SUPPLEMENTARY MATERIAL

Supplementary Methods

Generation and breeding of mice

arc*Pomc*$^{-}$. Mice carrying a floxed neo-cassette inserted in the neuronal *Pomc* enhancer neighborhood were generated as follows: The targeting vector was constructed with genomic sequences between -13 and -6.5 kb of mouse *Pomc* that were previously isolated (19), and a transgene construct harboring a 172 bp-deletion of *Pomc* neuronal enhancer nPE2 (18). A neomycin-resistance cassette (PGK-neo) flanked by *lox*P sites was inserted into a *Sph*I site located 40 bp upstream of the deleted nPE2 region (10.3 kb upstream of the *Pomc* transcriptional start site). The 5' and 3' recombination arms encompassed 3.0 kb and 3.3 kb, respectively. The targeting vector also included a *Herpes simplex I* thymidine kinase expression cassette (HSV-TK) downstream of the 3' arm to reduce the number of spurious recombination events. The targeting vector was linearized with *Kpn*I and electroporated into J1 ES cells (45), which were then propogated under positive-negative selection with G418 and ganciclovir. Clonal cells were screened for correct homologous recombination by Southern blot analysis of genomic DNA digested with *Eco*RV. Membranes were hybridized separately to [^{32}P]-radiolabeled 5' and 3' external probes cloned by PCR from *Pomc* sequences centered at positions -17.4 and -2.1 kb, respectively. Both probes hybridize to the same 15.5 kb band from the wildtype *Pomc* allele. Due to the presence of an internal *Eco*RV site in the targeting vector, the 5' and 3' external probes hybridize to 9.3 kb and 8.2 kb bands,

respectively, that are diagnostic for the correctly targeted mutant allele. Clone no. 2H4 with a normal karyotype (40, XY) was microinjected into e3.5 blastocysts derived from C57BL/6J mice to obtain germ-line transmission competent-male chimeras. Chimeric males were bred to C57BL/6 females to obtain heterozygous arc*Pomc*$^{+/-}$ mice. Integrity of the wildtype and mutant alleles was reconfirmed by Southern blots of tail genomic DNA and thereafter all mice were genotyped by PCR. Mice were backcrossed with C57BL/6 for 5 generations.

arc*Pomc*$^{+/+}$*.* To obtain mice lacking the neo-cassette from the start of embryonic development, arc*Pomc*$^{+/-}$ mice were mated with EIIa-Cre mice (24) [B6.FVB-Tg(EIIa-cre)C5379Lmgd/J; The Jackson Laboratory], which express Cre recombinase ubiquitously. Recombination in the offspring was ascertained by genomic PCR with primers delta 2.5 and delta 2.3 (27), which flank the floxed neo cassette and yield a 340-bp band for wild-type allele and a 309-bp band for the targeted allele lacking neo, while the allele with an intact neo cassette cannot be amplified. Mice lacking both the neo cassette and the EIIa-Cre transgene were backcrossed into C57Bl/6 for 5 generations giving rise to arc*Pomc*$^{+/+}$ mice

arcPomc$^{-/-}$*:POMC-EGFP.* To obtain mice lacking arcuate POMC expression with the cell-autonomous EGFP signal in POMC cells, arc*Pomc*$^{+/-}$ mice were crossed with heterozygous POMC-EGFP (19) mice. arc*Pomc*$^{+/-}$:POMC-EGFP mice were crossed with arc*Pomc*$^{+/-}$ mice to obtain arc*Pomc*$^{-/-}$:POMC-EGFP.

Immunohistochemistry

Mice were perfused with 4% paraformaldehyde in KPBS and brains were excised, postfixed in 4% paraformaldehyde/KPBS overnight at 4°C, and sectioned (50 µm) with a Vibratome 1000 (Ted Pella, Redding, CA). Brain slices were treated with 1% H_2O_2 in KPBS for 1 h, washed twice with KPBS, and incubated overnight at 4 °C with rabbit anti-rat ACTH antibody (1:10,000, A. Parlow, National Hormone, and Peptide Program) diluted in KPBS, 0.3% Triton X-100 and 2% normal goat serum. The next day, slices were washed in KPBS and incubated with biotinylated anti-rabbit IgG antibody (Vector) diluted 1:200 in KPBS, 0.3% Triton X-100 for 2 h at room temperature (RT). After washing in KPBS, slices were incubated with avidin/biotin-horseradish peroxidase complex (Vectastain Elite ABC kit; Vector) for 1 h at RT, washed in KPBS, and developed with 25 mg/ml of diaminobenzidine (DAB; Sigma) and 0.05% H_2O_2 in TBS (150 mM NaCl, 50 mM Tris–HCl, pH 7.5). Stained slices were mounted onto SuperFrost Plus slides (Fisher Scientific) and coverslipped with Permount (Fisher Scientific). Images were taken with an Olympus DP72 digital camera connected to a research microscope (Olympus BX51). The number of stained POMC neurons (positive for ACTH) in rescued mice was counted all across the arcuate nucleus and matched with equivalent slices of tamoxifen treated Arc-*Pomc*$^{+/+}$ mice to calculate percentage of recovery. Immunofluorescence was performed similarly as described above but the primary antiserum was detected with AlexaFluor555-coupled anti-rabbit IgG (1:1000; Molecular Probes, Invitrogen). After washing in KPBS, brain slices were mounted on Vectashield (Vector Labs). ACTH positive cells (red immunofluorescence) were co-

localized with endogenous EGFP fluorescence. Pituitary sections premounted onto glass slides were treated similarly for ACTH-like immunofluorescence.

Hypothalamic *Pomc* mRNA

Hypothalamic total RNA was prepared with Trizol (Invitrogen) and then treated with RNase-free DNase I (Ambion). First-strand cDNA synthesis was generated with random primers using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *Pomc* mRNA was identified by relative quantitative RT-PCR using TaqMan gene expression assay (Mm00435874_M1, Applied Biosystems) and normalized to 18S rRNA (HS99999901_S1, Applied Biosystems). Samples were run on a Rotor-Gene 6000 instrument (Corbett Life Science) and results were analyzed by the $2^{-\Delta\Delta C}$T relative quantitation method (46).

Serum glucose and hormone levels.

Blood for glucose, insulin, and leptin measurement was obtained by tail bleeding and collected between 10 am and 11 am following a 16-20 h fast. Samples were centrifuged at 1,000 g for 10 min, and the sera stored frozen at –20°C. The first drop of whole blood was used for glucose determination using a glucometer (OneTouch Ultra, LifeScan). Serum hormones were measured using mouse-specific leptin and insulin ELISA kits (Crystal Chem Inc.).

Additional References

45. Li, E., Bestor, T.H. & Jaenisch, R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**, 915-926 (1992).

46. Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408 (2001).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Detection of rescued POMC cells. Examples of coronal brain sections of *Pomc*$^{+/+}$, arc*Pomc*$^{-/-}$ and rescued arc*Pomc*$^{-/-}$:Cre-ERT treated with tamoxifen at P25. Immunohistochemistry with a rat anti-ACTH antibody was performed in order to quantify POMC cell recovery after treatment (See **Supplementary Table 1** for quantification). Arrows depict DAB stained POMC cells. 3V: 3rd ventricle.

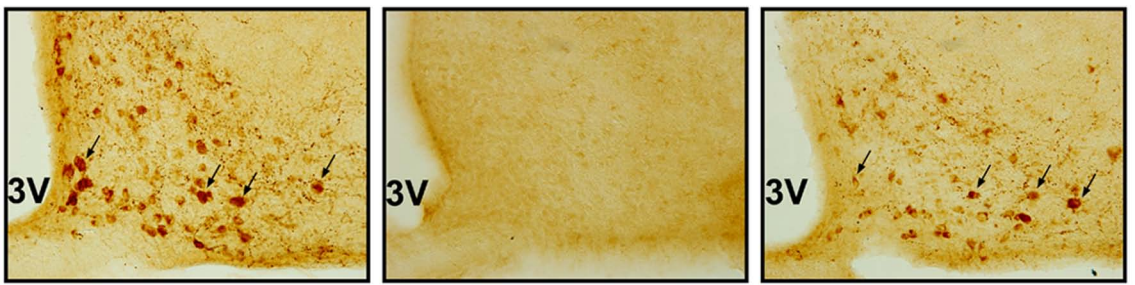
Supplementary Figure 2. Serial measurements of metabolic parameters before and 4 wk after tamoxifen treatment in a cohort of male P60 mice. a) Body composition measured by NMR. ***p<0.001, pair-wise comparison of body fat by Bonferroni's post-hoc test; n.s., not significant. b) Total locomotor activity in the horizontal plane measured by infrared beam breaks in the CLAMS chambers. The two line graphs show average hourly activity counts over the final 24-h period of a 72-h continuous measurement. The dark period between 6 PM to 6 AM is indicated by the black bar under the x-axis. The bar graph at the right shows the average hourly activity over the entire 12-h dark period. *p<0.05, effect of tamoxifen treatment in arc*Pomc*$^{-/-}$

:Cre-ERT mice. c) Oxygen consumption (VO_2) corrected to lean body mass (LBM) determined by indirect calorimetry in the CLAMS chambers. The two line graphs show average hourly VO_2 over the final 24-h period of a 72-h continuous measurement. The dark period between 6 PM to 6 AM is indicated by the black bar under the x-axis. The bar graph at the right shows the average hourly VO_2 over the entire 12-h dark period. None of the three genotypes of mice showed a significant change in VO_2 after tamoxifen treatment. All data shown are the mean ± SEM, n = 6 per genotype.

Supplementary Figure 3. Fat stores are improved after *Pomc* restoration. a) Determination of inguinal, gonadal and retroperitoneal fat pad and liver weights (in columns) of different groups of mice separated by sex and age (denoted on the left of each row), after treatment with vehicle (VEH) or tamoxifen (TAM). Bars show averages of 3-8 mice with the exception of male arc*Pomc*$^{-/-}$:Cre-ERT rescued with tamoxifen at P25 (n=2) that were excluded from statistical analysis. Error bars correspond to SEM. Letters a, b and c group mice that are not significantly different in a One way ANOVA analysis followed by Bonferroni's Post hoc test. b) Example of necropsies performed on obese arc*Pomc*$^{-/-}$ and rescued arc*Pomc*$^{-/-}$:Cre-ERT females treated at P25. Note differences in color and sizes of the liver (L), gonadal fat pads (G) and inguinal fat pads (I, already removed in the right side of each mice).

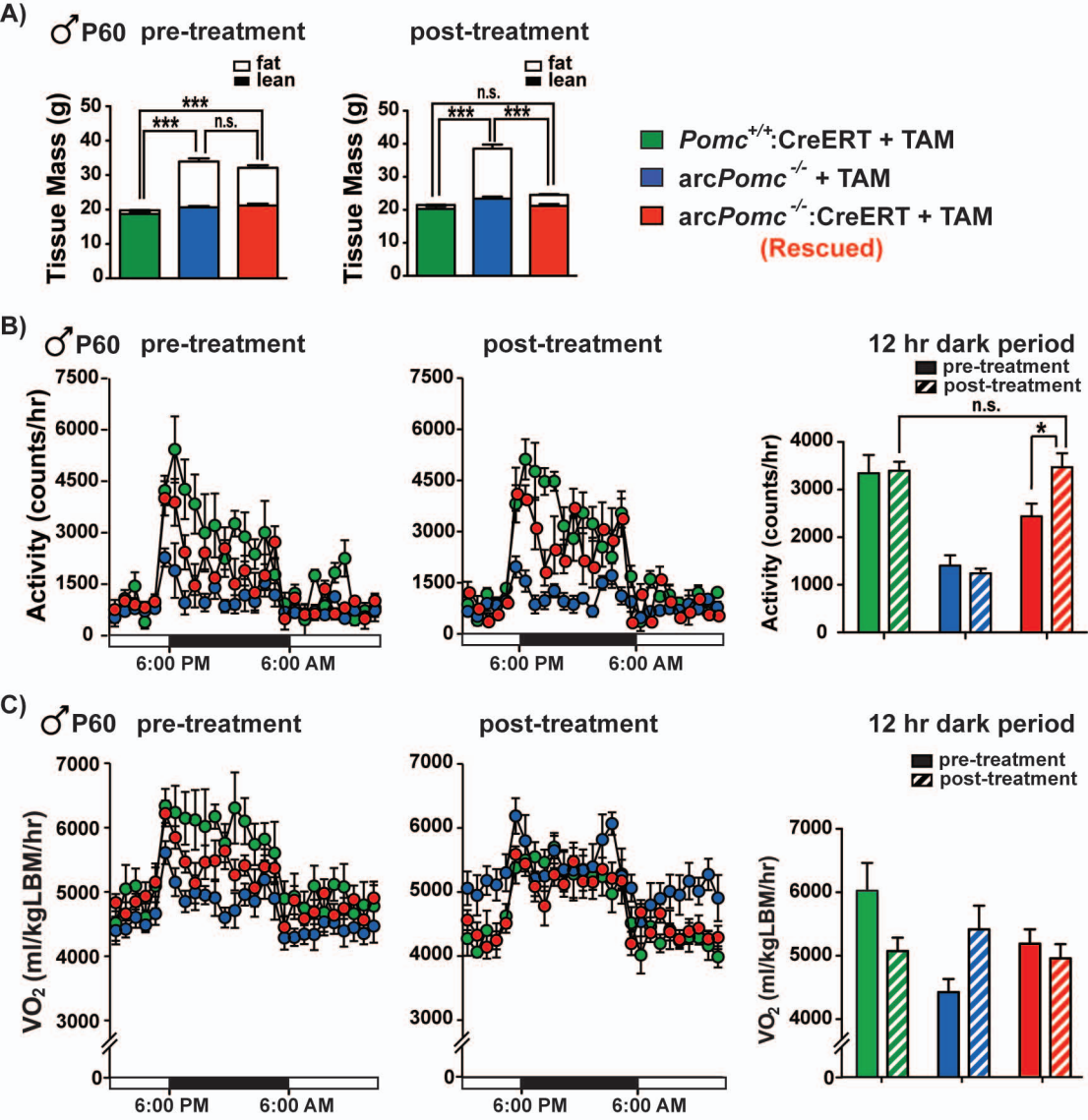
Supplementary Figure 4. *Pomc* rescue restores compensatory hyperphagia induced by food deprivation (absolute values). Both male (left) and female (right) mice treated with tamoxifen at P180 were subjected to 24-h of food deprivation at ages

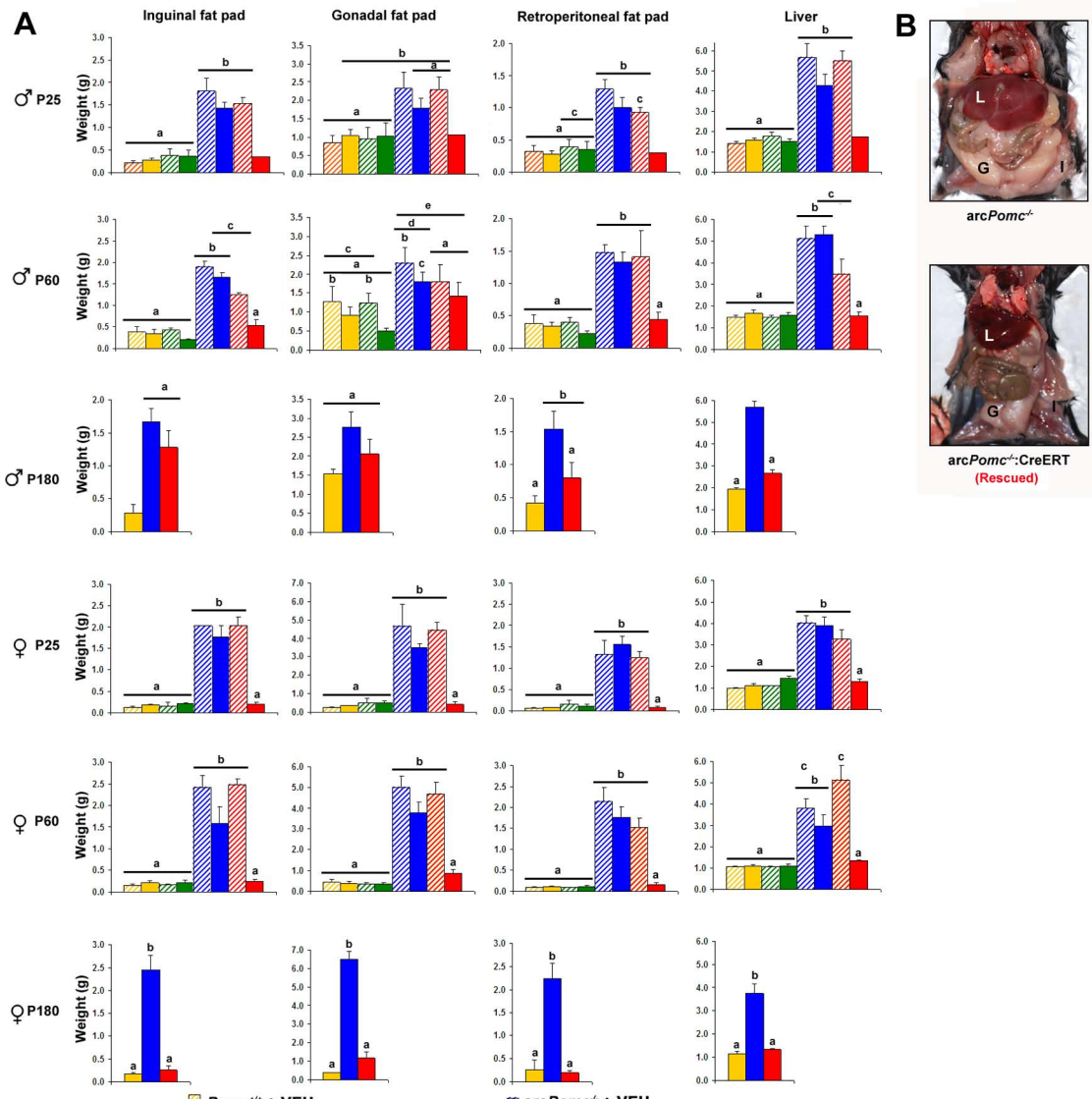
33-35 weeks (7-9 weeks after tamoxifen). Data correspond to the absolute values of food intake for the first 24-h of the same mice shown in **Figure 4** (n= 5-7). Pre-fasting bars represent average daily food intake of the same mice during 4 consecutive days before fasting. Error bars correspond to SEM. *p<0.05, ***p<0.001 (Repeated measures ANOVA).



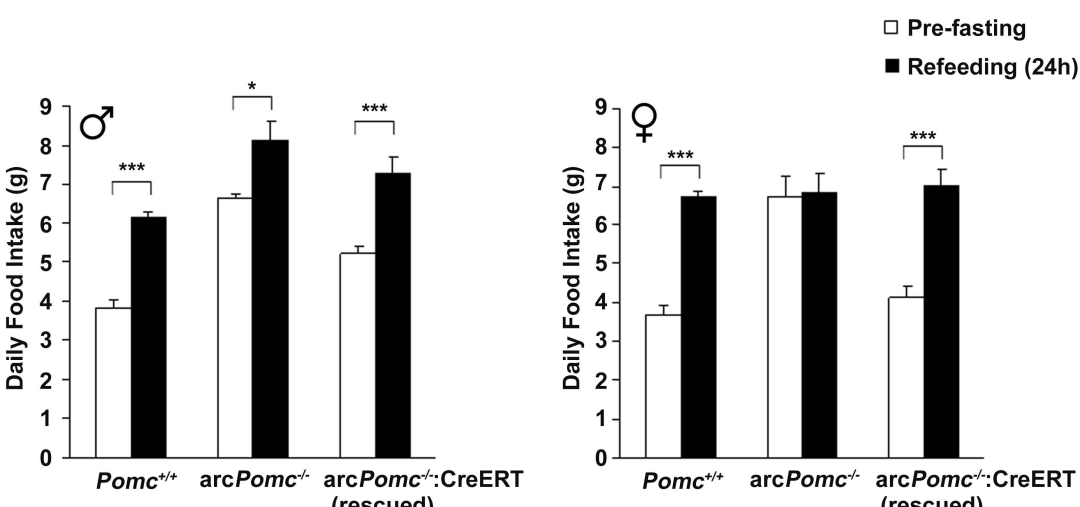
Pomc$^{+/+}$ arc*Pomc*$^{-/-}$ arc*Pomc*$^{-/-}$:Cre-ERT (rescued)

Supplementary Figure 1


A)
♂P60 pre-treatment
post-treatment
fat
lean
Tissue Mass (g)
***
n.s.
Pomc+/+:CreERT + TAM
arcPomc-/- + TAM
arcPomc-/-:CreERT + TAM
(Rescued)
B)
♂P60 pre-treatment
post-treatment
12 hr dark period
pre-treatment
post-treatment
Activity (counts/hr)
6:00 PM
6:00 AM
*
C)
♂P60 pre-treatment
post-treatment
12 hr dark period
VO2 (ml/kgLBM/hr)



Supplementary Figure 3



Supplementary Fig. 4

Supplementary Table 1. Assessment of POMC recovery in arc*Pomc*$^{-/-}$:Cre-ERT mice treated with tamoxifen at different ages

Sex	Age of TAM treatment	% of recovered POMC cells	% of *Pomc* mRNA expression
Male	P25	83 ± 11	56
	P60	72 ± 1	30 ± 6
	P180	70 ± 8	21 ± 2
Female	P25	68 ± 6	52 ± 10
	P60	78 ± 6	45 ± 8
	P180	74 ± 18	36 ± 2

At the end of the experiments, POMC recovery in the entire anterior to posterior extent of the arcuate nucleus was quantified either by anti-ACTH immuno-histochemistry (third column, for an example see **Supplementary Fig. 1**) or by qRT-PCR of *Pomc* mRNA (fourth column) in tissue from separate groups of mice. Age and sex-matched *Pomc*$^{+/+}$ mice treated with tamoxifen were used as 100% controls for the quantification of POMC cell number or *Pomc* mRNA levels. The numbers shown are averages of at least 3 mice with the exception of *Pomc* mRNA of P25 treated males (n=2, excluded from statistical analysis). One way ANOVA analysis showed no significant differences between the arc*Pomc*$^{-/-}$:Cre-ERT groups treated at three different ages for either POMC cell recovery [One way ANOVA, age + sex (group) effect: $F(5,12)=.35048$, $p=.87227$] or for *Pomc* mRNA expression [One way ANOVA, age + sex (group) effect: $F(5,17)=1.8598$, $p=0.15473$].