Sirtuin Activity in Dentate Gyrus Contributes to Chronic Stress-Induced Behavior and Extracellular Signal-Regulated Kinase 1/2 Cascade Changes in the Hippocampus

Supplemental Information

Supplemental Methods & Materials

Chronic Variable Stress (CVS)

Morning stressors were administered between 0830 and 1130, and afternoon stressors were administered between 1330 and 1630. Overnight stressors began immediately after cessation of the afternoon stressor and concluded with the start of the next day's morning stressor. CVS stressors consisted of warm swim (20 min at 31-33°C), cold swim (10 min at 16-18°C), cold room (1 hr at 4°C, two rats per cage without bedding), rotation (1 hr at 100 rpm), social isolation (overnight, one rat per cage), and social crowding (overnight, 6 rats per cage). All rats, control and CVS, were weighed every other day. On the morning of the 15^{th} day approximately 12 hours after administration of the last stressor (between 0900 and 1000), rats were killed by rapid decapitation, trunk blood collected and adrenal glands weighed. The hippocampus was extracted and subdivided into CA1, CA3 and dentate gyrus (DG) regions for histone and protein extraction.

Object Location

Rats were habituated to the open field comprised of black Plexiglas (90 x 90 x 45 cm) for 10 min daily on the final five days of stress prior to object location testing. The morning following the final day of stress (day 15), each rat was placed in the open field for a 3 min sample trial with two identical objects located 20 cm from the walls of the open field in the southeast and southwest corners. In the first object location experiment (Figure 1), the objects used were 2 water bottles (20 cm height x 7 cm diameter at base). In the sirtinol infusion experiment (Figure 7), 2 children's non-spilling cups (13 cm at highest point, 7 cm diameter at base, and 13 cm wide from handle to handle) were used. Upon

completion of the sample trial, rats were returned to their holding cages for a 30 min delay. After the delay period, each rat was returned to the open field with the same objects for a retention trial where one of the objects was positioned in a familiar location (i.e., southeast) of the field, while the other object was repositioned in a novel location (i.e., northwest). Investigation of an object was defined as attending to the object from a distance of 2 cm or less. The times spent investigating the objects in each location during both the sample and the retention trials were scored by an investigator blind to the conditions. The discrimination index was defined as the ratio of time spent investigating the object in the novel location during the first minute of the retention trial relative to the total amount of time spent exploring the objects.

Sirtinol Infusion

For sirtinol experiments, male Wistar rats (35 days of age; Harlan, Inc.) were housed as above and allowed 7 days to acclimate prior to surgery. Animals were randomly assigned to one of 6 experimental groups: control sham, control vehicle-treated, control sirtinol-treated, CVS sham, CVS vehicle-treated, and CVS sirtinol-treated. Alzet® osmotic mini-pumps (Durect Corp., Cupertino, CA) filled with either sirtinol (50 μM; Calbiochem, San Diego, CA) or 5% hydroxylpropyl β-cyclodextrin vehicle (CTD, Inc., High Springs, FL) were activated 40 hrs prior to implantation by incubating them in 0.9% saline at 37°C initiating immediate delivery of 0.25 μl/hr for the entire length of the experiment. Rats were anesthetized initially with ketamine/xylazine mixture (100 mg/kg body weight) and placed in the stereotaxic apparatus. Maintenance anesthesia was 1% isoflourane mixed with oxygen. An incision was made above the skull and bilateral guide holes were drilled above the DG at the following coordinates (Paxinos and Watson 2007 (1)): from bregma AP -3.2 mm; ML ± 1.6 mm, DV -5.0 mm. Cannulae (28 gauge stainless steel, 5.0 mm projection) were affixed to the skull using dental cement. The subcutaneous area between the scapulae was spread to create a pocket at the base of the rat's neck for

mini-pump placement. The infusion system was secured under the skin by closing the skin with surgical wound clips. Following surgery, animals were monitored and single-housed for 5 days to allow for recovery. After 5 days, animals were pair-housed, to minimize stress of isolation, and allowed an additional 6 days to recover prior to the start of CVS. A total of 56 animals underwent bilateral cannulation of the DG. One animal died during recovery from surgery. Additionally, two other animals were eliminated from all analysis due to secondary complications (1 animal had an infection around the placement site of the osmotic pump, 1 animal had an eye infection). At sacrifice, cannula placement was confirmed through direct visualization under a dissecting microscope during tissue extraction and hippocampal sub-dissection. Animals with cannula placement outside of the DG were removed from all analysis (n = 3). For the animals included in experimental analysis, the location of the infusion sites can be seen in Figure S1.

SIRT1 Activity Assay

Briefly, protein concentration was normalized across samples to a final concentration of 1 µg/µl. Samples were incubated with 100 µM of the SIRT1 acetylated (lysine 382) p53 substrate in the presence of 500 µM NAD^+ for 45 min at 37°C. Deacetylation of the SIRT1 substrate produces a fluorescence signal upon the addition of the developer compound. Fluorescence was read in clear, half-volume 96-well plates on SpectraMAX Gemini XS fluorescence plate reader (excitation 355 nm, emission 465 nm, gain = 85). Calculation of net fluorescence was obtained by subtraction of the blank control value from the triplicate mean of each sample's fluorescence readings.

Tissue Processing

The brain was removed and immersed in ice-cold artificial cerebrospinal fluid. The amygdala was blocked and dissected using a 1 mm punch. Medial prefrontal cortex was visually dissected. Then, 1 mm

coronal sections were made using a brain matrix (Braintree Scientific) and the hippocampus was subdivided into CA1, CA3 and DG regions under direct visualization for histone and protein extraction.

Histone Extraction

Tissue was processed in homogenization buffer containing (in mM): Sucrose 250, Tris, pH 7.5 50, KCl 50, PMSF 5, sodium butyrate 20, sodium orthovanadate 0.1. Samples were centrifuged @ 7700 *g* for 1 min. Pellet was resuspended in 0.4 N H_2SO_4 and centrifuged at max speed for 10 min at 4°C. Trichloroacetic acid + 4 mg/ml deoxychloric acid was added to the supernatant and centrifuged at max speed for 30 min at 4°C. Histones were extracted with cold acidified acetone and centrifuged at max speed for 5 min at 4°C. The histone pellet was resuspended in 10 mM Tris, pH 8.0 and stored at -80°C. Protein concentration was determined through Lowry protein assay and samples were normalized to 0.5 µg/µl. Histones were separated on a 15% gel and transferred to Immobilon for Western blotting and probed for the following antibodies: anti-acetyl lysine 12 (K12) H4 (1:1000, Cell Signaling) and anti-histone 3 (1:1000, Cell Signaling). The histone 4 antibody was undetectable; therefore all histone modifications were normalized to total H3 protein expression. Chemiluminescence was detected with either ECL (Amersham, UK) or Super Signal (Pierce, Rockford, IL). Blots were analyzed using Image J.

Western Blotting

Proteins were extracted using Chemicon's compartmental protein extraction kit. Protein concentration was determined through Lowry protein assay and samples were normalized to 0.5 µg/µl. Proteins were separated on a 10% SDS-PAGE gel. Blots were probed with the following antibodies from Cell Signaling Tech. unless otherwise noted: anti-phospho ERK1/2 antibody (1:1000), anti-ERK1/2 antibody (1:1000), anti-Bcl-2 antibody (1:1000), anti-phospho(S473)AKT (1:1000), anti-AKT (1:1000), anti-GAPDH antibody (1:1000), anti-acetyl-alpha-tubulin (Lys40) antibody (1:500), anti-tubulin (1:5000).

Chromatin Immunoprecipitation

Briefly, the DG region of hippocampus was micro-dissected in artificial cerebrospinal fluid containing protease inhibitors (1 mM PMSF, 1 μg/ml of protease inhibitor cocktail) and phosphatase inhibitors (1 mM Na_3VO_4 and 20 mM NaF). Tissue was incubated in 1% formaldehyde in PBS at 37°C for 10 minutes and homogenized in SDS lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS). Chromatin was sheared using a sonicator (Branson Sonifier 250). Lysates were centrifuged to pellet debris and supernatant was then diluted 1:10 in ChIP dilution buffer (16.7 mM Tris, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA). Immunoprecipitations were carried out at 4°C overnight with primary antibodies (anti-H4K12) or no antibody (control). Immunoprecipitated material was collected by protein A-agarose bead/salmon sperm slurry and sequentially washed with low salt buffer (20 mM Tris, pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), high salt buffer (same, including 500 mM NaCl, 1 mM EDTA), LiCl immune complex buffer (0.25 M LiCl, 10 mM Tris, pH 8.1, 1% deoxycholic acid, 1% IGEPAL-CA630, 500 mM NaCl, 2 mM EDTA), and finally TE buffer. Immune complexes were extracted in 1× TE with 1% SDS, and protein-DNA cross links were reverted by heating at 65°C overnight. DNA was extracted by phenol/chloroform/isoamyl alcohol after proteinase K digestion (100 μg; 2 hours at 37°C), and then ethanol precipitated.

Primer Sequences for Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Immunoprecipitated DNA was subjected to quantitative RT-PCR using primers specific for 150–200 bp segments corresponding to promoters upstream of the rat Bcl-2 or Zif268: Bcl-2 sense, 5’-GTCGGCACAATTGGTAGCTT-3’, Bcl-2 antisense, 5’-GGCTGGAAACTTCAGATGGA-3’; Zif268 sense, 5’-ATGGGCTGTTAGGGACAGTG−3’; Zif268 antisense, 5’-TTGGGGATTTAGCTCAGTGG-3’. The cumulative fluorescence for each amplicon was normalized to the input and CVS and control conditions compared using a student’s t-test. Actin sense, 5’-AGCCATGTACGTAGCCATCC-3’; Actin antisense, 5’-

GCTGTGGTGGTGAAGCTGTA-3'; Zif268 sense, 5'-GGGCTCCCCAGTTCCTCGGT-3'; Zif268 antisense, 5'-GGGTTGTTCGCTCGGCTCCC-3'; Bcl-2 sense, 5'-CGCAAGCCGGGAGAACAGGG-3'; Bcl-2 antisense, 5'-CGGGCGTTCGGTGCTCTCA-3'.

Statistical Analysis

Statistical analyses for molecular data were completed in Graph Pad 5 using analysis of variance (ANOVA) followed by Bonferroni or Tukey's *post-hoc* test comparisons when appropriate. The Student's *t*-test was used for pairwise comparisons. Analysis of behavioral data was completed in SPSS. Independent sample *t*-tests were performed to assess group differences in total object investigation times independent of location during both the sample trial and the retention trial, and to examine group differences in spatial memory during the retention trial. One-sample *t*-tests were performed to verify within-group spatial location memory, in which the percentage of time investigating the object in the novel location during the retention trial was compared to chance performance. Statistical significance was set at $p < 0.05$.

Supplemental Results

Physiological Markers of CVS

Rats subjected to CVS for 14 days demonstrated a significant decrease in body weight gain (Table S1) in response to chronic stress compared to handled controls. Two-way ANOVA revealed a significant interaction [$F_{(7,322)} = 98.6$, $p < 0.0001$] as well as a significant effect of condition [$F_{(1,322)} = 18.84$, $p < 0.001$] and day of protocol [$F_{(7,322)} = 493.8$, $p < 0.0001$] on total weight gain. Subsequent Bonferroni post-hoc tests confirmed a significant reduction in body weight gain by day 5 ($p < 0.05$) which persisted throughout the remainder of the experiment (days 7, 9, 11, 13, 15, $p < 0.0001$). Additionally, adrenal hypertrophy, another hallmark of chronic stress exposure, was seen in the CVS rats [Table S1; 19.9 ± 0.5

mg/100 g body weight (n = 24)] compared to control rats [14.2 ± 0.3 mg/100 g body weight (n = 24), p < 0.0001]. Finally, CVS-treated rats showed a significant increase in plasma corticosterone levels [Table S1; 70.1 ± 19.7 ng/ml (n = 10) compared to control rats with levels of 19.5 ± 3.3 ng/ml (n = 9), p = 0.03].

Effect of CVS on ERK1/2 Activation in the Amygdala and Prefrontal Cortex (PFC)

Interestingly, CVS caused a significant increase in ERK1/2 activation in the medial PFC (Figure S2A, 217 ± 30%, n = 5, compared to control 100 ± 16%, n = 4, $t_{(7)}$ = 3.2, p < 0.05). No significant effect of CVS was observed on Bcl-2 expression in the medial PFC (Figure S2B, 100 ± 15%, n = 4 compared to CVS 86 ± 7%, n = 4; $t_{(6)}$ = 0.8, p = 0.4). On the other hand, CVS had no significant effect on the ERK1/2 activation in the amygdala (Figure S2C; 100 ± 14%, n = 11 compared to CVS 111 ± 16, n = 10, $t_{(19)}$ = 0.5, p = 0.6). Since there was no effect on of CVS on ERK1/2 activation in the amygdala, we did not further investigate Bcl-2 expression. These results suggest that the cells of the various brain areas involved in stress exhibit a different response to CVS in terms of ERK1/2 activation.

Effect of CVS on Akt Pathway

The effect of CVS and sirtinol on the Akt pathway was also investigated (Figure S3). Western blotting revealed no significant effect of either brain region [$F_{(2,18)}$ = 0.9, p = 0.4] or condition [$F_{(1,18)}$ = 1.0, p = 0.34]. In addition, the effect of sirtinol infusion was investigated in the DG. We found no significant effect of CVS or sirtinol ($F_{(3,14)}$ = 1.4, p = 0.6) on phosphorylation of Akt in the DG. These results suggest that the effects of chronic stress in the hippocampus are not due to the effects on the Akt signaling cascade.

Table S1. Physiological Measures of Stress.

	Control	CVS
Adrenal Weight (mg/100 g Body Weight)	14.2 ± 0.3 (*n* = 12)	19.9 ± 0.5 (*n* = 24)***
Total Weight Gain (g)	59 ± 3 (*n* = 14)	28 ± 4 (*n* = 14)***
Corticosterone (ng/ml)	20 ± 3 (*n* = 9)	70 ± 20 (*n* = 10)*

CVS, chronic variable stress.
Data represented as mean ± SEM.
*$p < 0.05$ vs. Control.
***$p < 0.001$ vs. Control.

Table S2. Physiological Measures of Stress in Sirtinol Infusion Experiment.

	Control Sham	CVS Sham	Control Vehicle	CVS Vehicle	Control Sirtinol	CVS Sirtinol
Adrenal Weight (mg/100 g Body Weight)	15 ± 1 (*n* = 8)	19 ± 0 (*n* = 8)*	16 ± 0 (*n* = 8)	21± 1 (*n* = 8)**	15 ± 1 (*n* = 9)	19 ± 1 (*n* = 8)*
Total Weight Gain (g)	64 ± 4 (*n* = 8)	26 ± 4 (*n* = 8)***	61 ± 5 (*n* = 8)	31 ± 5(*n* = 8)***	63 ± 5 (*n* = 9)	21 ± 3 (*n* = 8)***
Corticosterone (ng/ml)	6 ± 0 (*n* = 8)	68 ± 30 (*n* = 7)*	9 ± 1 (*n* = 8)	66 ± 18 (*n* = 6)**	8 ± 1 (*n* = 9)	42 ± 17 (*n* = 8)*

CVS, chronic variable stress.
Data represented as mean ± SEM.
*$p < 0.05$ vs. Control Sham.
**$p < 0.01$ vs. Control Sham.
***$p < 0.001$ vs. Control Sham.

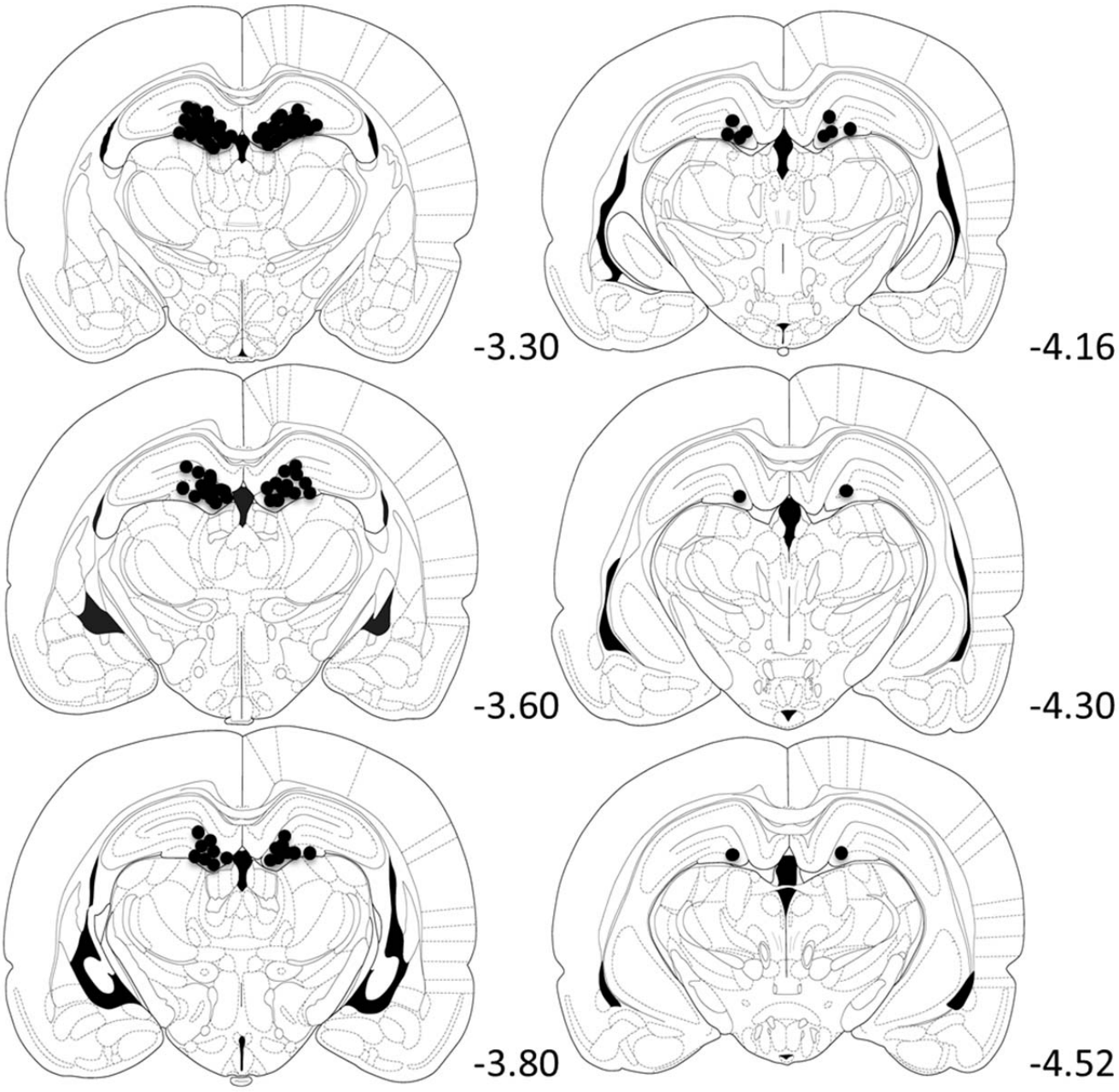

Figure S1. Schematic diagram demonstrating the location of the infusion sites into the DG that were included in sirtinol infusion study. Three animals were not used in the study as the cannulae were outside of the DG. DG, dentate gyrus. Rat brain coronal sections from Paxinos and Watson, reprinted with permission from Elsevier (1).

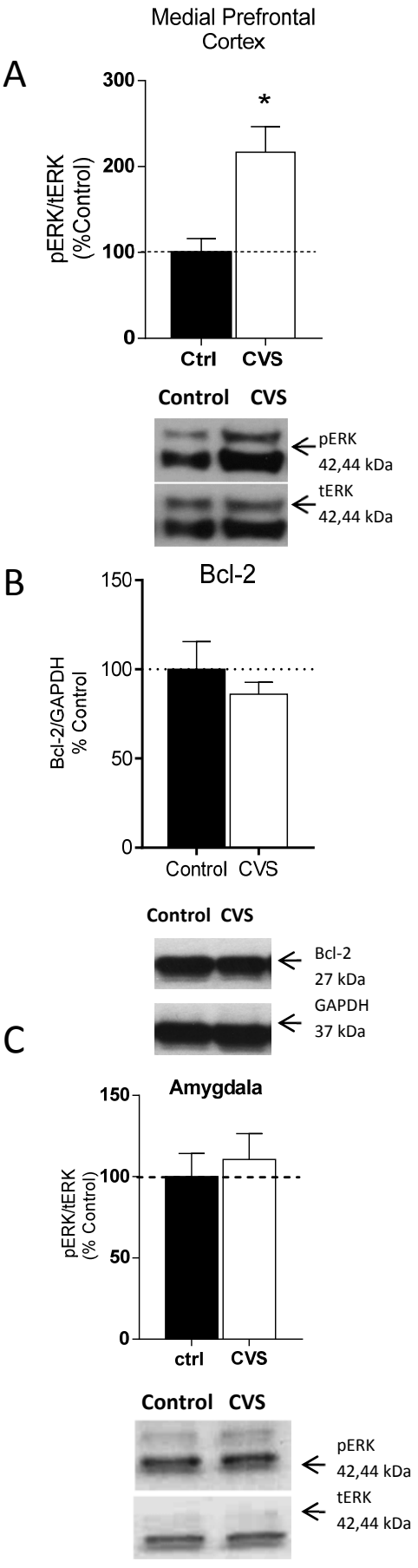

Figure S2. Activation of ERK1/2 in the medial prefrontal cortex (mPFC) region and amygdala of chronic variable stress (CVS) animals. (**A**) Quantification and representative Western blots of phosphorylated ERK1/2 (pERK) relative to total ERK1/2 (tERK) in the mPFC of control (Ctrl) and CVS-treated animals. CVS significantly increased ERK1/2 activation in the mPFC. (**B**) Quantification and representative Western blots of Bcl-2 expression relative to GAPDH in the mPFC of control and CVS-treated animals. (**C**) Quantification and representative Western blots of phosphorylated ERK1/2 relative to total ERK1/2 in the amygdala of control and CVS-treated animals. CVS had no significant effect on ERK1/2 activation in the amygdala. Data are shown as mean ± SE; **p* < 0.05; (*n* = 5-9 per group).

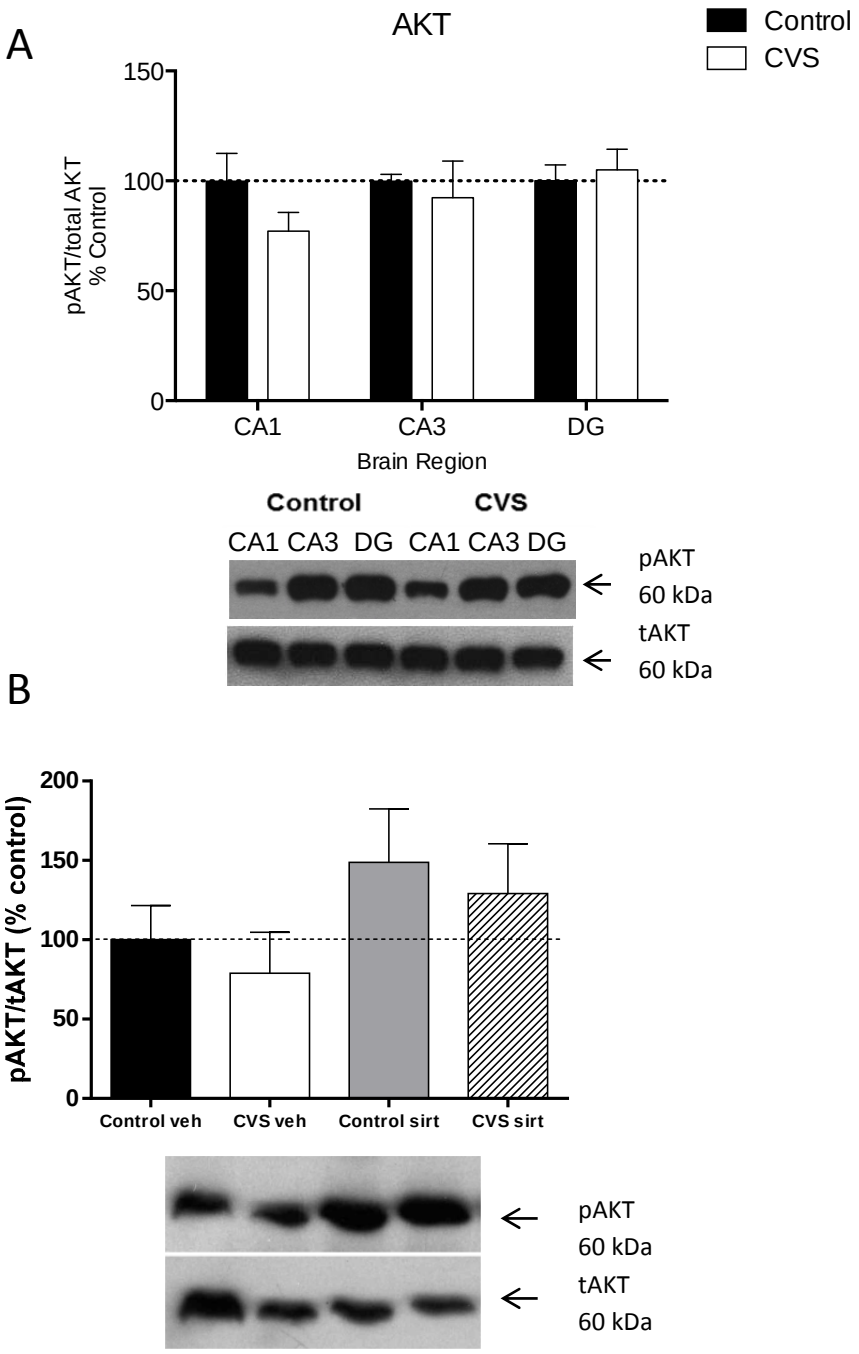

Figure S3. Effect of chronic variable stress (CVS) and sirtinol (sirt) on Akt activation in the hippocampus. (**A**) Quantification and representative Western blots of phosphorylated Akt (pAkt) relative to total Akt (tAkt) in the subregions of the hippocampus. CVS had no significant effect on Akt phosphorylation in any subregion. (**B**) Quantification and representative Western blots of phosphorylated Akt relative to total Akt in the dentate gyrus (DG) of control vehicle (veh), CVS vehicle, control-sirtinol and CVS sirtinol-treated rats. Neither CVS nor sirtinol had a significant effect on pAKT/tAKT in the DG. Data are shown as mean ± SE, (*n* = 4-5 rats per group).

Supplemental Reference

1. Paxinos G, Watson C (2007): *The rat brain in stereotaxic coordinates.* Amsterdam, Boston, Academic Press; Elsevier.