Supplementary online material for

Molecular therapy of obesity and diabetes using a physiological autoregulatory approach

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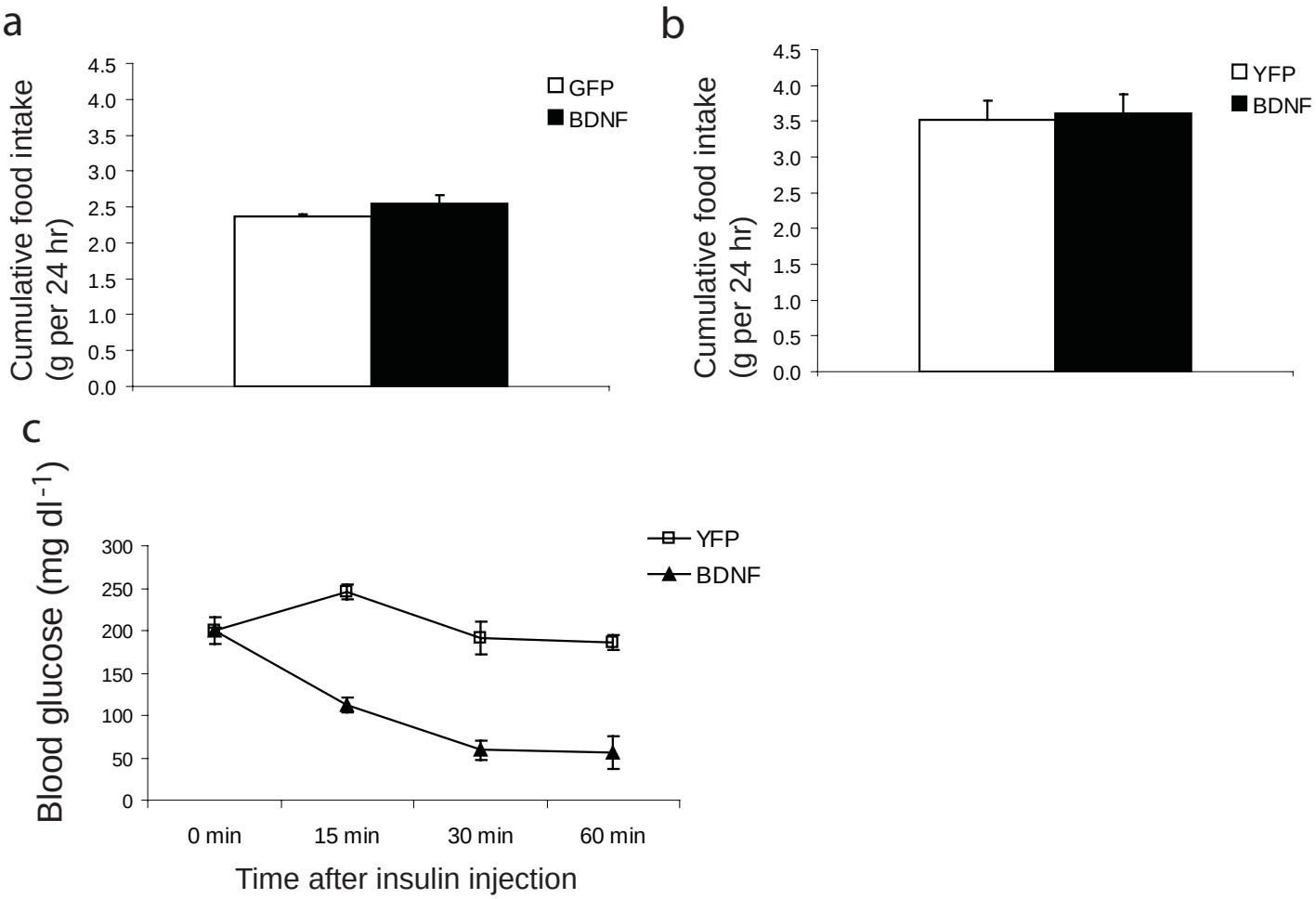
& Matthew J. During



Supplementary Figure 1. Food intake was not changed by hypothalamic gene transfer of BDNF in mice fed on standard diet (**a**) or high fat diet (**b**). (**c**) Hypothalamic gene transfer of BDNF improved insulin tolerance test in diet induced obesity model. Insulin was injected to mice without a fast and blood glucose concentration was measured (n = 4 YFP, n = 5 BDNF, $P < 0.0001$).

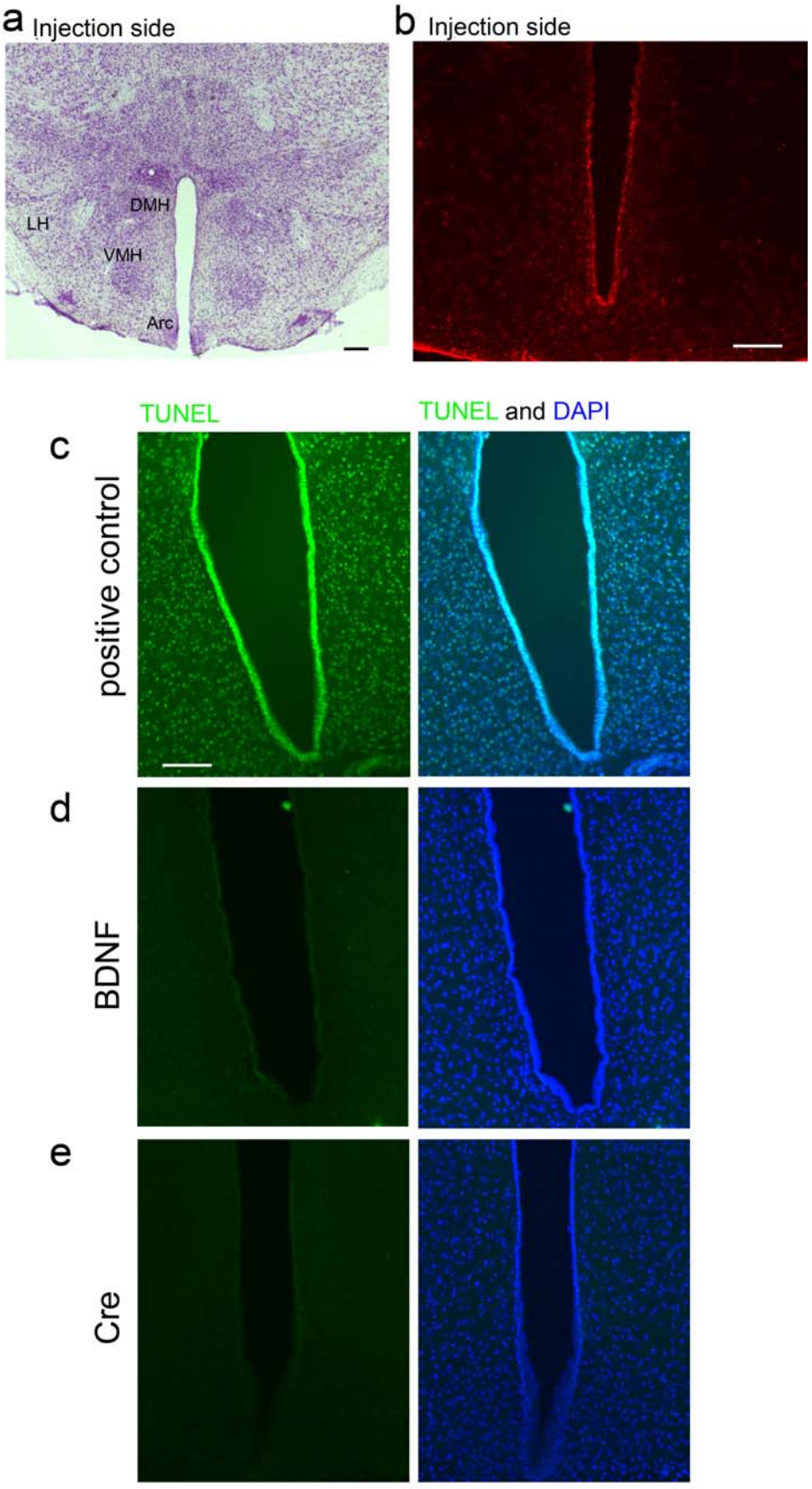
Supplementary Table. Effects of hypothalamic gene transfer of BDNF on various biomarkers in serum

Parameter	Standard diet#		High fat diet§	
	GFP	BDNF	YFP	BDNF
	(n = 10)	(n = 14)	(n = 8)	(n = 8)
IGF-1 (ng ml^{-1})	58.47 ± 3.38	15.05 ± 0.69*	62.02 ± 7.10	20.25 ± 3.51*
Leptin (ng ml^{-1})	0.613 ± 0.089	0.075 ± 0.002*	16.46 ± 2.27	0.989 ± 0.151*
Adiponectin (µg ml^{-1})	3.22 ± 0.13	5.15 ± 0.35*	3.07 ± 0.1	5.13 ± 0.15*
Leptin R (ng ml^{-1})	1.84 ± 0.08	3.59 ± 0.30*	3.10 ± 0.37	7.02 ± 0.83*
Insulin (ng ml^{-1})	1.27 ± 0.43	0.23 ± 0.03*	5.11 ± 1.14	0.74 ± 0.11*
Glucose (mg dl^{-1})	225.88 ± 13.24	189.18 ± 12.76^{+}	408.81 ± 30.86	212.03 ± 10.87*
Cholesterol (mg dl^{-1})	77.71 ± 2.14	65.29 ± 3.96*	174.09 ± 16.02	75.37 ± 3.36*
Triglyceride (mg dl^{-1})	70.05 ± 7.42	33.30 ± 3.39*	63.45 ± 8.00	49.25 ± 7.85

3 weeks after AAV injection; § 10 weeks after AAV injection

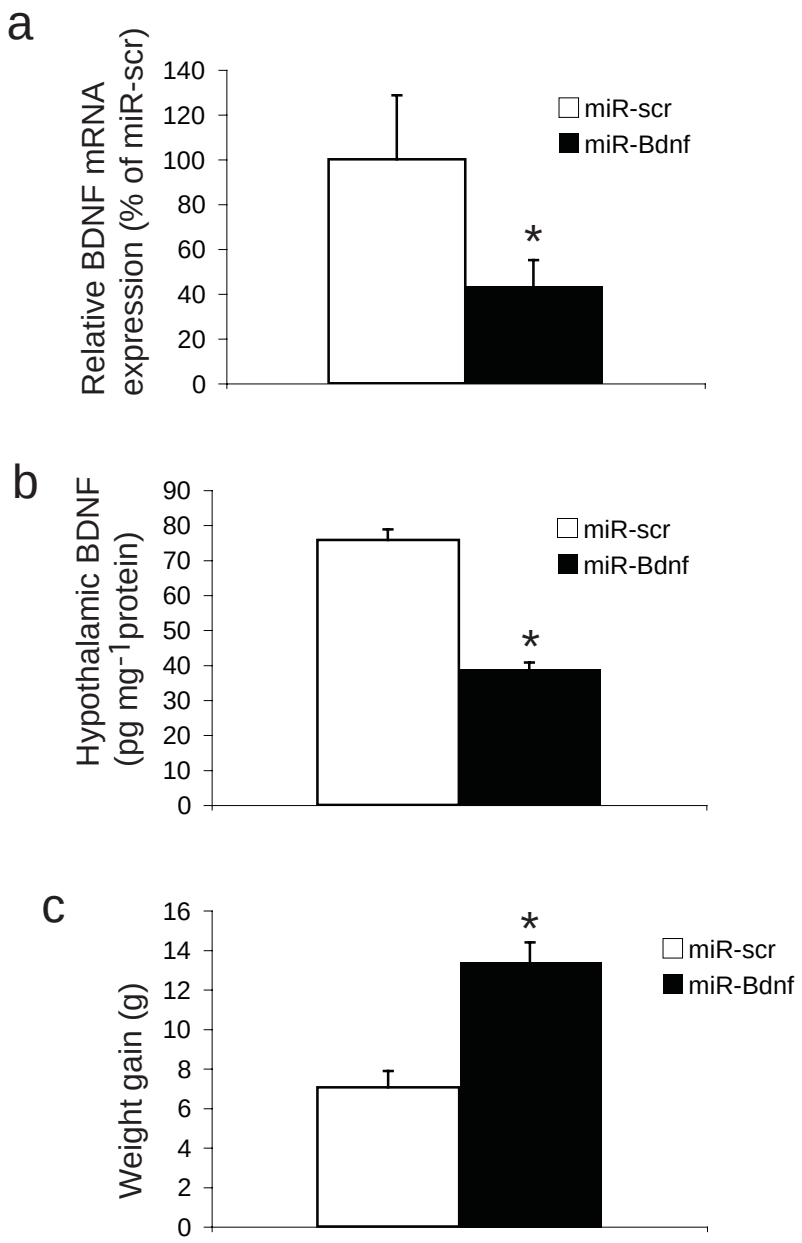
* $P < 0.05$ BDNF mice vs controls on respective diet; + $P = 0.062$

Supplementary Figure 2



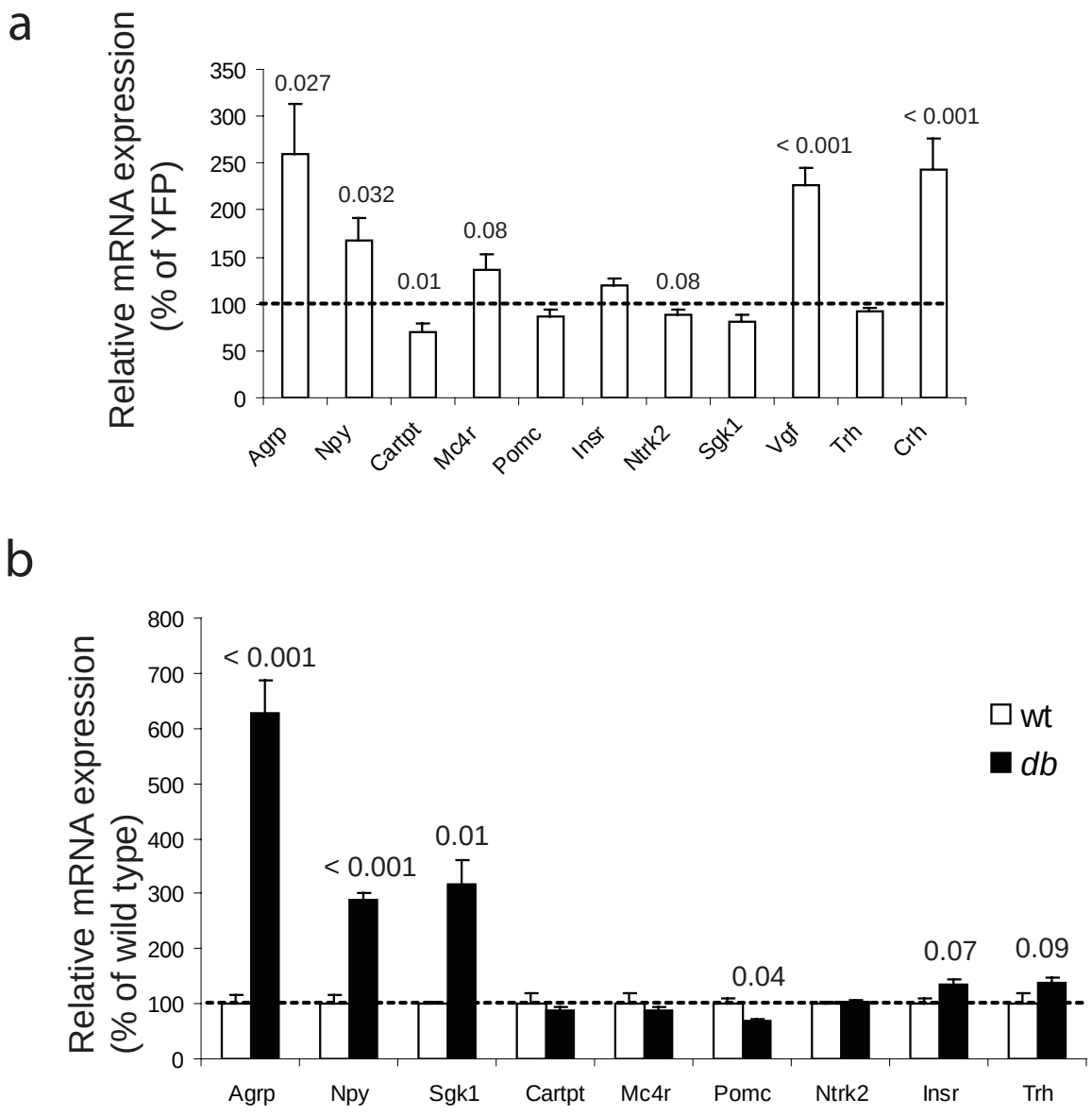
Supplementary Figure 2. rAAV mediated BDNF overexpression did not cause cytotoxicity. rAAV-BDNF was injected unilaterally to hypothalamus with no cell loss as shown in Nissl staining (**a**) no gliosis as shown in GFAP staining (**b**) compared to counterlateral side. TUNNEL assay showed no apoptosis in hypothalamus injected with rAAV-BDNF or rAAV-Cre. (**c**) TUNNEL assay positive control counterstained with DAPI (**d**) unilateral injection of rAAV-BDNF. (**e**) bilateral injection of rAAV-Cre. Scale bars: 200 μm.

Supplementary Figure. 3



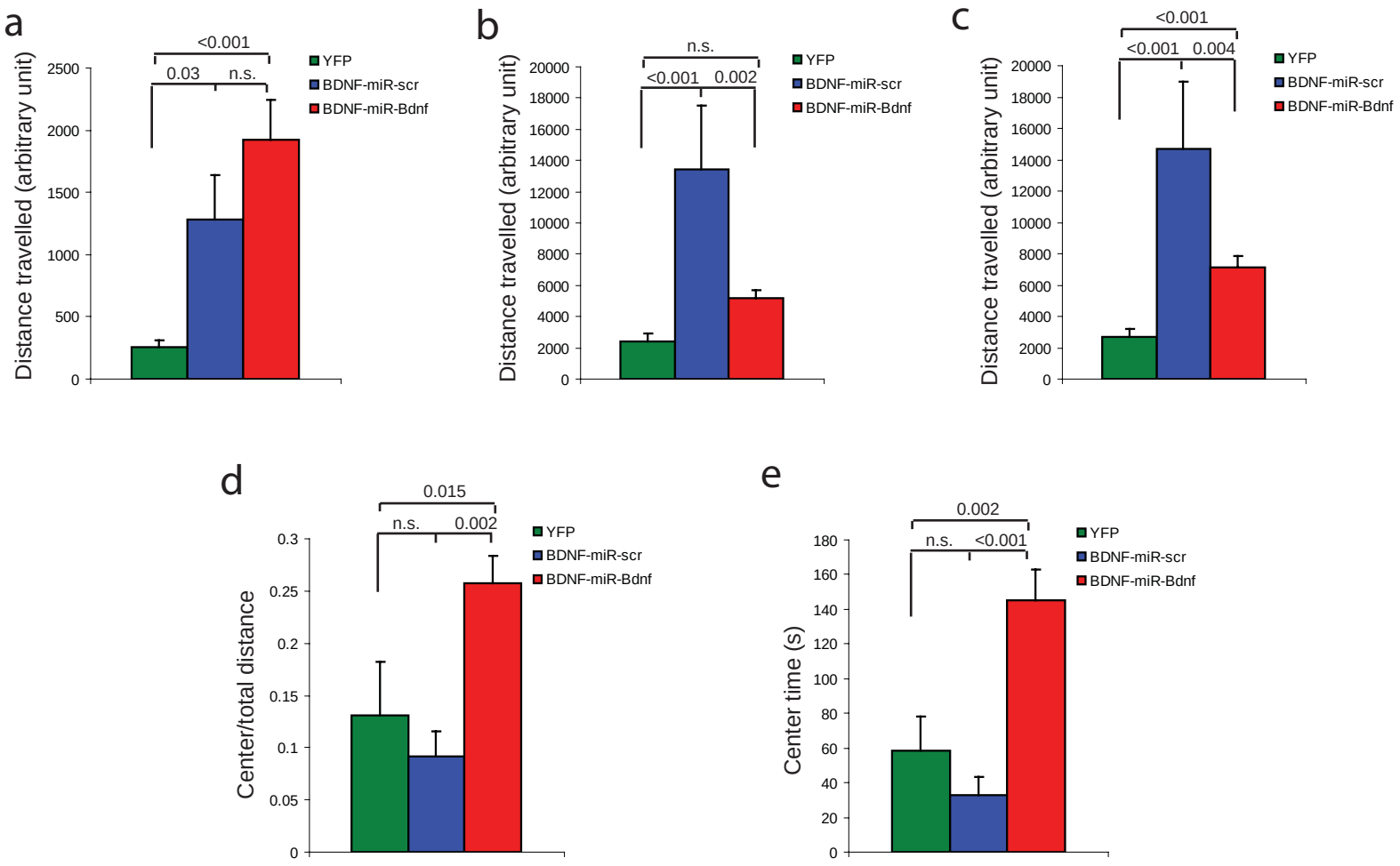
Supplementary Figure 3. MicroRNA vector knocked down hypothalamic BDNF expression and led to accelerated weight gain. *In vitro* experiments showed that the microRNA vector knocked down BDNF mRNA by 65% and protein levels by 80%. We further assessed the efficacy of this microRNA to BDNF by generating a rAAV vector with miR-Bdnf driven by CBA promoter. We also generated a control microRNA vector targeting a scrambled sequence (miR-scr) against no known genes. We injected rAAV vectors of miR-Bdnf or miR-scr bilaterally into the hypothalamus of wild-type mice and fed the mice on standard diet. Quantitative RT-PCR and ELISA showed that the miR-Bdnf vector significantly reduced BDNF expression in hypothalamus at both mRNA (**a**, * $P <$ 0.01) and protein levels compared to miR-scr (**b**, * $P < 0.01$). This reduction of BDNF expression in hypothalamus led to accelerated weight gain in miR-Bdnf mice by 26 days after injection (**c**, * $P<0.01$). n = 10–23 per group.

Supplementary Figure 4



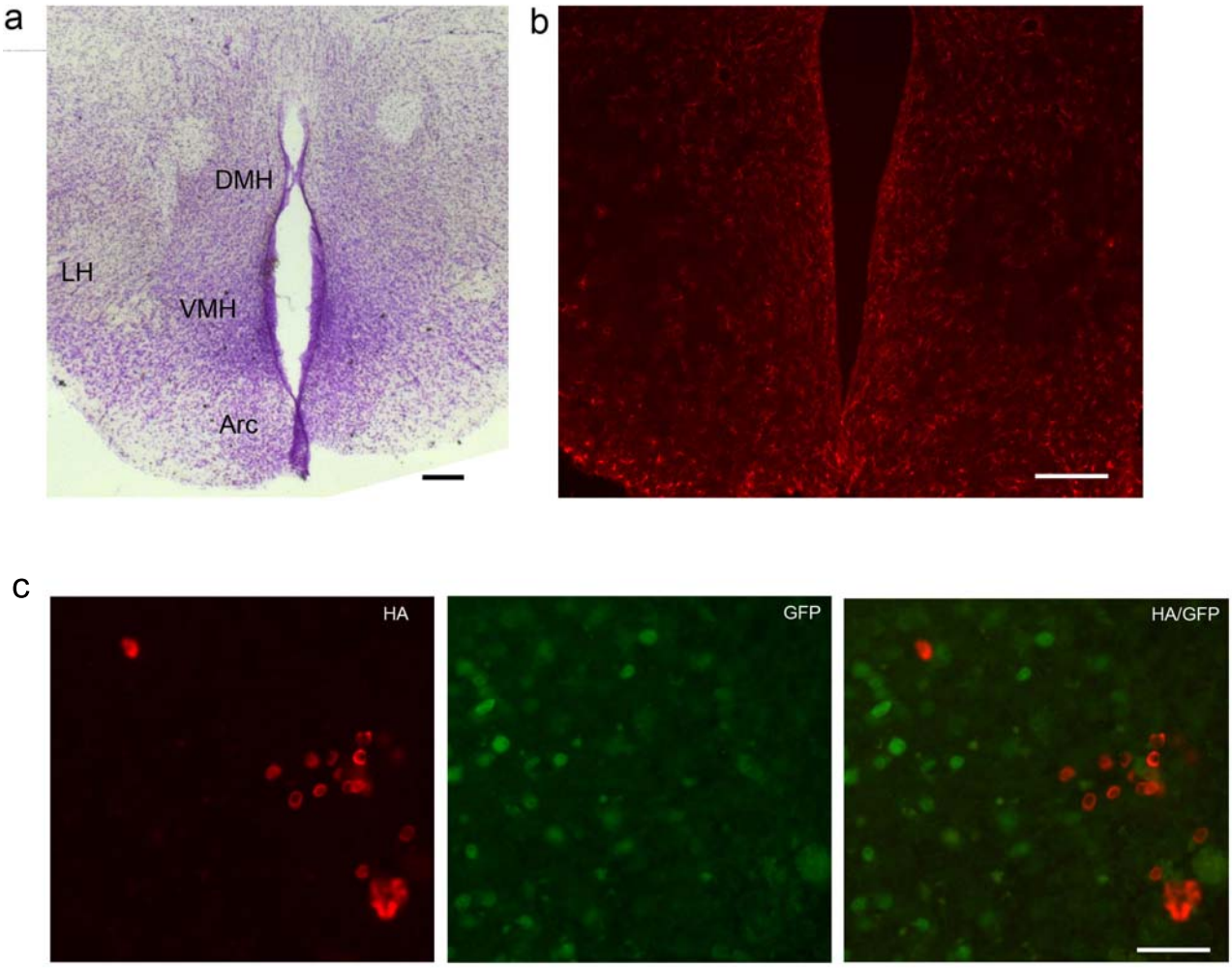
Supplementary Figure 4. (**a**) Hypothalamic gene expression profile of *db*/*db* mice treated with an autoregulatory BDNF vector. Relative mRNA expression levels of the indicated genes in hypothalamus are shown as percentage of control mice (n = 6 YFP, n = 9 BDNF-miR-Bdnf). (**b**) Hypothalamic gene expression profile of *db*/*db* mice compared to wild type mice. n = 6 *db*/*db*, n = 4 wild type. P values of significance or strong trend are shown on the bars.

Supplementary Figure 5



Supplementary Figure 5. Hypothalamic gene therapy with an autoregulatory BDNF vector improved mobility and exploration behavior of obese *db*/*db* mice. (**a**) Central distance. (**b**) Peripheral distance. (**c**) Total distance. (**d**) Ratio of central to total distance. (**e** Center time. n = 6 YFP, n = 5 BDNF-miR-scr, n = 9 BDNF-miR-Bdnf. *P* values are shown on the bars.

Supplementary Figure 6



Supplementary Figure 6. Injection of rAAV-GFP/Cre vector did not cause cell loss as shown with Nissl staining (**a**) or gliosis as shown by GFAP staining (**b**). Scale bars: 200 µm. (**c**) Double-staining of HA tag and GFP in hypothalamus of mice 4 months after first surgery (injection of AAV-flox-BDNF) and 3 months after second surgery (injection of AAV-GFP/Cre). HA (left) and GFP (middle) immunoreactivities were found in the same area of hypothalamus but no colocalization was observed (right). The majority of cells are GFP immunoreactive with fewer cells expressing HA consistent with the ~72% knockdown of BDNF protein levels observed in hippocamapal lysates. Scale bar: 50 µm.

Additional methods

Body weight and food consumption. We maintained the mice on a normal 12 h/12 h light/dark cycle with respective diet (NCD or HFD) and water *ad libitum* throughout the experiment. Body weight of individual mouse was recorded before injection and every 3–7 days after injection. Food consumption were recorded periodically after injection as the total food consumption of each cage housing 4–5 mice and represented as the average of food consumption per mouse per day.

Serum harvest and biomarker measurement. We collected blood from the retroorbital sinus 3-4 weeks after AAV injection. We anesthetized the mice of each group at the same time with ketamine (87 mg kg^{-1})/xylazine (13 mg kg^{-1}) followed by blood withdraw. All blood harvesting started at 10:00 am. We prepared serum by allowing the blood to clot for 30 min on ice followed by centrifugation. Serum was at least diluted 1:5 in serum assay diluent and assayed using the following DuoSet ELISA Development System (R&D Systems): mouse IGF-1, IGFBP-3, Leptin, Leptin R, Adiponectin/Acrp30. Insulin was measured using Mercodia ultrasensitive mouse insulin ELISA (ALPCO Diagnostic). Glucose was measured using QuantiChrom Glucose Assay (BioAssay Systems). Total cholesterol was measured using Cholesterol E test kit (Wako Diagnostics). Triglyceride was measured using L-Type test (Wako Dianostics).

BDNF expression quantification. We dissected hypothalami and prepared total RNA from half of the hypothalamic tissue and subjected it to quantitative RT-PCR. We calibrated the data of quantitative RT-PCR to the endogenous control gene *Eef2*. We

prepared lysates from the other half of the hypothalamic tissue and measured BDNF protein level using ELISA (BDNF Emax ImmunoAssay System, Promega). The BDNF protein level was calibrated to the total protein level.

Oil Red-O staining. We stained lipids in liver and white adipose tissue frozen sections using an Oil Red-O solution (Sigma).

Glucose tolerance test. We injected mice intraperitoneally with glucose solution (1mg glucose per kg body weight) after an overnight fast. We obtained blood from the tail at various time points. We measured blood glucose concentrations with a portable glucose meter (ReliOn Ultima).

Insulin tolerance test. We injected mice intraperitoneally with insulin (0.75 unit per kg body weight) at 2 pm without a fast. We obtained blood from the tail and measured the blood glucose concentration as described above.

Quantitative RT-PCR. We dissected liver, white adipose tissue and hypothalamus and isolated total RNA using RNeasy Mini Kit plus RNase-free DNase treatment (Qiagen). We generated first-strand cDNA using TaqMan Reverse Transcription Reagent (Applied Biosystems) and carried out quantitative PCR using an ABI PRISM 7000 Sequence Detection System with the Power SYBR Green PCR Master Mix (Applied Biosystems). We designed primers to detect the following mouse mRNA: *Bdnf*, *Npy*, *Agrp*, *Sgk1*, *Vgf*, *Insr*, *Lepr*, *Ntrk2*, *Cartpt*, *Pomc, Mc4r, Trh, Crh, Ucp1, Ucp2,Ucp3, Lep, Adipoq, Cycs,*

Fasn, Ppargc1a, Rb1, Pparg, Dio2, Acox1, Cpt1a, Gpam, Scd1, Srebf1. Primer sequences are available on request. We calibrated data to endogenous control *Actb* in liver and adipose tissue, *Eef2* in hypothalamus and quantified the relative gene expression using the equation $T_0/R_0=K$ x $2^{(CT,R-CT,T)}$. T_0 is the initial number of target gene mRNA copies, R_0 is the initial number of internal control gene mRNA copies, CT,T is the threshold cycle of the target gene, CT,R is the threshold cycle of the internal control gene and *K* is a constant.

rAAV-microRNA experiment. We randomly assigned 7 week old C57/BL6 mice to receive AAV-CBA-miR-Bdnf (*n* = 10) or AAV-CBA-miR-scr (*n* = 10). We injected 0.7 µl of AAV vectors ($1.4x10^{10}$ particles) bilaterally into the hypothalamus at the stereotaxic coordinates described above. We sacrificed the mice 30 days after vector injection and dissected the hypothalamus for Q-PCR and BDNF ELISA.

Immunohistochemistry. We perfused mice with 20 ml cold PBS followed by 50 ml 4% paraformaldehyde. Coronal brain sections (20 µm) were cut using a Leica freezing microtome and immunofluorescence staining was performed with the following antibodies: monoclonal antibody to HA tag (Covance, 1:250) followed by Cy3-conjugated secondary antibody (Jackson Immunoresearch, 1:400); polyclonal antibody to NPY (Chemicon, 1:8,000) followed by DyLight488-conjugated secondary antibody (Jackson Immunoresearch, 1:600); polyclonal antibody to GFAP (Dako, 1:500) followed by Cy3-conjugated secondary antibody (Jackson Immunoresearch, 1:400). Apoptosis was assessed by TUNEL assay using *in situ* Cell Death Detection Kit (Fluorescence, Roche)

according to manufacturer's instruction and counterstained with DAPI. We detected immunofluorescence with Zeiss Axioskop40 microscope and took pictures and processed the pictures with Zeiss AxioVision3.1 software. We processed confocal laser scanning with Zeiss 510 Meta Laser Scanning Confocal microscope.

Open field. To assess exploration and general motor activity, we placed mice individually into the center of an open square arena (60 cm x 60 cm, enclosed by walls of 48 cm). The mouse was allowed 10 min in the arena, during which time its activity was recorded and analyzed by Clever Systems TopScan Software (Clever Sys Inc, Vienna, VA). Specifically, we measured the distance traveled both in the center of the arena (36 cm x 36 cm), the total distance traveled and the time spent in the center of the arena. The total distance traveled provides a measure of exploratory activity while the time and distance ratio of arena center exploration provide preliminary indication of anxiety level. We cleaned the arena with 30% ethanol between trials to remove any odor cues.

Flox-BDNF vector. We generated flox-BDNF plasmid by inserting two lox P flanking the human BDNF-HA cDNA in the AAV vector. We packaged the following rAAV1 viral vectors: flox-BDNF, Cre recombinase fused to GFP, and empty vectors as control.

Metabolic studies. We measured energy expenditure and activity of mice using the Oxymax Lab Animal Monitoring System (Columbus Instruments). Individual mouse was allowed to be habituated to the instrument overnight and the physiological and behavioral parameters were monitored for 24 h (activity, food and water consumption, metabolic

performance and temperature). Oxygen consumption, carbon dioxide production and methane production were normalized to the body weight and corrected to an effective mass value according to the manufacturer's software.

Bone mineral density. We measured the volumetric bone mineral density (vBMD) by microcomputed tomography (μCT, Seimens Invion, Wright Center for Innovation, The Ohio State University).