

# Binge Drinking Decreases Corticotropin-Releasing Factor-Binding Protein Expression in the Medial Prefrontal Cortex of Mice

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**Background:** Dysregulation of the corticotropin-releasing factor (CRF) system has been observed in rodent models of binge drinking, with a large focus on CRF receptor 1 (CRF-R1). The role of CRF-binding protein (CRF-BP), a key regulator of CRF activity, in binge drinking is less well understood. In humans, single-nucleotide polymorphisms in *CRHBP* are associated with alcohol use disorder and stress-induced alcohol craving, suggesting a role for CRF-BP in vulnerability to alcohol addiction.

**Methods:** The role and regulation of CRF-BP in binge drinking were examined in mice exposed to the drinking in the dark (DID) paradigm. Using *in situ* hybridization, the regulation of CRF-BP, CRF-R1, and CRF mRNA expression was determined in the stress and reward systems of C57BL/6J mice after repeated cycles of DID. To determine the functional role of CRF-BP in binge drinking, CRF-BP knockout (CRF-BP KO) mice were exposed to 6 cycles of DID, during which alcohol consumption was measured and compared to wild-type mice.

**Results:** CRF-BP mRNA expression was significantly decreased in the prelimbic (PL) and infralimbic medial prefrontal cortex (mPFC) of C57BL/6J mice after 3 cycles and in the PL mPFC after 6 cycles of DID. No significant changes in CRF or CRF-R1 mRNA levels were observed in mPFC, ventral tegmental area, bed nucleus of the stria terminalis, or amygdala after 3 cycles of DID. CRF-BP KO mice do not show significant alterations in drinking compared to wild-type mice across 6 cycles of DID.

**Conclusions:** These results reveal that repeated cycles of binge drinking alter CRF-BP mRNA expression in the mPFC, a region responsible for executive function and regulation of emotion and behavior, including responses to stress. We observed a persistent decrease in CRF-BP mRNA expression in the mPFC after 3 and 6 DID cycles, which may allow for increased CRF signaling at CRF-R1 and contribute to excessive binge-like ethanol consumption.

**Key Words:** Corticotropin-Releasing Factor, CRF-Binding Protein, Ethanol, Binge Drinking, Drinking in the Dark.

THE NATIONAL INSTITUTE on Alcohol Abuse and Alcoholism (NIAAA) defines binge drinking as a pattern of drinking that results in blood ethanol concentrations (BECs) of 80 mg/dl or higher. Binge drinking has been linked to many adverse social and health consequences, including an increased risk of transitioning to alcohol dependence. Stress is a key environmental factor in the development of alcohol addiction and has been linked to binge drinking, drinking during dependence, and relapse to drinking after abstinence in clinical and preclinical models (Keys

et al., 2012; Lowery and Thiele, 2010; Phillips et al., 2015; Uhart and Wand, 2009). The key central nervous system regulator of the stress response is corticotropin-releasing factor (CRF). This 41 amino acid peptide mediates its effects through 2 G-protein-coupled receptors, CRF receptor 1 (CRF-R1) and CRF receptor 2 (CRF-R2), and its activity is modulated by CRF-binding protein (CRF-BP). Single-nucleotide polymorphisms in *CRHBP* and *CRHR1* have been associated with alcohol use disorder and stress-induced alcohol craving or consumption (Blomeyer et al., 2008; Enoch et al., 2008; Ray, 2011; Ray et al., 2013), supporting the role for these key molecules in vulnerability to alcohol addiction.

Dysregulation of the CRF system has been observed in rodent models of binge drinking and alcohol dependence, with a large focus in the literature on CRF-R1. For example, elevated CRF-R1 mRNA expression in the amygdala has been observed in rats with a history of alcohol dependence (Sommer et al., 2008). Furthermore, peripheral administration of CRF-R1 antagonists reduced binge drinking in a drinking in the dark (DID) paradigm (Sparta et al., 2008), as well as dependence-induced alcohol consumption (Funk

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et al., 2007). CRF receptor regulation of excessive alcohol consumption appears to be brain region-specific, with the amygdala, bed nucleus of the stria terminalis (BNST), ventral tegmental area (VTA), and medial prefrontal cortex (mPFC) as major sites of action. For example, injection of a CRF-R1 antagonist into the central nucleus of the amygdala (CeA), but not the basolateral amygdala (BLA), resulted in decreased binge drinking (Lowery-Gionta et al., 2012). Likewise, intra-VTA administration of a CRF-R1 antagonist led to decreased binge drinking (Sparta et al., 2013). Silberman and colleagues (2013) have shown enhanced CRF activation of VTA-projecting BNST neurons after alcohol withdrawal. Last, CRF neurons in the mPFC are up-regulated after abstinence from intermittent access to ethanol (EtOH) (George et al., 2012). Although less well characterized, CRF-R2 has also been implicated in binge drinking (Albrechet-Souza et al., 2015; Lowery et al., 2010) and alcohol dependence (Funk and Koob, 2007). While it is clear that CRF and the CRF receptors play a role in alcohol addiction, the role of CRF-BP has been less studied.

CRF-BP is a 37 kDa-secreted glycoprotein that binds CRF and the CRF-like ligand urocortin 1 with an equal or greater affinity than CRF receptors. It is estimated that 40 to 60% of CRF in the human brain is bound by CRF-BP (Behan et al., 1997), and CRF-BP colocalizes with CRF or CRF receptors at numerous sites (i.e., amygdala and BNST), suggesting potential sites of interaction in stress and reward pathways (Potter et al., 1992). Multiple roles for CRF-BP have been proposed. In cultured pituitary cells, CRF-BP attenuates CRF-R1 activity (Cortright et al., 1995; Potter et al., 1991; Sutton et al., 1995), suggesting an inhibitory role for CRF-BP. In support of this, CRF-BP deficient mice display increased anxiety (Karolyi et al., 1999), consistent with increased free levels of CRF. However, *in vivo* and slice studies have revealed a potential facilitatory role for CRF-BP, particularly in the VTA, with administration of the CRF-BP ligand inhibitor, CRF<sub>6-33</sub>, decreasing CRF-mediated potentiation of NMDA excitatory postsynaptic currents on VTA dopamine neurons (Ungless et al., 2003). Similarly, intra-VTA administration of CRF<sub>6-33</sub> decreased binge drinking (Albrechet-Souza et al., 2015) and CRF-induced relapse to cocaine seeking (Wang et al., 2007).

Thus, while a role for CRF receptors in binge drinking has been established, the role for CRF-BP, a key regulator of CRF receptor activity, has not been well characterized. Therefore, in this study, we sought to determine the role and regulation of CRF-BP in the DID mouse model of binge drinking. We examined the regulation of the CRF system, including CRF-BP, CRF-R1, and CRF mRNA expression, after repeated cycles of DID in brain regions of the stress and reward systems. Additionally, CRF-BP KO mice were utilized to determine the functional role of CRF-BP in modulating EtOH consumption in the DID paradigm.

## MATERIALS AND METHODS

### *Animals—3-Cycle DID Experiment*

Six- to 8-week-old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used for a 3-cycle DID experiment. Mice were maintained on a 14/10 light/dark cycle and had access to food and water *ad libitum*, except when noted. Mice were acclimated to single housing for >2 weeks prior to the start of DID. All mouse experiments were conducted according to National Institutes of Health guidelines for animal care and were approved by the University of Michigan Committee on Use and Care of Animals.

### *Animals—6-Cycle DID Experiment*

CRF-BP knockout mice (CRF-BP KO; Karolyi et al., 1999) were bred in our facility and have been backcrossed onto a C57BL/6J background for >17 generations. CRF-BP heterozygotes (Het) were crossed to generate wild-type, Het, and CRF-BP KO mice. To generate sufficient numbers of mice of similar age for the 6-cycle DID study, these wild-type progeny were crossed to generate wild-type mice, and Het × KO and KO × KO crosses were used to generate CRF-BP KO mice. Mice were 10 to 14 weeks old at the start of the experiment. These mice were switched on a 12/12 light/dark cycle to be more consistent with other DID studies in the literature. Mice had access to food and water *ad libitum*, except when noted, and were acclimated to single housing for >2 weeks prior to the start of DID. At the end of the 6-cycle DID experiment, the brains of the wild-type mice were used for *in situ* hybridization analyses.

### *Drinking in the Dark*

Male C57BL/6J mice (3-cycle DID;  $n = 12$  [6/group]) and male CRF-BP KO and wild-type controls (6-cycle DID; KO [EtOH:  $n = 17$ , H<sub>2</sub>O:  $n = 10$ ]; wild-type [EtOH:  $n = 13$ , H<sub>2</sub>O:  $n = 13$ ]) were tested in a DID protocol (Rhodes et al., 2005). On days 1 to 3, mice were given access to a single 50-ml centrifuge tube of 20% EtOH (v/v) for 2 hours, starting 3 hours into the dark cycle. On day 4, mice were given access to 20% EtOH for 4 hours. Control mice received a single 50-ml centrifuge tube of water instead of 20% EtOH. Mice received only water for the last 3 days (days 5 to 7) of each cycle. Repeated DID consisted of 3 or 6 cycles in total. Centrifuge bottles were fitted with a rubber stopper that contained a sipper tube with 2 ball bearings (Ancare Corp., Bellmore, NY). Two empty cages were placed in the experiment room, and each received a bottle of 20% EtOH during DID to control for spillage from the sipper tubes. These control volumes were averaged and subtracted from the experimental volumes before converting to g/kg. All bottles were weighed and recorded immediately before and after each drinking session.

### *Blood Ethanol Concentration*

For the 3-cycle DID experiment, 40  $\mu$ l of blood was collected via tail snip immediately after the drinking session on day 4 of cycle 3. For the 6-cycle DID experiment, 40  $\mu$ l of blood was collected on day 4 of cycle 5 to eliminate any potential effects of blood collection on gene expression 24 hours later. Blood samples were placed into a tube that contained 1.5  $\mu$ l 0.5M EDTA and centrifuged, and then, plasma was removed and stored at  $-20^{\circ}\text{C}$  until use. BECs were determined by an Analox alcohol analyzer (Analox Instruments, Atlanta, GA) for the 3-cycle DID experiment and alcohol dehydrogenase assay (Pointe Scientific Inc., Canton, MI; Cat. No.: A7504-39) for the 6-cycle DID experiment.

### Tissue Processing and In Situ Hybridization

Mice were euthanized 24 hours after 3 or 6 cycles of DID to assess neuroadaptive changes that occur after binge drinking cycles (including consumption and withdrawal) rather than the direct effects of EtOH consumption. Brains were removed, frozen in 2-methylbutane, and stored at  $-80^{\circ}\text{C}$  until use. Brains were sectioned via cryostat at  $14\ \mu\text{m}$  and collected in series of 6 slides (4 sections/slide). Every sixth slide was stained with cresyl violet to determine anatomical location and orientation. For each brain region of interest, adjacent slides were analyzed for CRF, CRF-R1, and CRF-BP mRNA expression using in situ hybridization, similar to what has been described previously (Herman et al., 1990; Seasholtz et al., 1991). Brain sections were postfixed in 4% paraformaldehyde for 1 hour and washed 3 times in  $2\times$  saline sodium citrate (SSC) buffer. Sections were then incubated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 minutes, washed 3 times in  $2\times$  SSC, dehydrated in EtOH, and air-dried. CRF, CRF-R1, and CRF-BP antisense cRNA riboprobes were generated with  $^{35}\text{S}$ -UTP and  $^{35}\text{S}$ -CTP (1,250 Ci/mmol; PerkinElmer Inc., Waltham, MA) from plasmids as described previously (pGem4ZPst578, Seasholtz et al., 1991; pTOPO CRH-R1, Westphal et al., 2009; mCRHBP666, Burrows et al., 1998). Sections were hybridized with the  $^{35}\text{S}$ -labeled riboprobes ( $2 \times 10^6$  cpm/slide) in 50% formamide hybridization buffer (Amersco, Framingham, MA) with 20 mM DTT overnight at  $55^{\circ}\text{C}$ . After hybridization, sections were washed 3 times with  $2\times$  SSC and treated with RNase A (200  $\mu\text{g}/\text{ml}$ ) for 1 hour at  $37^{\circ}\text{C}$ . Slides were then washed in decreasing salt solutions ( $2\times$ ,  $1\times$ , and  $0.5\times$  SSC), and a high-stringency wash was performed in  $0.1\times$  SSC at  $65^{\circ}\text{C}$  for 1 hour. Slides were then dehydrated in EtOH, air-dried, and exposed to BioMax MR autoradiography film (Carestream Health Inc., Rochester, NY) for 3 to 14 days depending on riboprobe and brain region.

### In Situ Hybridization Analyses

Autoradiography films were scanned and analyzed using densitometry in ImageJ (NIH, Bethesda, MD). A set of macros were utilized that enabled background to be selected and a mask created so that only signal  $>3.5$  SDs above background is measured. Mean optical density (mean OD), area, and integrated optical density (IOD; mean OD  $\times$  area of signal) were calculated for each brain region (left and right hemispheres). Brain regions of interest for in situ hybridization analyses were selected based on anatomical landmarks (Paxinos and Franklin, 2001) from adjacent cresyl violet-stained sections. Spatial expression profiles were generated for each probe and brain region of interest to map IOD signal spanning the rostral to caudal extent of each brain region. These expression profiles were used to determine Bregma coordinates (Paxinos and Franklin, 2001) for analysis of in situ hybridization experiments. If the IOD signal for a particular riboprobe varied significantly rostral to caudal, then the area where the signal was highest was also used for analysis.

### Statistical Analyses

Given that the in situ hybridization experiments were performed independently for each riboprobe, brain region, and cycle number (3 or 6 DID cycles), these data were analyzed via independent Student's *t*-tests. A 2-way repeated-measures analysis of variance (ANOVA) was used to analyze the DID data. When significant main effects were observed, Tukey HSD post hoc analyses were performed for multiple comparisons. All data are reported as means  $\pm$  SEM, and significant values were accepted at  $p < 0.05$  for all statistical tests.

## RESULTS

### Regulation of CRF, CRF-R1, and CRF-BP mRNA Expression After 3 Cycles of DID

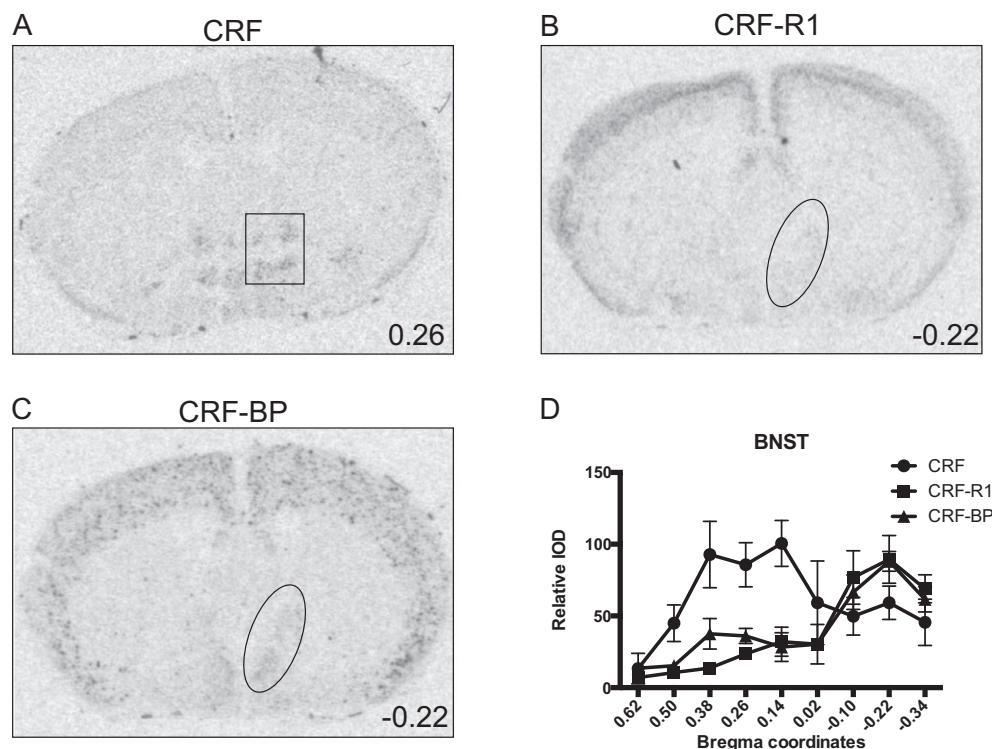
To determine how CRF-BP, CRF-R1, and CRF are regulated within the stress and reward systems after binge drinking, male C57BL/6J mice underwent a 3-cycle DID paradigm. On the fourth day of cycle 3, mice consumed an average  $5.45 \pm 0.24$  g/kg of EtOH and exhibited an average BEC of  $84.1 \pm 11.7$  mg/dl. Mice were sacrificed 24 hours later, and in situ hybridization experiments were performed to determine CRF, CRF-R1, and CRF-BP mRNA expression in the BNST, VTA, mPFC, and amygdala. Spatial expression profiles were generated for each riboprobe and brain region of interest to map IODs spanning rostral to caudal for each brain region. These expression profiles were used to determine Bregma coordinates for further analysis of the in situ hybridization data.

In the BNST, CRF, CRF-R1, and CRF-BP mRNA signal varied rostral to caudal (Fig. 1). Interestingly, CRF mRNA expression (IOD) was highest between Bregma coordinates 0.38 and 0.14 mm in anterior nuclei, whereas the expression of CRF-R1 and CRF-BP mRNA was highest more caudally in posterior nuclei between Bregma coordinates  $-0.10$  and  $-0.34$  mm. IOD signal from sections within these designated Bregma coordinates was averaged to generate one value per mouse for each riboprobe. Independent Student's *t*-tests revealed that there were no differences in CRF, CRF-R1, and CRF-BP expression between EtOH and control mice after 3 cycles of DID (Table 1; representative in situ autoradiograms in Fig. 1). Additionally, further analyses revealed that CRF expression was unaltered in the dorsal and ventral BNST nuclei of EtOH mice compared to controls (data not shown).

Expression profiles of CRF and CRF-R1 in the VTA did not show significant variation in expression, so signal was averaged across the rostral to caudal extent of the VTA. CRF and CRF-R1 expression in the VTA did not differ between EtOH and control mice (Table 1; representative in situ autoradiograms in Fig. 2). CRF-BP IOD was highest from Bregma coordinates  $-3.28$  to  $-3.52$  mm in the mid-to-posterior VTA, where IOD signal was averaged for analysis (Fig. 2). There was a trend for a decrease in CRF-BP IOD in EtOH mice compared to control mice, but this did not reach statistical significance (Table 1;  $t(8) = 2.25$ ,  $p = 0.055$ ). However, CRF-BP mean OD was significantly decreased in the VTA of EtOH mice compared to control mice ( $p < 0.05$ ; data not shown).

CRF, CRF-R1, and CRF-BP mRNA expression patterns in the prelimbic (PL) and infralimbic (IL) mPFC did not vary rostral to caudal, so signal was averaged from multiple sections to generate one value (representative autoradiograms in Fig. S1). Expression in the PL and IL mPFC was analyzed from coordinates 2.34 to 1.54 mm and 1.98 to 1.54 mm, respectively. Independent Student's *t*-tests revealed that CRF-BP IOD in the PL and IL mPFC was significantly decreased in EtOH mice compared to control mice (Table 1





**Fig. 1.** Representative in situ hybridization autoradiogram images for corticotropin-releasing factor (CRF) (A), CRF receptor 1 (CRF-R1) (B), and CRF-BP (CRF binding protein) (C) in the bed nucleus of the stria terminalis (BNST) of ethanol (EtOH)-treated mice 24 hours after 3 cycles of drinking in the dark (DID). Brain regions of interest are outlined in black. The coordinates for the autoradiograms are 0.26 mm for CRF and  $-0.22$  mm for CRF-R1 and CRF-BP, relative to Bregma. Spatial expression profiles were generated (D) to compare rostral to caudal patterns of CRF, CRF-R1, and CRF-BP mRNA expression in the BNST of EtOH-treated mice after 3 cycles of DID ( $n = 4$  to 5/probe; data represent the mean  $\pm$  SEM). Integrated optical density (IOD) values in panel D are plotted relative to the lowest value for each riboprobe. Intensities in panels A to D cannot be directly compared, as riboprobes were not equal in specific activity and exposure times were varied to yield optimal quantitative results.

**Table 1.** Summary of in situ hybridization data (represented as integrated optical density) for CRF, CRF-R1, and CRF-BP 24 hours after 3 cycles of Drinking in the Dark.

	CRF		CRF-R1		CRF-BP	
	H <sub>2</sub> O	EtOH	H <sub>2</sub> O	EtOH	H <sub>2</sub> O	EtOH
BNST	168.7 $\pm$ 11.4	158.0 $\pm$ 12.2	43.5 $\pm$ 4.8	39.9 $\pm$ 6.2	67.2 $\pm$ 3.0	80.8 $\pm$ 7.3
VTA	22.9 $\pm$ 3.9	26.0 $\pm$ 2.1	45.2 $\pm$ 2.2	50.1 $\pm$ 4.7	85.1 $\pm$ 9.2	61.1 $\pm$ 5.4 <sup>#</sup>
PL mPFC	4.4 $\pm$ 0.5	5.5 $\pm$ 0.9	27.9 $\pm$ 1.1	24.6 $\pm$ 1.3	45.9 $\pm$ 1.2	30.2 $\pm$ 3.2 <sup>#</sup>
IL mPFC	3.7 $\pm$ 0.5	4.4 $\pm$ 0.4	4.4 $\pm$ 0.8	3.1 $\pm$ 1.0	70.5 $\pm$ 5.4	50.1 $\pm$ 6.0 <sup>#</sup>
BLA/LA	ND	ND	83.3 $\pm$ 11.6	86.4 $\pm$ 9.8	49.1 $\pm$ 8.4	60.6 $\pm$ 10.7
CeA	101.1 $\pm$ 7.1	81.6 $\pm$ 7.2	36.0 $\pm$ 4.9	33.4 $\pm$ 5.1	4.8 $\pm$ 1.5	6.8 $\pm$ 2.1
BMA	ND	ND	108.0 $\pm$ 9.7	105.2 $\pm$ 9.4	10.6 $\pm$ 2.5	15.1 $\pm$ 4.0

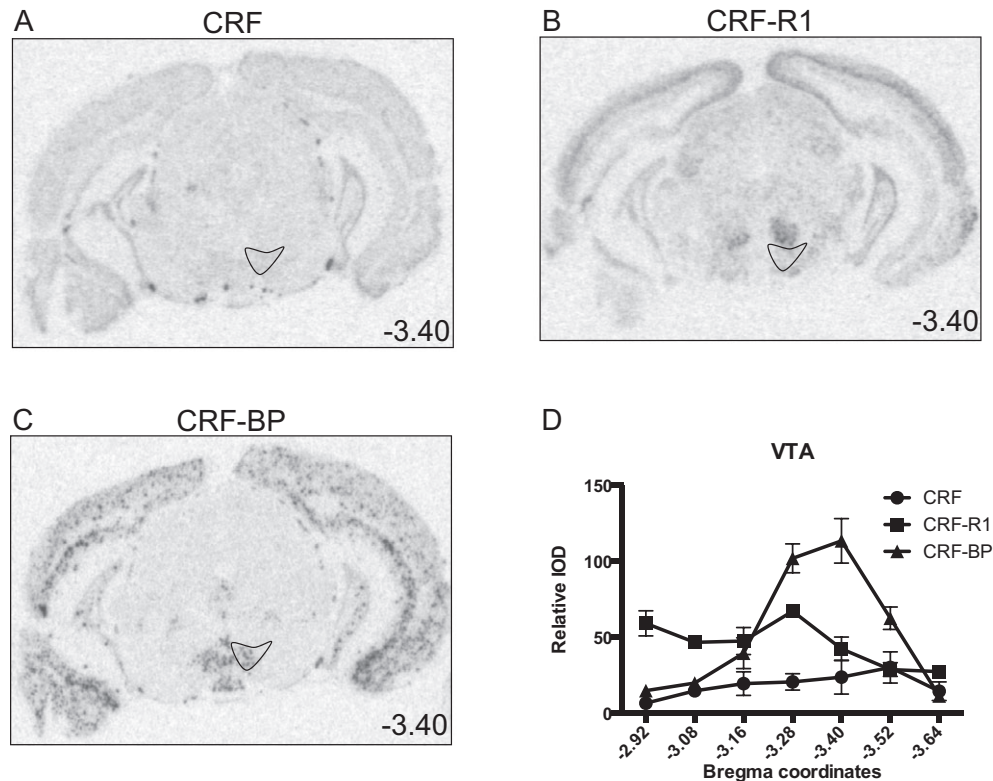
Data represent the mean  $\pm$  SEM ( $n = 4$ –5 per group). \* $p < 0.05$  and <sup>#</sup> $p = 0.055$  compared to respective water controls using independent Student's *t*-tests. Values within bolded lines represent independent experiments. Integrated optical density values should not be directly compared across independent experiments as riboprobe specific activity and exposure times are not equal. CRF, corticotropin-releasing factor; CRF-R1, CRF receptor 1; CRF-BP, CRF binding protein;

BNST, bed nucleus of the stria terminalis; VTA, ventral tegmental area; PL mPFC, prelimbic medial prefrontal cortex; IL mPFC, infralimbic medial prefrontal cortex; BLA, basolateral amygdala; LA, lateral amygdala; CeA, central nucleus of the amygdala; BMA, basomedial amygdala; ND, not detected.

and Fig. 3; PL,  $t(8) = 4.64$ ,  $p < 0.01$  and IL,  $t(8) = 2.51$ ,  $p < 0.05$ ). There were no significant differences in CRF and CRF-R1 mRNA expression levels in the mPFC between EtOH and control mice.

In the BLA/lateral amygdala (LA), CeA, and basomedial amygdala (BMA), CRF-R1 and CRF-BP expression were

analyzed from Bregma coordinates  $-0.94$  to  $-1.82$  mm, and in the CeA, CRF expression was analyzed from coordinates  $-0.82$  to  $-1.82$  mm (representative autoradiograms in Fig. S2). CRF mRNA was not detectable in the BLA/LA and BMA and therefore was not included in the analysis. There were some rostral to caudal variations in CRF,



**Fig. 2.** Representative in situ hybridization autoradiogram images for corticotropin-releasing factor (CRF) (A), CRF receptor 1 (CRF-R1) (B), and CRF binding protein (CRF-BP) (C) in the ventral tegmental area (VTA) of water-treated mice. Brain regions of interest are outlined in black. The coordinates for each autoradiogram are  $-3.40$  mm relative to Bregma. Expression profiles were generated (D) to compare rostral to caudal patterns of CRF, CRF-R1, and CRF-BP expression in the VTA of control mice after 3 cycles of drinking in the dark ( $n = 4$  to 5/probe; data represent the mean  $\pm$  SEM). Integrated optical density (IOD) values in panel D are plotted relative to the lowest value for each riboprobe.

CRF-R1, and CRF-BP signal in the amygdala; however, no significant changes in expression were observed in any of the amygdala nuclei after 3 cycles of DID (Table 1).

#### Regulation of CRF-BP mRNA Expression After 6 Cycles of DID

To test whether altered CRF-BP expression in the mPFC persists beyond 3 cycles of DID, male mice underwent a 6-cycle DID paradigm and in situ hybridization was performed on brains collected 24 hours after the last exposure to alcohol to determine changes in CRF-BP mRNA expression in the BNST, VTA, mPFC, and amygdala. Similar to 3 cycles of DID, there was a significant decrease in CRF-BP in the PL mPFC of EtOH-treated mice compared to controls (Table 2 and Fig. 4;  $t(10) = 2.6$ ,  $p < 0.05$ ). However, CRF-BP mRNA expression was unchanged in the IL mPFC of EtOH-treated mice after 6 cycles of DID. CRF-BP was not significantly altered in the BNST, VTA, and amygdala of EtOH-treated mice compared to controls (Table 2), similar to what was observed after 3 cycles of DID.

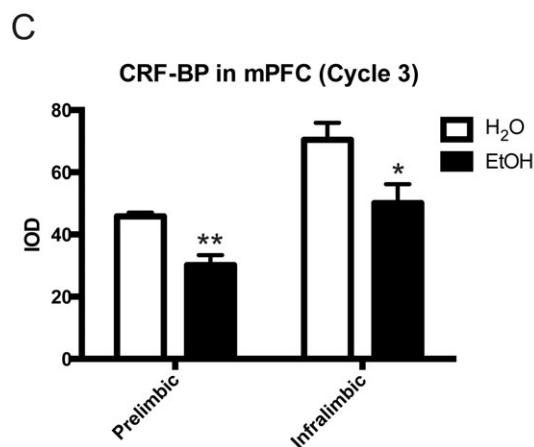
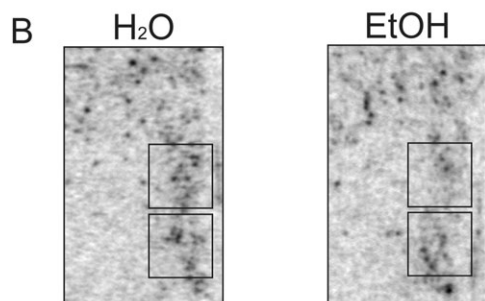
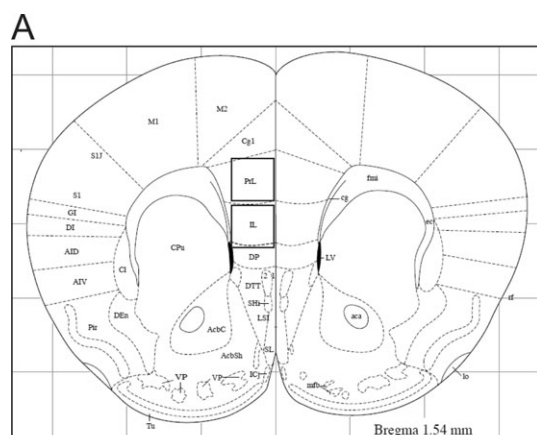
#### Functional Role of CRF-BP in DID Using CRF-BP KO Mice

To test the functional role of CRF-BP in binge drinking, CRF-BP KO mice (Karolyi et al., 1999) and wild-type mice

underwent a 6-cycle DID paradigm. Overall, alcohol consumption did not significantly differ between CRF-BP KO and wild-type mice across 6 cycles of DID, as revealed by a lack of a main effect of genotype in a 2-way repeated-measures ANOVA (Fig. 5; data shown for day 4 of each cycle). However, there was a significant main effect of cycle number,  $F(5, 140) = 6.97$ ,  $p < 0.0001$ , and a significant interaction effect,  $F(5, 140) = 2.46$ ,  $p < 0.05$ . Post hoc analyses revealed that wild-type mice drank significantly more EtOH on day 4 of cycle 5 compared to cycle 2 ( $p < 0.001$ ) and CRF-BP KO mice drank more EtOH on cycles 4, 5, and 6 compared to cycle 1 (cycles 4 and 5,  $p < 0.05$ ; cycle 6,  $p < 0.0001$ ), and cycle 6 compared to cycle 2 ( $p < 0.01$ ). CRF-BP KO and wild-type mice did not display significant differences in alcohol consumption at any of the 6 DID cycles. Additionally, there were no differences in average BEC between wild-type ( $78.5 \pm 11.7$  mg/dl) and CRF-BP KO mice ( $72.4 \pm 8.5$  mg/dl). These results suggest that the total absence of CRF-BP does not significantly alter binge drinking in the DID paradigm.

## DISCUSSION

In this study, we examined the regulation of CRF-BP, CRF-R1, and CRF mRNA expression in brain regions of the stress and reward systems after repeated cycles of binge



**Fig. 3.** Decreased CRF binding protein (CRF-BP) expression in the medial prefrontal cortex (mPFC) after 3 cycles of drinking in the dark (DID). Coronal section from the Paxinos and Franklin (2001) mouse brain atlas (A) at Bregma coordinate 1.54 mm and corresponding representative in situ hybridization autoradiogram images (B) comparing CRF-BP expression in ethanol (EtOH)-treated mice to water controls at 24 hours after 3 cycles of DID. CRF-BP integrated optical density (IOD) was significantly decreased in the prelimbic (PL) and infralimbic (IL) mPFC of EtOH-treated mice compared to water controls (C). The boxed areas for quantification of PL and IL mPFC were determined using the characteristics of cells in layer 2 of the cortex from adjacent cresyl violet-stained sections (Van De Werd et al., 2010). Data represent the mean  $\pm$  SEM. \* $p < 0.05$  and \*\* $p < 0.01$  compared to respective water controls using independent Student's *t*-tests.

drinking. While there were no detectable changes in CRF or CRF-R1 mRNA levels in amygdala, VTA, BNST, or mPFC after 3 cycles of DID, we provide the first evidence that repeated cycles of binge drinking alter CRF-BP mRNA expression in the mPFC. CRF-BP mRNA expression was significantly decreased in the PL and IL mPFC after 3 cycles

**Table 2.** Summary of in situ hybridization data (represented as integrated optical density) for CRF-BP after 6 cycles of DID.

	CRF-BP	
	H <sub>2</sub> O	EtOH
BNST	100.4 $\pm$ 9.0	90.1 $\pm$ 12.4
VTA	57.4 $\pm$ 3.2	52.9 $\pm$ 3.1
PL mPFC	63.3 $\pm$ 4.6	50.6 $\pm$ 1.8*
IL mPFC	99.2 $\pm$ 6.7	88.7 $\pm$ 3.4
BLA/LA	139.5 $\pm$ 18.3	177.5 $\pm$ 29.9
CeA	11.1 $\pm$ 0.9	13.3 $\pm$ 2.5
BMA	29.6 $\pm$ 4.3	37.7 $\pm$ 7.3

Data represent the mean  $\pm$  SEM ( $n = 4-6$  per group). \* $p < 0.05$  compared to respective water control using an independent Student's *t*-test. Values within bolded lines represent independent experiments. Integrated optical density values should not be directly compared across independent experiments as riboprobe specific activity and exposure times are not equal. CRF-BP, CRF binding protein;

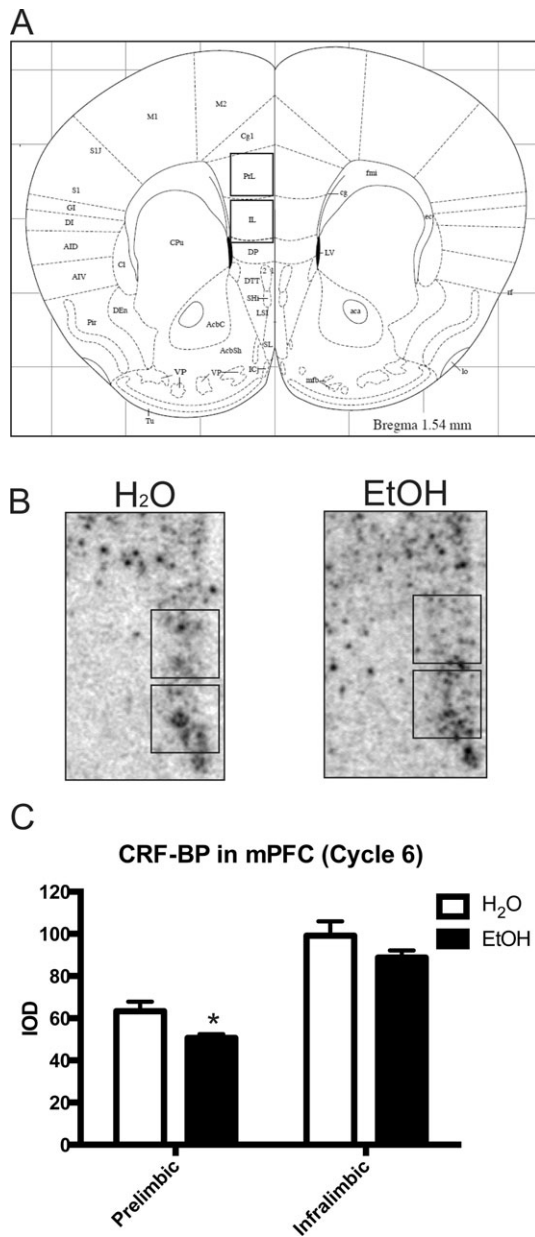
BNST, bed nucleus of the stria terminalis; VTA, ventral tegmental area; PL mPFC, prelimbic medial prefrontal cortex; IL mPFC, infralimbic medial prefrontal cortex; BLA, basolateral amygdala; LA, lateral amygdala; CeA, central nucleus of the amygdala; BMA, basomedial amygdala.

of DID and in the PL mPFC after 6 cycles of DID. Decreased CRF-BP may allow for increased CRF signaling at CRF-R1 in this region, well known for its roles in executive function, impulse control, and stress response regulation. We also examined binge drinking in CRF-BP deficient mice and observed no detectable differences from wild-type mice across 6 DID cycles.

The CRF system is widely expressed throughout stress and reward pathways (Chan et al., 2000; Van Pett et al., 2000). In the present study, we characterized CRF, CRF-R1, and CRF-BP mRNA expression in serial sections throughout the mPFC, BNST, VTA, and amygdala, key brain regions that have been implicated in excessive alcohol consumption (George et al., 2012; Lowery-Gionta et al., 2012; Silberman et al., 2013; Sparta et al., 2013). In the BNST, the expression of CRF-R1 and CRF-BP mRNA is highest in the caudal aspects of the BNST in posterior nuclei, whereas CRF mRNA expression is highest at more rostral coordinates in anterior nuclei. In the amygdala, CRF-BP mRNA is detected in CeA, BLA/LA, and BMA, major sites of CRF (CeA) and CRF-R1 (BLA/LA, CeA, and BMA) mRNA expression (Chan et al., 2000; Van Pett et al., 2000). In the mPFC, we found that CRF-BP mRNA is expressed at high levels in the PL and IL mPFC. CRF-R1 is highly expressed in the PL mPFC, but expressed at lower levels in the IL mPFC, and CRF expression is low throughout the mPFC. These expression profiles reveal sites of coexpression of CRF-BP with CRF or CRF-R1, predicting sites for interactions and modulation of CRF-mediated activities.

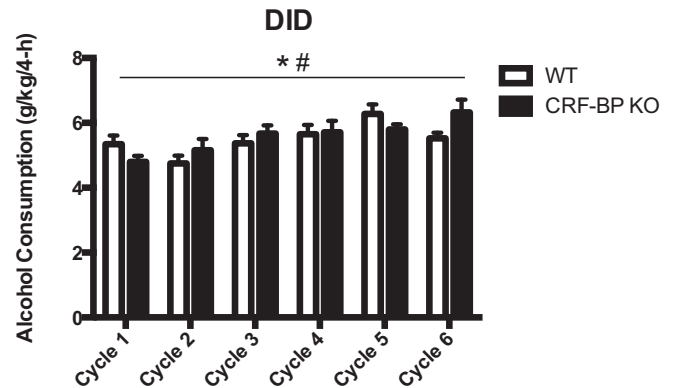
In the VTA, CRF-BP expression is highly expressed in the mid-to-posterior VTA, consistent with previous studies in rat (Wang and Morales, 2008). We detected CRF-R1 mRNA in a similar region within the mouse VTA (Fig. 2), whereas CRF mRNA is detected only at low levels throughout the





**Fig. 4.** Decreased CRF binding protein (CRF-BP) expression in the medial prefrontal cortex (mPFC) after 6 cycles of drinking in the dark (DID). Coronal section from the Paxinos and Franklin (2001) mouse brain atlas (A) at Bregma coordinate 1.54 mm and corresponding representative in situ hybridization autoradiogram images (B) comparing CRF-BP expression in ethanol (EtOH)-treated mice to water controls at 24 hours after 6 cycles of DID. CRF-BP integrated optical density (IOD) was significantly decreased in the prelimbic (PL) mPFC of EtOH-treated mice compared to water controls (C). The boxed areas for quantification of PL and infralimbic mPFC were determined using the characteristics of cells in layer 2 of the cortex from adjacent cresyl violet-stained sections (Van De Werd et al., 2010). Data represent the mean  $\pm$  SEM. \* $p < 0.05$  compared to respective water control using an independent Student's *t*-test.

VTA. While CRF mRNA expression is low in cell bodies of the VTA (Fig. 2; George et al., 2012), studies in rat have shown immunoreactive CRF peptide in axons and axon terminals that make contact with dopaminergic and non-dopaminergic neurons in the VTA, suggesting CRF release in this region (Tagliaferro and Morales, 2008). Additionally,



**Fig. 5.** Comparison of ethanol consumption between CRF binding protein knock out (CRF-BP KO) and wild-type (WT) mice after 6 cycles of drinking in the dark (DID). A 2-way repeated-measures ANOVA did not reveal a significant genotype effect; however, there was a significant main effect of cycle number and interaction. Data represent the mean  $\pm$  SEM on day 4 of each cycle (CRF-BP KO,  $n = 17$ ; WT,  $n = 13$ ). \* $p < 0.0001$  main effect of cycle number; # $p < 0.05$  interaction effect.

CRF-BP and CRF-R1 mRNA are expressed in dopaminergic neurons of the VTA (Refojo et al., 2011; Wang and Morales, 2008). Together, these results suggest that CRF may regulate the activity of dopamine neurons in the VTA via interactions with CRF-BP and CRF receptors. Future studies in our laboratory will utilize dual in situ hybridization techniques to characterize the coexpression of CRF-BP with CRF, CRF-R1, and other neurotransmitters/neuropeptides at the cellular level in the VTA, BNST, amygdala, and mPFC to provide further insight into the functional role of CRF-BP at each site in stress and reward pathways.

Dysregulation of the CRF system in the VTA has been previously linked to binge drinking. Sparta and colleagues (2013) observed increased CRF-R1 activity in the VTA after DID, as determined by increased CRF-R1-mediated potentiation of NMDA currents by CRF. Moreover, injection of a CRF-R1 antagonist into VTA reduced binge drinking during DID (Sparta et al., 2013). In the current study, we observed a trend ( $p = 0.055$ ) for a decrease in CRF-BP mRNA levels in the VTA after 3 cycles of DID. A decrease in CRF-BP expression in the VTA could lead to increased free CRF available for CRF-R1 activation, which may contribute to excessive alcohol consumption during DID. However, other studies suggest a facilitatory role for CRF-BP in the VTA, particularly via CRF-R2. For example, in vitro slice studies have shown that both CRF and CRF-BP are required for CRF-R2-mediated potentiation of NMDA currents in dopamine neurons of the VTA, an effect that occurred through the protein kinase C signaling pathway (Ungless et al., 2003). Similarly, pharmacological inhibition of CRF-BP via CRF<sub>6-33</sub> in the VTA reduced binge drinking (Albrechet-Souza et al., 2015) and CRF-induced relapse to cocaine seeking (Wang et al., 2007), possibly via an interaction with CRF-R2. CRF-R2 mRNA is detected in VTA by quantitative reverse transcription polymerase chain reaction (Korotkova et al., 2006; Ungless et al., 2003), but is not detected with

in situ hybridization under basal conditions (Van Pett et al., 2000; Stinnett G, unpublished data). Together, these data suggest that the effects of CRF-BP on CRF receptor signaling may depend upon the cellular context, with CRF receptor subtype and signaling pathway as possible determinants. Additional studies will be required to determine the interactions between CRF, CRF-BP, and CRF receptors in the VTA and their regulation by binge drinking.

Strikingly, the largest change in CRF-BP expression in binge drinking occurred in the mPFC, with a significant decrease in CRF-BP mRNA levels while CRF-R1 and CRF mRNA levels remained unchanged. The mPFC is responsible for executive function and regulation of emotion and behavior, and impairment of this region has been linked to excessive alcohol consumption (George et al., 2012; Goldstein and Volkow, 2011). The mPFC is interconnected with numerous brain regions of the stress and reward systems, including the amygdala, BNST, and VTA, and therefore may represent a potential site where the stress system can influence maladaptive behaviors such as excessive alcohol intake. In support of this, dysregulation of the CRF system in the mPFC has been linked to excessive alcohol consumption. Rats genetically selected to prefer alcohol displayed lower concentrations of CRF in the mPFC compared to nonpreferring rats, suggesting that CRF levels in this region may contribute to alcohol preference (Ehlers et al., 1992). In a separate study, George and colleagues (2012) found that abstinence from intermittent access to EtOH in rats recruited both CRF and GABA neurons in the mPFC and resulted in a disconnection between the mPFC and CeA. In humans, a variant in the CRF-R1 gene, *CRHR1*, was linked to increased right ventrolateral PFC activity, lower negative emotionality, and decreased binge drinking and alcohol-related problems (Glaser et al., 2014). The present study extends our current knowledge on the role of the CRF system in the mPFC in excessive alcohol consumption, revealing that binge drinking regulates CRF-BP mRNA expression in this region.

The mPFC is also sensitive to stress, playing a key role in the limbic forebrain circuit that regulates stress systems including the hypothalamic–pituitary–adrenal axis (HPA). Activation of the PL mPFC has been shown to dampen the HPA axis (Jones et al., 2011), whereas lesions of the PL mPFC enhance activation of the HPA axis (Radley et al., 2006). Jaferi and Bhatnagar (2007) determined that CRF receptors in the mPFC contribute to the regulation of the HPA axis, as administration of a nonselective CRF receptor antagonist decreased HPA activity after acute and chronic restraint stress. Acute and chronic administration of alcohol alters HPA axis activity, resulting in altered plasma corticosterone levels (Ellis, 1966; Richardson et al., 2008; Rivier, 1993). As stress, CRF, and glucocorticoids have been shown to regulate CRF-BP expression (reviewed in Westphal and Seasholtz, 2006), changes in CRF and/or corticosterone levels after binge drinking may contribute to altered CRF-BP mRNA expression in the mPFC. The observed decrease in CRF-BP mRNA expression could

lead to increased free CRF available to bind to and activate CRF-R1 receptors in the mPFC, which may contribute to excessive binge-like EtOH consumption. In support of this hypothesis, a recent study has shown that administration of a CRF-R1 antagonist into the mPFC attenuates the early life stress-induced increase in alcohol self-administration in an operant binge drinking paradigm (Gondré-Lewis et al., 2016).

It should be emphasized that the studies shown here evaluate CRF, CRF-R1, and CRF-BP mRNA levels. Changes in mRNA are not always revealed as changes in protein levels with a similar temporal pattern. It should also be noted that our expression studies examined mRNA changes at 24 hours after the last binge alcohol exposure to assess neuroadaptive changes rather than the acute effects of EtOH. This could account for differences between our data and other studies detecting changes in CRF mRNA or immunoreactivity at 0 to 2 hours after alcohol exposure (Funk et al., 2006; Lack et al., 2005; Lowery-Gionta et al., 2012). Finally, it should be noted that CRF peptide levels in axon terminals of projection neurons are not assessed by our cellular mRNA measures. This may be particularly important when considering CRF levels in areas enriched with CRF terminals, such as VTA, CeA, and BNST (Beckerman et al., 2013; Tagliaferro and Morales, 2008).

We found no difference in alcohol consumption between male CRF-BP KO mice and wild-type mice after repeated cycles of DID, indicating that the total absence of CRF-BP does not alter binge drinking in this paradigm. In contrast to these results, Albrechet-Souza and colleagues (2015) found that administration of the CRF-BP ligand inhibitor, CRF<sub>6-33</sub>, into the VTA, but not the CeA, decreased alcohol consumption in a DID paradigm, suggesting that CRF-BP may facilitate binge-like EtOH consumption. One significant difference between these studies is the method by which CRF-BP is inhibited. In the study by Albrechet-Souza and colleagues (2015), CRF-BP is site-specifically inhibited in the VTA or CeA using CRF<sub>6-33</sub>. In the present study a constitutive CRF-BP KO mouse model was utilized, resulting in global alterations in CRF signaling that could mask the influence of one particular brain region on alcohol drinking behavior. Additionally, the CRF-BP KO mice are deficient in CRF-BP throughout development; therefore, compensatory changes in CRF signaling could be occurring.

Overall, the current results expand our knowledge on the role of the CRF system in alcohol binge drinking. We discovered an enduring decrease in CRF-BP mRNA expression in the mPFC after both 3 and 6 DID cycles, reflecting a dysregulation of the CRF system that could contribute to escalated EtOH intake. We also demonstrated that CRF-BP KO mice do not display altered binge drinking across 6 cycles of DID. Future studies will utilize viral and genetic approaches to conditionally and site-specifically knockdown and/or overexpress CRF-BP to further elucidate its role in binge drinking and alcohol dependence.



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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## REFERENCES

- Albrechet-Souza L, Hwa LS, Han X, Zhang EY, Debold JF, Miczek KA (2015) Corticotropin releasing factor binding protein and CRF2 receptors in the ventral tegmental area: modulation of ethanol binge drinking in C57BL/6J mice. *Alcohol Clin Exp Res* 39:1609–1618.
- Beckerman MA, Van Kempen TA, Justice NJ, Milner TA, Glass MJ (2013) Corticotropin-releasing factor in the mouse central nucleus of the amygdala: ultrastructural distribution in NMDA-NR1 receptor subunit expressing neurons as well as projection neurons to the bed nucleus of the stria terminalis. *Exp Neurol* 239:120–132.
- Behan DP, Khongsaly O, Owens MJ, Chung HD, Nemeroff CB, De Souza EB (1997) Corticotropin-releasing factor (CRF), CRF-binding protein (CRF-BP), and CRF/CRF-BP complex in Alzheimer's disease and control postmortem human brain. *J Neurochem* 68:2053–2060.
- Blomeyer D, Treutlein J, Esser G, Schmidt MH, Schumann G, Laucht M (2008) Interaction between CRHR1 gene and stressful life events predicts adolescent heavy alcohol use. *Biol Psychiatry* 63:146–151.
- Burrows HL, Nakajima M, Lesh JS, Goossens KA, Samuelson LC, Inui A, Camper SA, Seasholtz AF (1998) Excess corticotropin releasing hormone-binding protein in the hypothalamic-pituitary-adrenal axis in transgenic mice. *J Clin Invest* 101:1439–1447.
- Chan RK, Vale WW, Sawchenko PE (2000) Paradoxical activational effects of a corticotropin-releasing factor-binding protein “ligand inhibitor” in rat brain. *Neuroscience* 101:115–129.
- Cortright DN, Nicoletti A, Seasholtz AF (1995) Molecular and biochemical characterization of the mouse brain corticotropin-releasing hormone-binding protein. *Mol Cell Endocrinol* 111:147–157.
- Ehlers CL, Chaplin RI, Wall TL, Lumeng L, Li TK, Owens MJ, Nemeroff CB (1992) Corticotropin releasing factor (CRF): studies in alcohol preferring and non-preferring rats. *Psychopharmacology* 106:359–364.
- Ellis FW (1966) Effect of ethanol on plasma corticosterone levels. *J Pharmacol Exp Ther* 153:121–127.
- Enoch MA, Shen PH, Ducci F, Yuan Q, Liu J, White KV, Albaugh B, Hodgkinson CA, Goldman D (2008) Common genetic origins for EEG, alcoholism and anxiety: the role of CRH-BP. *PLoS One* 3:e3620.
- Funk CK, Koob GF (2007) A CRF(2) agonist administered into the central nucleus of the amygdala decreases ethanol self-administration in ethanol-dependent rats. *Brain Res* 1155:172–178.
- Funk CK, O'Dell LE, Crawford EF, Koob GF (2006) Corticotropin-releasing factor within the central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn, ethanol-dependent rats. *J Neurosci* 26:11324–11332.
- Funk CK, Zorrilla EP, Lee MJ, Rice KC, Koob GF (2007) Corticotropin-releasing factor 1 antagonists selectively reduce ethanol self-administration in ethanol-dependent rats. *Biol Psychiatry* 61:78–86.
- George O, Sanders C, Freiling J, Grigoryan E, Vu S, Allen CD, Crawford E, Mandyam CD, Koob GF (2012) Recruitment of medial prefrontal cortex neurons during alcohol withdrawal predicts cognitive impairment and excessive alcohol drinking. *Proc Natl Acad Sci USA* 109:18156–18161.
- Glaser YG, Zubieta JK, Hsu DT, Villafuerte S, Mickey BJ, Trucco EM, Burmeister M, Zucker RA, Heitzeg MM (2014) Indirect effect of corticotropin-releasing hormone receptor 1 gene variation on negative emotionality and alcohol use via right ventrolateral prefrontal cortex. *J Neurosci* 34:4099–4107.
- Goldstein RZ, Volkow ND (2011) Dysfunction of the prefrontal cortex in addiction: neuroimaging findings and clinical implications. *Nat Rev Neurosci* 12:652–669.
- Gondré-Lewis MC, Warnock KT, Wang H, June HL Jr, Bell KA, Rabe H, Tiruveedhula VV, Cook J, Lüddens H, Aurelian L, June HL Sr (2016) Early life stress is a risk factor for excessive alcohol drinking and impulsivity in adults and is mediated via a CRF/GABA<sub>A</sub> mechanism. *Stress* 19:235–247.
- Herman JP, Wiegand SJ, Watson SJ (1990) Regulation of basal corticotropin-releasing hormone and arginine vasopressin messenger ribonucleic acid expression in the paraventricular nucleus: effects of selective hypothalamic deafferentations. *Endocrinology* 127:2408–2417.
- Jaferi A, Bhatnagar S (2007) Corticotropin-releasing hormone receptors in the medial prefrontal cortex regulate hypothalamic-pituitary-adrenal activity and anxiety-related behavior regardless of prior stress experience. *Brain Res* 1186:212–223.
- Jones KR, Myers B, Herman JP (2011) Stimulation of the prelimbic cortex differentially modulates neuroendocrine responses to psychogenic and systemic stressors. *Physiol Behav* 104:266–271.
- Karolyi IJ, Burrows HL, Ramesh TM, Nakajima M, Lesh JS, Seong E, Camper SA, Seasholtz AF (1999) Altered anxiety and weight gain in corticotropin-releasing hormone-binding protein-deficient mice. *Proc Natl Acad Sci USA* 96:11595–11600.
- Keyes KM, Hatzenbuehler ML, Grant BF, Hasin DS (2012) Stress and alcohol: epidemiologic evidence. *Alcohol Res* 34:391–400.
- Korotkova TM, Brown RE, Sergeeva OA, Ponomarenko AA, Haas HL (2006) Effects of arousal- and feeding-related neuropeptides on dopaminergic and GABAergic neurons in the ventral tegmental area of the rat. *Eur J Neurosci* 23:2677–2685.
- Lack AK, Floyd DW, McCool BA (2005) Chronic ethanol ingestion modulates pro-anxiety factors expressed in rat central amygdala. *Alcohol* 36:83–90.
- Lowery EG, Spanos M, Navarro M, Lyons AM, Hodge CW, Thiele TE (2010) CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57BL/6J mice independent of the HPA axis. *Neuropsychopharmacology* 35:1241–1252.
- Lowery EG, Thiele TE (2010) Pre-clinical evidence that corticotropin-releasing factor (CRF) receptor antagonists are promising targets for pharmacological treatment of alcoholism. *CNS Neurol Disord Drug Targets* 9:77–86.
- Lowery-Gionta EG, Navarro M, Li C, Pleil KE, Rinker JA, Cox BR, Sprow GM, Kash TL, Thiele TE (2012) Corticotropin releasing factor signaling in the central amygdala is recruited during binge-like ethanol consumption in C57BL/6J mice. *J Neurosci* 32:3405–3413.
- Paxinos G, Franklin K (2001) *The Mouse Brain in Stereotaxic Coordinates*. 2nd ed. Elsevier Academic Press, San Diego, CA.
- Phillips TJ, Reed C, Pastor R (2015) Preclinical evidence implicating corticotropin-releasing factor signaling in ethanol consumption and neuroadaptation. *Genes Brain Behav* 14:98–135.
- Potter E, Behan DP, Fischer WH, Linton EA, Lowry PJ, Vale WW (1991) Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins. *Nature* 349:423–426.
- Potter E, Behan DP, Linton EA, Lowry PJ, Sawchenko PE, Vale WW (1992) The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. *Proc Natl Acad Sci USA* 89:4192–4196.
- Radley JJ, Arias CM, Sawchenko PE (2006) Regional differentiation of the medial prefrontal cortex in regulating adaptive responses to acute emotional stress. *J Neurosci* 26:12967–12976.
- Ray LA (2011) Stress-induced and cue-induced craving for alcohol in heavy drinkers: preliminary evidence of genetic moderation by the OPRM1 and CRH-BP genes. *Alcohol Clin Exp Res* 35:166–174.

- Ray LA, Sehl M, Bujarski S, Hutchison K, Blaine S, Enoch MA (2013) The CRHR1 gene, trauma exposure, and alcoholism risk: a test of  $G \times E$  effects. *Genes Brain Behav* 12:361–369.
- Refojo D, Schweizer M, Kuehne C, Ehrenberg S, Thoeringer C, Vogl AM, Dedic N, Schumacher M, Von Wolff G, Avrabos C, Touma C, Engblom D, Schutz G, Nave KA, Eder M, Wotjak CT, Sillaber I, Holsboer F, Wurst W, Deussing JM (2011) Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1. *Science* 333:1903–1907.
- Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC (2005) Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiol Behav* 84:53–63.
- Richardson HN, Lee SY, Odell LE, Koob GF, Rivier CL (2008) Alcohol self-administration acutely stimulates the hypothalamic-pituitary-adrenal axis, but alcohol dependence leads to a dampened neuroendocrine state. *Eur J Neurosci* 28:1641–1653.
- Rivier C (1993) Female rats release more corticosterone than males in response to alcohol: influence of circulating sex steroids and possible consequences for blood alcohol levels. *Alcohol Clin Exp Res* 17:854–859.
- Seasholtz AF, Bourbonnais FJ, Harnden CE, Camper SA (1991) Nucleotide sequence and expression of the mouse corticotropin-releasing hormone gene. *Mol Cell Neurosci* 2:266–273.
- Silberman Y, Matthews RT, Winder DG (2013) A corticotropin releasing factor pathway for ethanol regulation of the ventral tegmental area in the bed nucleus of the stria terminalis. *J Neurosci* 33:950–960.
- Sommer WH, Rimondini R, Hansson AC, Hipskind PA, Gehlert DR, Barr CS, Heilig MA (2008) Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. *Biol Psychiatry* 63:139–145.
- Sparta DR, Hopf FW, Gibb SL, Cho SL, Stuber GD, Messing RO, Ron D, Bonci A (2013) Binge ethanol-drinking potentiates corticotropin releasing factor R1 receptor activity in the ventral tegmental area. *Alcohol Clin Exp Res* 37:1680–1687.
- Sparta DR, Sparrow AM, Lowery EG, Fee JR, Knapp DJ, Thiele TE (2008) Blockade of the corticotropin releasing factor type 1 receptor attenuates elevated ethanol drinking associated with drinking in the dark procedures. *Alcohol Clin Exp Res* 32:259–265.
- Sutton SW, Behan DP, Lahrachi SL, Kaiser R, Corrigan A, Lowry P, Potter E, Perrin MH, Rivier J, Vale WW (1995) Ligand requirements of the human corticotropin-releasing factor-binding protein. *Endocrinology* 136:1097–1102.
- Tagliaferro P, Morales M (2008) Synapses between corticotropin-releasing factor-containing axon terminals and dopaminergic neurons in the ventral tegmental area are predominantly glutamatergic. *J Comp Neurol* 506:616–626.
- Uhart M, Wand GS (2009) Stress, alcohol and drug interaction: an update of human research. *Addict Biol* 14:43–64.
- Ungless MA, Singh V, Crowder TL, Yaka R, Ron D, Bonci A (2003) Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. *Neuron* 39:401–407.
- Van De Werd HJ, Rajkowska G, Evers P, Uylings HB (2010) Cytoarchitectonic and chemoarchitectonic characterization of the prefrontal cortical areas in the mouse. *Brain Struct Funct* 214:339–353.
- Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, Prins GS, Perrin M, Vale W, Sawchenko PE (2000) Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* 428:191–212.
- Wang B, You ZB, Rice KC, Wise RA (2007) Stress-induced relapse to cocaine seeking: roles for the CRF(2) receptor and CRF-binding protein in the ventral tegmental area of the rat. *Psychopharmacology* 193:283–294.
- Wang HL, Morales M (2008) Corticotropin-releasing factor binding protein within the ventral tegmental area is expressed in a subset of dopaminergic neurons. *J Comp Neurol* 509:302–318.
- Westphal NJ, Evans RT, Seasholtz AF (2009) Novel expression of type 1 corticotropin-releasing hormone receptor in multiple endocrine cell types in the murine anterior pituitary. *Endocrinology* 150:260–267.
- Westphal NJ, Seasholtz AF (2006) CRH-BP: the regulation and function of a phylogenetically conserved binding protein. *Front Biosci* 11:1878–1891.

## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Representative in situ hybridization autoradiogram images for CRF (A), CRF-R1 (B), and CRF-BP (C) in the medial prefrontal cortex (mPFC) of water control mice.

**Fig. S2.** Representative in situ hybridization autoradiogram images for CRF (A), CRF-R1 (B), and CRF-BP (C) in the amygdala of EtOH-treated mice.