Supporting Information

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SI Materials and Methods

Mice. The mutant mouse strain harboring a conditional cAMP responsive-element binding (CREB)-1 null allele (Exon 10) flanked by LoxP recombination sites (CREBflox transgenic mice) and the transgenic strain expressing the Cre recombinase under the control of the CAMK2A promoter (CaMK2a-iCre BAC), both originally developed by Schütz and colleagues (1, 2), were obtained from the European Mouse Mutant Archive (EMMA ID:02151 and EMMA ID: EM01153, respectively), Monterotondo, Italy. Mice were bred and maintained in the animal facility of the Catholic University Medical School. Animals were housed in a 12-h light/12-h dark cycle at controlled temperature (25 °C ± 1 °C). Genotyping was performed by PCR of genomic DNA from ear punches, according to above referenced protocols. In experimental procedures, we compared mice homozygous for *floxed* CREB (CREB $^{loxP/loxP}$) and heterozygous for CamK-*Cre* [Brain CREB KO (BCKO) mice] with CREB $^{loxP/loxP}$ littermates not expressing the recombinase (control mice). All mice were in the C57BL/6 genetic background and were housed individually.

All experimental procedures were performed according to international standards of Animal Care and had been previously approved by the "Comitato Etico" (Ethical Committee) of Universita' Cattolica.

Frozen brains from Sirt1 KO mice and their littermates (3) were shipped from M.W.M.'s laboratory to Catholic University in dry ice, and were immediately put in liquid nitrogen upon arrival and stored until processing for protein and RNA extraction.

Animal Studies. ***Calorie restriction.*** For calorie restriction (CR) studies, the amount of food consumed by ad libitum (AL) mice was determined weekly, and CR mice were fed daily 80% of that value for the first week and 60% for the following 4 wk. Absolute food consumption AL was slightly (about 10%) higher in control than in BCKO mice, but the amount of chow per gram body weight comparable, because of the smaller size of the latter strain. CR in BCKO mice was calculated either on the AL feeding of mice of the same genotype, or on the AL consumption of control mice, with similar results. Body weight was monitored weekly.

Behavioral tests. All behavioral tests were conducted on male, 6 mo-old mice, during the dark cycle (the animals' active phase). Before each test, mice were acclimatized to the experimental room for at least 30 min. The animals were subjected to several basal tests of locomotion, exploration, and anxiety to exclude differences that could influence behavioral tests. The tests were performed from least to more stressful with a pause of at least 48 h between individual tests.

Novel object recognition task. During training sessions, two novel objects were placed into an open-field box 14 inches away from each other (symmetrically) and then the individual animal was allowed to explore for 10 min. An explorative behavior was scored when the head of animal was facing close (less than 1 inch away) to object or any part of the body except the tail was touching the object. The time spent to explore each object was recorded. The animals were returned to their home cages immediately after training. During the retention test, one of the familiar objects used during training was replaced by a novel object, and the animals were allowed to explore freely for 10 min. All objects were balanced in terms of physical complexity and were emotionally neutral. Moreover, the open-field and objects were thoroughly cleaned by 70% alcohol after each session to avoid possible odorant cues. A preference index, a ratio of the amount of time spent exploring any one of the two items (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure recognition memory. Two-way ANOVA (genotype X feeding regimen) and post hoc Tukey test were used to determine genotype and diet effects on the behavioral responses.

Resident-intruder paradigm. This test was performed according to Nelson et al. (4) with minor changes. The resident mice were housed individually 4 wk before the experiments, and the intruder mice were housed in groups of four. Adult, male stimulus mice (i.e., intruders) were introduced into the home cage of either a BCKO or a control adult male mouse. Intruder mice were marked on the tail with an indelible marker for purposes of identification. The bedding in the home cages remained unchanged for 10 d before testing. The latency to first aggressive encounter and the total number of aggressive encounters (bite attacks) initiated by the resident male were recorded. Aggression tests lasted 10 min and were conducted each day for 3 consecutive days at the same hour (normally 1500–1700 hours). Aggressive behaviors scored included chasing, biting, and offensive attacks. A novel pairing of animals was made for each consecutive test and intruder males were not used more than once per day. Raw or tied-ranked values were analyzed by two-way ANOVA test (genotype per feeding regimen), followed by Tukey post hoc test.

Long-term potentiation. Coronal hippocampal slices (400-μm thick) were prepared according to standard procedures (5) from three mice per experimental group. Slices were cut with a vibratome (VT1000S; Leica Microsystems) and incubated in the cutting solution [124 mM NaCl, 3.2 mM KCl, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 2 mM Na-pyruvate, and 0.6 mM ascorbic acid (pH 7.4, 95% O_2/5% CO_2)] at 30–32 °C for at least 60 min, and then stored in the same solution at room temperature until use. For electrophysiological recordings, slices were continuously perfused with artificial cerebrospinal fluid (aCSF) [124 mM NaCl, 3.2 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (pH 7.4, 95% O_2/5% CO_2)] in a submerged recording chamber. Flow rate was kept at 1.5 mL/min with a peristaltic pump (Minipuls 3; Gilson), and bath temperature was maintained at 30–32 °C with an in-line solution heater and temperature controller (TC-344B; Warner Instruments).

Field excitatory postsynaptic potentials (fEPSP) evoked by Schaffer collateral stimulation were recorded from the CA1 subfield of the hippocampus. For this purpose, a glass capillary microelectrode filled with aCSF (tip resistance 2–5 MΩ) and a stimulating bipolar tungsten electrode were positioned into the stratum radiatum of CA1. Hippocampal subfields and electrode positions were identified with the aid of 4× and 40× water immersion objectives on an upright microscope equipped with differential interface contrast optics under infrared illumination (BX5IWI; Olympus) and video observation (C3077-71 CCD camera; Hamamatsu Photonics). Data acquisition and stimulation protocols were performed with the Digidata 1440 Series interface and pClamp 10 software (Molecular Devices). Data were filtered at 1 kHz, digitized at 10 kHz, and analyzed both online and offline. Input/output curves were built by plotting the amplitudes of fEPSPs versus stimulus intensity. The stimulation intensity that elicited one-third of the maximal response was used for delivering test pulses and tetanus.

After 20–30 min of stable baseline responses to test stimulations delivered once every 20 s, long-term potentiation (LTP) was induced with a standard stimulation paradigm referred to as "teta-

nus" (four trains, 500 ms each, 100 Hz within the train, repeated every 20 s).

Responses to test pulses were then recorded every 20 s for 60 min to measure LTP. The magnitude of LTP was measured 60 min after tetanus and expressed as a percentage of baseline fEPSP peak amplitude. The mean values observed during the last 10 min of pretetanus recordings were considered to represent 100%. Reported fEPSP amplitudes at 60 min are averages from recordings obtained during the last 10 min of posttetanus recordings.

Immunohistochemistry. Anesthetized mice were transcardially perfused with Ringer's solution followed by 4% paraformaldehyde–lysine–periodate fixative solution. The brain was removed from the skull, postfixed overnight at 4 °C, and then transferred to a solution of 30% sucrose in PBS for 2 d. Sagittal or coronal brain sections (35-μm thick) were then cut with a vibratome (VT1000S; Leica Microsystems), and floated in ice-cold PBS. Sections were collected and stored until use in cryoprotectant at −20 °C.

For assessment of CREB expression, sections were blocked for 1 h at room temperature in 1% BSA solution containing 10% normal goat serum and 0.5% Triton X-100 (Sigma). The sections were then incubated for 48 h at 4 °C with the primary antibodies (rabbit monoclonal anti-CREB, 1:400; Cell Signaling Technology), washed several times in PBS, and incubated with secondary antibody (Alexa-488 donkey anti-rabbit IgG, 1:300; Invitrogen). Finally, brain slices were incubated with DAPI (0.5 μg/mL; Invitrogen) to stain cell nuclei, and the sections were mounted on glass slides and cover-slipped with ProLong Gold antifade reagent (Invitrogen).

Images were obtained with a fluorescence microscope (BX5I; Olympus) equipped with a professional digital compact camera (*CAMEDIA,* C5050 ZOOM; Olympus).

Chromatin Immunoprecipitation. ChIP assays were performed largely as described (6, 7). For all cell types, ~1 to 3×10^6 cells were used per ChIP. Briefly, at the end of stimulations formaldehyde (1%) was added directly to the medium for 10 min; afterward, medium was removed from treated cells and replaced with PBS containing protease inhibitors. Cells were rinsed twice and harvested in PBS, collected by centrifugation, and pellets were resuspended in 200 μL lysis buffer containing SDS (1%), Tris-HCl (pH 8.1, 50 mM), and EDTA (10 mM). Samples were sonicated on ice with eight 10-s pulses with a 10-s interpulse interval.

Cell debris was removed by centrifugation, and supernatants were precleared by incubation with protein-G Sepharose 4B beads (Sigma-Aldrich) for 1 h at 4 °C. Beads were collected by centrifugation and supernatants were subjected to immunoprecipitation. A fraction of the supernatant was used for total input control. The volume of each tube was adjusted to 2 mL with dilution buffer (0,01% SDS, 1% Triton X-100, 1,2 mM EDTA, 16,7 mM Tris HCl pH 8.1, 167 mM NaCl) and split in equal volumes for each immunoprecipitation; 2–4 μg of specific antibody or rabbit IgG was added overnight at 4 °C. Immune complexes were collected by incubation with protein-G Sepharose 4B beads for 2 h at 4 °C. Beads were collected and subjected to a series of seven sequential washes. Two washes each were performed in lysis buffer and then in washing buffer containing SDS (0.1%), Triton X-100 (0.5%), EDTA (pH 8.0, 2 mM), Tris-HCl (pH 8.1, 20 mM), and NaCl (150 mM). One wash was performed in lithium buffer containing LiCl (0.25 M), Nonidet P-40 (1%), deoxycholate (1%), EDTA (pH 8.0, 1 mM), and Tris-HCl (pH 8.1, 10 mM). Two final washes were performed in 1× TE (pH 8.1). Immune complexes were eluted from beads by vortexing in elution buffer containing SDS (1%) and NaHCO3 (pH 8.0, 0.1 M). NaCl was added (final concentration 0.33 M), and cross-linking was reversed by incubation overnight at 65 °C. DNA fragments were purified by using the Nucleospin extract II PCR purification kit (Macherey-Nagel). For PCR, specific sets of primers were designed that flank CRE regions within the upstream regulatory regions of the indicated genes. PCR conditions and cycle numbers were determined empirically for the different templates and primer pairs. Primers amplified fragments ranging in size from 200 to 400 bp. Primer sequences and PCR conditions are available on request.

Statistics. Datasets were compared by bifactorial (2 × 2) ANOVA or two-tailed Student *t* test where appropriate, using either raw or tied-ranked values. Threshold for significance was set at $P < 0.05$ unless indicated.

Reagents and Antibodies. Most of the Chemicals were obtained from Sigma-Aldrich. Forskolin (Fsk) was from BIOMOL/Enzo Life Sciences Inc.; NGF was a kind gift of Antonella Riccio (Medical Research Council, London, United Kingdom).

Polyclonal rabbit antibodies against CREB1 (cat#AB3006), p-Ser133-CREB (cat#06–519), anti-Sir2.1 (cat#07–131) and Histone H4 (cat#07–108), and the anti–Acetyl-Lysine mouse monoclonal antibody (cat.#05–515) were from Upstate Biotechnology/Millipore; anti-actin (goat polyclonal, cat #sc-1615 and sc-1616), anti Sirt-1 rabbit polyclonal (sc15404) and myc-tag monoclonal antibody (clone 9E10, sc-40) were from Santa Cruz Biotechnology; anti–Ac-H4K16 (H9164) and anti–Ac-H3K9 (H9286) rabbit anti-sera were from Sigma. Anti-Histone H3 mouse monoclonal antibody was from Abcam (ab10799).

The Ultrasensitive (Mouse) Insulin Elisa Kit (cat. # 80-INSMSU-E01) was purchased from Alpco Immunoassays.

The transfection reagent Lipofectamine 2000 was obtained from Invitrogen.

Cell Lines, Viral Vectors, and Plasmids. PC12 rat pheochromocytoma cells were obtained from ATCC. Cells were maintained in RPMI medium 1640 containing 10% (vol/vol) FCS and 5% (vol/vol) horse serum.

A ~450-bp genomic region surrounding two half-CREs located 1,998 and 2,012 bp downstream of the transcription start site in the mouse *Sirt1* gene was amplified by PCR with primers harboring KpnI (forward) and XhoI (reverse) restriction sites, and cloned in the MCS of the pGL3 luciferase reporter vector (Promega). The construct was verified by DNA sequencing.

Replication-deficient (ΔE1/ΔE3) human adenoviral vector type 5-expressing Cre recombinase under the transcriptional control of the cytomegalovirus promoter was constructed by homologous recombination in *Escherichia coli* using the Ad-Easy System (8). Full-length cDNA coding for the P1 bacteriophage Cre recombinase was derived from the pBS185 plasmid (9) and inserted into the pShuttle-CMV plasmid. Propagation in human embryonic kidney 293T cells and purification of the adenoviral vectors by cesium chloride gradient ultracentrifugation were performed as described previously (10). Viral titers were determined by serial dilution on 293 cells and were expressed as plaque-forming units per milliliter (PFU/mL). Adenoviral stocks were tested for the absence of replication-competent adenoviruses by a A549 cells assay (10).

A third-generation self-inactivating lentiviral vector (LV) expressing the human Sirt-1 was generated by cloning the full-length cDNA for Sirt-1 derived from the pYESir2-Puro plasmid (11) in replacement to the E-GFP cDNA into the pCCLsin. cPPT.hPGK.E-GFP.Wpre (phosphoglycerate kinase promoter–enhanced jellyfish GFP) construct, obtained from E. Vigna (Istituto Di Ricovero e Cura a Carattere Scientifico, Candiolo, Italy). Recombinant vesicular stomatitis virus-pseudotyped LVs were obtained as previously described (12).

The pYESir2-Puro plasmid encoding the human Sirt-1 cDNA in the pBabe-Y-Puro vector backbone was kindly provided by Michael Greenberg (Harvard Medical School, Boston, MA). Human CREB1 and CREB1-ΔLZ cDNAs inserted (EcoRI-

BglII) in the pCMV-Myc vector (Clontech Laboratories) were a gift of David Ginty (The Johns Hopkins University, Baltimore, MD). The pFC-PKA construct encoding the catalytic subunit of the c-AMP dependent protein kinase under the transcriptional control of the CMV promoter was obtained from STRATAGENE/Agilent Technologies.

Transfection of plasmids and siRNA duplexes into undifferentiated PC12 rat pheochromocytoma cells was performed using lipofectamine 2000, according to the manufacturer's recommendations.

Studies in Vitro. ***Culture and infection of primary neurons.*** Primary cultures of cortical and hippocampal neurons were obtained from E18 lox/lox mice embryos according to standard procedures. Briefly, for cortical neurons, cortices were dissected and incubated for 10 min at 37 °C in PBS containing 0.025% trypsin/0.01% EDTA (Biochrom AG). The tissue was then mechanically dissociated at room temperature (23–25 °C) using a fire-polished Pasteur pipette and the cell suspension was harvested and centrifuged at 100 × *g* for 8 min. The pellet was suspended in 88.8% (vol/vol) minimum essential medium (Biochrom), 5% FBS, 5% (vol/vol) horse serum, 1% glutamine (2 mM), 0.2% gentamicin (0.1 mg/mL) and glucose (25 mM). Cells were seeded on 6-, 12-, and 24-well plates precoated with poly-L-lysine (0.1 mg/mL; Sigma) at a density of 1×10^6, 5×10^5, 2.5×10^5 cells per well, respectively. At 24 h after plating, the culture medium was replaced with a medium containing 97.3% (vol/vol) neurobasal medium (Invitrogen), 2% (vol/vol) B-27 (Invitrogen), 0.5% glutamine (2 mM), and 0.2% gentamicin (0.1 mg/mL). After 72 h, the culture medium was replaced with a similar medium lacking glutamine and supplemented with 2 μM cytosine β-D-arabinofuranoside to inhibit glial cell proliferation. For hippocampal primary cultures, the hippocampi were dissected in ice cold HBSS and incubated with papain solution for 1 h at 37 °C with gentle shaking. Tissues were mechanically dissociated by gentle trituration and were plated at the same density described for cortical neurons.

The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 until experimental procedures.

For primary culture transduction neurons [days in vitro (DIV) 5] were infected with 10 PFU/cell of each adenovirus or lentivirus vector: half of the medium was replaced with fresh medium after 6 h and again after 48 h. After 6 d from infection the neurons (DIV11) were stimulated and harvested for mRNA and protein isolation or quantification of cell survival.

Analysis of cell survival. For MTT assay, neurons were seeded in 24-well plates (2.5×10^5/mL) and infected at DIV5 to delete CREB and/or overexpress SIRT1. After 6 d (DIV11) H_2O_2 (100 μM) was added to the medium and on the day of the assay (DIV12, after 24 h from the treatment) solutions were removed and replaced with fresh culture medium. MTT solution (1:10 dilution of the 5-mg/mL stock) was added and incubated at 37 °C for 3 h. The medium and MTT solution were then removed, and the converted dye was solubilized with 500 μL of acidic isopropanol (0.04 M HCl in absolute isopropanol). Absorbance was read at 570 nm. Data are the average of three wells, and the experiment was repeated three times with similar results.

PC12 differentiation. For differentiation into sympathetic neurons, PC12 cells were seeded at 2 to 5×10^4 cells per well in collagen-coated 12-well plates 16 h before transfection with 100 nmols of siCtrl or siSirt-1 RNA duplexes. After 3 d (day 0), medium was replaced and NGF was added at 50 ng/mL immediately and again after 36 h (day+1.5). At day +3 early differentiated cells (i.e., cells with processes longer than one cell body) were counted under the phase-contrast microscope. Percentage of differentiated cells was determined in 10 randomly chosen fields from different wells. Datasets were compared by two-tailed Student's *t*-test.

Analysis of gene expression. For mRNA isolation, retrotranscription and amplification, total RNA was extracted using QIAzol Lysis reagent (Qiagen) according to the the manufacturer's instructions. One to two micrograms of total RNA was reverse-transcribed by extension of oligodT (Fermentas) primers using M-MLV (USB) reverse transcriptase. PCR of the cDNA was performed using Taq Polymerase (Fischer) with the following pairs of primers:

PGC-1 (rat/mouse) Forward: GGAGACGTGACCACTGACA
PGC-1 (rat/mouse) Reverse: TCAATAGTCTTGTTCTCAAATG
SIRT1 (rat/mouse/human) Forward: TTTCATTCCTGTGAAAGTGATG
SIRT1 (rat/mouse/human) Reverse: CAAACTTGAAGAATGGTCTTG
nNOS Forward (rat/mouse): CTGTGACAACTCTCGATACAACATC
nNOS Reverse (rat/mouse): GAGTCTATAGTTGAGCATCTCCTGG
actin Forward (rat/mouse): GTCACCCACACTGTGCCCATCT
actin Reverse (rat/mouse): ACCGAGTACTTGCGCTCAGGA
PEPCK Forward (rat/mouse): GCAGCATGGGGTGTTTGTAGG
PEPCK Reverse (rat/mouse): AACAGCTCCTCCACGTTGACG
CREB Forward (rat/mouse/human): AGCCATCAGTTATTCAGTCTC
CREB Reverse (rat/mouse/human): AGTGCTTTTAGCTCCTCAATC

Sequences of primer sets used for peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) targets displayed in Fig. S2 can be provided by the authors upon request.

The PCR program used was: 2 min at 94 °C, followed by 33 cycles (30 s at 94 °C, 30 s at 56–60 °C, 40 s at 72 °C), followed by 7 min extension at 72 °C. A PCR was also performed on total RNA that had not been reverse-transcribed to control for the absence of genomic DNA in the RNA preparation. The products of the PCR reactions were resolved on a 1% agarose gel.

Luciferase Reporter Assay. Cells (PC12) were cotransfected (ratio 50:50:1) with the Sirt1-luc reporter, the PKA expression vector (or an empty control), and a plasmid encoding the *Renilla* luciferase under the control of the CMV promoter as an internal transfection control. Forty-eight hours later and after the appropriate stimulations, cells were lysed directly in the culture plate with Passive Lysis Buffer, and Firefly/*Renilla* luciferase activity measured with by a Dual Luciferase Assay Kit (Promega) and a portable luminometer (Junior LG 9509; Berthold Technologies), according to the manufacturer's instructions.

Protein Studies. For protein expression/phosphorylation studies cells were lysed in ice-cold lysis buffer (NaCl 150 mM, Tris-Hcl 50 mM pH 8; 2 mM EDTA) containing 1% vol/vol Triton X-100, 0.1% vol/vol SDS, 1:1,000 Protease Inhibitor mixture (Sigma), 1 mM Sodium Orthovanadate, 1 mM NaF, and 2 mM β-glycerophosphate. After 15 min on ice with occasional vortexing, cells were spun down at 22,000 × *g*, 4 °C to remove debris and unlysed cells, and supernatant quantified for protein content (DC Protein Assay; Bio-Rad), resuspended in 6× Laemmli buffer, boiled, and subdued SDS-PAGE.

For immunoprecipitation (or coimmunoprecipitation) studies, cells/tissues were lysed in low detergent (0.2% Nonidet P-40) buffer and lysates were precleared with empty protein G-sepharose 4B beads (Sigma) before being challenged with specific or control antisera and fresh protein G matrix. After 3 h incubation at 4 °C with continuous agitation, protein-G bound immunocomplexes were collected by centrifugation (22,000 × *g*,

30 s) and washed six times in immunoprecipitation buffer. Beads were finally resuspended in 40 μL of 1× Laemmli buffer and boiled. Eluted proteins were subjected to SDS-PAGE and immunoblotting.

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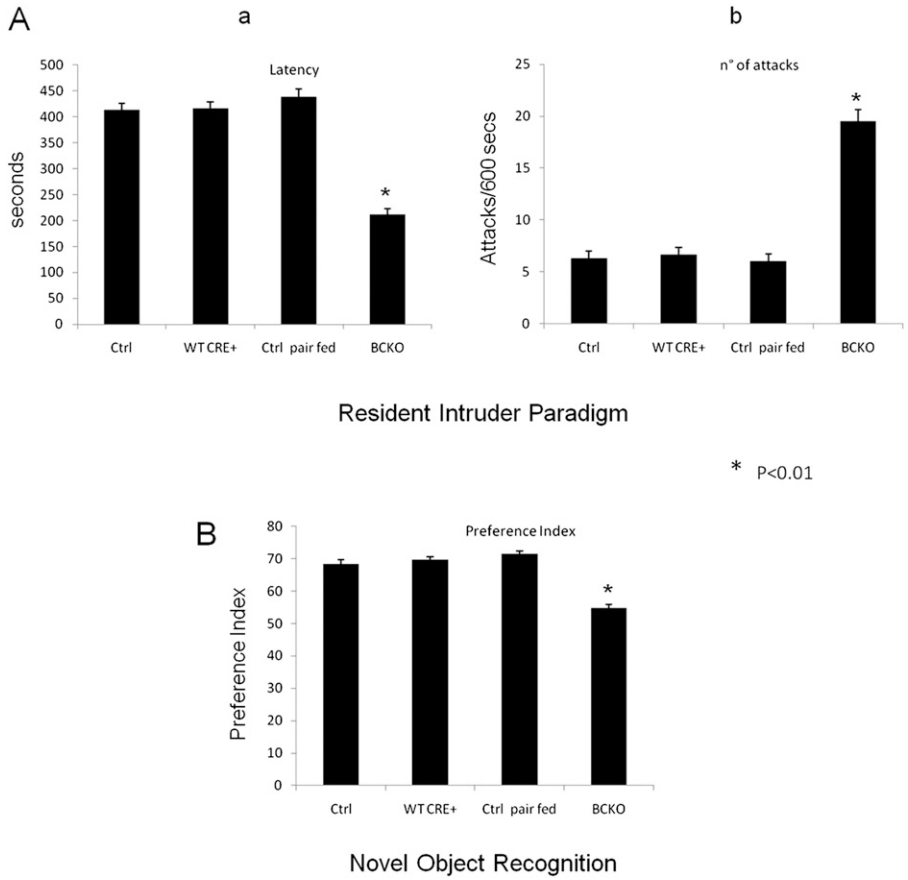

Fig. S1. (*A* and *B*) Behavioral differences between control (Ctrl) and BCKO mice are independent of Cre recombinase expression and AL food intake. The following groups of mice ($n = 6$) were compared: Ctrl and BCKO, as in Fig. 1; Cre+, CREB WT mice expressing the Cre transgene; Ctrl pair fed, Ctrl mice (CREB loxP/loxP Cre-) fed the same amount of chow as BCKO AL. The only significant difference is indicated by the asterisk ($P < 0.01$ by One-way ANOVA).

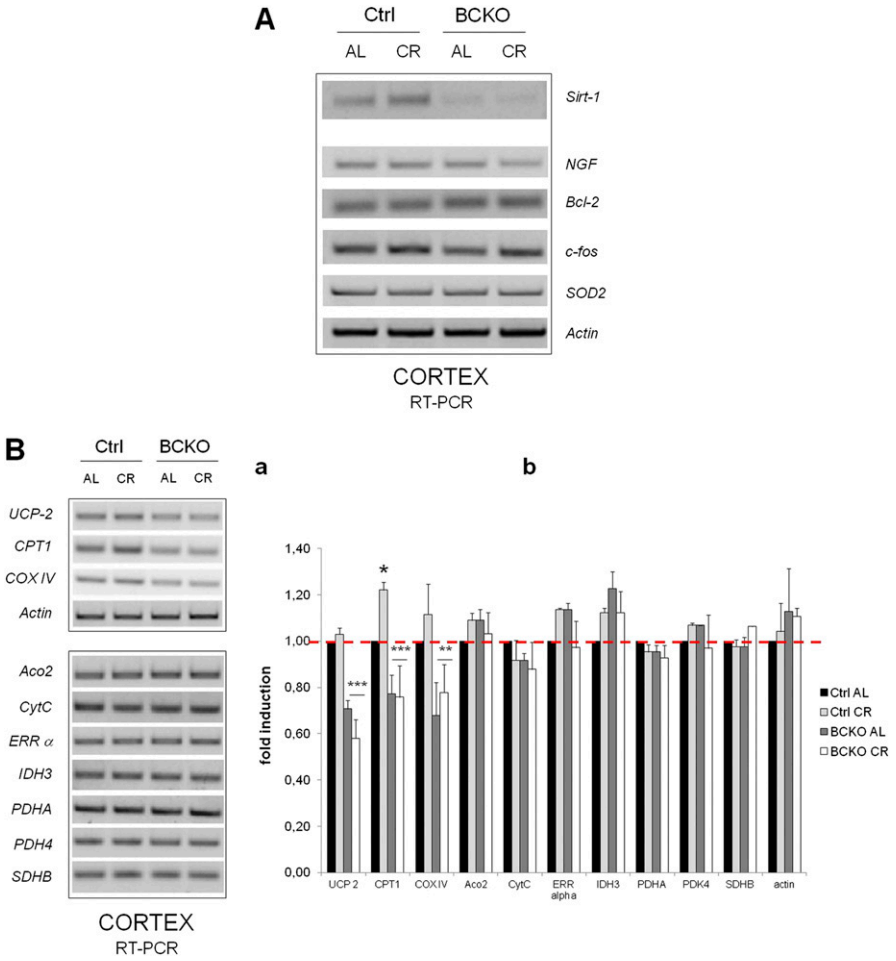

Fig. S2. (*A*) The cortical expression of some canonical CREB target genes is unaffected by CR and CREB deletion. RT-PCR analysis of brain cortex total RNA from AL and CR individual mice of the indicated genotypes. A number of putative CREB target mRNAs were retro-transcribed and amplified with specific primer sets. No relevant changes as a function of mouse genotype or feeding regimen were observed for *NGF*, *Bcl-2*, *c-fos*, and *SOD2*. Actin mRNA was amplified as input control. The expression pattern of *Sirt-1* in the same samples is also reported for comparison; note that the Sirt-1 gel displayed here is different from the one shown in Fig. 2*Ca*. (*B*) Effect of CREB deletion and CR on the expression of a number genes putatively regulated by PGC-1α in mouse cortex. (*a*) Expression of the indicated mRNAs was assessed by semiquantitative RT-PCR in individual mice representative of each genotype and feeding regimen. Transcripts that displayed the most evident changes by BCKO and/or CR were grouped in the *Upper* panel together with actin as input RNA control. The following genes were analyzed: Aconitase 2 (Aco2), Cytochrome oxidase subunit IV (COX IV), Carnitine Palmitoyl Transferase 1 (CPT 1), Cytochrome C (CytC), Isocitrate Dehydrogenase (IDH) 3, Pyruvate Dehydrogenase subunit A (PDHA), Pyruvate Dehydrogenase Kinase (PDK) 4, Succinate Dehydrogenase subunit B (SDHB), Estrogen recetor (ERR α), Uncoupling Protein (UCP) 2, actin. Genes were selected based on ref. 1. (*b*) Histogram displaying fold-changes of the mRNAs from *a* compared with the Ctrl AL group; values are mean± SD of $n = 3$ mice. Significant differences by two-way ANOVA are indicated. *$P < 0.05$ Ctrl AL vs. Ctrl CR; ** and ***: $P < 0.001$ and $P < 0.0001$ BCKO vs. Ctrl ("row effect").

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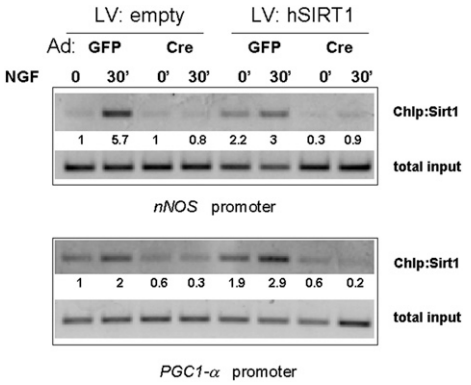

Fig. S3. Overexpression of hSirt-1 in CREB-deficient neurons does not rescue sirtuin interaction with CREB target promoters. CREBloxP/loxP hippocampal cells were infected with the indicated combinations of adenoviral (Ad-GFP or Ad-Cre) and lentiviral (LV-empty or LV-hSirt1) vectors to achieve deletion of CREB, overexpression of Sirt-1 or the two events in combination. Sirt1 binding to the promoter region of neuronal NO Synthase (nNOS) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) was determined by ChIP in resting and NGF-stimulated neurons. Note that basal and NGF-induced interaction of Sirt1 with both promoters is drastically reduced CREB-deleted cells irrespective of Sirt1 overexpression. Numbers are band densitometric values relative to lane 1. Amplification of total chromatin is displayed as input control.

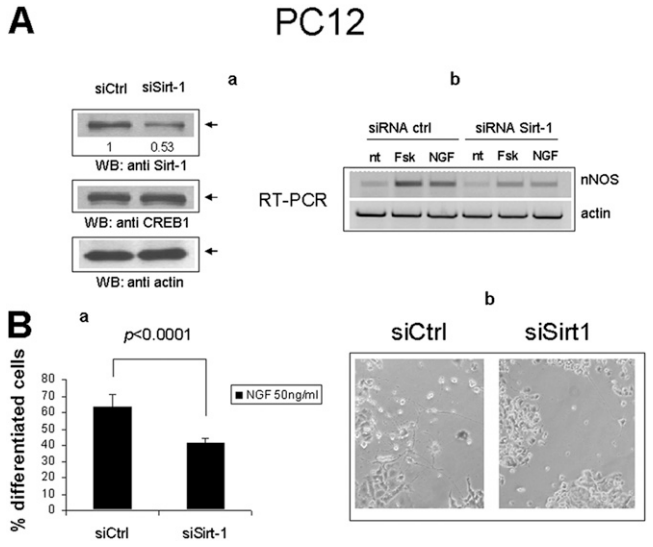

Fig. S4. Sirt1 knock-down inhibits induction of nNOS and differentiation of PC12 cells by NGF. (*Aa*) Western blot analysis of protein extracts from cells treated with Sirt-1 specific (siSirt-1) or irrelevant (siCtrl) RNA duplexes. Bands corresponding to Sirt-1, CREB, and actin are indicated by arrows. Numbers indicate relative optical density of the Sirt-1 bands. (*b*) Semiquantitative RT-PCR analysis of nNOS mRNA in naive PC12 cells 3 d after treatment with irrelevant or Sirt-1 specific siRNA duplexes. CREB-dependent stimuli (Fsk, 10 μM; NGF 50 ng/mL) are indicated. Actin amplification confirms equal input RNA throughout the lanes. (*Ba*) Differentiated cells were counted 72 h after exposure to NGF. Values are the mean percentage of differentiated cells over ten randomly chosen microscopic fields (about 1,000 total cells) ± SD. Datasets were compared by two-tailed Student *t* test. (*b*) Phase-contrast microphotographs of two representative microscopic fields. (Magnification: 100×.) Cells with processes longer than the cell body were scored as differentiated (details in *Materials and Methods*).

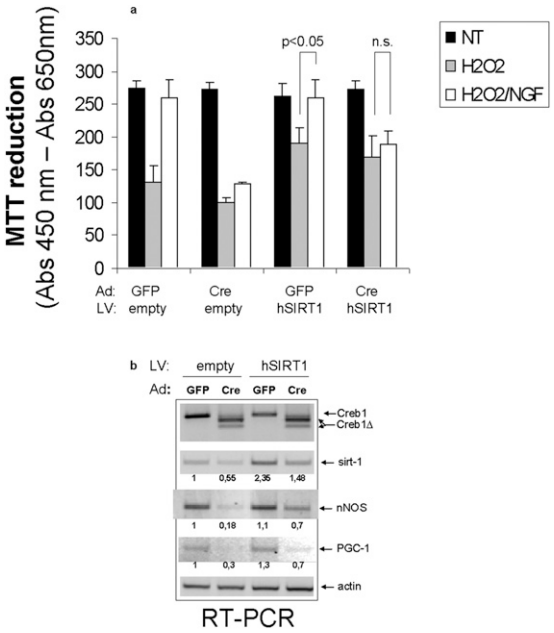

Fig. S5. Overexpression of human Sirt-1 does not rescue cell protection by NGF in CREB-deficient neurons. (*A*) Hippocampal neurons were infected with the same lenti/adenoviral mixtures as in Fig. S3; 3 d after infections cells were challenged with 100 mM hydrogen Peroxide (H_2O_2) in the presence or absence of NGF (50 ng/mL), or left untreated (NT). After 24 h of incubation, viability was determined by the MTT reduction assay. Figure representative of two independent experiments each performed in triplicate. Two relevant comparisons by Student's *t*-test are indicated. (*B*) Semiquantitative RT-PCR analysis of total RNA from NGF-stimulated hippocampal neurons infected as in *A*. Bands corresponding to *CREB1, Sirt-1, nNOS,* and *PGC-1α* are indicated by arrows. Primers for *Sirt-1* match perfectly both human and murine cDNA. Numbers are band intensities relative to the first lane. Picture representative of two independent experiments.

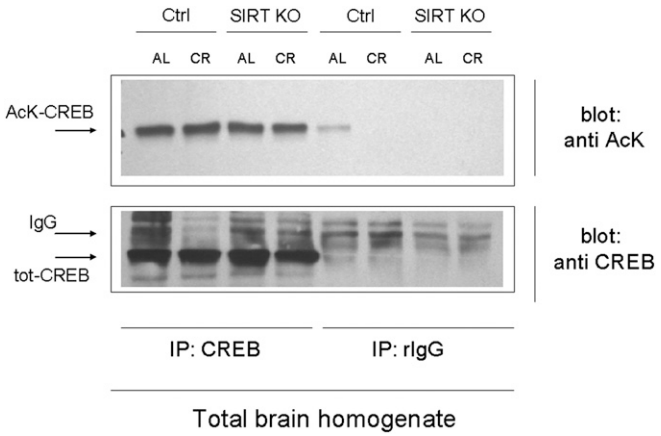

Fig. S6. Sirt-1 deletion and CR do not affect CREB acetylation in whole mouse brains. Brain homogenates from Ctrl and Sirt1 KO mice fed AL or under CR regimen were immunoprecipitated with an anti-CREB antiserum, and immunocomplexes immunostained with a mouse anti Acetyl-Lysine antibody (*Upper*) or with the same rabbit antiserum used for immunoprecipitation. Mock (rabbit IgG) immunoprecipitations were also performed as negative controls. Relevant bands, and an aspecific IgG band recognized by the secondary reagent, are indicated by arrows; samples are from one mouse for each strain per treatment. (*Upper*) A weak aspecific signal in lane 5 is probably because of spill-over from lane 4.

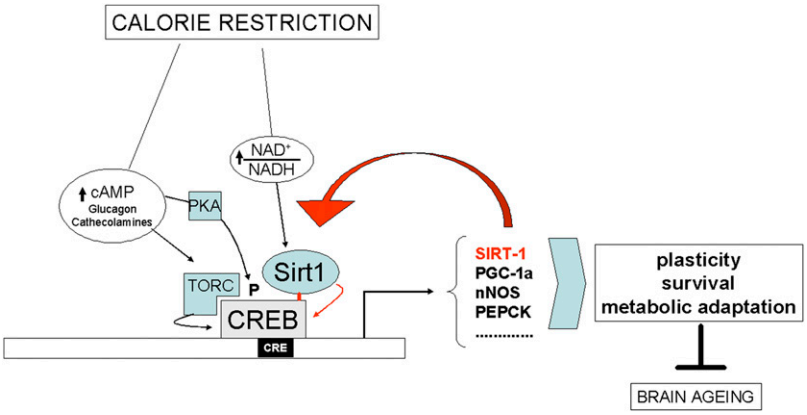

Fig. S7. Role of CREB and Sirt-1 in brain response to calorie restriction. Model depicting multiple mechanisms whereby reduced nutrient intake activates CREB; these include (*i*) metabolic activation of Sirt-1 molecules recruited to CREB-dependent promoters, (*ii*) hormone/cAMP-induced phoshorylation of CREB, and (*iii*) induction of the CREB coactivator TORC (1). CREB-induced genes enhance neuronal plasticity, survival and metabolic adaptation, thus delaying brain aging; up-regulation of Sirt-1 bolsters the protective response, establishing a feed-forward loop. Interactions demonstrated or suggested by the present work are highlighted in red.

1. Altarejos JY, Montminy M (2011) CREB and the CRTC co-activators: Sensors for hormonal and metabolic signals. *Nat Rev Mol Cell Biol* 12:141–151.