

# Cell-type specific deletion of *GABA(A) $\alpha$ 1* in corticotropin-releasing factor-containing neurons enhances anxiety and disrupts fear extinction

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Corticotropin-releasing factor (CRF) is critical for the endocrine, autonomic, and behavioral responses to stressors, and it has been shown to modulate fear and anxiety. The CRF receptor is widely expressed across a variety of cell types, impeding progress toward understanding the contribution of specific CRF-containing neurons to fear dysregulation. We used a unique CRF-Cre driver transgenic mouse line to remove floxed *GABA(A) $\alpha$ 1* subunits specifically from CRF neurons [CRF-*GABA(A) $\alpha$ 1* KO]. This process resulted in mice with decreased *GABA(A) $\alpha$ 1* expression only in CRF neurons and increased CRF mRNA within the amygdala, bed nucleus of the stria terminalis (BNST) and paraventricular nucleus of the hypothalamus. These mice show normal locomotor and pain responses and no difference in depressive-like behavior or Pavlovian fear conditioning. However, CRF-*GABA(A) $\alpha$ 1* KO increased anxiety-like behavior and impaired extinction of conditioned fear, coincident with an increase in plasma corticosterone concentration. These behavioral impairments were rescued with systemic or BNST infusion of the CRF antagonist R121919. Infusion of Zolpidem, a *GABA(A) $\alpha$ 1*-preferring benzodiazepine-site agonist, into the BNST of the CRF-*GABA(A) $\alpha$ 1* KO was ineffective at decreasing anxiety. Electrophysiological findings suggest a disruption in inhibitory current may play a role in these changes. These data indicate that disturbance of CRF containing *GABA(A) $\alpha$ 1* neurons causes increased anxiety and impaired fear extinction, both of which are symptoms diagnostic for anxiety disorders, such as posttraumatic stress disorder.

central amygdala | plasticity | HPA axis | short term synaptic depression | knockout

The neuropeptide corticotrophin-releasing factor (CRF) is involved in initiation of the endocrine, autonomic, and behavioral responses to stressors and its dysfunction is implicated in a variety of mood and anxiety disorders (1). Large populations of CRF-containing neurons are located within brain areas crucial for fear and anxiety, including the central amygdala (CeA), bed nucleus of the stria terminalis (BNST), and paraventricular nucleus of the hypothalamus (PVN). Although site-specific and conditional knockout approaches are increasingly being used (2, 3), the broad expression of CRF receptors and the varied cell types in which the CRF peptide is produced (4, 5) make it challenging to identify region and cell-type specific influences of CRF. One way to manipulate specific subpopulations of CRF neurons is to use mice with the Cre recombinase gene driven by the CRF promoter to allow different subgroups of CRFergic cells to be manipulated.

The interaction between CRF and GABA activity in brain structures important for fear and anxiety has been identified as a potential mechanism underlying anxiety disorders (6). CRF administration increases GABA release within the amygdala (7, 8) and causes deficits in *GABA(A)* receptor-mediated inhibitory transmission (9). Lifelong CRF overexpression leads to changes in *GABA* receptor subtype expression and sensitivity (10). Furthermore, GABAergic disinhibition or CRF excitation leads to long-term synaptic plasticity and increased excitability of neurons within the amygdala (11, 12). In the BNST, CRF application

enhanced *GABA(A)*-mediated transmission via postsynaptic activation of CRF1 receptors (13). The BNST is rich in GABA and CRFergic input originating from the CeA (14). Furthermore, CRF (15, 16) and GABA (17) signaling in the BNST are involved in anxiety. GABAergic neurons within the BNST also regulate the hypothalamic-pituitary-adrenal axis (HPA) responding to acute stress (18).

The actions of GABA receptors are defined by their component subunits. Research has pointed to an important role for the  $\alpha$ 1 subunit of the *GABA(A)* receptor in anxiety and fear memory. Fear training decreases *GABA(A) $\alpha$ 1* subunit containing neurons (19) and disruption of the *GABA(A) $\alpha$ 1* subunit receptor leads to enhanced synaptic plasticity in the lateral nucleus of the amygdala and enhanced auditory fear memory (20). *GABA(A) $\alpha$ 1* neurons can be found within the amygdala (21), BNST, and PVN (22), areas that also strongly express CRF. No studies to date have addressed the role of CRF containing *GABA(A) $\alpha$ 1* neurons in fear and anxiety disorders.

In the present study we combined mice with the *GABA(A) $\alpha$ 1* subunit flanked by loxP sites (floxed) with our previously developed CRFp3.0Cre line (23), resulting in a CRF neuron-specific deletion of the *GABA(A) $\alpha$ 1* gene [CRF *GABA(A) $\alpha$ 1* KO]. These animals were then examined in a variety of behavioral and physiological tests. Mice with CRF *GABA(A) $\alpha$ 1* KO were found to have enhanced anxiety-like behavior. Although these mice fear-conditioned normally, their extinction behavior was disrupted compared with littermate controls. Both the disrupted extinction and increased anxiety were rescued with administration of a CRF antagonist delivered systemically or given into the BNST. These animals also showed a lack of responsiveness to BNST administration of a *GABA(A) $\alpha$ 1* selective benzodiazepine. Electrophysiological findings indicate CRF *GABA(A) $\alpha$ 1* KO mice have a selective deficit in short-term synaptic plasticity. Taken together, these data show that disrupting this neuronal subtype affects baseline anxiety, fear extinction, and the electrophysiological response properties of these cells.

## Results

**Expression of Cre, CRF, and *GABA(A) $\alpha$ 1*.** CRF *GABA(A) $\alpha$ 1* KO and littermate Cre<sup>-</sup> control mice were compared at baseline using fluorescent in situ hybridization (FISH). Visual inspection of FISH-labeled sections (Fig. 1A–F) revealed that CRF-expressing neurons from CRF *GABA(A) $\alpha$ 1* KO mice were more often found without *GABA(A) $\alpha$ 1* mRNA compared with Cre<sup>-</sup> controls (Fig.

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1). Observation of in situ demonstrated decreased double-labeling in the BNST, PVN, and the CeA in the CRF *GABA(A) $\alpha$ 1* KO (Fig. 1 *G–L*) compared with Cre<sup>-</sup> mice.

Quantitative analyses were also performed with in situ hybridization (Fig. 1 *M–X*). Expression levels in the BNST (Fig. 1 *M–O*), PVN (Fig. 1 *Q–S*), and CeA (Fig. 1 *U–W*) from CRF *GABA(A) $\alpha$ 1* KO (Cre<sup>+</sup>) and Cre<sup>-</sup> animals were normalized to the same structure and compared using one-way ANOVA. Results from the BNST (Fig. 1*P*) [Cre<sup>-</sup> *n* = 8; Cre<sup>+</sup> *n* = 6; *F*(1, 12) = 6.654, *P* < 0.05]; PVN (Fig. 1*T*) [Cre<sup>-</sup> *n* = 8; Cre<sup>+</sup> *n* = 6; *F*(1, 12) = 6.464, *P* < 0.05], and CeA (Fig. 1*X*) [Cre<sup>-</sup> *n* = 7; Cre<sup>+</sup> *n* = 8; *F*(1, 13) = 7.192, *P* < 0.05] showed that CRF *GABA(A) $\alpha$ 1* KO animals expressed significantly more CRF mRNA under baseline conditions.

**Anxiety-Like Behavior.** We conducted a series of tests to determine whether baseline differences existed in anxiety-like behavior between Cre<sup>-</sup> (*n* = 6) and CRF *GABA(A) $\alpha$ 1* KO (Cre<sup>+</sup>, *n* = 9) mice (Fig. 2). One cohort of animals was examined on the elevated plus maze followed by an open-field test the following day. Time in the open arm of the plus maze was measured over 5 min. As shown in Fig. 2*A*, CRF *GABA(A) $\alpha$ 1* KO animals showed significantly decreased time in the open arm compared with Cre<sup>-</sup> controls [*F*(1, 13) = 6.920, *P* < 0.05]. There was no significant difference between Cre<sup>-</sup> and CRF *GABA(A) $\alpha$ 1* KO animals in total distance traveled in the plus maze (Fig. S1*A*), indicating the increased time on the open arm of the plus maze is not because of differences in general locomotor activity [*F*(1, 13) = 0.055, *P* > 0.05]. We also compared Cre<sup>-</sup> and CRF *GABA(A) $\alpha$ 1* KO animals in time spent in the center of an open field over 15 min (Fig. 2*C*). CRF *GABA(A) $\alpha$ 1* KO animals spent significantly less time in the center portion of the open field compared with Cre<sup>-</sup> mice [*F*(1, 13) = 8.246, *P* < 0.05]. However, no significant difference in the total distance traveled was shown between the groups, demonstrating general activity was similar (Fig. S1*B*) [*F*(1, 13) = 0.075, *P* > 0.05].

Animals also showed increased anxiety behavior when baseline startle was measured (Fig. 2*B*). Animals were exposed to 90-, 100-, 110-, and 120-dB noise bursts. No significant difference was seen at 90 dB [*F*(1, 13) = 1.512, *P* > 0.05] or 100 dB [*F*(1, 13) = 1.250, *P* > 0.05]. At higher decibel levels, 110 [*F*(1, 13) = 6.207, *P* < 0.05] and 120 dB [*F*(1, 13) = 5.601, *P* < 0.05] CRF *GABA(A) $\alpha$ 1* KO mice showed higher startle amplitude compared with Cre<sup>-</sup> animals further supporting disruption of CRF *GABA(A) $\alpha$ 1* as important for anxiety behavior. CRF Cre mice without floxed *GABA(A) $\alpha$ 1* KO showed no difference in anxiety measures compared to controls, indicating that the presence of the promoter alone did not effect anxiety behavior (Fig. S2 *A–C*).

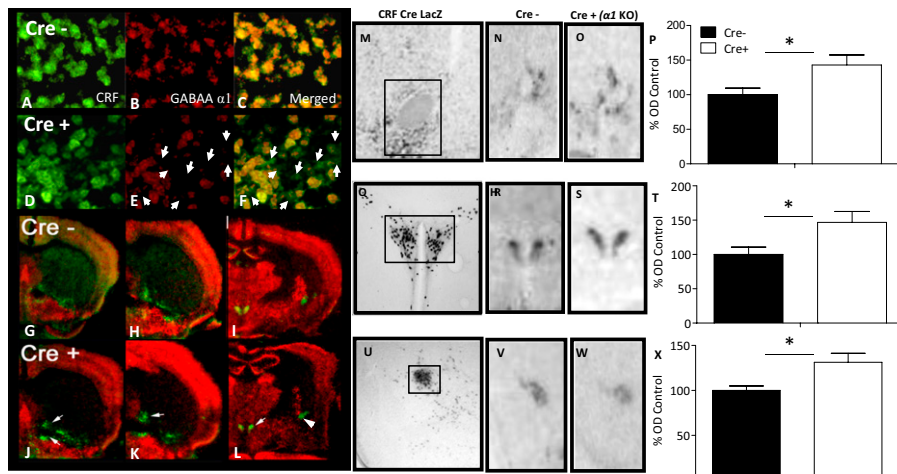
**Depression-Like Behavior.** Because CRF has been shown to play a role in depression, mice that were previously tested in baseline startle were assessed for differences in the tail suspension test, a common measure of depressive-like behavior. Using time spent immobile as a measure of depressive behavior, we found no significant differences between Cre<sup>-</sup> and CRF *GABA(A) $\alpha$ 1* KO mice [*F*(1, 13) = 0.054, *P* > 0.05], suggesting these mice do not have a depressive phenotype (Fig. 2*D*).

**Fear Conditioning and Extinction.** Fear conditioning and extinction were conducted with Cre<sup>-</sup> (*n* = 5) and CRF *GABA(A) $\alpha$ 1* KO (Cre<sup>+</sup>, *n* = 8) mice (Fig. 3). Animals were trained with five tone-shock pairings. Repeated-measures ANOVA with Trial and Genotype as factors showed that there was a significant main effect for Trial [*F*(4, 44) = 17.766, *P* < 0.05] indicating freezing increased over time during training. There was no interaction between Trial and Genotype [*F*(4, 44) = 1.896, *P* > 0.05] nor was there any difference between groups during training [*F*(1, 11) = 0.341, *P* > 0.05] or in shock reactivity (Fig. S3) [*t*(11) = 0.408, *P* = 0.52] between groups, indicating that Cre<sup>-</sup> and CRF *GABA(A) $\alpha$ 1* KO animals fear-conditioned equivalently and there were no differences in shock sensitivity.

Animals were extinguished with 30 tone presentations in the absence of a foot shock. To test for differences in memory consolidation, a repeated-measures ANOVA was conducted on freezing behavior during the first six tone presentations (Fig. 3*B*), with Trial and Genotype as factors. There was no significant main effect [*F*(4, 44) = 2.504, *P* > 0.05], interaction [*F*(4, 44) = 0.472, *P* > 0.05], or group effect [*F*(1, 11) = 4.178, *P* > 0.05], indicating no effect on fear memory consolidation. The same analysis was conducted across the complete 30-tone extinction session. Data were separated into five blocks of six trials with Block and Genotype entered as factors (Fig. 3*C*). Data from the extinction training session revealed a significant main effect for Block [*F*(4, 44) = 4.162, *P* < 0.05] with no Block-by-Genotype interaction [*F*(4, 44) = 0.846, *P* > 0.05]. The significant effect on Block indicates animals froze significantly less over extinction trials. We also found a significant between-subjects effect of Genotype [*F*(1, 11) = 21.834, *P* < 0.05] driven by CRF *GABA(A) $\alpha$ 1* KO mice freezing significantly more than Cre<sup>-</sup> mice during extinction training.

We conducted 2 d of 15-tone extinction tests. For both days the data were analyzed as during extinction training, except that data were separated into five blocks of three trials. Repeated-measures ANOVA on extinction test day 1 with Block and Genotype as factors showed there was a significant main effect of Block [*F*(4, 44) = 4.498, *P* < 0.05], Block-by-Genotype interaction [*F*(4, 44) = 7.177, *P* < 0.05] and a between-subjects group effect [*F*(1, 11) = 7.696, *P* < 0.05] (Fig. 3*D*).

**Fig. 1.** (*A–L*) In situ hybridization conducted on Cre<sup>-</sup> and CRF *GABA(A) $\alpha$ 1* (Cre<sup>+</sup>) mice under basal conditions. Cre<sup>-</sup> and Cre<sup>+</sup> CRF (green, *A* and *D*), *GABA(A) $\alpha$ 1* and (red, *B* and *E*) merged CRF and *GABA(A) $\alpha$ 1* (*C* and *F*) FISH images from the PVN. White arrows (*E* and *F*) indicate areas of CRF staining without coexpression of *GABA(A) $\alpha$ 1* in Cre<sup>+</sup> mice. Pseudocolor images from autoradiographic in situ hybridization of CRF (green) and *GABA(A) $\alpha$ 1* (red) in the BNST (*G*, *H*, *J*, and *K*), PVN and CeA (*I* and *L*) of Cre<sup>-</sup> and Cre<sup>+</sup> mice. Arrows indicate areas of more intense CRF staining in Cre<sup>+</sup> animals compared with Cre<sup>-</sup> mice. (*M–X*) LacZ staining with X-Gal demonstrates functional CRF activity in the BNST (*M*), PVN (*Q*), and CeA (*U*) of a CRFp3.0CreLacZ mouse. The box indicates the area sampled for data acquisition. Representative expression of CRF from Cre<sup>-</sup> (*N*) BNST, (*R*) PVN, (*V*) CeA and Cre<sup>+</sup> (*O*) BNST, (*S*) PVN, and (*W*) CeA in situ hybridization. Data from densitometry conducted on in situ hybridization shows increased CRF in (*P*) BNST, (*T*) PVN, and (*X*) CeA. Bars represent the mean of the densitometry measurements ( $\pm$  SEM) in all figures. \**P* < 0.05.

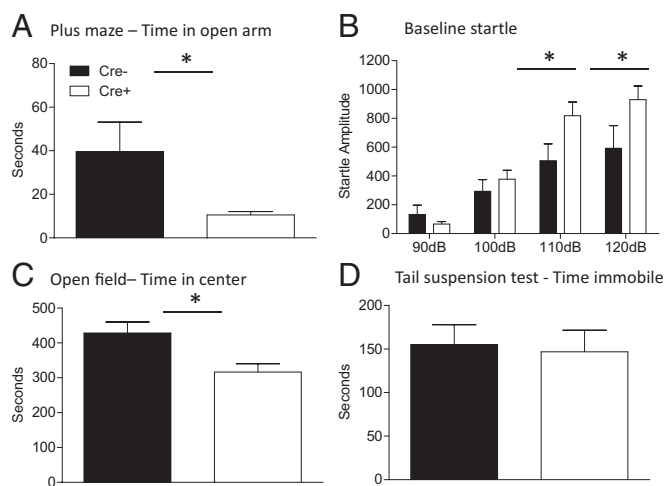


Data from the second extinction test (Fig. 3E) showed no significant main effect of Block [ $F(4, 44) = 0.959, P > 0.05$ ] nor a Block-by-Genotype interaction [ $F(4, 44) = 1.266, P > 0.05$ ]; however, there was a significant between-subjects group effect [ $F(1, 11) = 7.475, P < 0.05$ ], again driven by CRF *GABA(A) $\alpha$ 1* KO mice freezing significantly more than Cre<sup>-</sup> mice during extinction training. CRF Cre mice without floxed *GABA(A) $\alpha$ 1* KO showed no difference in fear training or extinction compared to controls, indicating that the presence of the promoter did not effect extinction behavior (Fig. S2 D–F). Altogether these data suggest CRF *GABA(A) $\alpha$ 1* KO disrupts extinction of conditioned fear.

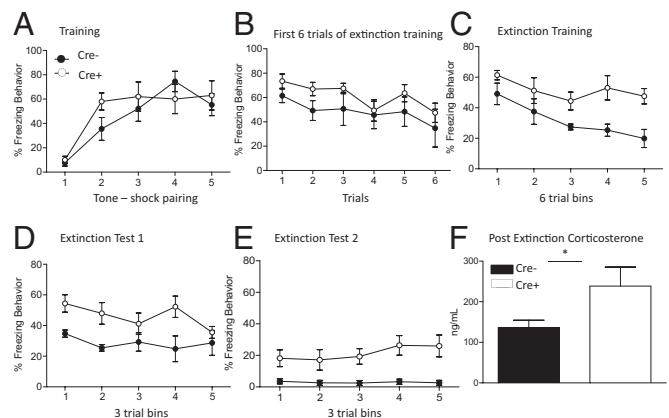
**Extinction and Plasma Hormone Measurement.** Corticosterone was also measured after extinction training in CRF *GABA(A) $\alpha$ 1* KO (Cre<sup>+</sup>,  $n = 7$ ) and Cre<sup>-</sup> ( $n = 7$ ) animals to assess if the HPA axis was engaged differentially by CRF *GABA(A) $\alpha$ 1* KO. A one-way ANOVA revealed a significant difference between the groups [ $F(1, 13) = 7.612, P < 0.05$ ]. Cre<sup>+</sup> animals showed a significant increase in corticosterone after extinction compared with Cre<sup>-</sup> controls (Fig. 3F). These findings suggest increased corticosterone levels may underlie the disrupted extinction seen in CRF *GABA(A) $\alpha$ 1* KO mice.

**Systemic Administration of the CRF Antagonist R121919 and Anxiety.** To assess whether the differences found in anxiety may be mediated by increased activation of CRF, we gave the CRF<sub>1</sub> receptor antagonist R121919 40 min before anxiety testing. If overactivation of CRF underlies the disruptions in anxiety behavior we would expect administration of an antagonist to ameliorate these effects.

One-way ANOVA conducted on the percentage of total time spent in the open arm of the elevated plus maze after R121919 injection showed a significant difference between groups (Fig. 4A) ( $F = 8.467, P < 0.05$ ). Planned comparisons between Cre<sup>-</sup> ( $n = 4$ ) and CRF *GABA(A) $\alpha$ 1* KO ( $n = 6$ ) mice given vehicle replicated our finding by showing CRF *GABA(A) $\alpha$ 1* KO spent significantly decreased time in the open arms ( $P < 0.05$ ). In contrast, planned comparisons between CRF *GABA(A) $\alpha$ 1* KO animals given R121919 ( $n = 5$ ) and Cre<sup>-</sup> vehicle controls showed no significant difference in percentage of time spent in the open arms ( $P > 0.05$ ). One-way ANOVA conducted on overall activity level during the testing session revealed no significant difference (Fig.



**Fig. 2.** Anxiety measures in CRF *GABA(A) $\alpha$ 1* KO (Cre<sup>+</sup>) and Cre<sup>-</sup> mice. (A) Cre<sup>+</sup> (white bar) mice spent less time in the open arm of the plus maze compared with Cre<sup>-</sup> (black bar) mice. (B) Baseline startle response was significantly increased at the higher decibel levels (110 and 120 dB) for Cre<sup>+</sup> compared with Cre<sup>-</sup> mice. (C) Time in the center of the open field was attenuated in Cre<sup>+</sup> mice compared with Cre<sup>-</sup>. (D) No difference between groups was shown in a test of depression-like behavior using the tail suspension test. \* $P < 0.05$ .



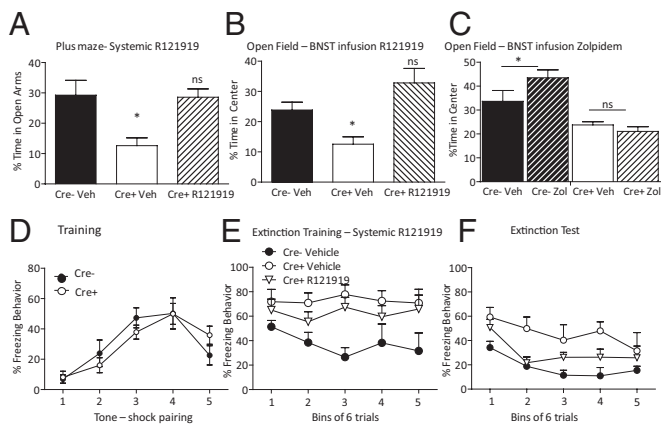
**Fig. 3.** Fear conditioning, extinction, and corticosterone in CRF *GABA(A) $\alpha$ 1* KO (Cre<sup>+</sup>) and Cre<sup>-</sup> mice. (A) Cre<sup>-</sup> (black circles) and Cre<sup>+</sup> (white circles) animals showed no difference in response to tone-shock pairings during training. (B) During extinction training there were no significant differences in freezing between the groups during the first six extinction trials. (C) When the entire extinction session was analyzed, Cre<sup>+</sup> mice showed significantly more freezing compared with Cre<sup>-</sup> animals. Extinction test (D) 1 and (E) 2 show Cre<sup>+</sup> animals continue to demonstrate significantly impaired extinction compared with Cre<sup>-</sup> controls. (F) Cre<sup>+</sup> animals show significantly increased corticosterone levels when measured after extinction. \* $P < 0.05$ .

S4.) [ $F(2, 12) = 0.268, P > 0.05$ ] suggesting that decreasing CRF in mice with augmented CRF returns anxiety to control levels.

**Intra-BNST Infusion of R121919 and Anxiety.** We targeted the BNST for local infusion of the CRF antagonist R121919 because of its involvement in anxiety behavior. Like systemic administration of R121919, BNST directed infusion just before the open-field test reverses the increase in anxiety in the knockout mouse. One-way ANOVA showed that there was a significant difference between groups in percent time spent in the open area during the open-field test (Fig. 4B) [ $F(2, 20) = 6.748, P < 0.05$ ]. Planned comparisons showed that Cre<sup>-</sup> vehicle-infused mice ( $n = 9$ ) spent significantly more of the test session in the center of the open field ( $P < 0.05$ ) compared with CRF *GABA(A) $\alpha$ 1* KO vehicle-infused animals ( $n = 6$ ). In contrast, planned comparisons show that Cre<sup>-</sup> vehicle-infused and CRF *GABA(A) $\alpha$ 1* KO R121919-infused animals ( $n = 8$ ) did not significantly differ in time spent in the center of the open field ( $P > 0.05$ ).

**Intra-BNST Infusion of Zolpidem and Anxiety.** To assess whether CRF *GABA(A) $\alpha$ 1* KO shifted the effectiveness of benzodiazepines, we administered Zolpidem (0.25 mg/mL, 0.5  $\mu$ L), a *GABA(A) $\alpha$ 1*-preferring benzodiazepine-site agonist into the BNST of Cre<sup>-</sup> and CRF *GABA(A) $\alpha$ 1* KO animals. One-way ANOVA indicated a significant between-groups effect (Fig. 4C) [ $F(3, 19) = 13.427, P < 0.05$ ]. Planned comparisons between Cre<sup>-</sup> vehicle ( $n = 5$ ) and Cre<sup>-</sup> Zolpidem ( $n = 5$ ) groups showed a significant increase in percentage time exploring the center area of the open field ( $P < 0.05$ ) for Cre<sup>-</sup> Zolpidem-infused mice. Planned comparisons revealed Zolpidem was unable to alter anxiety behavior in the CRF *GABA(A) $\alpha$ 1* KO mice (vehicle,  $n = 7$ ; and Zolpidem,  $n = 6, P > 0.05$ ). These findings indicate sensitivity to benzodiazepines is altered with CRF *GABA(A) $\alpha$ 1* KO in the BNST.

**Systemic CRF Antagonist and Extinction.** To determine whether the CRF system mediates the disruption in fear extinction, we administered R121919 systemically 40 min before extinction training. Cre<sup>-</sup> ( $n = 4$ ) and CRF *GABA(A) $\alpha$ 1* KO ( $n = 11$ ) animals previously run in plus-maze were trained with five tone-shock pairings (Fig. 4D). A repeated-measures ANOVA was run on training data with Trial and Genotype as factors. A significant within-subjects effect for Trial was found [ $F(4, 52) = 13.241, P < 0.05$ ], indicating increased freezing over the training session



**Fig. 4.** Reversal of anxiogenic and extinction deficit phenotype. (A) R121919 given to CRF *GABA(A)* $\alpha$ 1 KO (Cre<sup>+</sup>) animals (striped bar) systemically before plus-maze testing significantly increased time spent in the open arm. Cre<sup>+</sup> animals given vehicle (white bar) showed a significant decrease in percent time spent in the open arms of the elevated plus maze compared with Cre<sup>-</sup> vehicle-infused mice. In contrast, R121919-injected Cre<sup>+</sup> mice showed no difference from Cre<sup>-</sup> control (ns, not significant). (B) Infusion of R121919 into the BNST increased time spent in the open area of the open field in Cre<sup>+</sup> mice. (C) Cre<sup>+</sup> animals do not show a difference in open-field exploration when given an infusion of Zolpidem into the BNST, whereas Zolpidem increased time spent in the center of an open field in Cre<sup>-</sup> animals. \* $P < 0.05$ . (D) Neither Cre<sup>+</sup> nor Cre<sup>-</sup> mice show a significant difference in Pavlovian fear conditioning. (E) Systemic injection of R121919 to Cre<sup>+</sup> animals ( $\nabla$ ) before extinction training rescues the extinction deficit seen during extinction training. (F) Twenty-four hours later during extinction testing, we continue to see disrupted extinction in the Cre<sup>+</sup> vehicle mice ( $\circ$ ); however, the R121919-injected Cre<sup>+</sup> animals show no significant difference from Cre<sup>-</sup> vehicle-injected ( $\bullet$ ) animals. Cre<sup>+</sup> vehicle-infused ( $\circ$ ) animals again demonstrate significantly disrupted fear extinction.

across conditions. There was no significant Trial-by-Genotype interaction [ $F(4, 52) = 0.929, P > 0.05$ ] or Genotype effect [ $F(1, 13) = 0.012, P > 0.05$ ]. The lack of a difference during training replicates our previous finding that CRF *GABA(A)* $\alpha$ 1 KO disruption does not affect acquisition of a fear memory.

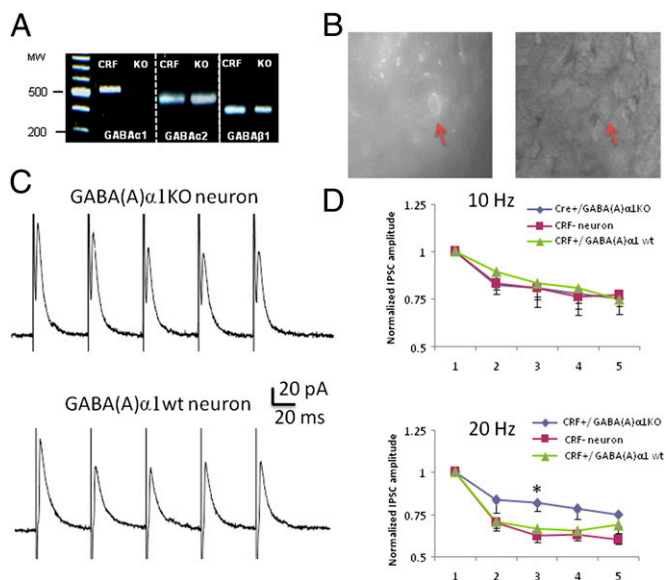
The following day, 40 min after animals received a systemic infusion of R121919, mice were exposed to 30 tones during extinction training. A repeated-measures ANOVA with Block and Drug as factors was conducted on the freezing data from extinction training grouped into five blocks of six tones each (Fig. 4E) and revealed no significant main effect for Block [ $F(4, 48) = 0.599, P > 0.05$ ] and no Block-by-Drug interaction [ $F(8, 48) = 0.940, P > 0.05$ ]. However, there was a significant between-subjects group effect [ $F(1, 1) = 4.898, P < 0.05$ ]. Tukey post hoc analysis showed that Cre<sup>-</sup> animals that received vehicle showed significantly less freezing behavior than CRF *GABA(A)* $\alpha$ 1 KO animals that received vehicle ( $P < 0.05$ ), replicating our earlier findings. A repeated-measures ANOVA was run on data from extinction testing 24 h later. We found a significant effect of Block [ $F(4, 48) = 9.081, P < 0.05$ ] but no significant Block-by-Group effect [ $F(8, 48) = 1.011, P > 0.05$ ]. A between-subjects group effect was found [ $F(1, 12) = 3.876, P < 0.05$ ] and follow-up Tukey post hoc analysis showed this effect was because of a difference between Cre<sup>-</sup> and CRF *GABA(A)* $\alpha$ 1 KO vehicle-injected mice ( $P < 0.05$ ). There was no difference between Cre<sup>-</sup> vehicle-injected and CRF *GABA(A)* $\alpha$ 1 KO R121919-injected mice ( $P > 0.05$ ). These data suggest that CRF neuronal disinhibition results in CRF receptor overactivation as a mechanism for the disrupted extinction seen in Cre<sup>+</sup> animals.

**Single-Cell RT-PCR.** BNST neurons from CRF *GABA(A)* $\alpha$ 1 KO mice were screened for their expression of the mRNA transcripts *GABA(A)* $\alpha$ 1,  $\alpha$ 2, and  $\beta$ 1 subunits and for 18S rRNA (a ubiquitous housekeeping ribosomal RNA). Here, we used the fact

that CRF *GABA(A)* $\alpha$ 1 KO neurons express Cre to selectively drive expression of an mCherry fluorophore in CRF neurons following local injection of a floxed mCherry AAV viral vector (see *Materials and Methods*). Using this combined methodology, and floxed *GABA(A)* $\alpha$ 1 mice as well as floxed-stop GFP reporter mice, we were able to identify cells that were CRF<sup>+</sup>/*GABA(A)* $\alpha$ 1 KO compared with CRF<sup>+</sup>/*GABA(A)* $\alpha$ 1 wild-type.

Cytosolic mRNA was pulled from five visually identified CRF-mCherry neurons from the BNST in CRF *GABA(A)* $\alpha$ 1 KO mice and screened for 18S rRNA (Fig. 5A). Three out of five neurons expressed 18S rRNA. These three neurons were then screened for *GABA(A)* $\alpha$ 1,  $\alpha$ 2 and  $\beta$ 1 transcripts. As expected, none of these putative CRF neurons expressed *GABA(A)* $\alpha$ 1 transcripts; however, they did express *GABA(A)* $\alpha$ 2 and  $\beta$ 1 transcripts indicating our deletion of *GABA(A)* $\alpha$ 1 did not result in gross disruption in *GABA(A)* $\alpha$ 2 and  $\beta$ 1 subunit expression in these neurons. We also screened for *GABA(A)* $\alpha$ 1 transcripts in 10 CRF neurons recorded from control CRF-Cre-GFP mice (23) and found that 8 of these neurons expressed *GABA(A)* $\alpha$ 1 mRNA, confirming that mouse BNST CRF neurons normally express *GABA(A)* $\alpha$ 1 mRNA transcripts.

**Potential Cellular Function of *GABA(A)* $\alpha$ 1.** We investigated the consequences of the *GABA(A)* $\alpha$ 1 KO on the intrinsic properties of BNST CRF neurons in an in vitro slice preparation. Here, we used the same mCherry AAV vector to identify CRF *GABA(A)* $\alpha$ 1 KO neurons and compared their basic membrane properties with that of mCherry<sup>-</sup> neurons. The physiological properties of CRF *GABA(A)* $\alpha$ 1 KO mCherry<sup>+</sup> neurons were not significantly



**Fig. 5.** Single neuron RT-PCR and electrophysiological recordings. (A) Image of an ethidium bromide-stained electrophoresis-separated agarose gel in which *GABA(A)* $\alpha$ 1 mRNA is expressed in CRF-CRE-GFP (with wild-type *GABA(A)* $\alpha$ 1) and not expressed in CRF-CRE-*GABA(A)* $\alpha$ 1 KO mice. We tested for expression of two other *GABA(A)* receptors types ( $\alpha$ 2 and  $\beta$ 1) and found the mRNA of both receptors are expressed in CRF-CRE-GFP and CRF-CRE-*GABA(A)* $\alpha$ 1 KO mice. MW represents molecular weight marker (magnification, 1 $\times$ ). (B) Representative image of recording site from CRF-expressing neurons identified with the floxed mCherry AAV. (C and D) *GABA(A)* $\alpha$ 1 KO affects short term plasticity of inhibitory transmission. Trains of five pulses at 10 or 20 Hz were delivered to induce IPSCs and determine short-term plasticity. (C) Representative traces show the IPSCs of a CRF Cre<sup>+</sup> [*GABA(A)* $\alpha$ 1 KO] neuron and mCherry Cre<sup>-</sup> [*GABA(A)* $\alpha$ 1 wild-type] neuron following five pulses at 20 Hz. (D) At 10 Hz, the short-term plasticity is not affected by *GABA(A)* $\alpha$ 1 KO, whereas at 20 Hz, a reduction of short-term synaptic depression is observed in CRF *GABA(A)* $\alpha$ 1 KO neurons compared with control neurons [mCherry<sup>-</sup> (CRF<sup>-</sup>) and CRF expressing neurons with wild-type *GABA(A)* $\alpha$ 1].

different from neighboring CRF *GABA(A) $\alpha$ 1* KO mCherry<sup>-</sup> neurons with the exception that they had a more hyperpolarized resting membrane potential (Table S1). We then compared the properties of visualized CRF *GABA(A) $\alpha$ 1* KO mCherry<sup>+</sup> neurons to those of CRF neurons recorded from CRF-Cre-GFP mice. No significant difference was found between these two groups of type III neurons, suggesting CRF *GABA(A) $\alpha$ 1* KO did not change the intrinsic physiological properties of CRF neurons.

We next examined the effect of the *GABA(A) $\alpha$ 1* KO on inhibitory postsynaptic currents (IPSCs) evoked in visually identified neurons by focal electrical stimulation of the BNST. No significant difference were observed across groups for the mean IPSC amplitude evoked by low-frequency (0.2 Hz) stimulation [CRF *GABA(A) $\alpha$ 1* KO mCherry<sup>+</sup> = 71.7  $\pm$  14.4 pA,  $n$  = 7; CRF *GABA(A) $\alpha$ 1* KO mCherry<sup>-</sup> = 76.9  $\pm$  13.3 pA,  $n$  = 6; CRF-Cre-GFP<sup>+</sup> = 62  $\pm$  3.4 pA,  $n$  = 15;  $F(2, 15) = 0.60$ ,  $P > 0.05$ ], or for the IPSC reversal potential [CRF *GABA(A) $\alpha$ 1* KO mCherry<sup>+</sup> = -65.0  $\pm$  1.2 mV; CRF *GABA(A) $\alpha$ 1* KO mCherry<sup>-</sup> = -64.6  $\pm$  1.5 mV;  $P > 0.05$ ]. We then examined the effect of the *GABA(A) $\alpha$ 1* KO on short-term synaptic plasticity using a train of five stimuli delivered at 10 or 20 Hz. In all neurons recorded, the amplitude of the evoked IPSC gradually reduced during repetitive stimulation (Fig. 5C). At 10 Hz, a two-way ANOVA indicated there was no significant difference in short-term synaptic depression elicited in CRF-expressing, *GABA(A) $\alpha$ 1* mCherry<sup>+</sup> KO neurons, CRF-CreGFP<sup>+</sup> neurons, or non-CRF-expressing mCherry<sup>-</sup> neurons [ $F(2, 70) = 0.5$ ,  $P > 0.05$ ]. However, at 20-Hz a two-way ANOVA indicated a significant difference among these three groups of neurons [ $F(2, 80) = 8.15$ ,  $P < 0.01$ ]. Bonferroni post hoc tests indicated that CRF *GABA(A) $\alpha$ 1* KO mCherry<sup>+</sup> neurons showed a smaller short-term synaptic depression in comparison with CRF *GABA(A) $\alpha$ 1* KO mCherry<sup>-</sup> neurons and CRF-Cre-GFP<sup>+</sup> neurons (Fig. 5C and D). These results suggest that the *GABA(A) $\alpha$ 1* subunit contributes to a frequency selective modulation of short-term synaptic plasticity in BNST CRF neurons.

## Discussion

The present study used a Cre-expressing transgenic mouse in which Cre is driven by a CRF-specific promoter to delete *GABA(A) $\alpha$ 1* only within CRF-expressing neurons [CRF *GABA(A) $\alpha$ 1* KO]. Radioactive and fluorescent in situ hybridization show CRF *GABA(A) $\alpha$ 1* KO increased expression of CRF within the PVN, CeA, and BNST. This type of knockdown provides a unique way to model disinhibition of CRF-containing neurons that have been shown to contribute to anxiety disorders (24, 25). We found that CRF *GABA(A) $\alpha$ 1* KO leads to increased anxiety and a persistent deficit in fear extinction. We also found that enhanced anxiety and disrupted fear extinction were rescued with application of the CRF antagonist R121919 systemically and when given into the BNST, indicating that directly attenuating CRF restores disrupted behavior to levels comparable to mice without the deletion.

We also found a significant increase in corticosterone after fear extinction. This finding suggests that the extinction phenotype of these animals does have a HPA axis contribution. Glucocorticoids (corticosterone in rodents and cortisol in humans) facilitate fear memory extinction (26–29). Because we did not measure baseline corticosterone, we cannot say whether this increase was a result of extinction training deficits or existed throughout fear training and fear extinction. However, if corticosterone was increased during fear memory formation or at baseline in the CRF *GABA(A) $\alpha$ 1* KO animals we would predict our knockouts would acquire fear faster than controls (e.g. ref. 30), whereas CRF *GABA(A) $\alpha$ 1* KO acquired fear during training equivalently compared to controls.

We also assessed CRF *GABA(A) $\alpha$ 1* KO on depressive-like behavior using the tail suspension test. Work has shown altered levels of multiple *GABA(A)* receptor subunits within the prefrontal cortex of depressed suicide victims compared with nondepressed controls (31). The same study showed CRF was decreased in some parts of the prefrontal cortex of depressed individuals. Our findings show no differences in one measure of depressive-like behavior, the

tail suspension test. Thus, our findings do not support CRF *GABA(A) $\alpha$ 1* KO as underlying one measure of depressive-like behavior. However, more comprehensive studies are needed to exclude a role for this neuronal subtype in depression.

The *GABA(A)* receptor is the target of a number of clinically important drugs, including benzodiazepines (32, 33). Zolpidem is a *GABA(A) $\alpha$ 1*-preferring benzodiazepine-site agonist. We infused Zolpidem into the BNST, where it effectively reduced anxiety in Cre<sup>-</sup> controls, as predicted. In contrast, when given into the BNST of CRF *GABA(A) $\alpha$ 1* KO mice, Zolpidem was ineffective at reducing anxiety. CRF *GABA(A) $\alpha$ 1* KO animals continued to show increased anxiety, even with BNST infusion of Zolpidem. This shift in effectiveness of Zolpidem may be due to the overall decrease in the number of *GABA(A) $\alpha$ 1* subunits within the BNST, because decreased *GABAergic* tone in this structure has been linked with heightened anxiety (17) and decreased Zolpidem effectiveness (34). However, the mechanism of this shift in effectiveness remains to be determined.

It is unclear if there is compensatory up-regulation of other subunits of the *GABA(A)* receptor in response to the CRF *GABA(A) $\alpha$ 1* KO. With any developmental knockout, there is concern that compensatory alterations in a multitude of other genes may occur. Single-cell RT-PCR shows, qualitatively, that  $\alpha$ 2 and  $\beta$ 1 are both expressed within *GABAergic* neurons lacking  $\alpha$ 1. In a mouse line without  $\alpha$ 1-subunit expression, compensation of other *GABA(A)* subunits was found, along with other compensatory changes (34, 35). It is unclear whether our cell-type specific disruption would have less dramatic compensatory effects. Determining compensation that occurs in these mice may offer interesting insights into *GABA(A)* receptor subunit dynamics, especially since the deletion is only within CRF containing neurons. This is an important and interesting question that requires future study.

Electrophysiological analysis demonstrated that compared with animals that expressed GFP in CRF neurons, CRF *GABA(A) $\alpha$ 1* KO mice showed reduced short-term synaptic depression. Short-term synaptic depression indicates a decrease in the probability of neurotransmitter release caused by recurring presynaptic activation (36) and may play a role in neural coding of information (37). While these findings are intriguing, it remains to be determined whether disrupted short-term synaptic depression in the BNST underlies our behavioral effects.

Disinhibition mediated by *GABA* or CRF-mediated hyperexcitability within the amygdala may act as a mechanism underlying disorders of anxiety (6). Work to date has been unable to address which subtypes of CRF<sub>ergic</sub> neurons contribute to dysregulation of anxiety. Global deletion of the *GABA(A)* receptor  $\alpha$ 1 subunit enhances auditory fear memory (20) or has no effect on anxiety (38). In the present work, when we specifically delete the *GABA(A) $\alpha$ 1* subunit in CRF neurons, we show enhanced anxiety and disrupted fear memory extinction, both of which are hallmarks of anxiety disorders, such as posttraumatic stress disorder. We also showed that the behavioral disruption in these mice was normalized by a CRF antagonist and sensitivity to a selective  $\alpha$ 1-acting benzodiazepine was disrupted. Finally, we demonstrated alterations in *GABAergic* responses in CRF-expressing neurons within BNST using in vitro electrophysiological recordings. These data indicate that increased CRF expression within this subtype of neurons leads to disrupted anxiety and fear extinction that can be rescued with pharmacological disruption of CRF.

This model of subtype-specific deletion of CRF-containing neurons adds to our understanding of the mechanism through which CRF<sub>ergic</sub> circuits contribute to anxiety-related psychological disorders. These findings also highlight the contribution cell-type specific genetic approaches can make to the functional dissection of circuits underlying complex behavior. Further understanding of cell-specific modulation of CRF neurons may also provide novel, more specific targets for therapeutic intervention into disorders of anxiety and fear.

## Materials and Methods

Further details beyond these materials and methods are provided in *S1 Materials and Methods*. Experimental methods were approved by the

Institutional Animal Use and Care Committee of Emory University. The transgenic mouse was made by crossing the CRFp3.0Cre transgenic mouse (23) with the GABA(A)  $\alpha 1$  floxed mouse (39, 40). For stereotaxic surgery, mice were anesthetized and received bilateral microinjections of 0.5  $\mu$ L 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. 41: AP = +0.5, ML =  $\pm$ 2.1, DV = -4.2 or cannulae were lowered to the same BNST coordinates for later drug delivery.

**Staining Procedures.** X-Gal staining was performed as in ref. 23. In situ hybridization was conducted as in ref. 42.

**Behavior. Elevated plus maze.** Open-arm entries, the total number of arm entries, and the total time spent in the open arms was recorded over 5 min. **Open field.** The center zone was located 6 cm from the perimeter of the chamber walls. Activity was monitored and data was collected over 10 min. **Baseline startle.** After 5-min acclimation, animals were given 10 startle stimuli at four stimulus intensities (90, 100, 110, 120 dB) with an interstimulus interval of 30 s in pseudorandom sequence and mean startle amplitudes were calculated. **Tail suspension test.** Animals were suspended by the tail for 6 min. Time spent immobile was recorded by an observer blind to the genotype of the animals. **Fear conditioning, shock reactivity, and extinction.** All groups received five conditioned-stimulus tones (30 s, 6 kHz, 74 dB) coterminating with

unconditioned-stimulus shocks (500 ms, 0.6 mA) with a 5-min intertrial interval (ITI). Shock reactivity data were collected from movement occurring during training. Extinction training and testing was conducted using 15 or 30 conditioned-stimulus tones (30 s, 6 kHz, 74 dB) in a novel context.

**Single-Cell RT-PCR and Electrophysiological Recordings.** The procedure used to determine mRNA transcript expression in single cells has been described in detail elsewhere (23, 43). BNST slices were obtained as previously described (44). Chr2mCherry CRE<sup>+</sup> neurons were selected under fluorescent illumination. Whole-cell patch clamp recordings were obtained with standard techniques (44).

**Statistical Analysis.** Data were analyzed using the one-way ANOVA or repeated measures with follow-up planned comparisons or Tukey post hoc tests when appropriate.

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