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Adolescent Alcohol Exposure-Induced Changes in Alpha-Melanocyte Stimulating Hormone and Neuropeptide Y Pathways via Histone Acetylation in the Brain During Adulthood

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Abstract

**Background:** Adolescent intermittent ethanol exposure causes long-lasting alterations in brain epigenetic mechanisms. Melanocortin and neuropeptide Y signaling interact and are affected by ethanol exposure in the brain. Here, the persistent effects of adolescent intermittent ethanol on alpha-melanocyte stimulating hormone, melanocortin 4 receptor, and neuropeptide Y expression and their regulation by histone acetylation mechanisms were investigated in adulthood.

**Methods:** Male rats were exposed to adolescent intermittent ethanol (2 g/kg, i.p.) or volume-matched adolescent intermittent saline from postnatal days 28 to 41 and allowed to grow to postnatal day 92. Anxiety-like behaviors were measured by the elevated plus-maze test. Brain regions from adult rats were used to examine changes in alpha-melanocyte stimulating hormone, melanocortin 4 receptor, and neuropeptide Y expression and the histone acetylation status of their promoters.

**Results:** Adolescent intermittent ethanol-exposed adult rats displayed anxiety-like behaviors and showed increased pro-opiomelanocortin mRNA levels in the hypothalamus and increased melanocortin 4 receptor mRNA levels in both the amygdala and hypothalamus compared with adolescent intermittent saline-exposed adult rats. The alpha-melanocyte stimulating hormone and melanocortin 4 receptor protein levels were increased in the central and medial nucleus of the amygdala, paraventricular nucleus, and arcuate nucleus of the hypothalamus in adolescent intermittent ethanol-exposed adult rats compared with adolescent intermittent saline-exposed adult rats. Neuropeptide Y protein levels were decreased in the central and medial nucleus of the amygdala of adolescent intermittent ethanol-exposed adult rats compared with adolescent intermittent saline-exposed adult rats. Histone H3K9/14 acetylation was decreased in the neuropeptide Y promoter in the amygdala but increased in the melanocortin 4 receptor gene promoter in the amygdala and the melanocortin 4 receptor and pro-opiomelanocortin promoters in the hypothalamus of adolescent intermittent ethanol-exposed adult rats compared with controls.

**Conclusions:** Increased melanocortin and decreased neuropeptide Y activity due to changes in histone acetylation in emotional brain circuitry may play a role in adolescent intermittent ethanol-induced anxiety phenotypes in adulthood.
**Significance Statement**

Adolescence is an important period for the maturation of the brain, and exposure to alcohol during this period can have long-lasting effects on brain biology and function. Here, we show that exposing rats to intermittent, binge-like alcohol exposure during adolescence increases the expression of the stress-related neuropeptide alpha-melanocyte stimulating hormone (α-MSH) and its receptor, the melanocortin 4 receptor (MC4R), and decreases expression of the anti-stress hormone neuropeptide Y (NPY) in emotional circuitry during adulthood. We also observe increased histone H3 acetylation, an epigenetic mark associated with increased transcription, at the promoters of both *Pomc* (the mRNA precursor to α-MSH) and *Mc4r* in the amygdala and hypothalamus of adult animals exposed to alcohol in adolescence. On the other hand, decreased histone acetylation at the *Npy* gene promoter is associated with decreased NPY levels in amygdala of adult rats after adolescent ethanol exposure. The imbalance between the melanocortin and NPY systems in emotional brain circuitry in adulthood after adolescent alcohol exposure may be related to changes in gene-specific histone H3 acetylation and possibly contributes to adult psychopathology.

**Keywords:** alcohol, adolescence, alpha-melanocyte stimulating hormone, melanocortin 4 receptor, neuropeptide Y, histone H3 acetylation

**Introduction**

Binge drinking in underage populations is a leading public health concern and societal issue (Brown et al., 2009; Witt, 2010; Patrick et al., 2013). Adolescence is a crucial period for brain maturation, involving the stabilization of synapse formation, grey matter integrity, and axonal projections (Keshavan et al., 2014). Adolescent exposure to alcohol affects the normal trajectory of the developing brain (Keshavan et al., 2014; Spear and Swartzwelder, 2014; Kyzar et al., 2016a). Early onset of alcohol use and adolescent binge consumption lead to an increased risk of alcohol use disorder and comorbid psychiatric diagnoses in adulthood (Grant and Dawson, 1997; DeWit et al., 2000; Witt, 2010).

Recently, stress-related molecules such as those involved in melanocortin signaling have emerged as a novel target of the effects of alcohol on the brain (Olney et al., 2014; Roltsch Hellard et al., 2017). Melanocortins are derived from a prohormone mRNA, pro-opiomelanocortin (Pomc), that is spliced to yield mature protein products including alpha-melanocyte stimulating hormone (α-MSH), along with β-MSH, γ-MSH, and adrenocorticotropic hormone. α-MSH exerts its effects by binding to and activating melanocortin receptors, such as melanocortin 3 receptor (MC3R) and MC4R. These receptors are G-protein coupled receptors that show wide distribution in key brain areas including the amygdala and hypothalamus (Kishi et al., 2003; Liu et al., 2003; Mountjoy, 2010; Doros et al., 2014). α-MSH induces anxiogenic behavior in rodents, and alcohol withdrawal-induced anxiety-like behavior is attenuated by central administration of a specific MC4R antagonist in adult rats (Kokare et al., 2006). Alcohol withdrawal increases immunoreactivity of α-MSH in specific neurocircuitry including the arcuate nucleus of the hypothalamus (ARC), paraventricular nucleus of the hypothalamus (PVN), and the central nucleus of the amygdala (CeA) (Kokare et al., 2008). These brain circuits are implicated in addictive processes (Koob and Volkow, 2010). The brain melanocortin system, including the lateral hypothalamus and ventral tegmental area, has been shown to be involved in the regulation of ethanol consumption (Olney et al., 2014; Shelkar et al., 2015; Sprod et al., 2016). On the other hand, neuropeptide Y (NPY) is an anti-stress molecule, and several studies suggest that lower NPY levels in the amygdala are associated with anxiety-like behaviors during withdrawal after chronic ethanol exposure and promote alcohol intake in adult rats (Badia-Elder et al., 2001; Thorsell et al., 2007; Gilpin et al., 2008a, 2008b; Zhang et al., 2010; Gilpin, 2012; Sparrow et al., 2012). In fact, α-MSH and NPY interact in opposing ways in the amygdala to regulate alcohol-related behaviors (Kokare et al., 2005).

Epigenetic processes are major regulators of ethanol action in the brain (Starkman et al., 2012; Kyzar and Pandey, 2015; Berkel and Pandey, 2017). Adolescent intermittent ethanol (AIE) exposure of rats causes long-lasting increases in anxiety-like and alcohol drinking behaviors and deficits in global histone H3K9 acetylation in the hippocampus and amygdala, as well as deficits in hippocampal neurogenesis, in adulthood (Vetrano et al., 2014; Pandey et al., 2015; Vetrano and Crews, 2015; Sakharkar et al., 2016). AIE-induced anxiety-like behavior in adulthood coincides with decreased dendritic spine density and expression of brain-derived neurotropic factor (BDNF) in the central (CeA) and medial nucleus of the amygdala (MeA) (Pandey et al., 2015), which are involved in the negative affective states of alcoholism (Koob and Le Moal, 2005; Koob and Volkow, 2010; Koob et al., 2014; Pandey et al., 2017).

The role of the melanocortin system and its regulation by histone acetylation mechanisms in the persistent effects of AIE in adulthood has not been explored previously. Also, the epigenetic regulation of NPY expression in the amygdala in adulthood after adolescent alcohol exposure is unknown. We therefore investigated the contribution of α-MSH and MC4R in the amygdala and hypothalamus and NPY in the amygdala to the lasting effects of AIE in adulthood. We measured mRNA expression of *Pomc* and *Mc4r* in the amygdala and hypothalamus. We measured NPY protein levels in the amygdala and α-MSH and MC4R protein levels in the amygdala and hypothalamus to identify nuclei-specific changes in AIE adult rats compared with AIS adult rats. In addition, we measured the occupancy of acetylated histone H3K9/14 in the promoter region of *Mc4r* and *Npy* in the amygdala and the *Mc4r* and *Pomc* promoter regions in the hypothalamus to better understand the epigenetic regulation of the melanocortin and NPY systems by AIE in adulthood.

**METHODS**

**Experimental Animals and Behavioral Testing**

Pregnant Sprague-Dawley rats were purchased from Harlan Laboratories and housed in a 12-hour-light/-dark cycle with...
drinking water and food ad libitum. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee. Male rat pups (n = 53) were weaned at postnatal day (PND) 21 and were group-housed (2 or 3 rats) with access to water and food. Rats were randomly assigned for exposure to adolescent intermittent ethanol (AIE) or normal saline (AIS) treatment. Rats received one dose of ethanol (2 g/kg, 20% v/v; AIE) or volume-matched saline (AIS) via i.p. injection per day for 2 consecutive days, followed by 2 days without ethanol or saline treatment for a total of 8 injections during PND 28 to 41, utilizing an exposure paradigm used by our laboratory (Pandey et al., 2015; Kyzar et al., 2016b; Sakharkar et al., 2016) and other laboratories (Fasciul et al., 2009; Alaux-Cantin et al., 2013). Both groups of rats were allowed to mature to PND 92 without further treatment and were subjected to the elevated plus maze (EPM) test for anxiety-like behaviors to replicate previous studies (Pandey et al., 2015; Kyzar et al., 2016b) as described by our laboratory and others (File, 1993; Pandey et al., 2006; Sakharkar et al., 2012).

Brain Tissue Collection

On PND 92 immediately after behavioral testing, animals were anesthetized (pentobarbital 50 mg/kg), and brain tissues were dissected and quickly frozen for biochemical studies. Some rats were perfused with normal saline followed by 4% paraformaldehyde solution prepared in phosphate buffer (pH 7.4) as described previously (Pandey et al., 2006, 2015). Brains were isolated and post-fixed overnight in paraformaldehyde and soaked in graded sucrose solutions (10%, 20%, and 30%). All brains were frozen and kept at −80°C until further use for either biochemical studies or immunohistochemical staining of α-MSH, MC4R, and NPY proteins.

mRNA Analysis of Pomc and Mc4r by Quantitative Real-Time PCR

Total RNA was extracted from amygdala and hypothalamic tissues from AIE and AIS adult rats using TRIzol reagent (Life Technologies) followed by the Qiagen miRNAeasy Mini protocol & DNase kit as described previously (Centanni et al., 2014; Kyzar et al., 2016b). Total RNA from each sample was reverse transcribed at 37°C for 2 hours using the high capacity cDNA RT kit (Life Technologies). The reaction was stopped by a 5-minute incubation at 85°C. Pomc and Mc4r mRNA levels were quantified by real-time polymerase chain reaction (PCR) using Maxima SYBR Green (Thermo Scientific). Hypoxanthine phosphoribosyltransferase 1 (Hprt1) was used as a reference gene, and PCR conditions for all primers were 3 minutes at 95°C followed by 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C for 40 cycles. The sequences for each primer set have been provided in Table 1. Quantitative PCR was performed using the Mx3000P qPCR system and analyzed with MxPro software (Agilent Technologies). The c(t) value of the gene of interest was corrected with the c(t) value of the respective control gene (c(t) control), and the respective fold changes were calculated using the 2−ΔΔc(t) method (Livak and Schmittgen, 2001) as reported earlier (Kyzar et al., 2016b). Data are presented as average fold change compared with AIS adult rats.

Gold Immunolabeling for α-MSH, MC4R, and NPY

Gold immunolabeling was employed for the measurement of NPY protein levels in the amygdala and α-MSH and MC4R protein levels in the amygdala and hypothalamus, as our laboratory has previously described (Pandey et al., 2008; Moonat et al., 2013; Sakharkar et al., 2014). Briefly, brains were cut into 20-μm-thick coronal sections and immunostained using antibodies against α-MSH (orb13589, Biorbyt Ltd; 1:200 dilution), MC4R (orb214232, Biorbyt Ltd.; 1:200 dilution), and NPY (22940, Immunostar; 1:500 dilution). Immunogold particles were quantified using the Computerized Image Analyzer (Loats Associates) connected to a light microscope. The threshold of a non-immunostained area was set to zero. After threshold determination, the software counted the number of immunogold particles/100 μm² area within 3 object areas from each of 3 bregma-matched brain sections from each rat at high magnification (100x). Values were averaged from 9 total object fields for each rat, and results are represented as number of immunogold particles per 100-μm² area.

Chromatin Immunoprecipitation (ChIP) Assay

Histone H3K9/14 acetylation at specific loci in the gene promoter regions was measured in the amygdala (Mc4r and Npy) and hypothalamus (Pomc and Mc4r) of AIE and AIS adult rats using ChIP as described previously (Moonat et al., 2013; Pandey et al., 2015; Kyzar et al., 2016b). We evaluated only the epigenetic state of the promoter regions where we observed a change in mRNA levels. Therefore, we did not quantify histone acetylation at the promoter of Pomc in the amygdala. Amygdala and hypothalamic tissue were fixed with methanol-free formaldehyde and subjected to DNA shearing via sonication. The resulting DNA-chromatin complex was immunoprecipitated with an antibody directed to acetylated H3K9/14 (06-599, Millipore), and the precipitated DNA was quantified using qPCR with a SYBR Green master mix (Bio-Rad) and primers designed for distinct regions of the Mc4r, Pomc, and Npy promoter regions (Table 1). After subtraction of input DNA amplification, the 2−ΔΔc(t) method (Livak and Schmittgen, 2001) was
Effects of AIE Exposure on Anxiety and α-MSH and MC4R Expression in the Amygdala and Hypothalamus in Adulthood

We reported recently that AIE induces anxiety-like behaviors in rats during adulthood (Pandey et al., 2015; Kyzar et al., 2016b; Sakhar kar et al., 2016). Here, we replicated our previous findings and found that AIE rats tested at PND 92 displayed decreases in the percentage of time spent in the open arms [t (20) = 5.37, P < .001] and in the percentage of open arm entries [t (20) = 6.37, P < .001] compared with AIS adult rats in the EPM (Figure 1). AIE adult rats displayed no change in closed arm entries compared with AIS adult rats. These results suggest that AIE produces anxiety-like behaviors in adulthood and further confirmed our published findings (Pandey et al., 2015; Kyzar et al., 2016b).

We measured the expression of Pomc and Mc4r mRNA in the amygdala of adult rats exposed to AIE or AIS using qRT-PCR. We found that mRNA levels of Mc4r were significantly [t (15) = -2.74, P < .05] increased in the amygdala of AIE adult rats compared with AIS adult rats (Figure 2). The mRNA expression of Pomc did not change significantly in the amygdala between the 2 groups. Similarly, the mRNA levels of both Pomc [t (9) = -2.56, P < .05] and Mc4r [t (9) = -2.47, P < .05] in the hypothalamus increased significantly in AIE adult rats compared with the AIS adult rats (Figure 2).

Epigenetic Regulation of Pomc and Mc4r Gene Expression following AIE

We recently reported that AIE causes a lasting increase in HDAC2 levels in the amygdala, leading to a decrease in acetylated histones globally (H3K9) and locally (H3K9/14) at important synaptic plasticity-related genes including Bdnf (Pandey et al., 2015). Given the involvement of histone acetylation mechanisms in the persistent effects of AIE, we measured occupancy of acetylated histone H3 lysine 9/14 (H3K9/14ac) at the Mc4r and Pomc promoter regions in the amygdala and hypothalamus of AIS and AIE adult rats using the ChiP assay. Here, we found that H3K9/14ac levels were significantly increased in the amygdala of AIE adult rats compared with AIS adult rats at 2 distinct regions [t (7) = -2.77, P < .05 for location 1; t (8) = -2.41, P < .05 for location 2] upstream of the transcription start site of Mc4r (Figure 5). Additionally, H3K9/14ac levels were elevated in the same 2 regions of the Mc4r promoter in the hypothalamus [t (12) = -3.13, P < .01 for location 1; t (12) = -5.67, P < .001 for location 2] and was also increased in 2 regions of the Pomc promoter of AIE adult rats compared with AIS adult rats [t (12) = -3.93, P < .01 for location 1; t (12) = -4.88, P < .001 for location 2].

Effects of AIE on the Expression of NPY and Histone Acetylation of Npy in Adulthood

We also measured histone acetylation levels at the Npy gene promoter region that overlaps with a CpG island as shown in Figure 6A. Levels of H3K9/14ac were determined using the ChiP assay. We observed a significant reduction [t (11) = 4.09, P < .01] in H3K9/14ac levels in the Npy gene promoter in the amygdala of AIE adult rats compared with AIS adult rats (Figure 6B). Further, NPY protein levels in the CeA [t (10) = 16.37, P < .001] and MeA [t (10) = 10.21, P < .001], but not in the BLA, were significantly decreased in AIE adult rats compared with AIS adult rats (Figure 6C-D). These results indicate that AIE condenses the...
chromatin by decreasing local histone acetylation that leads to a reduction in NPY expression in the amygdala at adulthood.

Discussion

The present study found that AIE increased mRNA and protein levels of MC4R in both the amygdala and hypothalamus of adult rats compared with AIS-exposed adult rats. Additionally, increased α-MSH protein in the CeA, MeA, ARC, and PVN, along with increased POMC mRNA in the hypothalamus, was observed in AIE adults compared with AIS adult rats. Epigenetic investigation of MC4R and POMC expression showed an increase in acetylated H3K9/14 in the promoter region of MC4R in the amygdala and both the MC4R and POMC promoters in the hypothalamus of AIE-exposed adult rats. AIE produced deficits in histone acetylation of the NPY gene promoter and decreased NPY levels in the CeA and MeA during adulthood. Taken together, these findings suggest that hyperactivity of the melanocortin system due to increased α-MSH and MC4R levels and hypoactive NPY may be involved in anxiety-like behavior seen here and elsewhere after AIE in adulthood (Pandey et al., 2015; Kyzar et al., 2016b; Sakharkar et al., 2016).

In this study, MC4R mRNA was increased in AIE-exposed adult amygdala and hypothalamic tissue, while POMC mRNA...
Figure 4. Effects of adolescent alcohol exposure on the protein levels of α-melanocyte stimulating hormone (α-MSH) and melanocortin 4 receptor (MC4R) in the hypothalamus of rats in adulthood. (A) Representative photomicrographs (scale bar = 50 μm) of α-MSH staining in the arcuate nucleus of the hypothalamus (ARC) and the paraventricular nucleus of the hypothalamus (PVN) of adolescent intermittent ethanol (AIE)- and saline (AIS)-exposed adult rats. (B) Bar diagram showing protein levels of α-MSH in ARC and PVN in AIE- and AIS-exposed adult rats as measured by gold immunolabeling. (C) Representative photomicrographs (scale bar = 50 μm) of MC4R staining in the ARC and PVN in AIE- and AIS-exposed adult rats. (D) Bar diagram showing protein levels of MC4R in ARC and PVN of AIS and AIE adult rats as measured by gold immunolabeling. Values are represented as the mean (±SEM) of the number of immunogold particles/100 μm². Values are significantly different from AIS-exposed adult group (**P < .01, Student’s t test, n = 6).

Figure 5. Effects of adolescent alcohol exposure on acetylated histone H3K9/14 occupancy at pro-opiomelanocortin (Pomc) and melanocortin 4 receptor (Mc4r) gene promoters at 2 locations as shown in upper panel. Rats exposed to adolescent intermittent ethanol (AIE) and saline (AIS) were used to measure for H3K9/14 occupancy at the Mc4r promoter region in the amygdala and at both the Mc4r and Pomc promoter regions in the hypothalamus in adulthood by chromatin immunoprecipitation (ChIP) assay. Values are represented as the mean ± SEM of the fold change derived from the AIS adult rats (*P < .05, **P < .01, ***P < .001 significantly different from control AIS rats, Student’s t test, n = 4-6 [amygdala], n = 7 [hypothalamus]).
was increased only in the hypothalamus compared with AIS-exposed control rats. Other groups have shown that chronic ethanol exposure suppressed \( \text{Pomc} \) mRNA levels in the mediobasal hypothalamus, while ethanol withdrawal gradually increased \( \text{Pomc} \) levels compared with nonexposed control adult rats (Rasmussen et al., 2002). Alcohol-preferring alcohol (AA) adult rats exhibited increased hypothalamic \( \text{Pomc} \) mRNA expression compared with nonpreferring alcohol (ANA) adult rats (Gianoulakis et al., 1992). Previous reports have noted an increase in melanocortin tone (i.e., increased \( \alpha \)-MSH) in rats during withdrawal after chronic alcohol exposure (Kokare et al., 2006, 2008). Interestingly, the MC4R antagonist HS014 attenuated ethanol withdrawal-associated anxiety-like behaviors in adult rats (Kokare et al., 2006). Conversely, direct injection of \( \alpha \)-MSH into the CeA leads to increased anxiety-like behaviors in rats (Kokare et al., 2005). Additionally, alcohol-dependent adult rats exhibit increased hypothalamic \( \text{Pomc} \) mRNA expression compared with nonpreferring alkoholic non-alcohol (ANA) adult rats (Gianoulakis et al., 1992). Previous reports have noted an increase in melanocortin tone (i.e., increased \( \alpha \)-MSH) in rats during withdrawal after chronic alcohol exposure (Kokare et al., 2006, 2008). Interestingly, the MC4R antagonist HS014 attenuated ethanol withdrawal-associated anxiety-like behaviors in adult rats (Kokare et al., 2006). Conversely, direct injection of \( \alpha \)-MSH into the CeA leads to increased anxiety-like behaviors in rats (Kokare et al., 2005). Additionally, alcohol-dependent adult rats exhibit hyperalgesia that is attenuated by both intracerebral and intranasal infusion of HS014, a selective MC4R antagonist, while HS014 has no effect on pain sensitivity in nondependent and alcohol-naive rats (Roltsch Hellard et al., 2017). Adult rats trained to self-administer ethanol in an operant conditioning paradigm exhibited enhanced \( \alpha \)-MSH immunoreactivity in the ARC, as well as the nucleus accumbens and bed nucleus of the stria terminalis (Shelkar et al., 2015). Notably, a previous study reported decreased \( \alpha \)-MSH immunoreactivity in the CeA, ARC, and PVN of adult rats at PND 63 after AIE (Lerma-Cabrera et al., 2013). Importantly, this group used a higher dose of ethanol (3 g/kg, i.p.) than the one used here, and the animals were allowed to grow only to PND 63 compared with PND 92 in this study. Therefore, the differences between these results could be related to the age and paradigm under which these measurements were performed after AIE.

The melanocortin system is intricately connected to stress and allostatic load (Chaki and Okuyama, 2005; Chaki and Okubo, 2007; Liu et al., 2007; Lim et al., 2012). Importantly, acute restraint stress and forced swimming stress both trigger \( \text{cfos} \) mRNA induction specifically in a high percentage of Pomc-positive neurons (Liu et al., 2007). Chronic stress causes synaptic changes in dopamine receptor D1-expressing medium spiny neurons in the nucleus accumbens that are mediated by MC4R activation and reversed by MC4R blockade (Lim et al., 2012). Additionally, intracerebral infusion of \( \alpha \)-MSH leads to enhanced immobility in the forced swim test that is attenuated by HS014 (Goyal et al., 2006), and chronic restraint increases Mc4r mRNA expression in the hypothalamus (Karami Kheirabad et al., 2015). MC4R blockade by intranasal infusion of HS014 attenuates anxiety-like behaviors and hypothalamic-pituitary-adrenal axis activity in a single prolonged stress model of posttraumatic stress...
disorder (Serova et al., 2013, 2014). Intracerebroventricular injection of HS014 also blocks social isolation-induced anxiety and depressive-like behaviors, indicating enhanced MC4R tone following stressful life events (Kokare et al., 2010). The increased α-MSH and subsequent activation of MC4R seen in the amygdala and hypothalamus in the present study may be involved in the anxiety-like behaviors in adulthood. Behavioral data showing that AIE produces anxiety-like behaviors in adulthood is similar to our previous studies (Pandey et al., 2015; Kyzar et al., 2016b; Sakharkar et al., 2016), but dissimilar to other studies where AIE appears to attenuate anxiety-like behaviors (Ehlers et al., 2011; Gilpin et al., 2012). The differences in these behavioral results could be related to the use of different rat strains as well as the duration and exposure method of ethanol paradigms.

The interaction between α-MSH/MC4R and other stress-related peptides such as NPY and corticotrophin-releasing factor (CRF), which controls the expression and release of Pomc mRNA and products, has been well documented (Marsh et al., 1999; Bell et al., 2000; Yamano et al., 2004; Kokare et al., 2005; Hsieh et al., 2011). Generally, α-MSH and NPY are functionally antagonistic with regard to food intake and anxiety- and depression-like behaviors, with α-MSH administration eliciting anxiogenic behaviors and NPY causing anxiolysis (Hansen and Morris, 2002; Kokare et al., 2005; Goyal et al., 2006). Like α-MSH, CRF is released in response to stress and is correlated with high-anxiety states (Radulovic et al., 1999; Shekhar et al., 2005). Brain stress systems involving CRF in the amygdala and hypothalamus play crucial roles in alcohol use and addiction (Koob, 2008, 2009; Koob et al., 2014).

The role of NPY, particularly in the amygdala and related structures, in response to alcohol has been extensively studied in adult animals (Heilig and Thorsell, 2002; Gilpin, 2012). Deficits in NPY in the CeA are associated with anxiety-like and alcohol drinking behaviors in rats in multiple alcohol exposure models (Badia-Elder et al., 2001; Thorsell et al., 2007; Gilpin et al., 2008a, 2008b; Zhang et al., 2010). Here, we observed for the first time that AIE significantly decreased NPY levels in CeA and MeA of rats in adulthood. These results suggest that AIE results in increased melanocortin activity and decreased NPY activity in the amygdala in adulthood. These findings are furthered by the observation that the combined treatment of NPY and HS014 (MC4R antagonist) significantly prevented chronic traumatic stress-induced anxiety-like behaviors (Sabbah et al., 2015). Another peptide known as agouti-related peptide (AgRP) is produced in NPY-containing neurons in the hypothalamus and acts as an endogenous antagonist at MC4R (Chen et al., 2006). Interestingly, AgRP infusion directly into the CeA of rats leads to an increase in Bdnf mRNA expression when measured 2 hours later (Boghossian et al., 2010), suggesting that antagonism of MC4R may lead to increased BDNF in the amygdala circuitry. BDNF mRNA and protein expression is decreased in the CeA and MeA of AIE adult rats (Pandey et al., 2015), and here we show increased expression of MC4R and its agonist α-MSH and decreased NPY expression in the amygdala. Therefore, it is possible that increased MC4R signaling in the amygdala along with a decrease in BDNF (Pandey et al., 2015) and NPY expression observed in AIE-exposed adult rats together might be operative in the anxiety phenotype seen in AIE adult rats (Figure 7).

Epigenetic mechanisms are involved in the lasting effects of AIE, and HDAC inhibitors are able to reverse the increased anxiety-like behavior and decreased activity regulated cytoskeleton-associated protein (Arc) and Bdnf gene-specific histone acetylation (H3K9/14 acetylation) observed in the amygdala of AIE-exposed adult rats (Pandey et al., 2015). Here, we report a paradoxical increase in histone acetylation at the Mc4r promoter in the amygdala of AIE adult rats compared with AIS adult rats, despite the globally increased HDAC2 and decreased H3K9 acetylation seen in the CeA and MeA of AIE adult rats (Pandey et al., 2015). This is possibly due to epigenetic mechanisms working differentially to activate brain pro-stress melanocortin systems, but

![Figure 7. Adolescent intermittent ethanol (AIE) exposure produces global deficits in histone acetylation due to increased histone deacetylase (HDAC) activity in adulthood, leading to a condensed chromatin structure in the amygdala (Pandey et al., 2015). This model indicates that neuropeptide Y (NPY) gene promoter histone acetylation is decreased, which is associated with decreased NPY expression in the amygdala by AIE in adulthood. On the other hand, AIE produces increased histone acetylation at pro-opiomelanocortin (Pomc) and melanocortin 4 receptor (Mc4r) gene promoters in the hypothalamus and at the Mc4r gene promoter in the amygdala in adulthood. This leads to increased melanocortin activity due to increased alpha-melanocyte stimulating hormone (α-MSH) and MC4R expression. This epigenetically mediated imbalance in NPY and melanocortin activity in the amygdala may be associated with AIE-induced anxiety-like behaviors seen in adulthood. Ac, acetylation, H3K9/14, Histone H3 lysine 9/14; TF, transcription factor.](image-url)
at the same time inhibiting genes involved in the regulation of synaptic plasticity and anti-stress activity (BDNF and NPY). This is in line with findings that global HDAC inhibition in the brain leads to increased expression of many genes, but also causes decreased expression of a subset of genes (Gräff et al., 2014). Other studies have shown that certain epigenetic enzymes serve dual functions as both transcriptional activators and repressors in the brain (Chahrour et al., 2008; Laurent et al., 2015). We also show that both Mc4r and Pomc show increased histone acetylation at two distinct loci within the promoter in the hypothalamus of AIE adult rats compared with AIS adult rats. Interestingly, fetal alcohol exposure decreases Pomc expression in the hypothalamus of both male and female rats, while also decreasing histone acetylation and increasing repressive H3K9 methylation and DNA methylation in the ARC (Govorko et al., 2012). The decrease in Pomc is additionally transferred intergenerationally to the offspring of male fetal alcohol-exposed rats. This finding potentially represents a divergence between fetal exposure and adolescent exposure to alcohol with regards to Pomc expression.

In summary, we report increased α-MSH and MC4R in the amygdala and hypothalamus of AIE-exposed adult rats that is possibly correlated with anxiety-like behavior and increased alcohol intake (Pandey et al., 2015). The increase in Mc4r expression in the amygdala and both Mc4r and Pomc expression in the hypothalamus of AIE-exposed adult rats may be caused by an increase in H3K9/14 acetylation in the promoters of these genes, which occurs despite a global decrease in histone H3K9 acetylation in the amygdala of AIE-exposed adult rats (Pandey et al., 2015). Interestingly, the anti-anxiety molecule NPY shows decreased expression due to deficits in histone acetylation of the Npy promoter in the amygdala after AIE in adulthood. The epigenetically regulated increase in melanocortin tone and decrease in NPY function may be responsible for the persistent effects of adolescent alcohol exposure on synaptic plasticity and adult psychopathology (Figure 7), and these represent important molecular targets for pharmacological intervention in alcohol addiction.

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Statement of Interest

S.C.P. reports that a US patent application entitled “Histone acetyl transferase activators and histone deacetylase inhibitors in the treatment of alcoholism” (serial number 60/848237 filed on September 29th, 2006) is currently pending. No biomedical financial interests or potential conflicts of interest were reported by the other authors.

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