Supporting Information

Liu et al. 10.1073/pnas.1202835109

SI Materials and Methods

Animals. Male mice were housed in groups of five per cage and allowed to acclimate to housing conditions under a 12/12-h light/dark cycle with free access to food and water. All animal procedures were conducted according to the National Institutes of Health guidelines and approved by the institutional animal care and use committee of the University of Texas Health Science Center at San Antonio.

Genotyping. Adipo$^{+/-}$ mice on a C57BL/6J genetic background were intercrossed to generate adiponectin-haploinsufficient mice and wild-type littermate controls (1). The offspring were genotyped by PCR amplication of tail DNA. The following primers were used to amplify a 482-bp product corresponding to the disrupted allele and a 260-bp fragment corresponding to the wild-type adiponectin allele: forward 5′-GGACCCCTGAACTTGCTTCAC-3′; reverse 5′-CACCCACAGTAATTCCATGGG-3′; and reverse 5′-GAATGGGCTGACCGCTTCCTCGTG-3′. PCR conditions for the reaction consisted of an initial denaturing step of 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 60 s.

Social Interaction. Mice were tested for two 2.5-min sessions in a 40 × 40 cm open field arena, with a 9-cm round wire basket located at one end of the arena in the interaction zone. In the first 2.5-min session (no target), the mouse was allowed to explore in the open arena with an empty wire basket. In the second 2.5-min session (with target), the mouse was reintroduced into the arena in the presence of an unfamiliar CD1 male mouse enclosed in the wire basket. The approach-avoidance behavior of the mice was recorded with a video tracking system in the absence and presence of an unfamiliar CD1 target. The time spent in the interaction zone (25 × 17 cm) was measured by using EthoVision 3.0 software (Noldus Information Technology).

Analysis of Distribution of Adiponectin Oligomeric Complexes. The oligomeric complex distribution of adiponectin in plasma was determined by using gel filtration with a Superdex 200 10/30 column using the AKTA FPLC system (GE Healthcare Bio-Sciences) as described elsewhere (2). Briefly, 100 μL plasma from a single mouse was loaded into the column and eluted with PBS (10 mM phosphate buffer; 137 mM NaCl; and 2.7 mM KCl, pH 7.4). The flow rate of the column was 0.5 mL/min and every 0.2-mL fraction was collected. Adiponectin elutes in fractions 33–56 with an equivalent volume of ~6.6–11.2 mL. Fractions were analyzed by Western blotting under reducing and denaturing conditions. Two-mercaptoethanol was added to the sample buffer. Samples were mixed with sample buffer and boiled for 5 min before electrophoresis on 12% acrylamide gels.

Adiponectin oligomeric complexes in plasma were also separated by SAS/PAGE under nonreducing/nondenaturing conditions. Samples were prepared using nonreducing sample buffer containing 2% (wt/vol) SDS, 50 mM Tris-HCl (pH 6.8), and 10% (vol/vol) glycerol, and separated by a 4–15% gradient gel (Bio-Rad Laboratories). After electrophoretic separation, proteins were immunoblotted with rabbit antisera to adiponectin, as described previously (2). IgG was used as loading control for plasma samples.

Cannulation Implantation and Microinjection. Mice were anesthetized and implanted with a guide cannula (C315GS; Plastics One) into the lateral ventricle using a procedure described previously with some modifications (3). The skull surface was first coated with Kerr phosphoric acid gel etchant (Kerr USA). After the guide cannula was inserted into the lateral ventricle (coordinates: 0.2 mm posterior, 1.1 mm lateral, and 2.7 mm ventral to the bregma), Kerr Prime was applied onto the skull and cannula surface. Then adhesive was brushed on top of the primer layer and light cured for 45 s with the VALO curing light (Ultradent Products). Finally, the dental cement was used to fill the area around the cannula and a dummy cannula was inserted into the guide cannula to maintain the cannula patency. Animals were individually housed, handled daily, and allowed to recover for 7 d after surgery. Microinjections were performed on conscious, unrestrained, freely moving mice in their home cages. Recombinant globular adiponectin (Phoenix Pharmaceuticals), full-length mouse adiponectin (R&D Systems), mouse monoclonal adiponectin antibody (Abcam), or mouse serum IgG (Sigma-Aldrich) in a total volume of 2 μL were infused into the lateral ventricle over 2 min at 30 min before behavioral tests (forced swim and tail suspension) or 1 h before social defeat. After the injection, an additional minute was allowed for diffusion and prevention of backflow through the needle track before the injector was withdrawn.

Behavioral Tests. All behavioral tests were performed during the late light phase except social interaction, which was measured in the early dark cycle, and sucrose preference that was measured every 24 h. Animals were transferred to the testing room and habituated to the room conditions for 3–4 h before the beginning of the behavioral experiments. Behavioral tests were conducted as described previously (4–7).

Learned helplessness test. This test was performed in a shuttle cage that was divided equally into two chambers with an auto-controlled guillotine door (Coulbourn Instruments). Learned helplessness (8) was induced in mice by administering 60 scrambled, inescapable foot shocks (0.3 mA shock amplitude, 6-s duration, 54-s average interval) over a 1-h session on two consecutive days. Control animals were exposed to the apparatus for the same period without receiving foot shocks. Escape performance was tested 24 h after the second foot shock session. Each mouse was given 30 shuttle escape trials with 25-s maximum duration and 30-s intervals. On the first five trials, a sound cue and the shock took place at the same time as the door to the safe compartment opened. For the remaining trials, the door opened 2 s after the shock was delivered. Each trial was terminated when the mouse crossed into the nonshock compartment. Latency to escape and the number of escape failures were recorded automatically by Graphic State software (Coulbourn Instruments).

Hot-plate test. Pain sensitivity was assessed by detecting the reflexes in response to a thermal stimulus using the hot-plate test. The surface of the hot plate was heated to a constant temperature of 55 °C. Mice were placed on the hot plate with surrounded wall. The duration of the test session was a maximum of 90 s. The latency to respond with hind paw lick or jump was recorded. The mouse was immediately removed from the hot plate and returned to its home cage. If the mouse did not respond, the test was terminated at the end of 90 s.

Sucrose preference test. Mice were habituated to drinking from two bottles of water for 1 wk before testing. To measure the preference for sucrose solution, the animals were singly housed and tested with a free choice of one bottle of water and one bottle of 1% sucrose. Water and sucrose intake were measured daily and the position of two bottles was switched every day. Sucrose

preference was calculated as the volume of sucrose intake over the total volume of fluid intake.

Locomotor activity. Mice were placed in an open field arena (40 × 40 × 40 cm) and allowed to freely explore for 2 h. A CCD camera was mounted above the open box for recording locomotor activity. For the pharmacological studies, locomotor activity was measured for 10 min in the home cage or in an open box at 30 min after intracerebroventricular (i.c.v.) injection. The open box consisted of a 60 × 60 cm open arena with 40-cm high walls. The entire test arena was adjusted to even illumination. Mice were placed in the center of the arena and allowed to freely explore. The total distance traveled was measured using a Noldus EthoVision 3.0 system.

Tail suspension test (TST). The apparatus consisted of a box (30 × 30 × 30 cm). The front of the box was open, and a bar was placed horizontally 1 cm from the top with an attached vertical bar hanging down in the center. Mice were individually suspended by the tail to the vertical bar with adhesive tape affixed 2 cm from the tip of the tail. A camera positioned in front of the TST box was used to record the animals' behavior for a 6-min test session. Immobility in this test was defined as the absence of any limb or body movements, except those caused by respiration.

Forced swim test. Mice were placed in a clear Plexiglas cylinder (25 cm high; 10 cm in diameter) filled to a depth of 15 cm with 24 °C water. A camera positioned directly above the cylinder recorded the 6-min swim session. For each test session, the first 2 min served as a habituation period. The immobility of the mice was measured during the last 4 min of the test. Immobility in this test was defined as the absence of any movement except for that required to keep the animal's head above water.

Visual cliff test. A box with the inner surface covered with checkerboard paper connected to a vertical drop of 0.7 m. A sheet of clear Plexiglas provides a solid horizontal surface despite the visual appearance of a cliff. The mouse was placed at the edge of the black floor, and the direction in which the mouse stepped was recorded. Each mouse was subjected 10 consecutive trials.

Olfactory function test. The olfactory function was examined using a buried food pellet test as described previously. After 24 h of food deprivation, a mouse was placed in a clean cage with a food pellet buried 0.5 cm below the surface bedding. The latency to grasp the food pellet was recorded.

RNA Extraction and Real-Time PCR. Total RNA was extracted from epididymal fat with TRIzol (Invitrogen) and cDNA was generated by using SuperScriptTM first-strand synthesis system (Invitrogen) (4, 5). Real-time PCR was performed using a Mastercycler ep realplex2 system (Eppendorf) and power SYBR green PCR master mix (Applied Biosystems). The primer sequences used to amplify each product are as follows: adiponectin, forward: 5′-CAGGCATCCCAGGACATCC-3′ and reverse: 5′-CCAAGAAGACCTGCATCTCCTTT-3′; 18S rRNA, forward 5′-CACGGACAGGATTGACAGAT-3′ and reverse 5′-CAAATCGCTCCACCAACTAA-3′. The amount of mRNA for adiponectin for each sample was normalized to 18S rRNA using the following formula: $2^{(\mathrm{CTadiponectin\text{-}CT\beta\text{-}18S})}$.

In Situ Hybridization. AdipoR1 and AdipoR2 cDNA fragments were amplified by RT-PCR using the primers as follows: AdipoR1 (721bp), forward: 5′-CCGCTCGAGGATCGGGCGCCCCTC-3′ and reverse: 5′-CGGGATCCCGAAGACCACCTTC-3′; AdipoR2 (912bp), forward: 5′-CCGCTCGAGTGTAACATGGGCCTCG-3′ and reverse: 5′-CGGGATCCCCAAAAGTGTGC-3′. These PCR amplified fragments were cloned into pBluescriptSKII+ vector (Stratagene). To generate radiolabeled cRNA probes, the plasmids were linearized and labeled with S^{35}-UTP and S^{35}-CTP (PerkinElmer) using the standard transcription system. In situ hybridization will be performed as described in our previous studies (4, 7, 9). The brain sections were fixed in 4% paraformaldehyde for 1 h, rinsed in 2× SSC (300 mM NaCl, 30 mM Na citrate, pH 7.2), then acetylated in 0.1 M triethanolamine (pH 8.0) with 0.25% (vol/vol) acetic anhydride for 10 min and dehydrated through a graded series of alcohol (50–100%). The tissue sections were incubated with 70 μL of diluted radiolabled probes at 55 °C overnight. The slides were rinsed in 2× SSC and incubated in RNase A buffer (200 mg/mL) for 1 h at 37 °C followed by a series of washes with increasing stringency (2×, 1×, 0.5× SSC). Finally, the slides were placed in 0.1× SSC at 70 °C for 1 h, rinsed in distilled water, dehydrated in a graded series of alcohol, and exposed to X-ray film.

Plasma Corticosterone, Glucose, Insulin, and Adiponectin Measurements. Plasma was separated after centrifugation at 2,500 × *g* for 15 min; the supernatant was stored at −70 °C until use. Plasma corticosterone concentrations were assayed using a radioimmuno assay. Briefly, 10 μL of plasma was heated at 70 °C for 30 min to denature corticosterone-binding protein. The samples were incubated overnight with a corticosterone antibody and [^{3}H] corticosterone (PerkinElmer). Free and bound corticosterone were separated by incubation with activated charcoal for 15 min. Plasma glucose levels were determined by using a glucometer (One Touch; Bionime Corp). Plasma insulin and adiponectin levels were measured by using mouse insulin ultrasensitive ELISA kits, or mouse adiponectin ELISA kits (Alpco Diagnostics).

1. Nawrocki AR, et al. (2006) Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists. *J Biol Chem* 281:2654–2660.
2. Liu M, et al. (2008) A disulfide-bond A oxidoreductase-like protein (DsbA-L) regulates adiponectin multimerization. *Proc Natl Acad Sci USA* 105:18302–18307.
3. Groseclose CH, Draughn RA, Tyor WR, Sallee FR, Middaugh LD (1998) Long-term intracranial cannula stabilization in mice with light cured resin composites. *J Neurosci Methods* 79:31–36.
4. Guo M, et al. (2012) Forebrain glutamatergic neurons mediate leptin action on depression-like behaviors and synaptic depression. *Transcult Psychiatry* 2:e83.
5. Liu J, Perez SM, Zhang W, Lodge DJ, Lu XY (2011) Selective deletion of the leptin receptor in dopamine neurons produces anxiogenic-like behavior and increases dopaminergic activity in amygdala. *Mol Psychiatry* 16:1024–1038.
6. Liu J, et al. (2010) Acute administration of leptin produces anxiolytic-like effects: A comparison with fluoxetine. *Psychopharmacology (Berl)* 207:535–545.
7. Yu T, et al. (2011) Cognitive and neural correlates of depression-like behaviour in socially defeated mice: An animal model of depression with cognitive dysfunction. *Int J Neuropsychopharmacol* 14:303–317.
8. Seligman ME, Maier SF (1967) Failure to escape traumatic shock. *J Exp Psychol* 74:1–9.
9. Liu J, et al. (2007) The melanocortinergic pathway is rapidly recruited by emotional stress and contributes to stress-induced anorexia and anxiety-like behavior. *Endocrinology* 148:5531–5540.

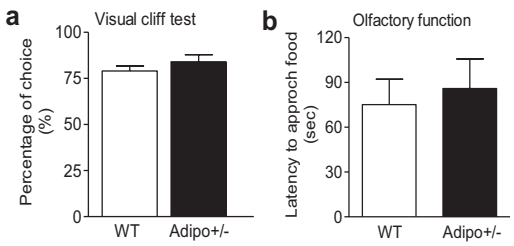

Fig. S1. Visual and olfactory functions of Adipo$^{+/-}$ mice. (*A*) Visual cliff test. Mice were subjected to 10 consecutive trials and showed no genotype difference ($t_{(23)} = 0.947$, $P = 0.354$). $n = 10–15$ per group. (*B*) Olfactory function test. Mice were tested for finding a food pellet buried below the surface bedding after 24 h of food deprivation. There is no genotype difference in olfactory functions ($t_{(19)} = 0.406$, $P = 0.689$). $n = 10–11$ per group. WT, wild-type. All data are expressed as mean ± SEM.

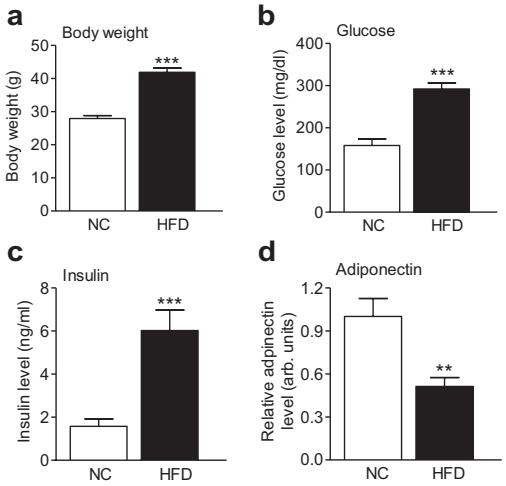

Fig. S2. Mice fed a high-fat diet display obesity, hyperglycemia, hyperinsulinemia, and hypoadiponectinemia. Body weight (*A*), plasma glucose (*B*), insulin (*C*), and adiponectin (*D*) levels were determined after 16 wk on normal chow diet (NC) or a high-fat diet (HFD). Adiponectin levels were measured by Western blotting. HFD significantly increased body weight ($t_{(17)} = 8.809$, $P < 0.001$), plasma glucose ($t_{(17)} = 6.449$, $P < 0.001$), and insulin levels ($t_{(17)} = 4.220$, $P < 0.001$) and reduced plasma total adiponectin levels ($t_{(12)} = 3.487$, $P < 0.01$) compared with NC. $n = 7–10$ per group. $^{**}P < 0.01$, $^{***}P < 0.001$. All data are expressed as mean ± SEM.