Supporting Information

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Materials and Methods

Preparation of Cortical Neuronal Cultures. Whole cortices were dissected from embryonic day 18 (E18) rats, dissociated in calcium and magnesium-free HBSS containing 0.125% trypsin for 15 min. After trituration, the neurons were plated at 0.4 million cells per well in 6-well plates or 2–4 million in each 150 mm plate in Dulbecco's modified Eagle's medium (DMEM, Invitrogen)/10% FBS and cultured at 37 °C, 5% CO_2, and 95% humidity overnight, and then changed to serum-free medium, Neurobasal plus B27 (Invitrogen). The medium was changed every 3 days. These cultures yielded virtually all neurons (data not shown).

MTR and MTG Staining of Cortical Neurons. Cortical neurons in 4-well chamber glass slides were incubated with MTR CM-H2XROS (Molecular Probes, final concentration 300 nM) and MTG (Molecular Probes, final concentration 60 nM) in 1× neurobasal without phenol red and 10 mM Hepes buffer for 15 min and washed twice with phenol red-free Neurobasal + 10 mM Hepes. Two to five Z-stack images for each slice were acquired randomly by using a Zeiss 510-LSM confocal microscope under exactly the same conditions. The longest dendrite from each neuron treated in each group was quantified by 510 metamorph software. The average dendrite lengths from quantified dendrites were not significantly different among the control and treated groups.

JC-1 Staining of Cortical Neurons. Cortical neurons in 4-well chamber glass slide were incubated with JC-1 (Molecular Probes, T3168, 10 μg/ml) in warm neurobasal without phenol red + 10 mM Hepes for 20 min at 37 °C and then washed twice. Two to five Z-stack images were randomly acquired by using a Zeiss 510-LSM confocal microscope under exactly the same conditions. The longest dendrite from each neuron treated in each group was quantified. The average dendrite lengths were not significantly different among the control and treated groups. The red and green fluorescent signals were analyzed by 510 metamorph software.

Determination of Mitochondrial Calcium by Rhod 2 Staining. Neurons were placed in MIC buffer (NaCl 130 mM, KCl 5.3 mM, $MgSO_4$ 0.8 mM, Na_2HPO_4 1 mM, Glucose 2 mM, Hepes 20 mM, Na-Pyruvate 1 mM, $NaHCO_3$ 2.5 mM, Ascorbic acid 1.0 mM, $CaCl_2$ 1.5 mM, BSA 1.5 mg/ml) for 10 min at room temperature and loaded with 4.0 mM dihydrorhod-2 in MIC buffer for 30 min at 37 °C. After washing twice, the fluorescent signals were measured by Zeiss LSM 510 microscopy (excitation at 543 nm and a 560 nm long pass filter was used for emission). The image-fields were randomly determined and images were captured every 20 sec. After 1 min and 40 sec of baseline measurements, 2 mM thapsigargin was added and images were taken for an additional 10 min. The regions of interest for quantification were the cell body and the longest dendrite. The average red fluorescent intensity of the cell in each time interval was determined by 510 Metamorph software. We calculated Fi/Fo before and after thapsigargin treatment.

Preparation of Mitochondrial Fractions from Cortical Neurons. Neurons were harvested in MitoBuffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 1 mM DTT and 1x protease inhibitors) and homogenized by tight glass homogenizers 20 times. One hundred microliters of the homogenized solution was saved as total homogenates. The rest of the solution was centrifuged at 800 × *g* for 10 min. The obtained pellet was saved as nuclear fraction. The supernatant was centrifuged again at 15,000 × *g* for 10 min. The resulting supernatant was transferred to another tube as cytosol fraction. The remaining second pellet (mitochondria) was dissolved with lysis buffer (10 mM Tris-HCL, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerophosphate) with phosphatase inhibitors mixture I and II (1:100) and protease inhibitor mixture (1:100) (Sigma). The final samples were kept on ice for 30 min, centrifuged at 140,000 × *g* for 10 min, and then the supernatants were stored at −80 °C as mitochondrial fractions.

in Situ Cell Death Detection Assay. We used the kit from Roche for detection and quantification of apoptosis at the single cell level based on labeling of DNA strand breaks (TUNEL assay) following the manufacturer's instructions. Briefly, cells were fixed in 4% paraformaldehyde in PBS for one hour at 15–25 °C and washed with PBS, then incubated in permeabilisation solution for 2 min on ice. Fifty microliter TUNEL reaction mixture was added to the sample and the slide was incubated at 37 °C for 60 min in the dark. The slide was rinsed 3 times and mounted with mounting media with DAPI. The pictures were randomly taken under the microscope and apoptotic and total cells were counted.

Western Blot Analysis. Protein concentrations were determined by using the BCA protein assay kit (Pierce). Equal amounts of proteins were subjected to 4–20% SDS/PAGE gels and separated by electrophoresis (Invitrogen), transferred to 0.45 μm pore-size PVDF membranes (Millipore) and immunoblotted with anti-GR (Abcam, 1:200), anti-Bcl-2 (Santa Cruz Biotechnology, sc-783, 1:200), anti-GADPH (Abcam, 1:500), anti-COX-1 (Molecular Probes, 1:200), anti-MR antibody (Affinity Bioreagents, 1:200), and anti-nucleus pore protein antibodies (Abcam, 1:300). HRP-conjugated anti-rabbit antibody and HRP-anti-mouse antibody (Amersham, and Vector Laboratories) were used as secondary antibodies. Immunoreactive bands were visualized by enhanced chemoluminescence (ECL+) (Amersham) and exposed to Kodak Biomax or Biolight film. The ECL signal intensities were quantified using a Kodak Image system. All data were analyzed by ANOVA or Student's *t* test.

Double Immunocytochemistry Staining of Cortical Neurons. Rat cortical neurons were fixed in 4% paraformaldehyde in PBS for one hour on ice. The cells were then blocked with 10% normal goat serum, 1% BSA, and 0.4% triton in PBS for one hour, followed by incubation with rabbit anti-GR (Abcam, 1:40) and mouse anti-COXIV (Molecular Probes, 1:100) at 4 °C overnight. The secondary antibodies were FITC-conjugated anti-mouse (Jackson Labs, for multiple staining, 1:50) and Cy3-conjugated anti-rabbit (Jackson Labs, for multiple staining, 1:150) antibodies. After washing, the cells were mounted onto slides with anti-fade mounting media (Molecular Probes). Z-stack imaging was acquired by using a Zeiss 510-Meta confocal microscope under exactly the same conditions. Neurons were randomly photographed with 5 images obtained for each slice to ensure that conditions for each slice for each group were the same. The longest dendrite from each neuron treated in each group was quantified. Colocalization efficiency of GR (red) and COXIV (green) at each longest dendrite was determined using 510-

Metamorph software, using exactly the same conditions for both control and experimental groups. The colocalization coefficient represents the percentage of red fluorescence (GR) on the green fluorescence (COXIV), using total red signal as 100% in the region of interest. Data were analyzed by Student's *t* test.

For double staining of GR and Bcl-2, rat cortical neurons were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were then blocked with 10% normal goat serum and 0.4% triton in PBS for one hour, followed by incubation with rabbit anti-GR (ABR Affinity Bioreagents, 1:60) and mouse anti-Bcl-2 (Abcam, 1:60) at 4 °C overnight. The secondary antibodies were Dylight-488-conjugated Donkey anti-mouse (Jackson Labs, for multiple staining,1:200) and Cy3-conjugated Donkey anti-rabbit (Jackson Labs, for multiple staining, 1:200) antibodies. After washing, the cells were mounted onto slides with anti-fade mounting media (Molecular Probes). Confocal images (0.5–0.8 μm in thickness) were acquired by using a Zeiss 510-LSM confocal microscope under exactly the same conditions. Neurons were randomly photographed with 5 to 10 images obtained for each slice to ensure that conditions for each slice for each group were the same. The cell bodies from all neurons in the image were extracted from the original image and the colocalization efficiency was quantified by 510-LSM software, using exactly the same conditions for both control and experimental groups. The colocalization efficiency represents the percentage of green fluorescence (Bcl-2) on the red fluorescence (GR). Data were analyzed by Student's *t* test.

1. Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council (1996) *Guide for the Care and Use of Laboratory Animals* (Superintendent of Documents, U.S. Government Printing Office, Washington, DC), p 140.

Corticosterone Treatment. All animal treatments, procedures, and care were approved by the NIMH Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Animals* (1). Animals received corticosterone in their drinking water in a final concentration of 50 μg or 400 μg corticosterone/ml of water for 3 weeks with a final ethanol concentration of 0.3% or 2.4%, respectively. Vehicle-treated animals received 0.3 or 2.4% ethanol in their drinking water for 3 weeks. Animals were weighed after the treatment period. For biochemical studies, the animals were decapitated and brain tissue was immediately processed for mitochondrial preparation. To assess the adequacy of the chronic corticosterone administration, thymus weight was determined.

Preparation of Mitochondrial Fraction from Brain Tissue. Prefrontal cortices were homogenized in 1 ml of homogenizing buffer (10 mM Hepes, pH 7.5, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA) in a 1 ml tissue grinder 4 times with loose and 8 times with tight grinder. One hundred microliters of tissue homogenates were saved as total homogenates. The rest of the homogenate was centrifuged at 2,000 $\times$ *g* for 4 min at 4 °C and the pellets were saved as nuclear fractions. The supernatants were centrifuged again at 12, 000 $\times$ *g* for 8 min at 4 °C. The second supernatants were saved as cytosol fractions. The pellets were resuspended as mitochondrial fractions and dissolved into 1 ml of homogenization buffer and centrifuged one more time at 12, 000 $\times$ *g* for 8 min. The pellets were resuspended into the lysis buffer and stored at -80 °C.

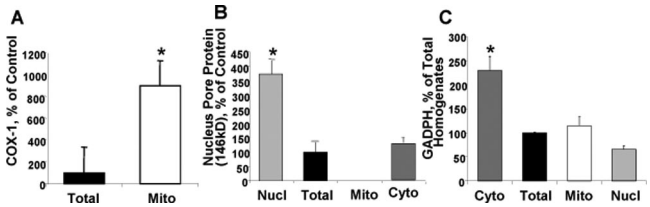

Fig. S1. Purity of mitochondrial fraction from cortical neurons. (*A*) Mitochondrial marker COX1 was enhanced 9-fold in the mitochondrial fraction. (*B*) Mitochondrial fraction showed low content of cytosol marker GADPH. (*C*) Absence of nuclear pore protein in the mitochondrial fraction (*Student's *t* test $P < 0.05$, # one-way ANOVA, $P < 0.01$, $n = 4$ for each group). This experiment was repeated.

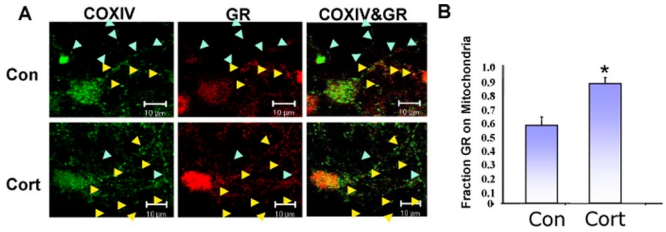

Fig. S2. Double immunostaining of cortical neurons with anti-GR and anti-COXIV antibodies after corticosterone treatment. (*A*) Cortical neurons (10–12DIV) were treated with 100 nM corticosterone for 1.5 h. Double immunostaining was performed with anti-GR and anti-COXIV antibodies. Green arrows indicate mitochondria without GRs and yellow arrows indicate mitochondria with GRs. (*B*) Quantification of GRs and COXIV colocalization. Colocalization efficiency of GRs (red) and COXIV (green) at each individual longest dendrite was determined using 510-Meta software. This experiment was repeated (*Student's *t* test, $P < 0.05$, $n = 10$).

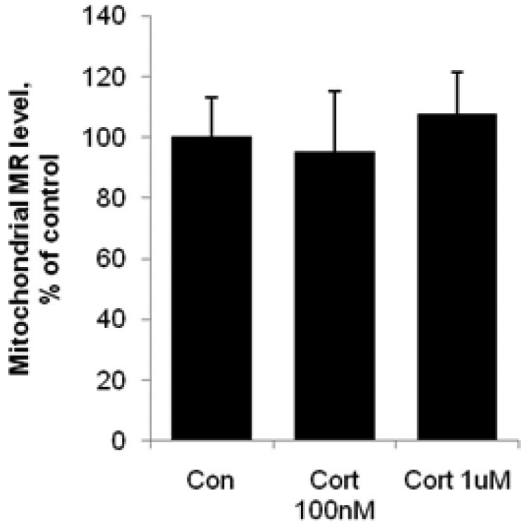

Fig. S3. Lack of translocation of MRs to mitochondria in response to corticosterone treatment. Cortical neurons (10–12DIV) were treated with 100 nM or 1 μM corticosterone for 1.5 h. Mitochondrial and nucleus fractions were isolated from the cortical neurons. Western blot analyses were performed with anti-MR antibody (n = 7–14 for each group). In contrast to what was observed with GRs, no mitochondrial translocation was observed for the MRs.

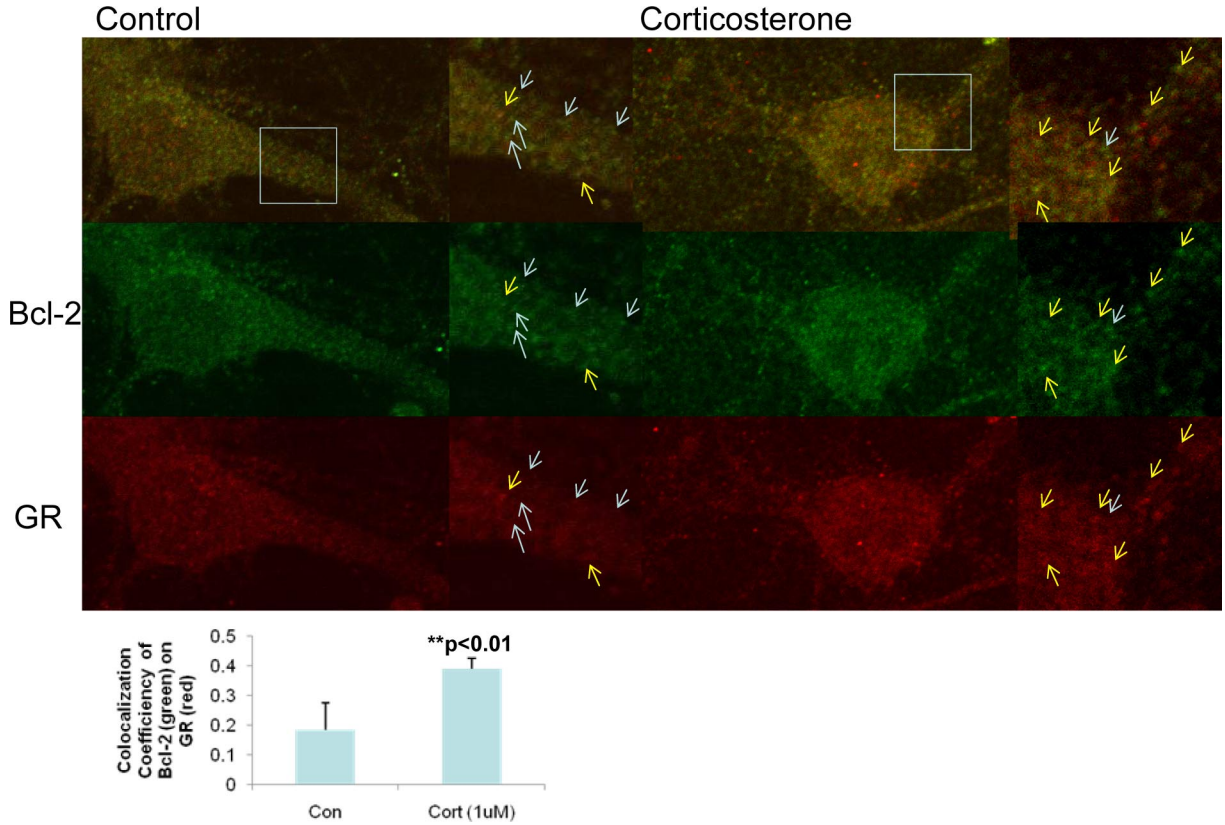

Fig. S4. Colocalization of GR and Bcl-2 after corticosterone treatment. Cortical neurons (12 DIV) were cultured in corticosterone-depleted medium (neurobasal without B27) for one day. The cells were then treated with corticosterone (1 μM) for one hour and fixed with 4% paraformaldehyde in PBS. Double immunostaining was performed with rabbit-anti-GR antibody and mouse-anti-Bcl-2 antibody. Images were taken by confocal microscope. Colocalization efficiency of GRs and Bcl-2 was analyzed by 510 LSM software. Light blue arrows indicate the Bcl-2 puncta (green) without GR (red). Yellow arrows indicate the puncta with both green (Bcl-2) and red (GRs). Note that after treatment with corticosterone, yellow puncta were increased (Student's *t* test, $^{**}P < 0.01$, $n = 15$). This experiment was repeated.