# Supporting Information

## Yau et al. 10.1073/pnas.1415219111

### SI Materials and Methods

**Forced Swim Test.** The FST, which has been widely used to probe depression-like behaviors in rodents, was conducted with minor modification as previously reported (1). In brief, the mouse was placed in a cylinder (height: 30 cm; diameter: 15 cm) of water (23–25 °C) and videotaped for 6 min, and the last 4-min session was scored by an observer blind to the treatment conditions. Mobility was defined as any movement beyond what was needed to keep the head above the water. Time spent in immobility was adopted as an indicator for behavioral despairs.

**Tail Suspension Test.** The TST, which was designed specifically for evaluating depression in mice, was conducted following the modified protocol by Gould et al. (1). Briefly, the mouse was suspended in the middle of a three-walled rectangular compartment (height: 55 cm; width: 15 cm; depth: 11.5 cm) with a climb-stopper placed around its tail before applying the tape. A 6-min session was videotaped and analyzed for immobility time by a blind observer. Small movements only confined to the front limbs and momentum-induced oscillations and pendulums following earlier mobility bouts were not regarded as mobility.

**Sucrose Preference Test.** The SPT has been applied to identify the behavior of anhedonia, which is the core symptom of clinical depressive patients (2). As previously described (3), individually housed mice were simultaneously supplied with one bottle of tap water and the other 2% (wt/vol) sucrose solution for 24 h. The positions of bottles were swapped 12 h later, and the consumption of water and sucrose solution was recorded by weighing bottles before and after the test. Preference to sucrose was presented as the percentage of the sucrose solution over the total weight of liquid consumed.

**Online Monitoring of Running Activity and Open Field Test.** The online running distance was monitored by the mouse activity wheel system (Lafayette Instrument) in 1-h intervals through the 14-d running period, during which mice received individual or paired housing (to mimic the wheel sharing condition), as designated. In addition, the open field test (OFT) was conducted to verify that WT and *adipo*$^{-/-}$ mice have a similar motor ability by measuring the moving distance and the average velocity during a period of 10 min.

**Intracerebroventricular Injection.** C57BL/6J mice were anesthetized using a mixture of Dormicum (6.25 mg/kg body weight; Roche) and 10% Hyponorm (1.25 mL/kg body weight; VetaPharma Ltd.). The anesthetized mice were placed in a stereotactic apparatus and injected with 2 μL of recombinant Ad-Adn ($10^8$ pfu) (4) or the same amount of control Ad-Luc into the ventricles (coordinates: posterior: 0.5 mm; lateral: 1 mm; ventral: 2.3 mm) at a rate 0.2 μL/min using the Hamilton syringe. The needle was withdrawn 5 min after the injection.

**Tail Vein Injection of Recombinant Trimeric Adiponectin Proteins.** *adipo*$^{-/-}$ mice were injected with either the phosphate buffer saline (vehicle) or recombinant trimeric adiponectin proteins (20 μg per mouse; Antibody and Immunoassay Services, HKU) through the tail vein as previously reported (5). Three hours after tail vein injection, approximately 5 μL of the CSF were collected by the cisterna magna puncture as previously described (6).

**Tissue Preparation.** Mice were deeply anesthetized with a mixture of ketamine and xylazine. Upon collection of trunk blood, they were sequentially perfused with 0.9% saline for 5 min and 4% (wt/vol) paraformaldehyde (PFA)/0.1 M PBS for 15 min. The isolated brains were postfixed in 4% PFA overnight at 4 °C and then transferred to 30% (wt/vol) sucrose solution until they sank. The brain slices (1-in-6 series, 40-μm thickness) were cryosectioned using a sliding freezing microtome (Thermofisher). The slices were stored in the cryoprotectant at −20 °C until use.

**Immunohistochemistry and Immuofluorescent Staining.** The sections were retrieved in citrate buffer (pH 6.0) at 95 °C for 30 min, followed by incubation in 2 N HCl for 30 min at 37 °C and 0.1 M borate buffer (pH 8.5) for 15 min. After washing in 0.01 M PBS, the sections were incubated overnight with the anti-BrdU (1:1,000; Abcam), followed by incubation with biotinylated goat anti-rat IgG (1:200; Dako). The BrdU staining was visualized with the peroxidase method (ABC system, Vector Laboratories) and diaminobenzidine kits (DAB kits, Sigma-Aldrich). For DCX or Ki67 staining, sections were incubated with rabbit anti-DCX (1:200; Abcam) or rabbit anti-Ki67 (1:1,000; Novocastra) antibody, respectively, followed by the biotinylated goat anti-rabbit IgG (1:200; Dako) and visualization with the same method mentioned above.

**Immuofluorescent Staining.** Immunofluorescent colabeling of BrdU and DCX was performed as previously reported (7, 8). After antigen retrieval, sections were incubated with primary antibodies overnight, and secondary antibodies for 2 h, including goat anti-rabbit IgG Alexa Fluor 488 and goat anti-rat IgG Alexa Fluor 568 (1:200; Invitrogen). The mounted sections were observed by fluorescent microscopy (Axioplan, Zeiss).

**Quantification of BrdU$^+$, Ki67$^+$, and DCX$^+$ Cells.** BrdU$^+$, Ki67$^+$, and DCX$^+$ cells (Fig. 3*B* and Fig. S3) were counted in the 1-in-6 series (from bregma −1.34 mm to −3.80 mm) using the optical fractionator system (grid size: 55 μm × 55 μm; counting frame: 35 μm × 35 μm) of StereoInvestigator (MicroBrightfield Inc.). Cells residing in the subgranular zone and granular cell layer of the dentate gyrus were counted, whereas those that appeared in the uppermost focal plane were excluded.

**Quantification of DCX/BrdU Colabeled Cells.** Quantification was performed in a blind manner as previously described (8). Fifty BrdU-positive cells were randomly selected for calculating the colabeling ratio with DCX, as the indicator for neuronal differentiation.

**Protein Extraction.** Brain tissues were freshly harvested after mice were killed. The whole hippocampus or the isolated dentate gyrus (9) were lyzed with RIPA buffer (Pierce) supplemented with a mixture of proteinase inhibitors and phosphatase inhibitors (Millipore) and phenylmethanesulfonyl fluoride (Sigma-Alrich). Samples were sonicated for 15 s with a 50% pulse and cleared by centrifugation (10,000 × *g*) at 4 °C for 30 min. Supernatant protein concentrations were quantified by the DC Protein Assay reagent (Bio-Rad).

**Western Blot Analysis.** Hippocampal homogenate containing 30 μg of protein per lane was separated by SDS/PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad). Nonspecific binding was blocked with 3% BSA dissolved in Tris·HCl buffer containing 0.1% Tween-20 for 1 h. Blots were then probed overnight at 4 °C with primary antibodies, followed by 1-h incubations with secondary antibodies conjugated to HRP, and then developed by chemiluminescence detection (Luminata

Forte, Millipore). The antibodies used for detection were as follows: mouse anti-β-actin (1:8,000; Abcam); and rabbit anti-Akt (1:2,000), mouse anti phospho-Akt$^{S473}$ (1:2,000), rabbit anti-p38MAPK (1:1,500), rabbit anti-phospho-p38MAPK$^{T180/Y182}$ (1:1,000), rabbit anti-AMPKα (1:1,500), rabbit anti-phospho-AMPKα$^{T172}$ (1:1,000), rabbit anti-Erk1/2 (1:1,500), and rabbit anti-phospho-Erk1/2$^{T202/Y204}$ (1:1,000; Cell Signaling Technology); and goat anti-mouse IgG-HRP (1:8,000) and goat anti-rabbit IgG-HRP (1:2,000; DAKO). Protein expression levels of ADNR1, ADNR2, and APPL1 in N2a cells and NPCs isolated from *adipo*$^{-/-}$ or WT mice were similarly determined with the following antibodies: mouse anti-ADNR1 (1:1,000) and goat anti-ADNR2 (1:1,000; Abcam), and rabbit anti-APPL1 (1:3,000; Antibody and Immunoassay Services, HKU).

**Immunoassays for Adiponectin, BDNF, and IGF-1.** The proteins were extracted from either whole hippocampal tissues or the isolated dentate tissue. The dentate region was isolated according to the method described by Hagihara et al. (9). Fresh tissues were collected 24 h after the 14-d running, and total proteins were extracted.

The levels of adiponectin, BDNF, and IGF-1 were determined using commercially available ELISA kits, including mouse adiponectin ELISA kits for detection in blood sample (Antibody and Immunoassay Services, Li La Shing Faculty of Medicine, The University of Hong Kong, Hong Kong) or in hippocampal tissue (Adipogen Corporation), Chemikine BDNF Sandwich ELISA Kit (Millipore), and Mouse/Rat IGF-1 Quantikine ELISA Kit (R & D Systems; Biovendor, Mediagnost).

**Culture of Mouse Neuroblastoma Cell Line N2a and Neural Progenitor Cell.** Transformed N2a cell line (ATCC) was maintained in DMEM high-glucose media containing 10% FBS, 2 mM L-glutamine, and 50 μg/mL gentamicin; cells were 1/5 subcultivated upon reaching 90% confluence. Passages 4–8 were used in the present study.

Primary NPCs were isolated from the dentate gyrus of the hippocampus of the WT or *adipo*$^{-/-}$ mice described previously (10) and were maintained in Neurobasal media containing 2% B-27 supplement, 20 ng/mL FGF-2, 20 ng/mL EGF, and 50 μg/mL gentamicin at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. Upon subculture, neurospheres were dissociated into a single cell suspension by treatment with TrypLE reagent and seeded into the Ultralow Attachment flasks (Corning Costar, Corning) at a density of $2 \times 10^4$ cells/mL. All of the reagents used in cell culture were obtained from Gibco, Invitrogen.

**Cell Proliferation Assays.** To quantify cell growth of N2a or progenitor cells after treatment, the MTT assay, which examines mitochondrial metabolic function, and CyQuant cell proliferation assay (Invitrogen), which quantifies nuclear DNA content, were adopted. The MTT assay and the CyQuant assay were conducted following the manufacturer's recommendation. The trimeric form of adiponectin (Antibodies and Immunoassays Services, The University of Hong Kong) was added to cells after overnight recovery to reach the final concentrations of 0–10 μg/mL, and assays were carried out 48 h after drug application. For the experiments involving NPCs, the 96-well plates were precoated with poly-L-ornithine and laminin to minimize cell detachment during bathing solution change.

**RT-PCR.** Total RNA isolated from progenitor cells or N2a cells or hippocampal tissue using the RNeasy Plus Mini Kit (Qiagen) was digested with DNase I to avoid possible genomic DNA contamination, and was subsequently reverse transcribed into cDNA templates by TaqMan Reverse Transcription Reagents (Invitrogen) following the manufacturer's instructions. PCR was performed with the Platinum PCR SuperMix reagent (Invitrogen) under the recommended conditions. Primers used for gene-specific amplification are shown in Table S1. PCR products were separated on 1% agarose gels containing 0.05% ethidium bromide. Bands were visualized under UV light, sized, and photographed by the Molecular Imager Gel Doc XR$^+$ System (Bio-Rad). The successfully amplified products were recovered by gel extraction and further verified by sequencing in the DNA Sequencing Facility of the University of Hong Kong.

**Real-Time Quantitative PCR.** The Taqman Gene expression assay was performed in quadruplicate for each cDNA sample. Assays were run with the Premix Ex Taq kit (Takara Biotechnology Co. Ltd.) on the MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad) using the following ramping protocol: 95 °C for 45 s, and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The 6-carboxyfluorescein (FAM) labeled dihydrocyclopyrroloindole tripeptide minor groove binder (MGB) Taqman probes used in the assays are listed in Table S2. The expression levels of indicated genes were all normalized to that of mouse N2a cells after $2^{-\Delta\Delta CT}$ calculation, using murine β-actin as the endogenous control.

**Confocal Microscopy.** N2a cells or neurospheres of WT and *adipo*$^{-/-}$ NPCs were seeded on poly-L-lysine-coated coverslips and allowed to attach firmly to the underlying surface for 4 h before fixation. Thereafter, cells were fixed with 4% PFA for 15 min and permeabilized with 0.1% saponin for 10 min, followed by blocking with 3% BSA-PBS for 1 h. Samples were probed with diluted primary antibodies in a humidified chamber overnight at 4 °C. Coverslips were rinsed with PBS three times and then incubated with fluorophore-conjugated secondary antibodies for 1 h. DAPI was added to counterstain the nuclei fluorescently. Coverslips mounted with fluorescent mounting medium (DAKO) were observed by confocal laser scanning microscope (LSM710; Carl Zeiss Microscope). Single layers of 0.5-μm thickness were photographed.

**Gene Knockdown by siRNA in N2a Cells.** N2a cells were plated into 96-well plates at $1 \times 10^4$ per well and permitted to attach to the bottom overnight. siRNA (10 pmol; Genepharma Co. Ltd.) targeting ADNR1 or ADNR2 (Table S3) was mixed with Lipofectamine RNAiMAX Transfection Reagent (0.5 μL; Invitrogen) and delivered into the cells following the manufacturer's instructions. The siRNA transfection was repeated on the third day with or without adiponectin treatment, and cells were ready for subsequent experiments 48 h after the second transfection to further enhance the transfection efficiency (Fig. S7).


1. Gould TD, et al. (2008) Involvement of AMPA receptors in the antidepressant-like effects of lithium in the mouse tail suspension test and forced swim test. *Neuropharmacology* 54(3):577–587.
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5. Wang Y, et al. (2006) Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. *Cancer Res* 66(23):11462–11470.
6. Liu L, Herukka SK, Minkeviciene R, van Groen T, Tanila H (2004) Longitudinal observation on CSF Abeta42 levels in young to middle-aged amyloid precursor protein/presenilin-1 doubly transgenic mice. *Neurobiol Dis* 17(3):516–523.
7. Yau SY, et al. (2012) Effects of voluntary running on plasma levels of neurotrophins, hippocampal cell proliferation and learning and memory in stressed rats. *Neuroscience* 222:289–301.
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9. Hagihara H, Toyama K, Yamasaki N, Miyakawa T (2009) Dissection of hippocampal dentate gyrus from adult mouse. *J Vis Exp*, 10.3791/1543.
10. Brewer GJ, Torricelli JR (2007) Isolation and culture of adult neurons and neurospheres. *Nat Protoc* 2(6):1490–1498.

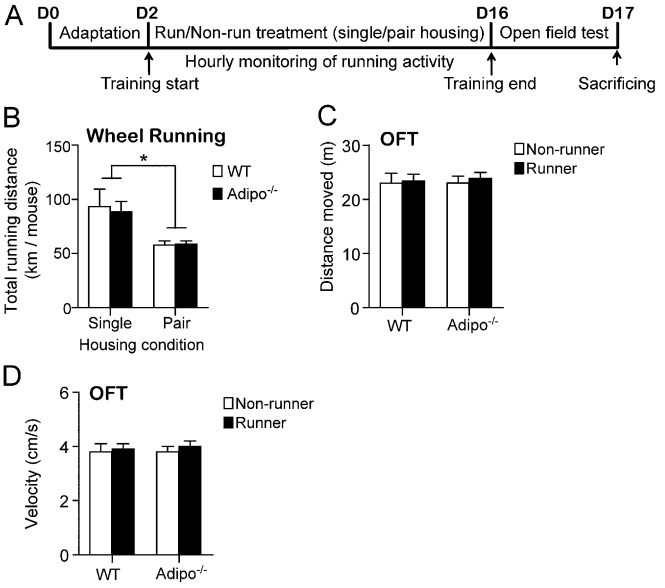


**Fig. S1.** Running distance and locomotor function of WT and *adipo*$^{-/-}$ mice. (*A*) Experimental timeline for monitoring the running activity of mice under various housing conditions and the OFT. (*B*) The total distance was automatically recorded by the wheel running system during the 2-wk treatments. *Main effect of housing: $P < 0.001$. $n = 8$–12 mice per group. (*C* and *D*) The locomotor ability assessed by the moving distance (*C*) and the average velocity (*D*) within the 10-min test session of the OFT was comparable among all groups receiving the pair housing. $n = 9$–10 mice per group.

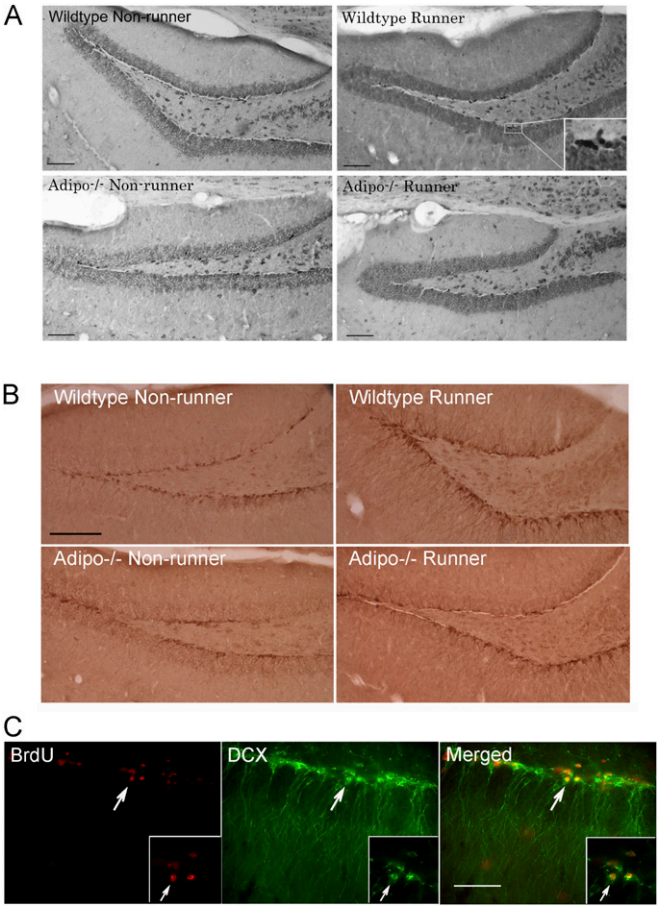


**Fig. S2.** Representative images of immunostaining. The brain samples collected from *adipo*$^{-/-}$ or WT mice receiving the 2-wk running or nonrunning treatment as described in Fig. 2*A* were sectioned coronally into 40-μm slices and subjected to immunostaining. (*A* and *B*) Representative images showing the proliferating (Ki67$^{+}$) cells (*A*) or immature neuronal (DCX$^{+}$) cells (*B*) in the hippocampal dentate gyrus. (*C*) Newborn cells differentiated into the neuronal lineage were identified as those positive for both BrdU and DCX staining (arrows). (Scale bars, 100 μm for *A*; 50 μm for *B* and *C*.)

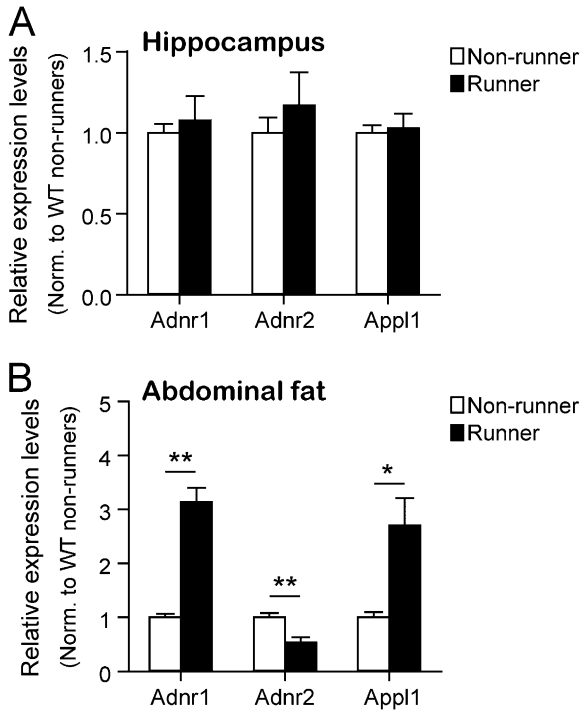


**Fig. S3.** Effects of running on the expression of adiponectin receptors and APPL1 in the hippocampus and adipose tissue of WT mice. (*A* and *B*) The hippocampal (*A*) or abdominal adipose (*B*) tissues were harvested from C57BL/6J mice receiving the 2-wk running or nonrunning treatment. The total RNA isolated from homogenized tissues was subjected to real-time PCR quantification for the mRNA expression of ADNR1, ADNR2, and the adaptor protein APPL1. The data were presented as fold changes over WT nonrunners using β-actin as the internal control. $*P < 0.05$; $**P < 0.005$. $n = 4$ mice per group.

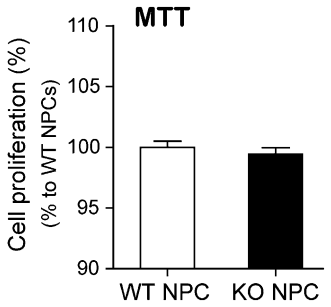


**Fig. S4.** Unaltered basal proliferation of neural progenitor cells isolated from *adipo*$^{-/-}$ mice. The passage-matched NPCs isolated from the hippocampi of the nontreated *adipo*$^{-/-}$ (KO) or WT mice were seeded at the same density and cultured for 48 h before the MTT assay for quantifying cell proliferation. $n = 4$ independent experiments for each group.

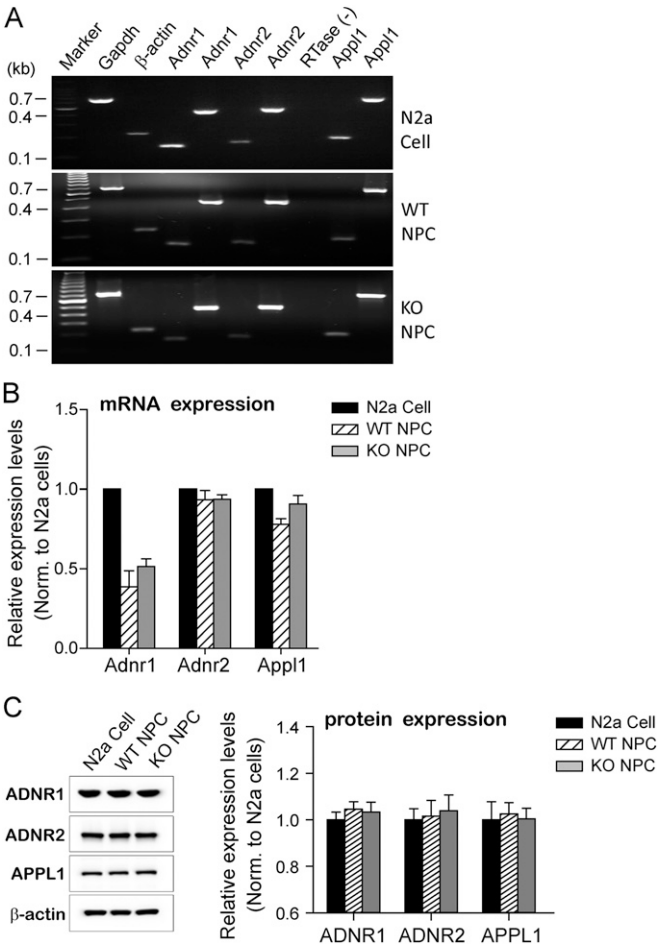


**Fig. S5.** Expression profile of ADNR1, ADNR2, and APPL1 on the N2a cell line and the primary NPCs. The total RNA and protein samples were extracted from the N2a cell line, as well as the hippocampal NPCs harvested from the nontreated *adipo*$^{-/-}$ (KO) or WT mice. (*A*) Presence of the message RNA of ADNR1, ADNR2, and APPL1 was confirmed by RT-PCR using gene-specific primers. (*B*) The relative expression levels of the targets were determined by real-time PCR. (*C*, *Left*) Representative immunoblotting images for ADNR1, ADNR2, APPL1, and the loading control β-actin. (*C*, *Right*) Semiquantitative analysis for ADNR1, ADNR2, and APPL1. The data were expressed as fold changes over the N2a cells. n = 4 independent experiments for each assay.

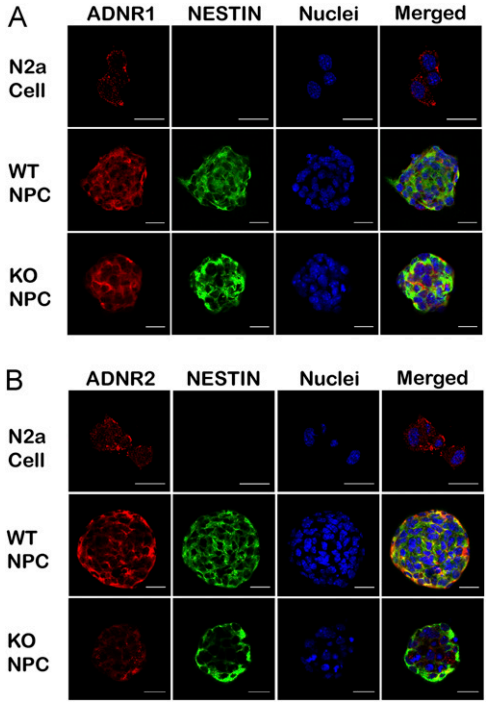


**Fig. S6.** Localization of ADNR1 and ADNR2 in the N2a cell line and the primary NPCs. (*A* and *B*) The monolayer of N2a cells and the spheres formed by the primary hippocampal NPCs isolated from the nontreated *adipo*$^{-/-}$ mice and WT mice were subjected to the immunostaining analysis with anti-ADNR1 (*A*) or anti-ADNR2 (*B*) antibodies, followed by analysis using confocal microscopy. Images were captured at a thickness of 0.5 μm. Nestin is the specific marker for NPCs. The proteins of ADNR1 (*A*) and ADNR2 (*B*) were expressed in these three cell preparations with similar localizations. (Scale bars, 20 μm.)

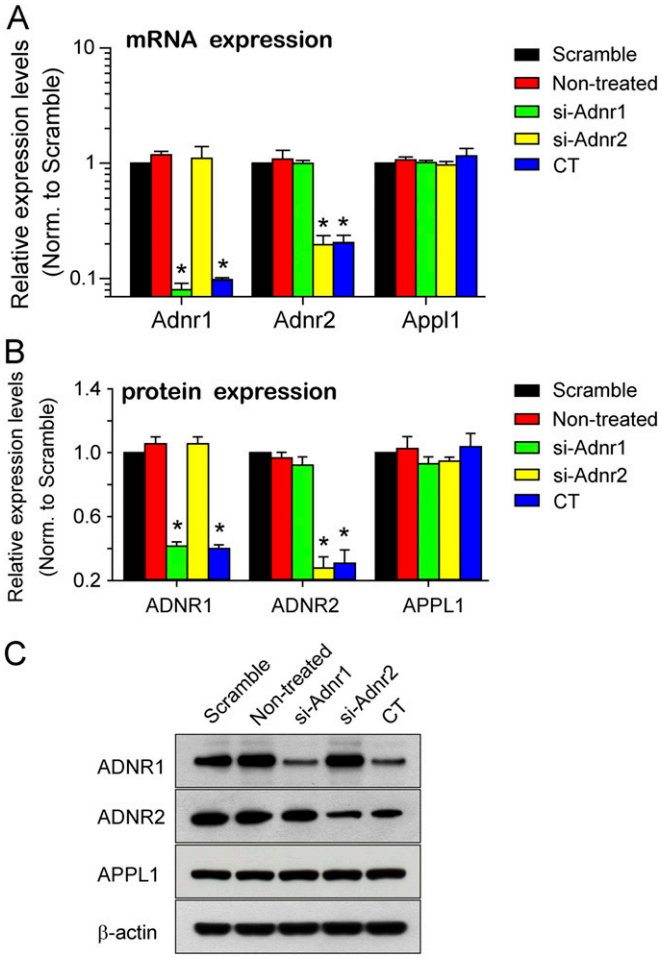


**Fig. S7.** Down-regulating adiponectin receptors by RNA interference in N2a cells. (*A*–*C*) N2a cells were transfected with siRNA against ADNR1 (si-Adnr1) or ADNR2 (si-Adnr2) for a period of 72 h, followed by evaluation of the knockdown efficiency at the mRNA (*A*) and the protein (*B* and *C*) levels by real-time PCR and semiquantitative immunoblotting, respectively. (*C*) Representative images for ADNR1, ADNR2, APPL1, and the loading control β-actin. $*P < 0.05$ vs. Scramble controls. $n = 4$ independent experiments for each assay. Nontreated, cells without transfection; CT, combined transfection with both si-Adnr1 and si-Adnr2.

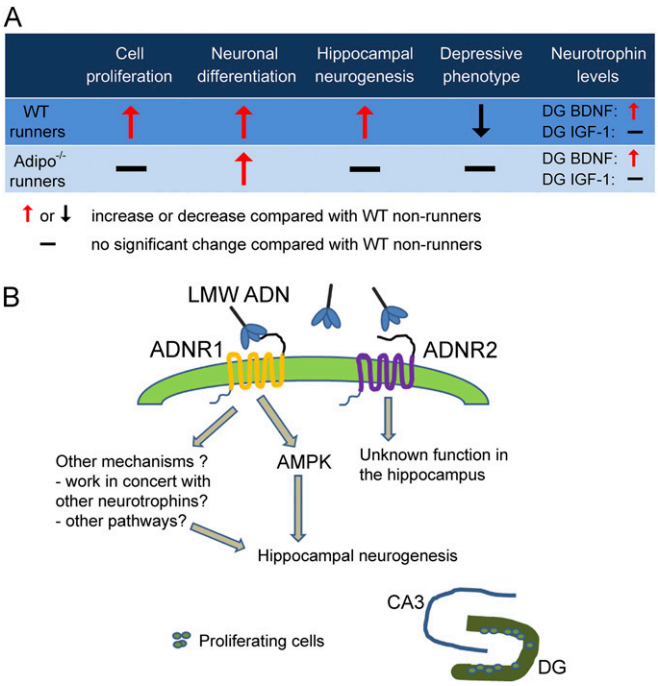


**Fig. S8.** Summary of the findings. (*A*) The tabulated data showing responses of Adipo$^{-/-}$ mice and WT littermates to the same running treatment in comparison with WT nonrunners. (*B*) The schematic diagram showing the potential neurogenic process in the dentate gyrus after exercise-triggered hippocampal ADN increase. DG, dentate gyrus; CA3, Cornu Ammonis region 3.

**Table S1. Gene-specific primers for RT-PCR**

| Target | | Forward primer | Reverse primer | Product size (bp) |
|---|---|---|---|---|
| GAPDH | | TCAACGGCACAGTCAAGG | GAAGTCGCAGGAGACAACC | 692 |
| β-actin | | GCTGTCCCTGTATGCCTCT | TTGATGTCACGCACGATTT | 222 |
| ADNR1 | (Pair 1) | GAAAGACAACGACTACCTGCTAC | CGTCAAGATTCCCAGAAAGAG | 154 |
| | (Pair 2) | AACTGGACTATTCAGGGATTGC | ACCATAGAAGTGGACGAAAGC | 446 |
| ADNR2 | (Pair 1) | CCACCATAGGGCAGATAGG | TGAACAAAGGCACCAGCAA | 169 |
| | (Pair 2) | CTCCTATGCCTTCCTTTCG | AACACTCCTGCTCTGACCC | 466 |
| APPL1 | (Pair 1) | GGTAGCCAGTGACCCTTTAT | CTCCTGCCACATCTCCAC | 194 |
| | (Pair 2) | AAGGCTGGATACCTAAATGCT | AGAACCAAGGAATCGGACA | 687 |

**Table S2. Inventoried Taqman gene expression assays for real-time PCR from Life Technologies Limited**

| Target | Assay ID of FAM-labeled MGB Probe |
|---|---|
| β-actin | Mm00607939_s1 |
| ADNR1 | Mm01291334_mH |
| ADNR2 | Mm01184032_m1 |
| APPL1 | Mm00507526_m1 |

**Table S3. Sequences of siRNA**

| Target | Nucleotide sequence (5′ to 3′) |
|---|---|
| ADNR1 | GACUGGCAACAUCUGGACAAA |
| ADNR2 | GCUUAGAGACACCUGUUUGUUAA |
| Scramble | AUUUAACUUCUGGUGACGAUACU |