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CRF_1 and CRF_2 Receptors are Required for Potentiated Startle to Contextual but not Discrete Cues

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Corticotropin-releasing factor (CRF) peptides and their receptors have crucial roles in behavioral and endocrine responses to stress. Dysregulation of CRF signaling has been linked to post-traumatic stress disorder, which is associated with increased startle reactivity in response to threat. Thus, understanding the mechanisms underlying CRF regulation of startle may identify pathways involved in this disorder. Here, we tested the hypothesis that both CRF_1 and CRF_2 receptors contribute to fear-induced increases in startle. Startle responses of wild type (WT) and mice with null mutations (knockout, KO) for CRF_1 or CRF_2 receptor genes were measured immediately after footshock (shock sensitization) or in the presence of cues previously associated with footshock (ie fear-potentiated startle, FPS). WT mice exhibited robust increases in startle immediately after footshock, which was dependent upon contextual cues. This effect was completely absent in CRF_1 KO mice, and significantly attenuated in CRF_2 KO mice. In contrast, CRF_1 and CRF_2 KO mice exhibited normal potentiation of startle by discrete conditioned cues. Blockade of both receptors via CRF_1 receptor antagonist treatment in CRF_2 KO mice also had no effect on FPS. These results support an additive model of CRF_1 and CRF_2 receptor activation effects on potentiated startle. These data also indicate that both CRF receptor subtypes contribute to contextual fear but are not required for discrete cued fear effects on startle reactivity. Thus, we suggest that either CRF_1 or CRF_2 could contribute to the increased startle observed in anxiety disorders with CRF system abnormalities.
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INTRODUCTION

The corticotropin-releasing factor (CRF) family of peptides, including CRF and the urocortins play a critical role in neuroendocrine and behavioral stress responses (for review see Hauger *et al*, 2006). Some anxiety disorders have been linked to abnormalities in CRF signaling. Post-traumatic stress disorder (PTSD) patients exhibit elevated levels of CRF in cerebrospinal fluid (Sautter *et al*, 2003; Bremner *et al*, 1997; Baker *et al*, 1999). Mutations in the CRF (Smoller *et al*, 2005) and CRF_1 receptor (Keck *et al*, 2008) genes have been associated with traits predictive for panic disorder and panic disorder diagnoses, respectively. Identifying CRF receptor signaling pathways that subserve anxiety and fear may further our understanding of how CRF pathology could contribute to these disorders.

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Corticotropin-releasing factor family peptides activate two known G-protein-coupled receptors, CRF_1 and CRF_2, which are expressed throughout the mammalian brain (for review see Risbrough and Stein, 2006). CRF_1 activation is required for both behavioral and endocrine responses to stress (for review see Hauger *et al*, 2006), supporting the clinical application of CRF_1 antagonists for anxiety and stress-related disorders (Steckler and Dautzenberg, 2006; Zorrilla and Koob, 2004; Holsboer and Ising, 2008). Involvement of CRF_2 receptors in stress and anxiety is less clear, and may depend on a number of factors including region of activation, baseline stress level, and type of behavior assessed (see Hauger *et al*, 2006 and discussion below). CRF receptor activation also modulates fear learning, although again, the specific contribution of CRF_2 is less clear. CRF_2 activation has been reported to either enhance or attenuate fear learning (Radulovic *et al*, 1999; Sananbenesi *et al*, 2003; Todorovic *et al*, 2007).

The defensive acoustic startle response consists of a series of involuntary reflexes elicited by a sudden, intense auditory or tactile stimulus (Graham, 1975; Yeomans *et al*, 2002). PTSD and panic disorder subjects exhibit exaggerated startle reactivity (e.g. Butler et al, 1990, Ludewig et al.

2005) predominantly in response to stressful environments or threat (eg oncoming shock, darkness; for review, see Grillon and Baas, 2003), although a direct link between CRF abnormalities and increased startle has not been established. In rodents, exogenous CRF administration increases startle, an effect that can be attenuated by anxiolytic treatment (Swerdlow *et al*, 1986, 1989). CRF_1 is required for exogenous CRF-induced increases in startle, as antagonism or null mutation of CRF_1 prevents CRF-induced increases in startle (Risbrough *et al*, 2003b, 2004). The role of CRF_2 in startle is less clear however. CRF_2 antagonism attenuates CRF-induced increases in startle; direct CRF_2 agonism however has minimal effects (Risbrough *et al*, 2003b, 2004). We have suggested previously that CRF_2 may be additive with CRF_1 in mediating CRF-induced potentiation of startle. A critical question remains however, are the functions of CRF_1 and CRF_2 observed after exogenous CRF treatment reproducible under physiological conditions that better model human anxiety and fear responses?

Contextual and discrete cues associated with an aversive stimulus can elicit exaggerated startle reactivity in humans, a phenomenon that may be exacerbated in PTSD and other anxiety disorders (Grillon and Baas, 2003; Stam, 2007). The enhancing effect of discrete fear cues on startle is termed fear-potentiated startle (FPS) and is found across rodents, primates, and humans (Brown *et al*, 1951; Winslow *et al*, 2002; Grillon *et al*, 1991). Studies examining the role of CRF receptors in FPS have been inconsistent, with CRF receptor antagonist treatment either reducing FPS (Swerdlow *et al*, 1989; Schulz *et al*, 1996) or having no effect on FPS (de Jongh *et al*, 2003). These inconsistencies may be because of a lack of receptor specificity of the pharmacological manipulations. Startle is also exaggerated immediately after footshock in rats and mice, a phenomenon originally termed 'shock sensitization' (Davis, 1989; Dirks *et al*, 2001). In rats, the shock sensitization effect is attributed to contextual fear learning (Richardson, 2000; McNish *et al*, 1997), and thus 'sensitization' may be a misnomer. Humans also exhibit increased startle reactivity after shock (Greenwald *et al*, 1998) and other stressors (eg social stress, Grillon *et al*, 2007). In rodents, shock has been shown to induce CRF receptor signaling (Bakshi *et al*, 2002; Ho *et al*, 2001; Le *et al*, 2002; Wang *et al*, 2005); hence, we reasoned that shock induced increases in startle may be attributable to CRF signaling. Here, we tested the hypothesis that CRF receptors contribute to fear induced increases in startle reactivity. To test our hypothesis, we examined the effect of CRF_1 or CRF_2 receptor null mutation on startle reactivity under two conditions, either under immediate response to footshock or in response to a discrete cue previously associated with footshock (FPS).

MATERIALS AND METHODS

Subjects

Separate naive cohorts of 2- to 3-month-old male and female CRF_1 (Timpl *et al*, 1998) or CRF_2 (Coste *et al*, 2000) WT and knockout (KO) mice bred in-house by heterozygous mating (backcrossed to C57BL/6J mice for more than 12 generations) were used for each experiment. Male C57BL/6J mice (Jackson Laboratories; 6- to 8-week-old on arrival) were used to test the dependence of shock induced increases in startle on contextual cues. Animals were housed 4 to a cage with food and water provided *ad libitum* and maintained in a climate-controlled room with a reverse 12-h light/dark cycle (lights on at 1700 hours). Animals were tested during the dark phase between 1000 and 1600 hours. Experiments were conducted in accordance with the 'Principles of Laboratory Animal Care' NIH guidelines and with local animal care committee approval.

Apparatus

Startle chambers and footshock apparatus (San Diego Instruments, San Diego, CA) are as described previously (Risbrough *et al*, 2003a). From startle stimulus onset, 65 consecutive 1 ms readings were recorded to obtain the peak amplitude of the animals' startle response to acoustic startle stimuli or the average response during the initial 65 ms of the footshock stimuli. Responses to these stimuli are presented in arbitrary units. A light + tone CS was delivered via a bare 5-W incandescent bulb located on the ceiling of the testing chamber (400 lux) and a Sonalert tone generator located at the far wall of the chamber (4 kHz, 70 dB). A 65 dB white-noise background was delivered throughout all sessions. Calibrations of stimuli and response sensitivity were conducted as described elsewhere (Risbrough and Geyer, 2005). Shocks presented outside of the chamber in control experiment 3 were presented in 4 FreezeMonitor chambers (San Diego Instruments). Each acrylic chamber (25 cm wide $\times$ 18 cm high $\times$ 21 cm deep) was inside a sound-attenuating box. A 15-W house light and small fan in the wall of the sound-attenuating box were on during all sessions. The shock grid floor consisted of 32 stainless steel rods wired to a shock generator for scrambled shock delivery.

Behavioral Testing

Fear-potentiated startle. Mice were tested over 4 consecutive days. On day 1, mice were tested initially with the FPS test session to evaluate any unconditioned effects of the CS on startle before pairing with the US. The session consisted of an initial eight startle pulses without the presence of the cue, four each of 100 and 105 dB 40-ms pulses, with an average ITI of 15 s (range: 7–23 s). This block was used to habituate and stabilize startle responding and was not used in the analysis of FPS. Then eight startle pulses in the presence of the CS and without the CS were presented in a pseudorandom order with an average ITI of 60 s (range: 30–90 s). Half of the startle pulse trials were 100 dB and the other half 105 dB. On days 2 and 3, mice received 10 training trials of CS–US pairings with an average ITI of 180 s (range: 130–230 s). Each training trial consisted of a simultaneous 30 s presentation of the light and tone stimuli (the compound CS), with a 0.4 mA footshock (250 ms) presented immediately at offset of the CS. The FPS test session was then repeated on day 4, 24 h after the last training session.

To test the effects of dual blockade of CRF_1 and CRF_2 receptors on FPS expression, a second cohort of CRF_2 KO mice were trained for FPS using the protocol above. On the pretraining test day, all mice were injected with vehicle to

allow for direct comparisons between pretraining and post-training tests as injection stress may increase unconditioned responses to the CS. On the post-training test day, the selective CRF_1 receptor antagonist NBI-30775 (also known as R-121919; a gift from Neurocrine Biosciences Inc., San Diego, CA) or vehicle was administered 30 min before FPS testing (day 4). Mice receiving drug or vehicle were matched for pretraining startle reactivity. NBI-30775 (20 mg/kg) was administered by intraperitoneal (i.p.) injection in a 5 ml/kg volume, using 3% Cremophor/sterile water as the vehicle. This dose and time point was efficacious in blocking exogenous CRF induced increases in startle in mice (Risbrough *et al*, 2003b, 2004).

Shock-potentiated startle. At least 24 h before shock exposure, mice were tested for baseline startle and assigned to Shock and No-Shock groups matched for baseline startle scores. On the test day, mice received a total of five brief startle sessions. After habituation to the startle chamber for 5 min, the first startle session was presented to examine preshock startle reactivity. At 30 s later, mice were presented with a block of five 0.2-mA shocks with an ITI of 60 s (30–90 s range). At 1 min after the last shock, the startle session was presented again. 1 min after the second startle session, a block of five 0.4-mA shocks was presented and followed by the third startle session. At 1 min later, a block of 5–0.8 mA shocks was presented, followed by the fourth startle session. All footshocks were 500 ms in duration.

The startle session consisted of two blocks: the first included nine each of 90, 105, and 120 dB startle pulses (40 ms), given in a pseudorandom order with an average ITI of 15 s (range: 7–23 s). The second block consisted of seven each of 105 and 120 dB pulse alone trials, and five each of four prepulse trials (71 or 75 dB 20 ms prepulse preceding either a 105 or 120 dB 40 ms pulse). The data for this second block (stimuli examining prepulse inhibition) are not presented here because they address different hypotheses. A 'no stimulus' trial in which data were recorded without startle stimuli occurred in the middle of each ITI to examine baseline activity.

To aid in interpreting the effects of shock on startle reactivity, we tested if shock induced increases in startle were context dependent. Two matched groups received either shock or no shock in the startle chambers (same-context group) and two matched groups received shock or no shock in fear conditioning chambers in another room (different-context group). All mice were initially tested for startle reactivity using the startle block described above and then immediately removed and placed in holding cages. Mice in the different-context group were then transported to an adjacent room for footshock presentations in fear conditioning chambers, and then immediately returned to the startle chambers for postshock testing. Mice receiving shock in the startle chambers (same context) were kept in holding cages in an adjacent room for 4 min before and after shock presentations to equate handling procedures across context. In both contexts, Shock groups were presented with five 0.4-mA footshocks (500 ms, 30–90 s ITI). No Shock controls underwent the exact same procedures as the shock groups however the shock grids were not activated.

Data Analysis

Fear-potentiated startle. A three-way ANOVA was completed with genotype as a between-subject factor and training (before or after training) and cue (startle trials with and without the presence of the CS) as within-subject factors. Initial analyses with startle intensity as a factor did not reveal any interactions with cue or genotype so this factor was dropped. % FPS was calculated to normalize for differences in startle during the no-cue trials, and analyzed using a two-way ANOVA with genotype as a between-subject factor and training as a within-subject factor (% FPS = ((mean 'cue' trial startle magnitude − mean 'no-cue' trial startle magnitude)/mean 'no-cue' trial startle magnitude) × 100). To assess NBI-30775 effects on FPS in CRF_2 WT and KO mice, a three-way ANOVA was completed on % FPS scores with genotype and treatment as between-subject factors and training as a within-subject factor.

Shock-potentiated startle. To examine initial preshock startle baseline across genotype, a three-way ANOVA with gene and shock as between-subject factors and startle intensity (90, 105, 120 dB) as within-subject factors was completed on data collected immediately before footshocks were presented. To assess the effects of the subsequent footshock on startle, a percentage change from preshock baseline scores ((postshock startle block − preshock startle block)/preshock startle block) × 100) was calculated. A four-way ANOVA with genotype and shock as between-subject factors and startle intensity and startle block (after 0.2, 0.4, 0.8 mA) as within-subject factors was completed on the percentage scores, followed by simple ANOVAs as appropriate. To assess context effects on shock induced increases in startle a two-way ANOVA with context exposure (same context *vs* different context) as a between-subject factor and startle intensity as a within-subject factor was completed.

The ANOVAs reported are collapsed across sex because initial analyses including sex as a factor revealed that, although female mice consistently showed significantly lower startle values than males, sex did not interact with gene, shock, or CS training. *Post hoc* analyses were completed (significance was considered as $\alpha < 0.05$) using Tukey's test or simple ANOVAs as appropriate.

RESULTS

Fear-Potentiated Startle

Experiment 1: fear-potentiated startle in CRF_1 WT and KO mice. After CS–US paired training, cue trials produced significantly higher startle reactivity compared to no cue trials, as supported by a significant interaction between training (pre *vs* post-training) and trial type (cue *vs* no cue) (training × trial type: $F(1,20) = 26.2$, $p < 0.0001$; data not shown). Accordingly, % FPS scores were significantly increased after training (Figure 1a, data collapsed across 100 and 105 dB intensities; training: $F(1,20) = 33.07$, $p < 0.0001$). There were no significant effects of CRF_1 genotype on startle magnitude or % FPS. There was also no significant effect of genotype on average shock reactivity ($F(1,20) = 1.2$, NS; data not shown).

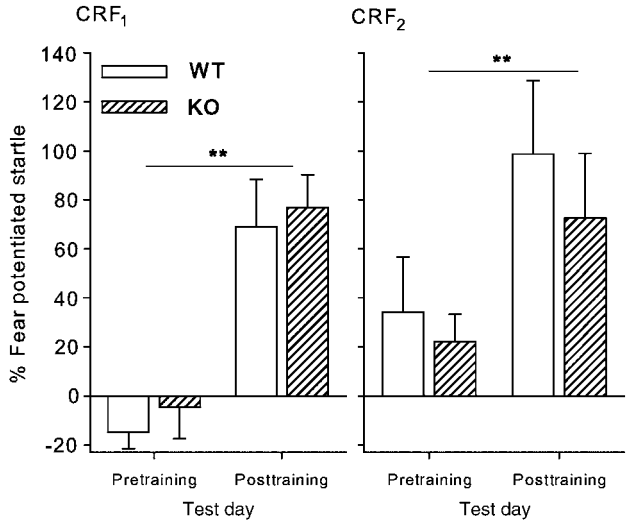

Figure 1 Potentiated startle induced by conditioned fear in CRF_1 and CRF_2 null mutation mice. Mice were tested for startle reactivity with and without the presence of the CS before (pretraining) and after 20 CS–US pairing trials (posttraining). Data are represented as mean ± SEM of % fear-potentiated startle (FPS). $N = 8$–19, $^{**}p < 0.01$ main effect of training on % FPS.

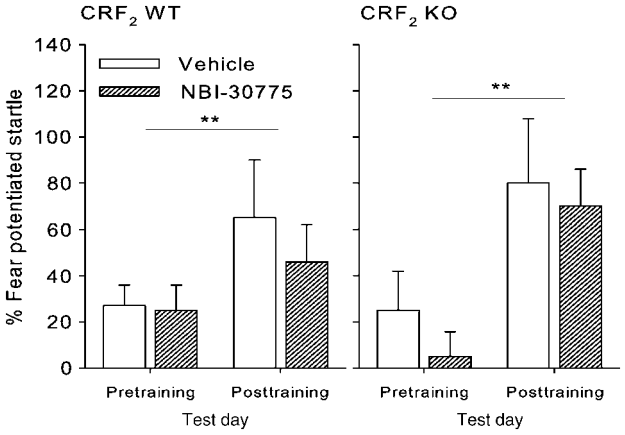

Figure 2 Effect of pharmacological blockade of CRF_1 receptors on potentiated startle induced by conditioned fear in CRF_2 null mutation mice. Mice were tested for startle reactivity with and without the presence of the CS before (pretraining) and after 20 CS–US pairing trials (posttraining). NBI-30775 (20 mg/kg, i.p.) or vehicle was administered 30 min before the posttraining test day only. Data are represented as mean ± SEM of % fear-potentiated startle (FPS). $N = 7$–13, $^{**}p < 0.01$ main effect of training on % FPS.

Experiment 2: fear-potentiated startle in CRF_2 WT and KO mice. As in experiment 1, training increased startle reactivity selectively in cue trials (data not shown; training × trial type: $F(1,31) = 5.64$, $p < 0.05$) and consequently increased % FPS (Figure 1b, data collapsed across 100 and 105 dB intensities; training: $F(1,31) = 5.26$, $p < 0.05$). CRF_2 KO mice exhibited no differences in startle or % FPS compared to WT mice. There was also no significant effect of genotype on average shock reactivity ($F(1,32) < 1$, NS; data not shown).

Experiment 3: fear-potentiated startle in CRF2 WT and KO mice with CRF_1 receptor antagonist treatment. To test for the possibility that either CRF_1 or CRF_2 receptors are sufficient for expression of FPS, we treated CRF_2 WT and KO mice with the selective CRF_1 receptor antagonist NBI-30775 on the post-training test day. NBI-30775 did not significantly alter % FPS in either WT or KO mice (Figure 2; main effect of training: $F(1,33) = 10.14$, $p < 0.005$, no interactions of training with genotype or drug).

Shock-Potentiated Startle

Experiment 1: shock-potentiated startle in CRF_1 WT and KO mice. CRF_1 WT and KO mice did not differ significantly in preshock baseline startle across the three startle intensities, although KO mice tended to have lower startle magnitude (Table 1; main effect of Genotype: $F(1,48) = 3.15$, $p = 0.08$). This preshock baseline was used to calculate a percentage change in startle after footshock to normalize for any small differences in startle reactivity. After shock, significant interactions of genotype with shock and startle intensity were found on % startle potentiation (Intensity × Genotype × Shock: $F(2, 96) = 5.11$, $p < 0.01$). To dissect the cause of these interactions, we conducted separate analyses for each genotype. As expected, the WT Shock group exhibited shock potentiation compared to the No-Shock group; this effect was most robust at the 90 dB startle intensity, with a peak of 119% increase from preshock baseline at the 0.4 mA block compared to peak increases of 35 and 16% at the 105 and 120 dB startle pulses (Figure 3, left panel; Table 1, Shock × startle intensity: $F(2,72) = 8.26$, $p < 0.001$). Likely because of ceiling effects at the higher intensities, shock induced increases were most robust at the lowest startle intensity (90 dB), thus data from the 90 dB pulse intensity trials will be presented in figures, percentage change at the 105 and 120 dB pulses are presented in Supplementary Table 1. In CRF_1 KO mice however, mice presented with shock showed no differences in startle potentiation compared to the No-Shock group (main effect of shock and interactions $F < 1$, NS). At the 90 dB intensity, CRF_1 KO mice had significantly less shock-potentiated startle than shocked WT mice, independent of shock intensity (Figure 3, left panel; main effect of Genotype: $F(1,24) = 4.82$, $p < 0.05$). To determine if the lack of potentiation in CRF_1 KO mice was because of reduced nociception, we examined jump responses during the shock stimuli. CRF_1 WT and KO mice exhibited similar jump responses to the footshock stimuli, with reactivity increasing with increasing current intensities (Table 2; Shock × Intensity: $F(2,96) = 3.24$, $p < 0.05$; Genotype: $F(1,48) < 1$, NS).

Experiment 2: shock-potentiated startle in CRF_2 WT and KO mice. As seen with CRF_1 null mutation, CRF_2 null mutation resulted in no significant differences in baseline startle (Table 1; Preshock: Genotype: $F(1,45) < 1$, NS). As in experiment 1, the shock increased startle reactivity in CRF_2 WT mice, with most robust effects at the 90 dB startle intensity (eg at the 0.4 mA shock block there was 113, 66, and 32% increase from preshock baseline at the 90, 105, and 120 dB startle intensities, respectively, Intensity × Block × Shock: $F(6,270) = 4.31$, $p < 0.001$, see Supplementary Table 1 for 105 and 120 dB data). *Post hoc* analysis at the 90 dB

Table 1 Baseline Startle Reactivity Immediately Before Footshock Stress

	CRF_1				CRF_2			
dB	**WT no shock**	**WT shock**	**KO no shock**	**KO shock**	**WT no shock**	**WT shock**	**KO no shock**	**KO shock**
90	119 ± 22	56 ± 13	96 ± 11	77 ± 12	131 ± 28	265 ± 61	167 ± 34	176 ± 44
105	372 ± 57	176 ± 28	302 ± 36	225 ± 69	308 ± 58	357 ± 52	292 ± 63	339 ± 73
120	459 ± 62	382 ± 118	399 ± 41	367 ± 117	472 ± 66	473 ± 64	438 ± 86	470 ± 87

CRF, corticotropin-releasing factor; KO, knock out; WT, wild type.
Measures are mean ± SEM of peak startle magnitude during 90, 105, and 120 dB startle pulses, presented in the startle session immediately before footshock stress. There was no significant difference between No-Shock and Shock group startle reactivity before footshock stress began. No significant effects of gene were observed; see 'Results' for details.

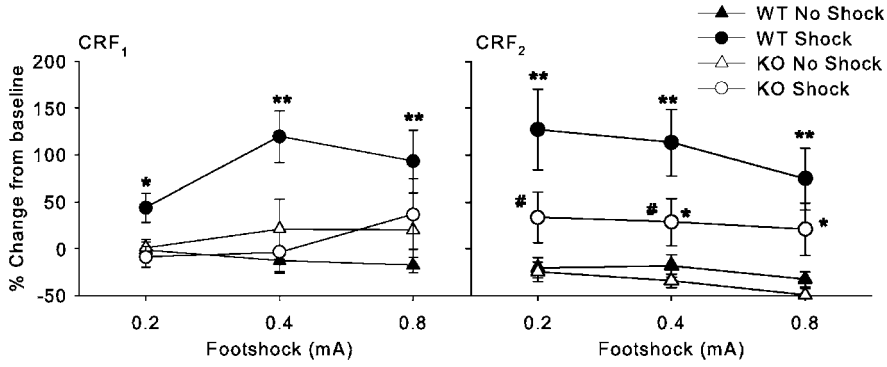

Figure 3 Potentiated startle induced by footshock in CRF_1 and CRF_2 null mutation mice. Effects of ascending footshock intensity on startle reactivity were assessed in CRF_1 and CRF_2 receptor wild-type and knockout mice (WT and KO). Startle reactivity was tested immediately before (preshock baseline) the initial shock block and immediately after each block of footshocks (footshocks were presented in ascending order, 0.2, 0.4, and 0.8 mA, five per block). Half of the animals in each genotype received shock (Shock group) and the other half did not receive shock (No-Shock group). Data are represented as mean ± SEM of % change from initial preshock baseline reactivity to 90 dB startle pulse. $N = 7$–19, $^*p < 0.05$, $^{**}p < 0.01$ *vs* respective No Shock control, $\#p < 0.05$ *vs*. WT Shock, Tukey's *post hoc* test.

Table 2 Footshock Reactivity in CRF_1 and CRF_2 WT and KO Mice

	CRF_1		CRF_2	
mA	**WT**	**KO**	**WT**	**KO**
0.1	—	—	111 ± 46	130 ± 34
0.2	232 ± 20	260 ± 65	295 ± 46	223 ± 43
0.4	313 ± 23	287 ± 21	389 ± 52	243 ± 52
0.8	263 ± 23	326 ± 31	344 ± 38	254 ± 37

CRF, corticotropin-releasing factor; KO, knock out; WT, wild type.
Measures are reported as mean ± SEM of average jump response over the initial 65 ms record window. In experiments 3 and 4, footshocks were varied in intensity (0.2, 0.4, 0.8 mA, 500 ms in duration, given in ascending order). In experiment 5, a separate test using only 0.1 mA (500 ms duration) shock was used in CRF_2 KO mice. No significant effects of gene were observed; see 'Results' for details.

intensity indicated that although both CRF_2 WT and KO mice exhibited increases in startle after shock (Figure 3, right panel, $p < 0.05$, Tukey's *post hoc* test), the magnitude of the increase in CRF_2 KO mice was significantly lower than WT mice (Figure 3, right panel, $p < 0.05$, Tukey's *post hoc* test). Because shock effects may cause an inverted U-shaped dose–response curve on startle reactivity (Davis and Astrachan, 1978; Borszcz *et al*, 1989), a separate experiment using lower shock levels was also conducted to test for the small chance that CRF_2 null mutation produced a leftward shift in the inverted shock response curve (ie with lower shock intensities CRF_2 null mutation would result in greater shock-potentiated startle). A 0.1 mA footshock intensity produced weak but significant increases in startle (mean ± SEM of % change from baseline at 90 dB: WT/No Shock = −8.3 ± 17.2, KO/No Shock = 16.2 ± 12.8, WT/Shock = 55.3 ± 39.9, KO/Shock = 32.3 ± 20.6) with no interaction with genotype or startle intensity (Shock: $F(1,27) = 7.65$, $p < 0.05$).

Experiment 3: dependence of shock induced increases in startle reactivity on contextual cues. To test if shock-potentiated startle was because of associative learning to contextual cues, we examined shock-potentiated startle in C57BL6J mice either in the same or different context in which they experienced shock. Unlike mice receiving shock in the startle chambers, mice given similar shock presentations outside of the startle chambers showed no significant increases in startle reactivity compared to No-Shock controls (90 dB pulse shown in Figure 4, 105 and 120 dB data not shown; Context × Shock: $F(1,43) = 5.65$, $p < 0.05$; Context × Shock × Pulse Intensity: $F(2,86) = 9.47$, $p < 0.001$).

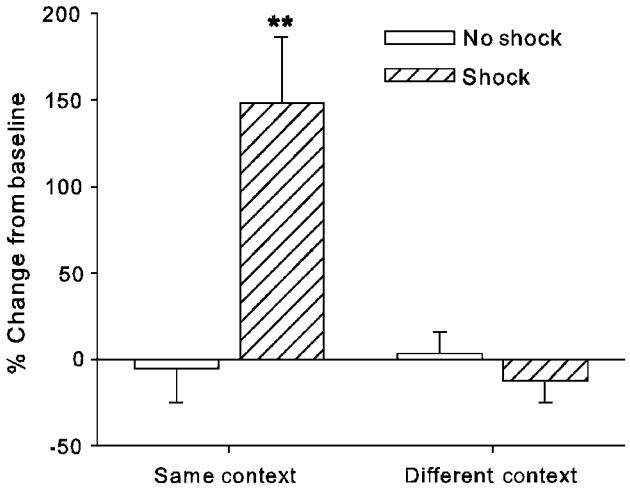

Figure 4 Effect of contextual cues on shock-potentiated startle in mice. Mice were initially tested for preshock baseline. Half of the mice were then moved to another room for footshock presentations (different context), whereas half were replaced into the startle chambers for footshock presentations (same context). Half of the mice in each context group received five 0.4-mA shocks ('Shock' group) or no footshock ('No Shock' group). After shock presentations all mice were replaced into the startle chambers to test postshock reactivity. Data are represented as mean ± SEM of % change from preshock baseline to 90 dB startle pulse. $N = 11–12$, $^{**}p < 0.01$ *vs* respective No-Shock control, Tukey's *post hoc* test.

DISCUSSION

Here we examined the role of CRF receptors in the potentiation of startle reactivity produced by either discrete or contextual fear cues. Unlike their WT littermates, CRF_1 KO mice failed to exhibit shock-potentiated startle. In contrast, CRF_2 KO mice exhibited increases in startle after footshock, although these responses were significantly reduced compared to WT mice. Neither CRF_1 nor CRF_2 KO mice exhibited significant changes in their jump response to footshock or in baseline (preshock) startle reactivity. These results support our hypothesis that CRF_1 and CRF_2 exert additive influences on startle potentiation. Conversely, both CRF_1 and CRF_2 KO mice exhibited normal acquisition and expression of FPS. Hence, startle increases elicited by discrete cues do not require either CRF_1 or CRF_2 signaling. These findings indicate a dissociation between the influences of CRF on context fear learning *vs* conditioned fear to discrete cues.

In this study, we found that CRF_1 and CRF_2 KO mice exhibited normal FPS (Figure 1). We also found that blockade of CRF_1 receptors in CRF_2 KO mice had no significant effects on FPS expression (Figure 2). The dose of NBI-30775 used was found previously to be effective in blocking the effects of exogenous CRF treatment on startle reactivity (Risbrough *et al*, 2004). These results are consistent with pharmacological studies indicating that non-selective CRF antagonists (de Jongh *et al*, 2003) and agonists (Risbrough and Geyer, 2005; Walker and Davis, 2002) have no effects on FPS. Others have observed blockade of FPS expression in rats with either α-helical CRF administration (Swerdlow *et al*, 1989) or the selective CRF_1 receptor antagonist CP-154,526 (Schulz *et al*, 1996). It is not immediately clear what differences could account for these disparate findings, although the previous studies were conducted in rats whereas the present studies are conducted in mice. Swerdlow *et al* (1989) trained rats with 30 pairing trials per day over 4 days, thus 'over-training' the rats in comparison to the present study, resulting in large increases (approximately 200%) in baseline startle. The FPS levels observed in Swerdlow *et al* (1989) were relatively weak compared to the present study, 21% increase *vs* 70–100%, respectively, perhaps because of the high baseline startle levels resulting from contextual learning. Schulz *et al* (1996) had fairly similar training methods as those presented here (20 pairing trials), although the shock level (1.2 mA) was three times stronger than the methods used here. Unfortunately, they did not report % FPS values or startle values, so it is difficult to directly compare our results. It is possible that prolonged training (Swerdlow *et al*, 1989) or very intense US stimuli (Schulz *et al*, 1996) result in stronger contextual learning influences on FPS, although these speculations are untested. We report here that context learning is reduced in CRF_1 and CRF_2 KO mice; hence, paradigms with greater contextual influences may be more sensitive to CRF receptor antagonist effects. We have also observed no effects of CRF_1 or CRF_2 receptor antagonist treatment on FPS expression in mice (VB Risbrough and MA Geyer, unpublished observations), making it more difficult to claim that compensatory effects alone could account for normal FPS expression in CRF_1 and CRF_2 KO mice. The present findings are also consistent with a recent report that CRF_1 receptor blockade in the amygdala has no effect on conditioned fear as assessed by freezing to discrete cues (Hubbard *et al*, 2007). Hence, taken together, our data support the hypothesis that neither CRF_1 nor CRF_2 receptors are required for cued FPS. It should be noted however that these results are not inconsistent with a modulatory role for CRF receptors on fear learning, in particular contextual fear learning (see below). CRF receptor activation before conditioning has been shown previously to influence fear learning, with septal CRF_2 receptor activation producing reductions in freezing to conditioned cues (Todorovic *et al*, 2007). Thus, it is possible that CRF_1 or CRF_2 null mutation may affect FPS under other protocols, specifically, when animals are stressed or treated with CRF before fear conditioning (Rau *et al*, 2005; Todorovic *et al*, 2007; Servatius *et al*, 2005).

Unlike FPS, mice lacking CRF_1 receptors showed a complete absence of shock-potentiated startle (Figure 3). This pattern of results is similar to the total absence of CRF treatment effects on startle reported previously in CRF_1 KO mice (Risbrough *et al*, 2004) or with CRF_1 antagonist treatment (Risbrough *et al*, 2003b). Conversely, CRF_2 KO mice exhibited increases in startle after shock; although the magnitude was attenuated significantly compared to WT mice (Figure 3). This overall pattern of effects is similar to findings using pharmacological manipulations. Previously, we showed that CRF_2 blockade via the antagonist Antisauvagine-30 attenuated the effects of high doses of rat/human CRF on startle (which activates both CRF_1 and CRF_2 receptors, but did not reverse the effects of low doses of CRF; Risbrough *et al*, 2003b), which are more selective for CRF_1 receptors. Taken together, our results indicate that shock- or CRF induced increases in startle require CRF_2 activation to reach maximal levels (Risbrough *et al*, 2004).

CRF_2 activation alone however does not appear to be sufficient to produce robust increases in startle. First, the present data indicate there were no residual increases in startle in shocked CRF_1 KO mice. These mice have intact CRF_2 receptors that are presumably being activated, as CRF_2 KO mice also show altered responses in this task. Second, previous reports indicate that selective CRF_2 agonists do not produce robust increases in startle (Risbrough *et al*, 2003b, 2004). Overall these data are in line with previous results that pharmacological blockade of CRF_1 and CRF_2 reduces shock-induced freezing (Bakshi *et al*, 2002). Taken together, these data also support our previous suggestion that startle-increasing effects of CRF_2 may require concomitant CRF_1 activation (Risbrough *et al*, 2004). Others have also suggested that anxiogenic effects of CRF_2 activation may require concurrent stress (Henry *et al*, 2006), which presumably activates CRF_1 receptors. It is important to note that CRF_1 and CRF_2 KO mice exhibited normal learning of cue associations with the unconditioned shock stimulus as measured by FPS, indicating that the shock stimulus retained its aversive properties in CRF_1 and CRF_2 KO mice.

As in rats, we found that shock induced increases in startle reactivity were dependent on the contextual cues, as mice tested immediately after footshock in a different context showed no significant increases in startle reactivity (Figure 4; Richardson, 2000). Because CRF_1 and CRF_2 null mutation modifies responses in this paradigm, it appears that activation of CRF_1 and CRF_2 receptors contributes to immediate context fear effects on startle reactivity, although the regions mediating these effects are unknown. Studies in rats suggest that the amygdala plays a permissive role in shock-potentiated startle; hence, the amygdala may be an important modulatory site of action for CRF receptor effects on this behavior as well (Van Nobelen and Kokkinidis, 2006; Sananes and Davis, 1992). Previous reports using freezing behavior support a modulatory role for CRF_1 and CRF_2 receptors in contextual fear learning when tested at longer time points after training. Stress or CRF-induced activation of hippocampal CRF_1 receptors enhances fear learning (Radulovic *et al*, 1999; Todorovic *et al*, 2007) whereas amygdala CRF_1 receptor activation contributes to long-term consolidation of fear learning (Roozendaal *et al*, 2002; Hubbard *et al*, 2007). Hippocampal injections of a CRF_2 antagonist block stressed induced increases in contextual fear learning (Sananbenesi *et al*, 2003). These CRF_2 effects were linked to secondary messenger signaling however, which is unlikely to be functioning in the immediate context fear produced by the shock stress. Shock-potentiated startle does not appear to require NMDA receptor activation or protein synthesis, necessary components for long-term (eg 24 h) fear memory storage (Van Nobelen and Kokkinidis, 2006). Hence, only tentative comparisons can be made between studies using context-conditioned freezing and startle, as these measures may be probing differential short- and long-term conditioned fear processes, as well as differential neural substrates (eg McNish *et al*, 1997).

Previous reports have shown consistently that the inactivation of CRF_2 receptors in the septum and the raphe produces anxiety-like effects in rodents (Bakshi *et al*, 2007; Henry *et al*, 2006; Hammack *et al*, 2003). Whole-brain inactivation is less consistent however, with pharmacological blockade inducing both anxiolytic (present data Pelleymounter *et al*, 2002; Takahashi *et al*, 2001) and anxiogenic-like effects (Kishimoto *et al*, 2000). Genetic null mutation has been reported to produce no effect or anxiolytic effects (Kishimoto *et al*, 2000; Bale *et al*, 2000; Coste *et al*, 2000) in approach-avoidance tests, and reductions in stress coping behavior, such as increased hypophagia in response to stress and increased immobility in the forced swim test (Coste *et al*, 2006; Bale and Vale, 2004). The present results are the first to our knowledge to show an anxiolytic-like effect of CRF_2 null mutation, thus being more consistent with septal or raphe CRF_2 receptor inactivation studies. This interpretation should be made cautiously however, as it is also possible that CRF_2 receptor activation mediates the aversive quality of the US (Sahuque *et al*, 2006) or could disrupt immediate fear learning. Evidence arguing against the latter interpretation is that cued fear learning was not affected by CRF_2 gene deletion, which would be expected to be altered if CRF_2 activation contributes to the aversive quality of the US. Future studies examining the contribution of CRF_2 to other forms of immediate learning are required to fully resolve whether CRF_2 is blocking cognition or defensive responding *per se*. CRF_2 receptor blockade also attenuates unconditioned increases in startle reactivity after CRF administration however, thus this contribution of CRF_2 effects on startle is observed in both conditioned and unconditioned potentiated startle models. In contrast to rodent approach-avoidance measures of anxiety (eg open field, plus maze, forced swim), defensive startle measures are not confounded by alterations in locomotor activity (Davis, 1990). CRF_2 ligands have robust effects on locomotor activity and grooming behavior (Todorovic *et al*, 2007; Valdez *et al*, 2003; Bakshi *et al*, 2007; Zhao *et al*, 2007), which may account for some inconsistent findings in models that are influenced by activity levels (eg grooming behaviors may compete with approach-avoidance behaviors). Unlike other lines of CRF_2 KO mice, this line exhibits normal approach-avoidance behavior (eg elevated plus maze or open field) and normal CRF expression (Bale *et al*, 2000; Bale and Vale, 2004; Coste *et al*, 2000). It has been suggested that the anxiety-like phenotype exhibited by other lines of CRF_2 KO mice may be in part because of an increased CRF expression in the amygdala (Bale and Vale, 2004). Nevertheless, this explanation seems unlikely to account for the present results, because increased CRF release would be expected to increase rather than decrease startle reactivity, as observed here.

Conclusions

The present data indicate that FPS to contextual cues requires CRF_1 activation, and also require CRF_2 activation to achieve maximal effects. Conversely, conditioned cue effects on startle using the same US (shock) do not require CRF_1 or CRF_2 signaling. These results suggest that CRF_2 blockade can attenuate immediate context learning effects on startle, but without altering effects of discrete conditioned cues. Although there is still continued controversy over the role of CRF_2 in anxiety, the present results provide more evidence that CRF_2 receptor activation can mediate increases in certain defensive behaviors.

The shock-potentiated startle task may be particularly relevant to clinical studies of startle reactivity, as human tests of potentiated startle are most commonly conducted over one session and assess immediate responses to threatening or painful stimuli, not long-term learned responses (eg 24 h in-between training and testing; Grillon, 2008). The findings that CRF receptors play a role in contextual FPS may also have relevance to PTSD. Exaggerated startle responses in PTSD subjects are most commonly observed in the laboratory in paradigms using stress or threat manipulations (for review see Grillon and Baas, 2003), and CRF abnormalities have been linked to PTSD (Baker *et al*, 1999; Bremner *et al*, 1997; Sautter *et al*, 2003; for review see Risbrough and Stein, 2006). Although no direct link between CRF pathology and startle abnormalities has been established, our results indicate that excessive CRF_1 or CRF_2 signaling could contribute to the exaggerated startle reactivity observed in PTSD. These studies support future examination of CRF receptor antagonists in clinical studies of context fear effects on potentiated startle.

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DISCLOSURE/CONFLICT OF INTEREST

Drs Hauger, Coste, Stenzel-Poore, Wurst and Holsboer have no potential financial conflicts to report. Over the past 3 years, Dr Geyer has received compensation from Acadia, Addex, Amgen, AstraZeneca, Jazz, Omeros, Organon and Wyeth-Ayerst and holds an equity interest in San Diego Instruments. Over the past 3 years Dr Risbrough has received compensation as a consultant from Arena Pharmaceuticals and San Diego Instruments.

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