Supporting Information

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SI Text

Animal Husbandry. All mouse protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committee of Washington University School of Medicine (St. Louis, MO). Mice were housed with a 12-h/12-h light/dark cycle with ad libitum access to rodent chow and water.

GFP Visualization in GFP Control Mice. After behavioral testing, GFP controls were anesthetized with 2.5% tribromoethanol. Mice were then perfused transcardially with PBS solution and 4% paraformaldehyde (PFA). Brains were then immersed in 5 ml agarose XI and sectioned on a vibratome (Technical Products International) at 200 μm. After cutting, sections were mounted onto slides and visualized using an Olympus IX-71 fluorescent microscope. Because the LV-GFP vector does not have a nuclear localization signal, GFP was seen in CeA output areas (e.g., white matter tracts and BnST).

Lentivirus Production. Briefly, the GFP or Cre (with a nuclear localization signal conferring segregation of the Cre expression to the nucleus of infected cells) containing plasmid was co-transfected with three packaging plasmids (PMDLg/pRRE, PMD.G, and pRSV-Rev) into HEK-293 cells. Viral supernatant was collected once 40 h after transfection, passed through a 0.2-μm filter, and concentrated by ultracentrifugation (25,000 rpm for 140 min at 4°C). Concentrated viral particles were resuspended in sterile Ringer solution and stored at −80°C until use.

In vitro titering of both the LV-Ef1a-GFP (LV-GFP) and LV-Ef1a-Cre (LV-Cre) was done in CHO cells that had been transfected with pβactin-loxp-stop-loxp-LacZ plasmid. Briefly, 1×10^5 CHO cells were transduced in six-well dishes with serial dilutions of virus. Forty-eight hours after transduction, cells were imaged for GFP (via fluorescence) or LacZ. For LacZ, CHO cells were washed with PBS and then fixed with 1% glutaraldehyde in PBS for 8 min at room temperature. Cells were then stained for LacZ for ≈8 h at 37°C in 1 ml X-gal/Fe cyanide solution. After staining, cells were washed three times with PBS and visualized using a light microscope. For each well of CHO cells, 10 random images were taken and the number of GFP- or LacZ-positive cells was determined. The resulting titer of the LV-GFP and LV-Cre ranged from 1×10^8 to 3×10^8 infectious units per mL.

Behavioral Analysis. ***Open field.*** Our open-field apparatus consisted of a Plexiglas box (76 × 76 × 30 cm). Each mouse was placed in a corner of the open field under low light conditions. Each trial lasted for 10 min, with one trial per mouse. Between sessions, the maze was rinsed with 70% ethanol and dried with paper towels. Time spent in the center square (10 × 10 cm) and total distance traveled were analyzed using Any-Maze software (Stoelting).

Conditioned fear. Conditioning capabilities were evaluated using a test of pavlovian fear conditioning that included CS/US training and contextual and auditory cued components. On day 1, individual mice were carried from a holding area to the testing apparatus. The conditioned fear training/contextual testing box was a standard grid box (20.3 × 15.9 × 10.0 cm; Med Associates) with a small vial of coconut oil, which served as an olfactory cue. During training, mice were put in the box for a 4.5-min trial. Training consisted of 2 min of basal exploration followed by 28 sec of white noise and 2 sec of white noise plus a 0.7-mA foot shock. Subsequently, postshock exploration was monitored for an additional 2 min. Freezing was monitored in 5-sec bins. Every 5 sec, an observer recorded the activity of the mouse. If the mouse was inactive during the bin (defined as the absence of all activity except respiration) a +1 was recorded. If the mouse was active, a 0 was recorded. Graphs express the percent of freezing bins (i.e., +1) over the course of a testing period. After training, mice were returned to their home cage. Apparatus was washed with 70% EtOH after it was used by each mouse. Six days after training, mice were put back in the original training chamber. Freezing was monitored as before for 5 min in the absence of auditory cues. The following day, mice were put in a novel box (27.9 × 13.9 × 7.2 cm) with a small vial of peppermint oil. Basal exploration was monitored for 2 min. This was followed by a 3-min period with the original white noise turned on. Apparatus was washed with Clidox (Pharmacal) after it was used by each mouse.

Immunohistochemistry For GR, NeuN, and Cre immunohistochemistry, CeAGRKO and GFP control mice were anesthetized with 2.5% tribromoethanol and perfused transcardially with PBS solution followed by 4% PFA. Brains were then embedded in paraffin, and 8-μm coronal sections were collected.

GR and NeuN Immunohistochemistry. Nonspecific binding for GR/NeuN was blocked with 3% normal goat serum (NGS) in PBS solution. Sections were incubated with primary antibody overnight at 4°C, washed with PBS solution, incubated with secondary antibody for 60 min at room temperature (NeuN, 1:200 alexa488-conjugated goat anti-rat IgG, Jackson ImmunoResearch; GR, 1:250 biotinylated goat anti-mouse IgG, Vector Laboratories), incubated in an avidin/biotin complex reagent (Vector Laboratories) for 60 min, washed with PBS solution, and incubated in a Cy-3 conjugated tyramide signal amplification reagent (Perkin-Elmer). Immunohistochemical sections were mounted with DAPI (Vector Laboratories) and visualized using an Olympus Bx-60 Microscope.

GR and Cre Immunohistochemistry. Nonspecific binding for GR was blocked with 3% NGS in TBS. Sections were incubated with GR primary antibody overnight at 4°C, washed with TBS, incubated with secondary antibody for 60 min at room temperature (1:250 biotinylated goat anti-mouse IgG), incubated in an avidin/biotin complex reagent for 60 min, washed with TBS, and incubated in a Cy-3 conjugated tyramide signal amplification reagent. Sections were then incubated in 3% NGS with 0.25% Triton-X 100 in PBS solution for 60 min. Sections were incubated with Cre primary antibody overnight at 4°C, washed with PBS, and incubated with secondary antibody for 60 min at room temperature (1:200 alexa488-conjugated goat anti-rabbit IgG). Immunohistochemical sections were mounted with DAPI (Vector Laboratories) and visualized using an Olympus Bx-60 Microscope.

Cre and NeuN Immunohistochemistry. For Cre and NeuN, nonspecific binding for Cre/NeuN was blocked with 3% NGS with 0.25% Triton-X 100 in TBS. Sections were incubated with primary antibodies overnight at 4°C, washed with TBS, incubated with secondary antibody for 60 min at room temperature (Cre, 1:200 alexa488-conjugated goat anti-rabbit IgG; NeuN, 1:250 biotinylated goat anti-mouse IgG), incubated in an avidin/

biotin complex reagent for 60 min, washed with PBS solution, and incubated in a Cy-3 conjugated tyramide signal amplification reagent. Immunohistochemical sections were mounted with DAPI (Vector Laboratories) and visualized using an Olympus Bx-60 Microscope.

cFos Immunohistochemistry. Brains were collected under basal conditions or 60 min after the end of conditioned fear training. Mice were deeply anesthetized with 2.5% tribromoethanol and then transcardially perfused with diethylpyrocarbonate (DEPC)–treated PBS solution, followed by 4% DEPC PFA. Isolated brains were postfixed in 4% PFA for 24 h, followed by immersion in 10% sucrose in DEPC PBS solution. Tissues embedded in OCT were cut into 30-μm sections on a cryostat and stored in 0.1 M NaAzide/PBS at 4°C until use. Nonspecific binding for cFos was blocked with 3% NGS in PBS solution. Sections were incubated with cFos primary antibody (1:20,000, Calbiochem; Ab-5 rabbit anti-cFos) overnight at 4°C, washed with PBS solution, blocked again with 3% NGS in PBS solution, incubated with secondary antibody for 60 min at room temperature (1:400 biotinylated goat anti-rabbit IgG), incubated in an avidin/biotin complex reagent for 60 min, washed with Tris saline solution, and incubated in a DAB reagent. Sections were air dried, dehydrated, counterstained with 0.5% methyl green, and mounted with Cytoseal (Richard-Allan Scientific). For quantitation of cFos-positive cells in CeAGRKO and GFP controls, at least two matched sections per mouse covering the anterior–posterior extent of the CeA, dBNST, and BLA were counted for the number of bilateral cFos-positive cells.

***In Situ* Hybridization.** For CRH mRNA evaluation, mice were deeply anesthetized with 2.5% tribromoethanol and then transcardially perfused with DEPC PBS, followed by 4% DEPC PFA. Isolated brains were postfixed in 4% PFA for 24 h, followed by immersion in 10% sucrose in DEPC PBS. Tissues embedded in OCT (Sakura Finetek) were cut into 15-μm sections on a cryostat and thaw mounted onto Superfrost plus slides (Fisher Scientific). An RNA probe complementary to mRNA for CRH was radiolabeled with a^{33}P-dUTP, hybridized to sections at an annealing temperature of 60°C, and washed, after hybridization, in 0.1 $\times$ SSC at 65°C for 30 min. Slides were exposed for 6 h to 7 days to Hyperfilm Max (Amersham Biosciences). Autoradiographic images were scanned at 2400 dots per inch on an Epson 1680 Pro scanner.

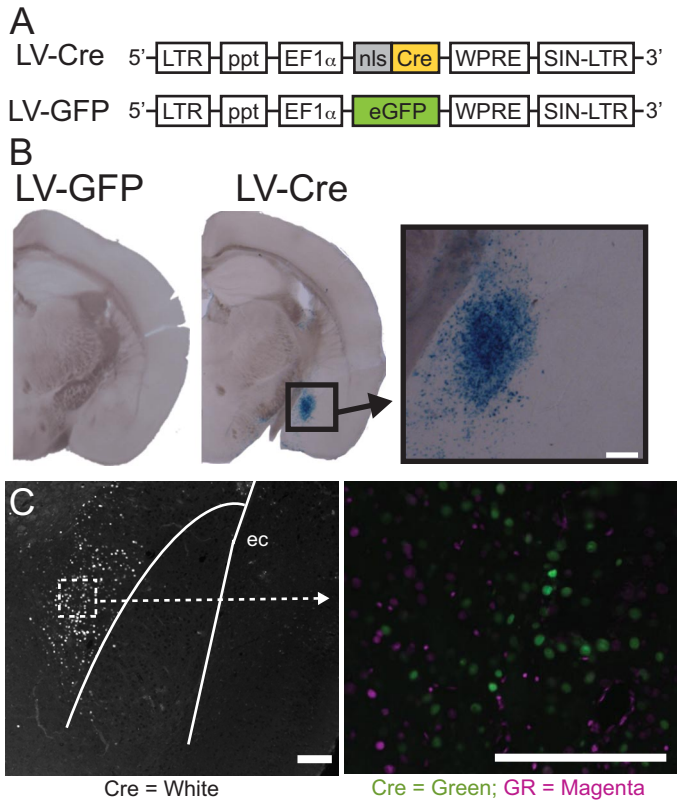

Fig. S1. Viral vectors targeting the CeA. (*A*) Illustration of lentiviral vectors containing Cre-recombinase (LV-Cre) or GFP (LV-GFP). (*B*) CeA is targeted with LV-GFP (*Left*) and LV-Cre (*Right*) in ROSA-26 reporter mice. LacZ expression (evidence of Cre) seen in CeA of LV-Cre–injected animal only (magnified CeA). (*C*) Immunohistochemical analysis of Cre and GR expression in the CeA of floxed-GR mice injected with LV-Cre. Left panel shows immunoreactivity for Cre (white cells). Prominent white matter separation (ec) of CeA and BLA shown with white line. Right panel is a magnification of the dotted white box in the left panel showing immunoreactivity for Cre (green) and GR (magenta). ppt, polypurine tract; EF1α, elongation factor 1-α; WPRE, woodchuck posttranscriptional regulatory element; SIN-LTR, self-inactivation long-terminal repeat; ec, external capsule. (Scale bars: 200 μm.)

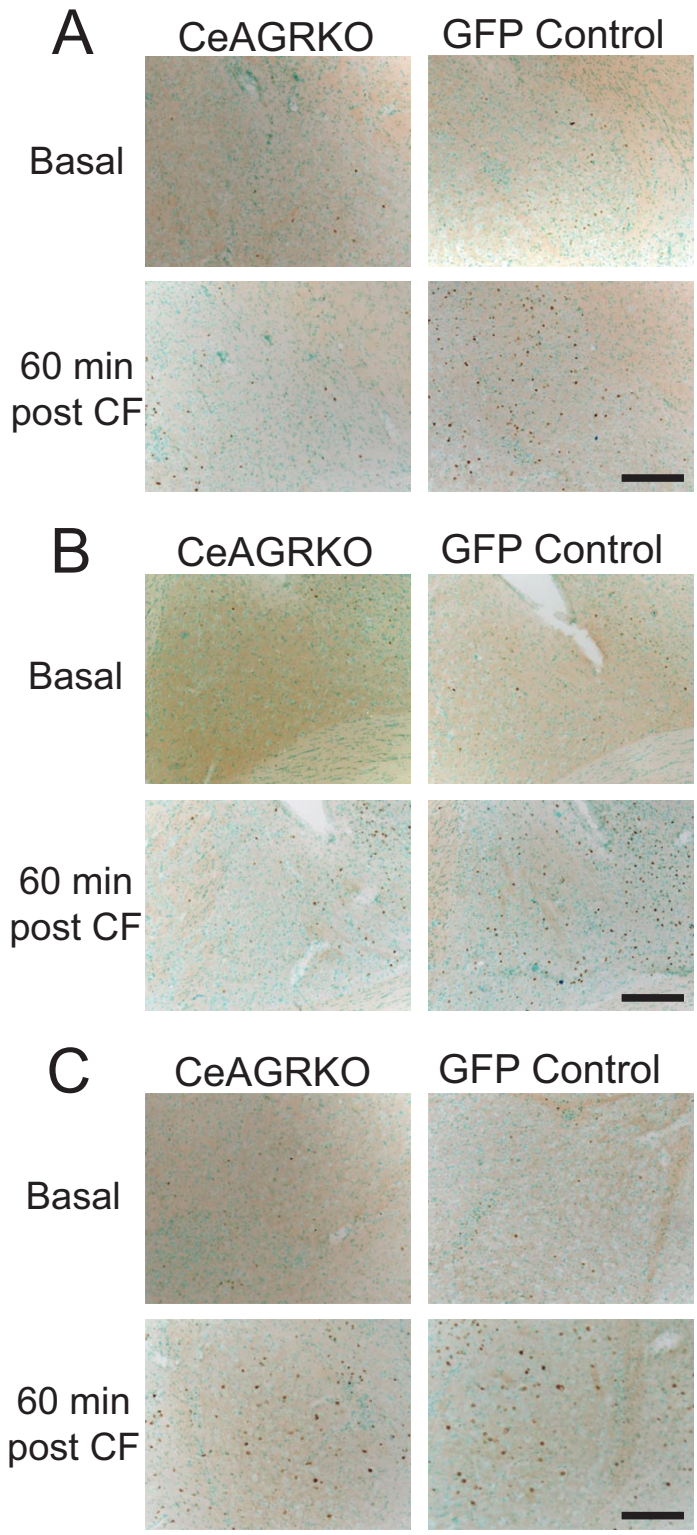

Fig. S2. cFos expression is altered in CeAGRKO mice in the CeA, BnST, and BLA. (*A*) Representative sections from the CeA show that CeAGRKO mice exhibit a reduced number of cFos-positive cells under basal conditions and 60 min following conditioned fear (CF) training compared with GFP controls. (*B*) Representative sections from the dorsal BnST (dBnST) show that CeAGRKO mice exhibit normal basal cFos expression but reduced cFos expression following conditioned fear training compared with GFP controls. (*C*) Representative sections from the BLA show that CeAGRKO mice exhibit normal basal cFos expression but reduced cFos expression following conditioned fear training compared with GFP controls. (Scale bars: 200 μm.)

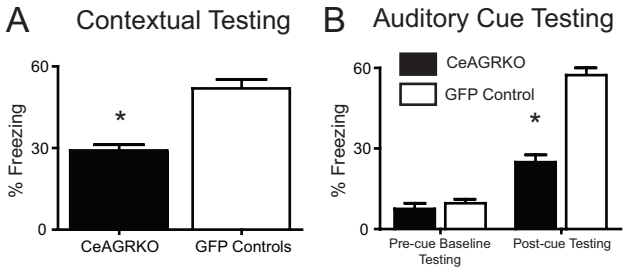

Fig. S3. ICV CRH delivered between training and testing does not rescue CeAGRKO conditioning deficit. (*A*) ICV delivery of CRH between training and testing does not cause an increase in contextual test freezing in CeAGRKO mice compared with GFP control mice. (*B*) ICV delivery of CRH between training and testing does not cause an increase in postcue auditory test freezing in CeAGRKO mice. Equivalent precue (baseline) freezing occurs in both groups. (*, $P < 0.001$ vs. GFP controls.)