SUPPLEMENTAL INFORMATION

Extended Experimental Procedures.

Quantitative RT-PCR (qPCR)

Proprietary sequence Taqman Gene Expression assay FAM/TAMRA primers (Applied Biosystems) used were: *Agrp* (Mm00475829_g1), *Atf4* (Mm00515324_m1), *Atf6* (Mm01295317_m1), *Beta-Actin* (4352933E), *Bip* (Mm00517691_m1), *Cart* (Mm00489086_m1), *Cat* (Mm00437992_m1), *Chop* (Mm00492097_m1), *Cpe* (Mm00516341_m1), *Crhr1* (Mm00432670_m1), *Drp1* (Mm01342903_m1), *Fis1* (Mm00481580_m1), *Gapdh* (Mm99999915_g1), *Gh* (Mm01258409_g1), *Gpx-1* (Mm00656767_g1), *Hprt* (Mm00446968_m1), *Mfn1* (Mm00612599_m1), *Mfn2* (Mm00500120_m1), *Ndufa9* (Mm00481216_m1), *Npy* (Mm00445771_m1), *Opa1* (Mm00453879_m1), *Pam* (Mm01293044_m1), *Pc1/3* (Mm00479023_m1), *Pc2* (Mm00500981_m1), *Pgc1*α (Mm01208835_m1), *Pit1* (Mm00476852_m1), *Pomc* (Mm00435874_m1), *Prcp* (Mm00804502_m1), *Sod2* (Mm01313000_m1), *Tpit* (Mm00453377_m1), *Tsh*β (Mm00437190_m1), *Ucp1* (Mm00494069_m1), *Xbp1s* (Mm03464496_m1).

Hypothalamic immunohistochemistry

Primary antibodies used were: rabbit anti-POMC precursor (1:1,000, Phoenix Pharmaceuticals), sheep anti-α-MSH (1:20,000, Chemicon), mouse anti-Mfn2 (1:200, Abcam) and rabbit anti-Tom20 (1:100, Santa Cruz Biotechnology). Secondary antibodies used were: chicken anti-rabbit 488 Alexa Fluor, donkey anti-sheep 488 Alexa Fluor, chicken anti-mouse 594 Alexa Fluor and goat anti-rabbit 594 Alexa fluor (1:400, Molecular Probes). Mfn2 immunoreactive signal was amplified using Biotinylated secondary anti-mouse (1:600, Abcam), Vecstatin A/B solution (1:365, Vector Laboratories), Biotinylated-tyramide (1:200, Perkin Elmer) and Streptavidin Alexa Fluor conjugate 610 (1:1,000, Molecular Probes).

Mitochondrial network complexity analysis in POMC neurons

POMCZ/EG mice were submitted to high-fat diet for 12 weeks and transcardially perfused. Immunohistochemitry for the mitochondrial marker Tom20 was performed as described above. A minimum of 15 ARC POMC GFP positive neurons from each mouse (n=3/group) were visualized. Analysis was performed using the Image J software. Briefly, acquired images were segmented by threshold to select the cellular area of study. Tom20 staining was submitted to background subtraction and filtering processes with Gausian Blur filter set at 0.8. Segmentatation of POMC neuron mitochondria was performed by local standard threshold using a radius of 7. Mitochondria of each POMC neuron were then subjected to particle analysis for acquiring aspect ratio (AR=major axis/minor axis) and circularity ($4\pi \cdot$area/ perimeter^2). Form factor (FF) was calculated as the inverse of circularity. An AR value of 1 indicates a perfect circle, and as mitochondria elongates and become more elliptical AR increases. A FF value of 1 corresponds to a circular unbranched mitochondrion, while higher FF values indicate a longer more branched mitochondrion. Particles smaller than 1.2 μm^2 were excluded. Images were taken with a Leica TCS SP5 laser scanning confocal system with a 63x oil immersion objective APO CS numerical aperture 1.4 equipped with a DMI6000 inverted microscope. GFP and AlexaFluor 594 images were acquired sequentially using 488-, 594-nm laser lines, an acousto optical beam splitter and emission detection ranges 500–550, 605–700 nm, respectively. The confocal pinhole was set at 1 Airy unit. Pixel size was 60 nm. 4x zoom was used in all images.

Electron microscopy and mitochondrial analysis

Control and *POMCMfn2KO* mice were transcardiacally perfused with 0.9% saline with heparine followed by fixative solution (paraformaldehyde 4%, gluteraldehyde 0.1%, picric acid 15% in phosphate buffer (PB) 0.1M, pH=7.4). Brains were removed and fixed overnight at 4°C with the same fixative without gluteraldehyde. Brains were washed vigorously with ice-cold PB 0.1 M, and sliced at 50 µm in a vibratome. Sections containing the ARC were stained for POMC (1:4,000; 48h at 4ºC with gentle shaking; Phoenix

Pharmaceuticals). After extensive washes, slices were incubated with secondary antibody, then with ABC and finally developed using 3,3'-Diaminobenzidine. After developing, slices were put in osmium tetroxide (1%, 15 min) and then dehydrated in an ethanol gradient. Uranyl acetate (1%) was added to 70% ethanol to enhance ultrastructural contrast. Slices were then embedded in Durcupan, cut in an ultra microtome and collected in grids for posterior analyzes. A Tecnai 12 Biotwin electron microscope was used to visualize the ultrastructure of the samples, and POMC neurons were imaged at 13,000x magnification for posterior offline analyzes. For mitochondria analyzes, random sections of POMC neurons cut throughout the middle of the cell body were analyzed. Most of these sections contained the nucleus. ImageJ software was used to manually outline each individual mitochondrion in the digital images. All samples were checked twice for consistency of mitochondria labeling. We used mitochondria cross-sectional area as a measurement of mitochondria size, and mitochondria aspect ratio (AR) as an index of mitochondria shape. Mitochondria density was estimated by dividing the number of mitochondria profiles by the cell area. Mitochondria coverage was estimated by dividing the total area of mitochondria (sum of all mitochondria profiles in a given cell) by the cell area. The same images were used for mitochondria-ER interaction analyzes. Slices containing POMC cytosol that were not cut across the middle of the cell were also included in this analysis. The number of mitochondria in juxtaposition to ER were counted and divided by the total number of mitochondria in the same cell to generate an index of mitochondria-ER interaction. All investigators were blinded to the experimental groups during the entire procedure.

Intracerebroventricular (i.c.v.) cannulation and treatments

I.c.v. surgery was carried out on 12 week-old male *POMCMfn2KO* mice and littermate controls. Mice were anaesthetized with a Ketamine/Xylazine cocktail and positioned in a stereotaxic frame (Kopf Instruments). The skull was exposed and a 26-gauge stainless steel guide cannula (PlasticsOne) was implanted into the third ventricle (midline 0 mm, 0.82 mm posterior from bregma, depth 4.8 mm from skull surface). The cannula was secured to the

skull with screws and dental cement and temporarily occluded with a dummy cannula (PlasticsOne). Post-surgery, the mice were singly-housed and given at least one week to recover. Prior to the study, cannula placement was verified by a positive dipsogenic response to angiotensin II (1 nmol in 1µL; Sigma-Aldrich). On experimental days, control and *POMCMfn2KO* mice were infused with: a) 2µl of either vehicle (aCSF; Tocris bioscience) or α-MSH (1nmol/µL; Sigma-Aldrich) 1h before lights-off; b) 2µl of either vehicle (Lipofundin; Braun) or Honokiol (37.6 mM; Sigma-Aldrich) 1h before lights-off. Food intake and body weights were measured over the subsequent 24h. In two independent set of studies, control and *POMCMfn2KO* mice were infused with 2µl of either vehicle (aCSF), Tauroursodeoxycholic acid (TUDCA; 2.5 µg/µl) (Calbiochem) or 4-phenyl butyric acid (4-PBA; 16.67 µg/µl) (Calbiochem) for 12 and 10 consecutive days (once a day, 1h before lights off), respectively. Food intake and body weights were recorded daily. I.c.v. injections were performed using a 30-gauge needle that extended 0.5 mm below the guide cannula (PlasticsOne), connected by cannula connector to a 5 µl Hamilton syringe and infused over 1 min using a microinfusion pump (Harvard Apparatus). Sixteen hours after the last chemical chaperone or vehicle injection, mice were either culled to dissect the hypothalamus or perfused for immunohistochemistry studies.

Western blotting

Western blot analysis was performed as previously described with minor modifications (Sebastian et al., 2012; Ozcan et al., 2009). Phospho-PERK immunoblotting was performed loading 100 µg of total protein. The following antibodies were used: Mfn2, XBP1s (1/1,000; Abcam); eIF2α, p-eIF2α, PERK and p-PERK (1/1,000; Cell Signaling); CHOP (1/1,000; Santa Cruz); ATF4 (1/1,000; Aviva Systems Biology); α-tubulin (1/8,000; Sigma); β-Actin (1/1,000; Sigma).

Mitochondrial respirometry

Mitochondrial function was assessed using high-resolution respirometry (Oroboros Oxygraph-2k, Oroboros Instruments), as described previously (Pesta and Gnaiger, 2012) and used by us in previous publications (Lindfors et al., 2011; Garcia-Roves et al., 2008; Holmstrom et al., 2012). Briefly, fresh hypothalamic arcuate-enriched microdissections were obtained from control and *POMCMfn2KO* mice. Tissue samples were mechanically permeabilized and homogenized in cold respiration media medium (0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose and 0.1% (w/v) bovine serum albumin, pH 7.1). The tissue homogenate was added to a 2 ml chamber to assess oxygen flux. Leak respiration was measured by adding malate (final concentration 2 mM) and pyruvate (10 mM), in the absence of ADP. Oxidative phosphorylation due to complex I substrates was quantified by the addition of ADP (5 mM, Oxphos I), followed by additions of glutamate (20 mM) and succinate (10 mM) for convergent electron flow through both complex I and II (Oxphos I+II). Subsequently, carbonylcyanide-4-(trifluoromethoxy)-phenyl-hydrazone (FCCP) was titrated to achieve maximum flux through the electron transfer system (ETS I+II) (1 µM). Finally, respiration was inhibited by the sequential addition of rotenone (0.1 µM) and antimycin A (2.5 µM), respectively. The remaining O_2 flux after inhibition with antimycin A (O_2 flux independent of the electron transfer system) was subtracted to calculate the different respiratory states. Oxygen flux values are expressed relative to protein content of the hypothalamic arcuate-enriched homogenate determined by Bradford method (pmol O2/sec/mg protein).

Carbonylated protein determination

Levels of carbonylated proteins in hypothalamic lysates from control and *POMCMfn2KO* mice were determined either by western blot (Oxyblot protein oxidation detection kit; Chemicon) or ELISA (Enzo Life Sciences) following manufacturers' instructions.

Generation of the adenoviral expression clone containing the full-length human Mfn2

Human Mfn2 cDNA was amplified with two primers bearing attB1 and attB2 gateway recombination sites using DNA polymerase (Biotools): attB1Mfn2 5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTCCCTGCTCTTCTCTCGA-3´; ttB2Mfn2 5´-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATCTGCTGGGCTGCA GGTACT-3´. PCR products were gel-purified and cloned into the pDONR221 vector using BP clonase (Invitrogen). Correct entry clones were identified by restriction analysis and fully sequenced. Positive clone was recombined using LR clonase (Invitrogen) with the vector pAd/CMV/V5-DEST to construct vector plasmids under the control of the human CMV promoter. Adenoviruses were obtained following standard protocols. Briefly, the construct was digested with PacI (New England Biolabs) and purified to expose the inverted terminal repeats (ITRs). The production and purification of the adenovirus was performed by the Vector Production Unit (UPV) of the Center for Animal Biotechnology and Gene Therapy (CBATEG) at the Universitat Autònoma de Barcelona. The expression clone was transfected into HEK 293 cells to produce an adenoviral stock and crude viral lysate was obtained. Purification was performed by double cesium chloride gradient and size exclusion chromatography. Virus infectious titer was determined by infecting HEK293 cells with serial dilutions of the purified vectors and immunocytochemical detection of hexon viral protein.

Adenoviral-mediated Mfn2 overexpression

C57BL/6 mice were fed either standard chow diet or high-fat diet (HFD) for 12 consecutive weeks (starting at 6 weeks of age). Animal experiments were conducted in accordance to the standards approved by the Faculty Animal Committee at the University of Santiago de Compostela, and the experiments were performed in agreement with the Rules of Laboratory Animal Care and International Law on Animal Experimentation. Mice were anesthetized by an intraperitoneal injection of Ketamine/Xylazine cocktail and placed in a stereotaxic frame (Kopf Instruments). Adenoviral vectors containing the full-length human Mfn2 (9.8×10^{10} pfu/ml) or null controls (1.8×10^{10} pfu/ml)

were injected in the ARC. The ARC was targeted bilaterally using a 32-gauge needle connected to a 1-μl syringe (Neuro-Syringe, Hamilton) and adenoviral vectors were delivered at a rate of 0.1 μl/min for 10 min (1 μl/injection site) according to the following coordinates: 1.5 mm posterior to the bregma, ± 0.2 mm lateral to midline, and 6 mm below the surface of the skull. Correct ARC adenoviral delivery was assessed by injecting Ad-GFP (3.1×10^{12} pfu/ml; Signagen Laboratories) and immunofluorescence analysis. After the procedure, the incision was closed with sutures, and mice were placed in a heated cage until they recovered from anesthesia. Body weight and food intake were measured daily for 11 consecutive days after the surgery. Average food intake was calculated from day 5 onwards to assure normalization of feeding behavior after surgery. Adiposity and insterscapular temperature was assessed at the end of treatment.

Supplementary figures

Supplementary Figure 1. Mitochondrial network complexity in POMC neurons is altered in DIO mice, Related to Figure 1.

Representative segmented images of mitochondria in individual POMC neurons from 18-week old male C57Bl/6 lean (A) and DIO (B) mice. A minimum of 45 POMC neurons from 3 different mice were analyzed.

(C-F) Expression of mitochondrial dynamics transcripts in the hypothalamus of lean and DIO mice at different time points during a high-fat diet time-course study (n=7-8/group/time point). Probe for *Hprt* was used to adjust for total RNA content. Data are expressed as mean ± SEM.

Supplementary Figure 2. Mfn2 deletion, pituitary-adrenal axis function and POMC neuron anatomy in *POMCMfn2KO* mice, Related to Figure 3.

(A) Representative confocal immunofluorescence images of the ARC from male *POMCZ/EG* indicator mice showing POMC neurons (green) and Mfn2 staining (red). Arrows show colocalization.

(B) PCR analysis of the recombination of the floxed allele in different tissues (MK: marker; H: hypothalamus; P: pituitary; C: cortex; K: kidney; L: liver; M:

muscle; F: fat; T:tail) from male control and *POMCMfn2KO* mice. A PCR reaction with IL-2 as internal control is also shown.

(C) Expression analysis of mitochondrial dynamics genes in the hypothalamus from 12-week old male control (n=6) and *POMCMfn2KO* (n=12) mice. Probe for *Hprt* was used to adjust for total RNA content.

(D) Expression analysis of representative pituitary genes in pituitaries from 12-week old male control (n=5) and *POMCMfn2KO* (n=5) mice. Probe for *Gapdh* was used to adjust for total RNA content.

(E) Plasma corticosterone levels under basal and stressed conditions in 6-week and 12-week old male control (n=10) and *POMCMfn2KO* (n=6) mice.

(F-H) Plasma concentrations of epinephrine, norepinephrine and dopamine in 12-week old male control (n=6) and *POMCMfn2KO* (n=7) mice.

(I) Representative images of the ARC from *POMCZ/EG* and *POMCMfn2KOZ/EG* mice showing POMC neurons.

(J) POMC neuron number and distribution throughout the ARC, and (K) POMC neuron area in 12-week old male control (n=3) and *POMCMfn2KO* (n=3) mice. 3V: third ventricle; ME: median eminence; ARC: arcuate nucleus of the hypothalamus; ns: not significant. Data are expressed as mean ± SEM. **P*<0.05.

Supplementary Figure 3. Pituitary-adrenal axis function and phenotypic characterization of *POMCMfn1KO* mice, Related to Figure 4.

(A) Expression analysis of mitochondrial dynamics genes in the hypothalamus from male control (n=6) and *POMCMfn1KO* (n=10) mice. Probe for *Hprt* was used to adjust for total RNA content.

(B) Expression analysis of representative pituitary genes in pituitaries from male control (n=6) and *POMCMfn1KO* (n=10) mice. Probe for *Gapdh* was used to adjust for total RNA content.

(C) Plasma corticosterone levels under basal and stressed conditions in male control (n=6) and *POMCMfn1KO* (n=10) mice.

(D-F) Plasma concentrations of epinephrine, norepinephrine and dopamine in male control (n=6) and *POMCMfn1KO* (n=10) mice.

(G) Body weight profile of control (n=6) and *POMCMfn1KO* (n=15) mice on chow diet.

(H) Body fat content in control (n=9) and *POMCMfn1KO* (n=8) mice.
(I) Plasma leptin in control (n=6) and *POMCMfn1KO* (n=10) mice.
(J) Daily food intake and (K) fast-refeeding test in control (n=6) and *POMCMfn1KO* (n=10) mice.
(L) Oxygen consumption, (M) energy expenditure and (N) interscapular temperature in control (n=9) and *POMCMfn1KO* (n=8) mice.
(O) Neuropeptide expression in the hypothalamus of control (n=6) and *POMCMfn1KO* (n=10) mice under fasting conditions. Probe for *Hprt* was used to adjust for total RNA content.
All studies were conducted in male 12-14 week old mice. Data are expressed as mean ± SEM. ns: not significant. **P*<0.05.

Supplementary Figure 4. Mitochondria-ER contacts and expression of hypothalamic ER stress markers in *POMCMfn2KO* mice, Related to Figure 5.
(A) Representative EM images of POMC neurons from non obese 6-week old control and *POMCMfn2KO* mice . Note enlarged, round mitochondria as well as diminished mitochondria-ER contacts in mutant mice.
(B) Quantification of mitochondria-ER interactions in control and *POMCMfn2KO* mice at 6 weeks of age (n=5/genotype).
(C-D) Immunoblot analysis of ER stress markers in the hypothalamus from control (n=5-7) and *POMCMfn2KO* (n=6-7) mice at 6 (C) and 12 (D) weeks of age. Representative images and densitometric quantification is shown. α-Tubulin was used as loading control. Data are expressed as mean ± SEM. **P*<0.05; ***P*<0.01; ****P*<0.001.

Supplementary Figure 5. Mfn2 deletion in POMC neurons alters mitochondrial respiratory capacity and enhances ROS production, Related to Figure 6.
(A) High-resolution mitochondrial respirometry of fresh hypothalamic arcuate-enriched microdissections from male control and *POMCMfn2KO* mice (n=8/genotype).
(B) Hypothalamic gene expression levels of Complex I subunit NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9 (Ndufa9) in control (n=8-10) and *POMCMfn2KO* (n=15) mice.

(C) Protein carbonylation (n=5/genotype) and (D-E) expression of ROS detoxification enzymes in the hypothalamus from control (n=8-10) and *POMCMfn2KO* (n=15) mice.

(F-G) Effects of i.c.v. administration of ROS scavenger honokiol in control (n=5) and *POMCMfn2KO* (n=5) mice on (F) food intake and (G) body weight gain.

(H) Carbonylated protein content in the hypothalamus from control (n=4) and *POMCMfn2KO* (n=4) mice after i.c.v. treatment with either vehicle or the chemical chaperon 4-PBA. Data are expressed as mean ± SEM.

All studies were conducted in male 12-14 week old mice. Data are expressed as mean ± SEM. V: vehicle. H: honokiol. **P*<0.05; ***P*<0.01; ****P*<0.001.

Supplementary Figure 6. Restoration of energy homeostasis in *POMCMfn2KO* mice by tauroursodeoxycholic acid (TUDCA) administration, Related to Figure 7.

Effects of 12-day i.c.v. TUDCA administration in 15-week old male control (n=5) and *POMCMfn2KO* (n=5) mice on:

(A) Daily food intake.

(B) Body weight gain.

(C) Adiposity.

(D) Plasma leptin.

(E) Expression of ER stress markers in the hypothalamus.

(F) *Pomc* mRNA expression.

(G) Hypothalamic POMC content.

(H) Hypothalamic α-MSH content.

(I) α-MSH/POMC ratio.

(J) Representative immunofluorescence images showing α-MSH staining in the PVN and (K) integrated density quantification (n=3/genotype/treatment).

Probe for *Hprt* was used to adjust for total RNA content. Data are expressed as mean ± SEM. **P*<0.05; ***P*<0.01; ****P*<0.001. eWAT: epididymal white adipose tissue; BW: body weight; V: vehicle. 3V: third ventricle. PVN: paraventricular nucleus.