Supplemental Methods and Materials

**Ex Vivo Electrophysiology**

**Preparation of ilPFC slices.** Approximately 16 h after the last stressor, animals were anesthetized with sodium pentobarbital and perfused with cold dissection solution [(in mM): 250 sucrose, 2.5 KCl, 25 NaHCO₃, 1.0 NaH₂PO₄, 6 MgCl₂, 0.5 CaCl₂, and 25 glucose] continuously bubbled with 95% O₂ / 5% CO₂. The prefrontal cortex was sectioned in 300 μm slices using a vibratome (7000smz-2; Campden Instruments, Lafayette, IN) and placed in a recovery solution containing (in mM): 92 NMDG, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 5 Na ascorbate, 2 thiourea, 3 Na pyruvate, 10 MgSO₄, and 0.5 CaCl₂ (1) for 15-20 min and then transferred to a chamber containing oxygenated artificial CSF (aCSF) solution [(in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.0 NaH₂PO₄, 1.0 MgCl₂, 2.0 CaCl₂, and 25 glucose] for ≥ 1 h at room temperature. After recovery, slices were transferred to a submersion-type recording chamber, mounted on the stage of an upright microscope (BX51WI, Olympus, Center Valley, PA) and perfused with oxygenated aCSF at room temperature.

**Patch clamp recordings.** Patch electrodes were constructed from thin-walled single-filamented borosilicate glass (1.5 mm outer diameter; World Precision Instruments) using a microelectrode puller (P-97; Sutter Instruments, Novato, CA). Pipette resistances ranged from 4 to 6 MΩ and seal resistances were >1 GΩ. To record miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs), electrodes were filled with an intracellular solution containing (in mM): 130 Cs-gluconate, 10 CsCl, 10 HEPES, 11 EGTA, 1.0 CaCl₂, and 2.0 MgATP, pH 7.2 (290-300 mOsm).
Whole-cell patch clamp recordings were obtained from layer V pyramidal neurons in the ilPFC, which are easily identifiable in the slice on the basis of somal morphology and the presence of a prominent apical dendrite, using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). mEPSCs and mIPSCs were recorded in the presence of tetrodotoxin (TTX; 500 nM) to block sodium channels and action potential discharge in the slice. mEPSCs were recorded at a holding potential of -70 mV and mIPSCs were recorded at a holding potential of 0 mV in the same cell (3 min each; \( n = 21-25 \) cells per group). Bath application of 10 µM gabazine (SR 95531 hydrobromide; Tocris, Bristol, UK) abolished mIPSCs at a holding potential of 0 mV, showing that the IPSCs observed using this protocol are mediated by GABA\(_{A}\)Rs (Figure 1). Currents were filtered at 4–6 kHz through a –3 dB, four-pole low-pass Bessel filter, digitally sampled at 20 kHz, and stored on a personal computer (ICT, Cincinnati, OH) using a commercially available data acquisition system (Digidata 1440A with pClamp 10.0 software; Molecular Devices).

**In Vivo Neuroanatomical Studies**

**Immunohistochemistry**

**Tissue Collection.** Animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% sodium phosphate-buffered paraformaldehyde. Brains were post-fixed in 4% sodium phosphate-buffered paraformaldehyde for 24 hours, then stored in 30% sucrose in DEPC-treated water at 4°C. Brains were sectioned on a microtome in 30-µm coronal sections (Leica, Buffalo Grove, Illinois).

**Immunohistochemistry.** For double-label glucocorticoid receptor (GR) and GAD67, PV, CCK, calretinin, or calbindin immunolabeling, free-floating sections stored in sodium phosphate-buffered, DEPC-treated cryoprotective solution were rinsed 5 times with 50 mM potassium phosphate buffered saline (KPBS) solution. Following all incubations, sections were rinsed with 50 mM KPBS 5 times unless otherwise indicated. Sections were blocked in 0.1% bovine serum
albumin (BSA) and 0.2% Triton-X in 50 mM KPBS for 1 h prior to incubation in rabbit polyclonal anti-GR (M-20) primary antibody and mouse antibodies targeting GAD67, PV, CCK, calretinin, or calbindin for approximately 18 h at 4°C. Sections were incubated in Cy3 anti-rabbit and Cy5 anti-mouse secondary antibodies (each at 1:500; JacksonImmuno, West Grove, PA) for 1 h at room temperature. For SST and GR co-immunolabeling, sections were incubated in rat monoclonal anti-SST for 24 h at 4°C before addition of rabbit anti-GR for an additional 24 h at 4°C. Sections were then incubated in Cy5 anti-rabbit and biotinylated anti-rat for 1 h at room temperature (each at 1:500; JacksonImmuno and Vector Laboratories, Burlingame, CA, respectively). Sections were next amplified with avidin- biotin complex (1:1000 in KPBS; 1 h at room temperature; Vector Laboratories) and then incubated with Cy3 strepavidin for 1 h at room temperature (1:250; JacksonImmuno). Sections were rinsed 4 times in 50 mM KPBS and mounted in 50 mM potassium phosphate-buffered solution and 1% gelatin onto ultrastick slides (Gold Seal, Portsmouth, NH) and coverslipped with polyvinyl alcohol anti-fading medium with DABCO (Sigma-Aldrich, St. Louis, MO).

For CaMKIIα and GAD65 immunolabeling, free-floating sections stored in sodium phosphate-buffered, DEPC-treated cryoprotective solution were washed and blocked for 1 h in 0.1% BSA without Triton-X. Sections were then incubated overnight in anti-CaMKIIα primary antibody for approximately 18 h at 4°C. Sections were next incubated in Cy3 donkey anti-mouse (1:500; JacksonImmuno) for 1 h at room temperature and then blocked for 1 h in 0.1% BSA and 0.2% Triton-X in 50 mM KPBS. Sections were then incubated in GAD65 primary antibody for approximately 18 h at 4°C. Next, sections were incubated in donkey anti-mouse Alexa 488 (1:500; JacksonImmuno), mounted, and coverslipped, as described above.

Imaging and Analysis. For analysis of GAD65 appositions onto CamKIIα-positive cells, 3 z-stacks (0.5 μm optical slice thickness) of each side of the ilPFC at the rostral (AP ~ +3.2 mm from bregma), middle (AP ~ +2.7 mm from bregma), and caudal (AP ~ +2.2 mm from bregma) extent, as defined by the rat stereotaxic brain atlas of Paxinos and Watson (2), were captured at
63x magnification with a Carl Zeiss Imager Z.1 (Carl Zeiss Microimaging, Thornwood, New York). Images were captured on the same day with the same settings, and a uniform threshold was applied to all images in a given brain region. The criteria for inclusion for each cell was: 1) definitive CaMKIIα-positive immunoreactivity, 2) the total z-plane of the soma visible within the z-stack, 3) sufficient separation from other cells in order to clearly identify appositions on that particular cell, as previously described (3). Immunoreactive cells were identified by scrolling through the z-stacks. All cells within the z-stack that met criteria were selected and analyzed. The total number of GAD65-positive boutons in apposition to the CaMKIIα-positive soma through the complete z-axis of each of the cells was quantified. Appositions were defined by immunoreactive boutons with absolutely no visible space between the bouton and the edge of the CaMKIIα-positive cell (see Figure 2). Immunoreactive labeling on the soma within a given z-plane was not included. To ensure that each bouton was only counted once, appositions were counted while scrolling back and forth through the z-stack. Due to insufficient immunoreactive labeling of the dendrites, only appositions onto CaMKII-positive soma were analyzed. The number of appositions for each cell was normalized to the cell volume. In order to estimate the volume of each cell, we used the unbiased “nucleator” method (4). Using Axiovision 4.4, we focused on the CaMKIIα-positive cell at the level of the nucleolus and measured the distance from the center to the edge of the cell along a randomly selected angle between 1-90 and again 90, 180, and 270 degrees away from the original angle. These radii measurements were then averaged and used to estimate cellular volume using the equation for the volume of a sphere: \( V = \pi r^3 \).

For analysis of GR colocalization with GAD67, calretinin, calbindin, CCK, SST, and PV, z-stacks of each side of the iIPFC were captured at \( \sim +3.2 \) mm (rostral), \( \sim +2.7 \) mm (middle), and \( \sim +2.2 \) mm (caudal) from bregma, as defined by Paxinos and Watson (2), at 20x magnification. The criteria for colocalization of GR with each of the interneuron subtypes was as follows: 1) at the largest part of the GAD-positive cell GR immunoreactivity must also be present,
2) the largest radius of the GAD-positive cell must match with the largest radius of the GR-positive cell, 3) the threshold for positive GR-immunoreactivity was set at 50% above background using Image J Analysis. All image capturing and quantification was conducted by an experimenter blinded to experimental treatments. After all quantification had taken place, images presented in the article were cropped and contrast and brightness were adjusted to enhance publication quality without altering the presence or absence of immunolabeling.

**Electron Microscopy**

**Tissue Collection.** Animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.1M PBS containing 0.1% sodium nitrite (vasodilator) until the right atrium was clear of blood and the liver started to blanch, followed by 4% paraformaldehyde + 2.7% picric acid and 0.2% glutaraldehyde in 0.1M PBS for 20 min. The brains were dissected and postfixed overnight in 4% paraformaldehyde + 2.7% picric acid and 0.2% glutaraldehyde in 0.1M PBS at 4°C. The next day, brains were rinsed 6X5 min with 0.1M PBS and stored in 0.1M PBS at 4°C, until sectioned.

**Immunohistochemistry.** Brains were super glued to specimen disc of Leica vibratome, sectioned at 50 µm, and collected in 0.1M PBS. A full series of sections were washed in 0.1M PBS 5X5 min and then incubated in 1% sodium borohydride / 0.1M PBS for 30 min. Sections were washed in 0.1M PBS 6 X 5 min and were then incubated in 3% hydrogen peroxide / 0.1M PBS for 30 min. Sections were again washed in 0.1M PBS 6 x 5 min, then washed in 0.1M PBS 2 X 30 min, washed in 0.1M PB 2 X 5 min, and then incubated in cryoprotectant consisting of 0.1M PB + 25% sucrose + 3.5% glycerol for 1 h. The tissue was transferred to vials (containing fresh cryoprotectant; which is able to withstand liquid nitrogen temperatures) and immersed sequentially in liquid borohydride (freon-refron at -35°C) for 5 min, then immediately into liquid nitrogen -120°C for 5 min. The frozen vials containing the sections were allowed to come to room temperature, the tissue was removed from the vials and rinsed in 0.1M PB 2 X 5 min, then
0.1 M tris buffered saline (TBS) for 5 x 5 min, and then blocked in TBS containing 0.8% BSA + 0.1% gelatin for 2 h. Sections were incubated in GAD 65 antibody (Hybridoma Bank; 1:500) in TBS containing 0.8% BSA and 0.1% gelatin overnight at room temperature. The following day, sections were washed in TBS + 0.08% BSA + 0.01% gelatin for 6 x 5 min and incubated in biotinylated goat anti mouse IgG (Vector Laboratories; 1:400) in TBS + .08% BSA + 0.01% gelatin for 1 h. Sections were then washed in TBS 3 x 5 min, incubated in avidin-biotin complex (Vectastain ABC Elite Standard; Vector Laboratories; Burlingame, CA; 1:800) for 1 h, washed in TBS 3 x 5 min, washed in PBS 3 x 5 min, and then incubated in diaminobenzidine tetrahydrochloride (DAB; made in 0.1M PBS) for 15 min. Sections were then washed in 0.1M PBS 5 x 5 min. Tissue was isolated from the ilPFC and processed for electron microscopy as follows: isolated tissue was incubated in 2% OsO₄ containing 1.5% potassium ferricyanide in 0.1M PBS for 45 min, dehydrated through 30, 50, 70, 75, 80, 90, 95% ETOH for 5 min each, and then dehydrated in 100% ETOH for 3 x 15 min. Isolated tissue was then incubated in propylene oxide for 2 x 10 min, ½ propylene oxide + ½ epon-araldite mixture, then pure epon-araldite mixture overnight. Isolated tissue was embedded and cured overnight at 60°C.
**Figure S1.** Lack of GR colocalization with calbindin and CCK interneurons. Panels A-D show the lack of colocalization of GR (red) and calbindin (green) (20x magnification) (A-B) and GR (red) and CCK (green) (5x magnification) (C-D). Further, CCK interneurons appear to be located mainly in layers II and III of the iIPFC. Arrows indicate calbindin or CCK neurons that lack GR colocalization. Scale bar = 50 μm.
Figure S2. Effect of chronic stress on GR and calretinin colocalization. Panels A-B and C-D show colocalization of GR (red) and calretinin (green) (20x magnification) and lack of GR (red) and calretinin (green) colocalization in subsets of neurons (20x magnification), respectively. Chronic stress does not affect GR colocalization with calretinin ($P > .05$) ($n = 6$ animals per group, 6 slices per animal). Arrows indicate calretinin-positive neurons that have or lack colocalization with GR. Scale bar = 50 μm.
Figure S3. Chronic stress impairs across- and within-phase errors in the DSWS without affecting overall time to acquire the task. (A) Panel A shows across-phase errors (visiting arms during the testing phase that were previously baited during the training phase) made in the DSWS task. (B) Panel B shows within-phase errors (revisiting arms during the testing phase) made in the DSWS task. The graphs are truncated to 7 days as there were no significant differences after day 5. Chronically stressed animals initially made significantly more across- and within-phase errors during the 3rd and 2nd and 3rd days of the DSWS task, respectively ($P < .05$). (C) Panel C shows average days to reach the criterion of visiting the 4 baited arms during the testing phase in 5 or fewer choices, 2 consecutive days in a row. There were no significant differences in days to reach criterion between control and chronically stressed animals ($P > .05$). $^*P < .05$. 
Table S1. Primary antibodies used in the present study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Immunogen</th>
<th>Dilution</th>
<th>Company, Species, Type, Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>N-terminus of the GRα of mouse origin</td>
<td>1:1000</td>
<td>Santa Cruz Biotech, rabbit, polyclonal antibody, M20</td>
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<td>GAD65</td>
<td>Adult rat glutamic acid decarboxylase, purified</td>
<td>1:100; 1:500</td>
<td>Developmental Studies Hybridoma Bank; mouse; monoclonal antibody</td>
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<tr>
<td>GAD67</td>
<td>Glutamic acid decarboxylase</td>
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<td>Millipore, mouse, monoclonal antibody, 1G10.2</td>
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<td>Frog muscle parvalbumin</td>
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<td>Sigma Life Science, mouse; monoclonal antibody, PARV-19</td>
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<td>Calretinin</td>
<td>Human calretinin-22k</td>
<td>1:500</td>
<td>Swant, mouse, monoclonal antibody, 6B3</td>
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<tr>
<td>Calbindin</td>
<td>Bovine kidney calbindin-D</td>
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<td>Sigma Life Science, mouse, monoclonal antibody, CB-955</td>
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<td>SST</td>
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<td>Millipore, rat, monoclonal antibody, YC7</td>
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<td>Partially purified full length native rat protein</td>
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<td>CCK</td>
<td>CCK-8 fragment 26-33</td>
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<td>Abcam; mouse, monoclonal antibody, HYB 345-02</td>
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Supplemental References


