Supplemental Data

Materials and Methods

Tissue collection and microdissection

After decapitation, trunk blood was collected in Eppendorf tubes, stored on ice and the serum was separated by centrifugation 1 hour later. The sera were stored at -80°C until further processing. Pituitary, liver and small intestine were removed and immediately frozen in powered dry ice. Brains were removed from the skull, snap frozen in -40°C isopentane. Coronal, 1 mm thick slice containing the paraventricular nucleus (PVN) and 2 mm thick slice containing the arcuate nucleus-median eminence (ARC-ME) region were cut with blades in precooled brain matrix (Electron Microscopy Sciences). ARC-ME and PVN hypothalamic areas were microdissected with punch needle from frozen sections placed on pre-cooled glass slides using the Palkovits Punch technique under a Stemi 508 stereomicroscope (Zeiss). The samples were collected in precooled Eppendorf tubes and stored at –80°C.

Taqman Real-time quantitative PCR

According to manufacturer's instructions total RNA was isolated from peripheral tissue samples with RNeasy Mini Kit (Qiagen), while RNeasy Lipid Tissue Mini kit (Qiagen) was used for RNA isolation from punched brain areas. Qiagen RNase-free DNase set (ref: 79254) was used to eliminate genomic DNA contamination. Total RNA (1µg) was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). cDNA concentration was determined with Qubit ssDNA assay kit. 10 ng cDNA was used in each Taqman reaction. Reactions were assayed on Viia 7 Real-time PCR instrument (Applied Biosystems), mRNA expression of target genes was detected with Taqman Gene expression probe sets using Taqman Fast Universal PCR Mastermix (Thermo Fisher Scientific). *Gapdh*

expression proved to be stable in all used challenge conditions (no significant difference between groups and low variance in groups) and was used as housekeeping.

5.55 ng of cDNA was used to preamplify the gene of interest and the housekeeping gene in small intestine samples in aged animals using Taqman Preamp Master Mix (Applied Biosystems). Reaction product was diluted 1:5 and subjected to Taqman PCR with equal volumes of diluted preamplification product in each reaction. *Hprt1* expression was proved to be stable in all used challenge conditions (no significant difference between groups and low variance in groups) and was used as housekeeping for preamplified samples. Accession numbers of used Taqman probes are listed in Supplemental Table 1. The sequence of the *dCpG Luciferase* probe has been previously published (12).

ProTrh mRNA in situ hybridization

Under anaesthesia, blood was collected from the caudal vena cava and then, the animals were decapitated. The brains were removed from the skull and frozen in chilled isopentane (24h fasting experiment) or on powdered dry ice (48h fasting experiment) Serial 12 μm thick coronal sections through the rostro-caudal extent of the PVN were cut on a cryostat (Leica CM3050 S, Leica Microsystems) and adhered to Superfrost Plus glass slides (Fisher Scientific Co.) to obtain five sets of slides, each set containing every fifth section through the PVN. The tissue sections were stored at -80°C until prepared for *in situ* hybridization histochemistry.

Every fifth section of the PVN was hybridized with a 741 base (corresponding to the 106-846 nucleotides of the mouse *proTrh* mRNA; BC053493) single stranded [^{35}S]UTP labeled cRNA probe for mouse *proTrh* following methods as previously described (15). In vitro transcription was performed using SP6 polymerase (Roche) and [^{35}S] α-UTP (Perkin Elmer). The hybridization was performed under coverslips in a buffer containing 50% formamide, a 2-fold concentration of standard sodium citrate (2XSSC), 10% dextran sulfate, 0.5% sodium

dodecyl sulfate, 250 µg/ml denatured salmon sperm DNA, and $3x10^5$ cpm of radiolabeled probe for 16h at 56°C. Slides were dipped into Kodak NTB autoradiography emulsion (Eastman Kodak, Rochester, N.Y.) diluted 1:1 in distilled water, and the autoradiograms developed after 4 (24h fasting) or 5 (48h fasting) days of exposure at 4°C. The slides were immersed in 0.005% cresyl violet acetate (Sigma-Aldrich) for 2 minutes to obtain fluorescent counterstaining of cell nuclei, dehydrated in ascending ethanol series and xylenes, and coverslipped with DPX mountant (Sigma-Aldrich). *In situ* hybridization autoradiograms were visualized under darkfield illumination using a Zeiss Axioimager M1 microscope equipped with MRc5 digital camera (Zeiss) and analyzed with Image J software (NIH). The background density points were removed by thresholding the image. The area covered by *proTrh* hybridization signal in the PVN was measured in four sections containing the mid-level of the PVN identified by the fluorescent counterstaining.

Determination of circulating TH and TSH levels

Serum free T4 (fT4) and free T3 (fT3) levels were measured with AccuLite CLIA Microwells kit (Monobind Inc., Lake Forest, CA USA) according to the manufacturer's instructions. Serum TSH was determined using a MILLIPLEXTM Rat thyroid panel (Millipore Corporation) and read on a Magpix (Millipore Sigma).

Deiodination assay of pituitary samples

In order to determine D2 activity, whole pituitary samples were homogenized with sonication in PE buffer (0.1M potassium phosphate, 1 mM EDTA, pH 6.9) containing 0.25M sucrose and 10 mM DTT. Protein content was determined with Bradford's method. Lysates containing 100 µg protein were assayed in the presence of freshly purified 100.000 cpm ^{125}I-T4, 1nM T4, 100

nM T3, 1mM PTU and 20 mM DTT for 180 min at 37 °C in duplicate followed by separation of released iodine and counting in a gamma counter.

Simple Western Blot (WES)

Microdissected ARC-ME samples were homogenized by sonication in 50 µl lysis buffer (50 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 M NaCl) containing 0.05 V/V% Triton, 1 mM DTT and 1 tablet/10.5 ml Roche complete protease inhibitor cocktail. Total protein content was determined with Qubit Protein Assay kit (Life Technologies). WES Simple Western capillary electrophoresis system (Protein Simple) was used to determine TRH-DE levels using the 12-230 kDa Separation module (ref: SM-W004). Samples were prepared according to manufacturer's instructions, using 1X sample buffer and 10 min heat denaturation on 70 °C. Samples were loaded onto WES in a concentration of 1,6 mg/ml. TRH-DE (ref: AF2985) and GAPDH (ref: AF5718) antibodies were obtained from R&D Systems, optimal dilutions were determined as 1:100 for GAPDH and 1:50 for TRH-DE. WES anti-goat detection module (DM-006) was used for multiplex detection. Optimal run settings for WES were determined as 32 min separation time, 60 min primary antibody incubation time, other settings were default of instrument. TRH-DE peak areas were normalized with GAPDH peak areas.

Data Analysis

Microsoft Excel and STATISTICA v13 was used to analyse data. Prism version 8.3 was used to prepare the graphs. Figures show Tukey Box-Plot; box represents the two middle quartiles, lower whisker represents lower quartile, upper whisker represents upper quartile, line represents median, dots represent outlier data. Figure legend "n" shows mice used in groups. Null hypothesis significance tests were conducted with 95 % level of confidence; Student's two sample two-sided t-test was used to analyse two groups; one-way analysis of variance

(ANOVA) followed by Tukey and Dunnett post-hoc test was used to compare more than two groups. Models were deemed adequate based on residual plots and residual normal plots. Used methods are detailed under each figure.