SUPPLEMENTAL TEXT AND FIGURES

EXPERIMENTAL PROCEDURES

Mice and primary neuronal cultures. All animal procedures were approved by the Animal Care and Use Committee of the National Institute on Aging Intramural Research Program. Breeding pairs of SIRT3-deficient mice were a generous gift from David Gius (then at the National Cancer Institute and now at Northwestern University). The colony was maintained, and littermate experimental animals were generated by breeding *Sirt3*+/- males with *Sirt3*+/- females. For genotyping, two complementary PCRs were performed on the genomic DNA from mouse tail biopsies or embryonic tissues. The first reaction used primers (forward: 5'ATCTCGCAGATAGGCTATCAGC3' and reverse: 5'AACCACGTAACCTTACCCAAG G3'), which flank the insertion site in *Sirt3* and produce a 336 bp PCR fragment for wild type (*Sirt3*+/+) and heterozygous (*Sirt3*+/-) mice. The second reaction primer set (forward: 5'CTGTGCTCGACGTTGTCACTG3'; reverse: 5'GATCCCCTCAGAAGAACTCGT3') targets the Neo marker in the insertion element and produces a 556 bp fragment in *Sirt3*+/- and homozygous (*Sirt3*-/-) mice (**Supplemental Figure 1A**). The genotypes were confirmed by RT PCR using two sets of specific primers targeting *Sirt3* cDNA (**Supplemental Figure 1B**). For some experiments, 6 month-old *Sirt3*-/- and *Sirt3*+/+ mice were housed individually with or without access to a running wheel. For primary cultures of embryonic cortical neurons, timed-pregnant mice were obtained by breeding *Sirt3*+/- male and female mice. Cortical cultures were prepared from embryonic day (E) 15 cerebral tissues. Briefly, pregnant mice were euthanized, embryo brains were removed, and the cerebral hemisphere was dissected in sterile Hank's Balanced Saline Solution (HBSS). The tail of each embryo was collected for genomic DNA extraction and genotyping. The brain tissue from each embryo was incubated in 0.25% trypsin-EDTA for 15 min at 37°C, and then transferred to MEM+ (Minimal Essential Medium supplemented with 10% fetal CloneIII bovine serum (HyClone, Logan, UT, USA)) and were dissociated by titration using a fire-polished Pasteur pipet. The dissociated cells were seeded into polyethyleneimine-coated plastic culture dishes or glass coverslips at a density of 60,000 cells/ cm^2. After a 4 hour incubation in MEM+ to allow for cell attachment, the medium was replaced with Neurobasal medium containing B27 supplements, 2 mM L-glutamine, antibiotic-antimycotic (Gibco) and 1 mM HEPES. The culture treatments included: L-Glutamic acid

(Glut), kainic acid (KA), N-methyl-D-aspartate (NMDA), MitoTEMPO and cyclosporin A (CsA). All the reagents were prepared as 500–1,000X stocks in dimethylsulfoxide or distilled water. Treatments were administered by direct dilution into the culture medium, and an equivalent volume of vehicle was added to control cultures.

Neurotoxin models of Huntington's disease and severe epileptic seizures. Methods for the 3-NPA model of HD and the kainic acid-induced seizure model of temporal lobe epilepsy have been described previously (Beal, 1995; Bruce-Keller et al., 1999; Guo et al., 1999). Briefly, for the 3-NPA model, a solution of 20 mg/ml 3-NPA (Sigma) in PBS was prepared and the pH was adjusted to pH 7.4 with NaOH, and the solution was sterilized by passing through a 0.22 μm filter disc. For evaluation of survival, mice were injected intraperitoneally with 30 mg/kg 3-NPA once daily (10 *Sirt3*+/+ mice and 10 *Sirt3*-/- mice; all 8 months old) at 9:00 AM. Mice were monitored every 3 hours until the end of the light period (6:00 PM). If a mouse died or was moribund before 6:00 PM, it was scored as dying on that day. If a mouse died after 6:00 PM and was found to be dead the next morning, it was scored as dying on the day it was found dead. In a separate experiment 8 month-old *Sirt3*+/+ mice and *Sirt3*-/- mice were injected intraperitoneally with PBS or 3-NPA (30 mg/kg/day) for 7 consecutive days (5 or 6 mice per group) at which time mice were tested on the rotarod and then euthanized and perfused transcardially with 4% paraformaldehyde in PBS. Brains were removed and processed for immunohistochemical analysis.. For intra-hippocampal KA infusion, mice were divided into six groups: *Sirt3*+/+ sham, *Sirt3*-/- sham; *Sirt3*+/+ KA; *Sirt3*-/- KA; *Sirt3*+/+ runners KA; *Sirt3*-/- runners KA. There were 5 mice/group. Intra-hippocampal injections of kainic acid were performed using a stereotaxic apparatus (RWD, Shenzhen, China) and a microinjection pump system (CMA, North Chelmsford, MA) in mice anesthetized with isoflurane. KA (0.5 μl of a 1 mM solution in PBS, pH 7.4) or 0.5 μl of PBS was infused into the dorsal hippocampus (AP, 2.0 mm; M, -2.2 mm; DV. -1.5 mm) unilaterally during a 3 minute period. The syringe needle was kept in the place for an additional 2 min after the injection was completed. One week after KA infusion mice were euthanized by anesthesia overdose and their brains were fixed by transcardial perfusion of PBS followed by 4% paraformaldehyde in PBS. Coronal sections containing the hippocampus (30 μm thickness) were collected and processed for histology and immunostaining.

Rotarod testing. Motor performance was evaluated using the ENV-577M Rotarod system (Med Associates, Georgia, VT) for mice before and after 7 consecutive days of 3-NPA intraperitoneally injections (30 mg/kg/day) . The time spent on the rotarod and the number of falls from the rotarod were measured for each mouse during 300 second trials (the speed of rod rotation was increased progressively from 3 to 30 rpm during the 300 second period). Mice were placed on the rotarod for three, 5 minute trials with 15 minutes rest between trials, and time until the first fall was recorded.

Histology and immunohistochemistry. Mice were anesthetized and perfused transcardially with PBS, followed by 4% paraformaldehyde in PBS, pH 7.4. Brains were postfixed for 2 d and then transferred to a solution of 30% sucrose in PBS for cryopreservation. After 3–5 d in cryopreservation solution, cryostat sections were cut in the coronal plane at a thickness of 30 μm. For 3-NPA model of HD, coronal sections containing the striatum were collected, and for the kainic acid-induced seizure model of temporal lobe epilepsy, coronal sections containing the hippocampus were collected on Superfrost Plus slides (VWR International) respectively. For NeuN immunostaining, brain sections were permeabilized and pre-incubated with blocking solution (0.2% Triton X-100, 10% normal goat serum) in PBS for 30 min and then incubated overnight with primary antibodies (Chemicon) diluted in the same blocking solution at 4°C. For NeuN only staining, cells were washed with PBS and incubated with FITC goat anti rabbit IgG secondary antibodies diluted in the same blocking solution for 2 h at room temperature. sections or coverslips were counterstained with propidium iodide (PI) (0.02% PI and 1% RNase in PBS) for 10 min; they were then washed with PBS and mounted on microscope slides by using anti-fade medium (Vector Laboratories). For double staining of brain tissue sections with SIRT3 and NeuN antibodies, and Tuj1 (Sigma) and GFAP (Chemicon) in cultured cells, the sections or cells were washed with PBS and incubated with FITC goat anti rabbit (for Sirt3) or mouse (for Tuj1) IgG (for Sirt3) and rhodamine goat anti rabbit (for GFAP) or mouse (for NeuN) secondary antibodies diluted in the same blocking solution for 2 h at room temperature.

Imaging and quantification of neuronal damage. All the images were acquired using the confocal with a Olympus Multiphoton Laser Scanning Microscope with 60x (NA, 1.42), 20x (NA, 0.75), 10x (NA, 0.4) or 4x (NA, 0.16) objectives. For the 3-NPA model, two methods were used to evaluate neuronal loss in coronal sections stained with propidium iodide and NeuN

antibody. First, the border of the region of cell damage indicated by loss of NeuN fluorescence were demarcated and the area quantified using Olympus Fluoview (FV10-ASW2.1) software. Second, the numbers of NeuN immunoreactive cells were counted in ten 200 x 200 μm bins along the dorsal – ventral dimension of the striatum. Two sections from each brain, located at the same rostro – caudal level for all mice. For the KA model, undamaged cells (cells in which the propidium iodide was distributed uniformly in the nucleus, compared to damaged cells with condensed nuclear DNA) in hippocampal regions CA1 and CA3 were counted in four coronal sections/brain, two located 60 μm anterior to the injection needle track and the other two 60 μm posterior to the needle track. Counts were made in a 200 μm x 50 μm area of CA1 and of CA3. Images were analyzed and plotted using Olympus Fluoview (FV10-ASW2.1) software, Sigma Plot software (Research Systems) or Graphpad Prism 5. All cell counts were performed by an investigator blinded as to the genotype and treatment history of the mice.

Immunoblot analysis and immunoprecipitation. Cultured cells or tissues were solubilized in SDS sample buffer, and the protein concentration in each sample was determined using a Bio-Rad protein assay kit with bovine serum albumin as the standard. Immunoblot analysis (30 μg of protein per lane) was conducted using 4–10% SDS gradient polyacrylamide gel followed by standard blotting procedure as described previously (Cheng et al., 2012; Hou et al., 2012). The primary antibodies included those that selectively recognize: SIRT3 or acetyl-lysine (1:1000, Cell Signaling Technology), SOD2 (1:1000, Sigma), cyclophilin D (CypD, 1:1000, Thermo Scientific), and actin (1:2000, Sigma). For immunoprecipitation analysis, tissues or cultured neurons were solubilized in lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.2, 1.0% Triton X-100, 1% deoxycholate, and 5 mM EDTA) with proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Equal amounts of protein extracts (500 μg) were pre-cleaned by incubating with 50 μl of protein A/G agarose (Santa Cruz) for 1 hour at 4°C on a rotating shaker and were then centrifuged to precipitate the beads. Supernatants (pre-cleaned protein lysates) were collected and incubated with 1.5 μg of antibodies against SOD2 (Sigma) or cyclophilin D (Thermo Scientific), and 50 μl protein A/G agarose beads for 4 hours at 4°C on a shaker. Equal amounts of supernatants (pre-cleaned protein lysates) were incubated with 1.5 μg normal rabbit IgG and 50 μl protein A/G agarose beads for 4 hours at 4°C on a shaker as a negative control.

Protein A/G beads were pelleted by centrifugation and washed four times with 1 ml of lysis buffer, the final pellets were eluted in protein loading buffer, and samples were vortexed and heated at 70°C for 10 min. Beads were precipitated by centrifugation, and supernatants were collected for immunoblot analysis using an acetyl lysine antibody (Cell Signaling Technology). Images of blots were analyzed using Image J software.

Quantitative reverse transcriptase PCR amplification. Total RNAs from mouse cortical or hippocampal tissues were extracted and purified using a RNeasy mini kit (Qiagen, Valencia, CA). The cDNAs were synthesized using 1 μg total RNA in a 20 μl reaction with a SuperScript III First-Strand kit (Invitrogen, Carlsbad, CA). Real-time PCR analysis was performed with a PTC 200 Pelthier Thermo Cycler and Opticon Monitor 3 software (BioRad). Each reaction included 100 ng cDNA as initial template and was performed in triplicate. PCR was performed under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The comparative C_t method was used to determine the normalized changes of the target gene relative to a calibrator reference. The actin levels were used as the internal control. The primers used in this study were as follows: *actin* (Forward: 5'-TAA AAC GCA GCT CAG TAA CAG TCC C-3' and Reverse: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3') and *Sirt3* (Forward: 5'-CCT GCA AGG TTC CTA CTC CA-3', Reverse: 5'-AGC CTT TCC ACA CCA TGA AC-3').

Cellular ROS measurement. The fluorescent reactive oxygen species (ROS) indicator 5-(and-6)-carboxy-2'7'-dichlorodihydrofluorescein diacetate (DCF) was used for measuring intracellular accumulation of ROS, and the mitochondrial superoxide indicator MitoSox Red was used for measuring mitochondrial superoxide levels. In brief, DCF or MitoSox Red was added to cells at a final concentration of 20 μM or 1 μM, respectively. The cultures were incubated at 37^{o}C for 30 min. After washing with pre-warmed culture medium, confocal images were acquired using a 40X water immersion objective on an Olympus Multiphoton Laser Scanning Microscope with DCF (488 nm excitation and 510 nm emission) and MitoSox Red (514 nm excitation and 580 nm emission). The images were acquired using identical parameters among different cultures. Fluorescence intensity was measured using Olympus Fluoview (FV10-ASW2.1) software.

Neuronal survival assay. After treatment for designated time periods, primary cultured neurons were stained with DNA-binding dye Hoechst 33342 by directly adding to the culture medium for 30 min to 1 hour. Each well (or plate) was observed and images of 3 or 4 randomly selected fields were acquired using a digital camera connected to Zeiss Fluorescence Microscope. The phase contrast and Hoechst staining images are simultaneously acquired using a 20X objective under the fluorescence microscope with the white light and UV light channels both on. The number of dead neurons with bright and condensed blue nuclei, and alive neurons with intact neuronal morphology with diffuse nuclear staining, were counted using NIH Image J software.

Mitochondrial isolation and mitochondrial swelling assays. Cortical tissues or cultured neurons were homogenized in mitochondria isolation buffer (250 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.3) and centrifuged at 850 g for 5 min at 4ºC. The pellet was discarded and the supernatant was centrifuged again at 13,500 g for 10 min. The pellet was resuspended in isolation buffer and centrifuged again at 13,500 g for 10 min. This step was repeated once more and the final pellet was re-suspended in isolation buffer without EGTA. Protein concentration was determined using a Bio-Rad protein assay kit. For mitochondrial swelling assays, isolated mitochondria at a concentration of 1 mg protein/ml were suspended in PTP buffer (200 mM sucrose, 10 mM HEPES, 5 mM KH_2PO_4, 10 μM EGTA, pH 7.3) supplemented with 10 mM succinate and 1.5 mM rotenone. The mitochondrial swelling was triggered by the addition of $CaCl_2$ (final concentration of 800 μM) to isolated mitochondria in the presence or absence of 1 μM cyclosporin A. Mitochondrial swelling was monitored by the decrease in light-scattering at 540 nm in a Perkin Elmer spectrophotometer (HTS 7000 plus) for 20 minutes.

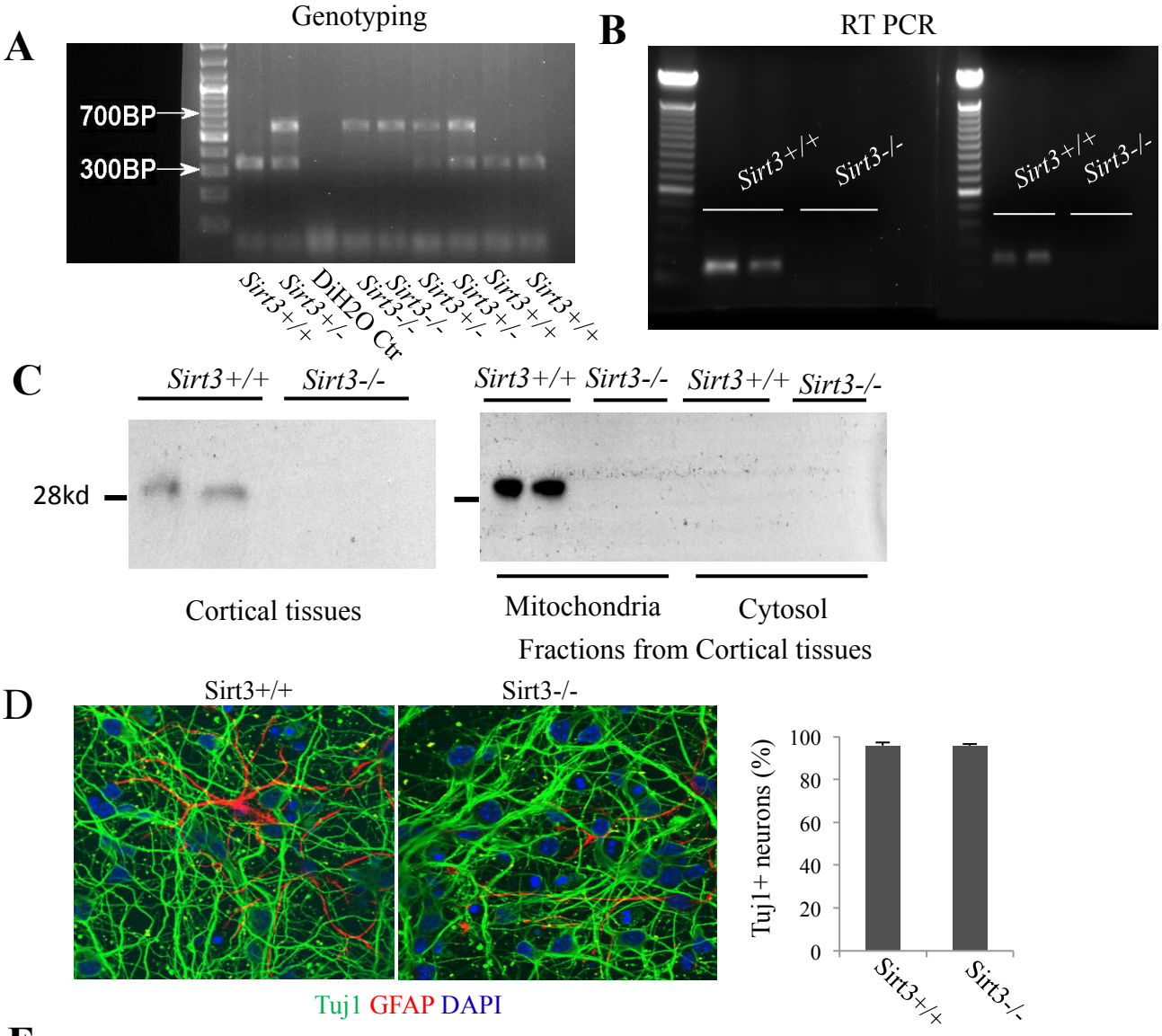
Determination of ATP levels. Fresh cortical and hippocampal tissues (from 10 month old mice) or primary cultured neurons were lysed in RIPA buffer and homogenized by passing through a 25 gauge syringe needle multiple times. The samples were then centrifuged at 10000 Rfc (g) for 10 min at 4^{o}C and the supernatants were processed for ATP measurements using bioluminescence detection kit for ATP measurement (Promega). In brief, 50 μl (tissues) or 30 μl (cells) of supernatant was transferred into a well of a black 96-well plate, and 150 μl reaction buffer (luciferin/luciferase/DTT buffer mix) was added to each well. The luminescence was

determined as relative luminescent units using a HTS 7000 plate reader (Perkin Elmer, Waltham, MA). Background levels were determined by analysis of RIPA from cell-free wells and were subtracted from the raw data. Experiments were performed in duplicate per sample. The protein concentrations from each sample determined were used to normalize the ATP level.

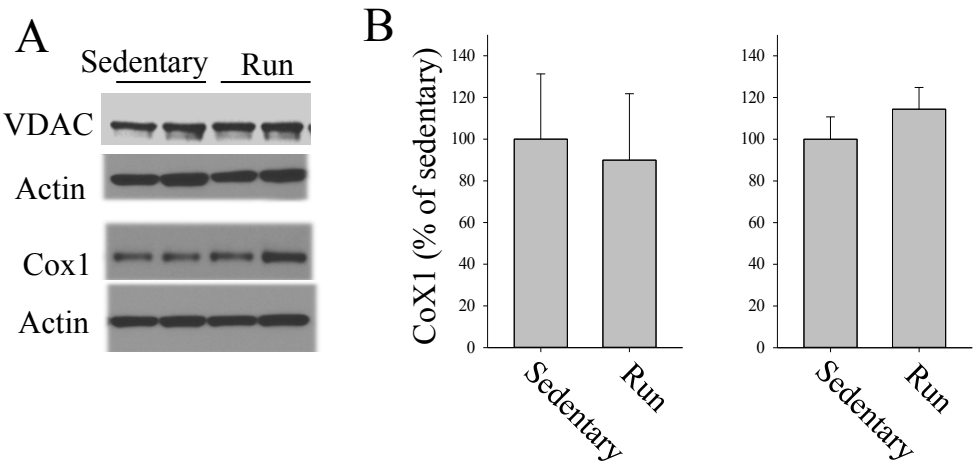
Adeno-associated virus (AAV) construction and packaging. pcDNA3.1-mSirt3 vector was purchased from Addgene. The PCR products of mSirt3 cDNA were ligated with PCR Blunt II TOPO vector and subcloned into AAV-IRES-GFP vector by EcoRI. For packaging of AAV, AAV-IRES-GFP or AAV-IRES- mSirt3-GFP were co-transfected with p-AAV-DJ vector and pHelper vector (Cell Biolab) into HEK293 cells (newly seeded and grown to approximately 60% confluency) at the molar ratio of 1:1:1 using a CalPhos™ Mammalian Transfection Kit (Clontech). 48 -72 hours after transfection, cells were collected and pelleted by centrifugation and high titer stocks of recombinant AAV viruses were purified using iodixanol gradient solutions and ultracentrifuged at 366585 Rcf (g) for 1 hour. The virus collected from the iodixanol gradient was concentrated and purified in sterile PBS using centrifugal filter units (Amicon Ultra). The concentrated virus was transduced into primary cultured neurons at a series of dilutions to observe GFP expression under fluroscence microscopy to estimate the best infection dilution ranges.

Calcium Imaging. Fluo-8 AM was used to measure the cytosolic Ca^{2+} concentration, and Rhod-2 AM was used to measure the mitochondrial Ca^{2+} concentration according to the manufacturer's instructions. Briefly, primary cultured neurons were incubated in the presence of Fluo-8 AM (2 μM) or Rhod-2 AM (2 μM) at 37 °C for 15–30 min. After washing with Locke's solution (in mM: NaCl 154, KCl 5.6, $CaCl_2$ 2.3, $MgCl^2$ 1.0, $NaHCO_3$ 3.6, HEPES 5, glucose 20), Fluo-8 and Rhod-2 fluorescence were measured in neuronal cell bodies. Confocal imaging used an Olympus Multiphoton Laser Scanning Microscope with a 60X, 1.3NA water immersion objective and a sampling rate of 1.5 s / frame with excitation at 488 nm and emission at BP505-540 nm for Fluo-8 AM and excitation at 559 nm and emission at >575 nm . Imaging experiments were performed at room temperature (24–26°C). Digital image processing used Olympus Fluoview (FV10-ASW2.1) software and Sigma Plot software (Research Systems), and user-designed programs.

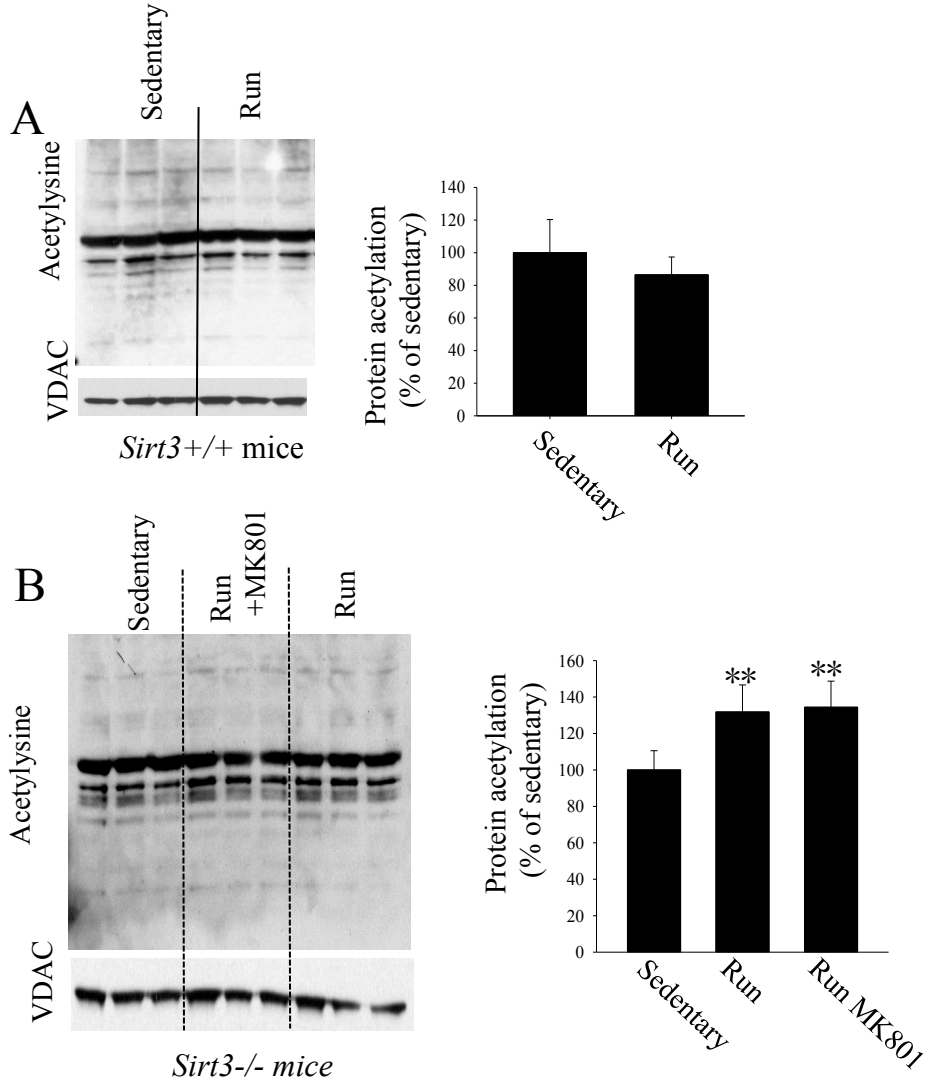
Statistical analyses. Statistical analyses were performed by one-way ANOVA or T-test using GraphPad Prism (GraphPad, San Diego, CA). Student Newman-Keuls post hoc tests were applied to detect statistical differences between groups. Data are expressed as mean ± SEM.

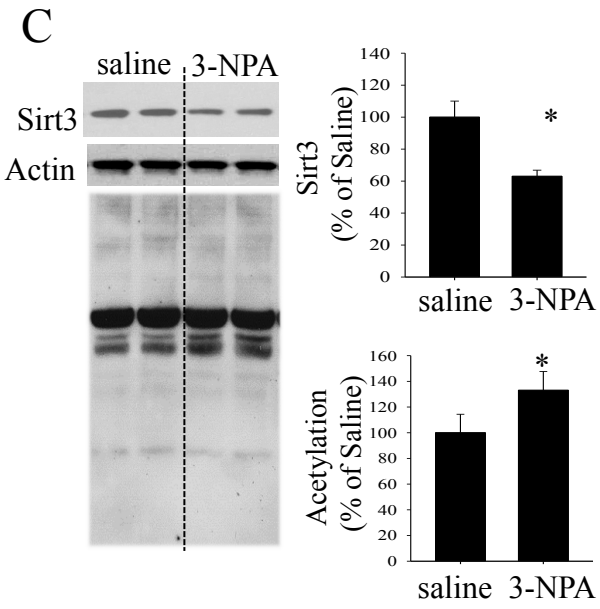


Supplemental Figure 1. Characterization of SIRT3 expression and subcellular localization in cortical neurons of wild type and *Sirt3*-/- mice (this Figure is related to Figure 1 in the manuscript). (**A**) Two complementary PCRs were performed on the genomic DNA from mouse tail biopsies, detecting WT (336 bp) and mutant alleles (Neo Marker) (556 bp). (**B**) Rt PCR using two sets of specific primers targeting *Sirt3* cDNA, confirming the lack of *Sirt3* mRNA in *Sirt3-/-* mice brains. (**C**) Immunoblots using an antibody against SIRT3 detected 28 kDa band which is absent in *Sirt3-/-* brains. Moreover, SIRT3 is present only in the mitochondrial fraction and not in the cytosolic fraction. (**D**) Representative confocal images of Tuj1 (green) and GFAP (red) immunostaining which are counterstained by DAPI (blue) in cultured *Sirt3+/+* and *Sirt3-/-* neurons. The right panel is a plot of the percentage of neurons (Tuj1$^+$ cells) quantified. Values are mean ± SEM (n = 3). (**E**) SIRT3 immunoreactivity in cultured cortical neurons demonstrates its mitochondrial location in the cell body and neurites. Bar = 10μm.

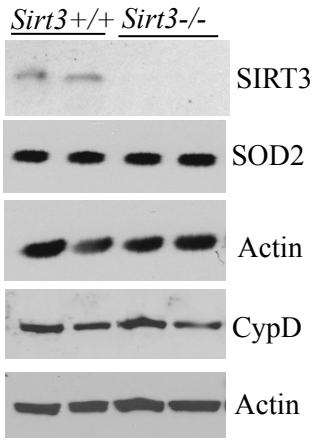


Supplemental Figure 2. Running does not alter levels of mitochondrial marker proteins (this Figure is related to Figure 5 in the manuscript). (A) Immunoblot analysis of voltage-dependent anion channel (VDAC) and cytochrome c oxidase subunit (COXI) from hippocampal tissue lysates of sedentary and running wild type mice. (**B**) Protein levels (densitometry analysis) were normalized to actin protein and expressed as percentage of sedentary mice.. Values are mean ± SEM (n= 4 mice).

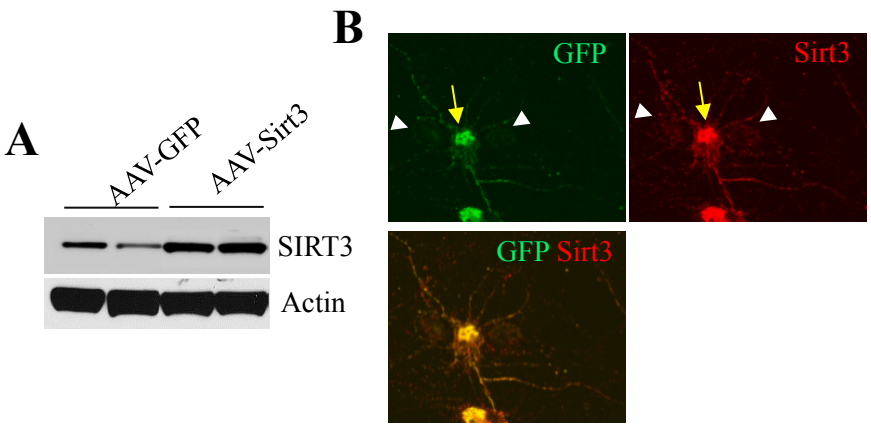




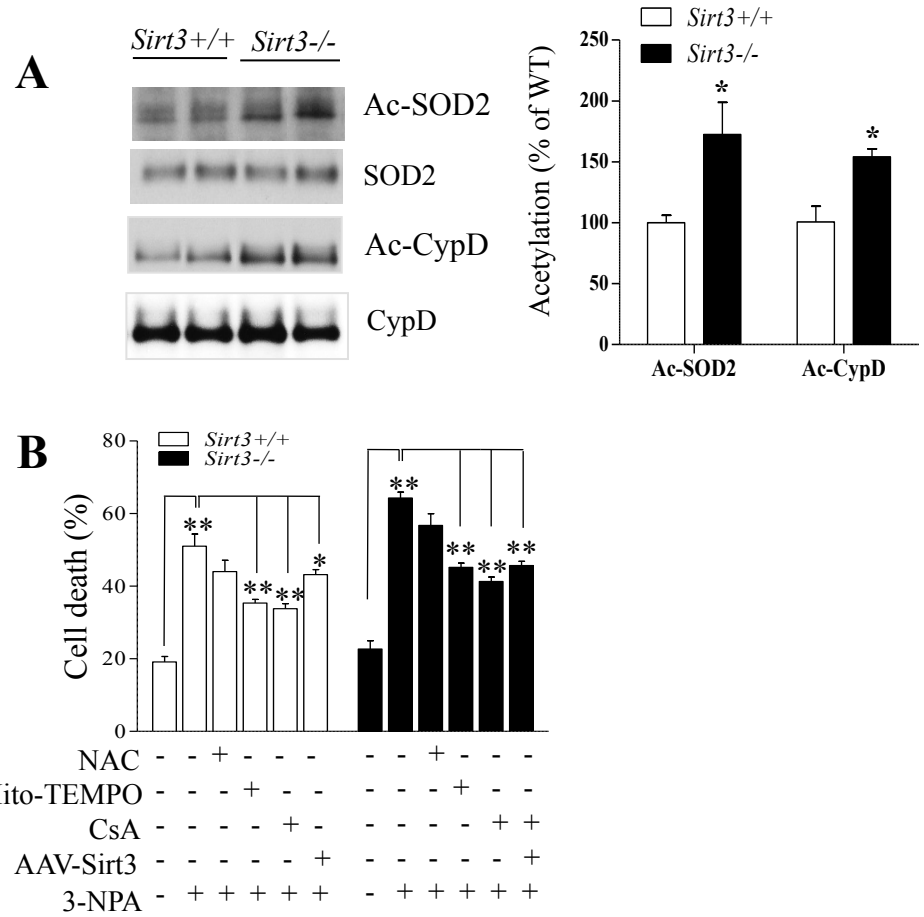
Supplemental Figure 3. Running significantly increases protein acetylation in mitochondrial fraction of cortical tissues of *Sirt3-/-* mice, but not in *Sirt3*+/+ mice (this Figure is related to Figure 6 in the manuscript). (**A**) Immunoblot analysis of protein acetylation in mitochondrial fractions of cortical tissue from *Sirt3*+/+ sedentary and runner mice (7days running). (**B**) Immunoblot analysis of protein acetylation in mitochondrial fractions of cortical tissues from *Sirt3-/-*sedentary, runner mice (7 days running) and runner mice with MK801 administrations. Actin was used as a loading control. The densitometry analysis for total protein acetylation levels was done the same way as shown in Figure 7. Total protein acetylation levels were normalized to actin protein and expressed as percentage of the value for either *Sirt3*+/+ (A) or *Sirt3-/-* (B) sedentary mice. Values are mean ± SEM (n= 4-6 mice). **p <0.01 (ANOVA with Student Newman–Keuls post-hoc tests)**.** (**C**) Immunoblot of SIRT3 and acetyllysine in hippocampal tissue samples from Sirt3+/+ mice 24 hours after intraperitoneal administration of either saline or 3-NPA (50 mg/kg body weight). Actin was used as a loading control. The total protein acetylation levels were quantified as described in Methods. The SIRT3 protein and total acetylated protein levels (densitometry analysis) (right panel) are normalized to actin protein levels and expressed as percentage of the value for *Sirt3*+/+ mice. Values are mean ± SEM (n = 4 mice). *p < 0.05 (Student's t-test).



Supplemental Figure 4 (this Figure is related to Figure 6 in the manuscript). SIRT3 deficiency does not affect the expression of SOD2 and CypD. Immunoblot analysis of SIRT3, SOD2 and cyclophilin D (CypD) in cerebral cortex tissue from 10 month-old *Sirt3*+/+ and *Sirt3*-/- mice. Actin was used as a loading control**.**



Supplemental Figure 5. Overexpression of SIRT3 and immunostaining of SIRT3 in primary cultured neurons after AAV-GFP or AAV-Sirt3-IRES-GFP infections (this Figure is related to Figure 7 in the manuscript). (A) Immunoblot analysis of SIRT3 protein levels in wild type cortical neurons 72 h after infection with AAV-GFP or AAV-Sirt3-IRES-GFP. (B) Images show examples of microsocope fields with one infected neuron (GFP+, arrow) and two uninfected neurons (GFP-, arrowhead) within one field showing SIRT3 immunoreactivity in wild type cortical neurons 72 h after infection with AAV-Sirt3-IRES-GFP. GFP+ neurons showed much stronger Sirt3 immunofluorescence (red) compared with uninfected neurons. The staining pattern is consistent with mitochondrial localization of the overexpressed SIRT3 protein.



Supplemental Figure 6. Inhibition of PTP and a mitochondria-targeted superoxide scavenger ameliorate the vulnerability of *Sirt3-/-* cortical neurons to 3-NPA (this Figure is related to Figure 7 in the manuscript). **(A)** Proteins in lysates of primary cultured cortical neurons from *Sirt3*+/+ and *Sirt3-/-* mice (8 days in culture) were immunoprecipitated with SOD2 or CypD antibodies, respectively, and subjected to immunoblotting with antibodies to acetyl-lysine (Ac-SOD2 and Ac-CypD). The blots were reprobed with SOD2 and CypD antibodies to control for protein loading. Protein levels (densitometry analysis) (right panel) are expressed as percentage of the value for *Sirt3*+/+ mice. Values are mean ± SEM (n = 6 mice). *p < 0.05, **p <0.01 (Student's t-test). (**B**) *Sirt3*+/+ and *Sirt3-/-* cortical neurons were pretreated with the indicated agents and then exposed to 10 mM 3-NPA (E) for 24 hours. Cell death was quantified as described in the Figure 1 legend. NAC, N-acetyl-L-cysteine (1 mM); mitoTEMPO, a mitochondrial superoxide scavenger (1 μM); CsA, the mitochondrial PTP inhibitor cyclosporin A (1 μM). Analysis of cell death was also performed in cultures of *Sirt3*+/+ and *Sirt3-/-* cortical neurons that had been infected with AAV-GFP or AAV-Sirt3-IRES-GFP) for 3 days and then exposed to 10 mM 3-NPA. Values are mean ± SEM (separate cultures from 4 or 5 mice; 6 images per culture were analyzed). *p<0.05 and **P<0.01 (ANOVA with Student Newman–Keuls post-hoc tests).