innervating interscapular BAT was identified, placed on the bipolar platinum-iridium electrode. Each electrode was attached to a high-impedance probe (HIP-511, *Grass Instruments*) and the nerve signal was amplified 10^5 times with a Grass P5 AC pre-amplifier. After amplification, the nerve signal was filtered at a 100- and 1000-Hz cutoff with a nerve traffic analysis system (Model 706C, *University of Iowa Bioengineering*). The nerve signal was then routed to an oscilloscope

(model 54501A, *Hewlett-Packard*) for monitoring the quality of the sympathetic nerve recording and to a resetting voltage integrator (model B600c, *University of Iowa Bioengineering*). BAT SNA measurements were made every 15 min for 6 h after ICV and VMH microinjections. To ensure that electrical noise was excluded in the assessment of sympathetic outflow, we corrected each SNA recording for post-mortem background activity

Blood biochemistry. We measured Plasma TSH, T3, T4, leptin and insulin using rat ELISA kits (*Crystal Chem Inc*). Kits for the measurement of glucose were obtained from *Roche***.**

Real-time quantitative PCR. We performed real-time PCR (TaqMan®; Applied Biosystems) as previously described [1, 3, 6, 8] , using the following sets of primers and probes (*GenBank* accession number is inside brackets after gene's name):

Adra2a (NM_012739): gene encoding adrenergic receptor alpha 2a (α2A-AR)

Forward primer: 5'-GGTGGTGATCGGCGTGTT-3'

Reverse primer: 5'-CGACGGCTATGAGCGTGTAG-3'

Probe: FAM-5'-TGGTGTGTTGGTTCCCGTTCTTTTTCA-3'-TAMRA

Adrb3 (NM_013108): gene encoding adrenergic receptor beta 3 (β3-AR)

Forward primer: 5'-CCTTCCCAGCTAGCCCTGTT-3'

Reverse primer: 5'-TGCTAGATCTCCATGGTCCTTCA-3'

Probe: FAM-5'-AACTCACCGCTCAACAGGTTTGATGGC-3'-TAMRA

Dio2 (NM_031720): gene encoding type 2 deiodinase (D2)

Applied Biosystems TaqMan® Expression Assays; Assay ID Rn00581867_m1

Fabp3 (NM_024162): gene encoding fatty acid binding protein 3 (FABP3)

Forward primer: 5'-ACGGAGGCAAACTGGTCCAT-3'

Reverse primer: 5'-CACTTAGTTCCCGTGTAAGCGTAGTC-3'

Probe: FAM-5'-TGCAGAAGTGGGACGGGCAGG-3'-TAMRA

Hprt (NM_012583): gene encoding hypoxanthine guanine phosphoribosyl transferase (HPRT)

Forward primer: 5'-AGCCGACCGGTTCTGTCAT-3'

Reverse primer: 5'-GGTCATAACCTGGTTCATCATCAC -3'

Probe: FAM-5'-CGACCCTCAGTCCCAGCGTCGTGAT 3'-TAMRA

Lipe (NM_012859): gene encoding hormone sensitive lipase (HSL)

Forward primer: 5'-CCAAGTGTGTGAGCGCCTATT -3'

Reverse primer: 5'-TCACGCCCAATGCCTTCT -3'

Probe: FAM-5'-AGGGACAGAGACGGAGGACCATTTTGACTC -3'-TAMRA

Lpl (NM_012598): gene encoding lipoprotein lipase (LPL)

Forward primer: 5'-CTGAAAGTGAGAACATTCCCTTCA-3'

Reverse primer: 5'-CCGTGTAAATCAAGAAGGAGTAGGTT-3'

Probe: FAM-5'-CCTGCCGGAGGTCGCCACAAATA-3'-TAMRA

Nrf1 (NM_001100708): gene encoding nuclear respiratory factor 1 (NRF1)

Forward primer: 5′-TGTTTGGCGCAGCACCTTT-3′

Reverse primer: 5′-CGCAGACTCCAGGTCTTCCA-3′

Probe: FAM-5'-ATGTGGTGCGCAAGTACAAGAGCATGATC-3′-TAMRA

Ppargc1a (NM_031347): gene encoding peroxisome-proliferator-activated receptor-gamma co-activator 1 alpha (PGC1α)

Forward primer: 5'-CGATCACCATATTCCAGGTCAAG-3'

Reverse primer: 5'-CGATGTGTGCGGTGTCTGTAGT -3'

Probe: FAM-5'-AGGTCCCCAGGCAGTAGATCCTCTTCAAGA-3'-TAMRA

Ppargc1b (NM_176075): gene encoding peroxisome-proliferator-activated receptor-gamma co-activator 1 beta (PGC1β)

Applied Biosystems TaqMan® Expression Assays;Assay ID Rn00598552_m1

Rplp0 (NM_001002): gene encoding ribosomal protein, large, P0 (RPLP0)

Forward primer: 5'-AGATGCAGCAGATCCGCAT-3'

Reverse primer: 5'-ATATGAGGCAGCAGTTTCTCCAG-3'

Probe: FAM-5'-AGGCTGTGGTGCTGATGGGCAAGAA-3'-TAMRA

Shbg (NM_001040): gene encoding sex hormone binding globulin (SHBG)

Applied Biosystems TaqMan® Expression Assays; Assay ID HS00168927_m1

Ucp1 (NM_012682): gene encoding uncoupling protein 1 (UCP1)

Forward primer: 5'-CAATGACCATGTACACCAAGGAA-3'

Reverse primer: 5'-GATCCGAGTCGCAGAAAAGAA-3'

Probe: FAM-5'-ACCGGCAGCCTTTTTCAAAGGGTTTG-3'-TAMRA

Ucp3 (NM_013167): gene encoding uncoupling protein 1 (UCP3)

Applied Biosystems TaqMan® Expression Assays;Assay ID Rn00565874_m1

In situ hybridization. Coronal hypothalamic sections (16 μm) were cut on a cryostat and immediately stored at –80°C until hybridization. We used specific oligos for AgRP, CART, FAS, NPY, POMC and TRH detection (*GenBank* accession number is inside brackets after gene's name):

Agrp (AF206017): *gene encoding* agouti-related protein; encoded **(**AgRP)

5'-CGACGCGGAGAACGAGACTCGCGGTTCTGTGGATCTAGCACCTCTGCC-3'

Cartpt (M29712): gene encoding cocaine and amphetamine-regulated transcript (CART)

5'-CCGAAGGAGGCTGTCACCCCTTCACA-3'

Fasn (NM_017332): gene encoding fatty acid synthase (FAS)

5'-GGGTCCATTGTGTGTGCCTGCTTGGGGTG-3'

Npy (M20373): gene encoding neuropeptide Y (NPY)

5'-AGATGAGATGTGGGGGGAAACTAGGAAAAGTCAGGAGAGCAAGTTTCATT-3'

Pomc (AF510391): gene encoding proopiomelanocortin (POMC)

5'-CTTGATGATGGCGTTCTTGAAGAGCGTCACCAGGGGCGTCTGGCTCTT-3'

Trh (NM_013046): gene encoding thyrotropin-releasing hormone (TRH)

5'-ATACCAGTTAGGGTGAAGATCAAAGCCAGAGCCAGCAGCAACCAA-3'

These probes were 3'-end labeled with ^{35}S-αdATP using terminal deoxynucleotidyl transferase (*Amersham Biosciences*). We performed in situ hybridizations as previously published [1–6]. Similar anatomical regions were analyzed using the rat brain atlas of Paxinos & Watson [9]. The slides from all experimental groups were exposed to the same autoradiographic film. All sections were scanned and the specific hybridization signal was quantified by densitometry using a digital imaging system (*ImageJ 1.33*)[1–6]. We determined the optical density of the hybridization signal and subsequently corrected by the optical density of its adjacent background value. A rectangle, with the same dimensions in each case, was drawn enclosing

the hybridization signal over each nucleus and over adjacent brain areas of each section (background). For the in situ analysis we used between 9 and 16 animals per experimental group. We used between 16 and 20 sections for each animal (4–5 slides with four sections per slide). The mean of these 16–20 values was used as the densitometry value for each animal.

Western blotting. We subjected hypothalamic total protein lysates were subjected to SDS-PAGE, electrotransferred them on a PVDF membrane and probed with the following antibodies: ACC, pACCα-Ser^{79}, AMPKα1 and AMPKα2 (*Upstate*); FAS (BD), tAMPKα, pAMPKα-Thr^{172}, pFoxO1-Ser^{256} (*Cell Signaling*); β-actin, pSTAT3-Tyr^{705} (*Abcam*) as previously described [2–6].

Immunohistochemistry. Enzymatic immunohistochemistry (*Dako EnVision™*), immunofluorescence and double labeling were performed as described [2, 6], using a rabbit anti-GFP (*Abcam*), rabbit anti-c-FOS (*Santa Cruz Biotechnology*), rabbit anti-pAMPKα-Thr^{172} (*Cell Signaling*) or goat anti-TRα (*Abcam*). We used 5–6 rats per experimental group. The specificity of Anti-pAMPKα-Thr^{172} antibody was shown by pre-absorption with 10 nmol ml^{-1} of control peptide (**Supplementary Fig. 5b**). Furthermore, anti-pAMPKα-Thr^{172} antibody specifically detected pAMPKα when hypothalamic samples were assayed by western blot, at the expected molecular weight (62 kDa) and no additional bands were observed (**Supplementary Fig. 5c**).

Enzymatic and malonyl-CoA assays. The CPT1 activity, the AMPKα1 and AMPKα2 phosphotransferase activities toward the AMARA peptide and the malonyl-CoA concentration were measured as previously described [2–6, 10]. For the AMPKα-CA and AMPKα-DN

experiments, malonyl-CoA was measured in the ventral hypothalamus, which was dissected under a microscope.

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