Behavioral/Systems/Cognitive

Corticotropin-Releasing Factor Receptors CRF_1 and CRF_2 Exert Both Additive and Opposing Influences on Defensive Startle Behavior

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The corticotropin-releasing factor (CRF) receptors (CRF_1 and CRF_2) are crucial mediators of physiological and behavioral responses to stress. In animals, CRF_1 appears to primarily mediate CRF-induced anxiety-like responses, but the role of CRF_2 during stress is still unclear. Here we report the effects of CRF_1 and CRF_2 on the magnitude and plasticity of defensive startle responses in mice. Startle plasticity is measured by inhibition of startle by sensory stimuli, i.e., prepulse inhibition (PPI), and is disrupted in patients with panic or posttraumatic stress disorders in which CRF neurotransmission may be overactive. Pharmacological blockade of CRF_1 reversed both CRF-induced increases in startle and CRF-induced deficits in PPI. CRF_2 blockade attenuated high-dose but not low-dose CRF-induced increases in startle and reduced PPI. Conversely, activation of CRF_2 enhanced PPI. CRF had no effect on startle and increased PPI in CRF_1 knock-out mice. These data indicate that CRF receptors act in concert to increase the magnitude of defensive startle yet in opposition to regulate the flexibility of startle. These data support a new model of respective CRF receptor roles in stress-related behavior such that, although both receptors enhance the magnitude of defensive responses, CRF_1 receptors contravene, whereas CRF_2 receptors enhance, the impact of sensory information on defensive behavior. We hypothesize that excessive CRF_1 activation combined with reduced CRF_2 signaling may contribute to information processing deficits seen in panic and posttraumatic stress disorder patients and support CRF_1-specific pharmacotherapy.

Key words: **CRH; antisauvagine-30; NBI-30775; R121919; urocortin; anxiety**

Introduction

The neuropeptide corticotropin-releasing factor (CRF) mediates many neuroendocrine and behavioral responses to stress (Vale et al., 1981; Rivier et al., 1983; Koob and Heinrichs, 1999). CRF acts in the brain at two distinct G-protein-coupled receptors, CRF_1 and CRF_2 (Perrin and Vale, 1999; Dautzenberg and Hauger, 2002). Although both pharmacological and CRF_1 knock-out (KO) mouse studies clearly support the anxiogenic effects of CRF_1 activation, the influences of CRF_2 activation on anxiety are less clear (Koob and Heinrichs, 1999; Bakshi and Kalin, 2000; Coste et al., 2001; Reul and Holsboer, 2002). Some evidence indicates that anxiety behaviors mediated by CRF_1 activation are reduced when CRF_2 is activated (Bale et al., 2000; Kishimoto et al., 2000; Coste et al., 2001; cf. Reul and Holsboer, 2002; Valdez et al., 2002). Nevertheless, other evidence indicates that CRF_2 is also capable of inducing anxiety-like behavior (Radulovic et al., 1999; Takahashi et al., 2001; Pelleymounter et al., 2002) and increasing certain defensive behaviors, such as conditioned freezing (Bakshi et al., 2002) and the startle reflex (Risbrough et al., 2003).

The startle response consists of a series of involuntary reflexes elicited by a sudden, intense auditory stimulus and is considered to be a defensive behavior evolved to protect the body from impact during attack (Graham, 1975; Yeomans et al., 2002). The magnitude of the response is highly plastic: fear-inducing stimuli or CRF administration increase startle (Brown et al., 1951; Swerdlow et al., 1986; Davis et al., 1997), whereas threat-reducing stimuli (Lang et al., 1990) or sensory input in the case of prepulse inhibition (PPI) (Graham, 1975) reduce startle. Across species, presentation of a neutral, nonstartling acoustic "prepulse" 30–300 msec before the startling stimulus reduces startle magnitude, possibly by requiring the organism to allocate attentional resources to process the prepulse and hence filter or "gate" the subsequent startling stimulus (Graham, 1975; Hoffman and Ison, 1980; Norris and Blumenthal, 1996; Swerdlow et al., 1999). Clinically, PPI is used as an experimental measure of sensory and

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cognitive information-processing mechanisms that are deficient in a number of neuropsychiatric disorders (Braff et al. 2001).

Recently, patients suffering from panic disorder or posttraumatic stress disorder (PTSD) have been shown to have disruptions in PPI (Grillon et al., 1996, 1998; Ludewig et al., 2002). Brain CRF hypersecretion and CRF_1 sensitization are hypothesized to contribute to some anxiety disorders (Dautzenberg and Hauger, 2002; Reul and Holsboer, 2002; Musselman and Nemeroff, 2003). Endogenous overexpression of CRF in transgenic mice or acute administration of CRF in rats reduces PPI (Conti et al., 2002; Dirks et al., 2002, 2003), although the mechanism whereby CRF receptor signaling modulates startle plasticity remains unknown. Understanding the mechanism of CRF-induced decreases in PPI may have important implications for describing specific brain system pathologies in anxiety disorder patients that exhibit disruptions in PPI. Hence, we examined the respective influences of CRF_1 and CRF_2 on the startle response and its sensitivity to inhibition by sensory information using both pharmacological techniques and CRF_1 KO mice.

Materials and Methods

Subjects

Male C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME) and male 129S6/SvEvTac mice from Taconic (Germantown, NY) aged 6–8 weeks on arrival were housed four per cage in a temperature-controlled (21–22°C) room under a reverse 12 hr light/dark cycle (lights off at 8:00 A.M.). The reverse light/dark cycle was used to minimize interactions with the stress of disruptions in diurnal cycles associated with testing during the sleep phase. Initial studies [antisauvagine-30 (ASV30) and urocortin 2] were performed in C57BL/6J mice (Risbrough et al., 2003). Subsequent studies (NBI-30775 and urocortin 3) used 129SvEv mice because these mice exhibit more robust PPI performance, which allows for an optimal baseline performance for detection of both disruptions and improvements in PPI performance (Crawley et al., 1997). Heterozygously bred CRF_1 receptor wild-type (WT) and KO mice (mixed C57BL/6J × 129SvEv background) (Smith et al. 1998) aged 4–5 months were housed similarly, except they were housed one per cage after surgery. All testing occurred from 10:00 A.M. to 6:00 P.M. and was conducted in accordance with the *Principles of Laboratory Animal Care*, National Institutes of Health guidelines, as approved by the University of California, San Diego, Veterans Affairs Medical Center, and Scripps Research Institute Animal Care Committees.

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. A dynamic calibration system was used to ensure comparable sensitivities across chambers. 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.

Drugs

Peptide infusions. In all experiments other than with CRF_1 receptor WT and KO mice, peptides were injected intracerebroventricularly using a free-hand method in lightly anesthetized mice as described previously (Pelleymounter et al., 2002; Risbrough et al., 2003). The injection volume was 5 μl. Pilot studies using dye injections instead of drug indicate that this method has ~95% accuracy for the ventricles, with dye (2 μl injected) being found in lateral, third, and fourth ventricles. Any mouse with either (1) a cerebral hematoma or (2) an injection site >0.5 mm away from bregma and behavioral performance 2 SD from the group mean was removed from analysis (7 of 162 total mice injected).

Free-hand intracerebroventricular injections were not possible in CRF_1 WT and KO mice because of their age and the thickness of their cranium. Hence, WT and KO 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). Cannulas were secured with one skull screw and dental cement. Drug injections and histologies were as described previously (Spina et al., 2000). In brief, injections were made 5–7 d after surgery in unanesthetized mice using a 30 gauge 8 mm injector (1 mm below the tip of the guide cannulas). Injection volume was 5 μl using gravity flow. Two weeks after testing, mice were anesthetized and 1 μl of dye was injected via the 8 mm injector using gravity flow. 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 noted 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).

Experiment 1, ASV30 versus human/rat-CRF. Human/rat (h/r)-CRF at 0.06 and 0.6 nmol (0.2 nmol = 1 μg of peptide; Bachem, Torrance, CA) and 3 nmol ASV30 (a gift from Neurocrine Biosciences, San Diego, CA) were diluted in water (Pelleymounter et al., 2002; Risbrough et al., 2003) and coadministered or given alone 1 hr before testing.

Experiment 2, NBI-30775 versus h/r-CRF. The selective CRF_1 receptor antagonist NBI-30775 (also known as R-121919; a gift from Neurocrine Biosciences) was diluted in 5% cremophor–sterile water and a 20 mg/kg dose was administered intraperitoneally both 10 min before and 60 min after h/r-CRF or artificial CSF (aCSF) administration (i.e., 110 and 60 min before test). This double-injection method was used to ensure a lasting effect of NBI-30775 over the 2 hr pretest and testing period.

Experiment 3, h/r-CRF in CRF_1 WT and KO mice. WT and KO CRF_1 mice received aCSF vehicle or 0.2 nmol of h/r-CRF and were tested 1 hr after injection.

Experiment 4, urocortin 2 and urocortin 3. Human urocortin 2–stresscopin-related peptide (2 and 6 nmol; Bachem) was dissolved in aCSF vehicle and administered 1 hr before the first test session. The mice were tested 1, 2, 3, and 4 hr after administration. This time course was chosen because some reports indicate that urocortin 2 can have effects up to 4 hr after administration (Valdez et al., 2002). Mouse urocortin 3 (0.8 and 2.4 nmol; Bachem) was dissolved in aCSF vehicle. The mice were tested 2, 4, and 6 hr after administration, because the urocortin 2 data indicated that a longer time window may be needed to detect time-dependent effects of CRF_2 receptor activation on PPI (see below).

Behavioral testing

For all acoustic startle sessions, the intertrial interval between stimulus presentations averaged 15 sec (range of 7–23 sec). A 65 dB background was presented continuously throughout the session. After placement into the startle chambers, a 5 min acclimation period preceded testing. Four to 10 d before drug testing, mice were tested briefly to measure baseline acoustic startle response and PPI. The mice were then assigned to drug groups (i.e., counterbalanced) so that all drug groups averaged similar startle response levels and PPI performance before drug testing. Startle pulses were 40 msec in duration, prepulses were 20 msec in duration, and prepulses preceded the pulse by 100 msec (onset–onset). In all prepulse trials, the interstimulus interval was 100 msec from onset of the prepulse to onset of the pulse. Testing parameters were varied slightly between each separate experiment to customize the parameters to keep baseline PPI performance consistent across different cohorts and strains (between 40 and 55% PPI on average). Thus, experiments in which the mice exhibited relative poor pretest performance, such as C57BL/6J mice, were presented some trials with louder prepulse stimuli and reduced startle stimuli than mice with relatively better pretest performance, such as 129SvEv mice (details below). Thus, in experiments using C57BL/6J (ASV30 vs h/r-CRF, urocortin 2) and CRF_1 WT and KO mice, acoustic startle sessions included two blocks. The first block tested acoustic startle

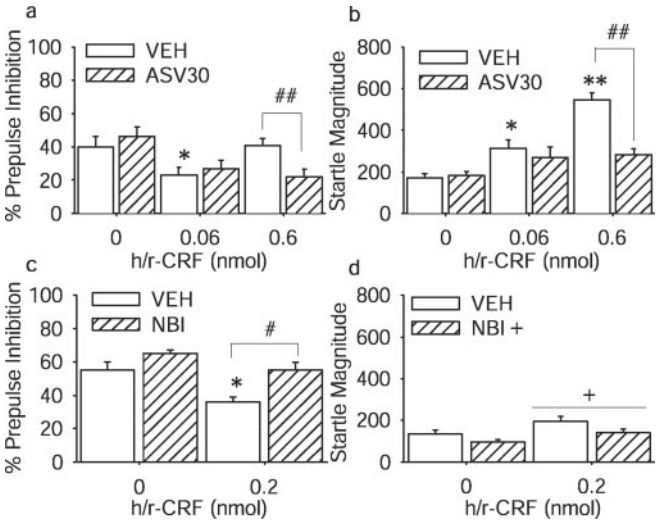

Figure 1. Effect of CRF on startle and prepulse inhibition during CRF_1 or CRF_2 receptor blockade. *a*, *b*, Vehicle (VEH), 0.06 or 0.6 nmol of h/r-CRF, and vehicle or 3 nmol of ASV30 were coadministered (5 μl, i.c.v.) in C57BL/6J mice 1 hr before prepulse inhibition (*a*) and startle (*b*) testing. $^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle/vehicle group; $^{\#\#}p < 0.01$ versus 0.6 nmol of h/r-CRF/vehicle group. *c*, *d*, Vehicle or 20 mg/kg (intraperitoneally) NBI-30775 (NBI) was administered 10 min before vehicle and 60 min after vehicle or 0.2 nmol of h/r-CRF (5 μl, i.c.v.) in 129SvEv mice. Prepulse inhibition (*c*) and startle (*d*) were tested 2 hr after h/r-CRF injection. $^{*}p < 0.05$ versus vehicle/vehicle group; $^{\#}p < 0.05$ versus 0.2 nmol of h/r-CRF/vehicle group; $^{+}p < 0.05$ main effect of NBI-30775 and main effect of h/r-CRF. Data are expressed as mean ± SEM.

response only and included three different acoustic stimulus intensities: 90, 105, and 120 dB. The data from this block in C57BL/6J mice were presented previously (Risbrough et al., 2003). The second block (data presented here) consisted of six each of 105 or 120 dB startle pulse intensities and five each of four different prepulse trials (73 and 81 dB preceding either a 105 or 120 dB pulse). In experiments in 129SvEv mice, the test sessions consisted of nine 120 dB startle pulses and six each of 69, 73, and 81 dB (urocortin 3) prepulse trials or 10 120 dB startle pulses and eight each of 73 and 81 dB (NBI-30775) prepulse trials.

Data analysis

In all experiments, the average startle magnitude over the record window (i.e., 65 msec) was used for all data analysis. Two mice were removed because of startle responses of <10 units during the 120 dB trials, which most likely indicates hearing loss. Either a two-way ANOVA with treatment (h/r-CRF) and pretreatment (ASV30 or NBI-30775) or genotype (WT or KO) or a one-way ANOVA with treatment (urocortin 2 or urocortin 3) as the between-subject factor, and prepulse and pulse intensity as within-subject factors, were used. *Post hoc* analysis followed significant main or interaction effects as appropriate. Unless specified otherwise, PPI data presented were collapsed across prepulse intensities, and startle reactivity data are shown at the 120 dB startle intensity (for detailed effects of CRF receptors on startle reactivity, see Risbrough et al., 2003).

Results

Experiment 1, ASV30 versus h/r-CRF: CRF_2 antagonism reduces PPI and decreases startle

Administration of h/r-CRF resulted in an inverted U-shaped dose–effect curve, with the low dose (0.06 nmol) of h/r-CRF decreasing PPI ($p < 0.05$; Dunnett's test), whereas a 10 times higher dose (0.6 nmol) had no effect on PPI (Fig. 1*a*) (h/r-CRF, $F_{(2,53)} = 7.65$; $p < 0.01$). The CRF_2 antagonist ASV30 reduced PPI when coadministered with the high 0.6 nmol dose of h/r-CRF ($p < 0.05$; Tukey's test) but had no effect when administered either alone or with the low dose of h/r-CRF (Fig. 1*a*) (ASV30 × h/r-CRF, $F_{(2,53)} = 4.13$; $p < 0.05$). In contrast to the effects on PPI, h/r-CRF dose dependently ($p < 0.01$; Tukey's test) increased acoustic startle magnitude (Fig. 1*b*) (h/r-CRF, $F_{(2,53)} = 19.96$; $p < 0.001$). Despite having no effects when given alone, ASV30 attenuated the increases in startle from the high but not low dose of h/r-CRF (Fig. 1*b*) (ASV30 × h/r-CRF, $F_{(2,53)} = 5.53$; $p < 0.01$).

The observation that the maximal effect of h/r-CRF on startle did not correspond with any effect on PPI indicates that the effects of CRF on startle and PPI are independent, thus confirming previous reports of CRF effects on startle plasticity (Conti et al., 2002; Dirks et al., 2002, 2003). To further evaluate the independence of the startle and PPI effects, we compared PPI across prepulse trials with different startle pulse intensities, thus more closely matching baseline startle values across prepulse performance (Brody et al., 2004). Comparisons of the PPI trials using a low-intensity startle stimulus (105 dB) in the 0.06 h/r-CRF group to PPI trials with a higher startle intensity stimuli (120 dB) in the vehicle group further confirmed the significant reduction in PPI at the 0.06 h/r-CRF dose group, even when the CRF group had a lower startle baseline relative to the vehicle group ($t_{19} = 1.8$; $p < 0.05$; vehicle, percentage PPI of 29 ± 5, 120 dB startle of 172 ± 21; 0.06 h/r-CRF, percentage PPI of 12 ± 9, 105 dB startle of 109 ± 32).

In vitro saturation binding studies indicate that ^{125}I-ASV30 binds to <0.1% of human CRF_1 recombinantly expressed in HEK cells (Higelin et al., 2001). In competitive binding experiments, ASV30 exhibits a 340- to 500-fold greater selectivity for CRF_2 over CRF_1 (Brauns et al., 2001; Dautzenberg et al., 2001). *In vivo* effects (some exerted by similar doses to the dose of ASV30 used in our experiments) are in sharp contrast to and/or do not interfere with CRF_1-mediated effects (Pelleymounter et al., 2002; Rivier et al., 2002; Hammack et al., 2003; Lu et al., 2003; Overstreet et al., 2004). Thus, current *in vitro* and *in vivo* data support the selectivity of ASV30 at the doses used for CRF_2 and not CRF_1 activity. *In vitro* studies indicate that h/r-CRF binds at CRF_2 with a K_i value that is 4- to 20-fold greater than the K_i value at CRF_1 (Dautzenberg et al., 1999; Perrin and Vale, 1999). Accordingly, the most parsimonious explanation for the present data are that the low h/r-CRF dose did not activate an observable CRF_2-mediated behavioral response, because ASV30 pretreatment did not alter the effects of this dose on PPI or startle. In contrast, ASV30 pretreatment prevented the effects of the high dose of h/r-CRF on PPI and startle. Thus, blockade of CRF_2 in the presence of high doses of agonist that no longer affect PPI may unmask a CRF_1 effect similar to that seen at lower CRF doses at which PPI deficits are observed. When tested with similar parameters in 129SvEv mice, which exhibit higher PPI performance (and are thus less likely to exhibit floor effects of CRF on PPI), ASV30 significantly potentiated 0.2 nmol h/r-CRF-induced deficits in PPI (percentage PPI: vehicle/vehicle, 72 ± 5; vehicle/CRF, 57 ± 5; ASV30/vehicle, 82 ± 5; ASV30/CRF, 32 ± 7; $p < 0.01$, vehicle/CRF vs ASV30/CRF). These data led us to hypothesize that the CRF_1 receptor is likely to mediate the low-dose CRF-induced deficits in PPI, although CRF_2 receptor activation may increase PPI. Accordingly, to test these hypotheses, we asked whether CRF_1 receptor blockade would attenuate or block CRF-induced decreases in PPI. To answer this question, we selected the 129SvEv strain of mouse, because their baseline PPI performance is more robust and consistent across testing (Crawley et al., 1997). We decided to use the 0.2 nmol dose in all additional experiments, because this appeared to be a reliable dose in both strains for startle effects (Risbrough et al., 2003) that would likely activate both CRF_1 and CRF_2.

Experiment 2, NBI-30775 versus h/r-CRF: CRF_1 antagonism increases PPI and decreases startle

Because these findings indicated that activation of CRF_2 increased PPI, we hypothesized that CRF_1 activation was responsible for the CRF-induced deficits in PPI and predicted that a CRF_1 antagonist would block CRF-induced decreases in PPI. As predicted, h/r-CRF decreased and NBI-30775 increased PPI (Fig. 1*c*) (h/r-CRF, $F_{(1,40)} = 19.07$, $p < 0.001$; NBI-30775, $F_{(1,40)} = 11.17$, $p < 0.01$). The main effect of NBI-30775 appeared to be attributable to NBI-30775 significantly increasing PPI in the h/r-CRF-treated group (Fig. 1*c*) ($p < 0.05$ h/r-CRF/vehicle vs h/r-CRF/NBI-30775; a priori Dunn's test). A similar test in C57BL6/J mice, although not statistically significant (possibly because of a smaller sample size), confirms that NBI-30775 attenuates the PPI disruption induced by 0.2 nmol h/r-CRF (percentage PPI: vehicle/vehicle, 38 ± 3; vehicle/CRF, 22 ± 5; NBI-30775/vehicle, 43 ± 5; NBI-30775/CRF, 34 ± 4). Startle reactivity was significantly increased after h/r-CRF treatment (Fig. 1*d*) (h/r-CRF, $F_{(1,40)} = 7.35$; $p < 0.01$), albeit more weakly than in experiment 1 (Fig. 1*b*), possibly attributable to the difference in pretest administration times chosen (experiment 1, 1 hr before test; experiment 2, 2 hr before test) (Risbrough et al. 2003). NBI-30775 treatment had the opposite effect, significantly reducing startle reactivity (Fig. 1*d*) (NBI-30775, $F_{(1,40)} = 6.01$; $p < 0.05$), confirming the effects of NBI-30775 on CRF-induced increases in startle in C57BL/6J mice (Risbrough et al., 2003). To further confirm the hypothesis that CRF_1 activation reduces PPI, we tested whether the effect of CRF on startle and PPI would be absent in CRF_1 gene mutation (CRF_1 KO) mice.

Experiment 3, CRF increases PPI but not startle in CRF_1 KO mice

CRF treatment (0.2 nmol) reduced PPI in WT mice (Fig. 2*a*), as seen previously. Strikingly, however, h/r-CRF treatment significantly increased PPI in the CRF_1 KO mice (Fig. 2*a*) ($p < 0.05$; Dunn's test) (h/r-CRF × gene, $F_{(1,24)} = 17.99$; $p < 0.001$). Thus, relative to its effect in WT mice, CRF had the opposite effect on PPI when CRF_1 had been genetically deleted. CRF also increased startle reactivity in the WT mice, and this effect was completely absent in the KO mice (Fig. 2*b*) (Dunn's test) (h/r-CRF × gene, $F_{(1,24)} = 7.86$; $p < 0.01$). In conjunction with the CRF_2 antagonist data, these data indicate that activation of CRF_2 increases startle inhibition by sensory input. The occurrence of increased PPI in CRF_1 KOs injected with CRF compared with WT mice could, however, result in part from compensatory changes in brain CRF and urocortin systems. For example, increased expression of CRF in the hypothalamic paraventricular nucleus has been observed in CRF_1 KO mice (Smith et al., 1998). To confirm that CRF_2 activation increases PPI in the normal system, we activated CRF_2 acutely in normal mice using the recently discovered peptides urocortin 2 and 3 that act preferentially at CRF_2 receptors.

Experiment 4, urocortin 2 and 3: CRF_2 agonism increases startle and PPI

Both urocortin 2 and 3 produced long-lasting effects on PPI, inducing significant increases in PPI across all time points and prepulse trials tested (Fig. 3) (data collapsed across time; urocortin 2, $F_{(2,28)} = 3.84$, $p < 0.05$; urocortin 3, $F_{(2,28)} = 3.79$, $p < 0.05$). These data confirm other reports of long-acting effects of CRF_2 activation on behavior (Valdez et al., 2002) and also confirm that CRF_2 activation increases PPI in both 129SvEv and C57BL/6J strains, although the baseline performance is markedly different across the two strains. Together, these data indicate the robustness of CRF_2-mediated effects across behavioral baseline, strain, and agonist used. Dunnett's *post hoc* analysis revealed that the 6 nmol dose of urocortin 2 and the 0.8 and 2.4 nmol doses of urocortin 3 significantly increased PPI relative to vehicle (Fig. 3). As shown in Table 1, the 2 nmol dose of urocortin 2 increased startle reactivity up to the second hour after injection ($p < 0.05$; Dunnett's test) (urocortin 2, $F_{(2,28)} = 4.64$, $p < 0.05$; time × urocortin 2, $F_{(6,84)} = 3.02$, $p < 0.01$). These data again confirm the dissociation between startle and PPI effects of CRF receptor activation, because the effect of urocortin 2 on PPI lasted at least 4 hr, whereas effects on startle were gone by the second hour after administration. Unlike urocortin 2 however, urocortin 3 did not affect startle responding at any of the doses or time points tested (Table 2).

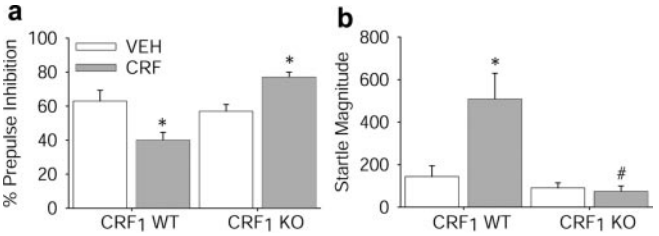

Figure 2. Effect of CRF on prepulse inhibition of the startle response and startle reactivity in CRF_1 gene deletion mice. CRF_1 WT and KO mice received vehicle or 0.2 nmol of h/r-CRF (5 μl, i.c.v.) 1 hr before prepulse inhibition (*a*) and startle (*b*) testing. $^*p < 0.05$ versus respective vehicle group. $^\#p < 0.05$ versus h/r-CRF WT group. Data are expressed as mean ± SEM.

Discussion

The present studies demonstrate that CRF_1 activation increases startle reactivity and reduces PPI. In contrast, CRF_2 activation increases startle reactivity (although only in combination with CRF_1 activation) but simultaneously enhances PPI. Hence, the extant hypotheses of additive or opposing functions of CRF receptors on behavior are not necessarily contradictory. Rather, we suggest a model in which the two CRF receptors exert complementary influences on the magnitude of defensive behaviors and opposing influences on the flexibility of the behavior in response to sensory input. Perhaps the most novel of the present findings is that CRF_1 and CRF_2 have opposing influences on information-processing mechanisms that regulate responses to stressors, findings that may have important implications for clinical anxiety and stress-induced psychiatric disorders.

The present data show that selective blockade of CRF_1 via NBI-30775 reverses CRF-induced deficits in PPI. In contrast, selective CRF_2 antagonism via ASV30 appears to potentiate CRF-induced deficits in PPI. Conversely, selective activation (via urocortin 2 or 3 or in CRF_1 KO mice) of CRF_2 increased PPI. Thus, both the pharmacological and genetic manipulations in the present study give strong evidence for the opposing influences of CRF_1 and CRF_2 on startle inhibition. It also seems clear that CRF_1 is the obligatory receptor for CRF effects on startle, because CRF_1 KO mice exhibited no effects of CRF on startle nor did the selective CRF_2 agonist urocortin 3. Although *in vitro* recombinant studies indicate similar selectivity, urocortin 2 may be less selective than urocortin 3 *in vivo* (Lewis et al., 2001; Valdez et al., 2003). For example, in rat pituitary cells endogenously expressing CRF_1, urocortin 2 was shown to increase cAMP at very high concentrations (Lewis et al., 2001). Thus, it is possible that the increase in startle reactivity by urocortin 2 may reflect a combination of strong CRF_2 activation with weak CRF_1 activation. ASV30 reduced the effects of high doses of CRF on startle but did not alter the effects of low doses of CRF on startle. This pattern of

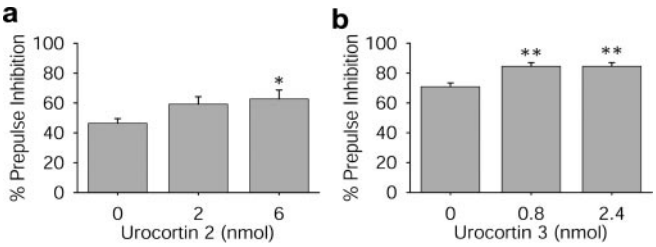

Figure 3. Effect of selective CRF_2 activation on prepulse inhibition of the startle response. *a*, Vehicle or urocortin 2 (2 and 6 nmol) was administered (5 μl, i.c.v.) in C57BL/6J mice. Data are collapsed across tests at 1, 2, 3, and 4 hr after administration. *b*, Vehicle or urocortin 3 (0.8 and 2.4 nmol) was administered (5 μl, i.c.v.) in 129SvEv mice. Data are collapsed across tests at 2, 4, and 6 hr after administration. $*p < 0.05$, $**p < 0.01$ versus vehicle. Data are expressed as mean ± SEM of 83 dB prepulse trials.

Table 1. Effect of intracerebroventricular urocortin 2 on acoustic startle responding to 120 dB pulse in C57BL/6J mice

Hour after injection	Vehicle	2 nmol	6 nmol
1	143 ± 10	234 ± 13*	168 ± 18
2	164 ± 14	202 ± 16	166 ± 22
3	158 ± 17	196 ± 15	137 ± 18
4	139 ± 15	164 ± 15	149 ± 24

$*p < 0.05$ versus vehicle at 1 hr after injection.

Table 2. Effect of intracerebroventricular urocortin 3 on acoustic startle responding to 120 dB pulse in 129SvEv mice

Hour after injection	Vehicle	0.8 nmol	2.4 nmol
2	209 ± 23	186 ± 16	194 ± 13
4	172 ± 18	172 ± 11	192 ± 8
6	168 ± 17	162 ± 12	177 ± 8

effects does not seem to support the possibility that ASV30 effects on startle are attributable to weak CRF_1 antagonism alone, because that should be expected to occur at low doses of competitive agonist, not just at the high dose of agonist. Thus, together, these data indicate that CRF_1 is obligatory for CRF effects on startle, and CRF_2 appears to have an additive influence only in the presence of CRF_1 activation.

The defensive nature of the startle response may be critical to understanding the biological relevance of CRF effects on startle plasticity. Possible functions of the startle reflex are reduction of the latency for defensive flight (Pilz and Schnitzler, 1996) or protection during unexpected predator attack from behind (Yeomans and Frankland, 1995; cf. Fendt, 1998). If CRF–urocortin released during threat produces more robust startle responses, perhaps it is not surprising that CRF_1 activation, in accordance with its activation of defensive behaviors, may also act to block any inhibition of the startle response from sensory input (e.g., from the prepulse). Thus, CRF_1 activation is "protecting" the behavior when it is theoretically needed most, during threat. On the other hand, CRF_2 activation increases the startle response further while enhancing the ability of sensory information to inhibit the response. Thus, with CRF_2 activation, startle responsiveness remains high if needed, but it can now be modulated by other external or internal influences. The idea that CRF_2 increases the flexibility of CRF_1-activated behaviors also supports the hypothesized function of this receptor to modulate the initial effects of CRF_1 activation during stress (Coste et al., 2001). Interestingly, both CRF administration and stress also reduce other forms of sensory inhibition or gating (Miyazato et al., 2000), which are also disrupted in PTSD patients (Gillette et al., 1997; Skinner et al., 1999). Thus, it is possible that CRF may be affecting information processing mechanisms per se, which in turn could modulate many responses other than just startle. Nevertheless, the benefits for the organism of CRF-induced reductions in gating remain unclear. We are currently exploring the possibility that CRF does not produce global "deficits" in gating but instead alters the tuning of gating mechanisms, such that gating is optimized for different stimulus conditions with CRF release compared with without it. For example, as with manipulations of dopamine (Swerdlow et al., 2003), CRF alters the temporal response function of PPI (e.g., prepulse to pulse intervals) (V. Risbrough, R. Hauger, and M. Geyer, unpublished observations).

Two extant hypotheses exist currently about the relative function of CRF receptors in anxiety-like responding, with data supporting either additive (Takahashi et al., 2001; Bakshi et al., 2002; Pelleymounter et al., 2002) or opposing (Radulovic et al., 1999; Bale et al., 2000; Kishimoto et al., 2000; Valdez et al., 2003) influences of CRF_2 on CRF_1 activation-induced behavior. The reports that activation of septal CRF_2 receptors specifically increases freezing and reduces exploration indicate that the influences of CRF_2 on anxiety-like behaviors may be region specific (Radulovic et al., 1999; Bakshi et al., 2002). The present findings of complementary effects of CRF_1 and CRF_2 on startle yet opposing effects on inhibition of startle (i.e., PPI) in the same animals may indicate that both models are possible within a given behavior, depending on the stimuli (e.g., prepulse or pulse alone) driving the behavior. CRF_2 activity on startle was not measurable in the present studies unless there was putative CRF_1 activity as well, which was not the case for CRF_2 effects on PPI. Therefore, measurable CRF_2 effects on behavior may sometimes depend on a minimal level of concomitant CRF_1 activity. On the other hand, it may be that CRF_2 effects on startle are dependent on more CRF_2 activation than are effects on PPI; perhaps urocortin 3 and 0.2 nmol of h/r-CRF in CRF_1 KO mice were not potent enough to increase startle via CRF_2 receptors alone. Thus, how much and in particular where endogenous CRF–urocortin agonist(s) are released during various stressors may be critical to understanding respective CRF receptor functions across various anxiety models.

Previous studies have indicated that CRF effects on startle responding are mediated by the hippocampus and bed nucleus stria terminalis (Davis et al., 1997), both of which express CRF_1 and CRF_2 (Van Pett et al., 2000). The anatomical substrates mediating the effects of CRF on startle inhibition, however, are unknown. Regions that modulate PPI and express CRF receptors include the amygdala, hippocampus, raphe nuclei, and the nucleus reticularis pontis caudalis (Van Pett et al., 2000; Swerdlow et al., 2001). Interestingly, dopamine infusions into the amygdala reduce PPI (for review, see Swerdlow et al., 2001), and dopamine terminals overlap with CRF-expressing neurons in the central amygdala (Eliava et al., 2003). Antipsychotic medications reduce the PPI deficits observed in CRF-overexpressing mice (Dirks et al., 2003), which is likely attributable to dopamine or serotonin receptor antagonism. It is also possible that the CRF effects on PPI are mediated by the modulation of monoamine release in other brain regions. For example, CRF infusion into the ventral tegmentum significantly increased dopamine turnover in the nucleus accumbens (Kalivas et al., 1987), a brain region in which increased dopamine release disrupts PPI (Swerdlow et al., 2001). CRF also influences serotonin transmission during stress via CRF receptors in the dorsal and median raphe (Price et al., 1998; Kirby et al., 2000; Lowry et al., 2000; Hammack et al., 2002; Pernar et al., 2004). Modulation of serotonin transmission certainly affects PPI, because 8-OHDPAT [8-hydroxy-2-(di-*N*-propylamino)-

tetralin], a 5-HT_{1A} agonist, reduces PPI when delivered to the median or dorsal raphe (Sipes and Geyer, 1995). Therefore, it is possible that CRF receptors modulate PPI via indirect effects on serotonergic or dopaminergic mechanisms.

The endogenous ligands mediating these effects on startle are also unknown. Urocortin-deficient mice exhibit reduced startle threshold but blunted responses to high-intensity stimuli (Wang et al., 2002), indicating that urocortin may mediate CRF_1 and/or CRF_2 effects on startle. Unlike urocortin-deficient mice, unstressed (vehicle) CRF_1 receptor KO mice had no baseline differences in startle or PPI, indicating that loss of CRF_1 does not alter startle responses during conditions of presumably low CRF release. Thus, it is possible that the reduced startle exhibited in urocortin-deficient mice is attributable to loss of urocortin activity at CRF_2 specifically. Future studies of CRF_2, urocortin 2, and urocortin 3 KO mice are warranted (as they become available) to answer whether loss of CRF_2 or its specific ligands results in patterns of startle behavior similar to those observed in urocortin KO mice.

The biologically active concentrations administered in the present studies (0.06–0.6 nmol/5 μl) are within the intracerebroventricular dose range for h/r-CRF effects on feeding, activity, and anxiety behaviors (Jones et al., 1999; Reyes et al., 2001). The exogenous CRF dose range required for efficacy is many-fold higher than stress-induced CRF release in the amygdala (11.5 fmol/50 μl) (Merlo Pich et al., 1995; Merali et al., 1998), calling into question the physiological relevance of exogenous CRF-induced behaviors. Unfortunately, comparisons of exogenous peptide versus endogenous peptide concentrations via microdialysis are difficult to interpret. For example, exogenous CRF concentrations that reach the potential brain regions of interest at the time point (e.g., 0.5–2 hr) in which behavioral measures are made are unknown and are likely to be much lower than the original concentration administered. Furthermore, the CRF dialysis recovery is 3% *in vitro* (for review, see Merali et al., 1998), indicating the *in vivo* dialysis measures are a great deal lower than the actual endogenous CRF concentrations at the synapse. The effects of exogenous CRF on startle and PPI, however, do mimic stress-induced effects on startle behavior, lending support to the physiological relevance to the present data. Acute or chronic stress has been shown to increase startle magnitude and reduce PPI in rats (Brake et al., 2000; Sipos et al., 2000; Faraday, 2002) and mice (Risbrough, Hauger, and Geyer, unpublished observations). Moreover, alcohol withdrawal, which induces CRF release (Merlo Pich et al., 1995), increases startle and reduces PPI in rodents (Rassnick et al., 1992) and humans (Keedwell et al., 2001).

Clinical studies have revealed gating deficits in patients with panic disorder (Ludewig et al., 2002) and PTSD (Grillon et al., 1996; Gillette et al., 1997). Hence, we suggest that CRF_1 receptor supersensitivity (possibly attributable to a deficiency in homologous desensitization mechanisms) (Dautzenberg and Hauger, 2002) may override CRF_2 receptor modulation of startle plasticity as indicated by reduced PPI. CRF has been implicated in particular in the production of panic attacks in patients, whereas high CRF levels in CSF and dysregulation of CRF-induced neuroendocrine responses occur in PTSD and possibly panic disorder (Holsboer et al., 1987; Bremner et al., 1997; Arborelius et al., 1999). Indeed, a recent preliminary report indicates that CSF concentrations of CRF are highest in PTSD patients that also exhibit pronounced disease severity and psychosis (Sautter et al., 2003). Thus, whether the PPI deficits incurred after stress in rodents or specifically in anxiety disorders in humans are mediated in part by CRF and pathophysiological presynaptic and postsynaptic CRF neurotransmission, respectively, remains to be determined. Schizophrenia patients also exhibit PPI deficits (Geyer and Braff, 1987; Braff and Geyer, 1990), and antipsychotic drugs block PPI deficits in CRF-overexpressing mice (Dirks et al., 2003), but there are conflicting data on whether disruption of the CRF system is an aspect of the pathophysiology particular to schizophrenia (Banki et al., 1987; Frederiksen et al., 1991; Lammers et al., 1995; Xing et al., 2004). Because so many different genetic (Geyer et al., 2002), pharmacological (Geyer et al., 2001), and neuroanatomical (Swerdlow et al., 2001) manipulations as well as disease states other than the anxiety disorders mentioned above (e.g., Tourette's syndrome, Huntington's disease, and obsessive compulsive disorder) (for review, see Braff et al., 2001) exert effects on sensorimotor gating and startle, it seems unlikely that all of these effects are attributable to one system (e.g., CRF) alone. Nevertheless, it is possible that stress exacerbates sensorimotor gating deficits in many patients, regardless of the specific underlying pathophysiology of information processing disturbances. Together with the present study, however, the converging data of CRF pathophysiology and PPI deficits in panic disorder and PTSD patients support the potential clinical use of CRF_1 antagonists in these particular clinical populations.

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