Supporting Information

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SI Materials and Methods

Animals. All animal protocols were approved by the local ethical committee. The experimental protocols followed the Principles of Laboratory Animal Care, and a specific authorization was issued by the Italian Ministry of Education (no. 129/2000-A) to the Consiglio Nazionale delle Ricerche Neuroscience Institute, where animals were housed. C57/BL6 male mice were raised in our in-house colony and used for all studies. Four experimental groups were analyzed: (*i*) young mice, referred to as YEE mice, born in the EE condition and killed at P50; (*ii*) adult mice, referred to as AEE mice, moved to EE cages at P50 and killed at P80; (*iii*) mice born in the EE condition, transferred to a standard cage at P50, and monitored until P80, referred to as EE/ST mice; and (*iv*) young mice reared since birth with free access to a running wheel, referred to as YW mice. Each group was compared with standard animals, namely, AST, YST, and ST/ST mice.

The enriched environment consisted of a large cage (44 × 62 × 28 cm) with a wire mesh lid containing several food hoppers, a running wheel, and differently shaped objects (tunnels, shelters, and stairs) that were repositioned once per day and completely substituted with others once per week. Every cage housed at least two dams, 10 pups, and two additional filler female mice. At weaning (P25), the female mice were moved to a different cage. A running wheel was installed on the lid of a standard cage for running wheel experiments. The standard environment consisted of a standard laboratory cage (26 × 42 × 18 cm) housing a maximum of four animals.

In both environmental conditions, food and water were available ad libitum. Food intake was monitored every other day, whereas BW measurements were obtained on a weekly basis. For all the experimental groups, monitoring of BW and food intake started from weaning (P22). At sacrifice, the brain, epigonadal WAT, and liver from each animal were dissected and processed for subsequent analysis.

Blood Collection and Plasma Assays. Blood samples were taken from the tail vein of animals that were fasted for 2 h, collected in EDTA-coated tubes, and centrifuged in a refrigerated microfuge. Plasma was collected and stored at −20 °C for subsequent assays.

Plasma glucose was measured with a One-Touch Ultra glucometer (LifeScan). Commercial ELISA kits were used to assess plasma levels of insulin (Linco Research, Inc.) and leptin (R&D Systems).

IpGTT and AUC Parameters. For IpGTTs, mice were fasted (given water only) for 2 h and then injected i.p. with 1 mg of glucose per gram of BW. Blood glucose was measured via tail vein bleeds at 0, 15, 60, and 120 min after injection using a One-Touch Ultra glucometer. Total AUC was calculated using the trapezoid model as an indication of insulin sensitivity.

Acute Leptin Injection. At the end of the EE or standard rearing period, leptin sensitivity of young animals was assessed. One hour before dark onset, food was removed. At dark onset (7:00 PM), mice received an i.p. injection of saline or murine leptin (3 mg/kg in 0.3 mL of PBS; Sigma) and were individually caged and given weighted chow food. Food intake was monitored 14 h after injection.

Isolation of Total RNA and Real-Time PCR. Total RNA was isolated from frozen tissues (epigonadal WAT and hypothalami from adult and young mice) with Tripure (Roche Molecular Biochemicals) or from formalin-fixed tissues (hypothalami from young and EE/ST or ST/ST mice) with an Agentcourt FormaPure Tube starter kit (Beckman Coulter). Its integrity was evaluated on formaldehyde denaturing agarose gel. RNA was then treated with Rnase-free Dnase (Roche Molecular Biochemicals) to remove any contaminating genomic DNA. First-strand cDNA synthesis was performed using oligo hexamers (Pharmacia).

Taq-Man quantitative PCR (50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min) was performed to amplify samples for leptin, cytokine signaling 3 (SOCS3), ObRb, NPY, POMC, and BDNF (protein-coding exon). The relative abundance of mRNAs was calculated with TATA binding protein mRNA as the invariant control. The assays were all purchased from Applied Biosystems.

Immunofluorescence. Animals were fasted for 2 h before receiving i.p. leptin at a dose of 3 mg/kg; 45 min later, they were anesthetized with an overdose of chloral hydrate. They were then transcardially perfused with PBS, followed by 4% (vol/vol) paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were quickly removed from the skull and postfixed in the same fixative at 4 °C for 4 h and then sunk in 30% (wt/vol) sucrose in 0.1 M PB at 4 °C. After cryoprotection, brains were frozen in isopentane and stored −80 °C. Forty-micrometer-thick coronal sections comprising the ARC were cut on a cryostat (Leica) and processed for immunofluorescence.

For vGAT and vGluT2 immunofluorescence, free-floating sections were blocked for 2 h at room temperature in 0.3% Triton X-100 and 10% (wt/vol) BSA in PBS and then incubated overnight at 4 °C with 1:1,000 rabbit anti-mouse vGAT or vGluT2 antibodies (Synaptic Systems) diluted in 0.1% Triton X-100 and 1% BSA in PBS. Sections were then revealed with 1:400 goat anti-rabbit secondary antibody conjugated to Alexa-568 diluted in 0.1% Triton and 1% BSA in PBS for 2.5 h at room temperature. For STAT3 and pSTAT3, sections were permeabilized in 100% (vol/vol) methanol at 4 °C for 5 min before blocking. The primary antibodies used were 1:250 rabbit anti-mouse STAT3 (Cell Signaling Technologies) and 1:250 rabbit anti-mouse pSTAT3 (Cell Signaling Technologies), and incubation was overnight at 4 °C. Sections were then revealed with the same secondary antibody solution described above. For double-immunofluorescence experiments, free-floating sections were prepared and incubated with blocking solution as above and then reacted with 1:1,000 anti-α-MSH sheep primary antibody (Calbiochem) or 1:1,000 anti-AgRP guinea pig primary antibody (AbCam), together with 1:1,000 anti-vGluT2 or 1:1,000 anti-vGAT rabbit primary antibody overnight at 4 °C. The secondary antibodies used were 1:400 goat anti-rabbit Alexa-568 (Molecular Probes), 1:400 donkey anti-sheep Alexa-488 (Molecular Probes), and 1:200 donkey anti-guinea pig DyLight-488 (Jackson ImmunoResearch). Incubation lasted 2.5 h at room temperature. Stained sections were mounted on glass slides, air-dried, soaked with VectaShield mounting medium (Vector Labs), covered with coverslips, and then stored at 4 °C in darkness to preserve the fluorescence signal.

Quantitative Analysis of Immunolabeled Cells and Puncta. For each group of immunofluorescence sections, optimal acquisition parameters (photomultiplier gain, intensity offset, and laser excitation intensity) were adjusted at the beginning of each experiment and held constant.

For quantification of pSTAT3- and STAT3-immunostained sections, at least five images for each experimental case were acquired with an Olympus confocal laser-scanning microscope

using FluoView software (Olympus). A 40× oil objective (N.A. = 1.3) guaranteeing coverage of the whole extension of each half of the ARC was used. The brightest focal plane for each section was chosen, and images from this one plane plus the two adjacent focal planes (z-step, 1 μm) were acquired. pSTAT3 and STAT3 images were stacked, and the number of immunoreactive cells was counted manually using MetaMorph software (Universal Imaging Corp.), with the operator blinded to the experimental case. Given that the ARC changes its area on the rostrocaudal axis, the area of the ARC in each section was measured and used to normalize cell counts. For vGAT and vGluT2, a 60× oil objective (N.A. = 1.4) was used, with a 2.5 digital zoom. To achieve an optimal resolution on the *z* axis, 15 sequential focal planes, spaced 0.125 μm apart, were acquired. Each focal plane was then saved as a single tagged image file format file in an 8-bit gray-scale mode. For vGAT and vGluT-2 immunoreactive puncta analysis, the images were processed using the "spots" function of Imaris software (Bitplane). The "spot quality threshold" and "minimum spot diameter" parameters were manually adjusted to optimize puncta detection, with the operator blinded to the experimental case. The "minimum spot diameter" was kept constant for all cases. For double-immunofluorescence quantification, single-cell images were acquired using a 60× oil objective and a 7.5 digital zoom. The number of focal planes, spaced 0.125 μm apart, was adjusted to comprise the entire extension of the cell soma on the *z* axis. The number of vGluT2- or vGAT-immunoreactive puncta contacting the soma of AgRP- or α-MSH-positive neurons was manually counted using MetaMorph software. Each punctum was considered to contact the cell soma when it was comprised within a distance of 1 μm from it.

Statistical Analysis. The number of mice in each experimental group is indicated in the figure legends. All values are expressed as the mean ± SEM. Pairwise comparisons of quantitative phenotypes between mice of different groups (e.g., standard vs. EE) were assessed by a two-tailed Student's *t* test. When more than two groups were analyzed, one-way ANOVA and two-way ANOVA, followed by a Bonferroni post hoc test for selected comparisons (e.g., standard vs. EE saline- or leptin-injected), were used. For immunofluorescence analyses, the cell count value obtained for each experimental case was normalized on the average value of the control group (standard injected with saline) to obtain the relative percentage of the group with respect to the control. Statistical evaluation of results was performed using GraphPad Prism version 3.00 for Windows (GraphPad Software).

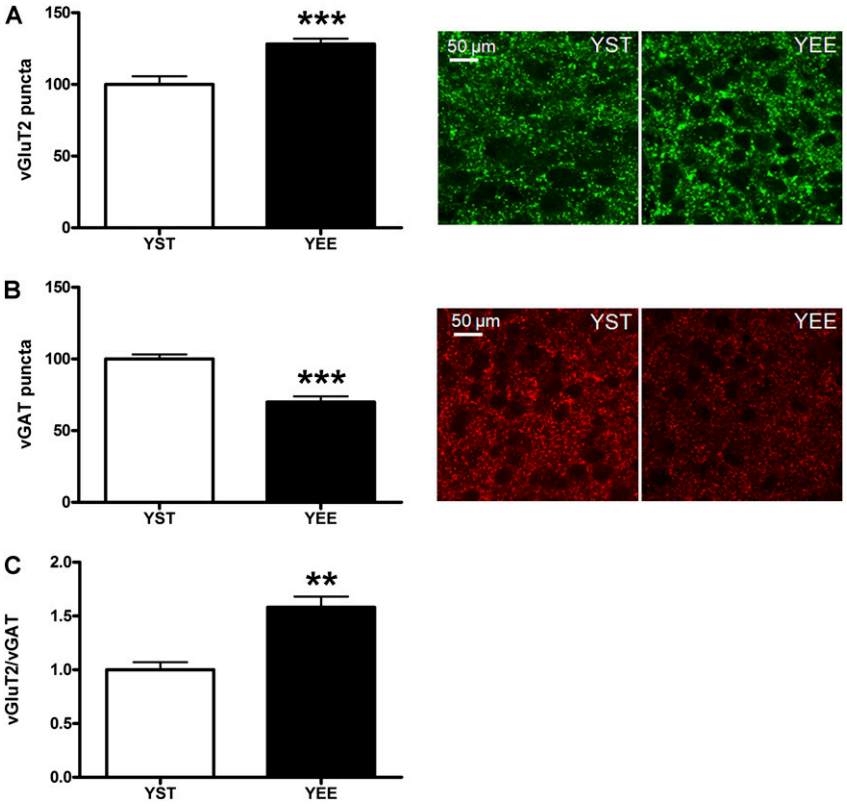

Fig. S1. Synaptic connectivity in the ARC of YEE and YST mice. (*A*) (*Left*) Increased total number of excitatory vGluT2-immunoreactive synapses in the ARC of YEE mice (YEE mice, $n = 13$ and YST mice, $n = 11$; *t* test, $^{***}P < 0.001$). (*Right*) Representative immunofluorescence showing vGluT2 expression in the ARC of YEE and YST mice. (Scale bar: 50 μm.) (*B*) (*Left*) Decreased total number of inhibitory vGAT-immunoreactive synapses in the ARC of YEE mice (*t* test, $^{***}P < 0.001$). (*Right*) Representative immunofluorescence showing vGluT2 expression in the ARC of YEE and YST mice. (Scale bar: 50 μm.) (*C*) Increased ratio between excitatory and inhibitory synapses in the ARC of YEE mice compared with YST mice (*t* test, $^{**}P < 0.01$).

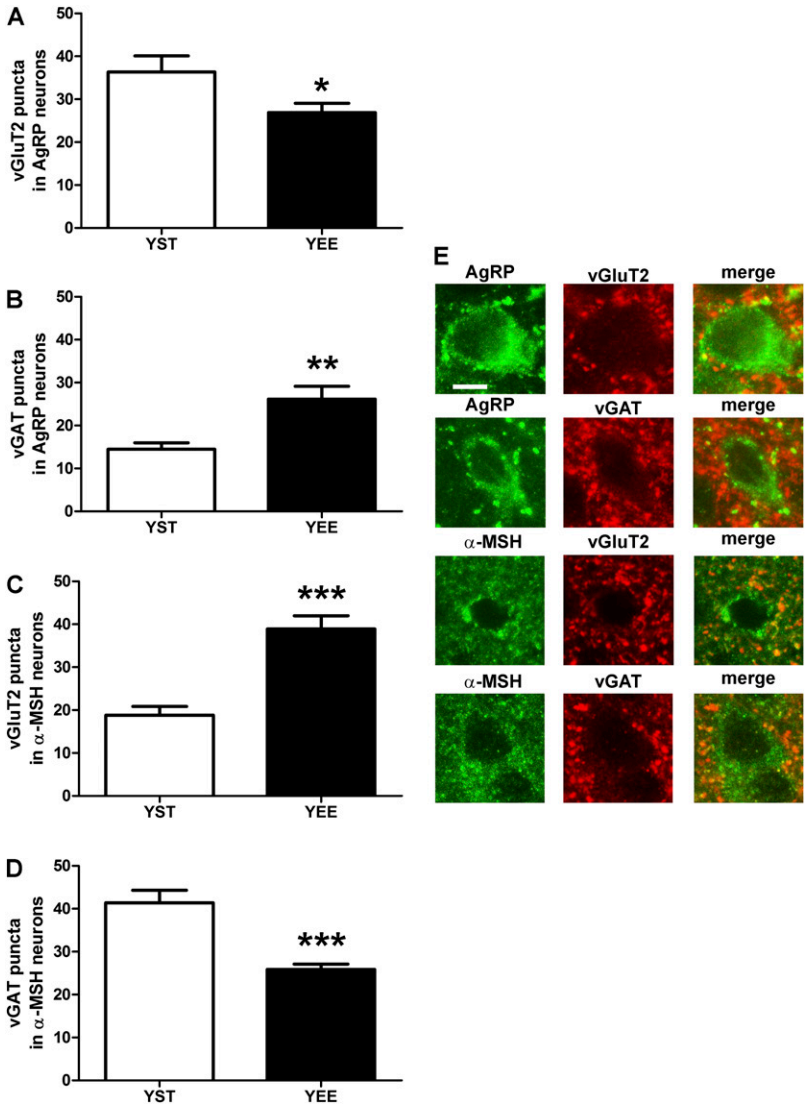

Fig. S2. Excitatory and inhibitory synapses on α-MSH and AgRP neurons in the ARC of YEE and YST mice. (*A*) Decreased number of excitatory vGluT2-immunoreactive synapses on AgRP neurons in the ARC of YEE (n = 9) and YST (n = 8) mice (t test, $^{*}P < 0.05$). (*B*) Increased number of inhibitory vGAT-immunoreactive synapses on AgRP neurons in the ARC of YEE (n = 7) and YST (n = 6) mice (t test, $^{**}P < 0.01$). (*C*) Increased number of excitatory vGluT2-immunoreactive synapses on α-MSH neurons in the ARC of YEE (n = 11) and YST (n = 9) mice (t test, $^{***}P < 0.001$). (*D*) Decreased number of inhibitory vGAT-immunoreactive synapses on α-MSH neurons in the ARC of YEE (n = 13) and YST (n = 9) mice (t test, $^{***}P < 0.001$. (*E*) Representative confocal microscopy images showing double immunofluorescence for AgRP or α-MSH (green) and vGluT2 or vGAT (red). (Scale bar: 10 μm.)

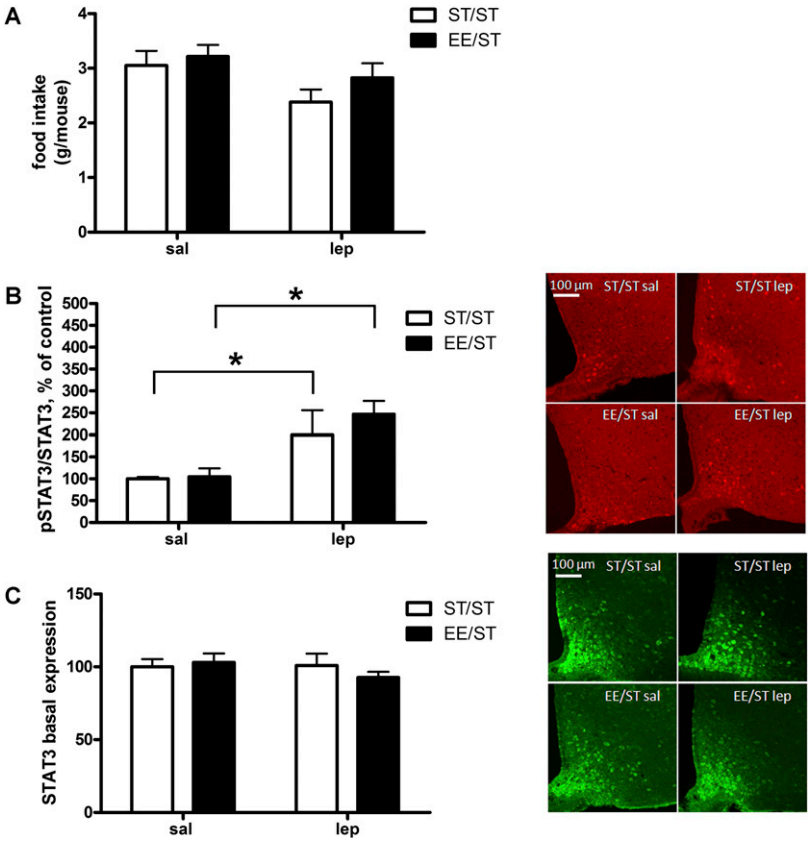

Fig. S3. Leptin response of ST/ST and EE/ST mice. (*A*) Food intake assessed 14 h after i.p. leptin injection in ST/ST (n = 12) and EE/ST (n = 19) mice. (*B*) (*Left*) Histogram of the ratio of pSTAT3-positive cells to the total number of STAT3-positive neurons 45 min after leptin (lep) or saline (sal) injection in the ARC of ST/ST (saline, n = 7; leptin, n = 5) and EE/ST (saline, n = 9; leptin, n = 10) mice (two-way ANOVA: rearing effect, NS, not significant; treatment effect, $P < 0.001$; interaction, NS. Bonferroni post hoc test in leptin vs. saline, $^*P < 0.05$). (*Right*) representative immunofluorescence showing pSTAT3 activation in the ARC after saline or leptin injection. (Scale bar: 100 μm.) (*C*) (*Left*) Total number of STAT3-positive neurons in the ARC of EE/ST and ST/ST mice after saline or leptin injection (two-way ANOVA, NS). (*Right*) representative immunofluorescence showing STAT3 expression in the ARC. (Scale bar: 100 μm.)

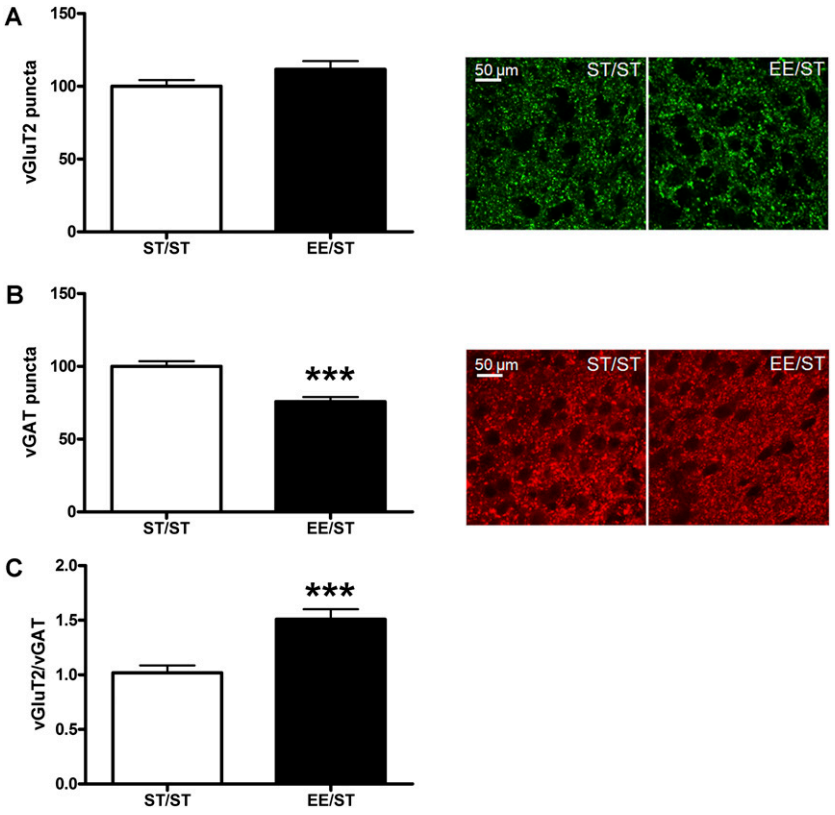

Fig. S4. Synaptic connectivity in the ARC of EE/ST and ST/ST mice. (*A*) (*Left*) Total numbers of excitatory vGluT2-immunoreactive synapses in the ARC of EE/ST ($n = 12$) and ST/ST ($n = 19$) mice are not significantly different (t test, $P = 0.297$). (*Right*) Representative immunofluorescence showing vGluT2 expression in the ARC of EE/ST and ST/ST mice. (Scale bar: 50 μm.) (*B*) (*Left*) Decrease in the total number of inhibitory vGAT-immunoreactive synapses in the ARC of EE/ST mice compared with ST/ST mice (t test, $^{***}P = 0.001$). (R*ight*) Representative immunofluorescence showing vGAT expression in the ARC of EE/ST and ST/ST mice. (Scale bar: 50 μm.) (*C*) Increased ratio between excitatory and inhibitory synapses in the ARC of EE/ST mice compared with ST/ST mice (t test, $^{***}P < 0.001$).

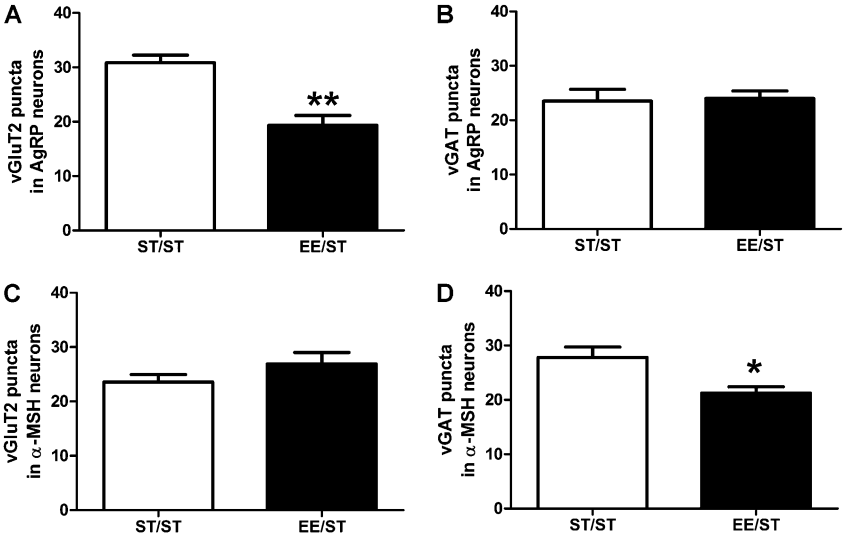

Fig. S5. Excitatory and inhibitory synapses in α-MSH and AgRP neurons in the ARC of EE/ST and ST/ST mice. (*A*) Decreased number of excitatory vGluT2-immunoreactive synapses in AgRP neurons in the ARC of EE/ST ($n = 4$) mice compared with ST/ST ($n = 4$) mice (t test, $^{**}P < 0.01$). (*B*) Lack of significant difference in inhibitory vGAT-immunoreactive synapses in AgRP neurons in the ARC of EE/ST and ST/ST mice. (*C*) Lack of significant difference in excitatory vGluT2-immunoreactive synapses in α-MSH neurons in the ARC of EE/ST and ST/ST mice. (*D*) Decreased number of inhibitory vGAT-immunoreactive synapses in α-MSH neurons in the ARC of YEE mice (t test, $^{*}P < 0.05$).

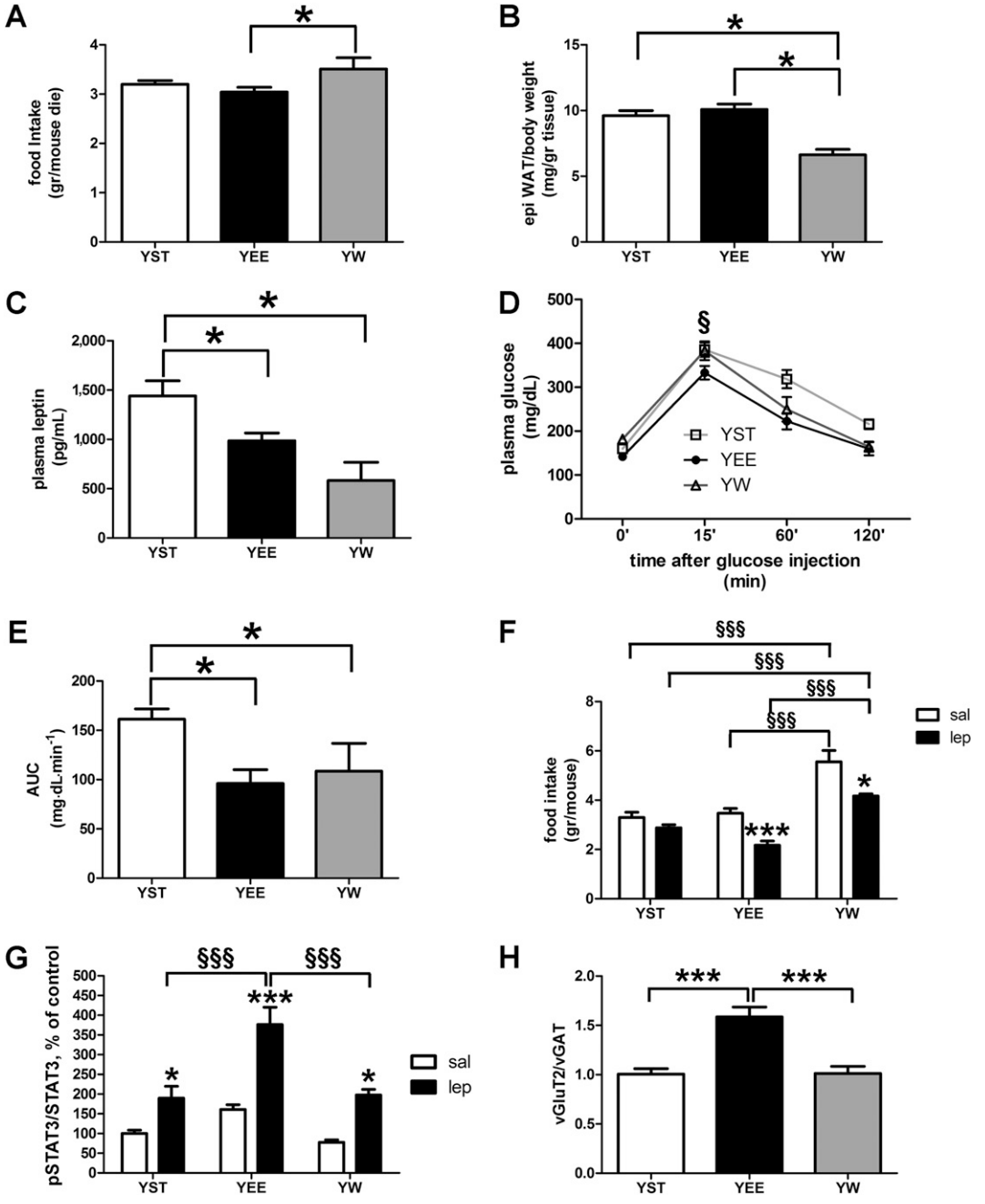

Fig. S6. Effects of physical exercise on metabolism, leptin response, and ARC synaptic connectivity in YW mice. (*A*) Average food intake between P25 and P50 (one-way ANOVA followed by Bonferroni post hoc test in YST vs. YW mice, $*P < 0.05$). (*B*) Weight of epididymal fat pad/BW (adiposity) (one-way ANOVA followed by Bonferroni post hoc test, $*P < 0.05$). (*C*) Plasma leptin at P50 (one-way ANOVA followed by Bonferroni post hoc test: $*P < 0.05$). (*D*) IpGTT results at the end of EE in YST, YEE, and YW mice (two-way ANOVA: rearing effect, $P < 0.001$; time effect, $P < 0.001$. Bonferroni post hoc test in YST vs. YW mice, $^{§}P < 0.05$). (*E*) AUC values (mg·dL·min^{-1} over a 120-min test) for the glycemic responses of YST, YEE, and YW mice shown in *D* (one-way ANOVA and Bonferroni post hoc test, $*P < 0.05$). (*F*) Food intake assessed 14 h after i.p. leptin injection in YST, YEE and YW mice (two-way ANOVA: treatment effect, $P < 0.0001$; interaction, $P < 0.0001$. Bonferroni post hoc test in saline vs. leptin, $*P < 0.05$; $***P < 0.001$ and Bonferroni post hoc test in YW vs. YEE or YST mice, $^{§§§}P < 0.001$). (*G*) Histogram showing the ratio of pSTAT3-positive cells to the total number of STAT3-positive neurons 45 min after leptin or saline injection in the ARC of YST, YEE, and YW mice (saline, $n = 3$; leptin, $n = 4$) (two-way ANOVA: rearing effect, $P < 0.001$; treatment effect, $P < 0.001$. Bonferroni post hoc test in saline vs. leptin, $*P < 0.05$, $***P < 0.001$ Bonferroni post hoc test vs. YEE mice, $^{§§§}P < 0.001$). (*H*) Ratio between excitatory and inhibitory synapses in the ARC of YW mice compared with YST and YEE mice (one-way ANOVA and Bonferroni post hoc test, $***P < 0.001$). Data for YST and YEE groups reported in *A*, *C*–*E*, and *G*–*I* have been redrawn from Figs. 3 *D* and *A*, 2 *A* and *B*, and 4 *A* and *C* and Fig. S3C, respectively.

Table S1. Metabolic assessment in AST and AEE mice

	AST (n = 16)	AEE (n = 14)
BW, g	25.7 ± 0.6	25.4 ± 0.4
Weight gain P50–P80, g	1.74 ± 0.42	1.99 ± 0.37
Fat pad (epididymal + perirenal), mg	397 ± 26	326 ± 14*
Liver, g	1.29 ± 0.04	1.25 ± 0.05
BAT, mg	121 ± 15	115 ± 11
Leptin, pg/mL	1,222 ± 93	927 ± 111*
Leptin/fat pads, pg·mL·mg	3.2 ± 0.22	2.9 ± 0.3
Average food intake, g·mouse·d	3.15 ± 0.06	3.61 ± 0.05***
Fasted insulin, ng/mL	0.72 ± 0.05	0.71 ± 0.16
Fasted glucose, mg/dL	124.7 ± 5.0	138.7 ± 5

Per t test, *$P < 0.05$; ***$P < 0.001$.

Table S2. Metabolic assessment in YST and YEE mice

	YST (n = 34)	YEE (n = 37)
BW, g	21.73 ± 0.40	21.79 ± 0.35
Weight gain P22–P50, g	12.97 ± 0.45	11.43 ± 0.48
Fat pad (epididymal), mg	208 ± 10	213 ± 11
Liver, g	1.28 ± 0.05	1.28 ± 0.06
BAT, mg	96 ± 11	81 ± 9
Leptin, pg/mL	1,406.6 ± 160.52	1,010.0 ± 81.86*
Leptin/epididymal fat pad, pg·mL·mg	7.09 ± 0.74	4.73 ± 0.42**
Fasted insulin, ng/mL	0.65 ± 0.1	0.53 ± 0.07
Fasted glucose, mg/dL	159.8 ± 6.87	141.9 ± 6.60

Per t test, *$P < 0.05$; **$P < 0.01$.

Table S3. Metabolic assessment in EE/ST and ST/ST mice

	ST/ST (n = 12)	EE/ST (n = 19)
BW, g	25.1 ± 0.5	27.3 ± 0.6**
Weight gain P50–P80, g	1.55 ± 0.36	3.97 ± 0.36***
Fat pad (epididymal), mg	0.38 ± 0.02	0.35 ± 0.02
Liver, g	1.52 ± 0.07	1.57 ± 0.06
BAT, mg	98 ± 8	103 ± 6
Leptin, pg/mL	2,259 ± 209	2,106 ± 148
Leptin/epididymal fat pad, pg·mL·mg	6.38 ± 0.51	7.13 ± 0.78
Average food intake, g·mouse·d	3.64 ± 0.06	4.29 ± 0.06***
Fasted insulin, ng/mL	0.71 ± 0.16	0.67 ± 0.17
Fasted glucose, mg/dL	162.6 ± 6	154.4 ± 5.1

Per t test, **$P < 0.01$; ***$P < 0.001$.