Supporting Information

Aveleira et al. 10.1073/pnas.1416609112

SI Materials and Methods

Western Blotting. Cells or whole hypothalamic tissue were lysed on ice in RIPA (radio-immunoprecipitation assay) buffer [50 mM Tris·HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.5% deoxycholate; 0.1% SDS; 200 μM phenylmethylsulphonylfluoride; 1 mM DTT, 1 mM Na_3VO_4; 10 mM NaF], supplemented with mini protease inhibitor mixture tablet (Roche). Lysates were incubated for 15 min at 4 °C, and the insoluble material was pelleted by centrifugation for 10 min at 16,000 × *g* and 4 °C. The protein concentration of each sample was determined by the bicinchoninic acid protein assay (Pierce Biotechnology). The samples were denaturated by adding 6× concentrated sample buffer [0.5 M Tris, 30% (vol/vol) glycerol, 10% (wt/vol) SDS, 0.6 M DTT, 0.012% bromophenol blue] and heating for 5 min at 95 °C. Samples were stored at −20 °C until use.

Equal amounts of total protein were loaded per lane and separated by electrophoresis in 4–12% (wt/vol) SDS polyacrylamide gels (SDS/PAGE). Proteins were then transferred electrophoretically in CAPS buffer [0.1 M CAPS, pH 11; 10% (vol/vol) methanol] to polyvinylidenedifluoride membranes (Millipore). The membranes were blocked with 5% (wt/vol) low-fat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris·HCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were incubated overnight with the primary antibodies at 4 °C. The primary antibodies used (all at a dilution of 1:1,000) were as follows: rabbit anti-LC3B, anti-SQSTM1, anti-mTOR, anti-phospho-mTOR (Ser2448), anti-RPS6K and anti-phospho-RPS6K (Thr389), anti-phospho-AKT (Ser473), anti-AKT, anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-p44/42 MAPK (ERK1/2), anti-phospho-PKA (Thr197) and anti-PKA from Cell Signaling. After three washes with TBS-T, the membranes were incubated for 1 h, at room temperature, with an alkaline phosphatase-linked secondary antibody, specific to rabbit IgG in a 1:20,000 dilution. Protein immunoreactive bands were visualized by chemifluorescence with the ECF substrate (GE Healthcare) in a VersaDoc Imaging System (Bio-Rad). The membranes were reprobed with a monoclonal anti–β-tubulin antibody (1:10,000; Sigma) for equal protein loading control. The optical density of the bands was quantified with the Quantity One Software (Bio-Rad). The results are normalized to β-tubulin and are expressed as the relative amount compared with control.

qPCR. Total RNA was isolated by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, cells were lysed, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers, and eluted with 30 μL of RNase-free water by centrifugation. Total RNA amount was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific), and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm. RNA samples were treated with RNase-free DNase (Qiagen) to eliminate any contamination with genomic DNA. Reverse transcription into cDNA was carried out by using the iScript Select cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was reverse transcribed into cDNA in a 20-μL reaction containing 1× reaction buffer, 1× random primers, and 50 units of reverse transcriptase. Reverse transcription reactions were performed in a thermocycler at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C for 5 min. cDNA samples were then stored at −20 °C until use.

qPCR was performed in an iQ5 thermocycler (Bio-Rad) by using 96-well microtiter plates and the QuantiTect SYBR Green PCR Master Mix (Qiagen). The primers for the target gene (rat NPY, NM-012614; mouse NPY, NM-023456) and the reference gene (rat HPRT, NM-012583; mouse HPRT, NM-013556) were predesigned and validated by QIAGEN (QuantiTect Primers). A master mix was prepared for each primer set, containing the appropriate volume of 2× QuantiTect SYBR Green PCR Master Mix and 10× QuantiTectPrimer. For each reaction, 18 μL of master mix were added to 2 μL of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 20 μL per well. Negative controls were performed without RNA sample, which was substituted by water. The reactions were performed according to the manufacturer's recommendations: 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. The melting curve protocol started immediately after amplification. qRT-PCR products were run by electrophoresis on a 2% (wt/vol) agarose gel containing GreenSafe (NZYTech), a DNA stain. The amplification efficiency for each gene and the threshold values for threshold cycle determination (Ct) were determined automatically by the iQ_5 Optical System Software (Bio-Rad). Relative mRNA quantification was performed by using the ΔCt method for genes with the same amplification efficiency. The results are expressed as the relative amount compared with control.

Neuropeptide Y Overexpression in the Mouse Hypothalamic Arcuate Nucleus by Stereotaxic Injection of AAV. Thirty male C57BL/6 mice weighing 20–24 g were randomly divided into two groups ($n = 15$ per group) and housed two per cage, under 12-h light/dark cycles. Mice were anesthetized with an i.p. injection of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) and placed on a stereotaxic frame. The ARC was defined by using The Paxino's Mouse Brain Atlas. Injection was performed bilaterally into the ARC: 0.5 mm lateral to the middle line, 1.65 mm posterior to the bregma and −5.8 mm ventral to the brain surface. The control group (AAV-GFP) received 1×10^9 v.g. per side of AAV-hSyn-EGFP-miR-ctr (v.g., viral genomes), in a final volume of 1.5 μL per side. The NPY overexpression group (AAV-NPY) received 3.6×10^9 v.g. per side of AAV-hSyn-NPY, in a final volume of 1.5 μL per side. Injection was performed at a rate of 0.5 μL/min with a 10 mL-Hamilton syringe attached to an automatic Pump Controller (WPI). Needle was kept in place for 5 min to minimize backflow. Mice were allowed to recover for 2 d.

Pair feeding and body weight gain analysis. Mice were housed in pairs and monitored for 1 mo after AAV injections. Because overexpression of ARC NPY increases food intake and body weight, leading to an obese phenotype (1), which can potentially affect autophagy regulation in the hypothalamus (2, 3), AAV-NPY mice were pair-fed to control AAV-GFP mice (given the same amount of food that AAV-GFP mice ate, daily: ~4–5 g/d). Each mouse was weighted every other day, for weight control.

Tissue and blood collection. All animals were euthanized 1 mo after the AAV injections, by an i.p. administration lethal dose of sodium thiopental (Braun). Animals from both groups were randomly selected either for collection of blood and hypothalamic tissue for protein extraction, or for whole brain removal for immunohistochemistry experiments. For tissue lysates and blood analysis, first, the blood was collected and serum was separated by centrifugation (2,000 × *g* for 15 min). Serum samples were kept at −20 °C until use. Then, after decapitation, hypothalami were individually collected and stored at −80 °C until use. For immunohistochemistry,

animals were intracardially perfused with 4% (wt/vol) paraformaldehyde/PBS fixative solution and, after decapitation, the brains were removed and cryoprotected in 25% (wt/vol) sucrose/0.1 M PBS solution for 48 h at 4 °C. Brains were then stored at −80 °C until use. For immunohistochemistry purposes, brains were cut at a cryostat-microtome (Leica CM3050S; Leica Microsystems Nussloch) in 25-μm coronal sections. Slices were collected and stored in 48-well trays, free-floating in 0.1 M PBS supplemented with 0.12 μmol/L sodium azide. The plates were stored at 4 °C until immunohistochemical processing.

Serum analysis. Glucose levels were measured by using Accu-Check Blood Glucose Sensor (Roche). Cholesterol and triglycerides were measured on an automated Synchron Clinical System (Beckman Coulter). Serum NPY levels were measured with an ELISA kit (Raybiotech) following the manufacturer's instructions.

Behavioral assessment. For the assessment of mice locomotor horizontal activity, open field tests were performed 29 d after AAV injections. Mice were acclimated into a test room for 1 h. Mice were placed in a 50 × 50 cm arena with 50-cm-high walls, and their movement activity was recorded for 40 min by using the Acti-Track System (Panlab). Mean values for each measure were calculated. Data were analyzed by using Student's unpaired *t* test with two-tailed *P* value, using Prism 5.0 (GraphPad Software). A value of $P < 0.05$ was considered significant. No alterations were found in the locomotor activity of AAV-NPY mice, 29 d after injection. The distance traveled (8,592 ± 2,060.7 cm) and mean velocity (3.5 ± 0.7 cm/s) of AAV-NPY mice was similar to AAV-GFP mice (9,050 ± 458.4 cm and 3.8 ± 0.2 cm/s, respectively).

Immunohistochemistry. Hypothalamic NPY and LC3B expression was assessed by immunohistochemistry on brain sections. Briefly, 25-μm brain coronal sections were blocked and permeabilized in PBS with 10% (vol/vol) goat serum and 0.3% Triton X-100, to label NPY, and in PBS with 10% (vol/vol) goat serum and 0.5% Triton X-100, to label LC3B. Brain slices were then incubated with polyclonal rabbit anti-NPY antibody (1:6,000; Sigma) or polyclonal rabbit anti-LC3B antibody (1:400; Cell Signaling) in the respective blocking solution, overnight at 4 °C. Sections were then incubated with goat anti-rabbit Alexa Fluor 594-conjugated secondary antibody (1:200; Invitrogen) for 2 h at room temperature. The nuclei were stained with Hoechst 33342 (2 μg/mL). After incubation, brain sections were washed and mounted in slides with Mowiol mounting medium and analyzed on a Zeiss Axiovert fluorescence microscope (Carl Zeiss).

Quantification of NPY immunoreactivity in the mouse hypothalamic ARC. Four weeks after AAV injection, NPY immunohistochemistry and imaging procedures were performed. Coronal sections of approximately equal spacing were sampled over the anterior–posterior extent of the hypothalamic ARC (Bregma −1.34 to −2.54) for NPY immunoreactivity determination (four mice per group). The ARC was defined by using The Paxino's Mouse Brain Atlas. For each mouse, the ARC of one hemisphere was delimited and the integrated density (the product of area and mean gray value; arbitrary units) of 12 sections was measured by using the Fiji (Fiji is Just ImageJ) software (National Institutes of Health). The integrated density values were summed to yield total integrated density values for each animal, and the mean of the total integrated density values was calculated for each experimental group. Analyses were done on one hemisphere from each section. The results are expressed as the relative amount compared with control.

1. Sousa-Ferreira L, et al. (2011) Moderate long-term modulation of neuropeptide Y in hypothalamic arcuate nucleus induces energy balance alterations in adult rats. *PLoS ONE* 6(7):e22333.
2. Las G, Serada SB, Wikstrom JD, Twig G, Shirihai OS (2011) Fatty acids suppress autophagic turnover in β-cells. *J Biol Chem* 286(49):42534–42544.
3. Ost A, et al. (2010) Attenuated mTOR signaling and enhanced autophagy in adipocytes from obese patients with type 2 diabetes. *Molecular Medicine* 16(7–8):235–246.