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Corticotropin-Releasing Factor (CRF) in Ventromedial Prefrontal Cortex Mediates Avoidance of a Traumatic Stress-Paired Context

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Abstract

Post-traumatic stress disorder affects 7.7 million Americans. One diagnostic criterion is avoidance of stimuli related to the traumatic stress. Using a predator odor stress conditioned place avoidance (CPA) model, rats can be divided into groups based on stress reactivity, measured by avoidance of the odor-paired context. Avoider rats, which show high stress reactivity, exhibit avoidance of stress-paired context and escalated alcohol drinking. Here, we examined the role of corticotropin-releasing factor (CRF), a neuropeptide that promotes anxiety-like behavior, in mediating post-stress avoidance and escalated alcohol drinking. CRF is expressed in the medial prefrontal cortex (mPFC). The dorsal and ventral sub-regions of mPFC (dmPFC and vmPFC) have opposing roles in stress reactivity and alcohol drinking/seeking. We hypothesized that CRF-CRFR1 signaling in the vmPFC contributes to stress-induced avoidance and escalated alcohol self-administration. In Experiment 1, adult male Wistar rats were exposed to predator odor stress in a CPA paradigm, indexed for avoidance of odor-paired context, and brains processed for CRF-immunoreactive cell density in vmPFC and dmPFC. In Experiment 2, rats were tested for avoidance of a context repeatedly paired with intra-vmPFC CRF infusions. In Experiment 3, rats were stressed, indexed, and tested for the effects of intra-vmPFC CRFR1 antagonism on avoidance and alcohol self-administration. Post-stress, Avoiders exhibited higher CRF cell density in vmPFC, but not dmPFC. Intra-vmPFC CRF infusion produced conditioned place avoidance. Intra-vmPFC CRFR1 antagonism reversed avoidance of odor-paired context, but did not alter post-stress alcohol self-administration. These findings suggest that vmPFC CRF-CRFR1 signaling mediates avoidance of stimuli paired with traumatic stress.

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Disclosure

All other authors report no biomedical financial interests of potential conflicts of interest.

Keywords

Post-traumatic stress disorder (PTSD); Avoidance; Alcohol Self-Administration; Medial Prefrontal Cortex (mPFC); Corticotropin-releasing factor (CRF); Anxiety

1. Introduction

Traumatic stress disorders (e.g. post-traumatic stress disorder; PTSD) affect 7.7 million Americans (National Institutes of Health, 2015). Major diagnostic criteria for PTSD include re-experiencing the traumatic event, increased arousal, negative affective state, and persistent avoidance of stimuli that are related to the traumatic stress (American Psychiatric Association, 2013). Additionally, approximately 22–43% of PTSD patients meet diagnostic criteria for alcohol use disorder (AUD; Blanco et al., 2013).

Persistent avoidance of stimuli that are related or perceived to be related to the traumatic stress is a major diagnostic criterion for PTSD in humans (American Psychiatric Association, 2013), and avoidance of trauma-related stimuli predicts subsequent onset of PTSD symptoms (Shin et al., 2015). Our lab uses a predator odor model of PTSD in which rats are divided into groups based on their stress reactivity, as indexed by avoidance of a predator odor-paired chamber (i.e., Avoiders and Non-Avoiders; Edwards et al., 2013). Using this model, our lab has previously shown that predator odor stress produces persistent avoidance of the predator odor-paired stimuli in some but not all animals (i.e., Avoiders), and that these animals escalate alcohol self-administration after stress (Edwards et al., 2013). Interestingly, these post-stress differences do not appear to be caused by differences in learning and memory. Non-Avoiders show no difference in appetitive conditioning and are able to acquire operant conditioning at the same rate as Avoiders prior to stress exposure (Edwards et al., 2013). Furthermore, these animals do not differ in baseline alcohol self-administration, anxiety-like behavior (Edwards et al., 2013), or nociception (Itoga et al., in press) prior to stress.

Corticotropin-releasing factor (CRF) mediates affective regulation and arousal after stress (Heinrichs & Koob, 2004). In addition to activating the hypothalamic-pituitary-adrenal (HPA) axis, CRF acts as a neuromodulator in extra-hypothalamic areas of the brain including the prefrontal cortex (PFC) and the extended amygdala (Koob & Heinrichs, 1999). Brain CRF is dysregulated in psychiatric disorders including anxiety disorders, depression, and drug use disorders (George & Koob, 2010). Central administration of CRF increases anxiety-like behaviors and autonomic stress responses (Dunn & Berridge, 1990; Fisher, 1989), and produces conditioned place avoidance (Cador et al., 1992). CRF1 receptors (CRFR1) mediate anxiety-like behavior and hyperarousal in stressed rats (Heinrichs and Koob 2004; Roltsch et al., 2014). In addition, CRFR1s mediate binge-like ethanol consumption in mice (Lowery-Gionta et al., 2012) and post-stress and post-dependent escalations in alcohol drinking in mice and rats (Lowery et al., 2007; Sommer et al., 2008). Prior work from our lab showed that systemic antagonism of CRFR1s reduces hyperalgesia, hyperarousal, and alcohol self-administration in stressed rats (Roltsch et al., 2014).

The medial PFC (mPFC) has a major role in cognitive and emotional regulation, and the mPFC sends highly organized efferent projections to sub-cortical regions important for affective and motivated behaviors. The mPFC exerts top-down regulation of subcortical sites that coordinate stress responses, including the extended amygdala, the paraventricular nucleus of the hypothalamus, and the dorsal raphe nucleus (Sotres-Bayon & Quirk, 2010; George & Koob 2010). Medial PFC projections to basolateral and central amygdala (CeA) are important for fear conditioning (Sotres-Bayon & Quirk, 2010) and are disrupted during alcohol withdrawal (George et al., 2012).

The dmPFC and vmPFC have opposing roles in various behaviors. For example, the dmPFC promotes fear learning and fear expression through its connections with the basolateral amygdala (BLA), and the vmPFC promotes fear extinction through connections with the intercalated cells, CeA, and/or basomedial amygdala (Sotres-Bayon & Quirk, 2010; Adhikari et al., 2015). Similarly, the dmPFC inhibit hypothalamic stress responses (Radley et al., 2006), whereas the vmPFC facilitates hypothalamic stress responses (Radley et al 2006). Past work from our lab showed that Avoider rats have increased activation, as measured by ERK phosphorylation, of the vmPFC, but not the dmPFC, following a stress reminder (Edwards et al., 2013). Furthermore, human imaging studies demonstrate that lesions of the vmPFC significantly decrease PTSD symptoms, suggesting a critical role for vmPFC in development of PTSD following traumatic stress (Koenigs & Grafman, 2008).

Recent studies demonstrate a role for CRF in the mPFC in mediating anxiety-like behavior (Miguel et al., 2014; Pentkowski et al., 2013). Specifically, low CRF doses infused into the mPFC increase anxiety-like behavior, while higher doses reduce anxiety-like behavior (Ohata & Shibasaki, 2011; Jaferi & Bhatnagar, 2007; Pentkowski et al., 2013). It is also worth noting that a recent study reports a role for CRF-CRFR1-PKA signaling in mPFC in acute stress-induced executive dysfunction (Uribe-Mariño et al., 2016). These studies examined the mPFC as a whole, but here we tested the effects of stress on CRF in vmPFC and dmPFC, due largely to their different afferents, efferents, and assigned functions (Heidbreder & Groenewegen, 2003).

The purpose of the current studies was to determine the effect of predator odor stress on CRF cell counts in mPFC sub-regions, to assess the aversive properties of CRF in vmPFC, and to determine the role of vmPFC CRF-CRFR1 signaling in avoidance of predator odor-paired stimuli and post-stress alcohol self-administration. We hypothesized that 1) predator odor stress would increase the density of CRF-immunoreactive (CRF-ir) cells in vmPFC of Avoiders, 2) CRF infusions into vmPFC would produce conditioned place avoidance (CPA) in rats, 3) CRFR1 antagonism in vmPFC would abolish avoidance of a predator odor-paired context, and finally that 4) Avoiders would exhibit increased alcohol self-administration that would be reversed by CRFR1 antagonism in the vmPFC.

2. Methods and Materials

2.1 General Methods

2.1.1 Animals—Specific-pathogen free adult male Wistar rats (N=152) (Charles River) weighing 225–250g at the time of arrival were housed in groups of two in a humidity- and

temperature-controlled (22°C) vivarium on a 12 h light/12 h dark cycle (lights off at 8 a.m.). Animals had *ad libitum* access to food and water throughout experiments. Rats were acclimated for one week before start of experiments and were handled daily prior to initiation of surgical and experimental protocols. Behavioral tests occurred during the dark period. All procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center and were in accordance with the National Institute of Health Guidelines.

2.1.2 Predator Odor Conditioned Place Avoidance—To index rats for avoidance, animals underwent a 4-day CPA procedure, as previously described (Edwards et al., 2013). Briefly, on the first day, rats were allowed 5 min to explore two conditioning chambers with distinct tactile (circles vs. grid rod floor) and visual (stripes vs. circles) cues. Sessions were videotaped and scored by an observer. Rats were assigned to predator odor stress and non-stress groups that were counterbalanced for magnitude of baseline preference for one chamber versus the other. For rats in the stress group, an unbiased and counterbalanced procedure was used to determine which chamber would be paired with predator odor exposure in individual rats. On the second day, rats were placed in one chamber without odor (neutral environment) for 15 min. On the third day, rats were placed in the opposite context for 15 min with a urine-soaked sponge (bobcat urine) placed under the floor of the chamber (predator odor environment), or no odor for control animals. On the fourth day, rats were again allowed to explore the two conditioning chambers in a 5 min video-recorded post-test. Avoidance was calculated as a difference score between post-conditioning time spent in odor-paired context and pre-conditioning time spent in odor-paired context. Rats that displayed a >10 sec decrease in time spent in odor-paired chamber were classified as ‘Avoiders’. Rats that displayed a <10 sec decrease in time spent in the predator odor-paired chamber were classified as ‘Non-Avoiders’. The 10s cut-off for Avoiders and Non-Avoiders is consistent with previous publications from our lab (e.g., Edwards et al., 2013; Itoga et al., in press; Whitaker & Gilpin, 2015). By not using a median split, our criterion for separating those two groups remains identical across studies. This 10-s cut-off has been used to show differences between groups in alcohol drinking (Edwards et al., 2013), nociception (Itoga et al., in press), and the corticosterone response to stress (Whitaker & Gilpin, 2015).

2.1.3 Immunohistochemical Staining and Counting of CRF cells in mPFC sub-regions—Rats (n=32) were deeply anesthetized with isoflurane, injected with chloral hydrate (35% 2ml), and then intracardially perfused with 4% paraformaldehyde/0.1M borate buffer, pH 9.5 as previously described (Richardson et al., 2006). Brains were post-fixed in the same fixative for 4 h at 4°C and submerged in the 20% sucrose/0.1M phosphate buffer, pH 7.4 for 48–72 hours before being snap-frozen in isopentane (2-methylbutane, Fisher Scientific) on dry ice. Brains were stored at –80°C, then coronally sectioned at 35 µm on a freezing microtome. Sections were stored in cryoprotectant (30% ethylene glycol, 30% sucrose and 1% polyvinyl pyrrolidone in 0.1M PBS) at –20°C until immunolabeling. Brain sections containing mPFC (from 3.72 mm to 2.52 mm relative to bregma) were sorted and labeled by rabbit anti-h/rCRF antiserum (1:5000, generously provided by Dr. Wylie Vale, Salk Institute) and biotin-conjugated goat anti-rabbit antiserum (1:200, Vector Laboratories) using free-floating immunohistochemistry procedure (Karanikas et al., 2013). CRF labeling

signal was developed with 3,3'-diaminobenzidine (DAB) and nickel (Vector Laboratories), and sections were mounted on slides for microscopic analysis.

Slides containing prefrontal sections were digitally scanned at high resolution (20×) under bright field illumination by Aperio ePathology (Leica Biosystems). Four coronal sections (spaced 350 µm apart) containing CRF immunoreactive (CRF-ir) cells and fibers were used to quantify CRF cell number in mPFC (Fig 2A). The dmPFC contained anterior cingulate cortex (AC) and dorsal prelimbic cortex (PrL); the vmPFC contained ventral PrL and infralimbic cortex (IL) (Figure 2A). Areas of interest were traced and measured using ImageScope software (Leica Biosystems), and all CRF-ir cells were counted within traced regions. Experimenters blind to the treatment group counted CRF-ir cells under 10× magnification of the slide images. The criteria for identifying CRF-ir cells were a clearly defined border of the soma and evidence of extended neurites.

2.1.4 CRF Conditioned Place Avoidance—Rats underwent a conditioned place avoidance procedure similar to the one described by Cador et al., 1992. Briefly, on day 1, rats underwent a 5 min video-recorded pre-test to explore two conditioning chambers with distinct tactile (circles vs. grid rod floor) and visual (stripes vs. circles) cues. Rat assignment to CRF dose groups was counterbalanced for context preference. Within dose groups, CRF infusions were paired with context for each rat in an unbiased counterbalanced design. On days 2, 4, and 6, rats were infused with sterile saline and immediately placed in one chamber for 15 minutes (neutral chamber). On days 3, 5, and 7, rats were infused with CRF (0, 0.05, 0.25, or 0.5 µg/0.5µl per side) and immediately placed in the opposite chamber for 15 minutes (CRF chamber). It is important to note that each individual rat was repeatedly infused with the same CRF dose across conditioning days (i.e., between-subjects dose-response). On day 8, rats underwent a 5 min video-recorded post-test to explore the two conditioning chambers. Avoidance was calculated as a difference score between post-conditioning time spent in CRF-paired context and pre-conditioning time spent in CRF-paired context.

2.1.5 Operant Self-Administration—Rats were trained to orally self-administer 10% w/v ethanol or water in a concurrent, two-lever, free-choice contingency that did not incorporate a sweet fading procedure, as previously described (Roltsch et al., 2014). Rats were first given a single 24-hr period of access to 10% w/v ethanol vs. water in the home cage to prevent neophobia upon presentation of ethanol in operant boxes. Rats were then given a single 15-h operant session to learn to press a single lever for water (right lever; FR1) in the presence of *ad libitum* food on floor of operant chamber. Rats were then allowed one 3-h two-lever operant session for 10% w/v ethanol (right lever; FR1) vs. water (left lever; FR1), one 2-h session, then one 1-h session, followed by daily 30-min session for ~15 days. All operant sessions after the initial 15-h session occurred in the absence of food. Rats with post-surgery baselines <10 alcohol presses in a 30-min session after vehicle infusion were excluded from further analysis.

2.1.6 Surgical Procedures—On the day of surgery, rats were anesthetized with isoflourane and mounted in a stereotaxic frame (Stoelting). An incision was made, small bilateral holes were drilled into the skull, and stainless steel guide cannulae (26 gauge)

lowered such that tips were 1mm dorsal to vmPFC (AP +2.8, ML $\pm$ 3.1, DV -3.9 from the skull at an angle of 30°). Guide cannulae were secured to the skull with metal screws and dental cement, the incision closed, and dummy cannulae inserted. Rats were allowed to recover and monitored daily for 1 week following surgery before initiating experimental procedures. Before sacrifice, animals were infused with Evans Blue to verify cannula placement (Figure 3B and 3D; Paxinos and Watson, 2005). Animals whose surgeries did not hit the target region bilaterally were excluded from analysis.

2.1.7 Infusion Procedure—Animals underwent sham infusions on days preceding infusion to acclimate them to the procedure. A Harvard instruments microinfusion pump was used for all infusions. Infusions were delivered via polyethylene tubing (PE20) connected to a 10 μ l Hamilton syringe. Infusions were administered bilaterally at a rate of 0.2 μ l/min over the course of 2.5 min via injectors (33ga, stainless steel) that extended 1 mm beyond the tip of the guide cannulae. Injection cannulae were left in the guide cannulae for an additional 1 min to allow for diffusion.

2.1.8 Drugs—The CRFR1 antagonist R121919 (generously supplied by Neurocrine, Inc.) was solubilized first in 1M HCl (10% final volume) then diluted into 2-hydroxypropyl-cyclodextrin (HBC; Sigma-Aldrich, 20% wt/vol final concentration in distilled water) and back-titrated with NaOH to pH 4.5. CRF (Sigma Aldrich, St. Louis, OM) was solubilized in sterile saline.

2.2 Experimental Protocols

2.2.1 Experiment 1. Effect of predator odor stress on CRF-ir cells in vmPFC and dmPFC—Rats were exposed to predator odor stress, indexed for avoidance, and separated into Control (n=10), Non-Avoider (n=12), and Avoider (n=9). Nine days post-stress, rats were deeply anesthetized with isoflurane, then injected with chloral hydrate (35% 2ml), and perfused intracardially. Brains were processed for CRF-ir cell density by immunohistochemical labeling as described in General Methods.

2.2.2 Experiment 2. Intra-vmPFC CRF-induced conditioned place avoidance—Rats (N=32) were implanted with bilateral cannulae targeted 1 mm dorsal to vmPFC. After one week of recovery, rats underwent the CRF CPA procedure described in General Methods.

2.2.3 Experiment 3. Effect of intra-vmPFC CRFR1 antagonism on post-stress avoidance and alcohol drinking—Rats were tested for baseline ethanol self-administration. Upon stabilization of operant responding, rats were implanted with bilateral cannulae targeted 1 mm dorsal to vmPFC. After one week of recovery, rats were re-tested for baseline self-administration with vehicle infusions, then exposed to predator odor stress, indexed for avoidance, and separated into Control (n=12), Non-Avoiders (n=15), and Avoiders (n=19). On post-stress days 2, 5, 8, and 11, rats were allowed to self-administer alcohol for 30 minutes, as described above. On post-stress day 12, rats were again tested for avoidance of the predator-odor paired chamber. Five minutes prior to each test, rats received

intra-vmPFC infusion of vehicle (20% HBC, 0.5 μ l) or R121919 (0.25 μ g/0.5 μ l); each rat received the same treatment (vehicle or R121919) throughout the experiment.

2.3 Statistical Analysis

Data are shown as mean $\pm$ SEM with the number of animals in each experiment indicated in Figure 1. In Experiment 1, CRF-ir cell density was analyzed with a mixed-design ANOVA where stress history was a between factor and bregma was a within-subjects factor. Also, overall mean CRF-ir cell number was analyzed with stress as the single between-subjects factor. In Experiment 2, avoidance of CRF-paired chamber was analyzed with a one-way ANOVA where drug dose was the treatment factor, and with trend analysis to determine whether the linear (change in dependent variable as a function of change in the independent variable) and/or quadratic (rate of change in the dependent variable as a function of change in the independent variable) components of responding were significantly affected by CRF dose. CRFR1 antagonist effects on avoidance were analyzed with two-way ANOVA where stress history and R121919 drug dose were the treatment factors. Differences in ethanol self-administration over time were analyzed with a three-way RM ANOVA where stress history and drug dose were the between-subject factors and time was the within-subjects factor. Post hoc analysis with the Tukey's HSD Test was used when appropriate. Statistical significance was set as $p < 0.05$.

3. Results

3.1 Predator odor stress increases CRF cell density in the vmPFC but not dmPFC of Avoiders

In Experiment 1, Avoiders exhibited higher density of CRF-ir cells relative to Non-Avoiders and unstressed Controls 9 days post-stress in the vmPFC ($F[2,28]=5.698$, $p=0.008$), but not dmPFC ($F[2,28]=1.550$, $p=0.230$) (Figure 2D, 2B). CRF-ir cell number in vmPFC and dmPFC was positively correlated with avoidance of the predator odor-paired chamber 24 h post-stress (dmPFC, $R^2=0.18$, $p=0.05$; vmPFC, $R^2=0.35$, $p=0.004$) (Figure 2C, 2E). Because there was no significant change in CRF immunoreactivity in the dmPFC of stressed rats, behavioral pharmacology studies were only conducted in the vmPFC.

3.2 CRF infusions into vmPFC produce conditioned place avoidance

In Experiment 2, rats underwent a CPA test using intra-vmPFC CRF infusion as the unconditioned stimulus. Compared to vehicle-infused rats, CRF-infused rats spent less time in the drug-paired chamber ($F[3,28]=2.862$; $p=0.054$; Figure 3A). Quadratic trend analysis revealed that the amount of time spent avoiding the CRF-paired chamber was related to dose of CRF, with lower doses producing more avoidance than higher doses ($p=0.054$).

3.3 CRFR1 antagonism in vmPFC reduces avoidance of stress-paired context

In Experiment 3, rats underwent a CPA procedure 1 d post-stress for indexing of avoidance behavior and again 12 d post-stress with or without antagonism of vmPFC CRFR1. A 1-way ANOVA of Day 1 avoidance data showed that Avoiders spent significantly more time avoiding the predator odor paired chamber compared to Non-Avoiders and Controls (Controls -5.94 ± 11.36 s; Non-Avoiders 25.24 ± 9.5 s; Avoiders -39.25 ± 6.21 s compared

to pre-conditioning baseline) ($F[2,43]=16.45$; $p<0.01$). A separate, 2-way ANOVA of Day 12 avoidance data revealed a main effect of stress on the amount of time spent avoiding the predator odor paired chamber. On Day 12 post-stress, Avoiders spent significantly less time than Non-Avoiders in the predator odor-paired chamber ($F[1,24]=18.06$; $p<0.01$) (Figure 3C), as would be expected based on the model and past reports from our lab (Whitaker & Gilpin, 2015). Based on the *a priori* hypothesis that CRFR1 antagonism would reduce avoidance, we analyzed day 12 (i.e., after R121919 treatment) avoidance scores in Avoider rats using a two-samples t-test where R121919 dose was the between-subjects factor. This analysis revealed that R121919 significantly reduced avoidance in Avoiders relative to vehicle-treated controls ($t(17)=2.09$; $p=0.026$) (Fig 3C). A separate two-samples t-test indicated that R121919 did not affect avoidance behavior in Non-Avoiders 12 days post-stress ($t(10)=0.4994$; $p=0.523$).

3.4 CRFR1 antagonism does not affect alcohol self-administration

In Experiment 3, rats were trained to self-administer 10% ethanol. The pre-surgery baseline was 42.08 ± 1.76 presses per 30 min session. After surgery, mean ethanol responding per 30-min session was 27.92 ± 1.62 presses. This decrease in ethanol self-administration was due either to surgery, the introduction of pre-operant sham infusions, or both. Once post-surgery operant responding stabilized, rats were stressed, then were allowed to self-administer alcohol on days 2, 5, 8, and 11 post-stress after infusion of either vehicle or R121919 into the vmPFC (Figure 4). When operant response data were analyzed as percent of baseline for each rat, (Figure 4), there was a trend for Avoiders to respond more for alcohol than the other two groups after predator odor exposure ($F[2,40]=2.99$; $p=0.06$). A 3-way RM ANOVA revealed no significant increases in raw operant alcohol lever presses across days after stress. There was also no significant main effect of R121919 treatment on operant alcohol self-administration ($F[1,40]=0.08$; $p=0.77$), nor was there a significant stress $\times$ drug interaction effect ($F[2,40]=1.28$; $p=0.29$).

4. Discussion

In this study, we tested the role of CRF in the vmPFC in mediating predator odor stress-induced avoidance and escalation of alcohol drinking. We demonstrated that 1) after predator odor stress, Avoiders have higher CRF cell density in vmPFC, but not dmPFC, that is positively correlated with avoidance of the predator odor-paired chamber 24 h after stress, 2) CRF infused into the vmPFC produces conditioned avoidance of a CRF-paired context, 3) CRFR1 antagonism in vmPFC reduces avoidance of a stress-paired context, and 4) CRFR1 antagonism in vmPFC does not reduce post-stress alcohol drinking.

The present study demonstrates a role for CRF in the vmPFC in stress-induced avoidance behavior. After exposure to predator odor stress, Avoiders exhibited higher CRF cell density 9 days post-stress. We assume that the difference CRF-ir cell counts are not pre-existing, but are instead caused by exposure to the predator odor. It is unlikely that random assignment of animals into stress and controls groups would select for animals with different numbers of CRF-ir cells in vmPFC. Moreover, the spread of the data in Avoiders and Non-Avoiders extends outside the spread of data in non-stressed Control animals. Increases in number of

CRF-ir cells within the vmPFC were correlated with more time spent avoiding the predator odor-paired chamber 24 hours post-stress, suggesting that more avoidance is related to higher CRF cell density in the vmPFC. We did not measure CRF release into the synapse, but it is notable that chronic CRFR1 antagonism in the vmPFC reduced avoidance behavior in Avoiders (Figure 3C). Overall, this suggests that avoidance of a stress-paired context is mediated, at least in part, by CRF signaling in vmPFC. This result seems to agree with previously published work from our group showing that exposure to a traumatic stress reminder increases ERK phosphorylation, a marker and mediator of neuronal activity downstream of CRFR1 signaling (Meng et al., 2011), in vmPFC of Avoiders compared to Non-Avoiders (Edwards et al., 2013).

Here, we used a conditioned place avoidance paradigm to show that CRF in vmPFC is aversive. We demonstrate that low CRF doses (0.05 µg/side) produce conditioned avoidance of a CRF-paired chamber compared to vehicle, while higher doses of CRF (0.25 and 0.5 µg/side) produced less avoidance. According to the literature, different doses of CRF in the mPFC have different effects on anxiety-like behavior in rodents. Specifically, low doses (0.02 – 0.05 µg/side) produce anxiety-like behavior (Jaferi & Bhatnagar, 2007; Ohata & Shibasaki, 2011), while high doses (0.2 – 1.0 µg/side) of CRF infused into the mPFC are anxiolytic (Ohata & Shibasaki, 2011; Pentkowski et al., 2013). In our study, the lowest CRF dose (0.05 µg/side), which matches anxiogenic CRF doses in the literature, produced the strongest avoidance of the CRF-paired chamber. Both the anxiogenic and anxiolytic effects of vmPFC CRF are thought to be mediated by activation of CRFR1 on glutamatergic pyramidal cells, because there are no CRFR2 in the vmPFC (Chalmers et al., 1995). It is possible that low and high CRF doses initiate different CRFR1 signaling cascades to produce opposite behavioral responses: for example, it has been suggested that the anxiogenic actions of low CRF doses may be mediated by protein kinase A signaling (Miguel et al., 2014), whereas the anxiolytic-like actions of high CRF doses may be mediated by protein kinase C signaling (Tan et al., 2004). Interestingly, CRFR1 antagonism in vmPFC reduced avoidance behavior in Avoiders, suggesting that physiological levels of CRF in vmPFC promote avoidance, and perhaps anxiety-like behavior. Further studies will determine the cellular mechanisms whereby CRF-CRFR1 signaling in vmPFC produces aversion.

The vmPFC projects to sub-cortical brain regions that control anxiety, fear, and drug addiction. The vmPFC facilitates hypothalamic stress responses and may regulate long-lasting anxiety through its indirect projections to the paraventricular nucleus of the hypothalamus and direct projections to bed nucleus of the stria terminalis, respectively (Radley et al., 2006; Motzkin et al., 2015). The vmPFC also sends projections to the basomedial amygdala and/or the intercalated cells of the amygdala to mediate fear extinction (Adhikari et al., 2015; Keifer et al., 2015; Sotres-Bayon and Quirk, 2010). In humans, damage to the vmPFC leads to disinhibition of the amygdala, which has been hypothesized to contribute to PTSD symptoms (Motzkin et al., 2015). These studies all support a role for dysregulation of the vmPFC in traumatic stress and anxiety disorders.

We also tested whether CRF-CRFR1 signaling in the vmPFC mediates escalation of alcohol drinking after stress. After stress, there was a trend toward stress group differences in

alcohol self-administration ($p=0.06$). Previously, we demonstrated that Avoider rats self-administer more alcohol than Non-Avoiders and unstressed Controls (Edwards et al., 2013). The current study may have failed to reach significance due to the stress caused by the drug infusion procedure 5 minutes before alcohol self-administration, despite the inclusion of post-surgery sham and vehicle infusions to acclimate animals to the infusion procedure. CRFR1 antagonism in vmPFC did not affect alcohol drinking. Although our data do not demonstrate a role for vmPFC CRF-CRFR1 signaling in post-stress alcohol drinking, the vmPFC is important for extinction and relapse of alcohol-seeking behavior. For example, the vmPFC inhibits drug-seeking following extinction (Van den Oever et al., 2010; Peters et al., 2008), and enhancement of mGluR5 signaling in the infralimbic cortex facilitates extinction of alcohol responding in rats (Gass et al., 2014). Pfarr et al. (2015) identified a specific vmPFC neuronal ensemble critical for inhibiting alcohol-seeking behavior after cue-induced reinstatement, whereas complete vmPFC inactivation does not affect extinction or cue-induced reinstatement of alcohol-seeking behavior (Willcocks & McNally, 2012; Pfarr et al., 2015). Our data suggest that CRF-CRFR1 signaling in vmPFC is not a critical mediator of non-extinguished alcohol self-administration after stress.

Overall, this study shows that stress alters CRF cell density in the vmPFC, but not dmPFC, of some but not all stressed animals. We also show that CRF in vmPFC is aversive and that CRF-CRFR1 signaling in vmPFC mediates avoidance of a predator odor-paired chamber but not escalation of alcohol self-administration after traumatic stress.

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References

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders: DSM-5. Washington, D.C: American Psychiatric Association; 2013.
2. Adhikari A, Lerner TN, Finkelstein J, Pak S, Jennings JH, Davidson TJ, Ferenczi E, Gunaydin LA, Mirzabekov JJ, Ye L, Kim SY, Lei A, Deisseroth K. Basomedial amygdala mediates top-down control of anxiety and fear. *Nature*. 2015; 527(7577):179–185. [PubMed: 26536109]
3. Blanco C, Xu Y, Brady K, Pérez-Fuentes G, Okuda M, Wang S. Comorbidity of posttraumatic stress disorder with alcohol dependence among US adults: Results from National Epidemiological Survey on Alcohol and Related Conditions. *Drug Alcohol Depend*. 2013; 132:630–638. [PubMed: 23702490]
4. Cador M, Ahmed SH, Koob GF, Le Moal M, Stinus L. Corticotropin-releasing factor induced a place aversion independent of its neuroendocrine role. *Brain Research*. 1992; 597:304–309. [PubMed: 1473001]
5. Chalmers DT, Lovenberg TW, De Souza EB. Localization of Novel Corticotropin-Releasing Factor Receptor (CRF₂) mRNA Expression to Specific Subcortical Nuclei in Rat Brain: Comparison with CRF₁ Receptor mRNA Expression. *J Neurosci*. 1995; 15(10):6340–6350. [PubMed: 7472399]

6. Dunn AJ, Berridge CW. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety of stress responsiveness? *Brain Research Reviews*. 1990; 15:71–100. [PubMed: 1980834]
7. Edwards S, Baynes BB, Carmichael CY, Zamora-Martinez ER, Barrus M, Koob GF, Gilpin NW. Traumatic stress reactivity promotes excessive alcohol drinking and alters the balance of prefrontal cortex-amygdala activity. *Transl Psychiatry*. 2013; 3:e296. [PubMed: 23982628]
8. Fisher LA. Corticotropin-releasing factor: endocrine and autonomic integration of responses to stress. *TIPS Reviews*. 1989; 10:189–193.
9. Gass JT, Trantham-Davidson H, Kassab AS, Glan WB Jr, Olive MF, Chander LJ. Enhancement of Extinction Learning Attenuates Ethanol-Seeking Behavior and Alters Plasticity in Prefrontal Cortex. *J Neurosci*. 2014; 34(22):7532–7574.
10. George O, Koob GF. Individual differences in prefrontal cortex function and the transition from drug use to drug dependence. *Neurosci Biobehav Rev*. 2010; 35(2):232–247. [PubMed: 20493211]
11. Heidbreder CA, Grenewegen HJ. The medial prefrontal cortex in the rat: evidence for a dorso-ventral distinction based upon functional and anatomic characteristics. *Neurosci Biobehav Rev*. 2003; 27:555–579. [PubMed: 14599436]
12. Heinrichs SC, Koob GF. Corticotropin-Releasing Factor in Brain: A Role in Activation, Arousal, and Affect Regulation. *J Pharmacol Exp Ther*. 2004; 311(2):427–440. [PubMed: 15297468]
13. Itoga CA, Roltsch-Hellard EA, Whitaker AM, Lu YL, Schreiber AL, Baynes BB, Baiamonte BA, Richardson HN, Gilpin NW. Traumatic Stress Promotes Hyperalgesia via Corticotropin-Releasing Factor-1 Receptor (CRFR1) Signaling in Central Amygdala. *Neuropsychopharmacology*. 2016 In press.
14. Jaferia A, Bhatnagar S. Corticotropin-releasing hormone receptors in the medial prefrontal cortex regulate hypothalamic-pituitary-adrenal activity and anxiety-related behavior regardless of prior stress experience. *Brain Research*. 2007; 1186:212–223. [PubMed: 18001698]
15. Karanikas CA, Lu YL, Richardson HN. Adolescent drinking targets corticotropin-releasing factor peptide-labeled cells in the central amygdala of male and female rats. *Neuroscience*. 2013; 249:98–105. [PubMed: 23628776]
16. Keifer OP, Hurt RC, Ressler KJ, Marvar PJ. The Physiology of Fear: Reconceptualizing the Role of the Central Amygdala in Fear Learning. *Physiology*. 2015; 30:389–401. [PubMed: 26328883]
17. Koenigs M, Huey ED, Raymont V, Cheon B, Solomon J, Wasserman EM, Grafman J. Focal brain damage protects against post-traumatic stress disorder in combat veterans. *Nature Neuroscience*. 2008; 11:232–237. [PubMed: 18157125]
18. Koob GF, Heinrichs SC. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res*. 1999; 848:141–152. [PubMed: 10612706]
19. Lowery EG, Sparrow AM, Breese GR, Knapp DJ, Thiele TE. The CRFR-1 receptor antagonist, CP-154,526, attenuates stress-induced increase in ethanol consumption by BALB/cJ mice. *Alcohol Clin Exp Res*. 2007; 32:240–248. [PubMed: 18162074]
20. Lowery-Gionta EG, Navarro M, Li C, Pleil KE, Rinker JA, Cox BR, Sprow GM, Kash TL, Thiele TE. Corticotropin Releasing Factor Signaling in the Central Amygdala is Recruited during Binge-like Ethanol Consumption in C57BL/6J Mice. *J Neurosci*. 2012; 32(10):3405–3413. [PubMed: 22399763]
21. Meng QY, Chen XN, Tong DL, Zhou JN. Stress and glucocorticoids regulated corticotropin releasing factor in prefrontal cortex. *Mol Cell Endocrinol*. 2011; 342:54–63. [PubMed: 21664419]
22. Miguel TT, Gomes KS, Nunes-de-Souza RL. Tonic modulation of anxiety-like behavior by corticotropin-releasing factor (CRF) type 1 receptor (CRFR) within the medial prefrontal cortex (mPFC) in male mice: Role of protein kinase A (PKA). *Hormones and Behavior*. 2014; 66:247–256. [PubMed: 24848364]
23. Motzkin JC, Philippi CL, Wolf RC, Baskaya MK, Koenigs M. Ventromedial Prefrontal Cortex is Critical for the Regulation of Amygdala Activity in Humans. *Biol Psychiatry*. 2015; 77:276–284. [PubMed: 24673881]
24. Motzkin JC, Philippi CL, Oler JA, Kalin NH, Baskaya MK, Koenigs M. Ventromedial prefrontal cortex alters resting blood flow to the bed nucleus of stria terminalis. *Cortex*. 2015; 64:281–288. [PubMed: 25569763]

25. <http://www.nimh.nih.gov>
26. Ohata H, Shibasaki T. Microinjection of Different Doses of Corticotropin-Releasing Factor into the Medial Prefrontal Cortex Produces Effects Opposing Anxiety-related Behavior in Rats. *J Nippon Med Sch.* 2011; 78(5):286–292. [PubMed: 22041875]
27. Ovari J, Leri F. Inactivation of ventromedial prefrontal cortex mimics re-emergence of heroin seeking caused by heroin reconditioning. *Neurosci Lett.* 2008; 444(1):52–55. [PubMed: 18706971]
28. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates.* San Diego, CA, USA: Elsevier Academic Press; 2005.
29. Pentkowski NS, Tovote P, Zavala AR, Litvin Y, Blanchard DC, Spiess J, Blanchard RJ. Cortagine infused into the medial prefrontal cortex attenuates predator-induced defensive behaviors and Fos protein production in selective nuclei of the amygdala in male CD1 mice. *Hormones and Behavior.* 2013; 64:519–526. [PubMed: 23845323]
30. Peters J, LaLumiere RT, Kalivas PW. Infralimbic prefrontal cortex is responsible for inhibiting cocaine seeking in extinguished rats. *J Neurosci.* 2008; 28(23):6046–6053. [PubMed: 18524910]
31. Pfarr S, Meinhardt MW, Klee ML, Hansson AC, Vengeliene V, Schöning K, Bartsch D, Hope BT, Spanagel R, Sommer WH. Losing Control: Excessive Alcohol Seeking after Selective Inactivation of Cue-Responsive Neurons in the Infralimbic Cortex. *J Neurosci.* 2015; 35(30):10750–10761. [PubMed: 26224858]
32. Radley JJ, Arias CM, Sawchenko PE. Regional Differentiation of the Medial Prefrontal Cortex in Regulating Adaptive Responses to Acute Emotional Stress. *J Neurosci.* 2006; 26(50):12967–12976. [PubMed: 17167086]
33. Richardson HN, Zorilla EP, Mandyam CD, Rivier CL. Exposure to repetitive versus varied stress during prenatal development generates two distinct anxiogenic and neuroendocrine profiles in adulthood. *Endocrinology.* 2006; 147(5):2506–2517. [PubMed: 16455779]
34. Roltsch EA, Bayes BB, Mayeux JP, Whitaker AM, Baiaomonte BA, Gilpin NW. Predator odor stress alters corticotropin-releasing factor-1 receptor (CRF1R)-dependent behaviors in rats. *Neuropharmacology.* 2014; 79:83–89. [PubMed: 24269607]
35. Shin KM, Chang HY, Cho SM, Kim NH, Kim KA, Chung YK. Avoidance symptoms and delayed verbal memory are associated with post-traumatic stress symptoms in female victims of sexual violence. *J Affect Disord.* 2015; 184:145–148. [PubMed: 26093033]
36. Sommer WH, Rimondini R, Hansson AC, Hippskind PA, Gehlert DR, Barr CS, Heilig MA. Upregulation of Voluntary Alcohol Intake, Behavioral Sensitivity to Stress, and Amygdala Crhr1 Expression Following a History of Dependence. *Biol Psychiatry.* 2008; 63:139–145.
37. Sotres-Bayon F, Quirk GJ. Prefrontal control of fear: more than just extinction. *Current Opinion in Neurobiology.* 2010; 20:231–235. [PubMed: 20303254]
38. Tan H, Zhong P, Yan Z. Corticotropin-releasing factor and acute stress prolongs serotonergic regulation of GABA transmission in prefrontal cortical pyramidal neurons. *J Neurosci.* 2004; 24(21):5000–5008. [PubMed: 15163692]
39. Uribe-Mariño A, Gassen NC, Wiesbeck MF, Balsevich G, Santarelli S, Solfrank B, Dourmes C, Fries GR, Masna M, Labermeier C, Wang XD, Hafner K, Schmid B, Rein T, Chen A, Deussing JM, Schimdt MV. Prefrontal cortex corticotropin-releasing hormone receptor 1 conveys acute stress-induced executive dysfunction. *Biol Psychiatry.* 2016 <http://dx.doi.org/10.1016/j.biopsych.2016.03.2016>.
40. Van den Oever MC, Spijker S, Smit AB, De Vries TJ. Prefrontal cortex plasticity mechanisms in drug seeking and relapse. *Neurosci Biobehav Rev.* 2010; 35:276–284. [PubMed: 19932711]
41. Whitaker AM, Gilpin NW. Blunted hypothalamo-pituitary adrenal axis response to predator odor predict high stress reactivity. *Physiology and Behavior.* 2015; 147:16–22. [PubMed: 25824191]
42. Willcocks AL, McNally GP. The role of medial prefrontal cortex in extinction and reinstatement of alcohol-seeking in rats. *Eur J Neurosci.* 2013; 37(2):259–268. [PubMed: 23106416]

Experiment 1 (Fig. 2) **Experiment 2 (Fig. 3)** **Experiment 3 (Fig. 3 & 4)** **Figure 1. Timelines for Experiments 1–3 and Corresponding Figures**

illustrates the experimental timeline for Experiments 1–4 described below.

Explanation of groups, treatments, experimental timelines, and analyses are contained in the *Materials and Methods* section. Experiments 1 and 3 used conditioned place aversion (CPA) paradigm to index the animals as Controls, Non-Avoiders, or Avoiders. The purpose of Experiment 1 was to generate tissue for *ex vivo* processing and analysis (Figure 2).

Experiments 2 and 3 were behavioral experiments in which animals received brain site-specific pharmacology (Figures 3 and 4).

Figure 2. Predator Odor Stress Increases CRF+ Cell Density in vmPFC of Avoiders

A. Representative images of examined sections (B1–B4) and CRF-ir cells in the mPFC. B. CRF-ir cell density in the dmPFC in 4 sections was not significantly changed after predator odor stress. Insert: Average CRF-ir cell density in the dmPFC. C. dmPFC CRF-ir cell density positively correlated with avoidance 1 day post-stress ($R^2=0.18$, $p=0.05$). C. Avoiders had significantly higher CRF cell density in 4 sections than Non-Avoiders and Controls in the cmPFC 9 days post stress ($*p=0.008$). Insert: Average CRF-ir cell density across 4 sections in the vmPFC. E. CRF cell density positively correlated with avoidance in

the vmPFC ($R^2=0.35$, $p<0.01$). Data are represented as Mean $\pm$ SEM. Control, white; Non-Avoiders, grey; Avoiders, black.

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Figure 3. CRF in vmPFC mediates avoidance behavior

A. Intra-vmPFC CRF infusions produced avoidance of a CRF-paired context 1 day post-containing ($p=0.054$). Quadratic trend analysis revealed time avoiding the chamber was related to the dose of CRF ($p=0.054$) with lower doses producing more avoidance than higher doses. B. cannula placements for Experiment 2 (White Dots – Misses; Black Dots – Hits; Images modified from Paxinos and Watson, 2005). Insert: Representative image of histological verification by Evan's Blue Dye. C. Avoiders spend significantly less time than Non-Avoiders in the predator-odor paired chamber relative to the pre-conditioning baseline 12 days post-stress ($p<0.05$). Antagonism of CRF1R receptors reduced the amount of time spent avoiding the predator odor-paired chamber in Avoider ($*p<0.05$). D. Cannula placements for Experiment 3. (White Dots – Misses; Black Dots – Hits; Images modified from Paxinos and Watson, 2005). Data are represented as Mean $\pm$ SEM. Non-Avoiders, grey; Avoiders, black.

Figure 4. Post-stress alcohol self administration does not differ across groups

Average percent of baseline self-administration per group across drinking days post-stress. After stress, there were no significant differences in alcohol self-administration between stress groups ($p=0.06$). Antagonism of CRF1Rs with R121919 in the vmPRC did not have a significant effect on alcohol self-administration ($p=0.77$). Data are represented as Mean $\pm$ SEM. Control, white color; Non-Avoider, grey color; Avoider, black color