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

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Abstract

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 (IL) 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 (VTA), bed nucleus of the stria terminalis (BNST), 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

Introduction

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 (Keyes 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 two 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 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. Lastly, CRF neurons in the mPFC are upregulated after abstinence from intermittent access to ethanol (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 40-60% of CRF in the human brain is bound by CRF-BP (Behan et al., 1997), and CRF-BP co-localizes 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_{6-33}, decreasing CRF-mediated potentiation of NMDA excitatory postsynaptic currents on VTA dopamine neurons (Ungless et al., 2003). Similarly, intra-VTA administration of CRF_{6-33} 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 the current 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 ethanol consumption in the DID paradigm.

Materials and Methods

Animals – 3-cycle DID experiment:

Six to eight-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 NIH 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 x KO and KO x KO crosses were used to generate CRF-BP KO mice. Mice were 10-14 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_2O: n=10); wild-type (EtOH: n=13, H_2O: n=13)) were tested in a DID protocol (Rhodes et al., 2005). On days 1-3, mice were given access to a single 50 mL centrifuge tube of 20% ethanol (v/v) for 2 hours, starting 3 hours into the dark cycle. On day 4, mice were given access to 20% ethanol for 4 hours. Control mice received a single 50 mL centrifuge tube of water instead of 20% ethanol. Mice received only water for the last 3 days (days 5-7) 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 two ball bearings (Ancare Corp., Bellmore, NY). Two empty cages were placed in the experiment room and each received a bottle of 20% ethanol 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 µL 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 µL 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 µL 0.5M EDTA, centrifuged, and then plasma was removed and stored at -20 °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. #: 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 ethanol consumption. Brains were removed, frozen in 2-methylbutane, and stored at -80 °C until use. Brains were sectioned via cryostat at 14 µm and collected in series of six 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 post-fixed in 4% paraformaldehyde for 1 hour and washed three times in 2x saline sodium citrate (SSC) buffer. Sections were then incubated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, washed three times in 2x SSC, dehydrated in ethanol and air-dried. CRF, CRF-R1, and CRF-BP antisense cRNA riboprobes were generated with ^{35}S-UTP and ^{35}S-CTP (1250 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}S-labeled riboprobes (2 x 10^6 cpm/slide) in 50% formamide hybridization buffer (Ameresco, Framingham, MA) with 20 mM DTT overnight at 55 °C. After hybridization, sections

were washed three times with 2x SSC and treated with RNase A (200 µg/mL) for 1 hour at 37 °C. Slides were then washed in decreasing salt solutions (2x, 1x, and 0.5x SSC) and a high-stringency wash was performed in 0.1x SSC at 65 °C for 1 hour. Slides were then dehydrated in ethanol, air-dried, and exposed to BioMax MR autoradiography film (Carestream Health Inc., Rochester, NY) for 3-14 days depending on riboprobe and brain region.

In situ hybridization analyses

Autoradiography films were scanned and analyzed using densitometry in ImageJ. A set of macros were utilized that enabled background to be selected and a mask created so that only signal greater than 3.5 standard deviations above background is measured. Mean optical density (mean OD), area, and integrated optical density (IOD; mean optical density x 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 t-tests. A two-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 ± 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 ± 0.24 g/kg of ethanol and exhibited an average BEC of 84.1 ± 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 (Figure 1). Interestingly, CRF mRNA expression (IOD) was highest between bregma coordinates 0.38 and 0.14 in anterior nuclei, whereas CRF-R1 and CRF-BP mRNA expression were highest more caudally in posterior nuclei between bregma coordinates -0.10 and -0.34. IOD signal from sections within these designated bregma coordinates were averaged to generate one value per mouse for each riboprobe. Independent student t-tests revealed that there were no differences in CRF, CRF-R1, and CRF-BP expression between ethanol and control mice after 3 cycles of DID (Table 1; representative *in situ* autoradiograms in Figure 1). Additionally, further analyses revealed that CRF expression was unaltered in the dorsal and ventral BNST nuclei of ethanol 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 ethanol and control mice (Table 1; representative *in situ* autoradiograms in Figure 2). CRF-BP IOD was highest from bregma coordinates -3.28 to -3.52 in the mid-to-posterior VTA, where IOD signal was averaged for analysis (Figure 2). There was a trend for a decrease in CRF-BP IOD in ethanol 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 ethanol 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 supplementary Figure 1). Expression in the PL and IL mPFC was analyzed from coordinates 2.34 to 1.54 and 1.98 to 1.54, respectively. Independent student t-tests revealed that CRF-BP IOD in the PL and IL mPFC was significantly decreased in ethanol mice compared to control mice (Table 1 and Figure 3; prelimbic, $t(8) = 4.64$, $p<0.01$ and infralimbic, $t(8) = 2.51$, $p<0.05$). There were no significant differences in CRF and CRF-R1 mRNA expression levels in the mPFC between ethanol 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, and in the CeA, CRF expression was analyzed from coordinates -0.82 to -1.82 (representative autoradiograms in supplementary figure 2). CRF mRNA was not detectible in the BLA/LA and BMA and therefore was not included in the analysis. There were some rostral to caudal variations in CRF, 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 ethanol-treated mice compared to controls (Table 2; Figure 4; $t(10) = 2.6$, $p<0.05$). However, CRF-BP mRNA expression was unchanged in the IL mPFC of ethanol-treated mice after 6 cycles of DID. CRF-BP was not significantly altered in the BNST, VTA, and amygdala of ethanol 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 two-way repeated measures ANOVA (Figure 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 ethanol on day 4 of cycle 5 compared to cycle 2 ($p<0.001$) and CRF-BP KO mice drank more ethanol 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 ± 11.7 mg/dL) and CRF-BP KO mice (72.4 ± 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 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 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, CRF-R1 and CRF-BP mRNA expression are 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 co-expression 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 (Figure 2), whereas CRF mRNA is detected only at low levels throughout the VTA. While CRF mRNA expression is low in cell bodies of the VTA (Figure 2 and 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, 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 co-expression 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_{6-33} 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 qRT-PCR (Korotkova et al., 2006; Ungless et al., 2003), but is not detected with *in situ* hybridization under basal conditions (G. Stinnett, unpublished data; Van Pett et al., 2000). 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 non-preferring 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 ethanol 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 non-selective 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 ethanol consumption. Hence, CRF-R1 antagonist administration into the mPFC may reduce ethanol consumption in binge drinking paradigms. 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 ethanol. This could account for differences between our data and other studies detecting changes in CRF mRNA or immunoreactivity at 0-2 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_{6-33}, into the VTA, but not the CeA, decreased alcohol consumption in a DID paradigm, suggesting that CRF-BP may facilitate binge-like ethanol consumption. One significant difference between these studies is the method by which CRF-BP is inhibited. In the study by Albrechet-Souza et al. (2015), CRF-BP is site-specifically inhibited in the VTA or CeA using CRF_{6-33}. 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 ethanol 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.

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Figure Legends

Fig. 1. Representative *in situ* hybridization autoradiogram images for CRF (A), CRF-R1 (B), and CRF-BP (C) in the bed nucleus of the stria terminalis (BNST) of ethanol-treated mice twenty-four hours after 3 cycles of DID. Brain regions of interest are outlined in black. The coordinates for the autoradiograms are 0.26 for CRF and -0.22 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 ethanol-treated mice after 3 cycles of DID (n=4-5/probe; data represent the mean ± SEM). IOD values in panel D are plotted relative to the lowest value for each riboprobe. Intensities in panels A-D cannot be directly compared, as riboprobes were not equal in specific activity and exposure times were varied to yield optimal quantitative results.

Fig. 2. Representative *in situ* hybridization autoradiogram images for CRF (A), CRF-R1 (B), and 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 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 DID (n=4-5/probe; data represent the mean ± SEM). IOD values in panel D are plotted relative to the lowest value for each riboprobe.

Fig. 3. Decreased CRF-BP expression in the medial prefrontal cortex (mPFC) after 3 cycles of DID. Coronal section from the Paxinos and Franklin (2001) mouse brain atlas (A) at bregma coordinate 1.54 and corresponding representative *in situ* hybridization autoradiogram images (B) comparing CRF-BP expression in ethanol-treated mice to water controls at 24 hours after 3 cycles of DID. CRF-BP IOD was significantly decreased in the prelimbic (PL) and infralimbic (IL) mPFC of ethanol-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 ± SEM. * $p<0.05$ and ** $p<0.01$ compared to respective water controls using independent student t-tests.

Fig. 4. Decreased CRF-BP expression in the medial prefrontal cortex (mPFC) after 6 cycles of DID. Coronal section from the Paxinos and Franklin (2001) mouse brain atlas (A) at bregma coordinate 1.54 and corresponding representative *in situ* hybridization autoradiogram images (B) comparing CRF-BP expression in ethanol-treated mice to water controls at 24 hours after 6 cycles of DID. CRF-BP IOD was significantly decreased in the prelimbic (PL) mPFC of ethanol-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 ± SEM. * $p<0.05$ compared to respective water control using an independent student t-test.

Fig. 5. Comparison of ethanol consumption between CRF-BP KO and wild-type (WT) mice after 6 cycles of DID. A two-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 ± 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.

Table 1.

CRF	CRF-R1	CRF-BP

	H_2O	EtOH	H_2O	EtOH	H_2O	EtOH
BNST	168.7 ± 11.4	158.0 ± 12.2	43.5 ± 4.8	39.9 ± 6.2	67.2 ± 3.0	80.8 ± 7.3
VTA	22.9 ± 3.9	26.0 ± 2.1	45.2 ± 2.2	50.1 ± 4.7	85.1 ± 9.2	61.1 ± 5.4#
PL mPFC	4.4 ± 0.5	5.5 ± 0.9	27.9 ± 1.1	24.6 ± 1.3	45.9 ± 1.2	30.2 ± 3.2*
IL mPFC	3.7 ± 0.5	4.4 ± 0.4	4.4 ± 0.8	3.1 ± 1.0	70.5 ± 5.4	50.1 ± 6.0*
BLA/LA	ND	ND	83.3 ± 11.6	86.4 ± 9.8	49.1 ± 8.4	60.6 ± 10.7
CeA	101.1 ± 7.1	81.6 ± 7.2	36.0 ± 4.9	33.4 ± 5.1	4.8 ± 1.5	6.8 ± 2.1
BMA	ND	ND	108.0 ± 9.7	105.2 ± 9.4	10.6 ± 2.5	15.1 ± 4.0

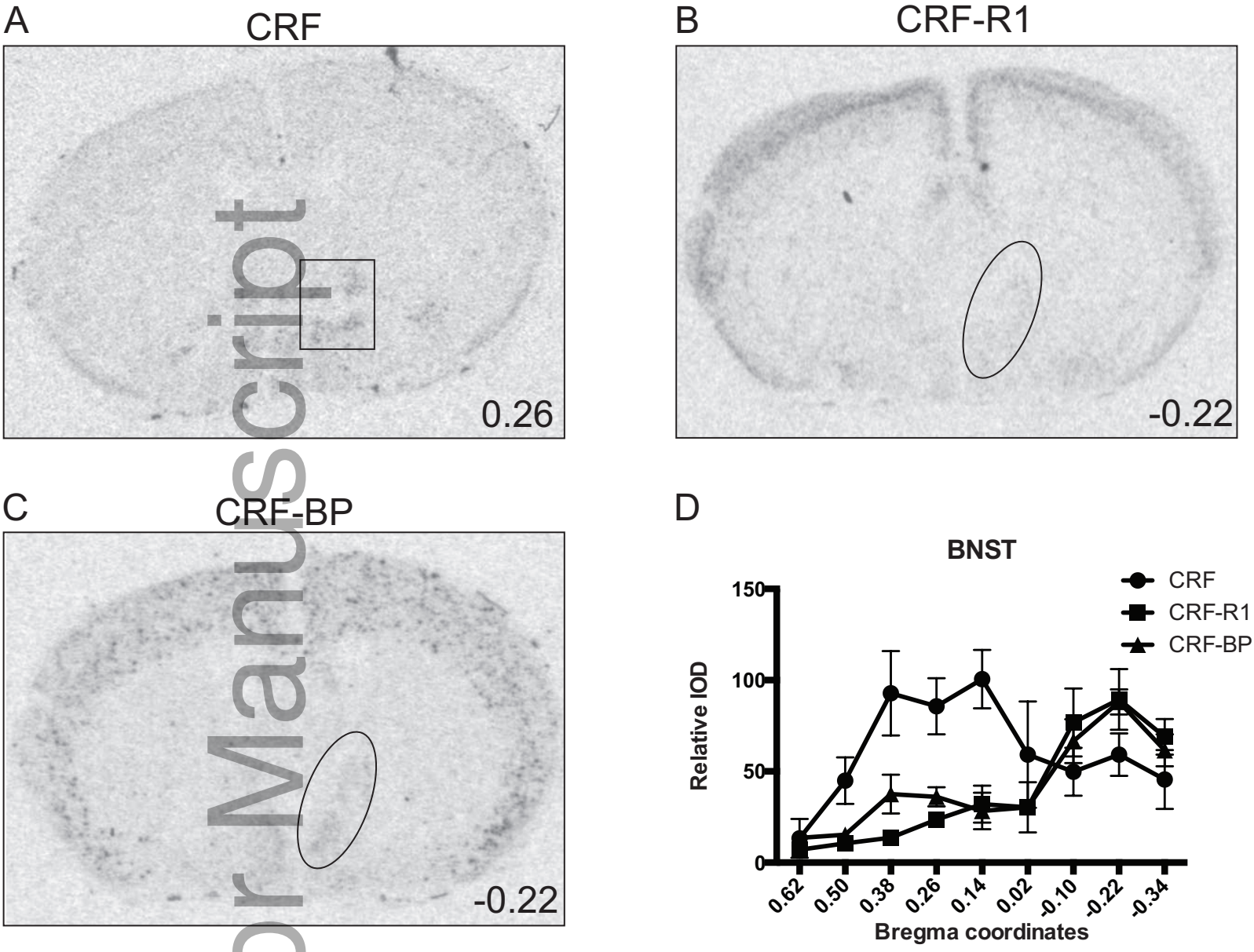
Table 2.

	CRF-BP	
	H_2O	EtOH
BNST	100.4 ± 9.0	90.1 ± 12.4
VTA	57.4 ± 3.2	52.9 ± 3.1
PL mPFC	63.3 ± 4.6	50.6 ± 1.8*
IL mPFC	99.2 ± 6.7	88.7 ± 3.4
BLA/LA	139.5 ± 18.3	177.5 ± 29.9
CeA	11.1 ± 0.9	13.3 ± 2.5
BMA	29.6 ± 4.3	37.7 ± 7.3

Table legends

Table 1. Summary of *in situ* hybridization data (represented as integrated optical density) for CRF, CRF-R1, and CRF-BP twenty-four hours after 3 cycles of DID. Data represent the mean ± SEM (n=4-5/group). * $p<0.05$ and # $p=0.055$ compared to respective water controls using independent student t-tests. Values within bolded lines represent independent experiments. IOD values should not be directly compared across independent experiments as riboprobe specific activity and exposure times are not equal. BNST, bed nucleus of the stria terminalis; VTA, ventral tegmental area; PL mPFC,

681 prelimbic medial prefrontal cortex; IL mPFC, infralimbic medial prefrontal cortex; BLA,
682 basolateral amygdala; LA, lateral amygdala; CeA, central nucleus of the amygdala;
683 BMA, basomedial amygdala; ND, not detected.
684
685 **Table 2.** Summary of *in situ* hybridization data (represented as integrated optical density)
686 for CRF-BP after 6 cycles of DID. Data represent the mean ± SEM (n=4-6/group).
687 * $p<0.05$ compared to respective water control using an independent student t-test.
688 Values within bolded lines represent independent experiments. IOD values should not be
689 directly compared across independent experiments as riboprobe specific activity and
690 exposure times are not equal. BNST, bed nucleus of the stria terminalis; VTA, ventral
691 tegmental area; PL mPFC, prelimbic medial prefrontal cortex; IL mPFC, infralimbic
692 medial prefrontal cortex; BLA, basolateral amygdala; LA, lateral amygdala; CeA, central
693 nucleus of the amygdala; BMA, basomedial amygdala.



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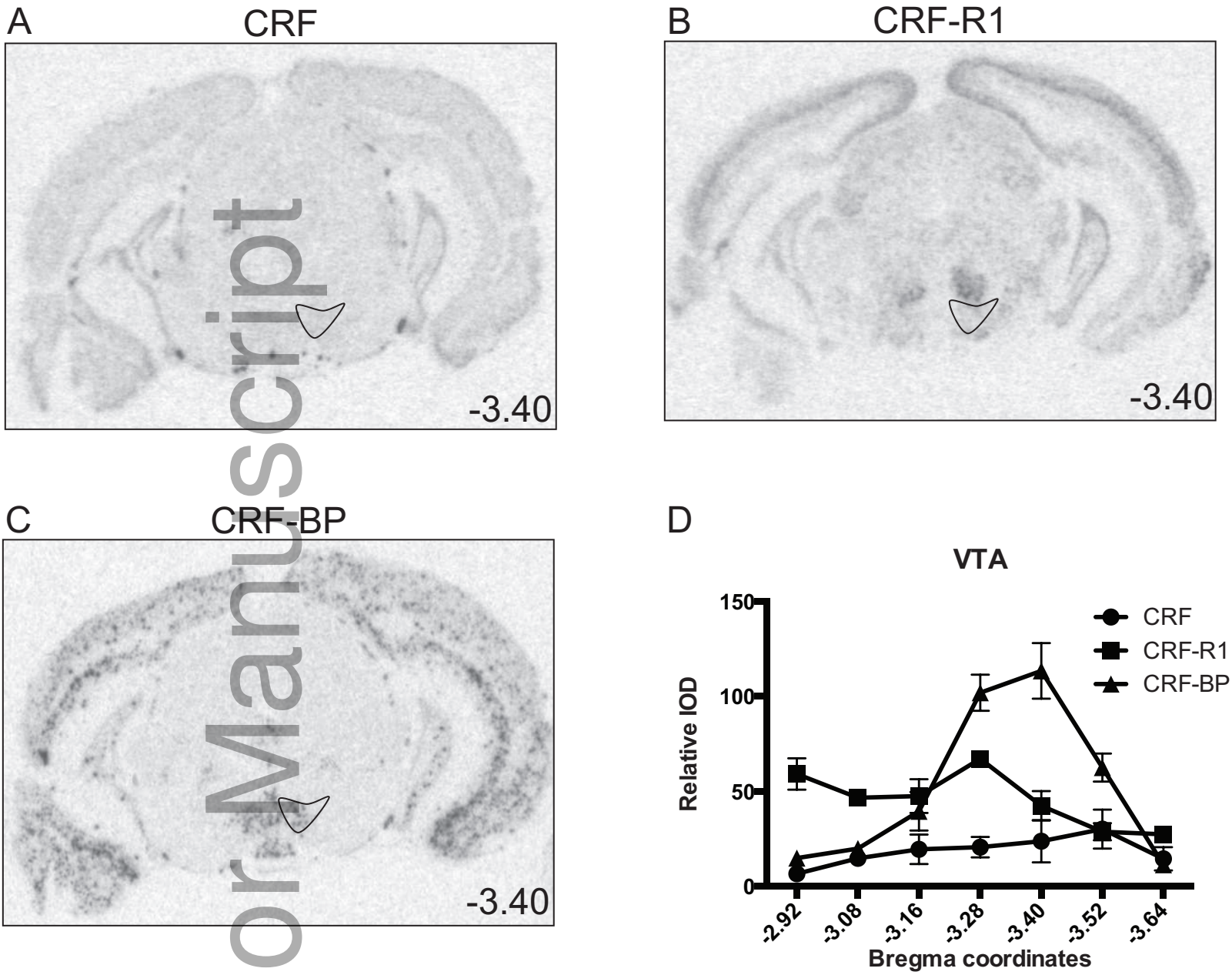

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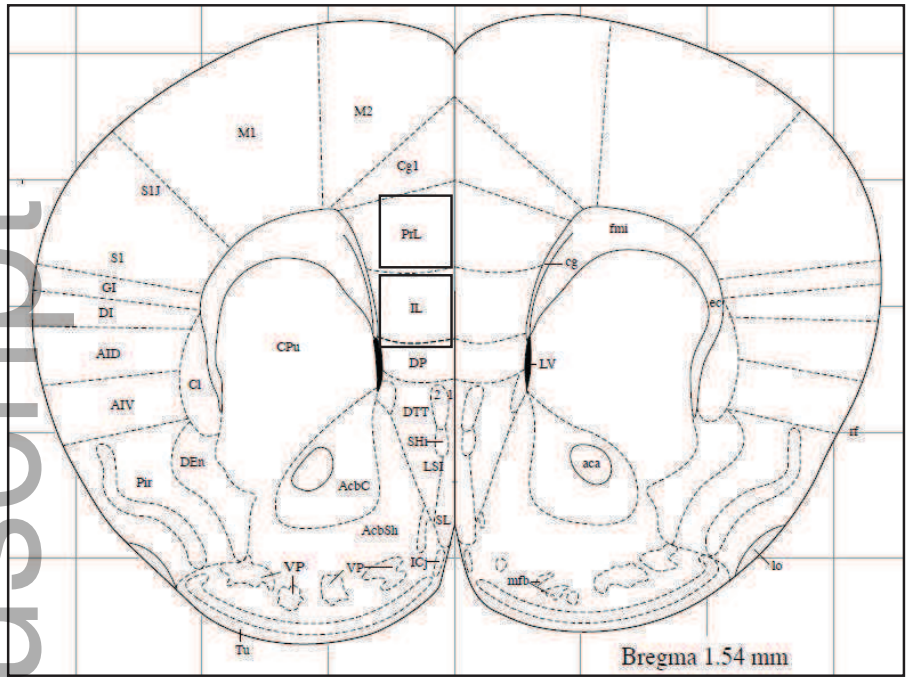

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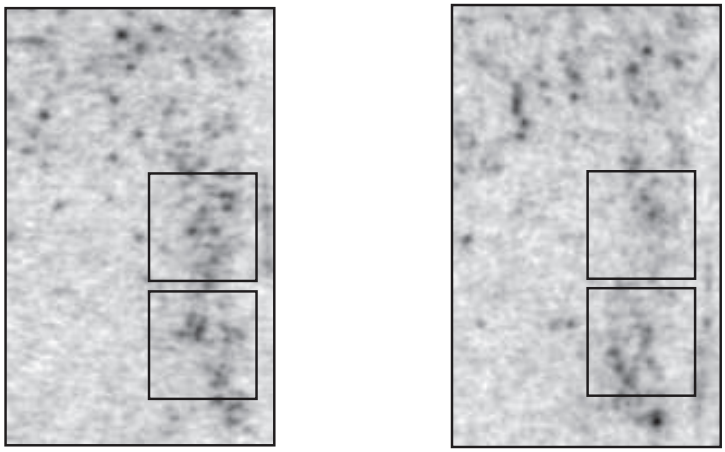

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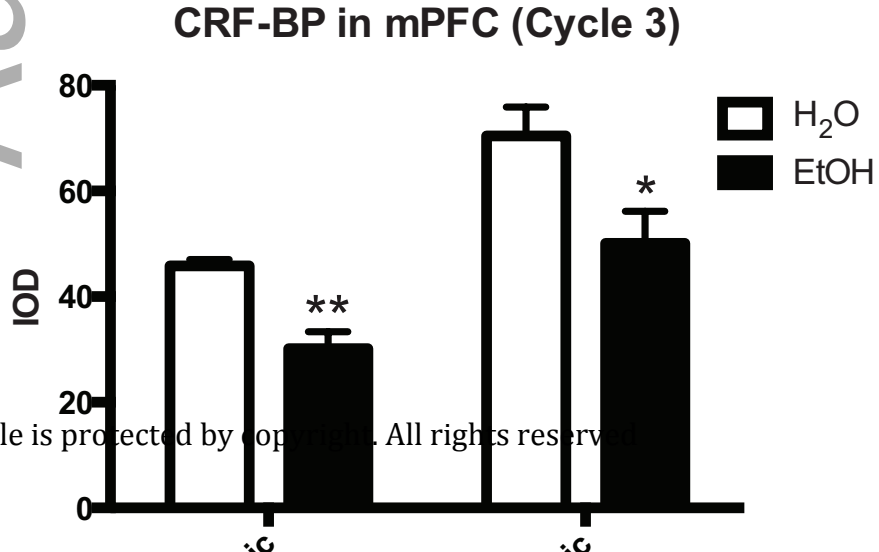

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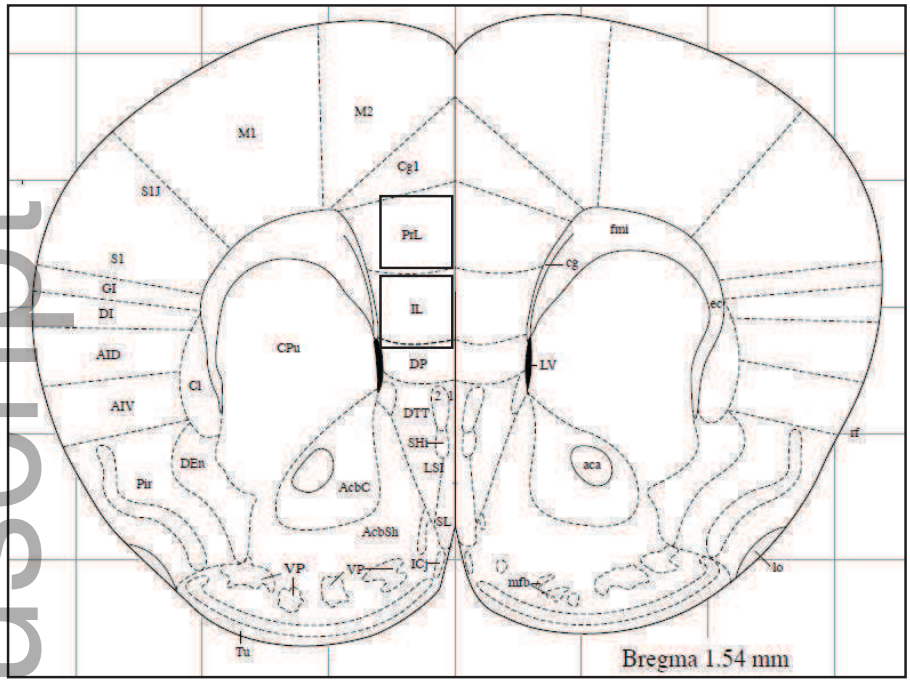

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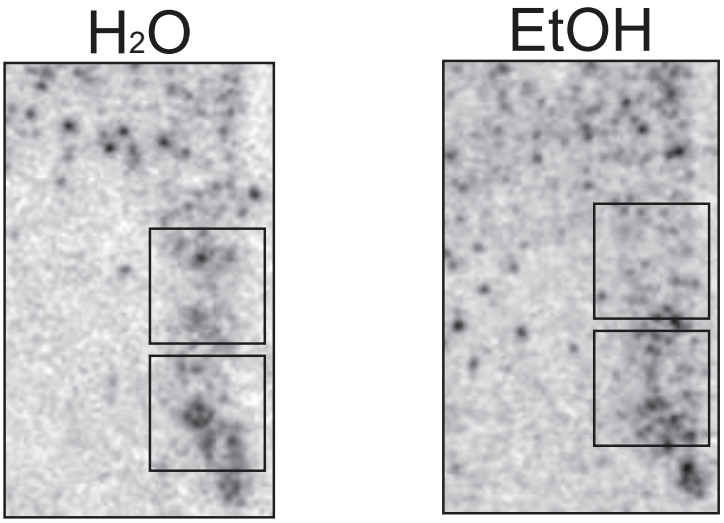

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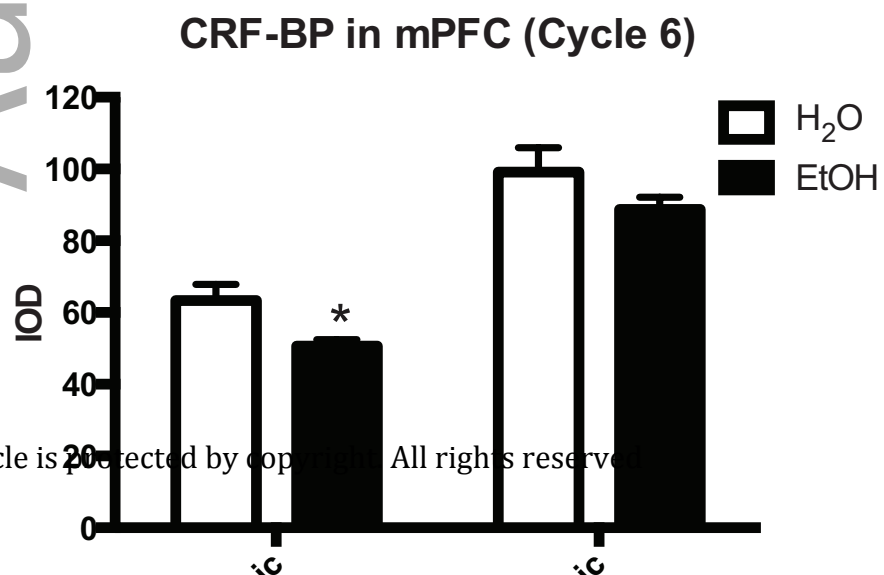

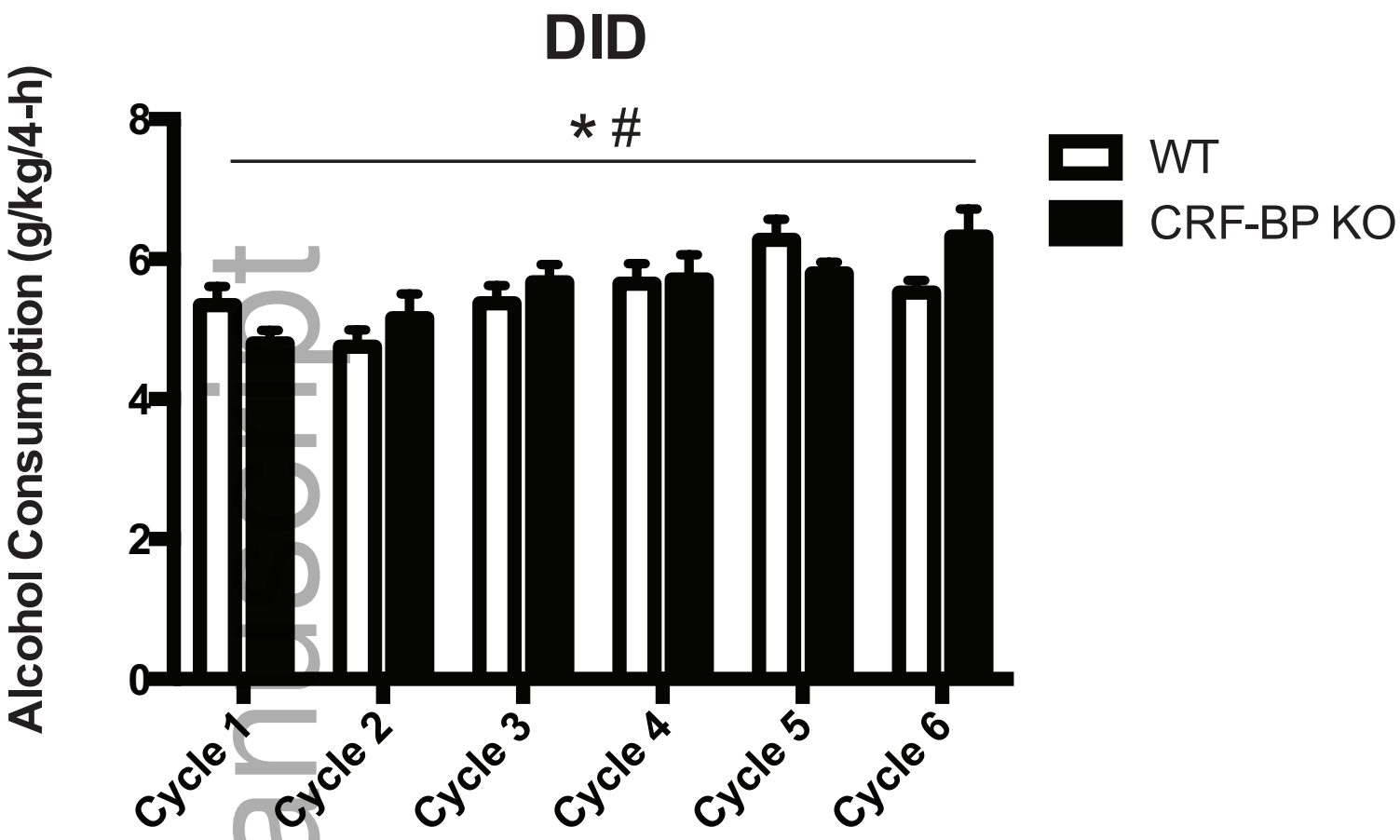

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