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Corticotropin-Releasing Factor in the Norepinephrine Nucleus, Locus Coeruleus, Facilitates Behavioral Flexibility

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Corticotropin-releasing factor (CRF), the stress-related neuropeptide, acts as a neurotransmitter in the brain norepinephrine nucleus, locus coeruleus (LC), to activate this system during stress. CRF shifts the mode of LC discharge from a phasic to a high tonic state that is thought to promote behavioral flexibility. To investigate this, the effects of CRF administered either intracerebroventricularly (30–300 ng, i.c.v.) or directly into the LC (intra-LC; 2–20 ng) were examined in a rat model of attentional set shifting. CRF differentially affected components of the task depending on dose and route of administration. Intracerebroventricular CRF impaired intradimensional set shifting, reversal learning, and extradimensional set shifting (EDS) at different doses. In contrast, intra-LC CRF did not impair any aspect of the task. The highest dose of CRF (20 ng) facilitated reversal learning and the lowest dose (2 ng) improved EDS. The dose–response relationship for CRF on EDS performance resembled an inverted U-shaped curve with the highest dose having no effect. Intra-LC CRF also elicited c-fos expression in prefrontal cortical neurons with an inverted U-shaped dose–response relationship. The number of c-fos profiles was positively correlated with EDS performance. Given that CRF excites LC neurons, the ability of intra-LC CRF to activate prefrontal cortical neurons and facilitate EDS is consistent with findings implicating LC-norepinephrine projections to medial prefrontal cortex in this process. Importantly, the results suggest that CRF release in the LC during stress facilitates shifting of attention between diverse stimuli in a dynamic environment so that the organism can adapt an optimal strategy for coping with the challenge.
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INTRODUCTION

Stress is generally thought to impair cognitive function (Arnsten, 2009; Holmes and Wellman, 2009; Marin *et al*, 2011). However, there is also evidence that stress enhances cognitive performance and it has been suggested that there is an inverted U-shaped relationship between stress intensity and cognitive performance (Beylin and Shors, 1998; de Kloet *et al*, 1999; Faraji *et al*, 2011; Luine *et al*, 1996). Although the effects of stress on cognition have been attributed to corticosteroids (de Kloet *et al*, 1999; McEwen, 2001; Sapolsky, 2000), they may also be mediated by corticotropin-releasing factor (CRF), the neuropeptide that orchestrates many aspects of the stress response (Bale and Vale, 2004). CRF acts as a neurohormone to initiate the cascade of pituitary adrenocorticotropin release and the subsequent release of adrenal corticosteroids that is the hallmark of stress (Vale *et al*, 1981). Additionally, extrahypophysial CRF acts as a neurotransmitter to promote autonomic and behavioral aspects of the stress response (Owens and Nemeroff, 1991; Valentino and Van Bockstaele, 2002). CRF may regulate cognitive processes by its modulation of the forebrain-projecting monoamine systems that are integral to these processes.

The major brain norepinephrine nucleus, locus coeruleus (LC), is one target of CRF neurotransmission (Valentino and Van Bockstaele, 2002, 2008; Van Bockstaele *et al*, 1996) that is thought to be important in cognition through its extensive hippocampal and cortical projections (Loughlin *et al*, 1986; Swanson and Hartman, 1976). LC neuronal discharge rate is positively correlated with arousal state (Aston-Jones and Bloom, 1981b; Berridge and Foote, 1991; Berridge *et al*, 1993). Additionally, LC neurons are phasically activated by salient stimuli and this activation often precedes orientation toward the stimulus (Aston-Jones and Bloom, 1981a; Foote *et al*, 1980). LC neuronal recordings in monkeys performing operant tasks have suggested that different patterns of LC discharge are associated with different cognitive processes (Aston-Jones and Cohen, 2005; Aston-Jones *et al*, 1999). Phasic LC discharge characterized by synchronously firing LC neurons

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that are responsive to discrete sensory stimuli is associated with focused attention and maintaining ongoing behavior with a known outcome. In contrast, a high tonic mode of activity with elevated spontaneous discharge rates, decreased synchrony, and diminished phasic responses to specific sensory stimuli is associated with hyperarousal, labile attention, and going off-task or changing behavior to seek an alternate outcome.

CRF increases LC neuronal firing rate and decreases the signal-to-noise ratio of the sensory response, biasing the mode of LC activity toward a high tonic state that would favor behavioral flexibility (Curtis *et al*, 1997; Valentino and Foote, 1987, 1988). Stress mimics these neuronal effects and this can be blocked by intra-LC administration of a CRF antagonist (Curtis *et al*, 2001; Valentino and Wehby, 1988; Valentino *et al*, 1991). The shift produced by CRF toward a high tonic mode of LC discharge and enhanced behavioral flexibility would be adaptive in a dynamic challenging environment.

The present study was designed to examine the effects of CRF in a rodent-based model for assessing cognitive flexibility, the attentional set shifting task (AST; Birrell and Brown, 2000; Lapiz and Morilak, 2006). The effects of different CRF doses administered intracerebroventricularly (i.c.v.) or directly into the LC (intra-LC) were examined. Because norepinephrine actions in the medial prefrontal cortex have been implicated in certain aspects of set shifting behavior, expression of the immediate early gene, c-fos, and the phosphorylated extracellular signal-regulated kinase ($p^{44/42}$ERK) were quantified here as indices of neuronal activation and correlated with task performance (Bondi *et al*, 2010; Lapiz and Morilak, 2006; McGaughy *et al*, 2008; Roberts *et al*, 1994; Tait *et al*, 2007).

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats (220–250 g; Charles River Laboratories, Wilmington, MA) were housed individually on a 12 h light/dark cycle with lights on at 0700 hours. Rats acclimated to the colony for a minimum of 5 days before surgery. Animal use and care was approved by the institutional animal care and use committee of the Children's Hospital of Philadelphia.

Experimental Design

After 5 days of acclimation, rats underwent surgery for stereotaxic implantation of cannula guides. They began a phase of food restriction 4 days after surgery and the training for the attentional set shifting procedure began after 5 days of food restriction, with a day of habituation, a day of training, and a day of testing as described below. Rats were transcardially perfused 15 min after completion of the last task.

Surgery

Rats were implanted with a cannula guide into lateral ventricle or bilateral cannula guides into the LC. Rats were anesthetized with isofluorane (2%) and positioned in a stereotaxic instrument with the head tilted at a 15° angle to the horizontal plane (nose down). A guide cannula (22 gauge) was implanted into the lateral ventricle as previously described (Valentino and Foote, 1988). For intra-LC injections, double guide cannulae (26 gauge, C/C dist. 2.2 mm, Plastics One, Roanoke, VA) were implanted with the following coordinates relative to lambda: AP −3.4 mm, ML ±1.1 mm, and DV 5.1 mm below the brain surface. Guide cannulae were affixed to skull and skull screws with cranioplastic cement. An obdurator was inserted into guide cannulae to prevent occlusion. Following 4 days of postsurgical recovery, rats were restricted to 10–15 g food per day, with 85% of free-feeding weight as a guideline, for the remainder of the experiment. Water remained available *ad libitum*.

Attentional Set Shifting Task

Procedures for the AST were similar to previous studies (Birrell and Brown, 2000; Lapiz-Bluhm *et al*, 2008; Liston *et al*, 2006). The testing apparatus was a custom-built white rectangular Plexiglas arena (inner dimensions: 75 × 40 × 30 cm) (Lapiz-Bluhm *et al*, 2008). Two ceramic pots (internal rim diameter 10 cm; depth 10 cm) were placed at one end of the arena. Each pot was distinguished by a pair of cues along two stimulus dimensions: (1) the medium contained within the pot and (2) an odor applied to the pot (Supplementary Table S1). Food reward (1/4 peanut butter chip) was placed at the bottom of one of the pots and buried with the digging medium. Beginning after 5 days of food restriction, the behavioral procedure was conducted over 3 days for each rat as follows:

Day 1: habituation. Rats were trained to dig reliably for food reward in the pots. Two unscented pots were placed in the home cage and baited, with the reward covered with increasing amounts of sawdust. Rats were required to dig for food within 5 min in order to move on to the next step. After rats learned to reliably retrieve the food from fully baited pots, they were transferred to the testing arena and given three consecutive trials to retrieve the reward from both sawdust-filled pots.

Day 2: training. Rats were trained to complete a series of simple discrimination tasks to a criterion of six consecutive correct trials, in which food was associated with one of two odors (eg, citronella *vs* lavender) and then one of the two digging mediums (green paper pellets *vs* Alpha-Dri bedding). All rats were trained using the same stimulus exemplars and in the same order. The positive and negative cues for each rat were randomly determined and equally represented.

Day 3: testing. Rats were tested on a series of five discriminations (Supplementary Table S1). The criterion to proceed to the next stage was the completion of six consecutive correct trials. Stage 1 was a simple discrimination (SD), in which the rat was required to discriminate between two digging media, only one of which predicted the food reward, in unscented pots. Stage 2 was a compound discrimination (CD) for which the same discrimination was required as in the SD, but irrelevant stimuli from a new dimension (odor) were introduced. Stage 3 was an intradimensional attentional shift (IDS), in which two new exemplars from each dimension were introduced, but the task-relevant dimension (medium) was unchanged. Stage 4

tested reversal learning where the reinforcement was associated with the alternate medium as in the previous IDS stage. Stage 5 involved an extradimensional attentional shift (EDS), in which two new exemplars from each dimension were introduced and the relevant dimension was also changed from medium to odor. The assignment of each exemplar in a pair as being positive or negative in a given stage, as well as the left–right positioning of the pots in the arena on each trial, were determined randomly in advance.

CRF Microinjection

Aliquots (10 μg) of ovine CRF (American Peptide Company, Sunnyvale, CA) were kept at −20 °C until use. On the day of the experiment, CRF was dissolved in artificial cerebrospinal fluid (ACSF) and ACSF or CRF were injected 10 min before beginning the AST. Microinjections were performed by lowering a stainless steel injector cannula (28 gauge for i.c.v. and 33 gauge for LC) with a length of 1 mm longer than the guide cannulae into the lateral ventricle or LC region. Animals received i.c.v. injections of ACSF (3 μl) or CRF (30, 100, and 300 ng in 3 μl ACSF) and bilateral intra-LC injections of ACSF (200 nl) or CRF (2, 6, or 20 ng in 200 ACSF). The i.c.v. doses of CRF are comparable to those used in other behavioral studies (Howard *et al*, 2008; Spina *et al*, 2002; Sutton *et al*, 1982). The intra-LC CRF doses are on the linear part of the CRF dose–response curve for increasing LC neuronal discharge and norepinephrine release in forebrain targets (Curtis *et al*, 1997; Page and Abercrombie, 1999). CRF or vehicle was infused over a 1-min period using a syringe pump and cannulae were left in place for an additional 60 s to minimize the backflow into the injection track. After 10 min, the rats were placed in the testing arena.

Histology

After completing the EDS component (15 min), rats were anesthetized with isofluorane and pontamine sky blue dye was injected through the i.c.v. (3 μl) or LC (200 nl) cannulae to verify placement. Rats were transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed, postfixed overnight, and placed in 30% sucrose with 0.1% sodium azide for at least 48 h. Frozen serial 30 μm coronal sections through the LC were cut on a cryostat and stained with neutral red to visualize cannulae placements. Animals were accepted for behavioral analysis and further cortical c-fos and $p^{44/42}$ERK determination only when one or both injection needle placements were located within the LC (Figure 1).

C-fos and $p^{44/42}$ERK Immunohistochemistry

Frozen serial 30 μm coronal sections through the frontal cortex were cut on a cryostat, collected into four wells, and stored at −20 °C in cryoprotectant until all of the brains were obtained so that sections could be processed for immunohistochemistry at the same time. Sections were rinsed to remove cryoprotectant and incubated in 0.75% H_2O_2 in phosphate buffer for 30 min. Sections were processed to visualize c-fos immunoreactivity as previously described (Carr *et al*, 2010), with the exception that the rabbit antibody directed against c-fos was obtained from

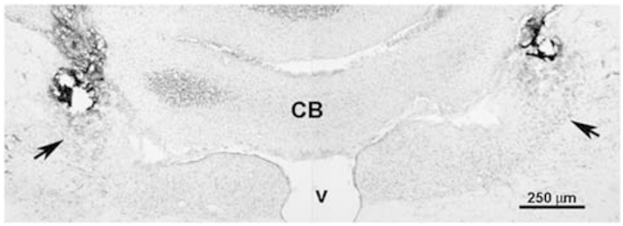

Figure 1 Brightfield photomicrograph of a section through the LC showing histological verification of the bilateral injection sites. The figure is a montage of right and left images of the same section. The section is counterstained with Neutral red. Arrows point to the LC. CB, cerebellum; V, ventricle.

Dr Paul Sawchenko (The Salk Institute, San Diego, CA) and used in a concentration of 1 : 20 000. Immunohistochemical visualization of $p^{44/42}$ERK was performed on different sections from the same rats using the rabbit monoclonal antibody raised against $p^{44/42}$ERK1/2 (1 : 1000, Cell Signaling no. 4370). This antibody specifically recognizes activated ERK, but it is not selective for the two isoenzymes, ERK1 and ERK2. The reaction was identical to that described above for c-fos with the exception that nickel was omitted from the DAB solution.

Data Analysis

Trials to reach criterion during each stage were recorded for each rat. The effects of different doses were analyzed using a two-way repeated measures ANOVA with stage as the within factor. The Student–Neuman–Keuls method was used *post hoc* to determine statistically significant differences between dose groups for a particular stage. Additionally, a comparison between stages within the ACSF group was done to verify differences between IDS and EDS stages.

Sections were visualized on a Zeiss Axiovert 25 and digital images were obtained using a Leica DFC 480 camera and imaging software by an individual blinded to the treatment group. Immunoreactive profiles were sampled in the same area of medial prefrontal cortex or orbitofrontal cortex of each section by creating a region-of-interest shape that was superimposed on all other sections in the same region (Figure 2). The c-fos profiles were counted within these areas using Image J. Immunoreactive $p^{44/42}$ERK profiles were counted manually. At least two sections per animal were used to count immunoreactive profiles and the number of profiles per section was averaged for each subject and the group mean determined from these values. Group data were compared using a one-way factorial ANOVA with *t*-test for *post hoc* analysis.

RESULTS

Effects of Intracerebroventricular CRF on Attentional Set Shifting

A total of 27 rats that were implanted with i.c.v cannula completed all stages of the AST. Rats administered 1000 ng CRF (i.c.v.) were unable to perform the task from the beginning stages, and hence the highest dose administered was 300 ng. The overall two-way repeated measures ANOVA indicated a trend for an effect of dose ($F(3, 23) = 2.8$, $p = 0.06$), an effect of stage ($F(4, 92) = 53.4$, $p < 0.001$), and a

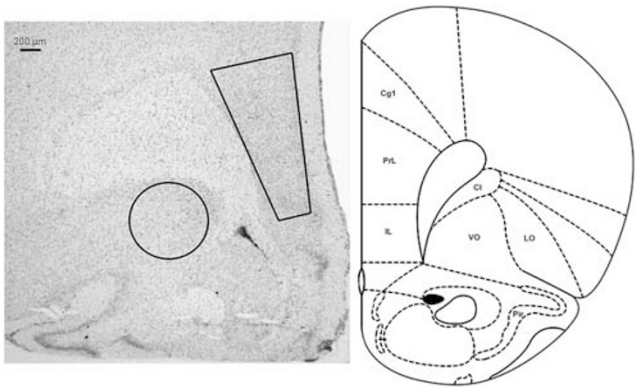

Figure 2 Region of prefrontal cortex in which immunoreactive profiles were quantified. The brightfield photomicrograph on the left shows a representative section through the frontal cortex at the level of the areas of prefrontal cortex in which immunoreactive cells were quantified. The region of interest in which cells were counted in the medial prefrontal cortex is drawn as a polygon that covers the prelimbic and infralimbic cortex. The region of interest in which cells were counted in the orbitofrontal cortex is drawn as a circle. The photomicrograph is juxtaposed to the representative section from the Rat Brain Atlas (Swanson, 1992). CG1, cingulate cortex; Cl, claustrum; IL, infralimbic cortex; LO, lateral orbitofrontal cortex; Pir, piriform cortex; PrL, prelimbic cortex; VO, ventral orbitofrontal cortex.

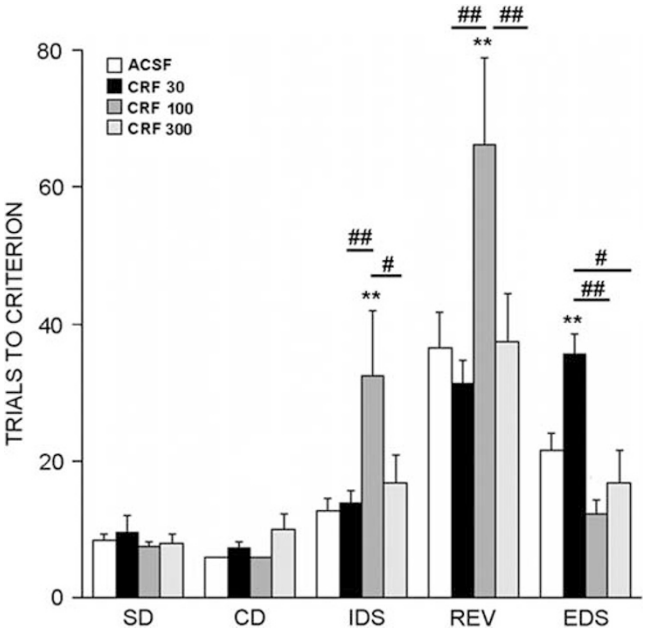

Figure 3 Intracerebroventricularly administered CRF (ng dose) impairs different components of the AST. The bars indicate the mean number of trials necessary to reach the criterion for simple discrimination (SD), compound discrimination (CD), intradimensional shift (IDS), reversal (REV), and extradimensional shift (EDS) components of the task. Bars are the mean of 4–10 rats for group. Vertical lines represent SEM. $^{**}p<0.005$, compared with ACSF; $^{\#}p<0.05$, $^{\#\#}p<0.005$ compared with other CRF doses.

dose × stage interaction ($F(12, 92) = 6.1$, $p<0.001$). Analysis of only ACSF rats indicated that the mean number of trials to reach criterion was greater for the EDS compared with the IDS stage ($p<0.05$, Student–Newman–Keuls method).

Figure 3 shows that CRF administered i.c.v. impaired different components of the task depending on the dose. CRF (100 ng, i.c.v.) impaired IDS ($p=0.002$) and reversal learning ($p<0.001$) and this effect diminished with a higher dose. Impairment of EDS was produced by the lowest dose of CRF (30 ng) but was not seen with higher doses ($p<0.005$).

Effects of Intra-LC CRF on Attentional Set Shifting

A total of 25 rats implanted with intra-LC cannula completed all stages of the task. The overall two-way repeated measures ANOVA indicated no effect of dose ($F(3, 21) = 1.3$), an effect of stage ($F(4, 84) = 51.6$, $p<0.001$), and a dose × stage interaction ($F(12, 84) = 3.2$, $p<0.001$). Analysis of only ACSF rats indicated that the mean number of trials to reach criterion was greater for the EDS compared with the IDS stage ($p<0.05$, Student–Newman–Keuls method).

The effects of CRF administered into the LC were markedly different from those administered i.c.v. (Figure 4). Particularly, no dose of CRF impaired performance in any of the stages. The highest dose of CRF (20 ng) improved reversal learning ($p=0.002$). There was an inverted U-shaped dose–response relationship for CRF effects on EDS performance. The lowest dose (2 ng) improved performance ($p<0.05$) and there was a trend for enhanced EDS performance after 6 ng CRF ($p<0.07$). However, these improvements reversed as the dose was increased to 20 ng.

Each CRF dose group had a number of misplaced injections. For the 2 and 6 ng doses, there were four cases

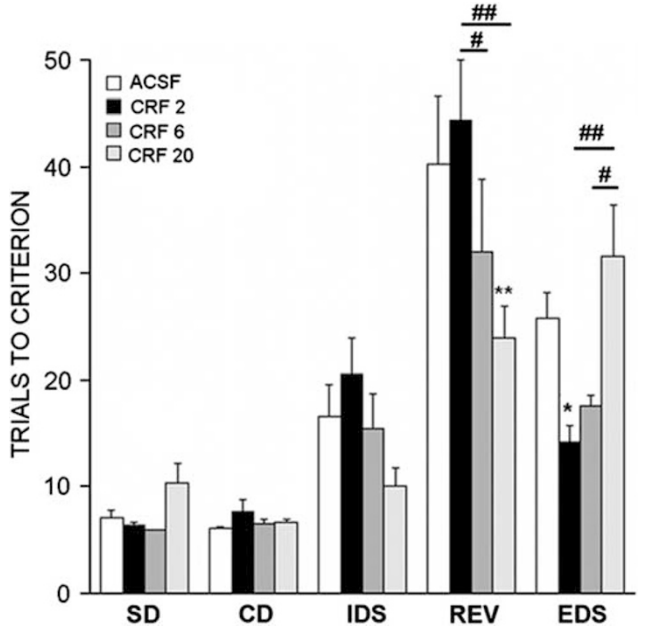

Figure 4 Intra-LC-administered CRF (ng dose) has differential effects on components of the AST. The bars indicate the mean number of trials necessary to reach the criterion for simple discrimination (SD), compound discrimination (CD), intradimensional shift (IDS), reversal (REV), and extradimensional shift (EDS) components of the task. Bars are the mean of 5–8 rats for group. Vertical lines represent SEM. $^{*}p<0.05$, $^{**}p<0.005$, compared with ACSF; $^{\#}p<0.05$, $^{\#\#}p<0.005$ compared with other CRF doses.

each in which the bilateral cannula assembly was shifted such that one cannula was lateral and the other was medial to the LC. For the 20 ng dose, there was one case in which the cannula assembly was shifted as described above and three injections were placed into the nearby dorsal raphe nucleus. These injections outside of the LC gave a very different pattern of responses and dose–response relationship compared with injections within the LC (Supplementary Figure S1).

Effects of Intra-LC CRF on C-Fos and $p^{44/42}$ERK Profiles in Medial Prefrontal Cortex

Figure 5 shows c-fos profiles in the medial prefrontal cortex in representative sections from rats administered ACSF or different CRF doses into the LC. There was a significant effect of intra-LC CRF dose on the number of c-fos-immunoreactive profiles in the medial prefrontal cortex ($F(3,14)=6.4$, $p<0.01$). Similar to the effect of CRF on EDS performance, the dose–response relationship for inducing c-fos expression resembled an inverted U-shaped curve with the 6 ng dose producing effects that were significantly different than ACSF ($p<0.05$) and 20 ng CRF ($p<0.001$; Figure 6a1). Although the 2.0 ng CRF dose effectively improved EDS performance, it did not produce a statistically significant increase in the number of c-fos profiles in the medial prefrontal cortex. Nonetheless, the number of c-fos profiles in medial prefrontal cortex was negatively correlated with the number of EDS trials to criterion as determined by both linear ($F(1,16)=9.3$, $p<0.01$) and log ($F(1,16)=18.9$, $p=0.0005$) transformation, consistent with a positive association between cellular activation in this region and EDS performance (Figure 6a2).

The CRF dose–response relationship for c-fos in the orbitofrontal cortex resembled that for the medial prefrontal cortex (Figure 6b1). There was a significant effect of intra-LC CRF dose ($F(3,14)=9.1$, $p<0.005$) with the 6 ng dose being associated with an increase in c-fos ($p<0.05$) and the 20 ng dose associated with a decrease ($p<0.05$) compared with ACSF-treated rats. The number of c-fos profiles in the orbitofrontal cortex was not linearly correlated with trials to criterion for reversal learning ($F(1,16)=3.1$, $p=0.1$) but there was a significant positive correlation between these end points upon log transformation of the data ($F(1,16)=6.2$, $p<0.05$) indicative of a negative association with performance (Figure 6b2). Interestingly, the CRF dose that improved reversal learning (20 ng) was associated with the least number of c-fos profiles in orbitofrontal cortex and a dose that had no effect on reversal learning was associated with increased c-fos expression in the orbitofrontal cortex.

Figure 7a shows representative sections of $p^{44/42}$ERK-expressing neurons in medial prefrontal cortex of rats administered ACSF or CRF (2 ng) intra-LC. CRF (2 ng) increased the number of $p^{44/42}$ERK-expressing neurons in the medial prefrontal cortex ($F(3,11)=6.1$, $p=0.01$). There was a trend for the number of $p^{44/42}$ERK profiles to be negatively correlated with EDS trials to criterion ($F(1,13)=4.3$, $p=0.057$; Figure 7b).

Because ERK is upstream from c-fos (Monje *et al*, 2005; Runyan *et al*, 2004), a correlation between the two end points was tested (Supplementary Figure S2). When all cases were considered, there was no correlation between the two measures ($r^2=0.12$; $F(1,13)=1.8$). However, omission of four cases with the highest number of fos profiles resulted in a highly correlated relationship between $p^{44/42}$ERK and c-fos expression ($r^2=0.73$; $F(1,9)=24$, $p<0.001$).

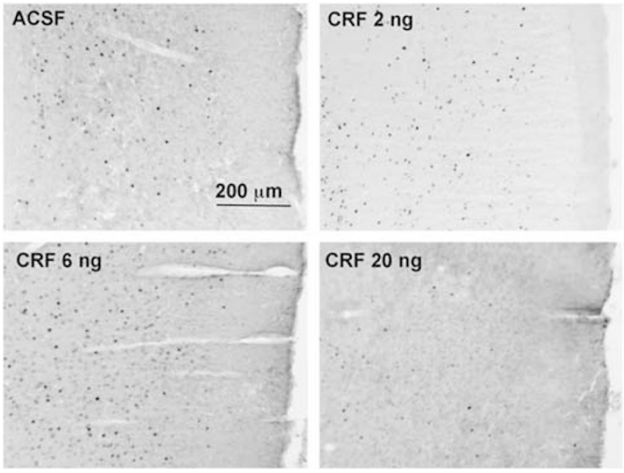

Figure 5 Effects of intra-LC CRF (ng dose) on c-fos expression in the medial prefrontal cortex. Photomicrographs of c-fos-immunoreactive profiles in medial prefrontal cortex of rats administered ACSF or different doses of CRF. Top is dorsal and right is medial.

DISCUSSION

This is the first report of the effects of the stress neuropeptide, CRF, on attentional set shifting behavior, an animal model of cognitive flexibility. CRF had qualitatively different effects depending on its route of administration. When administered into the lateral ventricle such that it could affect multiple brain regions, CRF generally disrupted different aspects of AST performance with an inverted U-shaped dose–response relationship. In contrast, when administered into the LC, CRF improved reversal learning and EDS performance. Given that the intra-LC doses of CRF also increase LC neuronal discharge rate and norepinephrine release in terminal fields (Curtis *et al*, 1997; Page and Abercrombie, 1999), these findings are consistent with other evidence for a role of norepinephrine in the medial prefrontal cortex in EDS (Lapiz and Morilak, 2006). Although a causal relationship between c-fos in the medial prefrontal cortex and EDS performance has not been established, the correlation between CRF effects on EDS performance and c-fos-immunoreactive profiles suggests that norepinephrine-elicited activation of prefrontal cortex neurons facilitates EDS performance. The inverted U-shaped dose–response relationship for CRF effects on both EDS behavior and c-fos expression may reflect the similar dose–response relationship for norepinephrine effects on cortical neuronal activity, where moderate concentrations facilitate transmission and high concentrations are inhibitory (Berridge and Waterhouse, 2003; Devilbiss and Waterhouse, 2000; Waterhouse *et al*, 1998). Together, the results suggest a model whereby low levels of

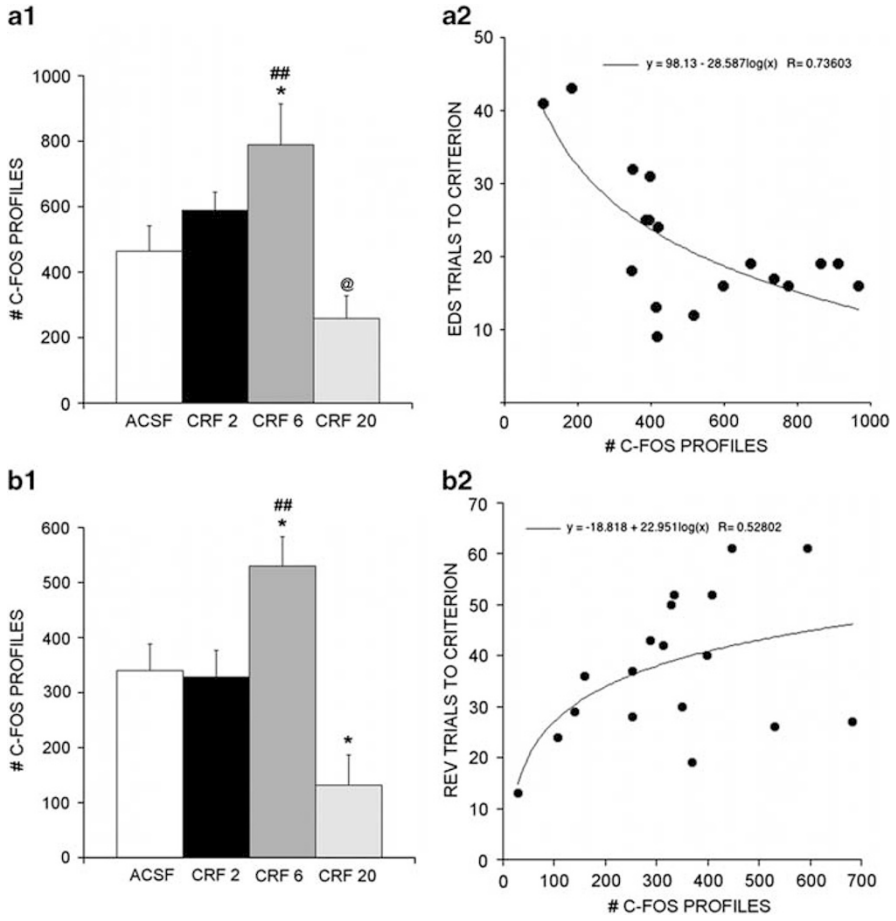

Figure 6 Quantification of c-fos in the medial prefrontal cortex and orbitofrontal cortex. (a1) Bars represent the mean number of c-fos profiles in the medial prefrontal cortex after injection of ACSF or different doses (ng) of CRF into the LC ($n = 4$–5 rats). $^{*}p < 0.05$ compared with ACSF, $^{\#\#}p < 0.005$, compared with CRF 20 ng, $^{@}p < 0.05$ compared with CRF 2 ng. (a2) Each point in the scatterplot represents the number of c-fos profiles in medial prefrontal cortex and trials to criterion during extradimensional set shifting for an individual rat regardless of treatment. The line represents the equation describing the relationship based on log transformation of the number of c-fos profiles. There was a significant negative relationship between number of c-fos profiles and trials to criterion indicating a positive relationship with performance on the task ($F(1, 16) = 18.9$, $p < 0.0005$). (b1) Bars represent the mean number of c-fos profiles in the orbitofrontal cortex after injection of ACSF or different doses (ng) of CRF into the LC ($n = 4$–5 rats). $^{*}p < 0.05$ compared with ACSF, $^{\#\#}p < 0.005$, compared with CRF 20 ng. (b2) Each point in the scatterplot represents the number of c-fos profiles in the orbitofrontal cortex and trials to criterion during reversal learning for an individual rat regardless of treatment. The line represents the equation describing the relationship based on log transformation of the number of c-fos profiles. There was a significant positive relationship between number of c-fos profiles and trials to criterion indicating a negative relationship with performance on the task ($F(1, 16) = 9.1$, $p < 0.05$).

CRF released in the LC during acute stress facilitate cognitive flexibility through a moderate activation of the LC-norepinephrine system. This would be adaptive in a life-threatening dynamic environment. On the contrary, excessive CRF, as may occur in pathological states, could have opposing effects by eliciting levels of norepinephrine that inhibit prefrontal cortex activity.

Effects of Intracerebroventricular CRF on Behavior

Intracerebroventricular CRF elicits active behaviors including increased locomotor activity in a familiar environment, grooming, burying, and aggressive behaviors (Eaves *et al*, 1985; Howard *et al*, 2008; Koob *et al*, 1984; Sutton *et al*, 1982; Tazi *et al*, 1987). In certain rodent models, CRF has anxiogenic effects expressed as effects in the elevated plus maze, enhanced conditioned freezing, decreased activity in open field, potentiated startle, and decreased punished responding (Britton *et al*, 1985; Cole and Koob, 1988; De Boer *et al*, 1992; Liang *et al*, 1992). In contrast, studies of the effects of CRF on cognitive processes are lacking. CRF has been reported to increase accuracy in the five-choice serial reaction time test (Ohmura *et al*, 2009). In the present study, the highest CRF dose that affected AST performance (100 ng, i.c.v.) is somewhat lower than doses that have previously been reported to produce behavioral effects (300–1000 ng, i.c.v.) (Spina *et al*, 2002) and rats administered 1000 ng CRF were unable to perform the task in the current study.

The lack of a monotonic dose–response relationship for CRF at any stage of the AST may reflect its actions at diverse sites that are accessed by i.c.v. CRF. For example, CRF facilitates conditioned learning when administered into the hippocampus but causes deficits in learning when administered into the lateral septum, the two sites that it would be likely to access via the lateral ventricle (Radulovic *et al*, 1999). CRF (100 ng, i.c.v.) directly inhibits the dorsal raphe-serotonin system, which would be detrimental to reversal

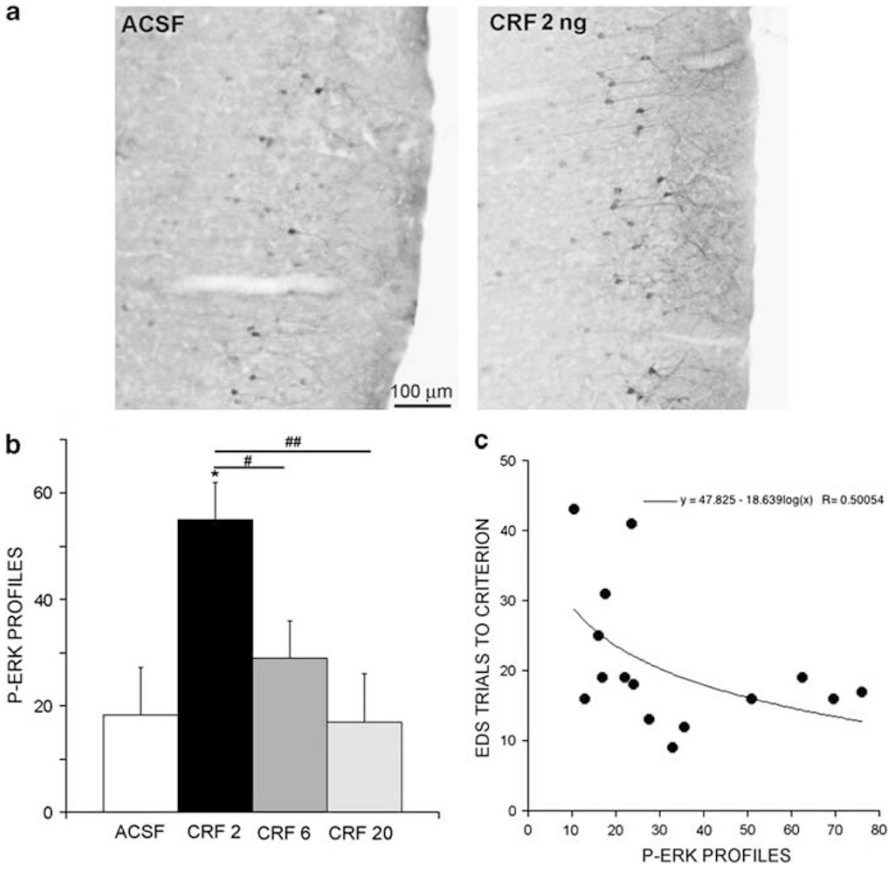

Figure 7 Expression of $p^{44/42}$ERK in medial prefrontal cortex induced by CRF injections into the LC. (a) Photomicrograph of $p^{44/42}$ERK-expressing cells in medial prefrontal cortex of rats administered either ACSF or CRF 2 ng. Top is dorsal and right is medial. (b) Bars indicate the mean number of $p^{44/42}$ERK profiles in the medial prefrontal cortex of rats administered ACSF or different doses (ng) of CRF into the LC ($n = 3$–5 rats). $^{*}p < 0.05$ compared with ACSF, $^{\#}p < 0.05$, $^{\#\#}p < 0.005$ compared with different doses of CRF. (c) The line represents the equation describing the relationship based on log transformation of the number of c-fos profiles. There was a negative relationship between number of $p^{44/42}$ERK profiles and trials to criterion indicating a trend of a positive relationship with performance on the task. $F(1, 13) = 4.3$, $p = 0.057$.

learning (Kirby *et al*, 2000; Price *et al*, 1998). However, higher doses (300 ng, i.c.v.) increase LC activity, which may counter some of these effects (see below).

Effects of Intra-LC CRF on Behavior

In contrast to the numerous studies on the behavioral effects of CRF administered i.c.v., studies on the behavioral consequences of intra-LC CRF are scant. One study reported increased activity by 100 ng CRF, both in a cage and in response to swim stress (Butler *et al*, 1990). All CRF doses used in the present study (2–20 ng) increase LC firing rate and extracellular norepinephrine levels in forebrain regions and are on the linear part of the CRF dose–response curve (Curtis *et al*, 1997; Page and Abercrombie, 1999). At the same time that CRF increases tonic LC firing rate, it decreases sensory-evoked phasic discharge (Valentino and Foote, 1987, 1988). A shift from phasic to high tonic LC activity is associated with increased arousal and a shift from the maintenance of ongoing behaviors that have known outcomes to going off-task in a search for alternate outcomes (Aston-Jones and Cohen, 2005). This should be expressed as an increase in behavioral flexibility and enhanced EDS performance in the AST. Consistent with this, idazoxan, which activates the LC-norepinephrine system by antagonizing α2-adrenergic receptors, facilitated attentional shifts (Devauges and Sara, 1990). In the present study, CRF, which activates the LC, also improved EDS performance. However, the CRF effect exhibited an inverted U-shaped dose response and was completely absent at a dose (20 ng) that remains effective at increasing tonic LC discharge rate and releasing norepinephrine in forebrain targets (Curtis *et al*, 1997; Page and Abercrombie, 1999). This suggests complex relationships between norepinephrine and target neurons involved in EDS.

The medial prefrontal cortex is a target region of the LC that is integral to behavioral flexibility and optimal EDS performance (Dias *et al*, 1996a, b; Milner, 1963). Prefrontal cortical networks generate and maintain representations of rules to guide behavior via the activity of recurrent networks that encode information about stimuli in their absence (Goldman-Rakic, 1995). Norepinephrine, derived solely from LC neurons, acts in the medial prefrontal cortex to strengthen connections between neurons with shared inputs (Wang *et al*, 2007). Antidepressants that increase norepinephrine levels improve EDS performance and, conversely, lesions of the LC-norepinephrine system impair performance (Bondi *et al*, 2010; Bondi *et al*, 2007; Lapiz

et al, 2007; McGaughy *et al*, 2008; Roberts *et al*, 1994). Similar to the behavioral effects of intra-LC CRF in the present study, the relationships between norepinephrine concentration and activity and functionality of prefrontal cortical neurons resemble an inverted U-shaped curve (Arnsten, 2009; Berridge and Waterhouse, 2003). This is thought to be due, in part, to the existence of multiple noradrenergic receptor subtypes with differential affinities for norepinephrine. For example, it has been proposed that activation of high-affinity α2-adrenergic receptors by moderate levels of norepinephrine is associated with optimal performance in prefrontal cortical-dependent working memory tasks because of enhanced activity and strengthened connections among task-relevant prefrontal cortex networks (Wang *et al*, 2007). Conversely, activation of low-affinity α1-adrenergic receptors by high norepinephrine levels has been associated with impaired performance in working memory tasks (Birnbaum *et al*, 1999). On the other hand, evidence for an involvement of α2-adrenergic receptors in stress-induced impairments in EDS performance and for α1-adrenergic receptors in the beneficial effects of norepinephrine reuptake inhibitors emphasizes that the role of various adrenergic receptors in specific cognitive functions is not clearcut (Bondi *et al*, 2010). Regardless of our knowledge of the adrenergic receptors involved, the biphasic (inverted U-shape) dose–response relationship for norepinephrine effects on forebrain neuronal activity is well documented (Berridge and Waterhouse, 2003). Because the CRF doses tested in this study are on the linear portion of the dose–response curve for LC activation and norepinephrine release (Curtis *et al*, 1997), a biphasic dose–response relationship for CRF effects on EDS performance must reflect the postsynaptic dose response to norepinephrine.

Effects of Intra-LC CRF on c-fos and $p^{44/42}$ERK

The CRF dose–response curves for c-fos and $p^{44/42}$ERK expression in the medial prefrontal cortex resembled that for facilitation of EDS behavior in being biphasic. The correlation between expression of these molecules with EDS performance implicates norepinephrine-induced activation of the medial prefrontal cortical neurons in the behavior. The relationship between the signaling molecules and EDS performance was best fit by a log transformation of the data underscoring the complexity of the relationship and suggesting that within a certain range, minimal increases in neuronal activation may have a large effect on performance. Although causality between prefrontal cortical neuronal activation as indicated by c-fos or ERK expression and improvement in EDS performance was not established here, others have demonstrated that pharmacological improvements in attentional set shifting in rats with medial prefrontal cortical lesions is associated with increased c-fos expression in spared neurons (Tait *et al*, 2009).

Although these experiments were not designed to elucidate the cellular signaling underlying the ability of the medial prefrontal cortex to facilitate EDS, the results suggest the potential involvement and interactions between $p^{44/42}$ERK and c-fos. A role for c-fos is supported by the high correlation between c-fos expression and EDS performance. On the other hand, the most behaviorally effective dose (2 ng) was the only one to increase $p^{44/42}$ERK expression. The ERK pathway in the prefrontal cortex has been implicated in consolidation and recall of recent memory (Leon *et al*, 2010). Evidence from trace fear conditioning studies also support a role for ERK in the prefrontal cortex in memory retention and memory for the relevancy of the training condition (Runyan *et al*, 2004). Given that $p^{44/42}$ERK is upstream of c-fos (Kim and Cochran, 2000; Monje *et al*, 2005), we speculate that norepinephrine in the prefrontal cortex engages a signaling cascade where the sequential expression of these molecules underlies the ability to optimize EDS performance. The strong correlation between $p^{44/42}$ERK and low-to-moderate levels of c-fos expression is consistent with this, and loss of this correlation with high c-fos expression may be explained as feedback inhibition of the ERK pathway by c-fos.

The finding that the highest CRF dose improved reversal learning is consistent with the concept that high tonic activity would promote going off-task and reduce perseverance. Supporting this notion, a previous study in monkeys found that high, but not low, doses of an α2-adrenergic agonist improved reversal learning in a visual discrimination task (Steere and Arnsten, 1997). Nonetheless, this finding was unexpected because performance in reversal learning is often attributed to serotonergic effects in the orbitofrontal cortex. It is possible that the enhanced reversal learning with this high dose of CRF was the indirect result of LC activation of the dorsal raphe-serotonin system. The dorsal raphe-serotonin system is thought to be under tonic activation by α1-adrenergic receptors (Baraban and Aghajanian, 1980; Bortolozzi and Artigas, 2003). Unlike the correlation between c-fos in the medial prefrontal cortex and EDS performance, c-fos in the orbitofrontal cortex was not positively correlated with reversal learning, and the effective CRF dose resulted in the least amount of c-fos expression in this region, whereas an ineffective dose was associated with increased fos expression. This suggests that alternate signaling cascades are involved in modulation of reversal learning by the orbitofrontal cortex.

CRF Modulation of LC Activity and Cognition During Stress

The present findings argue against the general idea that acute stress impairs cognition, at least through its effects on the LC-norepinephrine system. The levels of LC activation produced by CRF doses that improved EDS performance (2–6 ng) range from 25 to 60% above baseline (Curtis *et al*, 1997). In comparison, hypotensive stress, which increases LC discharge through CRF release in the LC, produces a similar magnitude of LC activation (Curtis *et al*, 2001; Page *et al*, 1993; Valentino *et al*, 1991). Similarly, exposure to predator odor increases LC discharge rate by 30–50% through a CRF-dependent mechanism (Curtis and Valentino, 2008). Both of these stressors also bias LC discharge toward a high tonic state. The present results suggest that a function of acute stress-elicited levels of CRF in the LC is to shift the mode of discharge toward a high tonic state in an effort to promote behavioral flexibility through its projections and impact on cells in the medial prefrontal cortex. Excessive CRF, which may be released with particularly severe stressors or in pathological states

where CRF is hypersecreted, would not improve, and could potentially impair, cognitive flexibility, possibly as a result of the inhibitory effects of norepinephrine on prefrontal cortical neurons.

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DISCLOSURE

The authors declare no conflict of interest.

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