Materials and Methods

Antibodies, Plasmids and Chemicals

Antibodies: anti-GluA1Ct (13011#314, rabbit) was custom-made by OriGene Technologies, Inc.; anti-GluA1Nt (MAB2263, mouse) and GluN1Ct (05-432, mouse) were purchased from Millipore; anti-PSD95 (73-028, mouse) was from NeuroMab; anti-tubulin (T2200, mouse) and GAPDH (G9545, mouse) were from Sigma-Aldrich; anti-SIRT2 antibodies were from Abcam (ab19388, rabbit, for immunostaining) or Cell Signaling (12650, rabbit, for immunoblotting); anti-FLAG was from Cell Signaling (8146, mouse, for immunoblotting) or Millipore (MAB3118, mouse, for co-IP,); anti-acetylated lysine (ab80178, rabbit) was from Abcam; anti-ubiquitin (ab7780, rabbit) and anti-GRIP1(ab25963, rabbit) was from Abcam.

Plasmids and siRNA: GluA1-4KQ mutant was generated from GluA1-GFP with the Agilent QuikChange Lightning Site-Directed Mutagenesis Kit; SIRT1 (#13812), SIRT2 (#13813) and SIRT3 (#13814) were obtained from the Eric Verdin lab at University of California, San Francisco, *via* their deposit at Addgene; Scrambled (sc-44230) and SIRT2 (sc-40989) siRNAs were purchased from Santa Cruz Biotechnology.

Chemicals: B2 (sc-202486) was purchased from Santa Cruz Biotechnology, and cycloheximide (C7698), AGK2 (A8231) and MG132 (M8699) from Sigma-Aldrich.

Transfection of neurons or human embryonic cells (HEK) 293T

Neurons at DIV 10-12 or HEK 293T cells that were split and cultured overnight were transfected with lipofectamine 2000 (Life Technologies) along with the target plasmids or siRNAs. Following the manufacturers' instructions, lipofectamine 2000 was mixed with target plasmids or siRNAs in 1X Dulbecco's Modified Eagle's Medium (DMEM, Corning) for 20 minutes at room temperature to

form the transfection complex. The transfection complex was then incubated with the neurons/293T cells in culture medium for another 4-hr at 37℃ in the cell culture incubator. Afterward, the medium with the transfection complex was replaced with fresh culture medium. For neurons, half of the fresh feeding medium was supplemented with conditioned feeding medium to minimize cell death. HEK 293T cells were cultured in the following medium: 1X DMEM with 10% FBS, 1% P/S and 1% L-Glutamine. To exogenously express the GluA1 subunit of AMPARs in HEK cells, a pRK5 plasmid vector carrying GluA1-GFP cDNA was transfected then incubated for another 48 hr before experiments.

Golgi impregnation

Golgi impregnation of the whole brains of C57BL/6J wild-type and SIRT2 knock-out (*Sirt2*$^{-/-}$) mice was performed with the FD Rapid GolgiStain^{TM} Kit (FD NeuroTechnologies, Inc), following the manufacturer's protocol. Briefly, mice of either sex at 12-15 weeks of age were sacrificed in a 4% CO_2 chamber for 5 min then the brains were immediately removed and rinsed in MilliQ water. Brains were retrieved and immersed in a Golgi-Cox solution containing potassium dichromate, mercuric chloride, and potassium chromate. The mixture of solutions was replaced once after 6 hours of initial immersion, then stored at room temperature in darkness for 2 weeks. After the immersion period in the Golgi-Cox solution, the embedded brains were transferred to a cryoprotectant solution and stored at 4℃ for at least 1 week in the dark before cutting. The brain slices were sectioned in the coronal plane at approximately 300 μm thickness on a vibratome. Brain slices were transferred onto gelatin-coated slides and were air dried at room temperature in the dark overnight before further processing. After drying, sections were rinsed with distilled water and stained in a developing solution and subsequently dehydrated with 50%, 75%, 95%, and 100%

ethanol. Finally, the sections were defatted in xylene substitute and mounted on coverslips with Permount mounting liquid (Fisher Scientific). Images were acquired with a Carl Zeiss Axiovert 200M microscope and AxioVision software (release 4.5). Each neuron was scanned under high magnification (63x, oil immersion, NA = 1.4) by varying the depth of the Z plane, to ensure that all parts of the cell (especially dendrites) were intact. The number and size of apical and basal spines, and the length of dendrites on hippocampal CA1 pyramidal neurons was measured blind to the genotype with Image J software. At least 20 neurons were selected from the CA1 region in each condition.

Hippocampal slice preparations

Mice were sacrificed by isoflurane anesthesia at age 6~8 wks. Brains were quickly removed and submerged in ice-cold oxygenated sucrose-replaced 1X ACSF cutting solution containing (in mM) 206 sucrose, 2 KCl, 2 $MgSO_4$, 1.25 NaH_2PO_4, 1 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 10 D-glucose, pH 7.4, 315 mOsm. Transverse slices (350 μm thick) were cut with a vibratome from the middle portion of each hippocampus. After dissection, slices were incubated in ACSF containing (in mM): 124 NaCl, 2 KCl, 2 $MgSO_4$, 1.25 NaH2PO_4, 2.5 $CaCl_2$, 26 $NaHCO_3$, 10 D-glucose, pH 7.4, 310 mOsm, in which brain slices were allowed to recover for at least 90 minutes before recording. A single slice was then transferred to the recording chamber and submerged beneath continuously perfused ACSF saturated with 95% O_2 and 5% CO_2. Slices were incubated in this chamber for 20 minutes before stimulation at room temperature (~24 ℃).

Electrophysiology

For whole cell patch-clamp recording on hippocampal slices, C57BL/6J wild-type and SIRT2

knock-out (*Sirt2*$^{-/-}$) mice (8-12 weeks old) of either sex were deeply anaesthetized with isoflurane followed with euthanasia by decapitation. After opening the skull, the brain was quickly removed and immersed into ice-cold (~4 ℃) sucrose slicing solution (Wang et al., 2015), containing (in mM) Sucrose 72, NaCl 83, KCl 2.5, NaH_2PO_4 1, $MgSO_47H_2O$ 3.3, $NaHCO_3$ 26.2, Dextrose 22, $CaCl_2$ 0.5, and saturated with 95% O^2 and 5% CO^2. Coronal slices of the hippocampus (350 – 400 μm in thickness) were prepared using a Leica VT1000S Vibratome. The slices were incubated in 1X ACSF (in mM: NaCl 126, KCl 3, NaH_2PO_4 1.25, $MgSO_47H_2O$ 2, $NaHCO_3$ 26, Dextrose 10 and $CaCl_2$ 2; pH 7.4) at 34 ℃ for 20 min, and then held at room temperature for another 30 min before recording. The slices could remain viable for 4-6 hours during recordings. The whole-cell patch-clamp recordings were performed at room temperature (~23 ℃). Before recording, the slices were transferred into a submerged recording chamber and perfused (2 mL/min) with oxygenated (with 95% O^2 and 5% CO^2) 1X ACSF. Recording electrodes were pulled from borosilicate glass capillaries (Sutter Instruments, Novato, CA) using a micropipette puller (Model P-97; Sutter Instruments, Novato, CA) with resistance of 4~7 MΩ when filled with internal solution (in mM: 120 CsGluconate, 5 $MgCl_2$, 0.6 EGTA, 30 HEPES, 4 MgATP, 0.4 Na_2GTP, 10 phosphocreatine-Tris, 5 QX-314; 290 mOsm; pH was adjusted at 7.2 with CsOH) (Lillis et al., 2015). Whole-cell patch clamp recordings were made from the CA1 pyramidal neurons visualized with an infrared and differential interference contrast camera (IR-DIC camera, Hitachi, Japan) in an upright Olympus microscope (Olympus, Tokyo, Japan). The signal acquisition was made through a Multiclamp amplifier (Multiclamp 700B; Molecular Devices, Sunnyvale, CA) along with the Clampex 10 software (Molecular Devices, Sunnyvale, CA). Signals were sampled at 10 kHz and filtered at 2 kHz. Data were digitized with an A/D converter (Digidata 1440A, Molecular Devices, Sunnyvale,

CA) for offline analysis. Membrane potential was corrected for the liquid junction potential of 13.7 mV. Neurons with negative resting membrane potential less than -60 mV were discarded. Neuron input resistance and patching access resistance were repeatedly monitored during the experiment. Cells with changes of more than 15–20% in access or input resistance were excluded from the analysis.

Standard field excitatory postsynaptic potentials (fEPSP) were recorded in the CA1 region of the hippocampus. A bipolar stimulating electrode (FHC Inc., Bowdoin, ME) was placed in the Schaffer collaterals to deliver test and conditioning stimuli. A borosilicate glass recording electrode filled with ACSF was positioned in the stratum radiatum of CA1, 200~300 μm from the stimulating electrode. fEPSP in CA1 were induced by test stimuli at 0.05 Hz with an intensity that elicited a fEPSP amplitude of 40~50% of maximum. Test responses were recorded for 30-60 minutes prior to beginning the experiment, to ensure stability of the response. To induce LTP, two consecutive trains (1 s) of high frequency stimulation (HFS) at 100 Hz separated by 20 s, a protocol that induces LTP lasting ~1.5 hr in wild-type mice were applied to the wild-type or SIRT2 KO slices. To induce LTD, 900 pulses at low frequency (1 Hz) stimulation (LFS) were applied to the slices. All LTP and LTD values represent fEPSP slopes measured 60 minutes after the conditioning stimulus, unless stated otherwise. The field potentials were amplified 100× using an Axon Instruments 200B amplifer and digitized with a Digidata 1322A digitizer. The data were sampled at 10 kHz and filtered at 2 kHz. Traces were obtained by pClamp 9.2 and analyzed using Clampfit 9.2.

Fear conditioning test

The fear conditioning tests consisted of two parts: context- and tone-dependent fear conditioning. Before the test, mice were habituated in a separate cage with a similar context as the testing cage

(bars on the floor, white and smooth walls, pine wood bedding with an 8% acetic acid odor) for three continuous days (5-min/day). On experiment day 1, in each trial, one mouse was placed into the testing cage and allowed to explore the cage freely for 3-min, followed by a 30 s tone (80 db) and an electrical foot shock (2 s, 0.6 mV) at the end of the tone. The tone and foot shock were repeated three times in each trial with three 1-min intervals. The procedure was repeated for every mouse in the experiment. The whole cage was thoroughly cleaned up with 70% ethanol and 8% acetic acid after every mouse to avoid potential olfactory distraction from other individuals. On experiment day 2, the mice were placed back into the same cage with exactly the same environment and odor to test their contextual fear memory without any foot shock. The mice were allowed to explore the cage for 5-min. On day 3, the context of the cage was completely changed (smooth floor, wall decorated by dots, corn bedding with 20% vanilla odor) and the mice were allowed to explore the cage freely for 3-min then another 2-min with the same tone played. The motion index and freezing percentages of the mice were recorded and analyzed by Video Freeze® system (Med Associates Inc.).

Novel object recognition test

The novel object recognition test protocol was adopted and modified from elsewhere (Gao et al., 2010). Briefly, the mice were habituated one by one in an open field arena (45 cm × 45 cm × 30 cm, made with PVC boards) for 3 continuous days (Day 1-3), 5 min per day. On the fourth day (Day 4), three identical blue-colored flasks (50 mL) were placed into the arena. The mice were trained repeatedly for 4 times on Day 4 to recognize these flasks. Each training session lasted 5 min. Object memory was probed on the fifth day (Day 5) when one flask was replaced by a yellow-colored beaker with similar size as the flask (50 mL). The arena was thoroughly wiped with 75% ethanol to

clean any odor cues between mice throughout all the sessions. The number of explorations (nose pokes) of the mice to each object were scored by experienced observers and expressed by the discrimination index (DI): $DI = [(N_{novel} - N_{familiar1}) + (N_{novel} - N_{familiar2})] / N_{total}$. Where N_{novel} refers to the number of times the mice explored the newly introduced object (yellow beaker), while $N_{familiar1}$ and $N_{familiar2}$ refer to the number of explorations of the other two old objects (blue flasks). N_{total} is the total number that all three objects were explored on the probe day.

Barnes maze test

The protocol of the Barnes Maze test is adopted and modified from previous studies (Sunyer et al., 2007). The maze itself was made from a circular, 0.75-inch thick, white plastic board with a 48-inch diameter. Twenty holes with a diameter of 2-inches were evenly spaced around the perimeter of the maze with a distance of 1-inch to the edge. The maze was mounted on a rotating pedestal that was 30 inches above the ground and could rotate at its center. The escape cage was made from a black plastic box with a ramp connected beneath the escape hole for easy access. Four bright ceiling lamps and a noisy buzzer were used as the aversive stimuli during the test. The maze and escape cage were thoroughly cleaned with 70% ethanol between testing sessions to avoid any olfactory cues and the maze was rotated randomly after every three mice to avoid intra-maze odor or visual cues.

During the test, the mice were habituated to the maze on day 1. Each time, one mouse was placed in the center of the maze, covered with an opaque cardboard chamber for 15 seconds then slowly guided to the escape hole with a 3-liter glass beaker. Three-min was given to the mouse to enter the escape hole on its own, if not, the mouse was nudged gently into the hole or placed directly into the hole. Afterward, the mouse was allowed to stay in the escape hole for 2-min. The

ceiling lights and white noise remained on while the mouse was exploring the maze and turned off immediately after they entered the escape hole. This procedure was repeated in all mice. On days 2-5, the mice were trained extensively to ensure a strong memory for the escape location (4 times/mouse/day, 16 times in total) to learn to enter the escape hole by themselves. On these trials, the mice were allowed to stay in the escape cage for 1-min in each training trial. Memory retention was probed 2 days later on day 7. During the probe session (day 7), the escape hole was covered and the spatial memory in the subject mice was tested. All behavior videos were captured by a Logitech c920 webcam. The primary latency, total latency, correct/error nose pokes, duration of stays in each quadrant and trace of locomotion were scored by experienced observers or the animal tracing program idTracker 2.1 blind to the animals' identity.

Supplemental References

Gao, J., Wang, W.Y., Mao, Y.W., Graff, J., Guan, J.S., Pan, L., Mak, G., Kim, D., Su, S.C., and Tsai, L.H. (2010). A novel pathway regulates memory and plasticity *via* SIRT1 and miR-134. Nature *466*, 1105-1109.

Lillis, K.P., Wang, Z., Mail, M., Zhao, G.Q., Berdichevsky, Y., Bacskai, B., and Staley, K.J. (2015). Evolution of Network Synchronization during Early Epileptogenesis Parallels Synaptic Circuit Alterations. J. Neurosci. *35*, 9920-9934.

Sunyer, B., Patil, S., Hoger, H., and Lubec, G. (2007). Barnes maze, a useful task to assess spatial reference memory in the mice. Nat. Protoc. *390*, 10-38.

Wang, Z., Neely, R., and Landisman, C.E. (2015). Activation of Group I and Group II Metabotropic Glutamate Receptors Causes LTD and LTP of Electrical Synapses in the Rat Thalamic Reticular Nucleus. J. Neurosci. *35*, 7616-7625.

Supplemental Figures (Figure S1-7) with legends

Figure S1

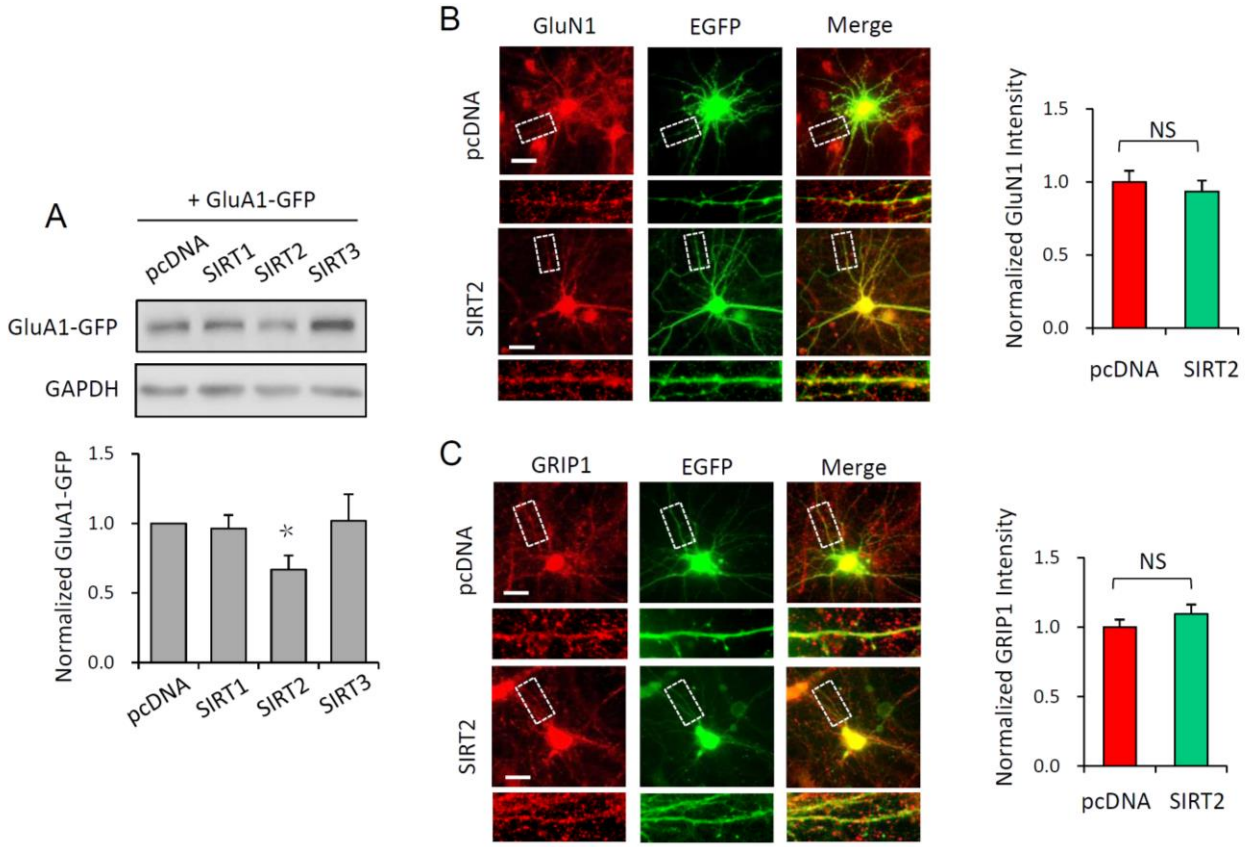

Figure S1 SIRT2 overexpression specifically down-regulates AMPAR expression without affecting other synaptic proteins, related to Figure 1. (**A**) Overexpression of SIRT2, but not SIRT1 or 3, dramatically decreased GluA1 subunit levels in HEK 293T cells (n = 4). Bar graphs represent mean ± S.E., $*P < 0.05$. One-way ANOVA with Tukey post-hoc test. (**B, C**) 72 hrs after co-transfection of SIRT2 with EGFP, neurons were immunostained for NMDAR subunit GluN1 (93.6 ± 7.4%, n = 12 cells/group, $P > 0.05$) (B) and AMPAR-associated protein GRIP1 (109.5 ± 6.2%, n = 11 cells/group, $P > 0.05$) (C). Bar graphs represent mean ± S.E. NS, not significant. Student's two-tailed t test. Scale bar, 20 μm.

Figure S2

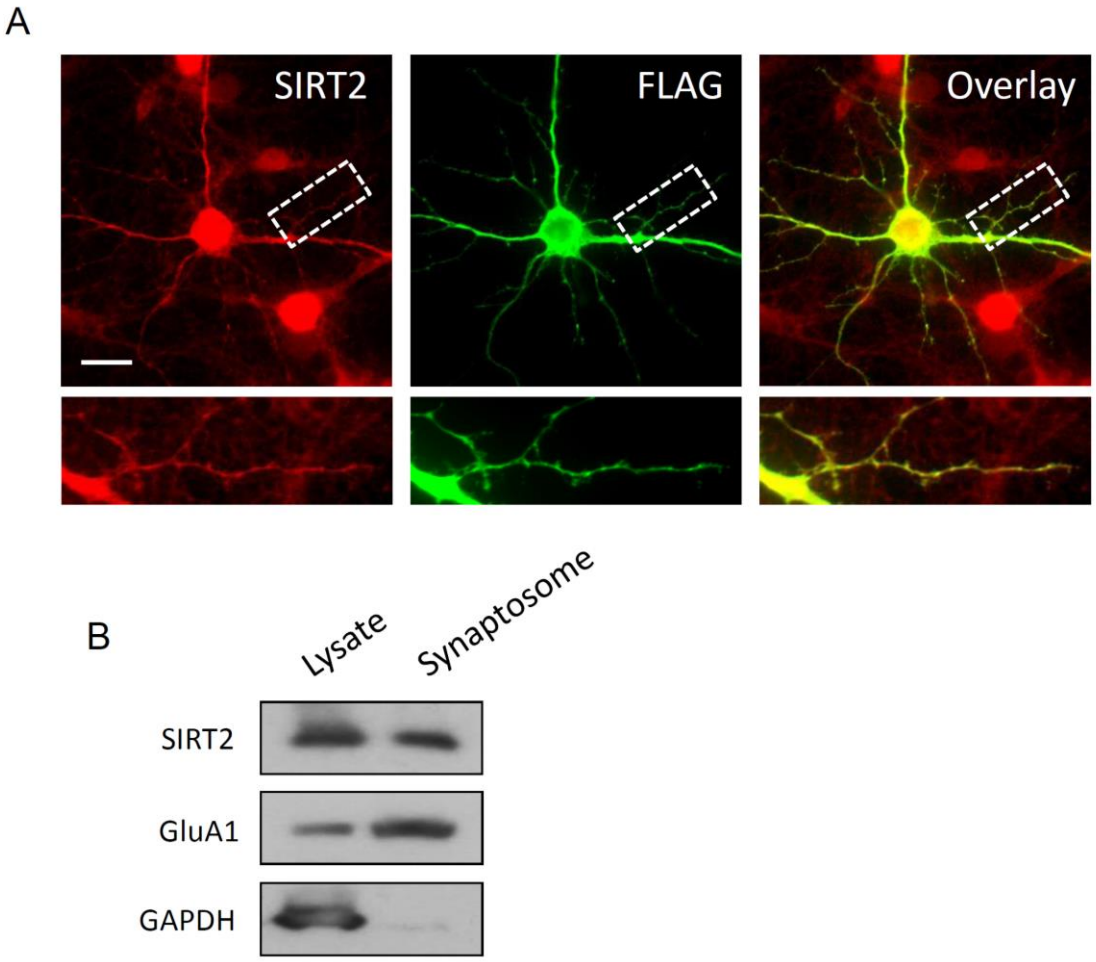

Figure S2 Subcellular distribution of SIRT2, related to Figure 1. (**A**) Co-immunostaining of SIRT2 and FLAG in cultured rat hippocampal neurons transfected with SIRT2-FLAG showing an overlapping pattern. Scale bar, 20 μm. (**B**) Synaptosome purification from rat hippocampal tissue showed that SIRT2 was not enriched, but did distribute in synapses. GluA1 showed enriched localization in the synaptosome and GAPDH was probed to indicate the purity of the synaptosome.

Figure S3

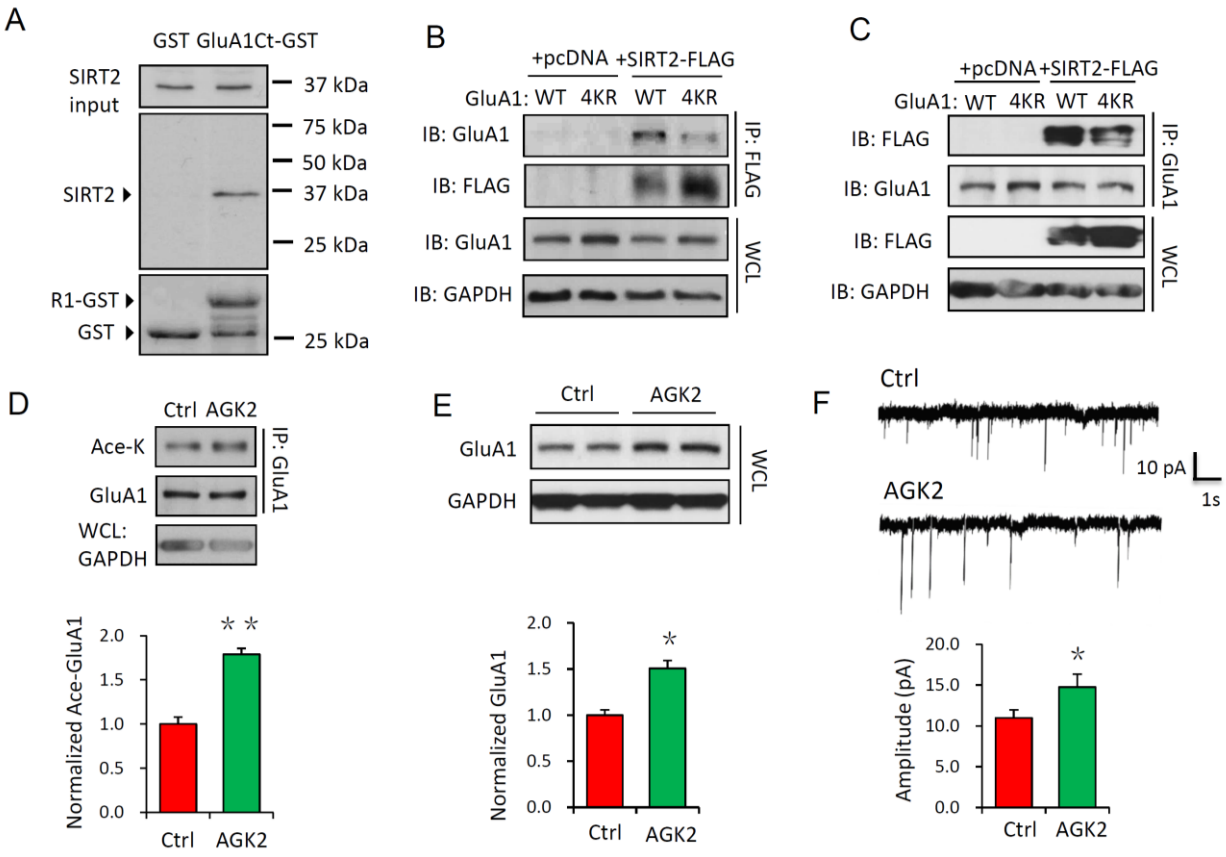

Figure S3 Lysine-dependent GluA1-SIRT2 interaction and the effects of SIRT2 inhibitor, AGK2, on GluA1 acetylation and expression, and miniature synaptic transmission, related to Figure 1, 2. (**A**) GST-tagged GluA1 C-terminus (R1Ct-GST) co-precipitated with SIRT2 using rat hippocampal lysate. (**B, C**) In HEK 293T cells, the physical interaction between GluA1 and SIRT2 was dramatically interrupted by the mutation of lysines to arginines (4KR) on the C-terminus of GluA1. (**D, E**) The chemical SIRT2 inhibitor, AGK2 (20 μM, 8 hrs), significantly increased the GluA1 acetylation and expression level in cultured cortical neurons (2-week old). (**F**) AGK2 (20 μM, 8 hrs) significantly increased the miniature synaptic transmission in cultured hippocampal neurons (2-week old). IP: immunoprecipitation, IB: immunoblotting, WCL: whole-cell lysates. Bar graphs represent mean ± S.E., $*P < 0.05$, $**P < 0.01$. Student's two-tailed *t* test.

Figure S4

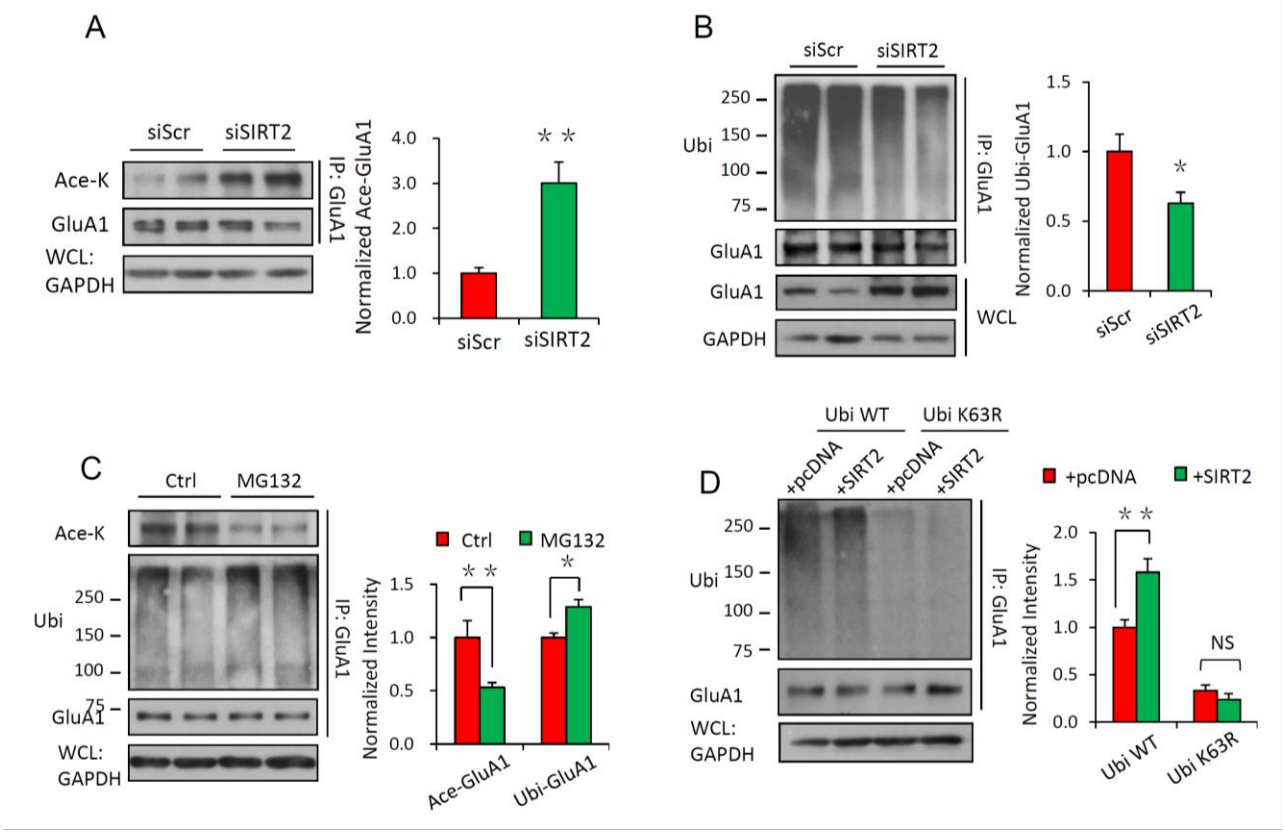

Figure S4. SIRT2 is a deacetylase of AMPAR and down-regulates AMPAR expression *via* the ubiquitination pathway, related to Figure 3. (**A, B**) Inhibition of SIRT2 by siRNA increased GluA1 acetylation (A) while decreased GluA1 ubiquitination (B) in HEK 293T cells (n = 4). IP: immunoprecipitation. WCL: whole-cell lysates. Bar graphs represent mean ± S.E., ＊$P < 0.05$, ＊＊$P < 0.01$. Student's two-tailed t test.

Figure S5

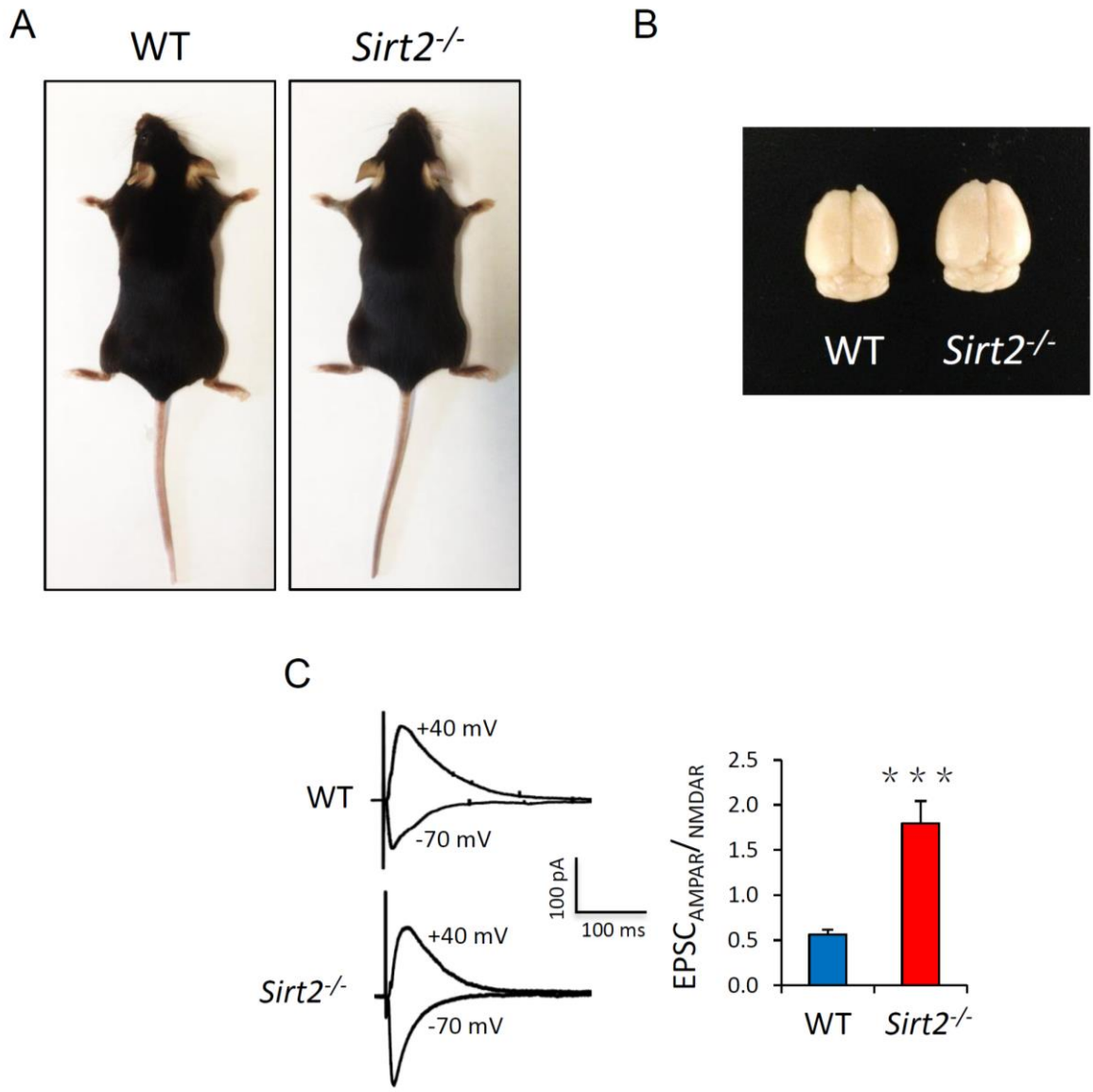

Figure S5 Animal growth, brain structure and AMPAR/NMDAR EPSCs in *Sirt2*$^{-/-}$ mice, related to Figure 5. (**A**) 12-wk old male C57BL/6J WT and *Sirt2*$^{-/-}$ mice had similar body size and (**B**) brain size. (**C**) Patch-clamp recordings of evoked excitatory postsynaptic currents (EPSC) in hippocampal slices showed increases in AMPAR current and an enhanced AMPAR/NMDAR EPSC ratio in *Sirt2*$^{-/-}$ mice compared to the WT mice (n = 8 cells/group). Bar graphs represent mean ± S.E., $***P < 0.001$. Student's two-tailed t test.

Figure S6

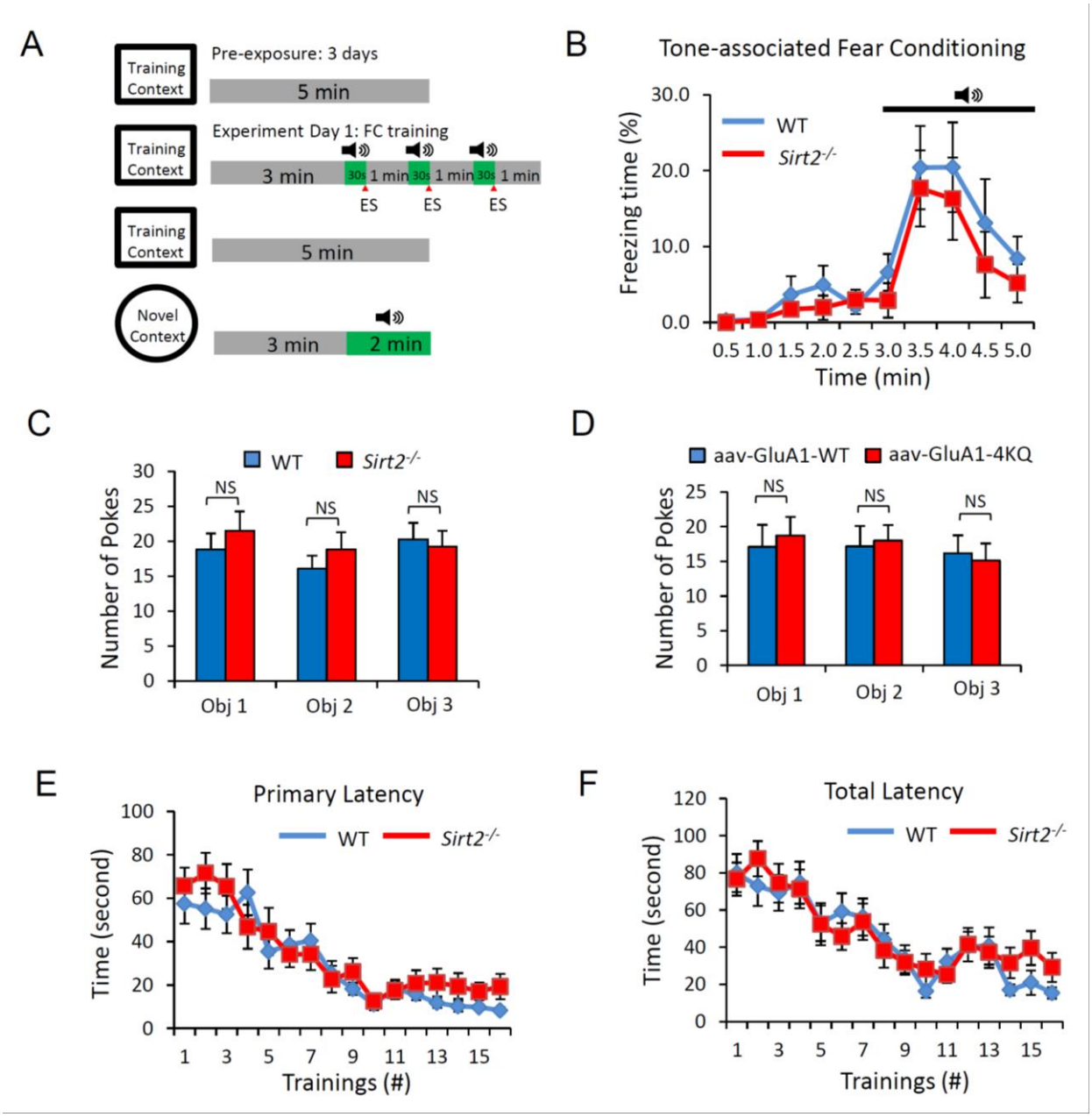

Figure S6 Behavioral assays utilized to test the fear, learning and spatial memory of WT, SIRT2 KO and AAV-infected mice, related to Figure 6. (**A**) The protocol for the fear conditioning tests used in this study (FC: Fear conditioning; ES: electric shock). Details of the test were provided in the Materials and Methods. (**B**) The WT and SIRT2 KO mice showed no significant difference in tone fear conditioning (n = 15 mice/group). (**C, D**) Pre-testing object preference. At the beginning of

the experiments, no difference was detected in object preference in WT or *Sirt2*$^{-/-}$ mice (*n* = 15 mice/group) (C), and mice with brain injection of GluA1-WT or GluA1-4KQ adenovirus (aav) (*n* = 12 – 15 mice/group) (D) during the pre-test exploration period in the training session. (**E, F**) WT and *Sirt2*$^{-/-}$ mice showed similar abilities to learn during the training phase. No significant difference was observed in primary latency (E) and total latency (F) between WT and *Sirt2*$^{-/-}$ mice. Primary latency refers to the time taken for the mice to find the target hole for the first time, and the total latency refers to the time the mice eventually got into the escape box (*n* = 15 mice/group). Bar graphs represent mean ± S.E., NS, not significant. Student's two-tailed *t* test.

Figure S7

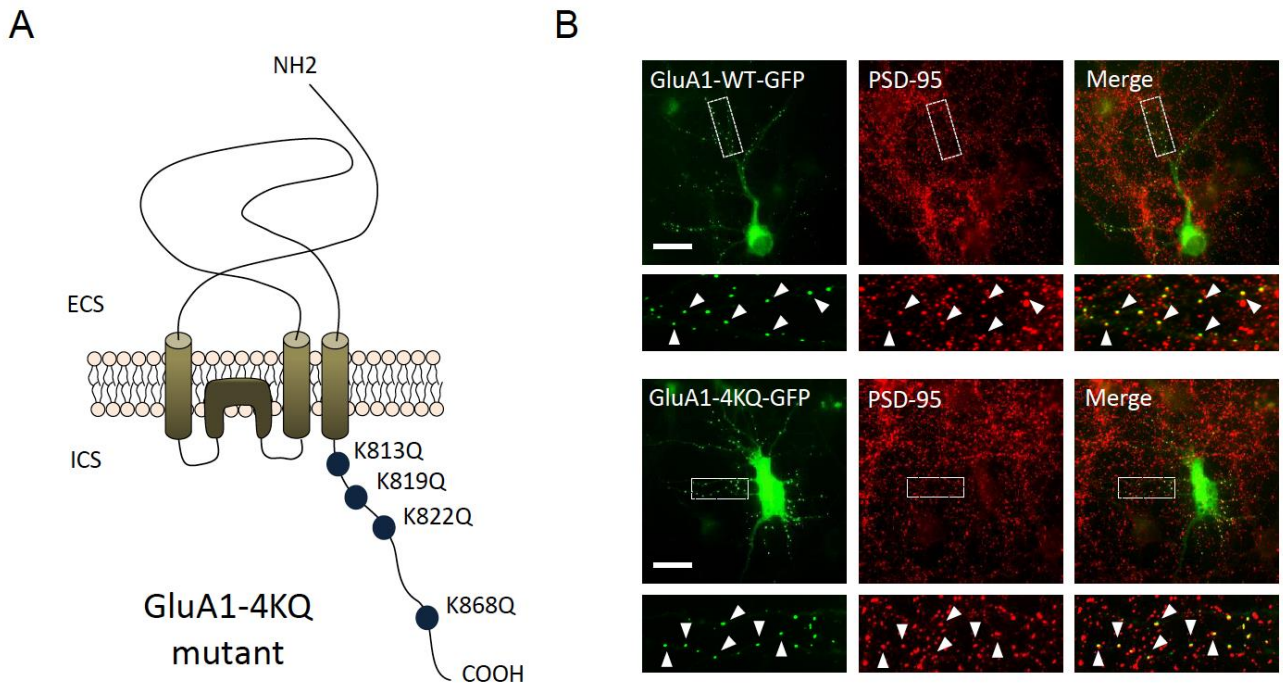

Figure S7 Synaptic expression of the GluA1 acetylation mimetic (GluA1-4KQ), related to Figure 7. (**A**) A schematic cartoon showing the structure of GluA1 acetylation mimetic (GluA1-4KQ) (ECS: extracellular space; ICS: intracellular space). (**B**) Synaptic expression of GFP-tagged GluA1-WT and 4KQ. PSD-95 protein was immunostained (red) to indicate the synaptic sites in cultured hippocampal neurons.