Supporting Information

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SI Materials and Methods

Production and Testing of the CRFp3.0Cre GABA α1 "Floxed" Transgenic Mouse. The corticotropin releasing factor (CRF)p3.0Cre transgenic mouse (1) was crossed with the GABA α1 floxed mouse (2, 3). The Gabra1-tm1Geh targeted knock-in mice, in a mixed C57/FVB background, possess loxP sites on both sides of the α1 exon encoding an essential transmembrane domain. These mice were mated until offspring were either positive or negative for Cre and all mice were positive for floxed GABA(A) α1, as verified by PCR. Cre^- littermates were used as controls in all experiments. Normal expression of the GABA(A) α1 gene is observed in mice that are homozygous for this floxed allele, and these mice do not display any deficiencies in GABA(A) α1 receptor. In the presence of Cre recombinase (CRE), the transmembrane domain of the *GABA(A) α1* gene is deleted, resulting in a nonfunctional α1 subunit (2).

In Situ Hybridization. We subcloned the α1 subunit for use as an in situ riboprobe. This plasmid was constructed by TOPO subcloning of a cDNA PCR product using the following customized primers: α1 sense, GGAGTGACGACTGTTCTGACTATG; α1 antisense, TTCTGGAACCACGCTTTTG (Sigma). Plasmid DNA sequencing (Iowa State University, Ames, IA) confirmed the subclone sequence and orientation. The resulting transcript corresponds to nucleotides 1,307–1,509 of the GABA(A)α1 cDNA, which is flanked by loxP sites and deleted by CRE recombinase. These nucleotides encode amino acids included in the second transmembrane domain and intercellular loop between transmembranes 3 and 4 (4).

Brains were rapidly removed following a lethal dose of anesthesia, sectioned at 20 μM, and placed onto SuperFrost Plus slides which were then stored at −80 °C until processing. The 35S-UTP labeled riboprobes were prepared from linearized clones as previously described and isolated using G50 Sephadex Quick Spin Columns (Roche Diagnostics) and diluted to a concentration of 100,000 cpm/lL in hybridization buffer consisting of 50% (vol/vol) deionized formamide, 10 mm DTT, 20 mm Tris, 300 mm sodium chloride, 5 mm EDTA, 10% (vol/vol) dextran sulfate, 1% Denhardt's solution, 0.5 mg/mL yeast RNA, and 10 mm NaH_2PO_4. Sections were then incubated overnight in humid chambers at 50 °C with 100 mL per slide of hybridization buffer covered with a Parafilm cover-slip. Following hybridization, slides were incubated in 50 °C 5× standard sodium citrate (SSC) until the cover-slips floated off. Sections were washed in 5× SSC/10 mm DTT at 50 °C for 30 min and treated with 50% (vol/vol) formamide/2× SSC/10 mm DTT at 56 °C for 30 min. Slides were next washed twice in wash buffer (Tris 10 mm, EDTA 5 mm, NaCl 100 mm) for 10 min at 37 °C, then treated with RNase A (20 mg/mL) in wash buffer for 30 min at 37 °C. Sections were dehydrated for 2 min in 30%, 50%, 70%, 85%, and 95% (vol/vol) ethanol containing 300 mm ammonium acetate, followed by 100% ethanol. Slides were air dried and exposed to Biomax MR autoradiographic film (Eastman Kodak) for 1–5 d.

FISH. Sections were prepared as for in situ hybridization and using the same probes as those from in situ hybridization experiments with the exception that FITC (CRF) and DIG (α1) RNA labeling mix (Roche) was used instead of S35. After hybridization at 65 °C for at least 12 h, slides were washed twice with 5× SSC buffer at 50 °C followed by a wash with 50% formamide/2× SSV buffer at 56 °C followed by RNase treatment and a series of washes. Sections were then blocked with 1% TNB buffer (1% BSA in TN) for 30 min, treated with peroxidase followed by anti-DIG antibody (1:500 dilution; Roche) for 1.5 h. Sections were rinsed and treated with Cy3 antibody (1:50 dilution; Roche) for 30 min in the dark. After further rinses, sections were similarly treated with anti FITC antibody (1:500 dilution; Vector Laboratories) followed by FITC tyramide (1:50) for amplification in a humid chamber in the dark. Finally, sections were stained with DAPI and cover-slipped with Vectashield (Vector Laboratories).

Stereotaxic Surgery. Mice were anesthetized by injections of Ketamine-Dormitor anesthesia before surgery. Mice used for electrophysiological recordings received bilateral microinjections of 0.5 μL of Cre-dependent floxed mCherry reporter virus via Hamilton microsyringe lowered to the following coordinates from bregma based on the mouse brain atlas of ref. 5: AP = +0.5, ML = ±2.1, DV = −4.2 and virus was infused over 10 min. Animals were sutured and remained in the home cage for at least 20 d before recording took place. Procedures were identical for cannulae placement with the exception that cannulae were lowered to the same bed nucleus of the stria terminalis (BNST) coordinates for later drug delivery. After surgery all animals received postoperative analgesic for 3 d and as needed.

Elevated Plus Maze. The elevated plus maze consisted of four arms (30 × 5 cm) elevated 50 cm from the floor. Lighting was provided by two 40-W fluorescent tubes in an overhead fixture providing 50 lx for closed arms and 150–200 lx for open arms. Each animal was placed into the center area of the elevated plus maze facing a closed arm and was permitted to explore the maze for a 5-min period. During this period, three measures are recorded: the number of open arm entries, the total number of arm entries, and the total time spent in the open arms. When the CRF antagonist R121919 was used it was given at 30 mg/kg 40 min before testing.

Open Field. The same animals that were tested in the elevated plus maze were tested in open field. Open-field behavior was conducted using MED PC open field boxes. The open field consisted of an arena in which the central zone was defined as the center compartment of the floor located 6 cm from the perimeter of the chamber walls. Activity was monitored using 24-beam infrared arrays across the base of each chamber wall (MED Associates, model OFA-MS). Activity data were collected via computer and analyzed with the MED Associates' Activity Monitor Data Analysis software. Over the 10-min test session multiple measures were recorded, including distance traveled and time in center and surround with a 50-ms resolution. All testing was conducted under standard room lighting conditions.

Baseline Startle. These animals were next tested in baseline startle in eight identical startle chambers (SR-LAB, San Diego Imaging). Mice were placed in the testing cylinder and after 5 min were given 10 startle stimuli at each of four different startle stimulus intensities (90, 100, 110, 120 dB) with an interstimulus interval of 30 s. All startle stimuli are presented in a pseudorandom sequence with the constraint that each stimulus intensity occurs only once in each consecutive four-trial block for a total of 10 trial blocks. The mean startle amplitudes were calculated for each mouse by computing the average startle response at each of four different startle stimulus intensities.

Fear Conditioning, Shock Reactivity, and Extinction. Animals that were previously tested in baseline startle and tail suspension were next tested for cue-dependent fear conditioning and extinction. All groups received five conditional-stimulus tones (30 s, 6 kHz, 74 dB) coterminating with unconditioned-stimulus shocks (500 ms, 0.6 mA) with a 5-min inter trial interval (ITI). Shock reactivity

data were collected from movement recorded by an accelerometer that occurred during the shocks delivered at training. The average of these values was compared across groups. During extinction training and testing mice received 30 conditioned-stimulus tones (30 s, 6 kHz, 74 dB) with a 90-s ITI in a novel context. The novel context differed in size, shape and lighting from the training context. In experiments in which the CRF antagonist R121919 was used, it was given 40 min before extinction at 30 mg/kg. Freezing was measured throughout training and testing using Freezeframe software (Coulbourn Instruments).

Plasma Hormone Measurement. Mice were fear-conditioned and received extinction as before. Thirty minutes after extinction training, brains were removed and trunk blood was collected into EDTA lined chilled tubes, centrifuged (7.6 rpm as $5.4 \times g$, 20 min, 4 °C), and plasma was collected and stored at −20 °C. Measurement of corticosterone concentrations was done by radioimmunoassay (MP Biomedicals) in quadruplicate.

Single-Cell RT-PCR. The procedure used to determine mRNA transcript expression in single cells has been described in detail elsewhere (1, 6). Briefly, the cytoplasm from neurons was aspirated into pipettes containing 5 μL of RNasefree patch solution by applying gentle negative pressure. The content of the patch pipette were then expelled into a microcentrifuge tube containing 5 μL of the reverse-transcription master mix, and then incubated in a thermal cycler (PTC-200 Peltier; MJ Research). Reverse transcription was performed in a final volume of 10 μL and stored at −20 °C before further processing. Reverse-transcriptase product was amplified in triplicate (6). Amplified cDNA from each cell was first screened for the expression of 18S rRNA as a positive control. Amplified cDNA was the subjected to another amplification step using 2 μL of cDNA from each cell as a template and 100 nM of GABAα1primer (accession no. AY574250, 511 bp). GABAα2 primer (accession no. P23576, 434bp) and GABAβ1primer (accession no. P15431, 397bp). The PCR master mix was comprised of 10× PCR Buffer, 3 mM MgCl2, 10 mM dNTPs, 2.5 U of Taq DNA Polymerase in a final volume of 20 μL. PCR was performed using a 10-min hot start at 95 °C followed by a 40-cycle program for 18S rRNA and GABAα1 (94 °C, 40 s; 56 °C, 40 s; 72 °C, 1 min). PCR products were separated by electrophoresis in 1% agarose gel and visualized by staining with ethidium bromide.

Electrophysiological Recordings. Mice were anesthetized by injections of Ketamine-Dormitor anesthesia before surgery. Mice used for electrophysiological recordings received bilateral microinjections of 0.5 μL of Cre-dependent floxed mCherry reporter virus [AAV5-doublefloxed-hChR2(H134R)-mCherry-WPRE-pA; gift of Karl Deisseroth, Stanford University, Stanford, CA], via Hamilton microsyringe lowered to the following coordinates from bregma based on the mouse brain atlas of ref. 5: AP = +0.5, ML = ±2.1, DV = −4.2 and virus was infused over 10 min. Animals were sutured and remined in the home cage for at least 20 d before recording took place. Procedures were identical for cannulae placement with the exception that cannulae were lowered to the same BNST coordinates for later drug deliver. After surgery all animals received postoperative analgesic for 3 d.

Mice were decapitated under deep isoflurane anesthesia (Abbott Laboratories), brains were rapidly removed, and BNST slices were obtained as previously described (7). Coronal slices (300 μm in thickness) containing BNST were cut using a Leica VTS-1000 vibrating microtome (Leica Microsystems) and incubated in artificial cerebrospinal fluid at room temperature.

BNST neurons were visualized using a Leica DMLFSA microscope equipped with infrared illumination and a 40× water-immersion objective (Leica Microsystems). ChR2mCherry CRE^+ neurons were selected under fluorescent illumination. Images were captured with a Hamamatsu Orca ER CCD camera (Hamamatsu) controlled by SimplePCI software (Compix). The selected cells were then visualized with infrared illumination and targeted for recordings. Whole-cell patch clamp recordings were obtained with standard techniques as previously reported (7) using Multiclamp700B amplifier, Digidata 1320A A–D interface, and pClamp 10.0 software (Molecular Devices). Standard protocols were used to determine the membrane properties.

We recorded from CRF-expressing neurons in floxed GABAα1 mice (identified by red fluorescence in CRF-Cre mouse infected with floxed stop AAV-mCherry virus); CRF^- neurons in these same mice (identified by nonfluorescence in the above AAV-invected CRF-Cre^+ mice); and separately in CRF neurons that express wild-type GABAα1 (identified as GFP+ cells in Crf-Cre^+ mice crossed with floxed-stop-GFP–expressing mice). Evoked inhibitory postsynaptic currents (IPSCs) were recorded by local stimulation with a concentric electrode in the presence of NMDA antagonist RS-CPP (10 μM), AMPA antagonist DNQX (20 μM), and GABAB antagonist CGP94532 (1 μM). To determine the reversal potential of IPSCs, membrane potentials were moved from −50 mV to −80 mV with 5-mV step. We used a short-term plasticity protocol to see whether GABA(A)α1 subunit deletion changes short-term plasticity. Here, five trains of five pulses at frequencies of 10 and 20 Hz were delivered every 5 s to induce IPSCs. To analyze, all IPSCs amplitudes were normalized to the first IPSC. A two-way ANOVA test and Bonferroni posttests were used to determine the effect of treatment and stimulation.

CRF Cre Line Controls. To control for the possibility that the CRF cells expressing Cre recombinase were affected independently of *GABA(A)α1* gene expression, we analyzed whether CRF Cre mice that were not crossed with the floxed *GABA(A)α1* strain showed any differences in anxiety, fear conditioning, or extinction. As shown in Fig. S1 *A* and *B*, CRF Cre^- ($n = 10$) and CRF Cre^+ ($n = 13$) mice did not show any differences in plus-maze behavior [time in open arm [$F(1, 21) = 0.503$, $P > 0.05$] or distance traveled [$F(1, 21) = 1.346$, $P > 0.05$)]. Animals were also tested in baseline startle [CRF Cre^- ($n = 11$) and CRF Cre^+ ($n = 14$)] and no differences were found at any decibel level tested (Fig. S3*C*) (all $P > 0.05$). A separate cohort of CRF Cre^+ ($n = 6$) and CRF Cre^- ($n = 10$) mice were fear-conditioned. Repeated-measures ANOVA revealed a significant increase in freezing over time during training [$F(1, 4) = 36.751$, $P < 0.01$] but no significant difference between groups [$F(1, 14) = 0.112$, $P > 0.05$], indicating animals trained equivalently (Fig. S1*D*). As shown in Fig. S1 *E* and *F*, these same animals were extinguished and tested and showed no significant differences between the groups during extinction [$F(1, 14) = 0.020$, $P > 0.05$] or extinction testing [$F(1,14) = 0.246$, $P > 0.05$]. These findings indicate that the significant behavioral changes we see when the CRF Cre animals are crossed with the floxed *GABA(A)α1* strain are a result of the interaction between Cre recombinase and loxP, leading to deletion of the *GABA(A)α1* gene in the CRF-expressing neurons.

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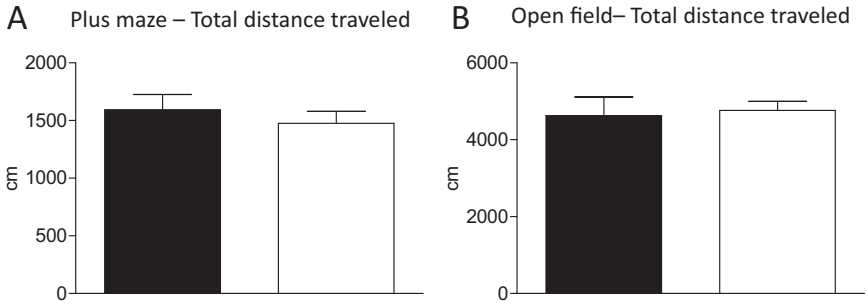

Fig. S1. (*A*) No difference was seen in total distance traveled in the plus maze. (*B*) Similarly, no difference was shown in total distance traveled in the open field.

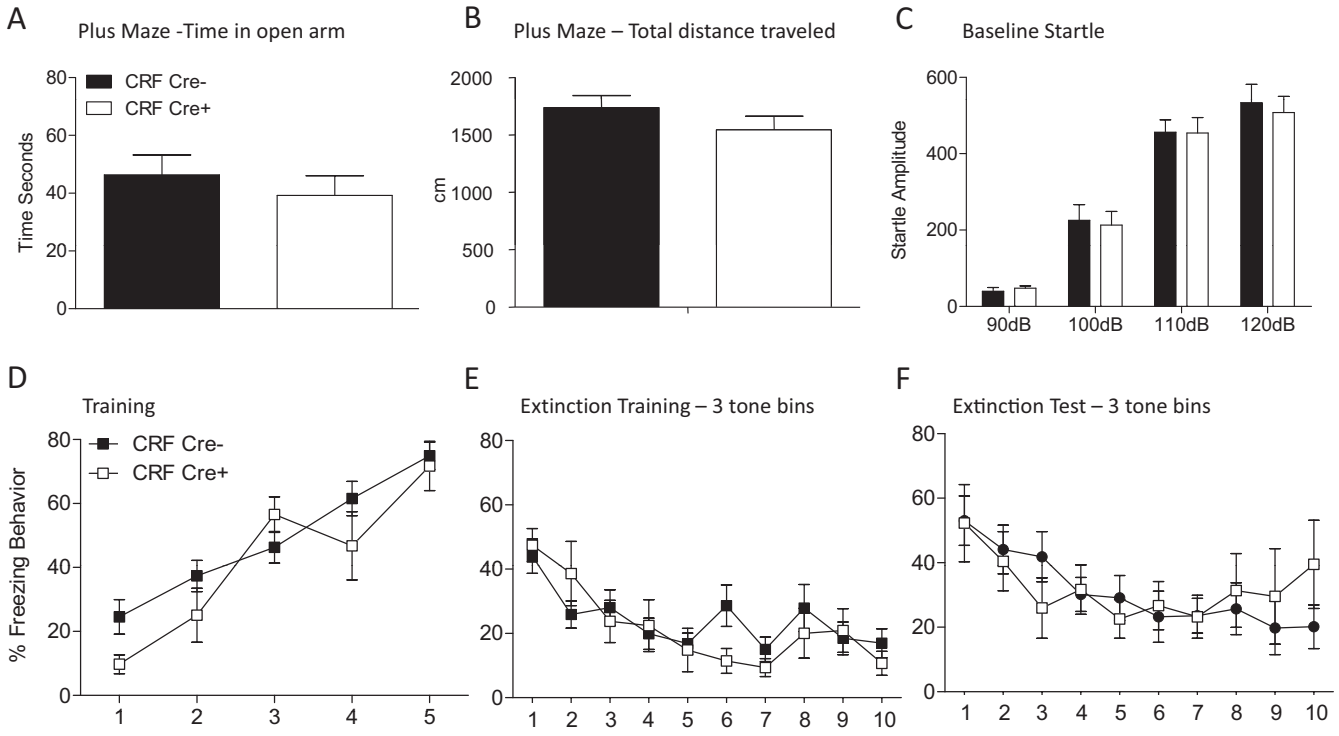

Fig. S2. CRF-Cre mice without floxed *GABA(A)α1* KO show equivalent anxiety and fear training and extinction behaviors. CRF-Cre^{-} (black bars, black square) and CRF-Cre^{+} (white bar, white square) animals show no differences on (*A*) the plus maze, (*B*) distance traveled in the plus maze, (*C*) baseline startle, and (*D*) fear conditioning or extinction behavior during either (*E*) extinction training or (*F*) testing.

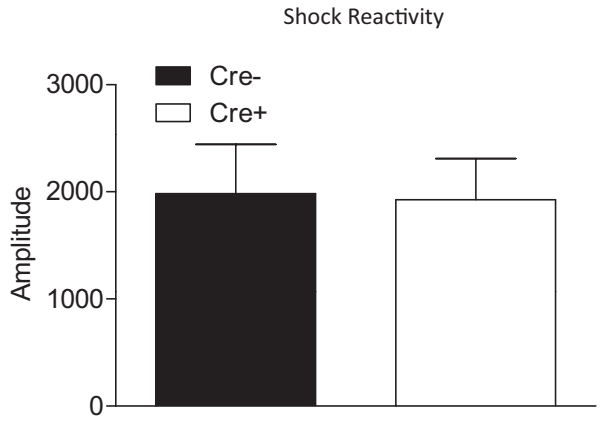

Fig. S3. Shock reactivity was measured from the mean displacement of the accelerometers in response to delivery of the shock. No differences were found in shock reactivity between groups during training indicating that Cre$^-$ (black bar) and CRF *GABA(A)α1* KO (Cre$^+$, white bar) animals had no gross differences in sensitivity to shock. Bars represent the mean of the densitometry measurements (± SEM).

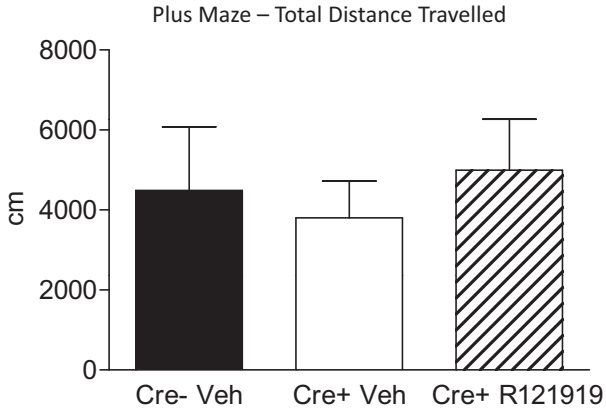

Fig. S4. The Cre$^-$ Vehicle (black bar), Cre$^+$ Vehicle (white bar) and R121919 (striped bar) groups did not differ in total distance traveled during the plus-maze test, indicating there were no differences in general locomotion between groups. Bars represent the mean of the densitometry measurements (± SEM).

Table S1. Membrane properties of GABA(A)α1 KO neurons

					Spike					
	n	RMP (mv)	Rm (MΩ)	τ (ms)	Amplitude (mV)	Fast AHP (mV)	Half-width (ms)	Threshold (mV)	Rise time (ms)	Decay time (ms)
CRF Cre KO mCherry$^-$	6	−58.2 ± 1.74	415 ± 65	22 ± 3.2	72 ± 5.4	−8.99 ± 2.48	0.75 ± 0.05	−33.4 ± 2.99	0.35 ± 0.15	0.77 ± 0.06
CRF Cre KO mCherry$^+$	7	−64 ± 2.0*	480 ± 73	23.4 ± 2.8	71 ± 0.9	−10.0 ± 1.26	0.88 ± 0.07	−32.9 ± 1.93	0.39 ± 0.02	0.96 ± 0.13
CRF Cre mCherry$^+$	4	−60.25 ± 2.3	261 ± 50	13.9 ± 1.8	73 ± 1.8	−7.8 ± 0.66	0.99 ± 0.02	−31.67 ± 1.24	0.42 ± 0.02	1.06 ± 0.04
CRF Cre GFP$^+$	15	−67.4 ± 0.44	337 ± 20	26.7 ± 1.6	70.3 ± 1.2	−4.96 ± 0.45	1.10 ± 0.03	−33.6 ± 0.5	0.45 ± 0.01	1.15 ± 0.04

Basic membrane properties of CRF *GABA(A)α1* KO mCherry$^+$ (CRF Cre KO mCherry$^+$), CRF *GABA(A)α1* KO mCherry$^-$ (CRF Cre KO mCherry$^-$), Crf Cre mCherry (CRF Cre mCherry), and CRF Cre GFP$^+$ BNST neurons were compared. Compared with nonfluorescing nearby neurons [CRF *GABA(A)α1* KO mCherry$^-$], neurons from animals that received mCherry virus but did not have the *GABA(A)α1* deletion (CRF Cre mCherry) or neurons that had endogenous CRF-tagged GFP (CRF Cre GFP) the physiological properties of CRF Cre KO mCherry$^+$ neurons were not significantly different from neurons that were CRF Cre KO mCherry$^-$ neurons, with the exception that Cre KO mCherry$^+$ neurons had a more hyperpolarized resting membrane potential (*$P < 0.05$). These recordings also revealed properties of visualized CRF Cre GFP$^+$ BNST neurons were not significantly different from CRF Cre KO mCherry$^+$ BNST neurons. No significant difference was found between these neurons, suggesting CRF *GABA(A)α1* KO did not change the intrinsic physiological properties of CRF neurons.