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CB1 receptor neutral antagonist treatment epigenetically increases neuropeptide Y expression and decreases alcohol drinking

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ABSTRACT

Alcohol consumption is mediated by several important neuromodulatory systems, including the endocannabinoid and neuropeptide Y (NPY) systems in the limbic brain circuitry. However, molecular mechanisms through which cannabinoid-1 (CB1) receptors regulate alcohol consumption are still unclear. Here, we investigated the role of the CB1 receptor-mediated downstream regulation of NPY via epigenetic mechanisms in the amygdala. Alcohol drinking behavior was measured in adult male C57BL/6J mice treated with a CB1 receptor neutral antagonist AM4113 using a two-bottle choice paradigm while anxiety-like behavior was assessed in the light-dark box (LDB) test. The CB1 receptor-mediated changes in the protein levels of phosphorylated cAMP-responsive element binding protein (pCREB), CREB binding protein (CBP), H3K9ac, H3K14ac and NPY, and the mRNA levels of *Creb1*, *Cbp*, and *Npy* were measured in amygdaloid brain structures. *Npy*-specific changes in the levels of acetylated histone (H3K9/14ac) and CBP in the amygdala were also measured. We found that the pharmacological blockade of CB1 receptors with AM4113 reduced alcohol consumption and, in an ethanol-naïve cohort, reduced anxiety-like behavior in the LDB test. Treatment with AM4113 also increased the mRNA levels of *Creb1* and *Cbp* in the amygdala as well as the protein levels of pCREB, CBP, H3K9ac and H3K14ac in the central and medial nucleus of amygdala, but not in the basolateral amygdala. Additionally, AM4113 treatment increased occupancy of CBP and H3K9/14ac at the *Npy* gene promoter, leading to an increase in both mRNA and protein levels of NPY in the amygdala. These novel findings suggest that CB1 receptor-mediated CREB signaling plays an important role in the modulation of NPY function through an epigenetic mechanism and further support the potential use of CB1 receptor neutral antagonists for the treatment of alcohol use disorder.

1. Introduction

The cyclic three stages (the binge/intoxication stage, the withdrawal/negative affect stage, and the preoccupation/anticipation or "craving" stage) of alcohol use disorder (AUD) are associated with adaptive changes in various neurocircuits, including the extended amygdala (Koob, 2003; Koob and Mason, 2016). Acute and chronic alcohol exposure has been shown to dysregulate several biological processes including epigenetic mechanisms in the amygdala (Pandey et al., 2017). This occurs particularly in the central nucleus of the amygdala (CeA), which is hypothesized to control the negative affective states experienced during alcohol abstinence, also known as the 'dark side' of alcohol addiction (Koob et al., 2014; Koob and Mason, 2016; Kyzar and Pandey, 2015; Pandey et al., 2017).

The endocannabinoid system, a key modulator of synaptic function, serves as a potential biological target for neurological and psychiatric disorders, including AUD (Castellano et al., 2003; Manzanares et al., 2018; Pava and Woodward, 2012; Piomelli, 2003; Vinod and Hungund, 2006). Several studies have shown that recruitment of the endocannabinoid system occurs in the specific limbic brain circuitry that regulates

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alcohol drinking as well as reinforcement (Manzanares et al., 2018; Pava and Woodward, 2012; Parsons and Hurd, 2015; Varodayan et al., 2016; Vinod and Hungund, 2006). Alcohol exposure has also been shown to dysregulate the central endocannabinoid system (González et al., 2002; Mitrirattanakul et al., 2007, Pava and Woodward, 2012; Vinod et al., 2006, 2010, 2012). Moreover, several studies have shown that CB1 receptor antagonists reduce voluntary alcohol consumption whereas activation of CB1 receptor promotes alcohol drinking in rodent models (Blednov et al., 2007; Colombo et al., 2005; López-Moreno et al., 2004; Pava and Woodward, 2012; Vinod et al., 2008a, 2008b). Relapse to alcohol drinking in rats can also be prevented by treatment with the CB1 receptor antagonist SR141716A (rimonabant) (Cippitelli et al., 2005; Maccioni et al., 2010). However, clinical use of rimonabant has been discontinued due to the resulting increased risk of anxiety and depression (Gueye et al., 2016; Kirilly et al., 2012), which may be due to its inverse agonistic property, extended half-life, and activity at other neuronal targets. Recent studies seem to support the potential utility of the CB1 receptor neutral antagonist AM4113 for treating tobacco, heroin, and cannabis dependence as well as binge alcohol drinking without producing anxiety- or depression-like behaviors in animal models (Balla et al., 2018; Gueye et al., 2016; He et al., 2019; Schindler et al., 2016).

The CB1 receptors are one of the most abundant neuromodulatory G-protein coupled receptors in the mammalian brain including in the amygdala and they are negatively coupled to adenylyl cyclase (AC) through G_i protein (Castillo et al., 2012; Howlett, 1998). The gene transcription factor cAMP-responsive element-binding protein (CREB) is the common convergence target of signaling cascades. It is regulated via phosphorylation at serine 133 by cAMP-dependent protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinases II and IV, and mitogen-activated protein kinase (Impey et al., 1999; Pandey, 2004; Silva et al., 1998; Soderling, 1999). Phosphorylated CREB (pCREB) then regulates the expression of cAMP-inducible genes, such as *Npy*, by interacting with chromatin (Lonze and Ginty, 2002; Mayr et al., 2001; McClung and Nestler, 2003; Pandey et al., 2017). Previous studies have shown an association between deficiency in CREB function and its related *Npy* gene in the CeA with anxiety-like and alcohol drinking behaviors in rodent models (Pandey et al., 2004, 2005; Zhang et al., 2010). It has been shown that pCREB recruits CREB binding protein (CBP) and p300 transcriptional regulators to chromatin for the modulation of histone acetylation and gene expression (Bannister and Kouzarides, 1996; Barrett et al., 2011; Berkel and Pandey, 2017; Kouzarides, 2007; Verdone et al., 2005). We recently showed that changes in chromatin dynamics via the acetylation of histones H3K9/14 (H3K9/14ac) at the *Npy* promoter modulate its expression in the amygdala, and that deficits in NPY expression in the CeA and the medial nucleus of the amygdala (MeA) promote higher alcohol intake and anxiety-like behaviors in rodent models of AUD (Kokare et al., 2017; Pandey et al., 2015; Sakharkar et al., 2014).

Although all of these studies have demonstrated the importance of the CB1 receptor and CREB related target gene, *Npy,* in alcohol-related behaviors, the molecular mechanisms through which endocannabinoid and NPY systems interact in the amygdala and regulate alcohol drinking remain unknown. We hypothesized that CB1 receptors have the ability to regulate chromatin remodeling via CREB/CBP signaling, leading to the regulation of NPY expression as well as alcohol drinking and anxiety-like behaviors. This study was designed to test the above hypothesis by examining the effect of the CB1 receptor neutral antagonist AM4113 on alcohol consumption and anxiety-like behavior, and the underlying epigenetic mechanisms of the interaction between CB1 receptors and NPY in the amygdala using an alcohol preferring mouse model.

2. Materials and methods

2.1. Animals

All of the experiments were conducted in adult male C57BL/6J mice (10–14 weeks old; Jackson Laboratory, USA) with body weights ranging from 24 to 30g at the time of the experiment. Mice were given unlimited access to water and standard mouse chow during the entire study. All procedures were conducted in accordance with the National Institutes of Health and Institutional Animal Care and Use Committees' guidelines.

2.2. CB1 neutral antagonist treatment

All mice in this study were injected intraperitoneally with AM4113 (1 mg/kg body weight) or vehicle (4% DMSO and 1% Tween-80 in saline) once daily for 3 days. This dose is based on our recent study on the pharmacokinetics of drug distribution and elimination rate in brain and blood in mice (Balla et al., 2018). For drinking and immunohistochemistry experiments, AM4113 was kindly provided by Drs. Alexandros Makriyannis and Kiran Vemuri of Northeastern University. For follow-up experiments on immunohistochemistry, biochemistry, and anxiety-like behavior, AM4113 was purchased from Cayman Chemical (No. 20581, Ann Arbor, MI). The drug and vehicle solutions were freshly prepared each day just before administration.

2.3. Alcohol consumption study

Mice were housed individually and habituated to a reverse light-dark cycle for a week prior to the test. Alcohol drinking behavior was measured using a two-bottle choice daily drinking paradigm following the acclimatization of mice to water and escalating concentrations of alcohol [3% (3 days), 6% (3 days), and 10% (6 days)] as previously described (Vinod et al., 2008a, 2008b). Animals were assigned to vehicle and AM4113 groups after alcohol habituation. They were then injected intraperitoneally with AM4113 (1 mg/kg) or vehicle (Balla et al., 2018) once daily for an additional 3 days. After 40 min of drug or vehicle treatment, mice were offered fresh bottles, one containing water and other containing 10% alcohol daily, and during this time consumption of alcohol and water of the previous day was also measured. The positions of the bottles were alternated each day to avoid side preference. Data were corrected for the loss of liquid due to spillage and evaporation by comparing weights of water and alcohol bottles in a control cage without a mouse. The mean alcohol intake is expressed as grams of alcohol/kg of body weight/day.

2.4. Anxiety-like behavior test

A separate cohort of ethanol-naïve mice was group-housed and habituated to regular handling and weighing for a week prior to test. Mice were injected with 1 mg/kg AM4113 or vehicle once daily for 3 days as described and tested for anxiety-like behavior 24 h following the final injection. Anxiety-like behavior was assessed in the light-dark box (LDB) apparatus, as previously described by us (Dong et al., 2018; Sakharkar et al., 2014). Briefly, mice were acclimated to the anxiety testing room for 1 h prior to being tested. Mice were tested individually and allowed to explore the LDB for 5 min. The LDB test sessions were video recorded and analyzed using ANY-maze behavioral video scoring software (Stoelting, Wood Dale, IL) which was programmed to track the center of the mouse to derive the following measurements: time spent in each chamber and entries into each chamber. The percentage of time spent in either the light or the dark compartment was then calculated for each mouse; more time exploring the light compartment is interpreted as decreased anxiety-like behavior (Sakharkar et al., 2014). We used the number of entries into the dark compartment as an index of general activity of the mouse.

2.5. Biochemical studies

For biochemical studies, alcohol-naïve adult mice were injected with either AM4113 (1 mg/kg i. p.) or vehicle (4% DMSO and 1% Tween 80 in saline, i. p.) once daily for 3 days. Twenty-four hours after the last

injections, mice were either perfused with saline followed by 4% paraformaldehyde or directly decapitated without perfusion under anesthesia (ketamine [100 mg/kg] and xylazine [10 mg/kg], i. p.) or isoflurane. Brains were collected and amygdaloid brain regions containing all major nuclei (central, medial, and basolateral amygdala) were immediately dissected out. For immunohistochemistry, brains were placed in fixative overnight at 4 °C and were then cryoprotected in 10%, 20%, and 30% sucrose (in 0.1 M PBS buffer, pH 7.4). All tissues were frozen at −80 °C until use.

2.6. Measurement of protein levels using immunohistochemistry

Gold immunolabeling procedure was employed to measure pCREB, CBP, NPY, H3K9ac, and H3K14ac protein levels in the amygdaloid brain structures as previously described by us (Pandey et al., 2004, 2008; Sakharkar et al., 2014). The bregma-matched coronal sections (20 μm-thick) were used and incubated in RPMI 1640 medium containing L-glutamine (Life Technologies, Grand Island, NY) for 30 min. Sections were incubated with 10% normal goat serum in PBS containing 0.25% Triton X-100 (PBST) for 30 min at room temperature. The sections were then incubated with antibodies against pCREB (1:500 dilution; 06–519, Millipore, Billerica, MA), CBP (1:200 dilution; sc-7300, Santa Cruz Biotechnology, Santa Cruz, CA), NPY (1:500 dilution; 22940, Immunostar, Hudson, WI), H3K9ac (06–942) and H3K14ac (06–911) (1:500 dilution; Millipore, Billerica, MA) in PBST containing 1% BSA for 18 h at room temperature. Sections were then washed with PBS containing 1% BSA and incubated with gold particle (1.4 nm)-conjugated anti-rabbit secondary antibody (1:200 dilution in PBS containing 1% BSA; Nanoprobes, Inc., Yaphank, NY) for 1 h at room temperature. The sections were washed with PBS containing 1% BSA and with distilled water. Gold particles were then silver enhanced (Ted Pella Inc., Redding, CA) followed by washing with water. Immunogold particles were visualized and counted using the Image Analyzer program (Loats Associates, Westminster, MD). The threshold for non-immunostained areas in the amygdalar region was set to zero. After threshold determination, the software counted the numbers of immuno-gold particles per 100 μm^2 brain area at higher magnification (100X). Three object areas of defined amygdaloid nuclei (CeA, MeA and the basolateral nucleus of the amygdala [BLA]) from each of the three brain sections for each animal were counted, and the values were averaged. Results are represented as number of immunogold particles per 100 μm^2 brain area.

2.7. Chromatin immunoprecipitation assay

The occupancy of CBP and acetylated histone (H3K9/14ac) at the *Npy* gene promoter was measured in the amgydala using the chromatin immunoprecipitation (ChIP) assay as previously described by us (Kyzar et al., 2017; Zhang et al., 2018). Briefly, amygdaloid tissues containing all major nuclei (CeA, MeA and BLA) were fixed in methanol free formaldehyde and subjected to DNA sonication to achieve DNA fragment size of 200–500 base pairs. The resulting DNA-chromatin complex was immunoprecipitated with 5 μg of either H3K9/14ac antibody (Millipore 06–599, Billerica, MA) or CBP antibody (Abcam 2832, Cambridge, MA) and then chromatin was eluted. DNA fragments were isolated and quantified by real-time quantitative PCR using primers designed within the promoter region of *Npy* gene (Set 1 F: ACTCGGGACTTCACAGGGTTTACA; R: GCGGTCCATGAGCTTCTGTATCTA; Set 2 F: TGCCCTTGCAGAGTTAGTCACAGT; R: AGACTCACCCAAATGTCCACAGCA; Set 3 F: GCGGGCCGCTTAGAATTGG; R: TTTATGGAGCGCCCTTGTCGC). Input DNA was used for the normalization as an internal control. After subtraction of the input DNA Ct value from the Ct value of each respective sample, the ΔΔCt method (Schmittgen and Livak, 2008) was used to determine the fold change in the levels of H3K9/14ac and CBP at the *Npy* gene promoter.

2.8. RNA isolation and real-time quantitative PCR

Total RNA was extracted from amygdala and was isolated using miRNeasy kit (Qiagen, Valencia, CA, USA). RNA was reverse transcribed using random hexamer primers and reverse transcriptase (Thermo Fisher Scientific, Waltham, MA,USA). Real-Time qPCR was performed on a CFX connect qPCR system (Bio-Rad, Hercules, CA, USA) with SYBR Green Master Mix (Thermo Fisher Scientific) using specific primers for *Npy* (F: CCAGACAGAGATATGGCAAGAG; R: GGGTCTTCAAGCCTTGTTCT), *Creb1* (F: TGTACCACCGGTATCCATGC; R: CCATGGACCTGGACTGTCTG), and *Cbp* (F: GACCTGGGATCTGCATGAAT; R: TGACTTGAGCTTGCCCTTGTT). Ct values for *Npy, Creb1, and Cbp* genes were normalized to the Ct value of Gapdh (F: CAATGTGTCCGTCGTGGATCT; R: GTCCTCAGTGTAGCCCAAGATG) as an internal control. Relative expression levels were calculated using the ΔΔCt method (Schmittgen and Livak, 2008).

2.9. Data analysis

The group differences in alcohol consumption were analyzed using a two-way repeated measures ANOVA followed by a Bonferroni post-hoc multiple comparison test. The effect of AM4113 on mean alcohol consumption, anxiety-like behavior and neurochemical data were evaluated by a Student t-test (two-tailed). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of AM4113 on alcohol consumption and anxiety-like behavior

We recently reported that AM4113 treatment decreases binge-like alcohol consumption in the limited-access drinking in the dark mouse model (Balla et al., 2018). Here, we used unlimited (24-h) two-bottle choice paradigm to examine the effect of daily treatment of AM4113 (1 mg/kg, i. p.) on alcohol consumption for three days. A two-way repeated measures ANOVA revealed a main effect of day ($F_{8,128} = 2.470$; $p = 0.0160$), a trend toward a main effect of AM4113 treatment on alcohol consumption ($F_{1,16} = 3.558$; $p = 0.0775$), and a significant interaction between AM4113 treatment and day ($F_{8,128} = 3.316$; $p = 0.0018$) (Fig. 1A). A Bonferroni post-hoc multiple comparison analysis showed a significant reduction in alcohol intake on day 7 ($t = 3.618$; $p = 0.0037$), day 8 ($t = 2.868$; $p = 0.0428$) and day 9 ($t = 2.877$; $p = 0.0417$) in AM4113 treated group compared to the vehicle treated control group. Treatment with AM4113 reduced mean daily alcohol consumption during the three treatment days compared to the vehicle treated group ($t_{16} = 2.966$; $p = 0.009$) (Fig. 1B). AM4113-treated mice spent more time exploring the light compartment of the LDB compared to vehicle-treated mice ($t_{21} = 2.104$; $p = 0.0476$) suggesting a small but statistically significant anxiolytic effect of AM4113 (Fig. 1C). Mice tested in the LDB do not differ in their general activity as entries to the dark compartment are not significantly different between treatment groups (Fig. 1D). These results suggest that AM4113 treatment is effective in attenuating continuous access alcohol consumption in male C57 mice and is anxiolytic in ethanol-naive mice. We then investigated the possible molecular mechanisms underlying these behavioral actions of the CB1 receptor neutral antagonist AM4113 as described below.

3.2. Effect of AM4113 on CREB signaling in the amygdala

Since CB1 receptors are negatively coupled to the cAMP second messenger pathway (Howlett, 1998; Pacher et al., 2006), we first examined the effect of AM4113 on protein levels of pCREB and CBP in amygdaloid brain structures. It was found that AM4113 treatment significantly increased the protein levels of pCREB in the CeA ($t_8 = 6.392$, $p < 0.001$) and MeA ($t_8 = 4.337$, $p = 0.002$) but not in the BLA as compared with the vehicle treated control group (Fig. 2A and B). In

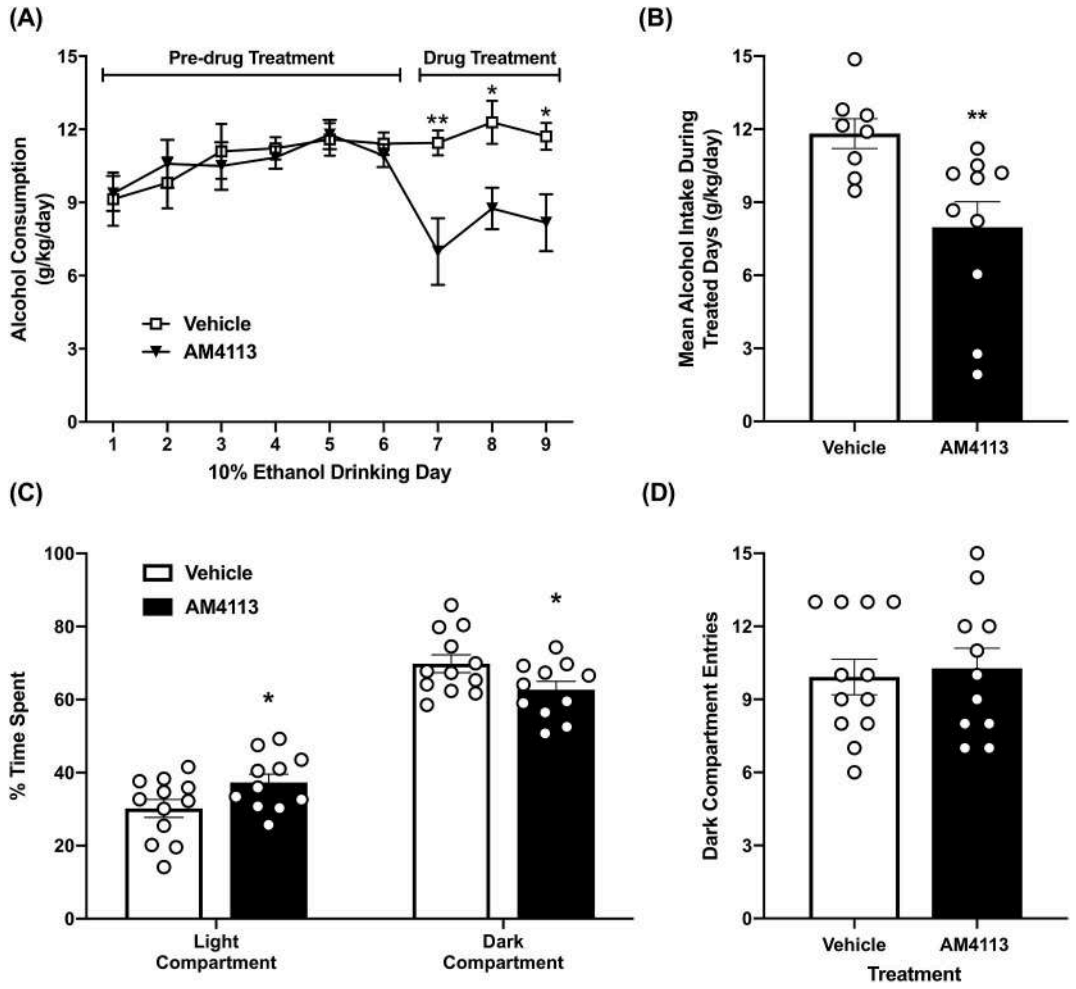

Fig. 1. Effect of AM4113 (1 mg/kg, i. p.) on alcohol drinking and anxiety-like behaviors in mice. (A) In a two-bottle free choice paradigm, AM4113 reduced consumption of 10% ethanol compared to vehicle treated group (*p < 0.05; **p < 0.01). (B) Bar graph shows the effect of AM4113 on mean alcohol intake (n = 8–10 in each group). (C) In the light-dark box (LDB) exploration test, AM4113 treatment increased time spent exploring the light compartment compared to vehicle treatment. (*p < 0.05). (D) AM4113 had no effect on general activity as entries to the dark compartment do not differ between AM4113-treated and vehicle-treated mice (n = 11–12 in each group). Overall values are represented as mean ± SEM and individual values are shown with open circles.

addition, we found a significant elevation in CBP levels in the CeA (t_{10} = 13.294, p < 0.001) and MeA (t_{10} = 13.350, p < 0.001) of AM4113 treated mice relative to the vehicle treated control mice (Fig. 2C and D). We also observed significant increases in mRNA levels of *Creb1* (t_{11} = 2.317, p = 0.041) and *Cbp* (t_{11} = 2.921, p = 0.014) in the amygdala after AM4113 treatment (Fig. 2E). These findings indicate that neutral antagonism of the CB1 receptor activates CREB and increases CBP levels in the amygdaloid structures of mice.

3.3. Effect of AM4113 on global histone acetylation in the amygdala

It has been shown that CBP has acetyltransferase activity that regulates gene expression by acetylation of histones (Bannister and Kouzarides, 1996; Berkel and Pandey, 2017). Thus, we examined the effects of AM4113 on the status of two epigenetic marks of transcriptional activation in the amygdaloid structures: H3K9ac and H3K14c. It was found that AM4113 treatment significantly increased the protein levels of acetylated histones in the CeA (t_{10} = 8.687, p < 0.001 for H3K9ac; t_{10} = 3.815, p = 0.003 for H3K14ac) and MeA (t_{10} = 9.743, p < 0.001 for H3K9ac; t_{10} = 4.276, p = 0.002 for H3K14ac) compared to the vehicle group (Fig. 3). There were no significant changes in the levels of these proteins in the BLA (Fig. 3). These results reveal that treatment with a neutral antagonist of CB1 receptors increased acetylation at two active epigenetic histone lysine marks, and further suggest that CB1 receptor modulation may impact downstream gene expression in the amygdala via global effects on the epigenome.

3.4. Effect of AM4113 on occupancy of acetylated histone and CBP at Npy gene promoter and NPY expression

NPY has been shown to regulate alcohol drinking behaviors (Pandey et al., 2003; Thiele et al., 1998; Robinson and Thiele, 2017), and higher levels of NPY in the CeA is associated with reduction in alcohol drinking and anxiety-like behaviors (Gilpin et al., 2008; Zhang et al., 2010). To determine the modulatory effects of CB1 receptor neutral antagonism on NPY expression through epigenetic mechanisms, ChIP analysis was performed using an H3K9/14ac and CBP antibodies at three locations of the *Npy* gene promoter in the amygdala (Fig. 4A) after AM4113 treatment. We found an elevation in H3K9/14ac levels at the *Npy* gene promoter location 1 (t_{10} = 2.567, p = 0.0280); location 2 (t_{10} = 3.248, p = 0.0088); location 3 (t_{10} = 2.528, p = 0.03) compared to vehicle control group (Fig. 4B). In addition, AM4113 treatment increased occupancy of CBP at the *Npy* gene promoter location 2 (t_{15} = 2.589, p = 0.0205) and location 3 (t_{16} = 4.193, p = 0.0007) but produced no change at location 1 (t_{16} = 0.6325, p = 0.5360) compared to the vehicle control group (Fig. 4C). Treatment with AM4113 also led to a significant increase in *Npy* mRNA levels in the amygdala as compared to the vehicle treated group (t_{11} = 3.641, p = 0.0039; Fig. 4D). This increase in *Npy*

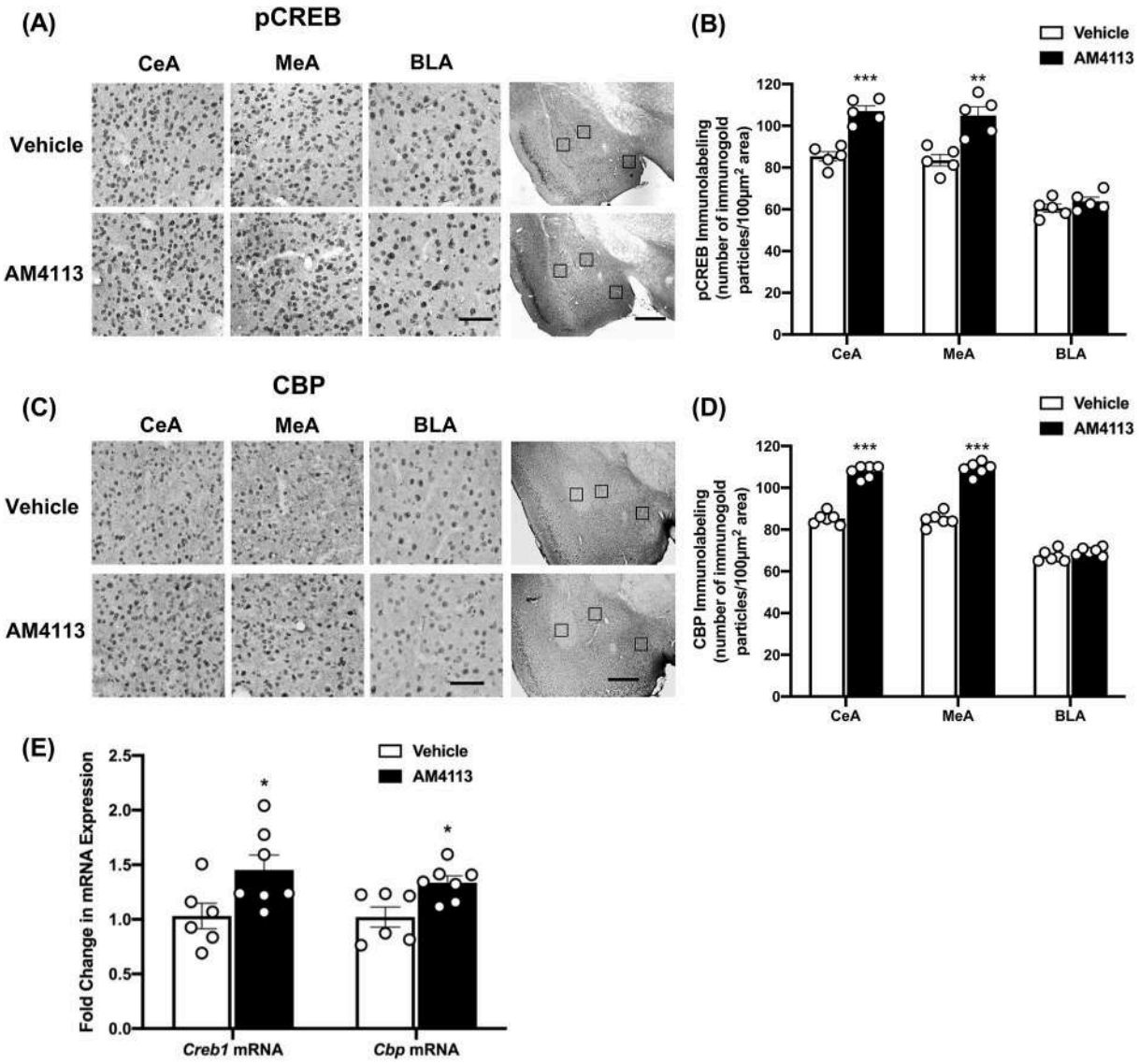

Fig. 2. Alteration in the protein levels of phosphorylated CREB (pCREB) and CREB binding protein (CBP) and the mRNA levels of *Creb1* and *Cbp* in amygdala of mice treated with AM4113 (1 mg/kg, i. p.) or vehicle. (A) Representative photomicrographs of high magnification (scale bar = 50 μm) show the gold immunolabeling of pCREB in the central (CeA), medial (MeA), and basolateral (BLA) nuclei of amygdala of AM4113 or vehicle treated mice. The locations of these amygdaloid areas are shown by box at low magnification (scale bar = 1 mm) images of amygdala of AM4113 or vehicle treated mice. (B) Bar graph depicts pCREB protein levels in amygdaloid brain regions after AM4113 treatment compared to vehicle group (**$p < 0.01$; ***$p < 0.001$; $n = 5$ in each group). (C) Representative photomicrographs of high magnification (scale bar = 50 μm) show the gold immunolabeling of CBP in CeA, MeA and BLA in mice treated with AM4113 or vehicle. The locations of these amygdaloid areas are shown by box at low magnification (scale bar = 1 mm) images of amygdala of AM4113 or vehicle treated mice. (D) Bar graph depicts levels of CBP protein in CeA, MeA and BLA following AM4113 treatment compared to vehicle group (***$p < 0.001$; $n = 6$ in each group). (E) Bar graph depicts *Creb1* and *Cbp* mRNA expression levels in the amygdala of mice treated with AM4113 or vehicle (*$p < 0.05$, $n = 6–7$ in each group). Individual values are shown with open circles and overall values are represented as mean ± SEM.

mRNA levels is associated with an enhancement of NPY protein expression in the CeA ($t_8 = 2.622$, $p = 0.031$) and MeA ($t_8 = 3.031$, $p = 0.016$), but not in the BLA of mice (Fig. 4E and F). These findings demonstrate that AM4113 treatment increases histone acetylation (H3K9/14ac) via increases in CBP at the *Npy* gene promoter, thereby increasing expression of *Npy* mRNA levels and ultimately NPY protein levels in the CeA and MeA. These molecular effects of AM4113 treatment in the amygdala may be responsible for reducing alcohol consumption and decreasing anxiety-like behavior.

4. Discussion

The goal of this study was to investigate the underlying molecular mechanisms of the attenuation of alcohol drinking behavior by CB1 receptor neutral antagonist AM4113. Our novel findings demonstrate that CB1 receptor blockade increases NPY expression via epigenetic mechanisms in the amygdala, which may be an important neuronal mechanism in the reduction of alcohol intake. Our data also underscore the ability of CB1 receptor signaling to modulate chromatin remodeling, and thereby regulate the expression of downstream genes. Particularly, we found that CB1 receptor blockade decreases alcohol consummatory and anxiety-like behaviors while producing increases in pCREB, CBP, global and *Npy*-specific histone acetylation, and NPY expression (Fig. 5).

Amygdaloid structures serve as important neuroanatomical substrates for anxiety and alcohol drinking behaviors (McBride, 2002; Koob et al., 2014; Koob and Mason, 2016; Pandey et al., 2006). The CeA represents a critical hub for the interaction of several neuromodulators such as NPY, corticotropin-releasing factor (CRF), and CB1 receptor

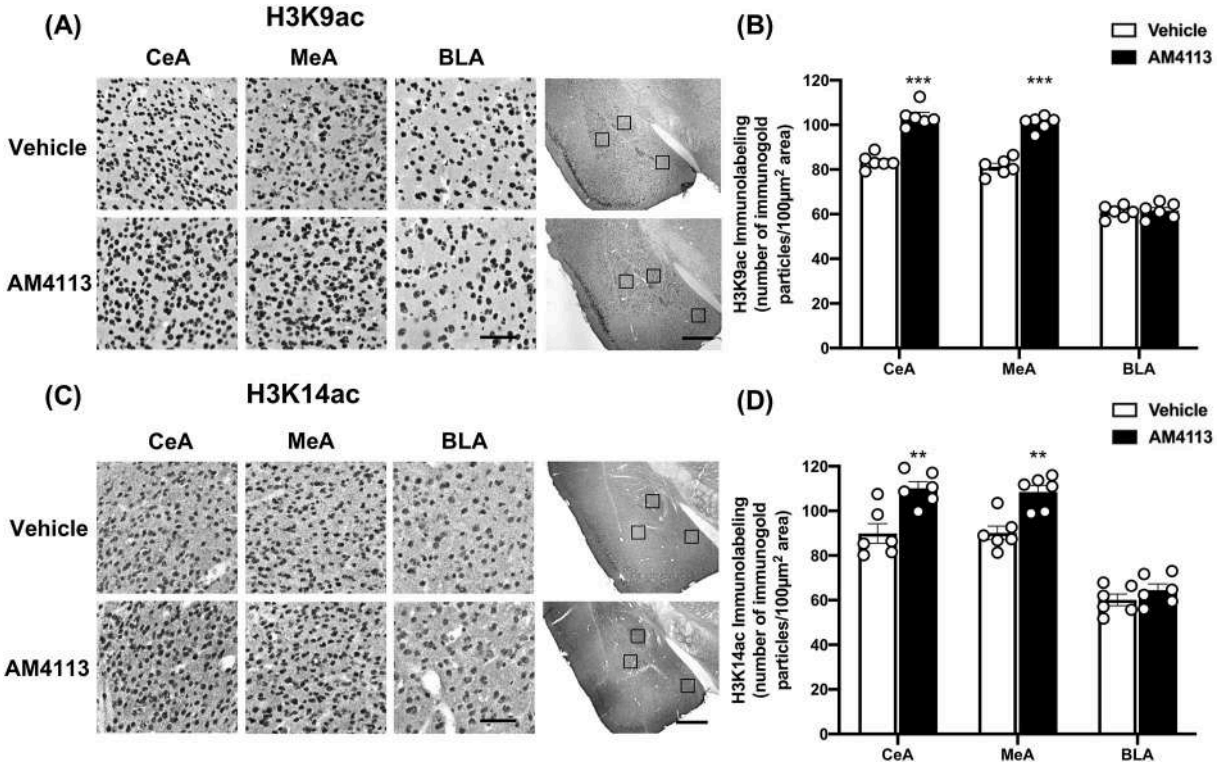

Fig. 3. Effect of AM4113 treatment on the global protein levels of acetylated histone H3K9 (H3K9ac) and acetylated histone H3K14 (H3K14ac). (A) Representative photomicrographs of high magnification (scale bar = 50 μm) of H3K9ac gold-immunolabeling in central (CeA), medial (MeA) and basolateral (BLA) nuclei of amygdala of mice treated with either vehicle or AM4113 (1 mg/kg, i. p.). The location of these amygdaloid areas are shown by box at low magnification (scale bar = 1 mm) images of amygdala of AM4113 or vehicle treated mice. (B) Bar graph shows levels of H3K9ac in CeA, MeA and BLA of AM4113 and vehicle treated mice (***$p < 0.001$; n = 6 in each group). (C) Representative photomicrographs of high magnification (scale bar = 50 μm) of H3K14ac gold-immunolabeling in CeA, MeA and BLA in mice treated with either vehicle or AM4113 (1 mg/kg). The locations of these amygdaloid areas are shown by box at low magnification (scale bar = 1 mm) images of amygdala of AM4113 or vehicle treated mice. (D) Bar graph shows levels of H3K14ac in CeA, MeA and BLA following administration of AM4113 compared to vehicle group (**$p < 0.01$; n = 6 in each group). Individual values are shown with open circles and overall values are represented as mean ± SEM.

signaling to regulate anxiety and alcohol drinking behaviors (Roberto et al., 2010, 2012; Gilpin, 2012). Optogenetic activation of projections from BLA to CeA produces anxiolytic effects, and inhibition of these projections causes anxiety-like behaviors (Tye et al., 2011). Furthermore, CeA neurons consist of mainly GABAergic projection and interneurons, and the lateral CeA sends projections to medial CeA internally; dynamic interactions between these synaptic arrangements also regulate anxiety and fear (Roberto et al., 2012; Gilpin, 2012; Janak and Tye, 2015). NPY and GABA are colocalized together and NPY modulates GABAergic neurotransmission in the CeA during alcohol exposure (Gilpin, 2012; Gilpin et al., 2011). Interestingly, GABAergic function in the CeA is also modulated by alcohol-induced disruptions of CB1 receptors (Varodayan et al., 2016). Our study extends these important functional studies in the CeA to the molecular level to suggest that CB1 receptor antagonist (AM4113) treatment upregulates NPY expression in the CeA and MeA to regulate anxiety and alcohol drinking behaviors, most likely via an epigenetic mechanism.

Deficits in NPY expression in the CeA are associated with alcohol-related behaviors, particularly an increase in alcohol intake (Gilpin et al., 2008; Thorsell et al., 2005, 2007; Zhang et al., 2010). For example, expression of NPY in the CeA of P rats is lower than that of alcohol non-preferring (NP) rats, and infusion of NPY into the CeA has been shown to attenuate alcohol consumption in P rats (Pandey et al., 2005; Suzuki et al., 2004; Zhang et al., 2010) and notably also reduces anxiety-like behaviors (Pandey et al., 2005; Zhang et al., 2010). Viral-mediated overexpression of the *Npy* gene in the CeA has also been shown to suppress alcohol preference in alcohol dependent rats, as well as in anxious rats (Gilpin et al., 2008: Primeaux et al., 2006; Thorsell et al., 2005, 2007) while additionally reducing anxiety-like behavior in the open-field test (Thorsell et al., 2007). We previously showed that deficits in NPY expression in the amygdala of P rats correspond with lower histone acetylation of the *Npy* gene as compared with NP rats (Sakharkar et al., 2014). Interestingly, deficits in *Npy* mRNA and protein levels in the CeA and MeA were normalized by increasing histone acetylation after treatment with a histone deacetylase inhibitor, trichostatin A (TSA). Behaviorally, anxiety and alcohol consumption in P rats were both attenuated by TSA treatment (Sakharkar et al., 2014). Ethanol withdrawal-induced decrease of NPY levels in the CeA and MeA can also be reversed by TSA treatment, which is associated with a reduction of anxiety-like behavior during ethanol withdrawal in rats (Pandey et al., 2008; Roy and Pandey, 2002). These data suggest that histone acetylation mediated changes in NPY expression in the amygdala are crucially involved in alcohol drinking and anxiety-like behaviors and our present data further highlights the importance of these histone acetylation mechanisms driving changes in amygdaloid NPY expression and subsequent behavior.

CREB also plays an important role in the neurobiological effects of alcohol and drugs of abuse (McClung and Nestler, 2003; Pandey, 2003). For instance, a decrease in amygdalar CREB function is causally linked to reduced NPY expression and increased alcohol drinking and anxiety-like behaviors (Pandey et al., 2004, 2005). These studies suggest that NPY is a CREB target and that decreased CREB function leads to a decrease in NPY, in turn promoting alcohol intake and anxiety-like behaviors (Pandey, 2003). We report here that CB1 receptor antagonism led to a reduction in alcohol consumption and anxiety-like behavior. Furthermore, inhibition of CB1 receptor function by AM4113 resulted in increased NPY expression via increases in both global and *Npy*-specific CBP and histone acetylation (H3K9/14ac) in the amygdala. The CB1

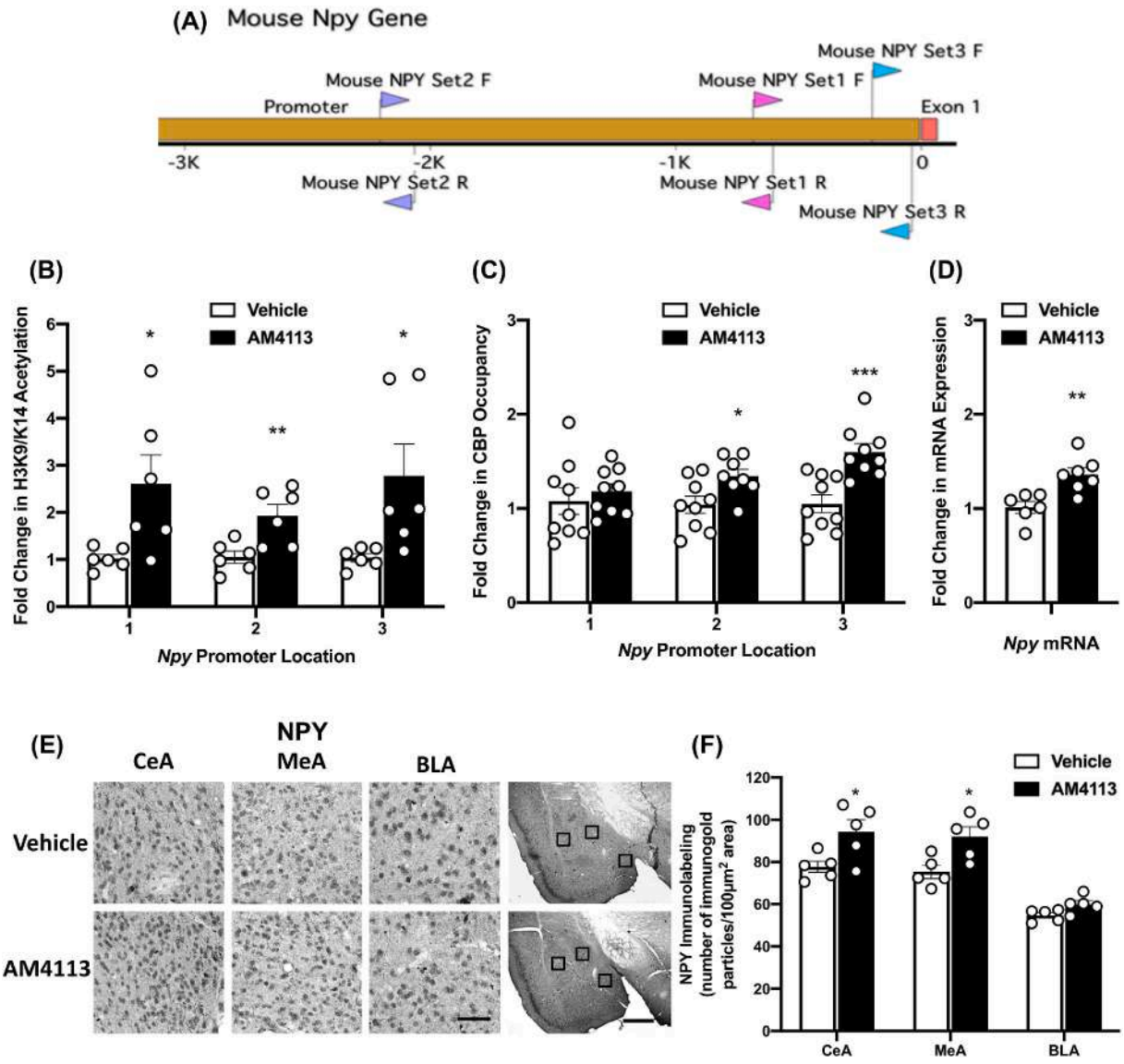

Fig. 4. Effects of AM4113 treatment (1 mg/kg, i. p.) on NPY-specific changes in CBP and H3K9/14ac as well as NPY protein and mRNA expression in the amygdala. (A) Gene diagram showing three locations of primers (location 1-primer set 1; location 2-primer set 2; and location 3-primer set 3) used to measure occupancy of CBP and H3K9/14ac at NPY gene promoter in amygdala using chromatin immunoprecipitation (ChIP) assay. (B) A significant increase in the occupancy of H3K9/14ac at all three locations was observed following treatment with AM4113 compared to vehicle treated group (*$p < 0.05$; **$p < 0.01$; $n = 6$ in each group). (C) A significant increase in the occupancy of CBP at locations 2 and 3 but not at location 1 of *Npy* promoter was observed following treatment with AM4113 compared to vehicle treated group (*$p < 0.05$; ***$p < 0.001$; $n = 8–9$ in each group). (D) The measurement of *Npy* mRNA level revealed an increase in *Npy* mRNA levels in amygdala following treatment with AM4113 (**$p < 0.01$; $n = 6–7$ in each group). (E) Representative photomicrographs of high magnification (scale bar = 50 μm) of gold immunolabeling of NPY in central (CeA), medial (MeA), and basolateral (BLA) nuclei of amygdala of mice. The locations of these amygdaloid areas are shown by box at low magnification (scale bar = 1 mm) images of amygdala of AM4113 or vehicle treated mice. (F) Bar graph shows NPY protein levels in CeA, MeA, and BLA of mice treated with AM4113 compared to vehicle treated group (*$p < 0.05$; $n = 5$ in each group). Individual values are shown with open circles and overall values are represented as mean ± SEM.

receptors are expressed in amygdala and are negatively coupled to the cAMP signaling pathway (Howlett, 1998; McDonald and Mascagni, 2001; Pacher et al., 2006; Stincic and Hyson, 2008). CREB appears to be a common denominator for the interaction of both CB1 and NPY signaling. This notion is supported by the findings that CB1 antagonism leads to increases in the mRNA levels of *Creb1* and *Cbp* and their protein levels in the amygdala. Thus, the decrease in ethanol consumption and anxiolytic effects produced by AM4113 are most likely due to the observed increase in CREB phosphorylation, as well as increased NPY expression in the CeA and MeA of mice.

The epigenetic modifications mediated by histone acetylation in the amygdaloid circuitry and other brain regions have been shown to regulate alcohol drinking behaviors (Moonat et al., 2013; Sakharkar et al., 2014; Starkman et al., 2012; Renthal and Nestler, 2008). In the present study, we observed increased acetylation of H3K9 and H3K14 globally in the CeA and MeA by AM4113 treatment. This somewhat concurs with the results of a previous study that associated lower H3K9ac in the CeA and MeA with a phenotype of higher alcohol intake in rodent models of AUD (Kokare et al., 2017; Moonat et al., 2013; Pandey et al., 2015). To further explore the relationship between histone acetylation and NPY expression after CB1 receptor blockade, ChIP assays revealed an increase in occupancy of CBP and H3K9/14ac at multiple sites in the *Npy* gene promoter in the amygdala. These changes were associated with increased mRNA levels in the amygdala and protein levels of NPY in the CeA and MeA, but not in the BLA of mice. The data indicate that blocking CB1 receptors leads to enhanced

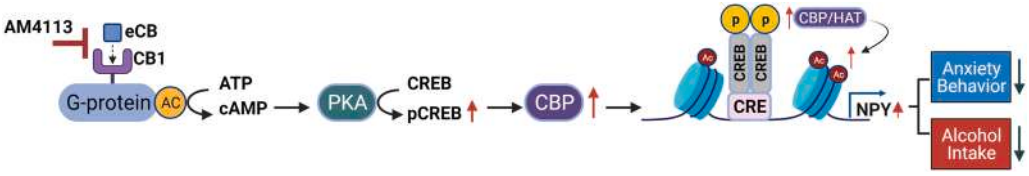

Fig. 5. Molecular mechanism of CB1 receptor-mediated attenuation of alcohol drinking and anxiety-like behavior. The blockade of CB1 receptors with neutral CB1 antagonist AM4113 leads to an increase in cAMP mediated CREB phosphorylation as these receptors are negatively coupled to adenylate cyclase (AC) and increase the levels of CREB binding protein (CBP). Direct or indirect activation of CBP/CREB signaling increases CBP and histone acetylation globally and at the *Npy* gene specifically leading to increased NPY expression in the amygdala. This CB1 receptor-mediated modulation of NPY expression through epigenetic modification via histone acetylation may be one of the important molecular mechanisms in the amygdala underlying regulation of alcohol consumption and anxiety-like behavior. eCB, endocannabinoid; CB1, Cannabinoid Receptor 1; cAMP, Cyclic adenosine monophosphate; PKA, Protein kinase A; CREB, cAMP-response element binding protein; pCREB, phosphorylated CREB; CBP, CREB binding protein; CRE, cAMP response element site; NPY, Neuropeptide Y.

phosphorylation of CREB which in turn activates CBP and leads to higher histone acetylation at the *Npy* gene promoter and enhancement of the transcriptional machinery of the *Npy* gene. Collectively, the present study clearly demonstrates enhancement of NPY expression at the transcriptional level is driven by epigenetic mechanisms after CB1 receptor blockade involving CREB-CBP mediated histone acetylation specifically in the CeA and MeA (Fig. 5).

Although CB1 receptors are highly expressed in the BLA (McDonald and Mascagni, 2001; Stincic and Hyson, 2008), their blockade did not produce any significant effect on the signaling cascade studied in this brain region. This finding seems to coincide with the results of an earlier study that showed that lesions to the CeA, but not to the BLA, caused decreased anxiety levels and alcohol intake in rats (Möller et al., 1997). The reason for this region-specific change is unclear at present, but it may be related to the existence of different cell types (Polepalli et al., 2020) and the output of presynaptic CB1-expressing cells onto postsynaptic neurons. While endocannabinoid signaling classically occurs in a retrograde manner (Castillo et al., 2012), emerging evidence suggests that interneurons can signal via an autocrine endocannabinoid mechanism (Bacci et al., 2004; Marinelli et al., 2009), and that astrocytes expressing CB1 receptors can modulate responses at more distant synapses (Navarrete and Araque, 2010). Since CB1 receptors are largely expressed presynaptically (Piomelli, 2003) and epigenetic alterations require access to DNA in the nucleus (Kouzarides, 2007), the epigenetic changes induced by CB1 receptor blockade observed in the current study may be due to autocrine CB1 receptor blockade or the cumulative effect of multiple actions of presynaptic CB1 receptors on postsynaptic signaling. Here, we report that treatment with AM4113 (1 mg/kg) produces subtle anxiolytic effects in LDB test in mice. This behavioral finding is in concordance with increased levels of NPY in the amygdala. One earlier study reported that lower (1 mg/kg) and higher doses of AM4113 (3 mg/kg or 10 mg/kg) produce no effects on anxiety-like behavior in the elevated plus maze in rats, but there is an antidepressant effect in the forced swim test at all three doses (Gueye et al., 2016). The differences in anxiety measures between the present findings and those of Gueye et al. (2016) could be related to differences in dose and duration of drug treatment as well as animal species and behavioral model. Nonetheless, these behavioral findings suggest that AM4113 treatment, unlike rimonabant (Kirilly et al., 2012), is devoid of liability to provoke anxiety or depression-like behaviors but still retains efficacy in decreasing alcohol intake in mice. The reduction of alcohol intake by CB1 neutral antagonist AM4113 treatment as observed here is consistent with the results of previous studies performed using CB1 inverse agonists (Femenía et al., 2010; Pava and Woodward, 2012; Vinod et al., 2008a; Wang et al., 2003; Zhou et al., 2016). Our recent study showed that AM4113 decreases binge-like alcohol consumption and alcohol-induced dopamine (DA) release in the nucleus accumbens (NAc) (Balla et al., 2018), which suggests that AM4113 may also regulate reward properties of alcohol drinking by blocking the release of accumbal DA. It remains to be examined whether AM4113 treatment is effective in reducing alcohol intake in female mice as well as treating alcohol dependence. Also, further study is needed to elucidate how epigenetic consequences of AM4113 treatment alter other amygdaloid gene network pathways or NPY signaling and non-neuronal immune responses in other brain regions of interest to addiction.

In summary, the present study identifies an important molecular mechanism by which CB1 receptors epigenetically regulate NPY function via histone acetylation in the amygdala. Furthermore, our findings suggest that AM4113 treatment increases CREB-CBP signaling and increases two active marks of histone acetylation both globally and specifically at the NPY promoter. Taken together, the data supports the potential use of CB1 receptor neutral antagonists for the treatment of excessive alcohol drinking.

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Author contributions

SCP and KYV conceived the study and designed experiments. RSD, KYV, BD, HZ, RB and HRK performed the experiments. RSD, HZ, KYV and HRK analyzed the data and prepared the figures. RSD, BLH, KYV and SCP interpreted the data. RSD, KYV and SCP wrote the manuscript. All authors read, edited and approved the final version of the manuscript. Overall, SCP supervised the study.

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