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Cell Type-Specific Modifications of Corticotropin-Releasing Factor (CRF) and its type 1 receptor (CRF₁) on Startle Behavior and Sensorimotor Gating

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Author Contribution

All authors revised the manuscript for intellectual content and approved the final version of the manuscript. Specific contributions to conception and interpretation of the study and acquisition of data are described below.

Elizabeth Flandreau: analyzed and interpreted data, created figures, wrote the manuscript and revised to include intellectual content from co-authors.

Victoria Risbrough: conceived and designed the study, acquired data for acoustic startle experiments, analyzed and interpreted the data and assisted in creating figures.

Ailing Lu: generated the CRF overexpression transgenic mouse line and prepared Nestin-Cre CRF overexpression mice actually used in the study.

Martin Ableitner: generated and characterized the CRF-COE-GABA mice.

Mark A Geyer: assisted in conception and design of the startle experiments.

Florian Holsboer: assisted in conception and design of the transgenic mouse lines.

Jan M Deussing: was instrumental in the conception and design of the startle experiments and transgenic mouse lines.

Conflict of Interest

Dr. Flandreau, Dr. Ableitner, Dr. Lu, and Dr. Deussing have no potential financial conflicts to report. Dr. Risbrough also had research support from Sunovion, Janssen, Pfizer, and Omeros. In the past 3 years, Dr Geyer has received consulting compensation from Abbott, Acadia, Addex, Cerca, Dart, Lundbeck/Otsuka, Merck, Neurocrine, Omeros, Takeda, and Teva, and holds an equity interest in San Diego Instruments. Dr. Holsboer is CEO of HMNC GmbH.

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Abstract

The corticotropin-releasing factor (CRF) family of peptides and receptors coordinates the mammalian endocrine, autonomic, and behavioral responses to stress. Excessive CRF production has been implicated in the etiology of stress-sensitive psychiatric disorders such as posttraumatic stress disorder (PTSD), which is associated with alterations in startle plasticity. The CRF family of peptides and receptors mediate acute startle response changes during stress, and chronic CRF activation can induce startle abnormalities. To determine what neural circuits modulate startle in response to chronic CRF activation, transgenic mice overexpressing CRF throughout the central nervous system (CNS; CRF-COE^{CNS}) or restricted to inhibitory GABAergic neurons (CRF-COE^{GABA}) were compared across multiple domains of startle plasticity. CRF overexpression throughout the CNS increased startle magnitude and reduced ability to inhibit startle (decreased habituation and decreased prepulse inhibition (PPI)), similar to previous reports of exogenous effects of CRF. Conversely, CRF overexpression confined to inhibitory neurons decreased startle magnitude but had no effect on inhibitory measures. Acute CRF receptor 1 (CRF₁) antagonist treatment attenuated only the effects on startle induced by CNS-specific CRF overexpression. Specific deletion of CRF₁ receptors from forebrain principal neurons failed to alter the effects of exogenous CRF or stress on startle, suggesting that these CRF₁ expressing neurons are not required for CRF-induced changes in startle behaviors. These data indicate that the effects of CRF activation on startle behavior utilize an extensive neural circuit that includes both forebrain and non-forebrain regions. Furthermore, these findings suggest that the neural source of increased CRF release determines the startle phenotype elicited. It is conceivable that this may explain why disorders characterized by increased CRF in cerebrospinal fluid (e.g. PTSD and major depressive disorder) have distinct symptom profiles in terms of startle reactivity.

Introduction

CRF is a 41 amino acid peptide, discovered for its role in activating the hypothalamic-pituitary-adrenal (HPA) axis, a primary endocrine response to disruption of body homeostasis and perceived threat (Vale et al., 1981). CRF acts centrally to coordinate autonomic and behavioral reactions to stress via extrahypothalamic actions in the brainstem and limbic system, respectively (e.g. (Hauger et al., 2009; Nemeroff and Vale, 2005). While critical in the face of real threat, overactive or inappropriate activation of CRF can have severe consequences for mental and physical health (Mitchell, 1998; Risbrough and Stein, 2006; Tache and Brunnhuber, 2008).

Increased release of CRF, as measured by elevated CRF concentration in cerebrospinal fluid (CSF), is observed in some patients with mood and anxiety disorders, most notably major-depressive disorder (MDD) and post-traumatic stress disorder (PTSD) (Kasckow et al., 2001). However the source of increased CRF is not clear, nor is it clear if different sources of CRF might be linked to specific symptom domains. Understanding the effects and mechanisms of CRF over-activation in the brain across differential neural circuits may provide a better understanding of its potential role in these disorders and their symptoms. A primary symptom of PTSD that is not common in depression is hyperarousal, which can manifest as increased acoustic startle reactivity at baseline (e.g. (Butler et al., 1990) and greater startle responses in aversive contexts, (reviewed in (Grillon and Baas, 2003; Risbrough, 2010)). PTSD may also be associated with decreased sensorimotor gating as measured by reduced habituation to repeated stimuli and reduced inhibition of startle as measured by prepulse inhibition (PPI) (reviewed in (Clark et al., 2009)).

The acoustic startle reflex (ASR) consists of a series of involuntary reflexes elicited by a sudden, intense auditory stimulus and the pathways mediating this reflex are analogous in rodent models and humans (Graham, 1975; Yeomans et al., 2002). The simple startle circuit begins in the auditory nerve and cochlear nuclei, continues through the caudal pontine reticular formation and on to motor neurons that elicit the physical startle response. Startle reactivity is modulated by forebrain limbic regions such as the hippocampus, amygdala, and bed nucleus of the stria terminalis (BNST), and by brainstem autonomic centers such as the locus coeruleus (LC; reviewed in (Koch, 1999; Swerdlow et al., 2001)).

In rodent models, stress or exogenous administration of CRF increases startle magnitude; this effect is mediated by activation of CRF₁ and CRF₂ receptors and can be attenuated by anxiolytics (Risbrough et al., 2003; Risbrough et al., 2004; Risbrough et al., 2009; Swerdlow et al., 1986). The CRF₁ and CRF₂ receptors are located throughout the neocortex, extended amygdala, and brainstem (Perrin and Vale, 2002; Risbrough and Stein, 2006). CRF peptide is produced in a variety of cell types including GABAergic interneurons and glutamatergic projection neurons, and is colocalized with other neurotransmitters and neuropeptides (Chen et al., 2004; Gallopin et al., 2006; Kubota et al., 2011; Sawchenko and Swanson, 1985). CRFergic circuits overlap with startle modulatory circuits at several points including the BNST, central nucleus of the amygdala (CeA), dorsal raphe, and LC (reviewed in (Koch, 1999; Swerdlow et al., 2001)).

The goal of the present experiment was to determine what neural circuits and cell types are sensitive to excessive CRF signaling effects on startle reactivity using selective genetic tools. We examined transgenic mice with differential CRF overexpression, i.e. within the entire central nervous system (CNS; CRF-COE^{CNS}) or confined to forebrain GABAergic neurons (CRF-COE^{GABA}). To determine the selective influence of forebrain CRF₁ receptors in acoustic startle we also used a mouse in which this receptor is deleted from CamKII α -producing neurons in the forebrain (forebrain CRF₁-KO). This strategy avoids the glucocorticoid confound of the traditional CRF₁-KO in that the receptor is still present in hypothalamus and pituitary. Loss of CRF₁ receptor expression is restricted to principal neurons of the anterior forebrain (Minichiello et al., 1999).

In the rodent brain, CRF is endogenously expressed in the paraventricular nucleus of the hypothalamus (PVN), CeA, BNST, olfactory bulb, cortex and brain stem nuclei. CRH-COE^{CNS} mice exhibit substantial elevations of CRF expression in neurons and glia throughout the CNS, in particular in the cortex, and hippocampus (Lu et al., 2008). The increased expression is gene-dose dependent; mice heterozygous for the conditional CRF expression unit at the ROSA26 (R26) locus show half expression compared to mice homozygous for the modified R26 allele following Cre recombinase-mediated induction of CRF expression. In CRF-COE^{GABA} mice, CRF is overproduced in inhibitory neurons of the anterior forebrain. In these mice, strong CRF overexpression is observed in the olfactory bulb, striatum, reticular nucleus, cortex, and hippocampus. Importantly, neither CRF-COE^{CNS} nor CRF-COE^{GABA} mice show alterations in their circadian HPA axis activity compared to control littermates (Lu et al., 2008).

In floxed CRF1 receptor mice harboring the CamKII α -Cre-gene, *CRF1* receptor expression is eliminated from CamKII α -expressing cells in the forebrain. Previous studies using these forebrain CRF₁-KO mice have shown reduced anxiety-like behavior, normal HPA axis reactivity under basal conditions, but prolonged HPA axis activation following stress exposure (Muller et al., 2003).

In the present study, we determined the effects of cell-specific CRF overexpression or loss of forebrain CRF1 receptor on startle plasticity. Based on the regional distribution of CRF we hypothesized that both CNS-specific and inhibitory neuron-specific sources of CRF overexpression may increase startle reactivity. This hypothesis is based on evidence for these models to increase CRF levels at the extended amygdala or at the reticular formation, both of which are sites of CRF modulation of startle behavior (Davis et al., 1997). We also tested the hypothesis that CRF1 receptor activation was required for the startle phenotypes observed in these different overexpression models. Additionally, given the anxiolytic-like profile of forebrain-specific CRF₁-KO mice and the requirement of CRF receptor activation in the BNST for CRF and stress-induced changes in startle, we also hypothesized that forebrain-specific deletion of CRF1 receptors would block CRF and stress-induced disruption of startle (Muller et al., 2003; Toth et al., 2013a)(Davis et al., 1997).

Materials and Methods

Animals

All mice were housed 2–4 per cage under standard laboratory conditions (22 $\pm$ 1 °C; 55 $\pm$ 5% humidity) with food and water available *ad libitum* under a 12 hr light/dark cycle (lights on at 0600 or 0800). Animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the Government of Bavaria. All mice were male and were 3–4 months old at the time of testing. Future research will also benefit from including female mice; the effects of CRF overexpression are expected to be even more robust in females (Toth et al., 2014). The extent of Cre-Recombinase activity from the Nestin-, CamKII α -, and Dlx-Cre mice has been reported (Lu et al., 2008).

Cell-Type Specific CRF Overexpression—Mice harboring a Cre recombinase-inducible CRF overexpressing allele have been described previously (Dedic et al., 2012; Lu

et al., 2008). Briefly, the conditional allele contains the CRF coding sequence preceded by a stop cassette, which is flanked by loxP sites (floxed-stop: “flop”). This Cre-inducible CRF expression allele was inserted into the constitutively active ROSA26 locus ($R26^{flopCRF}$). In the absence of Cre recombinase, no CRF is expressed from this transgene. In the presence of Cre recombinase, the floxed stop cassette is excised, allowing for the transcription of the CRF transgene. These conditional CRF overexpressing (CRF-COE) mice allow for the Cre-dependent overexpression of two different CRF dosages depending whether CRF is expressed from a single allele ($R26^{+/flopCrf}$, CRF-COE_{het}, het) or from both R26 alleles ($R26^{flopCrf/flopCrf}$ CRF-COE_{hom}, hom). The CRF-COE strain is on a mixed 129S2/Sv×C57BL/6J background.

Breeding to Cre recombinase strains to achieve cell-type specific expression was done as previously described (Lu et al., 2008). Briefly, to achieve CRF overexpression throughout the CNS (CRF-COE^{CNS}), the CRF-COE line was crossed with a mouse line expressing Cre recombinase driven by the Nestin promoter (*Nes-Cre*, (Tronche et al., 1999). In the *Nes-Cre* line, Cre recombinase expression is controlled by the Nestin promoter and neural enhancer, which drive Cre recombinase expression in neuronal and glial precursors as early as embryonic day 10.5 (Graus-Porta et al., 2001). For overexpression of CRF in inhibitory GABAergic neurons (CRF-COE^{GABA}), the *CRF-COE* line was crossed with a Cre driver in which the Dlx5/6 promoter drives expression of Cre recombinase in forebrain GABAergic neurons as early as embryonic day 10.5 (Monory et al., 2006). For both CRF-COE lines, respective littermates negative for the Cre recombinase but homozygous for the modified R26 allele were used as control subjects (CRF-COE^{Ctrl}). Mice were first characterized to test our hypotheses of CRF-OE-induced startle abnormalities and 1 week later began testing for the role of CRF1 receptors in these phenotypes using administration of the CRF1 receptor antagonist NBI-30775.

Forebrain-Specific CRF-R1 Knock out—A mouse line in which the CRF1 receptor is flanked by loxP sites, enabling conditional deletion of CRF, has been described previously (Muller et al., 2003). This mouse was crossed to the CamKIIα-Cre driver to disrupt CRF1 receptor expression in forebrain principal neurons (forebrain CRF₁-KO). Beginning between postnatal day 15 and 20, the CamKIIα promoter drives Cre recombinase expression in pyramidal cells of the neocortex, including the amygdala, and in the striatum and hippocampus; Cre expression is absent from inhibitory interneurons in this strain (Minichiello et al., 1999).

Genotyping

Genotyping was performed by PCR using primers: ROSA-1, 5'-AAA-GTC-GCT-CTG-AGT-TGT-TAT-3'; ROSA-5, 5'-TAG-AGC-TGG-TTC-GTG-GTG-TG-3'; ROSA-6 5'-GCT-GCA-TAA-AAC-CCC-AGA-TG-3' and ROSA-7, 5'-GGG-GAA-CTT-CCT-GAC-TAG-GG-3'. Standard PCR conditions resulted in a 398-bp wild-type and a 646-bp mutant PCR product, respectively. Animals with a premature deletion of the floxed transcriptional terminator sequence were identified by the occurrence of a 505-bp PCR product. The presence of Cre was evaluated using primers CRE-F, 5'-GAT-CGC-TGC-CAG-GAT-ATA-

CG-3' and CRE-R, 5'-AAT-CGC-CAT-CTT-CCA-GCA-G-3' resulting in a PCR product of 574 bp.

Acoustic Startle Parameters

Startle sessions were similar to those previously reported (Risbrough et al., 2004) and were constructed to assess multiple aspects of startle behavior: startle magnitude; threshold; habituation; and prepulse inhibition. Briefly, there were five blocks with a continuous 65 dB background noise. The first block had five 120 dB pulses; this block is typically used to assess initial startle reactivity as well as stabilize startle for assessment of PPI in the subsequent blocks. The second block had five each of 80, 90, 100, 110, and 120 dB pulses, which was meant to assess CRF overexpression effects on startle reactivity across both high and low pulse intensities. The third block had five each of prepulse intensities 67, 69, 73, or 81 dB preceding, by 100 ms, a 120 dB pulse. There was an additional trial with a prepulse of 73 dB preceding a 105 dB pulse and five each of 120 and 105 dB pulses without prepulses. This block assesses PPI in response to changes in prepulse and pulse saliency (i.e. loudness). The fourth block had a 81 dB prepulse followed by a 120 dB pulse with varying interstimulus interval (*ISI*) of 20, 70, 120, 360, or 1080 ms (five trials each). There were also seven 120 dB pulse alone trials. This block assesses the temporal processing window for prepulse effects on startle reactivity, since PPI is typically limited to a narrow temporal window (30–300 ms) that can be altered by CRF (Conti et al., 2009). The fifth block consisted of five 120 dB pulse trials. This final block helps assess overall habituation to the 120 dB pulses that are present in each block. For all parameters the inter-trial interval was an average of 15 s with a range from 7–23 s.

For the CRF₁ receptor antagonist trials in CRF-COE^{CNS} and CRF-COE^{GABA} mice, some parameters were changed based on the characterization results. This was in order to optimize the parameters that were most affected by CRF overexpression to test our hypotheses of effects of CRF excess or CRF₁ receptor impairment on startle behavior. In block 2, the 80 and 90 dB pulse trials were dropped from the first block since these pulses did not consistently induce detectable startle responses and thus were not useful for testing our hypotheses. For the assessment of PPI across varied ISIs, we also changed the prepulse intensity to 73 dB from 81 dB since higher prepulse intensities provided a broader behavioral range to detect disruptions induced by CRF overexpression (see supplemental material).

CRF₁ Receptor Antagonist in CRF-COE Mice

NBI-30775 (also known as R-121919; a gift from Neurocrine Biosciences, Inc., San Diego, Calif., USA) was administered 15 min prior to testing. In the CRF-COE^{CNS} mice, doses of 0.0, 2.0, or 20.0 mg/kg were administered by intraperitoneal (IP) injection in a 5 ml/kg volume in 3% Tween20 in saline. The order of the three doses was counterbalanced across 3 tests with 6 days between tests. In the inhibitory neuron-specific CRF-COE^{GABA} mice, the 2 mg/kg dose was not tested since it did not show significant efficacy in the CRF-COE^{CNS} mice. The dose order (0 and 20 mg/kg) was also counterbalanced with 7 days between tests.

oCRF in Forebrain CRF₁-KO Mice

Ovine CRF (oCRF) (Bachem, Torrance, CA) was administered ICV, 1 hr pre-test. 0.2 nmol oCRF was administered in a 5 µl volume of artificial CSF (aCSF) by gravity flow as described previously (Risbrough et al., 2003; Risbrough et al., 2004). For cannula placement, mice were anesthetized using a 90 mg/kg ketamine-10 mg/kg xylazine mixture and prepared with a 23 gauge 7 mm-length unilateral guide cannula in the lateral ventricle (flat skull; anteroposterior, -0.1 mm; mediolateral, -1.0 mm; dorsoventral, -1.5 mm below dura). Cannulae were secured with one skull screw and dental cement. Drug injections and histologies were as described previously (Toth et al., 2013b). In brief, CRF was infused 5–7 d after surgery in unanesthetized mice using a 30 gauge 8 mm injector (1 mm below the tip of the guide cannula). Two weeks after testing, mice were anesthetized and 1 µl of dye was injected via the 8 mm injector. Mice were immediately killed, and the brains were removed. As the brains were removed, presence of the dye in the fourth ventricle was noted. A coronal cut was made along the guide tract to reveal lateral and third ventricles, which were also examined for presence of dye, and brains were frozen and stored with cut side on slides for digital scanning. Only animals with verification of dye in all four ventricles were included in the analysis (30 of 30 mice implanted).

Shock-potentiated startle in Forebrain CRF₁-KO Mice

To examine the role of forebrain CRF₁ receptors in stress-induced increases in startle we tested the forebrain-CRF₁-KO mice in the shock-potentiated startle test as previously reported (Risbrough et al., 2009). This test was conducted in a separate cohort of mice using a different set of acoustic stimulus chambers which included shockers and footshock grids (see below). Briefly, at least 24 hr before shock exposure, mice were tested for baseline startle and assigned to Shock and No-Shock groups matched for baseline startle scores. After habituation to the startle chamber for 5 min, the first startle block was presented to examine preshock startle reactivity. Mice were presented with a startle block consisting of 9 trials of 90, 95, and 105 dB pulses (40 msec) before and immediately after a block of 5 0.4-mA shocks. Intertrial intervals between startle pulses were 7–23 s, and between shock presentations was 30–90 s.

Apparatus

Startle chambers (SR-LAB; San Diego Instruments, San Diego, CA) consisted of nonrestrictive Plexiglas cylinders 5 cm in diameter resting on a Plexiglas platform in a ventilated chamber. High-frequency speakers mounted 33 cm above the cylinders produced all acoustic stimuli, which were controlled by SR-LAB software. Piezoelectric accelerometers mounted under the cylinders transduced movements of the animal, which were digitized and stored by an interface and computer assembly. Beginning at startling stimulus onset, 65 consecutive 1 msec readings were recorded to obtain the peak amplitude of the animal's startle response. Chambers were calibrated to ensure comparable sensitivities. Sound levels were measured as described previously using the A weighting scale in units of decibels sound pressure level (Geyer and Dulawa, 2003). The house light remained off throughout all testing sessions. A different set of four startle chambers equipped with a footshock apparatus and footshock grid (San Diego Instruments, San Diego,

CA) were used for the shock-potentiated startle test as described previously (Risbrough et al., 2003; Risbrough et al., 2009). Calibrations of stimuli and response sensitivity were conducted as described (Risbrough and Geyer, 2005).

Data Analysis

Acoustic Startle—Two or three-way ANOVAs with genotype (CTRL, COE, or KO) and oCRF as the between-subject factors, and NBI-30775, prepulse intensity, ISI, or pulse intensity as within-subject factors, were used. In the CRF-COE^{CNS} mice, we had the *a priori* hypothesis that the high dose, 20 mg/kg, would be effective compared to the 2 mg/kg dose based on our past findings that this dose is effective in blocking exogenous CRF effects on startle (Risbrough et al., 2004) and thus we also report analyses comparing vehicle and the 20 mg/kg dose only. *Post hoc* analysis followed significant main or interaction effects as appropriate. Unless specified otherwise, startle reactivity data are shown at the 120 dB startle intensity. For PPI assessment, percentage of PPI was first calculated using the formula: $100 - ((\text{startle of the prepulse} + \text{pulse trials} / \text{startle in the pulse alone trial}) \times 100)$.

Shock-potentiated startle—A four-way ANOVA with genotype as between-subject factors and startle intensity and startle block (before and after 0.4 mA) as within-subject factors was completed, followed by simple ANOVAs as appropriate.

Results

The data are summarized in Table 1 to show the overall pattern of effect on either startle magnitude or prepulse inhibition.

CNS -Specific CRF-COE (CRF-COE^{CNS}) Mice

Characterization (Supplemental Figure 1)—Characterization data for the CRF-COE^{CNS} mice are detailed in the supplementary material. Overexpression of CRF throughout the CNS did not significantly alter acoustic startle but there was a trend ($p < 0.09$) toward reduced habituation to repeated blocks of 120 dB acoustic stimulus in homozygous CRF-COE^{CNS} mice. There was a significant interaction between genotype and prepulse intensity for CRF-COE^{CNS} mice ($F(8,120)=2.34$, $p < 0.05$) with CRF-COE^{CNS}_{hom} mice showing reduced PPI at the higher prepulse intensities. One mouse was removed from the analysis due to extraordinarily high values during non-stimulation trials indicating high artifact.

CRF₁ receptor antagonist treatment

Acoustic Startle (FIGURE 1): When comparing all 3 doses, there was a trend for a main effect of drug in the CRF-COE^{CNS} mice ($F(2,56)=2.42$, $p = 0.09$). The test of our *a priori* hypothesis that 20 mg/kg would be the most effective dose revealed a strong trend for a drug x gene interaction ($F(2,28)=3.15$, $p=0.0584$). Posthoc tests revealed significant elevations in startle magnitude at 100 and 110 dB pulses in vehicle-treated homozygous CRF-COE^{CNS} mice. However, in mice treated with 20 mg/Kg NBI-30775, there were no differences between heterozygous and homozygous CRF-COE^{CNS} groups compared to CRF-COE^{Ctrl} control littermates.

Prepulse Inhibition (FIGURE 2): Minimal effects of CRF-COE^{CNS} on PPI were observed in the varied prepulse intensity block (69, 73, and 81 dB), again with a trend for an effect of CRF overexpression at the high prepulse intensity (Gene x Intensity $F(4,58)=2.52$, $p=0.05$, Supplemental Figure 2). When keeping the prepulse intensity fixed at 81 dB and varying the ISI, a significant CRF-COE^{CNS} effect was observed, with a significant genotype x drug x ISI interaction ($F(16,240)=1.77$, $p < 0.05$). In vehicle-treated mice, CRF overexpression significantly decreased %PPI at the 70 and 120 msec intervals in homozygous CRF-COE^{CNS} mice and decreased %PPI at the 120, 260, and 1080 msec intervals in heterozygous CRF-COE^{CNS} mice ($p < 0.05$, Tukey's post hoc test). This effect was reduced following a 2 mg/kg dose of NBI-30775; significant PPI deficits were observed only at the 70 and 120 msec intervals and only in the homozygous CRF-COE^{CNS}. After treatment with 20 mg/kg NBI-30775, PPI performance of CRF-COE^{CNS} mice did not differ from controls.

Habituation to Startle (FIGURE 3): CRF-COE^{CNS} mice exhibited increased startle reactivity across all 5 blocks relative to their control littermates and this increase was reduced by CRF₁ receptor antagonist NBI-30775 treatment (Gene X Drug: $F(4,58)=2.82$, $p < 0.05$). Startle habituation (Main effect of Block: $F(4,116)=32.08$ $p < 0.0001$) was not influenced by drug or gene.

Forebrain GABAergic Neuron-Specific CRF-COE (CRF-COE^{GABA}) Mice (FIGURE 4)

Characterization (Supplemental Figure 3)—During the acoustic threshold block there was a significant gene x intensity interaction in startle magnitude ($F(5,95)=3.31$, $p < 0.01$) with reductions in startle specifically at the 120 dB pulse ($p < 0.05$, Tukey's post hoc test). Examining 120dB trials across the entire session there was a strong trend for decreased startle in CRF-COE^{GABA} mice ($F(1,19)=4.09$, $p = 0.057$). The CRF-COE^{GABA} mice exhibited no significant differences in startle inhibition measures (PPI or habituation).

CRF₁ receptor antagonist treatment—Startle magnitude at high pulse intensities was significantly decreased by CRF overexpression in inhibitory neurons (gene x intensity: $F(2,38)=3.80$, $p < 0.05$); however there was no effect of NBI-30775 treatment. As expected from the characterization data, post hoc analyses identified a significant decrease at 120 dB in both vehicle and NBI-treated CRF-COE^{GABA} mice compared to controls ($p < 0.05$ Tukey's post hoc test). Again, when examining startle habituation across the session, there was a strong trend for startle to be reduced across the session in CRF-COE^{GABA} mice ($F(1,20)=4.16$, $p = 0.0549$, Figure 4). NBI-30775 treatment also decreased startle overall ($F(1,20)=4.67$, $p < 0.05$) but had no interaction with genotype. PPI was unaffected by CRF overexpression in GABAergic forebrain neurons or NBI-30775 treatment (Supplemental Figure 4).

Forebrain-Specific CRF₁ Receptor Knockout (CRF₁-KO) Mice (FIGURE 5)

Ovine CRF Challenge—Ovine CRF was administered to control mice and forebrain-specific CRF₁-KO mice. As shown previously (Gresack and Risbrough, 2011), CRF-induced increases in startle were most apparent at the higher pulse intensities (drug x Intensity, $F(5,90)=9.24$, $p < 0.001$; 120 dB startle trials shown in Figure 5A, other intensities in supplemental materials). CRF infusions also reduced PPI (main effect of drug,

$F(1,18)=4.79$, $p < 0.05$) (Figure 5B shows the effect of CRF averaged across prepulse intensities). CRF-induced disruption of PPI depended on ISI (drug $\times$ ISI, $F(4,72)=3.28$, $p < 0.05$) as well as prepulse intensity (drug $\times$ PPI, $F(4,72)=2.64$, $p < 0.05$), likely due to floor effects at parameters that evoke relatively low PPI (e.g. low intensity and short ISIs; Supplemental Figure 5). However, forebrain-specific deletion of CRF₁ receptor had no significant effect on baseline startle or CRF-induced startle increases (main effect of gene, $F(1,18)<1$, NS) or on baseline PPI and CRF-induced PPI deficits (main effect of gene, $F(1,18)=1.13$, NS).

Footshock: To investigate the response to endogenous CRF release, we also examined the response of forebrain CRF₁-KO mice to shock-potentiated startle, which we have previously shown is absent in full CRF₁ receptor null mice (Risbrough et al., 2009). Similar to the response to exogenous CRF treatment, forebrain-specific deletion of CRF₁ receptors had no effect on shock-potentiated startle, with both groups exhibiting increased startle reactivity after foot shock (main effect of shock, $F(1,20)=22.99$, $p<0.001$). Figure 5C shows startle at the 105 dB level.

Discussion

To identify the impact of the CRF/CRF₁ system in specific cell types and brain regions on startle plasticity, we employed a series of transgenic mice overexpressing CRF either in the entire CNS (CRF-COE^{CNS}) or within GABAergic neurons (CRF-COE^{GABA}) or lacking the CRF₁ receptor within forebrain regions (forebrain CRF₁-KO). CRF-COE^{CNS} mice exhibit substantial elevations of CRF expression throughout the CNS (Lu et al., 2008). Based on previous pharmacological studies of exogenous CRF administration and studies of stress-induced endogenous CRF release, we hypothesized that CRF-COE would increase startle magnitude, reduce rate of habituation, and reduce PPI.

CNS-specific overexpression increased acoustic startle magnitude and reduced PPI in a gene-dose-dependent manner. This effect was due to CRF₁ receptor activation; the elevated startle and PPI disruption was attenuated by NBI-30775. These data support the hypothesis that life-long excessive CRF signaling in the CNS results in startle abnormalities, and that these abnormalities can be attenuated with acute CRF₁ receptor antagonist treatment. Conversely, CRF overexpression limited to inhibitory neurons significantly reduced startle reactivity at the 120dB pulse in block 1 and had no effects on inhibition or habituation of the startle response. CRF₁ receptor blockade did not attenuate these reductions in startle reactivity, suggesting that this phenotype is not via acute CRF₁ receptor activation. Finally, a role of forebrain CRF₁ receptor in CRF and stress-induced changes in startle was surprisingly not supported in our studies of forebrain-specific CRF₁-KO mice, suggesting that CRF₁ receptors on principal forebrain neurons are not required for CRF or stress effects on startle plasticity.

Our finding that CRF-COE^{CNS} induces reduced PPI is in agreement with other models of “lifelong” CRF overexpression. CRF overexpression under the Thy1 promoter has been shown to induce robust reductions in PPI that are reversible with acute CRF₁ receptor antagonist treatment (Groenink et al., 2008). A recent study from our group showed that life-

long CRF overexpression restricted to forebrain regions driven by the CamKII α promotor (no hypothalamic CRF overexpression in contrast to Thy1-CRF-OE and CRF-COE^{CNS} mice) also exhibited reductions in PPI (Toth et al., 2014), indicating that forebrain CRF overexpression is sufficient to induce PPI disruptions. It should be noted that this phenotype could also be produced by forebrain CRF overexpression restricted only to development (P2-P23), suggesting that sensorimotor gating is modifiable not only by acute CRF release, but also via adaptations induced by early life CRF exposure (Toth et al., 2014). The present data also indicate that lifelong CRF overexpression does not produce PPI disruptions under all circumstances, since CRF-COE^{GABA} had no effect on PPI. CRF expression levels are quite high in the brains of these mice (Lu et al., 2008), suggesting that a simple “dose” explanation for CRF exposure in these mice could not account for a lack of effects on PPI. CRF-COE^{GABA} mice do show some regional differences in CRF overexpression compared to CRF-COE^{CNS} mice. Notably, the overexpression in CRF-COE^{GABA} mice is restricted to forebrain structures and is absent from brainstem nuclei and the cerebellum.

The present data also indicate that CRF-induced disruptions in PPI are not via forebrain CamKII α -expressing neurons carrying CRF1 receptors, despite the role of this receptor population in the anxiety-inducing effects of CRF (Muller et al., 2003; Refojo et al., 2011). This finding was surprising given that forebrain CRF overexpression or direct infusions of CRF into the BNST reduces PPI (Lee and Davis, 1997; Toth et al., 2014) and that CRF1 receptors are required for CRF-induced disruption of PPI (Risbrough et al., 2004). An alternative explanation is that CRF1 receptors on cell types other than projection neurons (i.e. expressing CamKII α) are required for CRF effects on PPI (Refojo et al., 2011). Other potential circuits for CRF-induced disruption of PPI include non-forebrain regions that modulate/mediate PPI and express CRF receptors, such the raphe nuclei and the nucleus reticularis pontis caudalis (Swerdlow et al., 2001; Van Pett et al., 2000).

Loss of CRF1 receptors within principal cells of the forebrain also did not influence response to CRF-potentiated startle, suggesting that, like the effects on PPI, the effects of CRF on startle are not mediated by *CRF1* receptors within this cell population. This result is surprising given that startle magnitude is increased by CRF injection directly into the BNST, an effect that is blocked by CRF receptor antagonists into the BNST (Davis et al., 1997; Lee and Davis, 1997). Forebrain-specific deletion of CRF1 receptors also had no influence on shock-potentiated startle, a behavior that is completely blocked by full KO of CRF1 receptors (Risbrough et al., 2009). There is some evidence that shock-potentiated startle (also termed context-potentiated startle) is mediated by *CRF1* receptors in the CeA (Walker et al., 2009). While future studies are needed to determine whether CRF1 receptors in non-CamKII α -expressing neurons contribute to this phenotype, these data support the hypothesis that activation of CRF receptors in the BNST and CeA are sufficient but not necessary for CRF or shock-potentiated startle, respectively. Previous studies with this forebrain CRF₁-KO line have shown that anxiety-like behavior in the light dark box (LDB) and elevated plus maze (EPM) is reduced (Muller et al., 2003). These data dissociate the role of CRF1 receptors on startle versus avoidance-based anxiety-like behavior.

While further studies are needed to determine the specific cell type and region of CRF1 receptors responsible for the effects of CNS-specific CRF overexpression on startle, one

candidate is the LC. This hindbrain nucleus is the primary source of central norepinephrine and previous studies have shown that norepinephrine mediates the effects of CRF on startle magnitude (Gresack and Risbrough, 2011). In contrast, CRF effects on PPI are not mediated by norepinephrine, thus other circuits are likely involved in CRF effects on sensorimotor gating. CRF₁ receptors are expressed in numerous hindbrain regions involved in the startle reflex including the dorsal and ventral cochlear nucleus and the pontine reticular nucleus (Justice et al., 2008). CRF₁ receptor is also expressed on GABAergic cells of the globus pallidus (Refojo et al., 2011); GABAergic cells from the globus pallidus have been shown to influence PPI via projections to the pedunculo pontine tegmentum (Takahashi et al., 2007). CRF₁ receptors on GABAergic cells of the reticular thalamic nucleus or medial septum could also be involved (Refojo et al., 2011).

Intriguingly, overexpression of CRF in GABAergic neurons in the present study reduced startle magnitude, which did not require CRF₁ receptor activation. Given that this effect was not due to CRF₁ receptor activation, and acute CRF₂-specific activation either has no effect or facilitates startle reactivity induced by CRF or stress (Risbrough et al., 2003; Risbrough et al., 2004; Risbrough et al., 2009), the most parsimonious explanation is that unique compensatory changes over development resulted in this phenotype. Studies are ongoing to delineate the compensatory changes in CRF₁ and CRF₂ expression and binding in response to CRFOE in inhibitory vs. excitatory neurons. The transgenic mouse using the Thy1 promoter to overexpress CRF in postnatal and adult neurons also yielded reduced startle magnitude in some but not all reports (Dirks et al., 2002; Groenink et al., 2008), and these mice have reductions in CRF₁ receptors in thalamus, striatum, cortex, and septum (Korosi et al., 2006). Overexpression of CRF restricted to adulthood also result in alterations in CRF₁ receptor expression and possibly accounts for “paradoxical” anxiolytic effects of CRF overexpression in some reports (Regev et al., 2011). An alternative explanation is that the CRF overexpression in GABAergic neurons is favoring CRF signaling at regions which control anxiolytic-like effects of CRF₁ and/or CRF₂ receptors, such as the globus pallidus (Refojo et al., 2011; Sztainberg et al., 2011). Further research is needed to test these alternative hypotheses.

A number of neuropsychiatric disorders exhibit CRF hypersecretion, however these disorders exhibit different startle phenotypes. The present findings and previous reports taken together support the hypothesis that distinct neuronal sources of CRF hypersecretion could “drive” the specific startle phenotypes across these disorders. In particular, our results show that CRF from GABAergic neurons is unlikely to mediate these effects given the inability to mimic the phenotype when CRF overexpression was restricted to inhibitory neurons. Similarly, it is likely that CRF effects on PPI and startle do not require CRF₁ receptor activation at CamKII α expressing cells in the forebrain. Future studies must overcome some of the limitations of the present experiment, which include the potential for effects of ectopic CRF overexpression and possible compensation due to “life-long” CRF₁ receptor loss or CRF peptide overexpression. Future studies using CRFOE and CRF₁ gene deletion across specific neural circuits and cell types will be critical in identifying the specific neural circuits that play a role in stress-induced changes in these symptom domains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Rationale:* Excessive CRF production has been implicated in the etiology of stress-sensitive psychiatric disorders such as posttraumatic stress disorder (PTSD), which is associated with alterations in startle plasticity.
- *Goal:* To determine what neural circuits modulate startle in response to chronic CRF activation
- *Method:* Transgenic mice lacking the CRF1 receptor in forebrain principle neurons, or mice overexpressing CRF throughout the central nervous system (CNS; CRF-COE^{CNS}) or restricted to inhibitory GABAergic neurons (CRF-COE^{GABA}) were compared across multiple domains of startle plasticity.
- *Result:* CRF overexpression throughout the CNS increased startle magnitude and reduced ability to inhibit startle (decreased habituation and decreased prepulse inhibition (PPI)), while CRF overexpression confined to inhibitory neurons decreased startle magnitude but had no effect on inhibitory measures.
- *Result:* Acute CRF receptor 1 (CRF₁) antagonist treatment attenuated only the effects on startle induced by CNS-specific CRF overexpression and specific deletion of CRF1 receptors from forebrain principal neurons failed to alter the effects of exogenous CRF or stress on startle.
- *Conclusion:* Each neural source of increased CRF may yield unique startle phenotypes; this may have implications for disorders characterized by increased CRF in cerebrospinal fluid.

Figure 1. Acoustic Startle Magnitude in CRF-COE^{CNS} Mice Treated with NBI-30775

There was a strong trend for a gene x drug interaction and *post hoc* analysis revealed that heterozygous CRF-COE^{CNS} mice display elevated startle (A), which was reduced by the CRF₁ receptor antagonist NBI-30775 at the 20 mg/kg (C) dose.) * = $p < 0.05$ vs. Ctrl, Tukey's *post hoc* test. The 2.0 mg / Kg dose is also shown (B). Data are presented as arbitrary units (AU). N = 10–11 per group.

Figure 2. Prepulse inhibition with varying interstimulus interval (ISI) in CRF-COE^{CNS} mice treated with NBI-30775

A prepulse of 16 dB reduced the startle response to a 120 dB pulse in control mice (Ctrl). This PPI was reduced in vehicle-treated heterozygous (Het) and homozygous (Hom) CRF-COE^{CNS} mice (A). The decrease in PPI was attenuated by 2 mg/kg (B) and 20 mg/kg (C) NBI-30775. * = $p < 0.05$, ** = $p < 0.001$ vs. Ctrl, Tukey's post hoc test. N = 10–11 per group.

Figure 3. Habituation to Startle Reactivity in CRF-COE^{CNS} Mice Treated with NBI-30775
 All mice showed significant habituation to startle reactivity with repeated exposure. There was a significant interaction between genotype and drug with elevated startle magnitude across the 5 startle blocks in vehicle-treated CRF-COE^{CNS} mice (**A**), reduced by 2 mg/kg (**B**) and 20 mg/kg (**C**) of NBI-30775. N = 10–12 per group.

Figure 4. Startle reactivity in CRF-COE^{GABA} mice

CRF-COE^{GABA} mice exhibit decreased startle magnitude when treated with vehicle (A) or 20 mg/Kg NBI-30775 (B). * = $p < 0.05$, ** = $p < 0.001$. N = 10–11 per group.

Figure 5. oCRF administration in mice lacking CRF₁ receptors in forebrain regions
 CRF elevated acoustic startle (**A**) and reduced PPI (**B**). However, forebrain-specific deletion of CRF₁ receptors had no significant effect on baseline- or CRF-induced startle increases or PPI deficits (figure B depicts PPI averaged across pulse intensities). Deletion of CRF₁ receptors also had no effect on endogenous CRF release in shock-potentiated startle at the 105 dB level (**C**). * = $p < 0.05$, ** = $p < 0.001$ indicating main effect of CRF collapsing across genotype. Note that CRF infusion and shock-potentiated startle experiments were conducted in different acoustic startle chambers resulting in slightly different baseline magnitude readings. N = 5–6 per group for the oCRF experiment; N = 11 per group for the shock experiment.

Table 1

Table 1 depicts the effects of genotype and / or 20 mg/Kg NBI-30775 on startle magnitude at varying pulse intensities (block 1 of the startle paradigm) or at the 120dB across all 5 blocks of the startle paradigm and on prepulse inhibition with varying pulse intensity (block 3 of the startle paradigm) or varying ISI (block 4 of the startle paradigm). Direction of effect is indicated by up or down arrows. ANOVA results are included for all trends ($p < 0.1$) and significant ($p < 0.05$) differences. Only *post hoc* effects with $p < 0.05$ are described.

		STARTLE MAGNITUDE		PREPULSE INHIBITION	
		Block 1 Varied Pulse Intensity (80–120 dB)	Block 1–5 Habituation All 120dB Trials	Block 3 Varied Prepulse Intensity (69–81 dB)	Block 4 Varied ISI (20–1080 ms)
CNS CRF-COE	Characterization	NS	$\uparrow$ Gene x Block $p < 0.1$ <i>post hoc</i> $p < 0.05$ at block 3,5	$\downarrow$ Gene x Intensity $p < 0.1$, <i>post hoc</i> $p < 0.05$ at 73/105 dB*	$\uparrow$ Gene x ISI $p < 0.05$ <i>Post hoc</i> $p < 0.05$ at 20 ms*
	Effect of CRFOE	$\uparrow$ Gene x Drug $p < 0.1$ <i>post hoc</i> $p < 0.05$ at 100 and HOdB	$\uparrow$ Gene x Drug $p < 0.05$	$\downarrow$ Gene x Intensity $p < 0.1$	$\downarrow$ Gene x Drug x ISI < 0.05 , <i>post hoc</i> $p < 0.05$, 0.01 at ISI 70–1080 ms
	Effect of Drug (20mg/Kg NBI-30775)	(–)Attenuated COE-induced increases Gene x Drug $p < 0.1$	(–)Attenuated COE-induced increases Gene x Drug $p < 0.05$	NS	(–)Attenuated COE-induced decreases Gene x Drug x ISI < 0.05
GABA CRF-COE	Characterization	$\downarrow$ Gene x Intensity $p < 0.01$ <i>post hoc</i> $p < 0.05$ at 120 dB**	$\downarrow$ Gene ANOVA $p < 0.1$	NS	NS
	Effect of CRFOE	$\downarrow$ Gene x Intensity $p < 0.05$ <i>post hoc</i> $p < 0.05$ at 120 dB	$\downarrow$ Gene ANOVA $p < 0.1$	NS	NS
	Effect of Drug	No effect of drug	$\downarrow$ Main effect of Drug $p < 0.05$	NS	NS
Forebrain CRFj-KO	CRF-potentiated startle & reduced PPI	No effect of genotype	No effect of genotype (data not shown)	No effect of genotype	No effect of genotype
	Shock-Potentiated Startle	No effect of genotype	N/A	N/A	N/A

* Note change to 81dB for further tests based on characterization data suggesting CRFOE has strongest effects on PPI at high intensity prepulse trials.

** Note 80 and 90 dB pulse trials were dropped from this study due to low levels of startle activation.