Supplementary Methods

Animal care

Care of all animals was within institutional animal care committee guidelines and all procedures were approved by local government authorities and were in accordance with NIH guidelines. Mice were housed in groups of 3-5 at 22-24°C using a 12hrs light/12hrs dark cycle with lights on at 6 a.m. Animals were fed regular chow food (Teklad Global Rodent # T.2018.R12) containing 53.5% of carbohydrates, 18.5% of protein, and 5.5% of fat (12% of calories from fat). The Animals had ad libitum access to water at all times.

Generation of AgRP\textsuperscript{DTR} and POMC\textsuperscript{DTR} mice

AgRP-Cre and POMC-Cre mice were mated with iDTR\textsuperscript{+/-} mice, and a breeding colony was maintained by mating resulting double heterozygous AgRP-Cre iDTR\textsuperscript{+/-} (AgRP\textsuperscript{DTR}) and POMC-Cre iDTR\textsuperscript{+/-} (POMC\textsuperscript{DTR}) mice with wildtype mice. Only animals from the same mixed background strain generation were compared to each other.

Genotyping

Animals were genotyped for the presence of various transgenes by PCR on DNA isolated from tail biopsies as previously described. Animals were genotyped for the presence of the iDTR allele with primers 5’-GGCTACTGCTGACTCTCAACATT-3’ and 5’-TCATGGTGCCGAATTCGAT-3’, with primers 5’-TGGCTCAATGTCCCTCCCTTG-3’, 5’-CACATAAGCTCATCGTATAAG-3’ and 5’-GAGATATCTTITAACCCTGATC-3’ for the presence of the POMC-Cre-transgene, with primers 5’-ATGTTTAGCTGGCCCAAATG-3’ and 5’-CCCTAAGGATGAGGAGAGAC-3’ for the presence of the AgRP-Cre-transgene, with primers 5’-ATCCTCTGTGTCAGTTCCTT-3’ and 5’-CGTGGCCGTATTCCATTCC-3’ for the presence of the ROSA26-ß-Gal-transgene. Moreover, animals were genotyped for deletion of the STOP cassette on DNA isolated from tail biopsies, since both for a minority of
POMC-Cre and AgRP-Cre-transgenic mice, activity of the Cre-recombinase has been detected either in germ line or as a mosaic in somatic cells. Mice exhibiting detectable deletion for the STOP-cassette on DNA isolated from tail biopsies indicative of germ line or mosaic deletion were therefore sacrificed and not included in the study (27% of AgRP<sup>DTR</sup> and 8% of POMC<sup>DTR</sup> mice).

RNA

Measurements of mRNA levels were carried out by quantitative RT-PCR on RNA extracted from dissected hypothalamic tissue. Total RNA for each hypothalamus was quantified by spectrophotometry after purification using TRIzol reagent (peqLab). 200 ng of each total RNA sample was reverse-transcribed, then PCR-amplified using TaqMan Principles ABI Prism 7700 Sequence Detection System. Efficiency for the primers was estimated from standard curves made with serial cDNA dilutions.

Analytical procedures

Blood glucose values were determined from whole venous blood using an automatic glucose monitor (GlucoMen®; A. Menarini Diagnostics). Insulin, leptin and corticosterone levels in serum were measured by ELISA using mouse standards according to manufacturer’s guidelines (Insulin Mouse Ultrasensitive ELISA, DRG Instruments GmbH; Mouse Leptin Immunoassay, Quantikine; Corticosterone Enzyme Immunoassay Kit, Assay Designs Inc.).

Food intake determination

Food intake was measured in a regular cage using racks, which were weighed daily, and daily food intake was calculated as the average intake of chow within the time stated or measurements were performed with a Comprehensive Laboratory Animal Monitoring System (CLAMS, Oxymax Windows 3.0.3; Columbus Instruments).
Statistical methods

Datasets were analyzed for statistical significance using a two-tailed unpaired student’s T-Test.

Tissue preparation for immunohistochemistry and immunohistochemical procedures

AgRP<sup>DTR</sup> and POMC<sup>DTR</sup> mice were mated with Rosa<em>Arte1</em> reporter mice. AgRP<sup>DTR/LacZ</sup>, POMC<sup>DTR/LacZ</sup> and wildtype reporter mice were anesthetized, and then perfused transcardially with 40 ml of 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4). The hypothalami were dissected and soaked in 25% sucrose overnight for cryoprotection. Then, 25-μm thick free-floating coronal sections were cut through the paraventricular nucleus (PVN) and the arcuate nucleus (ARC) using a freezing microtome (Leica). The sections were collected in PBS (pH 7.4). The sections were treated with 1.0% sodium borohydride for 30 min, then 0.5% Triton X-100 in 0.5% H<sub>2</sub>O<sub>2</sub> in PBS for 15 min and in 2% normal horse serum in PBS to block the non-specific antibody binding. Following pretreatments, the sections were placed in sheep α-β-gal antiserum at 1:16,000 dilution for 2 days at 4°C, followed by treatment in Cy3-conjugated donkey anti-sheep IgG (1:100; Jackson) for 2h. After rinses in PBS, the sections were mounted and coverslipped with Vectashield mounting medium (Vector).

Ultrastructural analysis of AgRP and POMC perikarya

Mice were anesthetized and perfused transcardially with saline followed by 100 ml Somogyi-Takagi fixative containing 4% paraformaldehyde and 0.8% glutaraldehyde in 0.1 M phosphate buffer (PB). The brains were dissected and post-fixated in glutaraldehyde free fixative for at least 2 h at 4°C and washed in 0.1M PB. Tissue blocks containing the ARC were dissected from each brain. 50 μm-thick vibratome sections were cut and washed in 0.1
To eliminate unbound aldehydes, sections were incubated in 1% sodium-borohydride for 15 min, and then rinsed in PB. Subsequently, sections were incubated in rabbit anti-AgRP antisera (dilution 1:3000 in PB) for 24 h at room temperature followed by incubation in biotinylated goat anti-rabbit immunoglobulin (dilution 1: 250; Vector Laboratories) for 2 h at room temperature. Then, sections were incubated in avidin-biotin-complex (2 h, room temperature; ABC Elite Kit, Vector Laboratories) and the tissue-bound peroxidase was visualized by a diaminobenzidine reaction. After the immunostaining, the sections were osmicated (15 min in 1% osmium tetroxide in PB) and dehydrated in increasing ethanol concentrations. During the dehydration, 1% uranyl-acetate was added to the 70% ethanol to enhance ultrastuctural membrane contrast. Dehydration was followed by flat-embedding in Araldite. Ultrathin sections were cut on a Leica ultra microtome, collected on Formvar-coated single-slot grids and analyzed with a Tecnai 12 Biotwin (FEI Company) electron microscope.