Covalent Histone Modifications in Nucleus Accumbens

and Amygdala of Mice: A Role in Alcoholism

BY

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THESIS

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BGS

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LIST OF ABBREVIATIONS

(+/+)	Wild-type Littermate Mice
(+/-)	CREB Deficient Mice
α	Alpha
A	Adenine
Ab	Antibody
AD	Alzheimer's Disease
ADP	Adenosine Diphosphate
ALD	Alcohol Liver Disease
ANOVA	Analysis of Variance
APA	American Psychiatric Association
AVP	Arginine Vasopressin
β	Beta
B.C.	Before Christ
BDNF	Brain-Derived Neurotrophic Factor
BLA	Basolateral Nucleus of Amygdala
BNST	Bed Nucleus of the Stria Terminalis
bp	Base pairs
BSA	Bovine Serum Albumin
C	Cytosine
C-terminus	Carboxyl-terminus
C57	C57BL/6J Mouse
CA	California

LIST OF ABBREVIATIONS (continued)

Ca^{2+}	Calcium Ion
CaMKs	Ca^{2+} calmodulin-dependent protein kinases
CBP	CREB-Binding Protein
CeA	Central Nucleus of Amygdala
cm	Centimeter
CNS	Central Nervous System
ChIP	Chromatin Immunoprecipitation
Cyclic-AMP	Cyclic Adenosine Monophosphate
CRE	Cyclic AMP Responsive Element
CREB	Cyclic AMP Responsive Element-Binding Protein
CRF	Corticotrophin-Releasing Factor
Δ	Delta
DA	Dopaminergic
DBA	DBA/2J Mouse
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DSM	Diagnostic and Statistical Manual of Mental Disorders
EHMT1	Euchromatic Histone-Lysine-N-Methyltransferase 1
EPM	Elevated Plus Maze
Erk 1/2	Extracellular Signal-Regulated Kinases 1/2
Fig(s)	Figure(s)

LIST OF ABBREVIATIONS (continued)

GABA	γ-aminobutyric acid
g	Gram
G	Guanine
H1	Histone Linker Protein H1
H2A	Histone Protein H2A
H2B	Histone Protein H2B
H_2O	Water
H3	Histone H3
H4	Histone H4
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDMs	Histone Demethylases
HMTs	Histone Methyltransferases
hr	Hour
IC_{50}	Half-Maximal Inhibitory Concentration
IL	Illinois
IN	Indiana
I.G.	Intragastrointestinally
I.P.	Intraperitoneally
JARID1C/SMCX	X-Linked Mental Retardation Gene
K	Lysine
K-8	Lysine 8 Residue

LIST OF ABBREVIATIONS (continued)

K-9	Lysine 9 Residue
K+	Potassium Ion
KID	Kinase Inducible Domain
kg	Kilogram
MAPK	Mitogen-Activated Protein Kinase
MA	Massachusetts
MD	Maryland
Mg^{2+}	Magnesium Ion
ME	Maine
MeA	Medial Nucleus of Amygdala
MeDIP	Methyl-DNA Immunoprecipitation
mg	Milligram
ml	Milliliter
mRNA	Messenger RNA
ml	Milliliter
mV	Millivolt
M	Molar
MBF	MicroBrightField
MD	Maryland
MO	Missouri
n	number

LIST OF ABBREVIATIONS (continued)

n-saline	Normal Saline
N-terminal	Amino-terminal
Na+	Sodium Ion
NAc	Nucleus Accumbens
NeuN	Neuron-Specific Nuclear Protein
NGS	Normal Goat Serum
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NIH	National Institute of Health
NP	Non-Preferring
NPY	Neuropeptide Y
N-terminus	Amino-Terminus
NY	New York
OD	Optical Density
ODN	Oligodeoxynucleotide
OR	Oregon
P	Alcohol-Preferring
p300	E1A binding protein or EP300
pAkt	Phosphorylated Akt
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PBST	0.1% Tween in Phosphate Buffered Saline
PCAF	p300/CBP-Associated Factor

p-CREB	Phosphorylated CREB
PFA	Paraformaldehyde
PKA	Protein Kinase A
PKC	Protein Kinase C
P-value	Probability Value
R	Arginine
RNA	Ribonucleic Acid
RSTS	Rubinstein-Taybi Syndrome
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SD	Sprague-Dawley
SEM	Standard Error of the Mean
Ser-133	Serine Residue 133
T	Thymine
TSA	Trichostatin A
μm	Micrometer
VTA	Ventral Tegmental Area
WKS	Wernicke-Korsakoff syndrome
w/v	Weight/Volume

SUMMARY

Epigenetic mechanisms play an important role in the regulation of gene expression. C57BL/6J (C57) mice consume higher amounts of ethanol compared to DBA/2J (DBA) mice. Alterations in cyclic-AMP responsive element binding protein (CREB) function in specific brain regions have been implicated in alcohol drinking behaviors. Previous studies have shown that CREB protein levels are innately lower in the shell of the nucleus accumbens (NAc shell) of C57 mice compared to DBA mice. CREB recruits CREB-binding protein (CBP), which has intrinsic histone acetyltransferase (HAT) activity. It may be possible that epigenetic mechanisms due to HAT-induced chromatin remodeling may be involved in a genetic predisposition to excessive alcohol drinking behaviors observed in C57 mice compared to DBA mice. Therefore, we examined innate differences in CBP levels, histone H3 and H4 acetylation, and H3 methylation in NAc and amygdaloid brain regions of C57 and DBA mice using the gold-immunolabeling histochemical procedure. We also investigated colocalization of acetylated H3 with neuron-specific nuclear protein (NeuN) using double-immunofluorescence labeling in the NAc of C57 and DBA mice.

We found lower levels of CBP and acetylated histone H3 (K-9) and higher methylated histone H3 (K-9) in the NAc shell, but not core, of C57 mice compared to DBA mice. Acetylated H3 predominantly localized to NeuN-positive neurons in the NAc of C57 and DBA mice. In addition, abnormal acetylation was specific to H3, since no differences in the levels of acetylated H4 (K-8) were observed in the NAc of C57 mice compared to DBA mice. There were also no

differences in protein levels of CBP, acetylated H3 or H4, or dimethylated H3 in amygdaloid structures between C57 and DBA mice.   These results suggest that innately lower levels of CBP, and lower acetylation and higher methylation of histone H3 (K-9) in the NAc shell may be operative in abnormal gene expression involved in the excessive alcohol drinking behaviors of C57 mice compared to DBA mice.

In order to determine the effects of ethanol exposure on brain chromatin remodeling in C57 mice compared to DBA mice, we examined the effects of voluntary ethanol exposure on levels of CBP, acetylated histone H3 and methylated histone H3 in the NAc and amygdala of C57 mice exposed to ethanol compared to control mice using the gold- immunolabeling histochemical procedure. We also determined the effects of acute ethanol exposure on protein expression of CBP, acetylated histone H3, methylated histone H3, and brain-derived neurotrophic factor (BDNF), a neurotrophin implicated in alcohol abuse disorders, in the NAc and amygdala of C57 and DBA mice. In addition, we determined baseline levels of neuronal marker (NeuN) and the effects of acute ethanol exposure on the number of NeuN-positive neurons in the shell and core of the NAc of C57 and DBA mice.

We found that voluntary ethanol exposure significantly increased levels of CBP and acetylated histone H3 in the NAc shell, but not NAc core or amygdaloid structures [central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), and basolateral nucleus of amygdala (BLA)] of C57 mice compared to control mice. We also found that voluntary ethanol exposure significantly

decreased levels of methylated histone H3 in the NAc shell, but not core, and also decreased levels of methylated histone H3 in the amygdala (CeA, MeA, or BLA) of C57 mice compared to control mice. Acute ethanol exposure was found to increase levels of CBP, acetylated histone H3, and BDNF in the NAc shell, but not core or amygdaloid structures of C57 mice compared to control mice. Acute ethanol exposure was also found to decrease levels of methylated histone H3 in the NAc shell, but not core or amygdaloid structures of C57 mice compared to control mice. Interestingly, acute ethanol exposure increased levels of CBP, acetylated histone H3, and BDNF in both the NAc shell and core of DBA mice compared to controls, while having no effects on amygdaloid structures (CeA, MeA, or BLA). In addition, acute ethanol exposure was found to decrease levels of methylated histone in both the shell and core of the NAc of DBA mice compared to controls, while also having no effects on amygdaloid structures (CeA, MeA, or BLA). We also measured the effects of acute ethanol exposure on levels of NeuN in the NAc shell and found no significant differences between of C57 and DBA mice. Also, there were no baseline differences in the number of NeuN-positive neurons in the NAc shell between C57 and DBA mice. These findings suggest that ethanol exposure has a profound effect on brain chromatin remodeling in both C57 and DBA mice; and voluntary and acute ethanol exposure elicit differential effects on chromatin remodeling in the NAc (shell and core) and amygdala (CeA, MeA, BLA) of C57 and DBA mice.

It has been shown that abnormal CREB functioning in the amygdala may be related to anxiety and alcohol abuse disorders. We examined the possible

direct role of CREB in amygdaloid chromatin remodeling and how epigenetic aberrations may affect anxiety-like and alcohol drinking behaviors of CREB deficient (+/-) mice compared to wild-type littermate (+/+) mice. We also determined the effects of a histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), on chromatin remodeling in the amygdala and anxiety-like and alcohol drinking behaviors of CREB deficient (+/-) mice compared to wild-type (+/+) littermates. We found that levels of phosphorylated CREB (p-CREB), CBP, and acetylated histones H3 (K-9) and H4 (K-8) were lower in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermates. Interestingly, while the levels of p-CREB were lower in the NAc shell and core of CREB deficient (+/-) mice compared to wild-type (+/+) littermates, the levels of CBP, and acetylated histones H3 and H4 were not significantly different in the NAc structures of these mice. CREB deficient (+/-) mice have been shown to exhibit higher anxiety-like behaviors and consume larger amounts of alcohol compared to their wild-type (+/+) littermates. We found that treatment with TSA corrected deficits in CBP and histone (H3 and H4) acetylation in CREB deficient (+/-) mice, while there were no observable effects of TSA on these measures in the amygdaloid structures of wild-type littermate (+/+) mice. In addition, treatment with TSA was also able to attenuate anxiety-like and alcohol drinking behaviors of CREB deficient (+/-) mice. The results of this study suggest that partial deletion of the CREB gene produces aberrant chromatin remodeling in the amygdala, which may be involved in baseline anxiety-like behaviors and higher

alcohol intake of CREB deficient (+/-) mice compared to wild-type (+/-) littermates.

Our studies are important in understanding the cellular and molecular basis for alcoholism. We show how that CBP HAT-induced histone modifications in the specific neurocircuitries in the brain may be involved in the actions of ethanol. We also show that deficits in CREB and CBP produce aberrant chromatin architecture in the brain, which may be responsible for the phenotype of anxiety and alcohol preference. Manipulating the chromatin structure by pharmacological inhibition with HDAC inhibitors may also prove to be important in treating anxiety and alcohol abuse disorders.

I.   LITERATURE REVIEW

VI. <u>Alcoholism: An Overview</u>

A. <u>A Brief History of Alcohol Consumption and Abuse</u>

Alcohol is a ubiquitous product that has served several different functions throughout history. Evidence of the existence of alcoholic beverages dates all the way back to the Stone Ages, circa 10,000 B.C. up to present day, with roles ranging from enjoyment amongst friends, to dietary or bartering uses and for religious purposes (Hanson, 1995). Ancient Egyptian hieroglyphics demonstrate the importance of alcoholic beverages to those early civilizations (Bard, 1999). While consumption of alcohol continued over several generations and was highly regarded by peoples throughout history, alcohol was banned in the U.S. during the 1930's due to prohibition, which made the manufacture and sale of alcohol illegal (Cherrington, 1925). Today, consumption of alcohol is a worldwide phenomenon, with numerous bars, taverns, and watering holes that line the streets all over the world. It is currently debated if alcohol serves a therapeutic or medicinal function or is harmful to humans. Several literature publications suggest that moderate consumption of alcohol may increase cognition, reduce coronary artery-related events such as myocardial infarction, and prevent the onset of both diabetes and dementia (Klatsky, 1987; 2007; Koskinen et al., 1994; Franz et al., 2002; Ruitenberg et al., 2002; Espeland et al., 2005; Mukamal et al., 2006; Lakshman et al., 2009). In addition, alcohol has been shown to exhibit anxiolytic effects (Henniger et al., 2002; Schneier et al., 2009; National Institute of Health (NIH), 2010). However, while it is possible that alcohol can serve a

variety of beneficial purposes, it has also been shown that moderate to excessive alcohol consumption can damage several organs, including the stomach, liver, and kidneys, and can also be a major factor in the development of breast and colorectal cancers (Kune and Vitetta, 1992; Vamvakas et al., 1998; Hoek and Pastorino, 2004; Zima and Kalousova, 2005; Boffetta and Hashibe, 2006; Zhang et al., 2007; Bongaerts et al., 2008; Boyle and Boffetta, 2009). Chronic alcohol consumption has profound effects on the brain that manifest as difficulty walking, blurred vision, slurred speech, slowed reaction times, and impaired memory (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2004). Alcohol use disorders have been linked with memory impairment and neurodegeneration in direct proportion to the amount of alcohol consumed, with excessive amounts of alcohol consumed in a short period of time (e.g. binge drinking) leading to blackouts or periods of memory loss (White, 2003; Crews and Nixon, 2009). Heavy drinking over long periods of time can also cause severe brain damage as a direct result from alcohol abuse or indirectly from poor health or severe liver damage (Harper, 2009; Wang et al., 2010b). However, even heavy social drinkers who have no specific neurological or hepatic problems still show signs of regional brain damage and cognitive dysfunction (Harper, 2009). Vitamin B1 (thiamine) deficiency appears to affect the cerebellum and occurs in about 80 percent of alcoholics, with some of these individuals developing a more severe brain disorder called Wernicke-Korsakoff syndrome (WKS) (NIAAA, 2004; Mulholland, 2006; Guerrini et al., 2009). WKS prodromes as the acute and potentially reversible neurological disorder Wernicke encephalopathy, which if left

untreated can develop into Korsakoff syndrome, a severe neurological disorder

characterized by retrograde amnesia (Zahr et al., 2011). In spite of its known

adverse effects, alcohol consumption is often misused by the general population,

leading to alcohol abuse and alcoholism.

B. **Definition of Alcoholism by The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and The National Institute on Alcohol Abuse and Alcoholism Current Statistics on Alcoholism**

According to the Diagnostic and Statistical Manual of Mental Disorders

(DSM-IV) the formal diagnostic criteria for an individual to be an alcoholic

requires symptoms in at least three of seven of the following categories: (1)

tolerance; (2) withdrawal symptoms or use of alcohol to avoid or relieve

withdrawal; (3) drinking more than intended; (4) unsuccessful attempts to cut

down on use; (5) excessive time related to alcohol (obtaining, hangover); (6)

impaired social or work activities due to alcohol; and (7) continued use despite

physical or psychological consequences (American Psychiatric Association

(APA), 1994). Alcohol abuse is a drinking pattern of drinking without physical

dependence that results in personal harm affecting one's health, interpersonal

relationships, or ability to work; whereas dependency on alcohol, or alcoholism,

is a chronic disease ((APA), 1994). Prior to the use of the phrase "alcoholism",

the conditions of the disease were designated by labels such as intemperance,

inebriety, and habitual drunkenness (Keller and Doria, 1991). As early as 1785,

Dr. Benjamin Rush described the effects of habitually extreme alcohol

intoxication as a disease and an addiction (Katcher, 1993). During the mid-

nineteenth century, Swedish physician, Dr. Magnus Huss, first coined the term "alcoholism" to describe excessive alcohol consumption and referred to it as a chronic, relapsing disease (Lader, 1983). Opposition to alcoholism as a disease even during the earliest years of observance as a disease, was rampant (Brown, 1985). Even today with all the data from numerous studies, alcoholism and other psychiatric disorders are still not well met or accepted by the general public. Psychiatric disorders are often misunderstood by the general population; and severe social consequences and stigma often accompany these debilitating diseases (Bukstein et al., 1989; Kessler et al., 1995; 1997; 1998). According to the World Health Organization, each year alcoholism results in approximately 2.5 million deaths and 320,000 young people between the ages of 15 and 29 die from alcohol-related causes, which make up approximately 9% of all deaths in this age group. Alcoholism is a chronic relapsing psychiatric disease characterized by physical dependence and tolerance to alcohol that together cause a strong craving to drink accompanied by the inability to stop drinking.

C. **Alcohol and the Brain**

   a. **Functional Neuroanatomy of the Nucleus Accumbens (NAc) and Amygdala**

      i. **Anatomy of the NAc**

The nucleus accumbens (NAc), sometimes referred to the NAc septi, is a structure located in the basal forebrain (Zahm, 2000). The NAc exists at the lateral base of the septal complex and extends to the junction of the caudate and putamen, where it joins the striatal nuclei (Zahm, 2000). The NAc is a neuroanatomically important structure that receives afferents from the amygdala, projects to the globus pallidus, and appears to functionally link the limbic system with the basal ganglia (Zahm and Heimer, 1990; O'Donnell et al., 1997; Tzschentke and Schmidt, 2000). The NAc is part of the ventral striatal complex and serves as a critical brain region where stimuli originating from the limbic system interact with motor circuitry to regulate rewarding and goal-directed behaviors (Mogenson et al., 1980; Groenewegen et al., 1996). As a functional part of the striatal complex, the NAc receives extensive excitatory afferents from the cerebral cortex and thalamus (Nicola et al., 2000; Zahm, 2000). The NAc then sends out projections to the ventral pallidum, which primarily innervates the mediodorsal and other thalamic divisions, thus completing cortico-striato-pallidal-thalamocortical loops (Zahm and Heimer, 1990; O'Donnell et al., 1997). These important neurocircuitries are critical in the modulation of behavioral responses to reward and alterations in synaptic plasticity within this complex circuitry are strongly associated with the development of addictive disorders (Kalivas et al.,

2005; Robbins et al., 2008; Carlezon Jr. and Thomas, 2009). The NAc is divided into two major sectors: the core, which is the central part of the NAc and is located lateral and caudal to and continuous with the dorsal striatum and surrounding the anterior commissure; and the shell, which is contained within the ventral and medial portions of the NAc (Zahm and Brog, 1992). The core and shell of the NAc consist of mainly 90% medium spiny neurons, while the rest are cholinergic interneurons and parvalbumin cells (Kawaguchi et al., 1995; Meredith, 1999). The projections from the NAc core and shell differ in their cellular characterization and morphology, projection patterns, and functions (Heimer et al., 1991; Meredith et al., 1992; Jongen-Relo et al., 1994; Meredith et al., 1996).

1. Shell of NAc

The shell of the NAc has been associated with the rewarding properties of drug and alcohol abuse (Koob et al., 1998a; Misra and Pandey, 2003; Carlezon Jr. and Thomas, 2009). A recent study determined the involvement of the NAc with ethanol reinforcement and also suggested a relationship between the genetic selection for high alcohol preference and sensitivity of the NAc to the reinforcing effects of ethanol (Engleman et al., 2009). The authors found that ethanol was self-administered into the NAc shell, but not the core, of both alcohol-preferring (P) rats and Wistar rats, suggesting the NAc shell is a neuroanatomical structure that functions in mediating the reinforcing actions of ethanol; in addition, compared to Wistar rats, the NAc shell of P rats was found to be more sensitive to the reinforcing effects of ethanol (Engleman et al., 2009).

Dopamine levels have been shown to increase in the NAc after ethanol administration in rats, however, until recently the contributions of the core and shell subregions of the NAc to this response have been unclear (Franklin et al., 2009). Howard et al. determined the effects of intravenous ethanol infusion of dopamine in the shell and core of the NAc (Howard et al., 2008). The authors found that the shell of the NAc had a stronger response than the core to intravenous ethanol administration, and that dopamine in the shell increased in a dose-dependent manner between 0.5-1.0 g/kg, but higher ethanol doses reached a plateau (Howard et al., 2008). Environmental stimuli have also been shown to be involved in the regulation of cyclic-AMP responsive element (CRE)-mediated transcription within the shell of the NAc, and changes in cyclic-AMP responsive element binding protein (CREB) activity in this brain region alter gating between emotional stimuli and behavioral responses (Barrot et al., 2002). These findings suggest the importance of CREB functioning in the NAc shell in the regulation of addiction disorders. Our studies of CREB functioning in the regulation of chromatin remodeling will contribute to our current understanding of the epigenetic processes that occur in the NAc shell of mice and how alcohol can alter these processes.

2. <u>Core of NAc</u>

The NAc core plays a critical role in determining the incentive value of reward, but does not participate in the timing of reward delays (Galtress and Kirkpatrick, 2010). Lesions of the NAc core induced persistent impulsive choice in rats, suggesting that the NAc core may be a key element in the

neuropathology of impulsivity, which has been linked with relapse to alcohol abuse (Cardinal et al., 2001; Dom et al., 2006). Dopamine in the NAc shell and core play differential roles in behavior and addiction (Di Chiara, 2002). NAc dopamine has been shown to increase in both shell and core during intravenous cocaine self-administration (Ito et al., 2000). The core and shell of the NAc have been suggested to have opposing roles in reinstatement of food-seeking behavior; where the shell facilitates alterations in behavior in response to changes in the incentive value of conditioned stimuli, the core enables reward-related stimuli to bias the direction and vigor of instrumental responding (Floresco et al., 2008). A study found that the functional integrity of the core, but not the shell, of the NAc was necessary for conditioned cue-induced reinstatement of cocaine seeking behavior (Fuchs et al., 2004). Reinstated ethanol-seeking in rats has also been shown to be modulated by environmental context and requires the NAc core (Chaudhri et al., 2008). Ethanol-seeking behavior triggered by environmental context was attenuated by blocking dopamine D1 receptors in the NAc shell and core in rats (Chaudhri et al., 2009). A recent study highlighted an important role for the NAc core in cue-induced ethanol-seeking and suggested that the shell was required in mediating the influence of context on conditioned ethanol-seeking (Chaudhri et al., 2010). NAc core action potential firing was enhanced after abstinence from long-term alcohol intake, due to inhibition of small-conductance calcium-activated potassium channels (Hopf et al., 2010). These results may explain alcohol cravings that drive relapse.

ii.   <u>Anatomy of Amygdala</u>

The amygdala, or the amygdaloid complex, is part of the basal ganglia and is sometimes referred to as the archistriatum (Moreno and Gonzalez, 2007). The striatum is an anatomical phrase short for corpus striatum; this brain area refers collectively to the neostriatum (caudate and putamen), paleostriatum (globus pallidus), and archistriatum (amygdala) (Swanson and Petrovich, 1998). The amygdala is a large, well-defined brain structure composed of a group of many nuclei (Sah et al., 2003). These nuclei are grouped into three divisions that are interconnected and share common functions. The three nuclei of the amygdala are the central nucleus of amygdala (CeA), medial nucleus of the amygdala (MeA), and basolateral nucleus of the amygdala (BLA) (Swanson, 2003). The amygdala is located in the rostral tip of the temporal lobe, deep to the piriform cortex of the uncus and is continuous with the ventrolateral portion of the putamen (Alheid et al., 1998). The amygdala makes essential connections with the limbic and olfactory systems of the brain (Swanson and Petrovich, 1998). The neurocircuitry of the amygdala does not contain loop circuits but instead afferent and efferent projections make this area the functional part of the limbic system (Price, 2003). The efferent projections from the amygdala to the hypothalamus and brainstem, which course through both the stria terminalis and ventral amygdalofugal pathway, originate from the centromedial nuclear group, the superficial nuclear group, and the accessory basal nucleus of the basolateral nuclear group (McDonald, 1998).

The amygdala is involved in many limbic system functions, most of which also involve other limbic structures (Price, 2003). The modulation of autonomic responses is one direct function of the amygdala (Sah et al., 2003). These modulations of autonomic activity occur in two ways. The first is by modulating hypothalamic activity through the extensive reciprocal connections between the amygdala and the hypothalamus; the second is through the direct projections from the amygdala to autonomic centers in the brain stem (McDonald, 1998; Shu et al., 2003). The amygdala alters autonomic function based on learning and past experience (McDonald, 1998). The BLA, with its extensive neocortical connections, is the receiving station for the instinctive or learned experience (McDonald, 1998). The CeA and MeA are the source of the efferents with their projections to the brain stem and hypothalamus, respectively (McDonald, 1998).

The amygdala is the emotional processing center in the brain, including fear processing (Pessoa and Adolphs, 2010). An individual can, for example, develop anxiety during withdrawal from ethanol exposure, subsequently leading to relapse (Koob 2003a; Pandey 2004). The sounds and sights associated with drinking alcohol, such as a bar/tavern setting can trigger autonomic responses, such as increases in heart rate, sweating, or respiration (Tiffany and Conklin, 2000). Under these circumstances, the autonomic changes are not in response to the body's internal physiology but in response to past drinking experiences, which over time represent a learned response (Tiffany and Conklin, 2000). The amygdala is the center responsible for the final integration of these learned or conditioned responses (Koob, 2003a).

The 'extended amygdala' represents a common neuroanatomical substrate that regulates the negative emotional states associated with withdrawal, thereby promoting negative reinforcement of addictive behaviors (Heimer and Alheid, 1991; Koob, 2003b). The extended amygdala is composed of the CeA, bed nucleus of the stria terminalis (BNST), and a transition zone in the shell of the NAc (Koob, 2003b; Koob and Volkow, 2010). In general, the traditional nomenclature of the amygdaloid structures (CeA, MeA, and BLA) are predominantly used in describing neuroanatomy, however, the extended amygdaloid neurocircuitry is more often cited in the addiction field (Koob, 2003b; Prakash et al., 2008).

1. <u>Central Nucleus of Amygdala (CeA)</u>

The CeA is an ovoid-shaped structure located dorsal and lateral to the MeA, and in the dorsal central aspect of the amygdala (De Olmos et al., 2004). CeA projections to the brainstem and hypothalamus may be critical for reflexive, classically conditioned responses (McDonald, 1998). The cells in the CeA closely resemble the medium spiny neurons of the adjacent striatum (McDonald, 1998). Several studies have implicated the CeA in regulation of the the effects of alcohol and alcohol drinking behavior in rodents (McBride, 2002). The c-fos protooncogene is often used as a marker for cellular activity and mapping of functional neuronal pathways (Dragunow and Faull, 1989). Intraperitoneal (i.p.) injection of 1.5-3.0 g/kg ethanol into Sprague-Dawley (SD) rats increased c-fos immunoreactivity 2-4 hours later in the CeA, which was still evident 16 hours post-injection (Chang et al., 1995). High doses of ethanol (2 g/kg) increased c-

fos immunoreactivity in the CeA of rats (Morales et al., 1998). Studies on the innate differences in neuropeptide Y (NPY) in the CeA have been shown between rats selectively bred for high and low alcohol preference, suggesting a potential involvement in alcohol preference and anxiety (Hwang et al., 1999; Pandey et al., 2005b). In a recent study from our lab, P rats were bilaterally cannulated targeting the CeA and infused with NPY; NPY signaling in the CeA was found to regulate alcohol drinking and anxiety-like behaviors of P rats (Zhang et al., 2010). Levels of corticotropin releasing factor (CRF), an important polypeptide hormone and neurotransmitter involved in the stress response, within the CeA have also been shown to mediate enhanced ethanol self-administration in withdrawn, ethanol-dependent rats (Funk et al., 2006).

2. **Medial Nucleus of Amygdala (MeA)**

The MeA is located at the brain surface in the dorsomedial corner of the temporal/piriform lobe, just lateral to the optic tract (McDonald, 1998). It lies dorsal to the superficial cortex-like nuclear group and exhibits a cell-sparse molecular layer that contains fibers from the main and accessory olfactory bulbs, and a deeper cell-dense portion (McDonald, 1998). The cells of the MeA are mostly spiny, however there is a lower spine density compared with central lateral amygdaloid neurons (McDonald, 1998). Studies from our lab indicate a role for both CeA and MeA brain-derived neurotrophic factor (BDNF) signaling in the regulation of alcohol-drinking and anxiety-like behaviors (Pandey et al., 2004; 2006). In addition, histone deacetylase (HDAC)-induced histone modifications in the CeA and MeA may play a critical role in rapid tolerance to the anxiolytic

effects of ethanol (Sakharkar et al., 2011). Aberrant NPY expression in the MeA could contribute to alcohol preference of P rats (Caberlotto et al., 2001; Pandey et al., 2005b). The MeA has also been identified as a critical brain region involved in substance P neurotransmission, a receptor system implicated in anxiety, depression, and other stress-related disorders (Ebner et al., 2004). Arginine vasopressin (AVP) has important roles in the regulation of stress-related behaviors during chronic stress and AVP-immunoreactive and synthesizing cells are present in the MeA (Urban et al., 1990; Albeck et al., 1997). The MeA has direct main efferents to the hypothalamus and a recent study indicated the hypothalamic AVP system as a potential therapeutic target for treating anxiety and depression associated with cocaine addiction (Sah et al., 2003; Zhou et al., 2011). The MeA may also play a key role in the acute responses to emotional environmental stimuli due to recent findings that show lesions of the MeA differentially influence stress responsivity and sensorimotor gating in rats (Vinkers et al., 2010).

3. <u>**Basolateral Nucleus of Amygdala (BLA)**</u>

The BLA is a central component of the brain's fear/anxiety circuitry and acts as the primary input nuclei of the amygdala. The BLA receives extensive inputs from sensory, limbic, insular cortex, and thalamic nuclei (Aggleton et al., 1980). In turn, the BLA provides major excitatory input to the neighboring CeA, NAc, and also has extensive reciprocal connections with the medial prefrontal and orbitofrontal cortices (Nose et al., 1991; Groenewegen et al., 1996). Projections from the amygdala to the striatum originate from the BLA and terminate primarily in the ventral and medial portions of the striatum, including the NAc (McDonald, 1998). Projections of the BLA to the ventral striatum in the rat are important in controlling instrumental behavior dependent on stimulus-reinforcement associations (Everitt and Robbins, 1992). The BLA exhibits two major cell types: spiny pyramidal-like neurons and spine-sparse stellate neurons (McDonald, 1998). Alcohol has been shown to inhibit spontaneous activity of BLA projection neurons in the rat (Perra et al., 2008). Alcoholism affects many genes in the BLA of adult human males involving synaptic transmission, neurotransmitter transport, structural plasticity, metabolism, energy production, transcription and RNA processing, and the circadian cycle; decreased expression of these genes may contribute to neurodegeneration and anxiety, which underlie the continuance of alcohol abuse and chronic relapse (Kryger and Wilce, 2010).

b. <u>Mesolimbic Dopaminergic System and Addiction</u>

The neural circuitry associated with the euphoric or rewarding actions of alcohol that lead to the development of addictive behaviors is the mesolimbic dopaminergic (DA) pathway (Imperato and Di Chiara, 1986; Weiss et al., 1993; McBride et al., 1995). Often referred to as the brain's "reward" pathway, reciprocal DA projections originating from the ventral tegmental area (VTA) to the NAc, limbic system, and orbitofrontal cortex are hypothesized to undergo modifications by drug and ethanol use and abuse (Imperato and Di Chiara, 1986; Wise and Bozarth, 1987; Weiss et al., 1993; Robbins and Everitt, 1996; 1999; Hyman and Malenka, 2001; Chao and Nestler, 2004). Central to the rewarding aspects of alcohol addiction is the NAc, which is a nuclear mass found in the rostroventral part of the ventral striatum bordered medially by the septum and ventrally by the olfactory tubercle (Haines, 2008). The shell and core of the NAc receive different inputs from several brain areas, including the hippocampus, amygdala, prefrontal cortex, thalamus, ventral pallidum, and VTA (Di Chiara, 2002). The various limbic and subcortical structures that project to the shell and core structures of the NAc are both strongly and reciprocally interconnected (Jongen-Relo et al., 1994; Di Chiara, 2002). The feelings of euphoria associated by consumption of alcohol and drugs are related to increased synaptic DA transmission in the reward pathway intertwined with changes in numerous neurotransmitters including γ-aminobutyric acid (GABA), glutamate, serotonin, opioid peptides, and cannabinoids (Wise and Bozarth, 1987; Wise, 1998; Koob, 2000; Koob, 2003a; Chao and Nestler, 2004; Nestler, 2004).

Several studies have shown that decreased activation of the mesolimbic DA pathway in the NAc occurs during acute drug and ethanol withdrawal (Rossetti et al., 1992; Weiss et al., 1992; 1996). In addition, neuroadaptations to chronic drug exposure have been linked with decreased functioning of the neurotransmitter systems implicated in the acute reinforcing effects of drug abuse (Koob and Volkow, 2010). The dopamine hypothesis of drug addiction proposes a hypodopaminergic state during withdrawal linked with decreased motivation for nondrug-related stimuli and increased sensitivity to the abused drug (Melis et al., 2005). Decreased dopamine functioning may be related to withdrawal symptoms such as those observed in humans like fatigue and depression; in addition to those observed in animals including decreased motivation for natural reward and decreased locomotor activity (Pulverenti and Koob, 1993; Barr and Phillips, 1999).

Several animal models are currently used to study the genetic basis for alcoholism (Li et al., 1993; 2001). C57BL/6J (C57) mice innately consume higher amounts of alcohol compared to DBA/2J (DBA) mice (Belknap et al., 1993; Meliska et al., 1995; Podhorna and Brown, 2002). C57 mice are not innately anxious, however, their high alcohol drinking behaviors provide a means to study the motivational aspects of alcohol reward, such as craving (Belknap et al., 1993; Misra and Pandey, 2003). Several studies have shown how dopamine functioning of C57 mice may differ from DBA mice, which may be an important factor in increased alcohol preference of C57 mice compared to DBA mice. Ethanol increased the firing of VTA DA neurons and increase extracellular DA

concentrations in the NAc (Brodie et al., 1990; Appel et al., 2003). Studies have also shown that ethanol-induced excitation of VTA DA neurons from DBA mice were twice as sensitive compared to neurons from C57 mice, suggesting that a lower excitability of VTA DA neurons by ethanol may be related to the high alcohol consumption of C57 mice compared to DBA mice (Cabib et al., 1990; Puglisi-Allegra and Cabib, 1997; Brodie and Appel, 2000). Thus, innate differences in VTA DA projections to the shell of the NAc may be involved in excessive alcohol drinking behaviors of C57 mice. In addition, C57 mice were also shown to have decreased dopamine content and turnover in the terminals of the mesolimbic and mesostriatal dopamine neurons compared to DBA mice, suggesting that a genetically determined hypodopaminergic function in these pathways may play a role in a genetic predisposition to high voluntary intake of ethanol (George et al., 1995).

Several studies have also examined differences in an alcohol preference paradigm between C57 and DBA mice. An experimental paradigm involving continuous ethanol access demonstrated that orally administered ethanol was a more effective reinforcer in C57 mice than in DBA mice (Risinger et al., 1998). In a study involving a limited access paradigm, C57 mice were found to consume more ethanol compared to DBA mice (Le et al., 1994). In our studies we used C57 mice and DBA mice as one animal model to elucidate epigenetic mechanisms in the NAc and amygdala that may be operative in the processes of alcoholism. The NAc and amygdala of C57 and DBA mice have been studied extensively, however, it has not yet been established how these neuroanatomical

structures differ between these two strains of mice in regards to chromatin remodeling and alcoholism. We also used a model of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice to study a possible role for CREB in chromatin remodeling in the NAc and amygdala. To evaluate the effects of ethanol exposure on chromatin remodeling in the NAc and amygdaloid regions of our different mice models we used a relative oral-self administration from a bottle containing alcohol versus one containing water, a so-called "two-bottle free-choice" paradigm (Rhodes et al., 2005). In 1980, Cicero proposed several criteria that an animal model of alcoholism should satisfy: (1) ethanol should be self-administered orally; (2) the amount of ethanol consumed should elevate blood ethanol concentrations to pharmacologically significant levels; (3) ethanol should be consumed primarily for its pharmacological effects, rather than calories, taste, or smell; (4) ethanol should be positively reinforcing; (5) chronic ethanol consumption should produce metabolic and functional tolerance; and (6) chronic ethanol consumption should produce physical signs of dependence (Cicero, 1980). The two-bottle free-choice drinking paradigm is widely used in the field and represents a model in which the mouse exhibits a preference for drinking ethanol compared to water (McClearn and Rodgers, 1959; Belknap et al., 1993; Metten et al., 1998). In this model, consumption is measured after 24 hours over a several day time period in order to determine a relative preference for ethanol versus water in the rodent (Rhodes et al., 2005). In these studies, we deemed the two-bottle free-choice paradigm as a reasonable measure of the effects of ethanol exposure on C57 and DBA mice due to evidence in the

literature demonstrating the effectiveness of the procedure (Dole and Gentry, 1984). We also used the same drinking paradigm in our CREB deficient (+/-) mice model to determine effects of HDAC inhibitor treatment on chromatin remodeling and alcohol drinking behaviors. Please see below for details of epigenetic mechanisms in alcoholism.

c. __Positive and Negative Affective States Associated with__

__Alcohol Intake__

Early evidence demonstrated an increased hereditary risk for developing alcoholism (Goodwin et al., 1974; Cotton, 1979). More recent studies also reinforce that genetic factors are involved in the predisposition to alcoholism, which can play a role in the development of alcohol tolerance and dependence (Cloninger, 1987; Phillips et al., 1994; Foroud and Li, 1999; Koob, 2000; Thome et al., 2000; Radel and Goldman, 2001; Enoch et al., 2003; Pandey, 2003; 2004; Pandey et al., 2008b). Many alcoholics drink either for the rewarding effects of alcohol or to suppress the aversive effects of alcohol withdrawal, or for a combination of the two (Lewis, 1996; Koob, 2003a). The rewarding or reinforcing aspects of alcohol drinking include anticipation of positive outcomes from consumption, such as improved mood and a relaxed state, while the negative aspects or negative reinforcing properties of alcohol drinking include reduction of withdrawal symptoms, stress, pain, and anxiety (Koob 2003a; Grusser et al., 2006). Both the positive and negative affective states of alcohol drinking lead to the development and maintenance of the addictive behavioral pattern observed in alcoholism (Pandey, 2004; Moonat et al., 2010). The positive or euphoric effects of ethanol, associated with activation of the brain's reward circuitry, primarily dominate upon first exposure to ethanol (Koob et al., 1998a). However, after prolonged exposure to ethanol, especially in individuals that have a pre-existing anxiety disorder, the negative reinforcing effects of ethanol, such as self-medication for anxiety during withdrawal, may take over and play a major role in

maintaining excessive drinking behaviors (Kushner et al., 2000; Koob and Le Moal, 2008; Moonat et al., 2010).

Both the NAc and amygdala, particularly the CeA, are thought to play important roles in the processes of alcohol addiction (Yoshimoto et al., 1991; Weiss et al., 1993; McBride, 2002; Pandey et al., 2006; Zhang et al., 2010). In the NAc, ethanol stimulates the release of both dopamine and serotonin (Yoshimoto et al., 1991). The mesolimbic DA system is activated by acute administration of opioids, ethanol, and nicotine (Di Chiara and Imperato, 1988). Chronic drug abuse has been suggested as a critical component in the activation of the NAc reinforcement circuitry (Koob, 1992; Nestler, 2005; Koob and Volkow, 2010). Many of the brain's reward systems converge on the NAc, a region richly innervated by excitatory, inhibitory, and modulatory afferents representing the circuitry necessary for selecting adaptive motivated behaviors (Ikemoto, 2007). All drugs of abuse, including alcohol, when administered acutely, decrease brain stimulation reward thresholds and when administered chronically increase reward thresholds during withdrawal (Koob and Volkow, 2010).

While the VTA-NAc has been implicated in regulating the positive affective states of alcohol dependence, amygdaloid structures have been shown to play a primary role in regulating the anhedonic or dysphoric reactions associated with the negative affective stages of alcoholism (Koob, 2000; 2003b). The extended amygdala is comprised of the BNST, the CeA, and a transition zone in the medial subregion of the NAc (Alheid and Heimer, 1988; Alheid et al., 1998; Koob, 2000; 2003b). The extended amygdala receives numerous afferents from limbic

structures, such as the BLA and hippocampus, and sends efferents to the medial ventral pallidum and lateral hypothalamus (Koob, 2003b). Structures in the extended amygdala serves as neuroanatomical substrates for many psychiatric conditions, including drug and alcohol abuse (Koob, 2000; 2003b; McBride, 2002; Pandey, 2004). Alcoholism is a chronic relapsing disorder and anxiety may serve as a major factor in negatively promoting relapse (Willinger et al., 2002; Koob 2003a). There is a large body of evidence that suggests an important relationship between stress, anxiety, and alcohol abuse disorders (Kushner et al., 2000; Roberts et al., 2000; Silberman et al., 2009). Several clinical studies have shown a significant comorbidity between anxiety and alcohol abuse (Merikangas et al., 1996; Swendsen et al., 1998; Conway et al., 2006). The negative affective state associated with withdrawal from ethanol may be related to neuroadaptations that occur involving modulation of stress pathways. The brain stress system is mediated by CRF (Stengel and Tache, 2010). CRF is thought to play an important role in regulating voluntary ethanol intake (Richardson et al., 2008). CRF is activated during withdrawal from chronic abuse of drugs and alcohol, with a commonality in elevated adrenocorticotropic hormone, corticosterone, and amygdala CRF during acute withdrawal (Koob and Volkow, 2010). The CeA is an important site for both the acute positive reinforcement of ethanol addiction and for the negative reinforcement associated with abstinence and withdrawal (Koob, 2003a). CRF release into the CeA is increased in alcohol-dependent animals and appears to contribute to alcohol withdrawal-related anxiety, which was shown to be reduced by CRF type 1

receptor antagonists injected into the CeA (Rassnick et al., 1993; Merlo-Pich et al., 1995). CRF also contributes to increased alcohol consumption in dependent animals due to increased ethanol self-administration, which was reduced by CRF type 1 receptor antagonists or by deletion of the CRF type 1 receptor itself (Overstreet et al., 2004; Chu et al., 2007; Funk et al., 2007).

In our experiments here, we examined anxiety-like behaviors in a CREB deficient (+/-) mouse model compared to wild-type (+/+) littermate mice using the elevated-plus maze (EPM) test. The EPM test is a widely used model for testing anxiety-like behavior in rodents due to their aversion to open spaces, termed thigmotaxis, or the tendency of rodents to remain close to vertical surfaces, presumably as an innate behavior reflecting taking cover from predators (Montgomery, 1955; Grossen and Kelley, 1972; Treit et al., 1993; Hogg, 1996). The plus-shaped apparatus is elevated approximately 40-70 cm from ground level and consists of two open arms and two arms enclosed with walls about 40-70 cm tall (File, 1993). Reduction in anxiety-like behaviors in the EPM is indicated by an increase in percent open arm entries and percent time spent in the open arm compared with percent closed arm entries and percent time spent in the closed arm (Pellow et al., 1985). Total number of arm entries is also monitored as a measure of general activity of the rodents (Pellow et al., 1985). Using the EPM test as a means to measure the efficacy of pharmacological drugs on anxiety-like behaviors in rodents has been demonstrated (Pellow and File, 1986). In addition, the use of the EPM test to measure anxiety-like behaviors in the mouse has been established (Lister, 1987). Several studies

have used the EPM test to measure anxiety-like behaviors related to alcohol abuse disorders (Lapin, 1993; Wilson et al., 1998; Pandey, 2003; Pandey et al., 2003; 2006). In addition, treatment with the HDAC inhibitor, trichostatin A (TSA), has been shown to prevent development of alcohol withdrawal-related anxiety in rats as measured by the EPM exploration test (Pandey et al., 2008a). Since the EPM is well established in the alcohol research field, we used the EPM exploratory test in our studies to observe anxiety-like behaviors and determine effects of HDAC inhibition by TSA on anxiety-like behaviors in CREB (+/-) mice compared to wild-type (+/+) littermate mice.

d. **CREB, BDNF, and Alcoholism**

It is well established that CREB protein functioning is important in the regulation of synaptic plasticity during long-term memory formation and development of addiction (Pandey et al., 2004; Carlezon et al., 2005; Abel and Zukin, 2008; Alberini, 2009). CREB belongs to the bZIP superfamily of transcription factors. The most abundant CREB isoforms, CREBα and CREBΔ, contain a kinase inducible domain (KID) that has a serine residue at position 133 (Ser-133) (Lonze and Ginty, 2002). CREB mediates the activity of several signaling cascades, including cyclic-AMP-dependent protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), and mitogen-activated protein kinases (MAPKs) (Silva et al., 1998; Impey et al., 1999; Soderling, 1999; Lonze and Ginty, 2002). Phosphorylated CREB (p-CREB) binds to an eight base pair (bp) palindromic sequence (5'-TGACGTCA-3'), called the CRE, that is present in the regulatory region of a variety of genes whose transcription is regulated by cyclic-AMP (Deutsch et al., 1988; Lonze and Ginty, 2002; Cha-Molstad et al., 2004). Transcription of these genes is activated only when CREB is phosphorylated at Ser-133 and binds to the CRE (Lonze and Ginty, 2002). Studies from several labs, including ours, implicate a critical role for CREB in the regulation of alcohol-related behaviors (Asher et al., 2002; Pandey 2003; 2004; Acquaah-Mensah et al., 2006). Alcohol-induced degeneration due to binge drinking was associated with decreased CREB functioning in the hippocampus (Crews and Nixon, 2009). Sensitivity to the effects of ethanol has been associated with CREB transcriptional activity in the cerebellum (Acquaah-

Mensah et al., 2006). Abnormal CREB functioning in the amygdala, which is an important brain region implicated in regulating emotions, has also been linked with a predisposition to anxiety and alcoholism (Pandey et al., 2005b; Wand, 2005; Zhang et al., 2010). Amygdaloid CREB levels were significantly decreased in rats undergoing withdrawal after chronic ethanol exposure (Pandey et al., 2003; 2008b).

CREB signaling is also important in the regulation of downstream expression of genes implicated in addiction, such as BDNF (Janak et al., 2006; Moonat et al., 2010). BDNF is a neurotrophin that is essential for both the survival and differentiation of neurons (Hyman et al., 1991; Poo, 2001). BDNF stimulates many signal transduction cascades by binding to high affinity tropomyosin-related kinase B (TrkB) receptors, resulting in activation of extracellular signal-regulated kinases (Erk1/2), which can then which can phosphorylate CREB and activate CREB-dependent transcription (Impey et al., 1999; Shaywitz and Greenberg, 1999; Sweatt, 2001). In addition, BDNF and TrkB are CREB target genes (Tao et al., 1998; Deogracia et al., 2004). The BDNF gene contains nine short 5' non-coding exons in rodents and humans, which can undergo alternative splicing to generate several different gene transcripts; the alternatively spliced mRNA transcripts contain distinct promoter regions that code for the same protein and allow for temporal and spatial specificity of BDNF expression (Timmusk et al., 1993; Liu et al., 2006; Tsankova et al., 2006).

BDNF is readily inducible by neuronal activity and has important implications in development, neurite outgrowth, and synaptic plasticity (Poo, 2001; Messaoudi et al., 2002). Aberrant BDNF functioning has been implicated in several psychiatric disorders, including schizophrenia, depression, anxiety, and alcohol addiction (Angelucci et al., 2005; Starkman and Pandey, 2007; Davis, 2008). Studies have shown that chronic drug or alcohol use resulted in changes in BDNF mRNA levels and the phosphorylation state of CREB during ethanol exposure and its withdrawal in the piriform cortex, hypothalamus, hippocampus, and amygdala (Pandey et al., 2001; 2003; 2006; 2008b; Meredith et al., 2002; Filip et al., 2006; Prakash et al., 2008). It is possible that decreased BDNF expression in specific brain regions is linked with ethanol dependence and increased ethanol preference. A study from our lab found that deletion of the CREB gene in mice reduced BDNF expression in the brain and also provoked anxiety-like and alcohol drinking behaviors (Pandey et al., 2004). BDNF-haplodeficient mice displayed a higher preference for ethanol compared to wild-type littermates (Hensler et al., 2003; Jeanblanc et al., 2006). Ethanol differentially regulated BDNF expression in the NAc of C57 mice compared to DBA mice (Kerns et al., 2005). Acute alcohol exposure increased BDNF levels in the rat hippocampus and striatum and BDNF has also been shown to be a homeostatic factor in regulating alcohol consumption (McGough et al., 2004; Jeanblanc et al., 2006). On the other hand, chronic ethanol exposure produced a decrease or no change in BDNF levels in the rat hippocampus and forebrain regions (MacLennan et al., 1995; Tapia-Arancibia et al., 2001; Miller et al., 2002).

Using antisense oligodeoxynucleotides (ODNs), our lab established a role for BDNF in ethanol preference. BDNF expression was reduced by BDNF antisense ODNs infused into the CeA and MeA of SD rats, which resulted in increased ethanol consumption and anxiety-like behaviors; in contrast, BDNF co-infusion with antisense ODNs rescued these behaviors (Pandey et al., 2006). These results were supported by another study done in our lab that found P rats innately expressed lower levels of BDNF in the CeA and MeA compared to non-preferring (NP) rats (Prakash et al., 2008; Moonat, et al. 2011). Baseline BDNF protein levels were also found to be lower in the NAc of P rats compared to NP rats (Yan et al., 2005). It is possible that BDNF may play a neuroprotective role during ethanol exposure. A functional CRE in the human BDNF gene promoter region has been shown to modulate BDNF expression and upregulation of BDNF gene expression produced neuroprotective signals through TrkB and promoted cell survival under conditions of cell stress (Fang et al., 2003).

B. <u>Epigenetics and Alcoholism</u>

      In 1953, two scientific papers written by James Watson and Francis Crick changed science as we know it by revealing the structure of the DNA double helix, the macromolecule that carries genetic information and propagates this stored information from one generation to the next (Watson and Crick, 1953a; 1953b). The elucidation of this important structure paved the way for the in depth study of DNA, leading to the "central dogma" encapsulating the processes involved in translating the genetic template required for life; i.e. *DNA → RNA → Protein* (Crick, 1958; 1970). In brief, DNA serves as the template by which RNA is transcribed and then translated into protein. These initial findings on the structure of DNA revealed a wealth of knowledge concerning the study of genetics; in 2003 the Human Genome Project determined the sequence of the human genome in its entirety, including the shocking revelation that a large portion of our genome (98%) exists as "non-protein-coding" DNA, the purpose of which has not fully been unraveled (Schmutz et al., 2004). The landmark discovery by Watson and Crick yielded information that the genetic code is made up of simply four nucleotides: guanine (G), cytosine (C), adenine (A), and thymine (T), which is consistent throughout humanity and life in general. But how does the individuality of people and animals prevail in such a dramatically different fashion? In addition to the genetic code, there exists an "epigenetic" code, which may provide some answers to the indelible question of how each human being or animal exists and is distinguishable as irreplaceable, individual creatures, each containing their own unique thoughts, behaviors, and ideas. A

wealth of recent literature has sparked a new found interest in "epigenetics", a word first communicated in 1957 by Conrad Waddington, who described how identical genotypes could express a wide variety of phenotypes over a lifetime of development (Waddington, 1957). Waddington's theories on epigenetics have taken on additional meaning over the years to specifically explain how epigenetic regulation of gene expression involves changes in gene transcription through chromatin remodeling without changing the DNA sequence (Jaenisch and Bird, 2003). An article by Gosden and Feinberg describes genetics and epigenetics in a rather clever way, coining each phrase as "nature's pen-and-pencil set" (Gosden and Feinberg, 2007). In this article, the "pen" is representative of the ineradicable ink that is staunchly transcribed and passed on to offspring, whereas the "pencil" describes the epigenetic modifications of DNA by addition of methyl groups to cytosine nucleotides, in addition to covalent histone protein modification, around which the DNA is wrapped around. Whereas the genome refers to the relatively immutable sequence of the DNA, the epigenome is highly variable and undergoes dramatic changes throughout life, starting at the earliest stages of development. Just as genetic information is inherited, epigenetic principles are also important in governing heredity and chromosomal segregation.

1. <u>Introduction to Epigenetics</u>

One of the most important functions of DNA is to carry the information stored in genes that specifies the expression of all the RNA and proteins of an organism and passes this information to offspring. The genomes of eukaryotic organisms are packaged into chromosomes, which are long linear DNA molecules associated with many proteins and RNA molecules that are required for the complex processes of gene expression (Alberts et al., 2007). In general, human and animal cells contain 46 chromosomes- 22 pairs of homologous chromosomes, each inherited from maternal and paternal chromosomes, and 2 sex chromosomes, XY in males and XX in females (Alberts et al., 2007). In a diploid cell, genes will typically contain two alleles, each of which can be several alternative forms of a gene and occupy their corresponding locus on homologous chromosomes (Alberts et al., 2007).

Epigenetics describes the direct methylation of DNA and the covalent modification of histone proteins, around which the DNA is wrapped; both mechanisms that work in concert to remodel the chromatin structure and regulate gene expression in the cell (Kornberg, 1974; Olins and Olins, 1974). Epigenetic regulation of gene expression involves changes in transcriptional activity that occurs due to the remodeling of chromatin found in the nucleosome. The nucleosome is a dynamic structure discovered in 1974 that is found in the nucleus of eukaryotic cells (Kornberg, 1974; Olins and Olins, 1974). The nucleosome is a protein-DNA complex containing approximately 147 bp of DNA wrapped around an octamer of histone proteins that make up the histone core

(Smith, 1991; Jenuwein and Allis, 2001). This core structure is also referred to as the histone fold domain, which is located near the carboxyl (C)-terminus of each histone protein, and consists of a central heterotetramer of histones H3 and H4, and two heterodimers of histone H2A and H2B (Luger et al., 1997). Each nucleosomal core particle is coupled together by a single histone linker protein H1, which binds to each nucleosome, making contacts with both DNA and protein (Pruss et al., 1995; Woodcock, 2006). Lysine (K) and arginine (R) residues make up the majority of the amino acids found in the histone amino (N)-terminal "tail" region, that projects out from the histone core and contains these highly basic side chains with positive charges that in effect neutralize the particularly negatively charged DNA backbone (Mersfelder and Parthun, 2006). Hydrophobic interactions and salt bridges also occur within the nucleosome (Luger and Richmond, 1998). Together, these reciprocal actions allow for diverse sequences of DNA to bind to the nucleosomal core (Luger and Richmond, 1998). The majority of covalent modifications occur at the N-terminal tail of histones and include methylation, acetylation, phosphorylation, ubiquitylation, and ADP-ribosylation (Smith, 1991; Jenuwein and Allis, 2001). The most recognized and well established of these epigenetic modifications include histone acetylation and histone and DNA methylation, which control the accessibility of chromatin to essential transcriptional machinery and thereby play a crucial role in the regulation of gene expression levels in the cell (Abel and Zukin, 2008). Acetylation of histone H3 at K-9, in conjunction with phosphorylation at Ser-10, cause the DNA that is wrapped around the histone

core to exist in a relaxed conformation (Cheung et al., 2000). This open

conformation allows for easier transcriptional factor access to the DNA, which in

turn, increases transcriptional activity and gene expression levels in the cell

(Berger, 2007). Methylation of specific histone tail amino acids has also been

linked with activation of transcription (Bauer et al., 2002). However, methylation

is more often associated with gene silencing (Jaenisch and Bird, 2003).

Replacement of acetyl groups located on histone H3 protein at K-9 with methyl

groups (mono-, di-, or trimethylated groups) condenses the chromatin

architecture and prevents transcription factors from accessing the DNA, thus

resulting in repressed gene transcription (Jenuwein and Allis, 2001). Some

residues that are acetylated and/or phosphorylated have also been associated

with decreased gene expression however, for the most part these epigenetic

marks are associated with activation of transcription (Jaenisch and Bird, 2003).

2. Role of Chromatin Remodeling in Regulation of Neuroplasticity

a. Histone Acetylation: HATs and HDACs

Histone modifying proteins include histone acetyltransferases (HATs) and HDACs, which are major players involved in remodeling the chromatin structure and regulating gene expression (Roth et al., 2001; de Ruijter et al., 2003). Regulation of histone acetylation is important during formation of memories in the hippocampus (Levenson et al., 2004). Alterations in histone acetylation have recently been shown to be associated with age-dependent memory impairment in mice (Peleg et al., 2010). A possible role for CREB in epigenetic chromatin remodeling may be through its activation. The activated and phosphorylated form of CREB, p-CREB, recruits another transcription factor called CREB-binding protein (CBP), which has intrinsic HAT capabilities (Chrivia et al., 1993; Kalkhoven, 2004). CBP, along with its transcriptional coactivator p300, enzymatically remodels the nucleosome through the transfer of acetyl groups to histones, thereby allowing the chromatin to exist in a relaxed conformation (Chrivia et al., 1993; Martinez-Balbas et al., 1998; Hsieh and Gage, 2005; Liu et al., 2008). CBP has been shown to be an essential proponent for both short-term and long-term memory formation and consolidation (Korzus et al., 2004; Chen et al., 2010). A recent study in a mouse model for Alzheimer's disease (AD) expressing abnormal CREB functioning demonstrated how brain viral delivery of CBP increased BDNF levels and was able to improve learning and memory deficits (Caccamo et al., 2010). Interestingly, alterations in both memory capacity and responses to stress have also been observed in a knockout mouse

model of p300/CBP-associated factor (PCAF), a CBP homolog that also contains HAT activity (Kalkhoven, 2004; Maurice et al., 2008). Loss of CBP function has increasingly become a target for the treatment of neurological disorders, including Rubinstein-Taybi syndrome (RSTS), Huntington's disease, AD, and amyotrophic lateral sclerosis (Rouaux et al., 2004; Klevytska et al., 2010; Selvi et al., 2010). Mutations in the CBP gene are prevalent in RSTS (Alarcon et al., 2004; Bartsch et al., 2005; Roelfsema et al., 2005). Exon deletions in genes that code for p300 and CBP have been detected in children with RSTS (Tsai et al., 2011). Changes in CBP functioning described in these neurological disorders may be similar to the neuroadaptations that occur during the processes of alcoholism (**Figure 1**). Acute ethanol may cause nucleosomal remodeling through the recruitment of HATs, such as CBP, and the decreased activity of HDACs (Pandey et al., 2008a). Overall, the sum of these modifications results in increased acetylation of histone proteins and the loosening of the chromatin structure, which allows for transcription factors to bind more easily to the DNA, thereby increasing gene expression levels and decreasing anxiety (Moonat et al., 2010). It is possible that chronic ethanol exposure may also affect chromatin remodeling, which can lead to a neuroadaptive state that requires ethanol to maintain homeostasis. Upon withdrawal from chronic ethanol exposure HDACs are activated while HAT activity decreases, which leads to increased methylation, a condensed chromatin structure, and the concomitant silencing of gene expression and anxiogenesis (Pandey et al., 2008a).
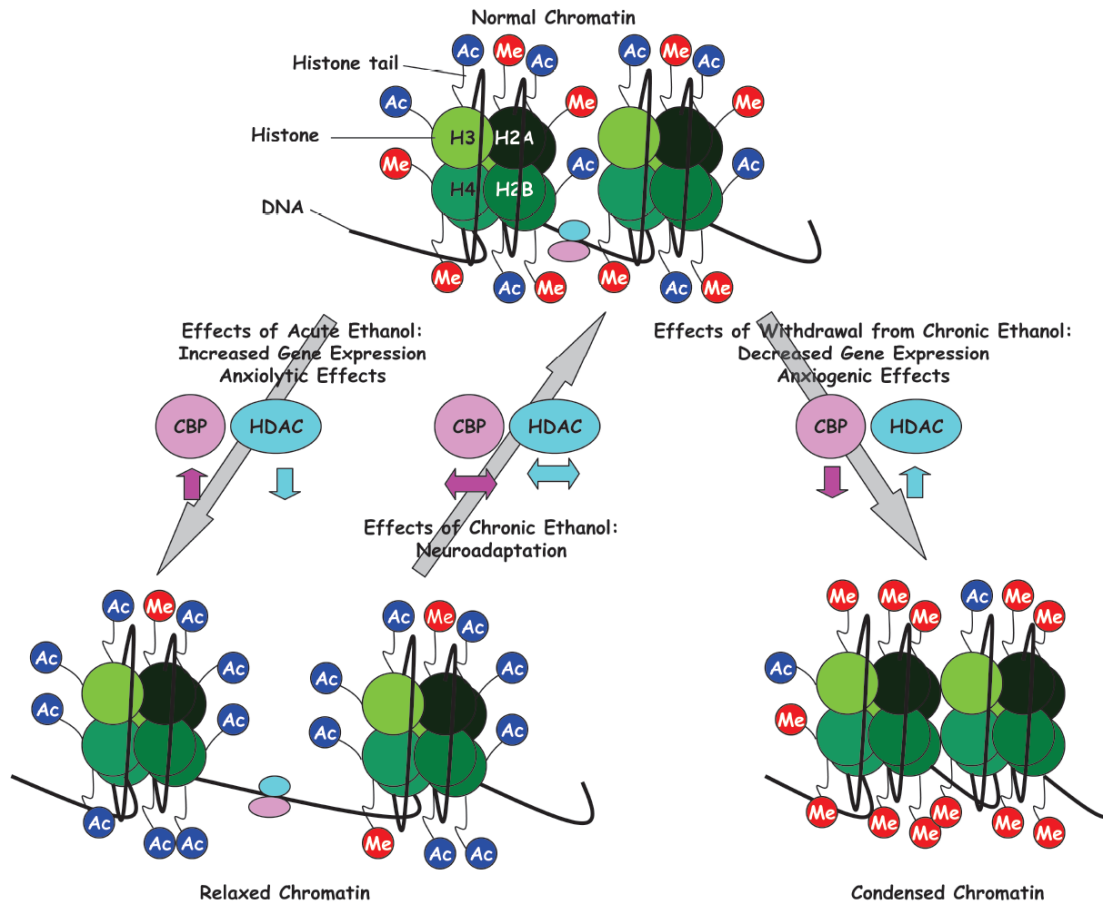
Figure 1.   Possible effects of ethanol on chromatin remodeling: acute and chronic ethanol exposure and withdrawal. Acute ethanol exposure initiates nucleosomal remodeling through the activation of histone acetyltransferases (HATs), such as cyclic-AMP responsive element binding (CREB)-binding protein (CBP) and the decreased activity of histone deacetylases (HDACs). These alterations in enzymatic function increase acetylation of histones, relax the chromatin structure, and temporarily increase gene expression levels in the cell and decrease anxiety. On the other hand, chronic ethanol exposure can have long-term effects due to persistent changes in the overall chromatin structure leading to a neuroadaptive state. Ethanol withdrawal symptoms may be related to increased recruitment of HDACs and decreased CBP activation, which results in decreased histone acetylation, thereby condensing the chromatin structure, silencing gene expression, and increasing anxiogenesis (Pandey et al., 2008a).

Abbreviations: Ac, acetylation; CBP, Cyclic-AMP responsive element binding protein (CREB) binding protein; H2A, histone protein H2A; H2B, histone protein H2B; H3, histone protein H3; H4, histone protein H4; HATs, histone acetyltransferases; HDACs, histone deacetylases; Me, methylation.

HDACs are an important type of protein involved in chromatin remodeling; they utilize their enzymatic capabilities to oppose the activity of HATs by inducing the removal of acetyl groups, thereby resulting in a condensed chromatin conformation and decreased gene expression levels in the cell (Grunstein, 1997; Turner, 2002; Hsieh and Gage, 2005). There are currently two distinct families of HDACs, the Sirtuins (class III), which are a family of NAD+-dependent HDACs, and the classical HDAC family (de Ruijter et al., 2003). Members of the classical HDAC family include class I (isoforms: HDAC 1, 2, 3, and 8) and class II HDACs (isoforms: HDAC 4, 5, 6, 7, 9, and 10) and class IV HDACs (HDAC 11) (de Ruijter et al., 2003; Voelter-Mahlknecht et al., 2005). The catalytic domain of HDACs contains approximately 390 conserved amino acids, while the active site is a tubular pocket with a wide bottom (Finnin et al., 1999). Removal of an acetyl group occurs via a charge-relay system consisting of two adjacent histidine residues, two aspartic acid residues, and one tyrosine residue (Finnin et al., 1999; Buggy et al., 2000). Reversible acetylation at the epsilon-amino group of lysine at the conserved domain of core histones plays an important role in the regulation of chromatin structure and transcriptional activity (Grunstein, 1997; Strahl and Allis, 2000). A Zn^{2+} ion found in the active site of HDACs is displaced by HDAC inhibitors, which in turn inactivates the charge-relay system (Marks et al., 2000). TSA is an HDAC inhibitor that inhibits class I and II HDAC isoforms, but not class III HDACs (Sirtuins) (Yoshida et al., 1995; Blander and Guarente, 2004; Villar-Garea and Esteller, 2004; Dokmanovic and Marks, 2005). The structural conformation of TSA makes it an ideal fit into the HDAC active site, in

that it contains a hydroxamic acid group and a five-carbon atom linked to a phenyl group (Finnin et al., 1999). TSA is also one of the most potently reversible HDAC inhibitors to date, with an IC_{50} in the low nanomolar range (Yoshida et al., 1990). All HDACs are thought to be approximately equally sensitive to inhibition by TSA, which is why TSA is considered a pan-HDAC inhibitor (de Ruijter et al., 2003).

For several years, HDAC inhibitors have been promising anti-cancer agents (Dokmanovic and Marks, 2005; Kelly and Marks, 2005; Dickinson et al., 2010). More recently, HDAC inhibition has been suggested to be a novel treatment for psychiatric disorders (Kazantsev and Thompson, 2008). TSA has potential uses in the treatment of Huntington's disease, multiple sclerosis, and RSTS (Urdingulo et al., 2009). TSA treatment was able to restore memory deficits in CBP transgenic mice that contained HAT deficiencies (Korzus et al., 2004). A recent study showed how inhibitors of class I HDACs were able to reverse contextual memory deficits in a mouse model of AD (Kilgore et al., 2010). In addition, HDAC inhibitor treatment has been shown to have effective antidepressant actions (Tsankova et al., 2006; Covington et al., 2009). Several studies regarding the modulatory effects of HDAC inhibition on cocaine abuse have recently been published, suggesting a role for HDACs in the modulation of cocaine addiction (Romieu et al., 2008; Febo et al., 2009; Malvaez et al., 2010; Wang et al., 2010c).

Even though the use of HDAC inhibitors in the treatment of psychiatric disorders and drugs of abuse is relatively new, HDACs as a therapeutic target for

alcoholism are very promising. Epigenetic mechanisms may be regulating the

processes associated with alcohol abuse and dependence and HDAC inhibitors

can serve as novel treatments for alcoholism. Current treatments for alcoholism

inhibit reward mechanisms in the brain or reduce withdrawal symptoms

associated with alcohol dependence (Littrell, 1991). However, alcohol relapse

rates are commonplace and current treatment are often accompanied by side

effects such as nausea (Gorman, 2007). HDAC inhibitors have been shown to

be promising therapeutic agents in the treatment of drug and alcohol abuse

(Kumar et al., 2005; Pandey et al., 2008a). Recent studies from our lab suggest

a role for HDACs in regulating alcohol tolerance and dependence (Pandey et al.,

2008; Sakharkar et al., 2011). HDAC activity was increased in the amygdala of

alcohol-withdrawn rats, which was accompanied by decreased CBP levels,

histone acetylation, and NPY gene expression (Pandey et al., 2008a). These

data suggest that epigenetic modifications involving CBP result in a refinement of

the amygdaloid chromatin structure, which may be a contributing factor affecting

the expression of genes implicated in alcohol abuse disorders. The studies

detailed here will expand upon these recent epigenetic studies by determining

the effects of TSA on the levels of certain chromatin remodeling proteins in the

brain and if HDAC inhibition by TSA can reverse the anxiety-like and alcohol

drinking behaviors in a mouse model.

b. <u>Histone Methylation</u>

Methylation of the genome is another important mechanism by which the cell regulates transcriptional activation or repression. Methylation of histones is mediated by histone methyltransferases (HMTs), whereas histone demethylation occurs via histone demethylases (HDMs) (Cheung and Lau, 2005; Margueron et al., 2005; Wysocka et al., 2006; Agger et al., 2008; Shukla et al., 2008). The degree of methylation of these specific amino acid residues, which can be either mono-, di-, or tri-methylated, corresponds to the overall chromatin structure (Peters et al., 2003). Tightly packed areas of the chromatin, or heterochromatin, are often highly methylated and transcriptionally inactive, whereas loosely packed DNA, or euchromatin, has areas abundant with transcriptional activity (Kouzarides, 2007). Histone methylation has been shown to be important in the regulation of memory formation (Gupta et al., 2010). Aberrations in histone methylation at K residues have been implicated in several different psychiatric disorders (Akbarian and Huang, 2009). For instance, mutations within genes encoding euchromatic histone H3 K-9-specific methyltransferase, EHMT1, and the H3 K-4-specific demethylase JARID1C/SMCX, have been associated with autism and mental retardation, respectively (Tahiliani et al., 2007; Balemans et al., 2010). Histone methylation, specifically H3-K-4 methylation at gene promoter regions, has also been linked with glutamatergic receptor expression levels, which can be associated with schizophrenia, since a hallmark of this disease is altered glutamate receptor functioning (Chavez-Noriega et al., 2002; Stadler et al., 2005; Conn et al., 2009).

Not only does alcohol exposure affect the brain leading to neuropsychiatric disorders, it has been shown that alcohol can also have serious debilitating effects in other organs and during development. In vivo studies have shown increased histone acetylation in lungs, spleen, and testes of rats treated acutely with ethanol (Kim and Shukla, 2006). Alcohol has also been shown to have deleterious effects on the liver associated with epigenetic changes. Alcoholic liver disease (ALD) is a debilitating process associated with chronic alcohol abuse (Beier and McClain, 2010). A study in primary rat hepatocytes indicated that ethanol induced acetylation of histone H3 at K-9 (Park et al., 2003). In addition, exposure of hepatocytes to ethanol was associated with distinct histone H3 methylation patterns at K-9 and K-4, which correlated with up- and down-regulation of gene expression, respectively (Pal-Bhadra et al., 2007).

C. **Summary and Rationale**

Studies in our lab have shown that C57 mice, compared to DBA mice, expressed lower levels of CREB and p-CREB in the shell of the NAc, a brain region implicated in the reward mechanisms of alcohol use and abuse (Misra and Pandey, 2003; Pandey, 2004). From the literature review, it is reasonable to suggest that changes in CBP-HAT affecting chromatin remodeling in the NAc of C57 mice may be operative in the innate preference for excessive consumption of ethanol in these mice compared to DBA mice. Recent findings from our lab indicate that aberrant chromatin remodeling due to abnormal histone acetylation in the amygdala of rats was involved in the process of alcohol tolerance and dependence (Pandey et al., 2008a; Sakharkar et al., 2011). Since CREB and p-CREB are lower in the NAc of C57 mice compared to DBA mice, these data suggest that low CREB function in the NAc may lead to the decreased recruitment of CBP causing lower histone acetylation and altering gene expression, thereby predisposing C57 mice to high alcohol drinking behaviors. However, the innate differences in chromatin remodeling in the NAc of alcohol-preferring C57 mice compared to non-preferring DBA mice are currently not known.

Studying gene expression in the intact organism is important in determining a gene's biological role. The genotype is the specific set of alleles forming the genome of an organism, whereas the phenotype describes the visible character of the organism. In order to determine the role of CREB in chromatin remodeling we used an animal model containing a genotype of CREB-

haplodeficiency or a CREB-haplodeficient mouse [CREB deficient (+/-) mouse]

model to study chromatin remodeling and also to observe if these mice express

an alcohol drinking and anxiety-like phenotype. CREB deficient (+/-) mice

containing targeted gene mutations encoding for CREB have been developed

(Bourtchuladze et al., 1994; Hummler et al., 1994; Maldonado et al., 1996;

Blendy et al., 1996) and have been used by our lab to establish a role for CREB

in alcohol drinking and anxiety-like behaviors (Pandey et al., 2004). The

mutations in the CREB deficient (+/-) mice result in deletion of two highly

abundant isoforms of CREB (α and Δ) (Hummler et al., 1994; Blendy et al.,

1996). Our studies begin to elucidate the mechanisms by which CREB may

regulate CBP-HAT activity in different brain regions of CREB deficient (+/-) mice

compared to their wild-type (+/+) littermates.

D. <u>Specific Aims</u>

       The overall objectives of the current studies are to examine innate differences in chromatin remodeling (CBP and histone modifications) in the NAc and amygdala of alcohol-preferring C57 mice compared to non-preferring DBA mice and to determine the effects of ethanol exposure on chromatin remodeling in the NAc and amygdala of C57 mice compared to DBA mice. Another objective is to determine the direct role of CREB in the regulation of nucleosomal chromatin remodeling of histone proteins in the NAc and amygdala and its role in anxiety-like and alcohol drinking behaviors using a CREB knockout mouse model. These objectives will be achieved with the following specific aims:

1. Innate aberrant chromatin remodeling in the NAc is operative in abnormal gene expression that may be involved in the excessive alcohol-drinking behaviors of C57 mice compared to DBA mice.

    a. Determine innate differences in CBP, acetylated histone H3 (K-9), acetylated histone H4 (K-8), and methylated histone H3 (K-9) in the NAc (shell and core) and amygdaloid (CeA, MeA, and BLA) brain regions of C57 and DBA mice.

    b. Determine primary localization of acetylated histone H3 (K-9) in the NAc shell of C57 and DBA mice.

2. Ethanol exposure induces histone modifications in the NAc and amygdala of C57 and DBA mice, which may be involved in the processes of alcohol addiction.

    a. Determine effects of voluntary ethanol exposure on levels of CBP and acetylated and methylated histone H3 (K-9) proteins in the NAc and amygdala of C57 mice compared to controls.

    b. Determine effects of acute ethanol exposure (2 g/kg; i.p.) on levels of CBP, acetylated and methylated histone H3 (K-9) proteins, and BDNF in the NAc and amygdala of C57 mice compared to DBA mice.

    c. Determine effects of acute ethanol exposure (2 g/kg) on the number of NeuN-positive neurons in the NAc (shell and core) of C57 mice compared to DBA mice.

3. CREB plays a crucial role in amygdaloid chromatin remodeling and epigenetic aberrations may augment anxiety-like and alcohol drinking behaviors in CREB deficient (+/-) mice compared to wildtype (+/+) littermate mice.

   a. Determine baseline protein levels of p-CREB, CBP, and acetylated histones H3 (K-9) and H4 (K-8) in the amygdala (CeA, MeA, and BLA) and NAc of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.

   b. Determine the effects of HDAC inhibition, using TSA, on anxiety-like and alcohol drinking behaviors of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.

   c. Determine the effects of HDAC inhibition, using TSA, on amygdaloid levels of CBP, and acetylated histones H3 (K-9) and H4 (K-8) in CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.

II. INNATE DIFFERENCES IN CHROMATIN REMODELING IN THE NUCLEUS ACCUMBENS AND AMYGDALA BETWEEN ALCOHOL-PREFERRING C57BL/6J (C57) MICE AND NON-PREFERRING DBA/2J (DBA) MICE

A. Abstract

Covalent histone modifications play an important role in gene expression. C57BL/6J (C57) mice consume higher amounts of ethanol compared to DBA/2J (DBA) mice. Cyclic-AMP responsive element binding protein (CREB) levels have been shown to be innately lower in the shell of the nucleus accumbens (NAc) of C57 mice compared to DBA mice. CREB recruits CREB-binding protein (CBP), which has intrinsic histone acetyltransferase (HAT) activity; therefore, we examined innate differences in CBP, histone H3 (K-9) and histone H4 (K-8) acetylation, and histone H3 (K-9) methylation in NAc and amygdaloid neurocircuitries of C57 and DBA mice using the gold-immunolabeling histochemical procedure. We also determined the colocalization of acetylated histone H3 (K-9) with neuron-specific nuclear protein (NeuN) using double-immunofluorescence labeling in the NAc of C57 and DBA mice. We found lower levels of CBP and acetylated H3 (K-9) and higher methylated H3 (K-9) in the shell, but not core, of the NAc of C57 mice compared to DBA mice. Acetylated H3 (K-9) predominantly localized to NeuN-positive neurons in the NAc of C57 and DBA mice. In addition, lower acetylation was specific to H3 (K-9), since no differences in the levels of acetylated H4 (K-8) were observed in the NAc of C57 mice compared to DBA mice. There were also no differences in protein levels of

CBP, acetylated H3 (K-9) or H4 (K-8), or methylated H3 (K-9) in amygdaloid structures between C57 and DBA mice.   These results suggest that innately lower levels of CBP, and lower acetylation and higher methylation of H3 (K-9) in the NAc shell may be operative in abnormal gene expression involved in the excessive alcohol drinking behaviors of C57 mice compared to DBA mice.

B. Introduction

Many alcoholics drink either for the rewarding effects of alcohol or to suppress the aversive effects of alcohol withdrawal, or for a combination of the two (Lewis, 1996; Koob, 2003a). Many studies suggest that genetic factors play a role in the development of addictive disorders, including alcoholism (Cloninger, 1987; Thome et al., 2000; Radel and Goldman, 2001; Enoch et al., 2003). Several animal models have been developed to study the genetic basis for alcoholism (Li et al., 1993; 2001). Ethanol-preferring C57BL/6J (C57) mice and ethanol non-preferring DBA/2J (DBA) mice represent two genetically different strains of mice frequently used to study alcoholism. C57 mice consume higher amounts of ethanol compared to DBA mice and have also been shown to respond differently to the rewarding aspects of ethanol consumption (Belknap et al., 1993; Meliska et al., 1995; Brodie and Appel, 2000; Misra and Pandey, 2003).

The neural circuitry associated with the euphoric or rewarding responses to ethanol that can lead to the development of addictive behaviors is the mesolimbic dopaminergic (DA) system (Imperato and Di Chiara, 1986; Weiss et al., 1993; McBride et al., 1995; Robbins and Everitt, 1999). DA projections

originate from the ventral tegmental area (VTA) to limbic regions of the brain, namely the shell of the nucleus accumbens (NAc) and prefrontal cortex (Robbins and Everitt, 1996; Tzschentke and Schmidt, 2000). Long-term changes in DA pathways within these brain areas have been associated with repeated drug and ethanol use (Wise and Bozarth, 1987; Robbins and Everitt, 1996; 1999; Hyman and Malenka, 2001; Chao and Nestler, 2004). Cyclic-AMP responsive element binding protein (CREB) is a gene transcription factor that has also been implicated in alcoholism (Pandey 2003; 2004; Pandey et al., 2004). Lower levels of CREB and its phosphorylated form (p-CREB) have been reported in the shell, but not core, of the NAc in C57 mice compared to DBA mice (Misra and Pandey, 2003; Pandey, 2004). Activation of CREB via phosphorylation recruits CREB-binding protein (CBP), which contains intrinsic histone acetyltransferase (HAT) activity (Silva et al., 1998; Mayr and Montminy, 2001; Hsieh and Gage, 2005). The activity of HATs, which are proteins that modify the chromatin architecture by adding acetyl groups to different amino acids, is opposite to that of histone deacetylases (HDACs), which enzymatically remove acetyl residues (Grunstein, 1997; Yang and Seto, 2007). Histone acetylation by HATs can loosen DNA-histone interactions to allow binding of transcriptional machinery, thereby increasing transcription (Grunstein, 1997; Verdone et al., 2005).   Conversely, HDACs cause the chromatin to exist in a condensed form and inhibit gene transcription (Vogelauer et al., 2000; Dokmanovic and Marks, 2005; Moonat et al., 2010).

In addition to acetylation, other important covalent histone modifications include methylation, phosphorylation, and ubiquitylation (Smith, 1991; Jenuwine and Allis, 2001). Methylation of histones and DNA is often associated with reduced gene expression (Cedar and Bergman, 2009). Methyltransferases and demethylases are highly specific and can respectively add or remove methyl groups, either directly to cytosine residues located on DNA or to specific lysine or arginine residues located on histone protein amino (N)-terminal tail regions (Okano et al., 1999; Bestor, 2000; Cheung and Lau, 2005; Margueron et al., 2005; Wysocka et al., 2006; Agger et al., 2008). It has been suggested that HDACs may engage in crosstalk with histone methyltransferases (HMTs) to silence gene transcription via the HDAC-induced removal of acetyl groups, followed by the subsequent addition of methyl groups by HMTs (Zhang and Reinberg, 2001; Cedar and Bergman, 2009).

We have shown that baseline levels of both CREB and p-CREB were lower in the shell of the NAc of C57 mice compared to DBA mice. However, it is unknown how the chromatin architecture varies in the NAc and amygdala between C57 and DBA mice. We hypothesized that aberrant CREB signaling in C57 mice affects gene expression in the NAc due to innate differences in the chromatin structure, which can segue into changes in behaviors, such as the increased alcohol drinking behaviors of C57 mice compared to DBA mice. We therefore examined baseline differences in CBP levels and histone acetylation and methylation in the specific neurocircuitries of C57 mice compared to DBA mice.

C. **Materials and Methods**

1. **Animals**

Adult male C57BL/6J (C57) and DBA/2J (DBA) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used in these studies.   Animals were housed in a temperature-controlled room with a 12-hr light and 12-hr dark cycle and provided with food and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee and concurred with recommendations in the *Guide for the Care and Use of Laboratory Animals*.

2. **Gold-Immunolabeling of CBP, Acetylated Histones H3 (K-9) and H4 (K-8), and Dimethylated Histone H3 (K-9) in NAc and Amygdala of C57 and DBA Mice**

C57 and DBA mice were anesthetized (pentobarbital 50 mg/kg, i.p.) and perfused intracardially with 30-50 ml of normal saline, followed by 30-50 ml of 4% ice-cold paraformaldehyde (PFA) fixative prepared in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post-fixed overnight in PFA at 4°C. After post-fixation, brains were cryoprotected using a sucrose gradient (10, 20, and 30%) prepared in 0.1 M phosphate buffer (pH 7.4). Brains were frozen and 20 μm coronal sections were collected using a cryostat, which were used for gold immunolabeling. The cellular expression of CBP (C-20) (SC-583; Santa Cruz Biotechnology, Santa Cruz, CA), acetylated histones H3 (K-9) and H4 (K-8) (Millipore, Billerica, MA), and dimethylated histone H3 (K-9) (Cell Signaling Technology, Danvers, MA) in amygdaloid brain regions [central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), and basolateral nucleus of

amygdala (BLA)] and NAc structures (shell and core) of C57 and DBA mice was determined using the gold-immunolabeling histochemical procedure, as previously described by us (Pandey et al., 2001; 2005b; 2006). In essence, sections were washed using phosphate-buffered saline (PBS) (2 x 10 min) and then incubated with RPMI 1640 medium (Life Technologies, Grand Island, NY) for 30 minutes at room temperature, followed by incubation with 10% normal goat serum (NGS) prepared in PBS containing 0.25% Triton X-100 (PBST) for an additional 30 minutes at room temperature. Sections were then blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. Sections were incubated with antibodies against CBP (C-20) (1:200), acetylated histone H3 (K-9) (1:500), acetylated histone H4 (K-8) (1:500), and dimethylated histone H3 (K-9) (1:200) prepared in 1% BSA diluted in PBST, overnight (approximately 16 hours) at 4^oC. After 2 x 10 minute washes with PBS, followed by 2 x 10 minute washes with 1% BSA in PBS, sections were incubated with gold particle (1.4 nm)-conjugated anti-rabbit secondary antibody (Nanoprobes, Yaphank, NY) for CBP, acetylated histones H3 (K-9) and H4 (K-8), and dimethylated histone H3 (K-9) (1:200 dilution in 1% BSA in PBS) for one hour at room temperature. Sections were washed 3 times each in 1% BSA in PBS followed by 3 washes using double distilled water. Gold particles were then silver-enhanced (Ted Pella, Redding, CA) for 12 to 20 minutes and then washed several times using tap water in order to stop the developing reaction. Negative controls were also prepared in the same manner, however, primary antibodies were not incubated with these sections. After washings, sections were mounted

on slides, dehydrated in alcohol and xylene, and finally coverslipped using

Permount (Sigma-Aldrich, St. Louis, MO). Gold-immunolabeled proteins [CBP,

acetylated histones H3 (K-9) and H4 (K-8), and dimethylated histone H3 (K-9)]

were quantified using the Loats Image Analysis System (Loats Associates Inc.,

Westminster, MD) connected to a light microscope that calculated the number of

gold particles/ 100 μm^2 area at high magnification (100x) of the brain structure

desired. The threshold of each image was set up to ensure that an area without

staining gave zero counts. Under this condition, gold particles in the defined

areas (3 fields in each section) of 3 adjacent brain sections (20 μm) of each

mouse (n = 5-6) were counted and values averaged for each mouse.

3. Double-Immunofluorescence Labeling of Acetylated Histone H3 (K-9) and NeuN in NAc Shell of C57 and DBA Mice

We determined the cellular localization of acetylated histone H3 (K-9) and anti-NeuN (Millipore, Billerica, MA) in the NAc shell of C57 and DBA mice (n=3) using immunofluorescence double staining. Briefly, brains were collected in the same manner as described above for gold immunolabeling histochemical procedure. Coronal brain sections (20 μm) were obtained using a cryostat and placed in PBS (0.01 M, pH 7.4). Sections were then rinsed 3 x 10 minutes in PBS, blocked with 10% NGS diluted in PBST for 30 minutes and further blocked with 1% BSA prepared in PBST for 30 minutes. Sections were incubated with primary antibodies against acetylated histone H3 (K-9) (1:200) and NeuN (1:500) diluted in PBST containing 2% NGS overnight at 4°C. Negative control sections were prepared without incubation with primary antibodies. Brain sections were then washed with PBS followed by incubation with Alexa Fluor-488 dye conjugated goat anti-rabbit and Alexa Fluor-568 dye conjugated goat anti-mouse (Invitrogen, Molecular Probes, Inc., Eugene, OR) secondary antibodies (1:500 dilution with PBST containing 2% NGS) to localize acetylated histone H3 (K-9) and NeuN, respectively, for 2 hours in the dark at room temperature. Sections were then washed with PBS 3 x 10 minutes, mounted on glass slides and then coverslipped with Vectashield Mounting Medium (Vector Labs, Burlingame, CA). Images were obtained using confocal microscopy (LSM 510; Carl Zeiss Inc., Thornwood, NY).

4. <u>Statistical Analysis</u>

Student's t-test was used to detect significant differences between C57 and DBA mice (n = 5-6). A p-value < 0.05 was considered significant.

D. Results

1. **Baseline CBP Levels in NAc and Amygdaloid Structures of C57 and DBA Mice**

CBP protein levels were measured in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of C57 and DBA mice using the gold-immunolabeling histochemical procedure. Figure 2A indicates the distribution patterns of CBP-positive cells in NAc structures of C57 and DBA mice. It was found that protein levels of CBP were significantly ($p<0.001$) lower in the shell, but not core, of the NAc in C57 mice compared to DBA mice (Fig. 2C). However, there were no significant differences observed in CBP levels in any amygdaloid brain structures (CeA, MeA, or BLA) of C57 mice compared to DBA mice (Figs. 2B & 2C). These data indicate that C57 mice have innately lower CBP levels in the shell, but not core of the NAc, or amygdaloid brain structures compared to DBA mice.

Figure 2. **Baseline CBP levels in NAc and amygdaloid structures of C57 and DBA mice.** C57BL/6J (C57) mice innately express lower levels of CREB-binding protein (CBP) in the shell, but not core of the nucleus accumbens (NAc) or amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] compared to DBA/2J (DBA) mice. **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of CBP gold immunolabeling in the NAc shell and core of C57 and DBA mice. **B:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of CBP gold immunolabeling in the CeA, MeA, and BLA of C57 and DBA mice. **C:** Bar diagram demonstrates the quantified results of CBP in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5-6 mice in each group. *P<0.001, significantly different from DBA mice (Student's t-test).

2. **Baseline Acetylated Histones H3 (K-9) and H4 (K-8) Protein Levels**

in NAc and Amygdaloid Structures of C57 and DBA Mice

CBP contains intrinsic HAT activity and can add acetyl groups to specific lysine residues located at the amino-terminal of histone proteins (Grunstein, 1997; Chan and La Thangue, 2001). To determine if decreased CBP levels are associated with decreased levels of histone acetylation in specific brain regions, we examined levels of acetylated histone proteins H3 (K-9) and H4 (K-8) in the NAc and amygdala of C57 mice compared to DBA mice. It was found that acetylated histone H3 levels were significantly ($p<0.001$) lower in the shell, but not the core of the NAc of C57 mice compared to DBA mice (Figs. 3A & 3C). There were no significant differences observed in acetylated histone H3 protein levels in amygdaloid brain regions (CeA, MeA, or BLA) (Figs. 3B & 3C). Interestingly, we did not observe any significant differences in protein levels of acetylated histone H4 in either the NAc (Figs. 4A & 4C) or amygdaloid (Figs. 4B & 4C) brain structures in C57 mice compared to DBA mice. Since there were no observable differences in the acetylation levels of histone H4 at the K-8 residue, these data suggest that lower acetylation of the K-9 residue of histone H3 in the NAc shell of C57 mice may be operative in the phenotype of higher ethanol intake compared with DBA mice.

Acetylated Histone H3
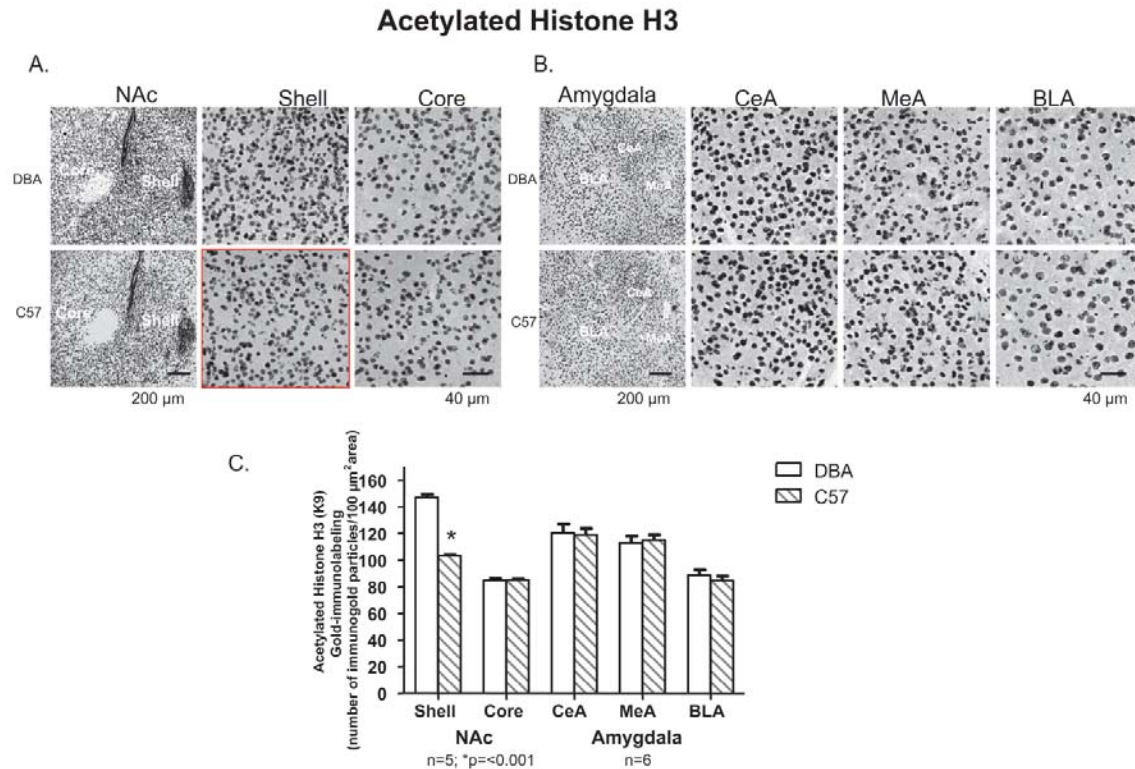
Figure 3.   Baseline acetylated histone H3 (K-9) protein levels in NAc and amygdaloid structures of C57 and DBA mice. C57BL/6J (C57) mice innately express lower levels of acetylated histone H3 (K-9) in the shell, but not core of the nucleus accumbens (NAc) or amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] compared to DBA/2J (DBA) mice.   **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H3 (K-9) gold immunolabeling in the NAc shell and core of C57 and DBA mice.   **B:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H3 (K-9) gold immunolabeling in the CeA, MeA, and BLA of C57 and DBA mice.   **C:** Bar diagram demonstrates the quantified results of acetylated histone H3 (K-9) in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5-6 mice in each group. *P<0.001, significantly different from DBA mice (Student's t-test).

Figure 4.   Baseline acetylated histone H4 (K-8) protein levels in NAc and amygdaloid structures of C57 and DBA mice. There were no significant differences in the levels of acetylated histone H4 (K-8) in the nucleus accumbens (NAc) shell or core or amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of C57BL/6J (C57) mice compared to DBA/2J (DBA) mice. **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H4 (K-8) gold immunolabeling in the NAc shell and core of C57 and DBA mice. **B:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H4 (K-8) gold immunolabeling in the CeA, MeA, and BLA of C57 and DBA mice. **C:** Bar diagram demonstrates the quantified results of acetylated histone H4 (K-8) in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5-6 mice in each group.

3. **Baseline Methylated Histone H3 (K-9) Protein Levels in NAc and Amygdaloid Structures of C57 and DBA Mice**

Previous studies have shown that the K-9 residue located on histone H3 can be covalently modified by both HATs and histone methyltransferases (HMTs) (Turner, 2000; 2002; Stewart et al., 2005). While HATs add acetylated groups to K residues, HMTs add mono-, di-, or tri-methylated groups to either the same or different K residues (Stewart et al., 2005). Since we observed lower levels of acetylation at the K-9 residue of histone H3 in the shell of the NAc of C57 mice compared to DBA mice, we investigated methylation levels at the same K-9 residue of histone H3. Figures 5A & 5C show, that in contrast to the decreased acetylation levels of histone H3 (K-9) observed in the shell of the NAc in C57 mice compared to DBA mice, dimethylated histone H3 (K-9) levels were significantly (p<0.001) increased in the shell of the NAc in C57 mice compared to DBA mice. We did not observe any significant differences in methylated histone H3 (K-9) protein levels in the NAc core (Figs. 5A & 5C) or amygdaloid brain structures (Figs. 5B & 5C). Taken together, these results demonstrate that C57 mice express innately higher levels of methylation and innately lower levels of acetylation at the K-9 residue of histone H3 in the shell of the NAc compared to DBA mice.

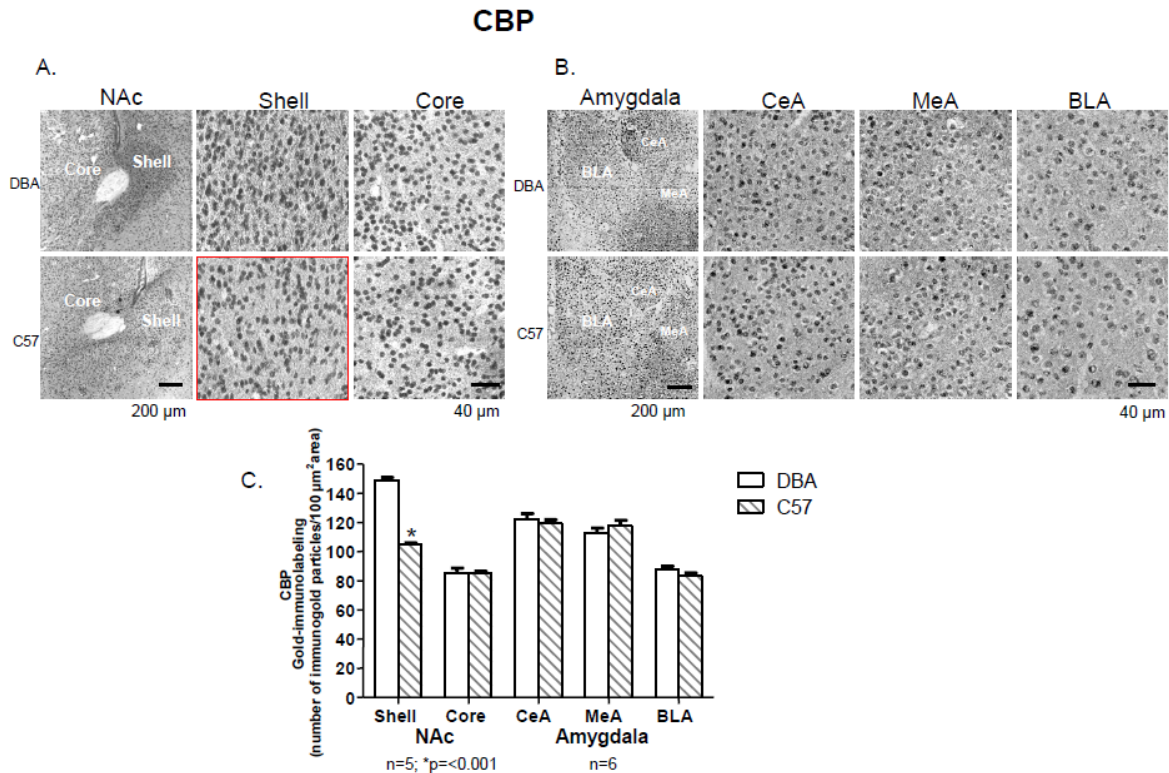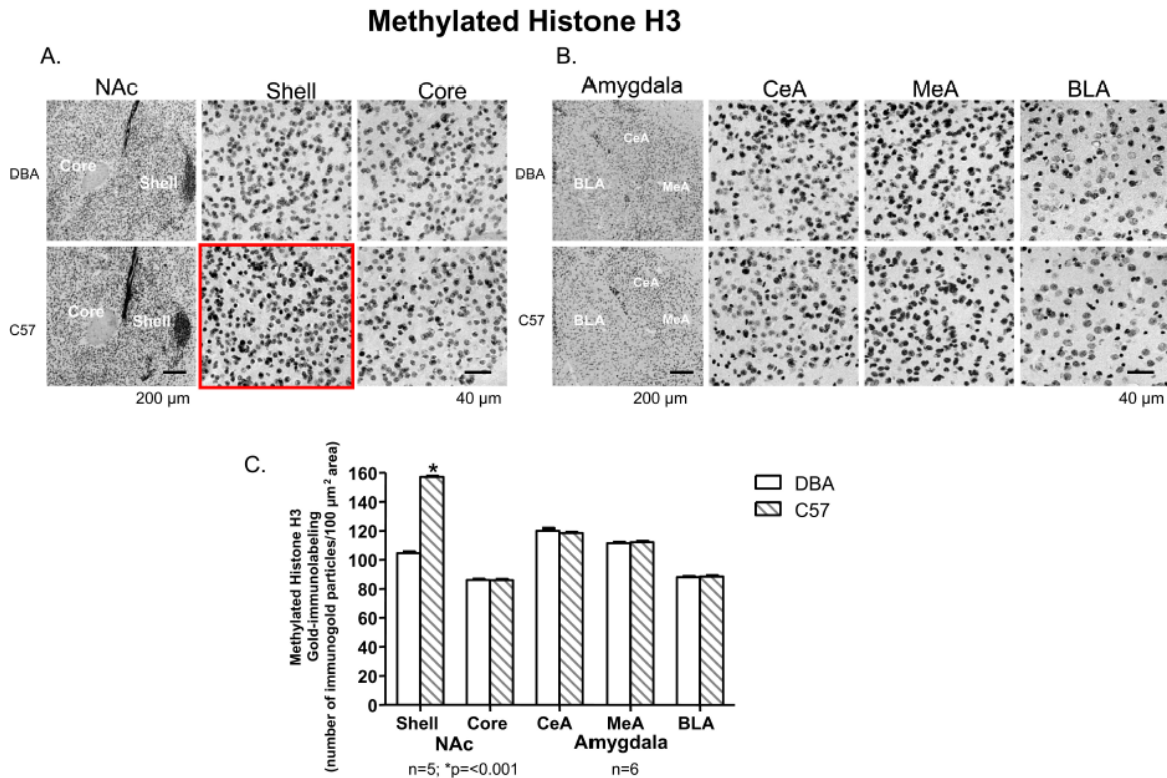**Figure 5.   Baseline methylated histone H3 (K-9) protein levels in NAc and amygdaloid structures of C57 and DBA mice.** C57BL/6J (C57) mice innately express higher levels of dimethylated histone H3 (K-9) in the shell, but not core of the nucleus accumbens (NAc) or amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] compared to DBA/2J (DBA) mice. **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of dimethylated histone H3 (K-9) gold immunolabeling in the NAc shell and core of C57 and DBA mice. **B:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of dimethylated histone H3 (K-9) gold immunolabeling in the CeA, MeA, and BLA of C57 and DBA mice. **C:** Bar diagram demonstrates the quantified results of dimethylated histone H3 (K-9) in terms of number of immunogold particles/100 μm^2 area.   Values are mean ± SEM of 5-6 mice in each group.   *P<0.001, significantly different from DBA mice (Student's t-test).

4. **<u>Colocalization of Acetylated Histone H3 (K-9) and NeuN in NAc of</u>**

   <u>C57 and DBA Mice</u>

In order to determine the cellular localization of acetylated histone H3 (K-9) in the NAc of C57 and DBA mice, we performed immunofluorescence double staining of acetylated histone H3 (K-9) and NeuN. It was found that the majority of acetylated histone H3 (K-9) colocalized with NeuN in the shell of the NAc (Fig. 6), suggesting that acetylated histone H3 (K-9) is mainly localized to and expressed by neurons in the NAc shell. However, some acetylated H3-positive cells were not colocalized with NeuN, suggesting that other types of cells, such as microglia or astrocytes, may also express acetylated H3 (K-9) in the NAc shell brain region. A similar pattern of colocalization was also observed in the NAc core of C57 and DBA mice (data not shown).
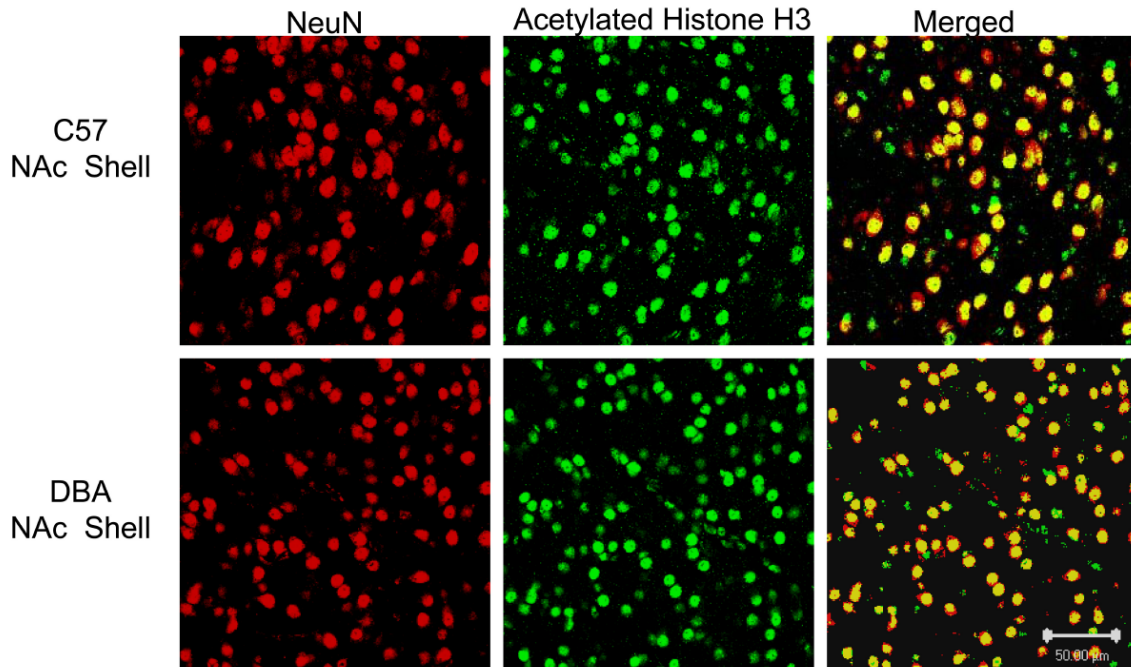
Figure 6. **Colocalization of acetylated histone H3 (K-9) and NeuN in NAc of C57 and DBA mice.** Immunofluorescence double staining (scale bar, 50 μm) to visualize colocalization of acetylated histone H3 (K-9) and neuronal nuclei (NeuN) in the shell of the nucleus accumbens (NAc) of C57BL/6J (C57) mice and DBA/2J (DBA) mice (3 mice in each group). The majority of acetylated histone H3 (K-9) colocalized with NeuN in the shell of the NAc, suggesting that acetylated histone H3 (K-9) is mainly expressed by neurons in the NAc shell. However, some acetylated histone H3-positive cells did not colocalize with NeuN, suggesting that microglia or astrocytes may also express acetylated histone H3 (K-9) in the shell of the NAc.

E. Discussion

The novel findings of this study are that lower levels of CBP occur in the shell of the NAc in C57 mice compared to DBA mice. Lower CBP levels were associated with significantly lower H3 acetylation at K-9 and significantly higher H3 methylation at K-9. We did not find significant differences in CBP, H3 (K-9) acetylation or H3 (K-9) methylation in the core of the NAc or in amygdaloid structures (CeA, MeA, or BLA). Interestingly, we found that H4 acetylation at K-8 was similar in NAc and amygdaloid structures of C57 mice compared to DBA mice. These results correlate with findings from our previous study that showed lower levels of CREB and p-CREB in the shell of the NAc, but not core of the NAc, or amygdala of C57 mice compared to DBA mice (Misra and Pandey, 2003). Together, these findings provide a general understanding of covalent histone modifications in the NAc and amygdala of C57 mice compared to DBA mice.

The NAc has been implicated in reward, drug self-administration, and the reinforcing properties of alcohol and other drugs of abuse (Rassnick et al., 1992; Spanagel and Weiss, 1999; Olive et al., 2001). The mesolimbic DA pathway mediates the rewarding aspects of ethanol and other drugs of abuse (Koob, 1992; Ortiz et al., 1995; Nestler, 2005). Ethanol excites dopamine neurons in the VTA, which leads to the release of dopamine in the NAc (Yoshimoto et al., 1991; Brodie et al., 1999; Boileau et al., 2003). Several studies have suggested the shell of the NAc to be more relevant than the core in alcohol drinking behaviors in rodents (Rodd-Henricks et al., 2002; Misra and Pandey, 2006; Engleman et al.,

2009). For example, ethanol is self-administered into the NAc shell but not the

NAc core in alcohol-preferring (P) rats and Wistar rats (Engleman et al., 2009).

Ethanol also induces a more profound release of dopamine in the shell of the

NAc in P rats compared to alcohol-avoiding rats (Bustamante et al., 2008).

Repeated local exposure of ethanol produced neuroadaptations in posterior VTA

DA neurons that project to the NAc shell, leading to a sustained increase in

sensitivity of these neurons to the stimulating and rewarding effects of ethanol in

rats (Ding et al., 2009). Furthermore, ethanol has also been shown to incite a

more robust excitation of DA VTA neurons in DBA mice compared to C57 mice

(Brodie and Appel, 2000). The results of these later studies suggest that the

shell of the NAc of C57 mice may be innately less sensitive to VTA DA firing,

thereby requiring C57 mice to consume more ethanol than DBA mice in order to

achieve a comparable reward response.

As stated above, our previous findings indicate decreased expression of

CREB and p-CREB in the shell of the NAc (Misra and Pandey, 2003). Here, we

found that baseline CBP levels were also lower in the shell of the NAc of C57

mice compared to DBA mice. It is possible that decreased CBP in the shell of the

NAc may be related to decreased levels of CREB, as our previous findings have

shown (Misra and Pandey, 2003). Phosphorylation of CREB on Ser-133 leads to

the recruitment of CBP to the transcriptional machinery, thereby initiating cyclic

AMP-regulated gene expression (Chrivia et al., 1993; Silva et al., 1998). CBP

and its coactivator, the closely related p300, are important components of the

transcriptional machinery that function to regulate gene expression (Chawla et

al., 1998). CREB is involved in the regulation of several cAMP-inducible genes, including neuropeptide Y (NPY), enkephalin, and dynorphin (Chance et al., 2000; Mayr and Montminy, 2001; Pandey et al., 2003). Baseline levels of dynorphin, enkephalin, and NPY are decreased in the NAc of C57 mice compared to DBA mice (Ng et al., 1996; Ploj et al., 2000; Misra and Pandey, 2003; Hayes et al., 2005). It is reasonable to speculate that lower levels of CBP, and a related decrease in H3 acetylation (K-9) and increase in H3 methylation (K-9) in the shell of the NAc, may be responsible for decreased expression of genes related to these key peptides in C57 mice compared to DBA mice and may be operative in the excessive alcohol drinking behaviors of C57 mice. We also found that acetylated histone H3 (K-9) was mainly colocalized with NeuN in the NAc shell, suggesting that observed innate differences in acetylation and methylation may alter neuronal gene expression leading to the increased alcohol preference phenotype of C57 compared to DBA mice.

The amygdala is another brain region associated with the motivational characteristics of drug and alcohol abuse (Koob et al., 1998a; Pandey, 2003). The findings from a recent study in our lab suggest that changes in amygdaloid chromatin structure of rats may be involved in the processes of alcohol tolerance and dependence (Pandey et al., 2008a; Sakharkar et al., 2011). Here, we found similar levels of CBP and H3 (K-9) acetylation and methylation in the CeA, MeA, and BLA of C57 mice compared to DBA mice. These results correlate with our previous study in which levels of CREB, p-CREB, and NPY were similar in amygdaloid structures of C57 compared to DBA mice (Misra and Pandey, 2003),

suggesting that excessive alcohol drinking of C57 mice may be related to decreased histone acetylation due to decreased CBP recruitment (Levine et al., 2005). A recent study using CBP-haplodeficient mice demonstrated that CBP activation of developmental genes correlated with histone acetylation, while decreased CBP functioning and associated histone acetylation resulted in abnormal cognitive development that persisted at adulthood (Wang et al., 2010a). Another study showed that CBP-related deficits in histone acetylation corresponded with impaired learning and memory, both of which were significantly ameliorated by HDAC inhibitor treatment (Alarcon et al., 2004). Interplay between acetylation and methylation at K-9 of histone H3 may play a critical role in the regulation of chromatin architecture, leading to altered gene expression levels in the cell (Strahl and Allis, 2000; Nakayama et al., 2001; Thatcher and LaSalle, 2006). In contrast to innately lower H3 (K-9) acetylation in the shell of the NAc of C57 mice compared to DBA mice, we found that C57 mice expressed innately higher methylation at K-9 of histone H3 in the NAc shell of C57 mice compared to DBA mice. A recent study showed that contextual fear conditioning was associated with increased H3 methylation at K-9 (Gupta et al., 2010). In this study, the authors inhibited HDACs using sodium butyrate, resulting in decreased histone H3 methylation in the hippocampus of mice following 1 hour of fear conditioning. These data demonstrate that H3 methylation may be coupled to HDAC inhibition in the hippocampus of mice and changes in methylation may facilitate long-term memory formation. In addition, ethanol exposure in hepatocytes resulted in decreased H3 K-9 methylation,

which was associated with downregulated gene expression (Pal-Bhadra et al., 2007). It is possible that the epigenetic modifications that we observed in histone H3 may be responsible for the regulation of genes involved in the development of alcoholism. Taken together, these epigenetic modifications suggest that a chromatin remodeling-related mechanism may be involved in contributing to a genetic preference for higher ethanol consumption observed in C57 and not DBA mice. This notion is further supported by recent findings indicating aberrant chromatin remodeling in NAc may be involved in the individual differences in alcohol drinking behaviors of mice (Wolstenholme et al., 2011).

Another interesting finding of this study was that significant changes in both acetylation and methylation occurred on histone H3 (K-9), but not histone H4 (K-8), suggesting chromatin remodeling due to H3 histone modifications may play a role in alcohol drinking behaviors. It has been shown that the acute effects of cocaine were predominantly associated with changes in H4 acetylation, whereas chronic effects were associated with changes in H3 acetylation (Kumar et al., 2005). These data indicate that specific epigenetic modifications occur on either histone H3 or H4 and depend on the length of drug exposure. Furthermore, specific patterns of histone acetylation, such as deacetylation of H3 but not H4, may be regulated by targeting HDACs to methylated chromatin (Gregory et al., 2001). It is not clear whether this phenomenon is due to the specific recruitment of HDACs or because of compensatory mechanisms that can selectively recruit HATs. Nonetheless, our data suggest that covalent modifications of histone H3, specifically in the NAc shell of C57 mice, play an

important role in alcohol preference. However, several key questions still remain and need to be addressed in future studies, including how exposure to ethanol may elicit changes in the chromatin architecture and related gene expression in the NAc and amygdala and if HDAC inhibition can modulate the excessive alcohol drinking behaviors of C57 mice.

In conclusion, these results provide novel evidence that alcohol-preferring C57 mice display aberrant chromatin remodeling, specifically in the shell of the NAc, compared with non-preferring DBA mice. Innately lower CBP expression in the shell of the NAc, but not core, or amygdaloid structures of C57 mice compared to DBA mice may lead to decreased acetylation and increased methylation of H3 at K-9. Innately different chromatin remodeling machineries in these different strains of mice (C57 and DBA) may be a reflection of the differential expression of genes involved in regulating reward pathways in the NAc and may therefore be responsible for the apparent differences in alcohol preference between C57 and DBA mice.

F. Significance

The observed innate differences in the levels of CBP, acetylated histone H3 (K-9), and methylated histone H3 (K-9) in the NAc shell may be a critical factor contributing to a genetic preference for alcohol consumption by C57 mice compared to DBA mice. Acetylated histone H3 (K-9) mainly localized to neurons, as observed by immunofluorescence double staining of acetylated histone H3 (K-9) with NeuN. Taken together, our results reveal a novel role for NAc chromatin remodeling in alcohol drinking behaviors.

III. EFFECTS OF ACUTE AND VOLUNTARY ETHANOL EXPOSURE ON CHROMATIN REMODELING IN THE NUCLEUS ACCUMBENS AND AMYGDALA OF C57 AND DBA MICE

A. Abstract

Accumulating evidence suggests that genetic factors may play a critical role in alcohol drinking behaviors. C57BL/6J (C57) mice have an innate preference for alcohol consumption compared to DBA/2J (DBA) mice. Alterations in cyclic-AMP responsive element binding protein (CREB) functioning in the brain have been implicated in alcohol drinking behaviors. Studies in our lab have shown that C57 mice innately express lower levels of CREB and its phosphorylated form, p-CREB, in addition to lower levels of neuropeptide Y (NPY), a neurotrophin also implicated in alcohol abuse disorders, in the shell, but not core of the nucleus accumbens (NAc) or amygdala, compared to DBA mice. Epigenetic modifications may also play important roles in the regulation of neuroplasticity leading to increased alcohol drinking behaviors. We demonstrated in Specific Aim 1 that C57 mice innately expressed lower levels of the histone acetyltransferase (HAT) CREB-binding protein (CBP) and acetylated histone H3 (K-9), and innately higher levels of methylated histone H3 (K-9) in the shell of the NAc but not core or amygdala compared to DBA mice. Aberrant chromatin remodeling in the shell of the NAc may be involved in increased alcohol preference of C57 mice compared to DBA mice; it is also possible that innate differences in chromatin remodeling result in diverging responses to ethanol exposure in C57 and DBA mice. In order to determine the effects of

ethanol exposure on brain chromatin remodeling in C57 mice compared to DBA mice, we examined the effects of voluntary ethanol exposure on levels of CBP, acetylated histone H3 (K-9) and methylated histone H3 (K-9) in the NAc and amygdala of C57 mice exposed to ethanol compared to C57 control mice using the immunolabeling histochemical procedure. We also determined the effects of acute ethanol exposure on protein expression of CBP, acetylated histone H3 (K-9), methylated histone H3 (K-9), and brain-derived neurotrophic factor (BDNF), a neurotrophin implicated in alcohol abuse disorders, in the NAc and amygdala of C57 and DBA mice. In addition, we measured baseline numbers of NeuN-positive neurons and then examined the effects of acute ethanol exposure on the numbers of NeuN-positive neurons in the shell and core of the NAc of C57 and DBA mice. We found that voluntary ethanol exposure significantly increased levels of CBP and acetylated histone H3 (K-9) in the shell, but not core of the NAc or amygdaloid structures of C57 mice compared to control mice. Voluntary ethanol exposure significantly decreased levels of methylated histone H3 (K-9) in the shell, but not core of the NAc, and also decreased levels of methylated histone H3 (K-9) in the amygdala [central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), and basolateral nucleus of amygdala (BLA)] of C57 mice compared to control mice. Acute ethanol exposure increased levels of CBP, acetylated histone H3 (K-9), and BDNF in the shell, but not core of the NAc or amygdaloid structures of C57 mice compared to control mice. Acute ethanol exposure decreased levels of methylated histone H3 (K-9) in the shell, but not core of the NAc or amygdaloid structures of C57 mice compared to control mice.

Interestingly, acute ethanol exposure increased levels of CBP, acetylated histone H3 (K-9), and BDNF in both the shell and core of the NAc of DBA mice compared to controls, while having no effects on amygdaloid structures (CeA, MeA, or BLA). In addition, acute ethanol exposure decreased levels of methylated histone in both the shell and core of the NAc of DBA mice compared to controls, while eliciting no effects in amygdaloid structures (CeA, MeA, or BLA). Baseline numbers of NeuN-positive neurons were similar in the NAc shell and core between C57 and DBA mice and acute ethanol exposure had no effects on the number of NeuN-positive neurons in the NAc shell or core of C57 and DBA mice. These findings suggest that ethanol exposure has a profound effect on brain chromatin remodeling in both C57 and DBA mice; and voluntary and ethanol-induced chromatin remodeling in the NAc (shell and core) and amygdala (CeA, MeA, BLA) of C57 and DBA mice may play a role in the development of alcoholism in both strains of mice.

B. <u>Introduction</u>

   Recently, a prominent role for epigenetics in the regulation of ethanol's actions has emerged (Pandey et al., 2008a; Shukla et al., 2008; Sakharkar et al., 2011). Epigenetic control of chromatin remodeling appears to be one way in which transcriptional regulation of gene expression occurs in the brain (Borrelli et al., 2008). The nucleosome core particle is the fundamental repeating unit of chromatin and consists of 147 bp of DNA wrapped around an octamer of core histone proteins (H2A, H2B, H3, and H4) (Jenuwein and Allis, 2001). Variations in the nucleosome structure elicited by different mechanisms cause subtle changes in the chromatin architecture that correlate with either an open or closed chromatin state (Strahl and Allis, 2000). These open and closed chromatin states also correlate with euchromatin and heterochromatin, which most often is associated with increased and decreased gene expression in the cell, respectively (Berger, 2007). Covalent histone modifications, including the most well documented: phosphorylation, methylation, and acetylation, work together to modify the chromatin fiber (Grunstein, 1997). Acetylation has long been correlated with increased transcriptional activity, likely as a result of weakening of histone-DNA interactions due to neutralization of the positively charged lysine residue by addition of acetyl groups (Bauer et al., 1994). In contrast, hypoacetylated states and hypermethylated states of histones have been associated with gene silencing (Turner, 2000; Kouzarides, 2002; Cheung and Lau, 2005). Acetylation and methylation result from the respective enzymatic addition or removal of acetyl and methyl groups to histone proteins. Acetylation

is mediated by histone acetyltransferases (HATs), while histone deacetylases

(HDACs) remove acetyl groups (Roth et al., 2001; de Ruijter et al., 2003).

Methylation affects both histone proteins and DNA and is mediated by histone

methyltransferases (HMTs) and demethylases (Zhang and Reinberg, 2001;

Kouzarides, 2002). Cyclic-AMP responsive element binding protein (CREB) has

been implicated as a regulator of addictive behaviors, including alcoholism

(Nestler, 2004; Pandey et al. 2005a). Activation and phosphorylation of CREB

results in the recruitment of CREB-binding protein (CBP), which is a HAT (Mayr

et al., 2001). In our current study, we chose to study C57BL/6J (C57) and

DBA/2J (DBA) mice because C57 mice innately prefer alcohol consumption

compared to DBA mice, in addition to innately expressing lower levels of CREB,

p-CREB, and neuropeptide Y (NPY) in the shell of the nucleus accumbens (NAc)

but not core or amygdaloid structures (Misra and Pandey, 2003). The NAc and

amygdala are important limbic brain regions implicated in the processes of

alcoholism and addiction (Di Chiara, 2002; Boileau et al., 2003; Koob 2003a;

Pandey et al., 2003; Bustamante et al., 2008; Carlezon Jr. and Thomas, 2009;

Zhang et al., 2010). However, the regulatory effects of ethanol exposure on

epigenetic mechanisms in the NAc and amygdala of C57 and DBA mice have not

been clearly elucidated. What is currently known is that ethanol results in the

selective acetylation of histone H3 at the lysine (K)-9 residue in primary cultures

of rat hepatocytes, hepatic stellate cells, and also in rats acutely treated with

ethanol in vivo (Park et al., 2003; Kim and Shukla, 2005; 2006). Acetylation

induced by ethanol may be mediated through HAT activity. A study by Park et

al., demonstrated the involvement of HATs in ethanol-induced acetylation of histone H3 at K-9 in hepatocytes, which correlated with increased gene expression (Park et al., 2005). On the other hand, ethanol was also shown to relay distinct methylation patterns of histone H3 at K-9, which were associated with decreased gene expression levels in hepatocytes (Pal-Bhadra et al., 2007).

From Specific Aim 1, we found that C57 mice express lower baseline levels of the HAT, CBP, and acetylated histone H3 (K-9) in the NAc shell, but not core, compared to DBA mice. In contrast to lower levels of acetylated histone H3 (K-9), we also demonstrated in Specific Aim 1 that C57 mice express higher methylation of histone H3 (K-9) in the shell of the NAc, but not core, compared to DBA mice. We did not observe any changes in the levels of any of these proteins in amygdaloid structures (CeA, MeA, or BLA). Therefore, we reasoned that voluntary ethanol exposure may affect the expression levels of these proteins in the shell of the NAc of C57 mice. We extended the studies of Specific Aim 1 to demonstrate the effects of voluntary ethanol exposure on levels of CBP, acetylated histone H3 (K-9), and methylated histone H3 (K-9) in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of C57 mice exposed to ethanol (C57 + Ethanol) compared to controls (C57 + Water). We chose to study a C57 mice model of voluntary ethanol exposure, and not DBA mice, since C57 mice have repeatedly demonstrated a pattern of voluntary ethanol consumption greater than 10 g/kg/day, whereas DBA mice consumed less than 2 g/kg/day (Yoneyama et al., 2008). We found that voluntary ethanol exposure increased levels of CBP and acetylated histone H3 (K-9) in the shell but not core of the

NAc, while at the same time decreasing levels of methylated histone H3 (K-9) in the shell but not core of the NAc. Interestingly, while voluntary ethanol exposure had no significant effects on levels of CBP or acetylated histone H3 (K-9) in amygdaloid structures, we did find that voluntary ethanol decreased levels of methylated histone H3 (K-9) in the CeA, MeA, and BLA.

Acute responses to ethanol have a predictive value regarding long-term ethanol drinking behaviors in both humans and animal models (Schuckit, 1994; Metten et al., 1998). Therefore, since voluntary ethanol exposure had significant effects on protein levels of CBP, and acetylated and methylated histone H3 (K-9) protein levels, we also wanted to determine the effects of acute ethanol exposure on the levels of these proteins in C57 mice compared to DBA mice. Moreover, since brain-derived neurotrophic factor (BDNF) has been implicated in alcohol abuse disorders and is also a downstream target of the CREB signaling pathway, we also determined the effects of acute ethanol exposure on BDNF expression levels (McGough et al., 2004). In addition, we measured NeuN expression levels in both the shell and core of the NAc in order to establish that any observed changes in the levels of the aforementioned proteins were not due to differences in neuronal numbers in these specific brain regions. Similar to voluntary ethanol exposure, we found that acute ethanol exposure increased levels of CBP and acetylated histone H3 (K-9) in the shell, but not core of the NAc or amygdaloid structures of C57 mice compared to control mice. Acute ethanol exposure decreased methylated histone H3 (K-9) in the shell, but not core of the NAc or amygdaloid structures of C57 mice compared to control mice. In addition, C57

mice expressed innately lower levels of BDNF in the NAc shell, but not core or amygdaloid structures compared to DBA mice; acute ethanol exposure increased the levels of BDNF in the NAc shell but not core or amygdaloid structures of C57 mice compared to control mice. The effects of acute ethanol exposure differed in DBA mice compared to C57 mice. We found that in DBA mice, acute ethanol exposure increased levels of CBP, acetylated histone H3 (K-9), and BDNF in both the shell and core of the NAc, while there were no observable effects in the amygdala. We also found that methylated histone H3 (K-9) was decreased in both the shell and core of the NAc of DBA mice compared to controls after acute ethanol exposure, while no observable effects were found in the amygdala. Similar to voluntary ethanol exposure, acute ethanol exposure had no significant effects on the number of NeuN-positive neurons in the NAc shell or core structures between C57 and DBA mice. Our results suggest that the NAc is an important part of the reward neurocircuitries of both C57 and DBA mice and that several discrete mechanisms may contribute to the differential activation of the shell and core of the NAc between C57 and DBA mice, which may result in the divergent alcohol drinking behaviors between these two strains of mice.

C. **Materials and Methods**

1. **Animals**

Adult male C57BL/6J (C57) and DBA/2J (DBA) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-controlled room with a 12-hr light and 12-hr dark cycle and provided with food and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee and concur with recommendations in the *Guide for the Care and Use of Laboratory Animals*. Adult C57 mice were used to determine the effects of voluntary ethanol intake on chromatin remodeling proteins [CBP, acetylated histone H3 (K-9), and methylated histone H3 (K-9)]. Adult C57 and DBA mice were used to determine effects of acute ethanol, injected intraperitoneally (I.P.), on protein levels of CBP, acetylated histone H3 (K-9), methylated histone H3 (K-9), BDNF, and the number of NeuN-positive neurons.

2. **Gold-Immunolabeling of CBP, Acetylated Histone H3, Dimethylated Histone H3, BDNF, and NeuN in NAc and Amygdala of C57 and DBA Mice**

C57 and DBA mice were anesthetized (pentobarbital 50 mg/kg, i.p.) and perfused intracardially with 200 ml of n-saline, followed by 300 ml of 4% ice-cold paraformaldehyde (PFA) fixative prepared in 0.1 M phosphate buffer (pH 7.4). Brains were removed following perfusion and post-fixed overnight in PFA at 4°C. After postfixation, brains were cryoprotected using a sucrose gradient (10, 20, and 30%) prepared in 0.1 M phosphate buffer (pH 7.4). Brains were then frozen and 20 μm coronal sections were collected using a cryostat, which were used for gold immunolabeling. The cellular expression of CBP (C-20) (SC-583; Santa Cruz Biotechnology, Santa Cruz, CA), acetylated histone H3 (K-9) (Millipore, Billerica, MA), dimethylated histone H3 (K-9) (Cell Signaling Technology, Danvers, MA), brain-derived neurotrophic factor (BDNF H-117) (Santa Cruz Biotechnology, Santa Cruz, CA), and neuron-specific neuronal marker (NeuN) (Millipore, Billerica, MA) in amygdaloid structures (CeA, MeA, and BLA) and NAc structures (shell and core) of C57 and DBA mice was determined using the gold-immunolabeling histochemical procedure, as previously described by us (Pandey et al., 2001; 2005b; 2006). In essence, sections were washed using phosphate-buffered saline (PBS) (2 x 10 min) and then incubated with RPMI 1640 medium (Life Technologies, Grand Island, NY) for 30 minutes at room temperature, followed by incubation with 10 % normal goat serum (NGS) prepared in PBS containing 0.25% Triton X-100 (PBST) for an additional 30 minutes at room

temperature. Sections were blocked with 1% bovine serum albumin (BSA) in

PBS for 30 minutes at room temperature. Sections were incubated with

antibodies against CBP (1:200), BDNF (1:200), NeuN (1:500), acetylated histone

H3 (K-9) (1:500), and dimethylated histone H3 (K-9) (1:200) prepared in 1% BSA

diluted in PBST, overnight (approximately 16 hours) at 4^oC. After 2 x 10 minute

washes with PBS, followed by 2 x 10 minute washes with 1% BSA in PBS,

sections were incubated with gold particle (1.4 nm)-conjugated anti-rabbit

secondary antibody (Nanoprobes, Yaphank, NY) for CBP, acetylated histone H3

(K-9), dimethylated histone H3 (K-9), and BDNF (1:200 dilution in 1% BSA in

PBS) or gold particle (1.4 nm)-conjugated anti-mouse secondary antibody for

NeuN (1:200 dilution in 1 % BSA in PBS) for one hour at room temperature.

Sections were washed 3 times each in 1 % BSA in PBS followed by 3 washes

using double distilled water. Gold particles were silver-enhanced (Ted Pella,

Redding, CA) for 12 to 20 minutes and washed several times using tap water in

order to stop the developing reaction. Negative controls were also prepared in

the same way however, primary antibodies were not incubated with these

sections. After washing, sections were mounted on slides, dehydrated in alcohol

and xylene, and coverslipped using Permount (Sigma-Aldrich, St. Louis, MO).

Gold-immunolabeled proteins [CBP, BDNF, acetylated histone H3 (K-9), and

dimethylated histone H3 (K-9)] were quantified using the Loats Image Analysis

System (Loats Associates Inc., Westminster, MD) connected to a light

microscope that calculated the number of gold particles/100 μm^2 at high

magnification (100x) of the brain structure desired. The threshold of each image

was set up to ensure that an area without staining gave zero counts. Under this condition, gold particles in the defined areas (3 fields in each section) of 3 adjacent brain sections of each mouse were counted and values were averaged for each mouse. The number of NeuN-positive neurons in the NAc of C57 and DBA mice was quantified using the Neurolucida program (MBF Bioscience, MicroBrightField, Inc., Chicago, IL). The number of NeuN-positive neurons in the shell and core of the NAc was counted under low magnification (20x) in defined areas (3 fields in each section for both shell and core). Under this condition, the number of NeuN-positive neurons in each of the defined areas was counted and values were averaged for each mouse (n=5).

3. <u>Voluntary Ethanol Drinking Paradigm of C57 Mice</u>

Ethanol preference was measured using a modified two-bottle free-choice paradigm described previously by us (Misra and Pandey, 2003) and others (Thiele et al., 1998; 2000). Mice were habituated to drink tap water equally from two bottles (approximately 1.5 weeks). Fresh bottles were given everyday at 5pm and drinking was measured to ensure that the mice continued to drink equally from both bottles; the placement of the bottles was switched every day to prevent a positional habit from forming. Once a pattern of drinking water equally from both bottles was established, mice were provided with 7% ethanol (95% ethanol diluted in tap water) in one bottle and tap water in the other bottle for three consecutive days, followed by 9% ethanol for the next six days. At the end of nine days of ethanol exposure (10th day morning), animals were anesthetized, perfused, and their brains dissected out for immunohistochemical analysis.

4. <u>**Acute Ethanol Injections of C57 and DBA Mice**</u>

On the day prior to the start of the experiment, animals were re-weighed and placed in separate cages. C57 and DBA mice were injected (i.p.) with 20% ethanol (95% w/v ethanol diluted in sterile saline, up to a final concentration of 2 g/kg, depending on the body weight of the animal). Briefly, 20 g of 95% w/v ethanol was measured on a laboratory scale and diluted to 100 ml of normal saline (n-Saline) (0.9%; sterile); yielding a final ethanol solution concentration of 0.2 g/ml. Control C57 and control DBA mice were injected with sterile saline (10 μl/g of body weight). The first set of C57 and DBA mice received injections at 9:00 am and the rest of the animal sets followed in 15-minute increments. C57 mice and DBA mice were separated into two groups: the ethanol treated group and the saline (control) treated group. One hour post-injection, animals were anesthetized, perfused, and brains were dissected out for immunohistochemical analysis. Blood was collected from each mouse at the time of brain collection and analyzed for blood alcohol content using the Analox Alcohol Analyzer (Analox Instruments, Lunenberg, MA).

5. **Statistical Analysis**

For voluntary ethanol consumption studies (C57 + Water/Water; C57 + Ethanol/Water), we used n= 6 mice per group for immunohistochemical analyses. We applied the Student's t-test function using SigmaStat statistical software to detect significant median differences between the following groups used for the voluntary ethanol consumption studies: C57 + Water and C57 + Ethanol. For acute ethanol injection studies, we analyzed multiple groups [C57 + n-Saline (10 μl/g); C57 + ethanol (2 g/kg); DBA + n-Saline (10 μl/g); DBA + Ethanol (2 g/kg)] by one-way analysis of variance (ANOVA) using SigmaStat; we used n= 5 C57 and n= 5 DBA mice for the gold-immunolabeling studies. All post-hoc comparison procedures were analyzed using Tukey's test. P-values < 0.05 were considered significant and data in the figures were expressed as mean ± standard error of the mean (SEM).

D. <u>**Results**</u>

    1. <u>**Effects of Voluntary Ethanol Exposure on CBP Levels in NAc**</u>

        <u>**and Amygdaloid Structures of C57 Mice**</u>

        The effects of voluntary ethanol exposure on CBP protein levels were

measured in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of

C57 mice using the gold-immunolabeling histochemical procedure. Figure 7A

indicates the distribution patterns of CBP-positive cells in NAc structures of C57

mice that voluntarily consumed ethanol (C57 + Ethanol) and water (C57 +

Water). It was found that voluntary ethanol exposure significantly ($p < 0.001$)

increased protein levels of CBP in the shell, but not core, of the NAc in C57 mice

compared to controls (C57 +Water) (Figs. 7A & 7B). Interestingly, voluntary

ethanol exposure had no significant effects in the amygdala, as there were no

significant differences observed in CBP levels in any amygdaloid brain structures

(CeA, MeA, or BLA) of C57 mice compared to control mice (Figs. 8A & 8B).

These data indicate that voluntary ethanol consumption by C57 mice increased

CBP levels in the NAc shell, but not core, or amygdaloid brain structures

compared to control C57 mice.
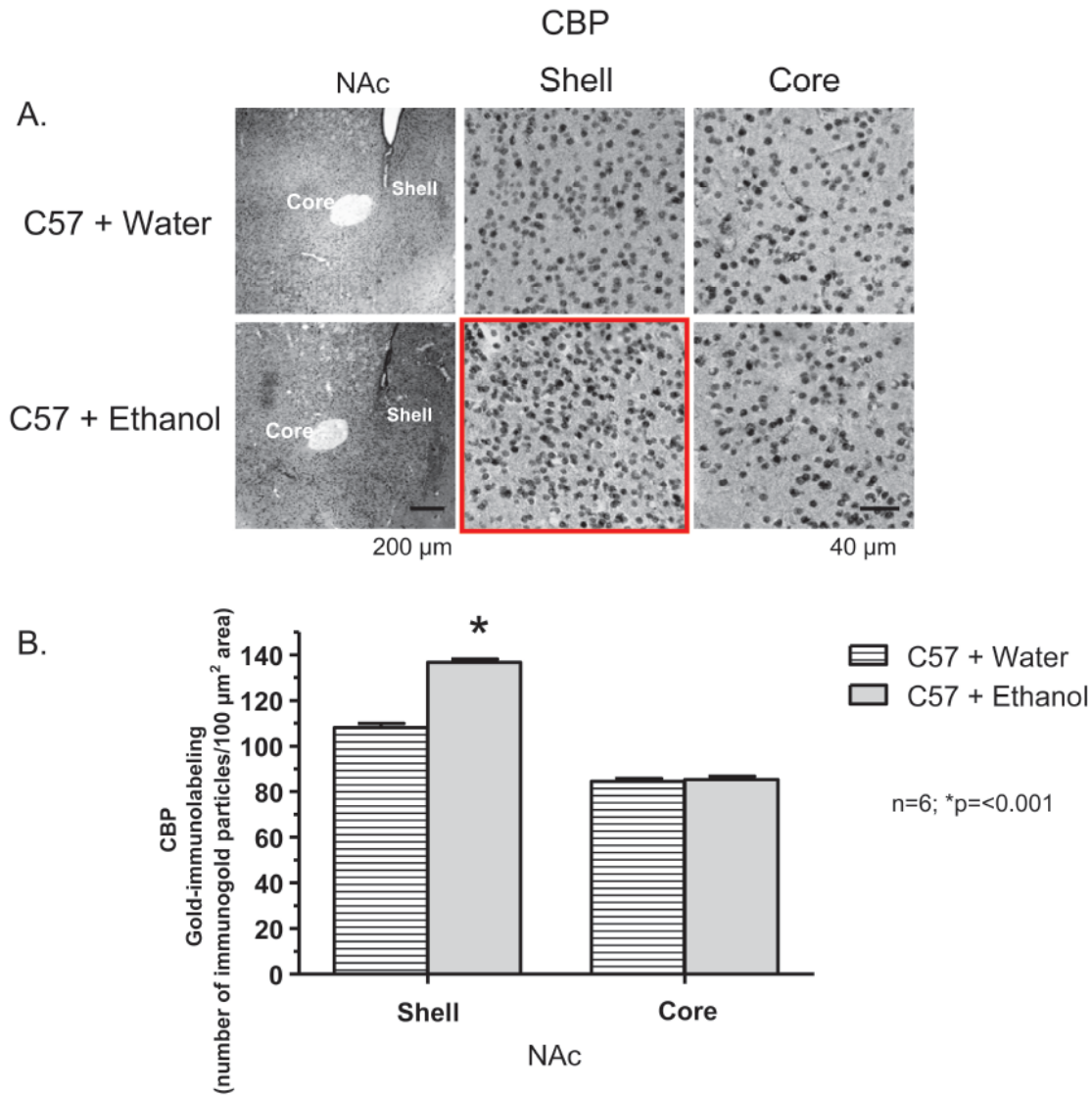
Figure 7. Effects of voluntary ethanol exposure on CBP levels in NAc of C57 mice. Voluntary ethanol exposure significantly increased levels of CREB-binding protein (CBP) in the shell, but not core of the nucleus accumbens (NAc) of C57BL/6J (C57) mice (C57 + Ethanol) compared to C57 control mice (C57 + Water). **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of CBP gold immunolabeling in the shell and core of the NAc of C57 mice exposed to ethanol (C57 + Ethanol) and C57 control mice (C57 + Water). **B:** Bar diagram demonstrates the quantified results of CBP in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 6 mice in each group. *P<0.001, significantly different from C57 control mice (C57 + Water) (Student's t-test).

Figure 8. **Effects of voluntary ethanol exposure on CBP levels in amygdaloid structures of C57 mice.** Voluntary ethanol exposure had no significant effects on the levels of CREB-binding protein (CBP) in amygdaloid structures [central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), and basolateral nucleus of amygdala (BLA)] of C57BL/6J mice (C57 + Ethanol) compared to C57 control mice (C57 + Water). **A:** Shows low magnification (scale bar, 200 μm) also high magnification (scale bar, 40 μm) photomicrographs of CBP gold immunolabeling in the CeA, MeA, and BLA of C57 mice exposed to ethanol (C57 + Ethanol) and C57 control mice (C57 + Water). **B:** Bar diagram demonstrates the quantified results of CBP in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 6 mice in each group.

2. <u>**Effects of Voluntary Ethanol Exposure on Acetylated Histone**</u>
<u>**H3 (K-9) Protein Levels in NAc and Amygdaloid Structures of**</u>
<u>**C57 Mice**</u>

The effects of voluntary ethanol exposure on levels of acetylated histone H3 (K-9) were measured in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of C57 mice using the gold-immunolabeling histochemical procedure. Figure 9A indicates the distribution patterns of acetylated histone H3 in NAc structures of C57 mice exposed to ethanol (C57 + Ethanol) and C57 control mice (C57 + Water). It was found that voluntary ethanol exposure significantly ($p < 0.001$) increased levels of acetylated histone H3 (K-9) in the shell, but not core, of the NAc in C57 mice compared to controls (C57 + Water) (Figs. 9A & 9B). Interestingly, voluntary ethanol exposure had no significant effects in the amygdala, as there were no significant differences observed in acetylated histone H3 (K-9) protein levels in any amygdaloid brain structures (CeA, MeA, or BLA) of C57 mice compared to controls (Figs. 10A & 10B). These data indicate that voluntary ethanol consumption by C57 mice increases levels of acetylated histone H3 (K-9) in the shell, but not core of the NAc, or amygdaloid brain structures compared to control mice.
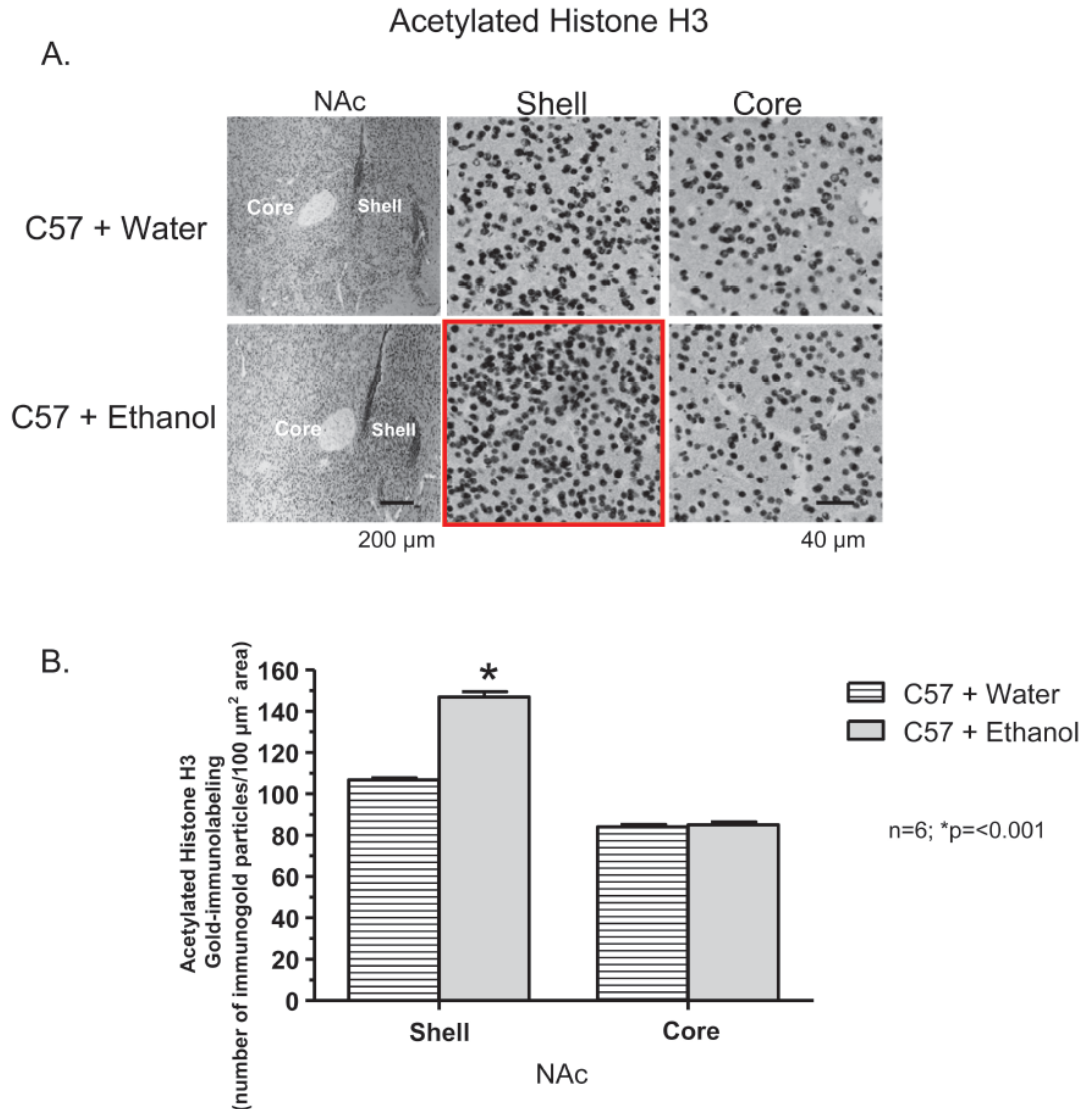
Figure 9. **Effects of voluntary ethanol exposure on acetylated histone H3 (K-9) protein levels in NAc of C57 mice.** Voluntary ethanol exposure significantly increased levels of acetylated histone H3 (K-9) in the shell, but not core of the nucleus accumbens (NAc) of C57BL/6J (C57) mice (C57 + Ethanol) compared to C57 control mice (C57 + Water). **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H3 (K-9) gold immunolabeling in the shell and core of the NAc of C57 mice exposed to ethanol (C57 + Ethanol) and C57 control mice (C57 + Water). **B:** Bar diagram demonstrates the quantified results of acetylated histone H3 (K-9) in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 6 mice in each group. *P<0.001, significantly different from C57 control mice (C57 + Water) (Student's t-test).

Figure 10. **Effects of voluntary ethanol exposure on acetylated histone H3 (K-9) protein levels in amygdaloid structures of C57 mice.** Voluntary ethanol exposure had no significant effects on the levels of acetylated histone H3 (K-9) in amygdaloid structures [central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), and basolateral nucleus of amygdala (BLA)] of C57BL/6J (C57) mice (C57 + Ethanol) compared to C57 control mice (C57 + Water). **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H3 (K-9) gold immunolabeling in the CeA, MeA, and BLA of C57 mice exposed to ethanol (C57 + Ethanol) and C57 control mice (C57 + Water). **B:** Bar diagram demonstrates the quantified results of acetylated histone H3 (K-9) in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 6 mice in each group.

3. Effects of Voluntary Ethanol Exposure on Methylated Histone H3 (K-9) Protein Levels in NAc and Amygdaloid Structures of C57 Mice

The effects of voluntary ethanol exposure on levels of methylated histone H3 (K-9) were measured in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of C57 mice using the gold-immunolabeling histochemical procedure. Figure 11A indicates the distribution patterns of methylated histone H3 (K-9) in NAc structures of C57 mice exposed to ethanol (C57 + Ethanol) and C57 control mice (C57 + Water). It was found that voluntary ethanol exposure significantly ($p<0.001$) decreased levels of methylated histone H3 (K-9) in the shell, but not core, of the NAc in C57 mice compared to controls (C57 +Water) (Figs. 11A & 11B). Interestingly, voluntary ethanol exposure also significantly ($p<0.001$) decreased levels of methylated histone H3 (K-9) in amygdaloid brain structures (CeA, MeA, or BLA) of C57 mice compared to controls (Figs. 12A & 12B). These data indicate that voluntary ethanol consumption decreased levels of methylated histone H3 (K-9) in the shell, but not core of the NAc, and also decreased methylated histone H3 (K-9) levels in amygdaloid brain structures of C57 mice compared to control mice.
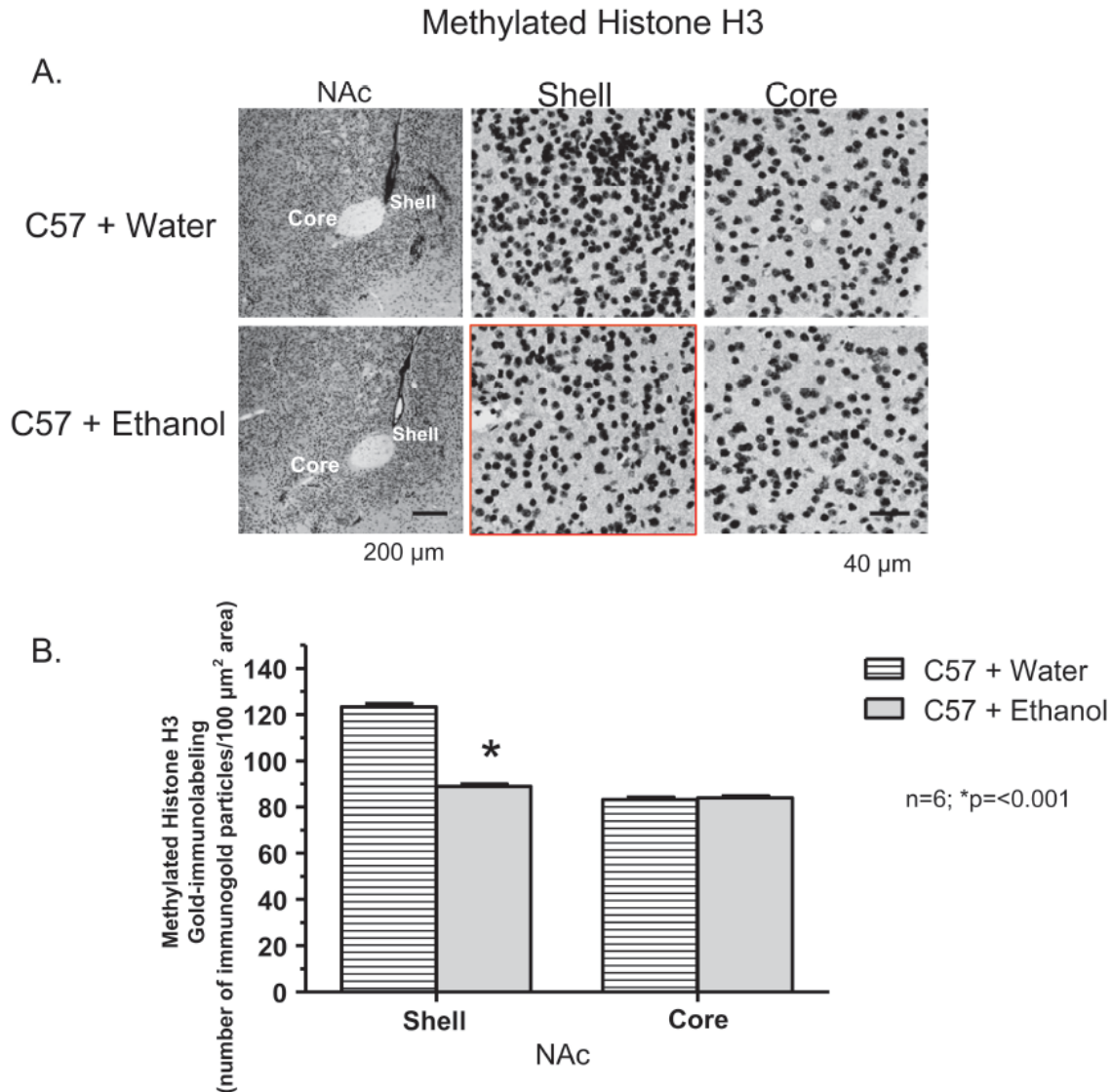
Figure 11.   Effects of voluntary ethanol exposure on methylated histone H3 (K-9) protein levels in NAc of C57 mice. Voluntary ethanol exposure significantly decreased levels of dimethylated histone H3 (K-9) in the shell, but not core of the nucleus accumbens (NAc) of C57BL/6J (C57) mice (C57 + Ethanol) compared to C57 control mice (C57 + Water). **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of dimethylated histone H3 (K-9) gold immunolabeling in the shell and core of the NAc of C57 mice exposed to ethanol (C57 + Ethanol) and C57 control mice (C57 + Water). **B:** Bar diagram demonstrates the quantified results of dimethylated histone H3 (K-9) in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 6 mice in each group. *P<0.001, significantly different from C57 control mice (C57 + Water) (Student's t-test).
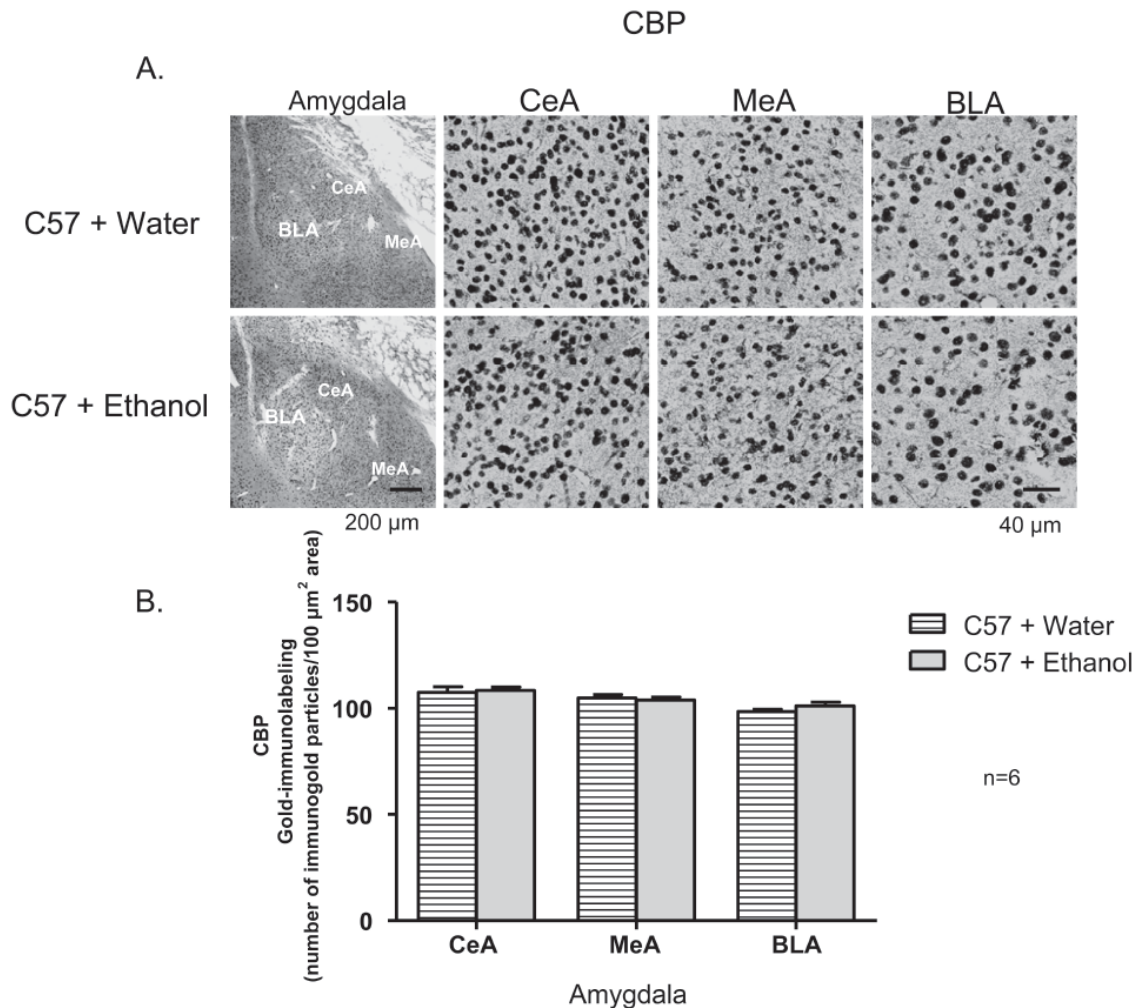
Methylated Histone H3

Figure 12. Effects of voluntary ethanol exposure on methylated histone H3 (K-9) protein levels in amygdaloid structures of C57 mice. Voluntary ethanol exposure significantly decreased levels of dimethylated histone H3 (K-9) in amygdaloid structures [central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), and basolateral nucleus of amygdala (BLA)] of C57BL/6J (C57) mice exposed to ethanol (C57 + Ethanol) compared to controls (C57 + Water). **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of dimethylated histone H3 (K-9) gold immunolabeling in the CeA, MeA, and BLA of C57 mice exposed to ethanol (C57 + Ethanol) and C57 control mice (C57 + Water). **B:** Bar diagram demonstrates the quantified results of dimethylated histone H3 (K-9) in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 6 mice in each group. *P<0.001, significantly different from C57 control mice (C57 + Water) (Student's t-test).

4. Effects of Acute Ethanol Exposure on CBP Levels in NAc and Amygdaloid Structures of C57 and DBA Mice

The effects of acute ethanol exposure on CBP levels were measured in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of C57 (blood ethanol levels: mean ± SEM, n=5; 193 ± 6.6) and DBA (blood ethanol levels: mean ± SEM, n=5; 236 ± 24) mice using the gold-immunolabeling histochemical procedure. Figure 13A indicates the distribution patterns of CBP in the NAc of C57 and DBA mice injected with saline (C57/DBA + Saline) or ethanol (2 g/kg) (C57/DBA + Ethanol). The results reconfirmed our previous findings from Specific Aim 1, in that C57 mice express lower levels of CBP in the shell, but not core of the NAc compared to DBA mice; there were no significant differences in CBP levels in amygdaloid structures between C57 and DBA mice (Figs. 13A & 13B). Acute ethanol exposure also significantly ($p < 0.001$) increased levels of CBP in the shell, but not core, of the NAc in C57 mice (C57+ Ethanol) compared to controls (C57 + Saline) (Figs. 13A & 13B). Interestingly, acute ethanol exposure significantly ($p < 0.001$) increased levels of CBP in both the shell and core of the NAc in DBA mice (DBA + Ethanol) compared to controls (DBA + Saline) (Figs. 13A & 13B). Acute ethanol exposure had no significant effects on levels of CBP in the amygdala (CeA, MeA, or BLA) of C57 or DBA mice (Fig. 13B). These data indicate that acute ethanol exposure increased levels of CBP in the shell, but not core of the NAc of C57 mice compared to control mice; and also increased CBP in both the shell and core of the NAc of DBA mice compared to control mice.
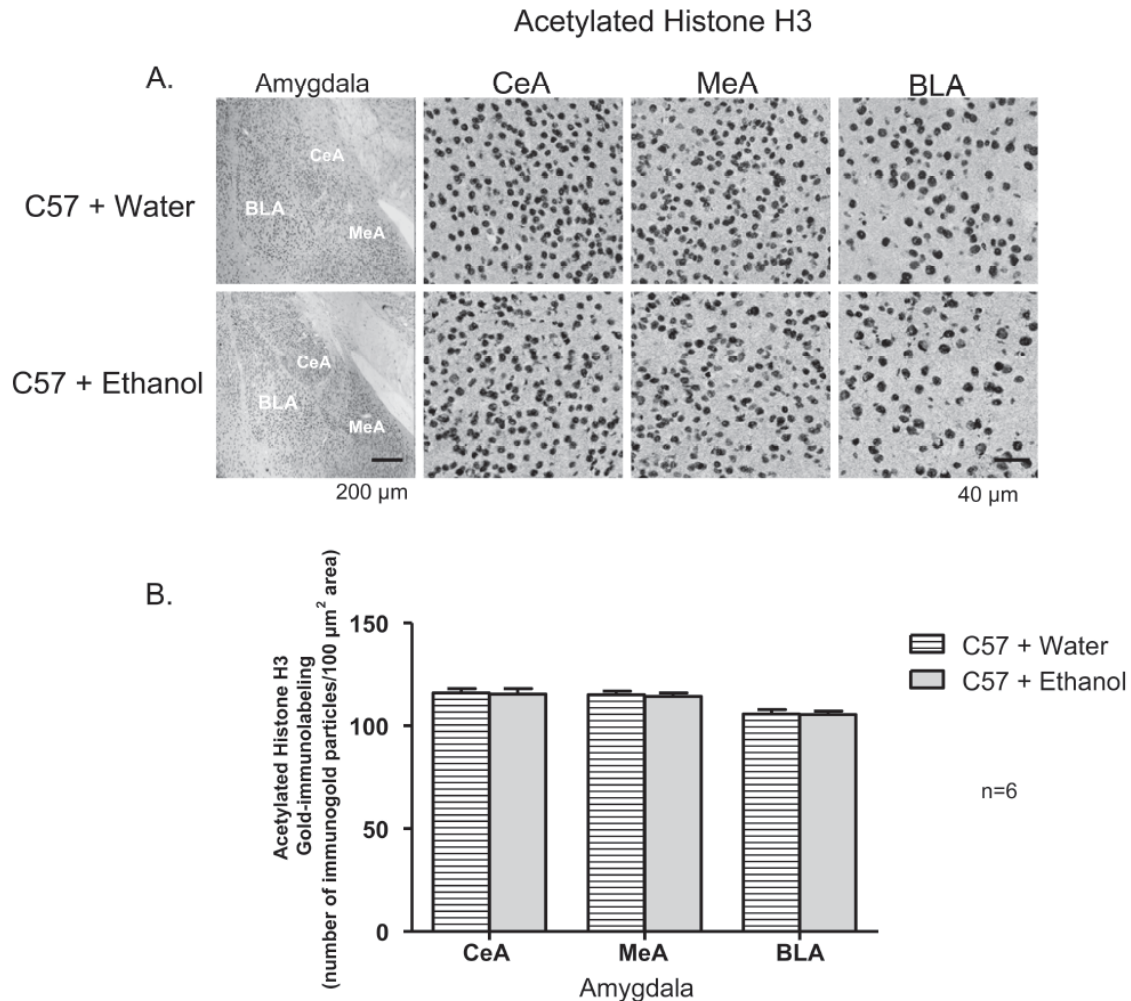
CBP

A.

B.
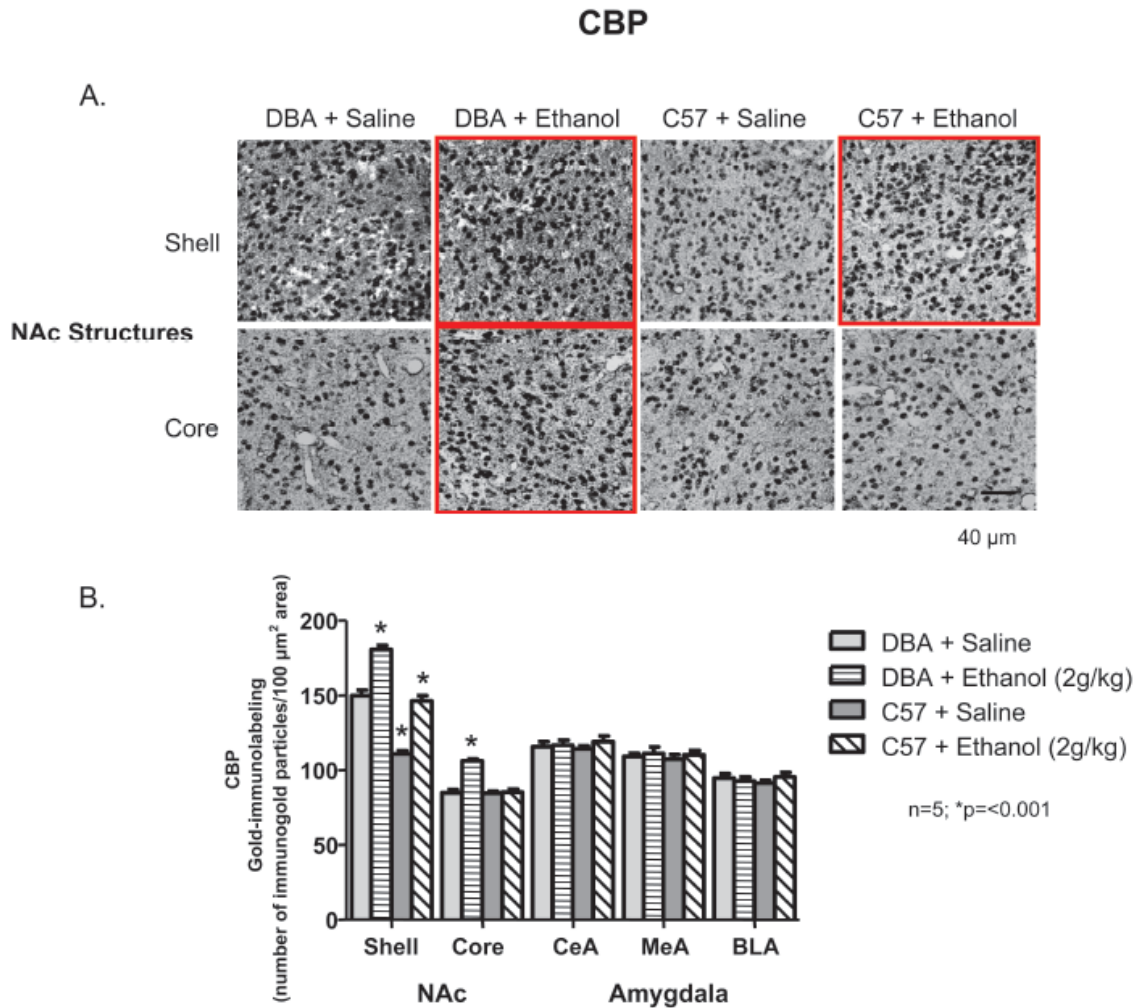
Figure 13. Effects of acute ethanol exposure on CBP levels in NAc and amygdaloid structures of C57 and DBA mice. Acute ethanol exposure (2 g/kg) significantly increased levels of CREB-binding protein (CBP) in the shell, but not core of the nucleus accumbens (NAc) of C57BL/6J (C57) mice (C57 + Ethanol) compared to control C57 mice (C57 + Saline). Acute ethanol exposure (2 g/kg) significantly increased levels of CBP in both the shell and core of the NAc of DBA/2J (DBA) mice (DBA + Ethanol) compared to DBA control mice (DBA + Saline). Acute ethanol exposure (2 g/kg) had no significant effects on CBP levels in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of C57 or DBA mice. We also reconfirmed that C57 mice innately express lower levels of CBP in the shell, but not core of the NAc compared to DBA mice. A: Shows high magnification (scale bar, 40 μm) photomicrographs of CBP gold immunolabeling in the NAc shell and core regions of C57 mice exposed to ethanol (C57 + Ethanol), C57 control mice (C57 + Saline), DBA mice exposed to ethanol (DBA + Ethanol), and DBA control mice (DBA + Saline). B: Bar diagram demonstrates the quantified results of CBP in the NAc (shell and core) and CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group. *P<0.001, significantly different from C57 control mice (C57 + Saline) or DBA control mice (DBA + Saline) [(One-way analysis of variance (ANOVA) followed by post-hoc comparison by Tukey test].

5. <u>Effects of Acute Ethanol Exposure on Acetylated Histone H3</u>

<u>(K-9) Protein Levels in NAc and Amygdaloid Structures of C57</u>

<u>and DBA Mice</u>

The effect of acute ethanol exposure on acetylation of H3 (K-9) was

measured in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of

C57 and DBA mice using the gold-immunolabeling histochemical procedure.

Figure 14A indicates the distribution patterns of acetylated H3 (K-9) in NAc

structures of C57 and DBA mice injected with saline (C57/DBA + Saline) or

ethanol (2 g/kg) (C57/DBA + Ethanol). We reconfirmed our previous findings

from Specific Aim 1, in that C57 mice express lower levels of acetylated H3 (K-9)

in the shell, but not core of the NAc compared to DBA mice; there were no

significant differences in levels of acetylated H3 (K-9) in amygdaloid structures

between C57 and DBA mice (Figs. 14A & 14B). Acute ethanol exposure

significantly ($p<0.001$) increased acetylation of H3 (K-9) in the NAc shell, but not

core, of C57 mice (C57+ Ethanol) compared to controls (C57 + Saline) (Figs.

14A & 14B). Interestingly, acute ethanol exposure significantly ($p<0.001$)

increased acetylation of H3 (K-9) in both the shell and core of the NAc in DBA

mice (DBA + Ethanol) compared to controls (DBA + Saline) (Figs. 14A & 14B).

Acute ethanol exposure had no effects on acetylation of H3 (K-9) in the

amygdala (CeA, MeA, or BLA) of C57 or DBA mice (Fig. 14B). Acute ethanol

exposure increased levels of acetylated H3 (K-9) in the shell, but not core of the

NAc of C57 mice compared to controls, and increased acetylated H3 (K-9) in

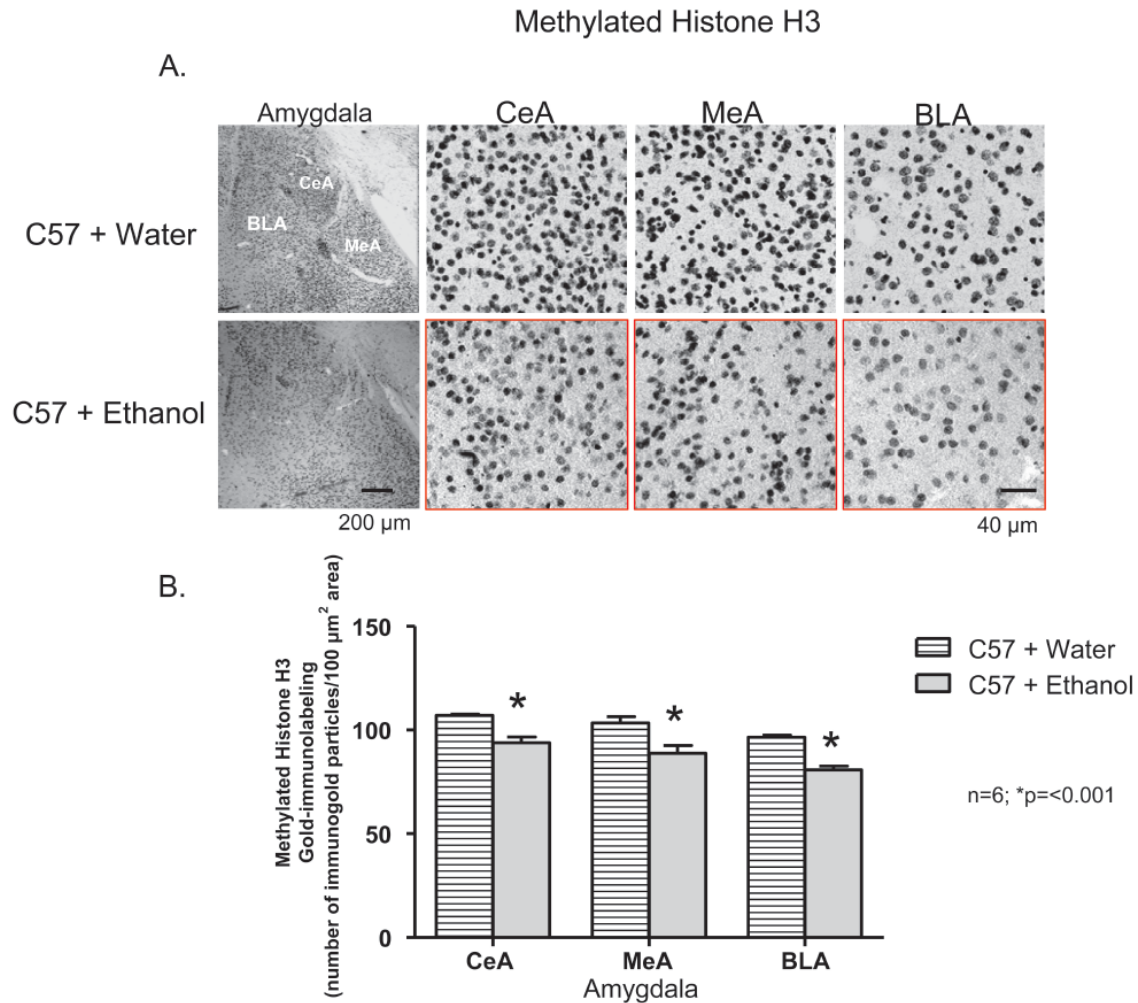both the shell and core of the NAc of DBA mice compared to controls.

Acetylated Histone H3

Figure 14. **Effects of acute ethanol exposure on acetylated histone H3 (K-9) protein levels in NAc and amygdaloid structures of C57 and DBA mice.** Acute ethanol exposure (2 g/kg) significantly increased levels of acetylated histone H3 (K-9) in the shell, but not core of the nucleus accumbens (NAc) of C57BL/6J (C57) mice (C57+Ethanol) compared to control C57 mice (C57+Saline). Acute ethanol exposure (2 g/kg) significantly increased levels of acetylated histone H3 (K-9) in both the shell and core of the NAc of DBA/2J (DBA) mice (DBA + Ethanol) compared to DBA control mice (DBA + Saline). Acute ethanol exposure (2 g/kg) had no significant effects on acetylated histone H3 (K-9) levels in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of C57 or DBA mice. We also reconfirmed that C57 mice innately express lower levels of acetylated histone H3 (K-9) in the shell, but not core of the NAc compared to DBA mice. A: Shows high magnification (scale bar, 40μm) photomicrographs of acetylated histone H3 (K-9) gold immunolabeling in the NAc shell and core regions of C57 mice exposed to ethanol (C57+ Ethanol), C57 control mice (C57+Saline), DBA mice exposed to ethanol (DBA + Ethanol), and DBA control mice (DBA + Saline). B: Bar diagram demonstrates the quantified results of acetylated histone H3 (K-9) in the NAc (shell and core) and CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group. *P<0.001, significantly different from C57 control mice (C57 + Saline) or DBA control mice (DBA + Saline) [(One-way analysis of variance (ANOVA) followed by post-hoc comparison by Tukey test].

6. **Effects of Acute Ethanol Exposure on Methylated Histone H3 (K-9) Protein Levels in NAc and Amygdaloid Structures of C57 and DBA Mice**

The effects of acute ethanol exposure on levels of methylated H3 (K-9) were measured in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of C57 and DBA mice using the gold-immunolabeling histochemical procedure. Figure 15A indicates the distribution patterns of methylated H3 (K-9) in NAc structures of C57 and DBA mice injected either with saline (C57/DBA + Saline) or with ethanol (2 g/kg) (C57/DBA + Ethanol). The results reconfirmed our previous findings from Specific Aim 1, in that C57 mice express higher levels of methylated H3 (K-9) in the shell, but not core of the NAc compared to DBA mice; there were no significant differences in levels of methylated H3 (K-9) in amygdaloid structures between C57 and DBA mice (Figs. 15A & 15B). Acute ethanol exposure significantly ($p<0.001$) decreased levels of methylated H3 (K-9) in the shell, but not core, of the NAc in C57 mice (C57+ Ethanol) compared to controls (C57 + Saline) (Figs. 15A & 15B). Interestingly, acute ethanol exposure significantly ($p<0.001$) decreased levels of methylated H3 (K-9) in both the shell and core of the NAc in DBA mice (DBA + Ethanol) compared to controls (DBA + Saline) (Figs. 15A & 15B). Acute ethanol exposure had no effects on methylated H3 (K-9) in the amygdala (CeA, MeA, or BLA) of C57 or DBA mice (Fig. 15B). Acute ethanol exposure decreased methylation of H3 (K-9) in the NAc shell, but not core, of C57 mice compared to controls; and decreased methylated H3 (K-9) levels in both the shell and core of the NAc of DBA mice compared to controls.

Methylated Histone H3

A.

B.
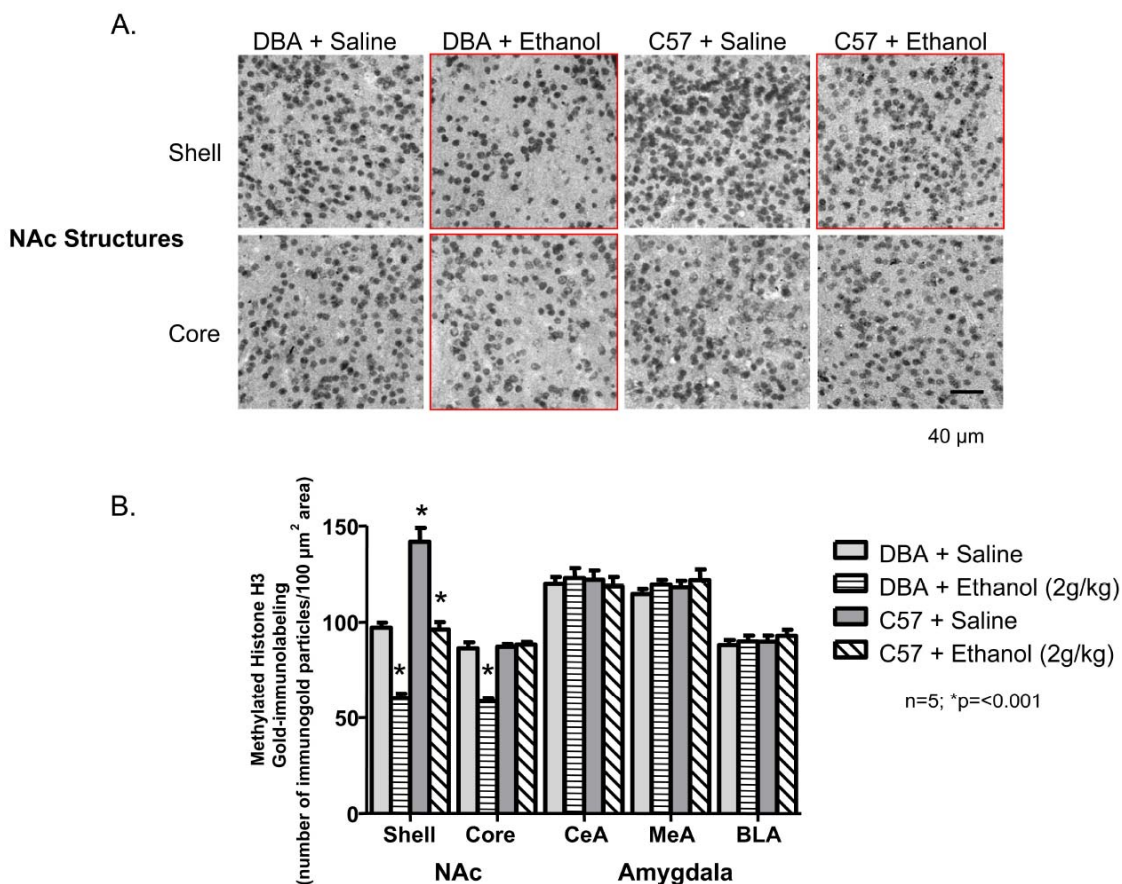
Figure 15. **Effects of acute ethanol exposure on methylated histone H3 (K-9) protein levels in NAc and amygdaloid structures of C57 and DBA mice.** Acute ethanol exposure (2 g/kg) significantly decreased levels of dimethylated histone H3 (K-9) in the shell, but not core of the nucleus accumbens (NAc) of C57BL/6J (C57) mice (C57+Ethanol) compared to control C57 mice (C57+Saline). Acute ethanol exposure (2 g/kg) significantly decreased levels of dimethylated histone H3 (K-9) in both the shell and core of the NAc of DBA/2J (DBA) mice (DBA+Ethanol) compared to DBA control mice (DBA+Saline). Acute ethanol exposure (2 g/kg) had no significant effects on dimethylated histone H3 (K-9) levels in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of C57 or DBA mice. We also reconfirmed that C57 mice innately express higher levels of dimethylated histone H3 (K-9) in the shell, but not core of the NAc compared to DBA mice. A: Shows high magnification (scale bar, 40μm) photomicrographs of dimethylated histone H3 (K-9) gold immunolabeling in the NAc shell and core regions of C57 mice exposed to ethanol (C57+Ethanol), C57 control mice (C57+Saline), DBA mice exposed to ethanol (DBA +Ethanol), and DBA control mice (DBA+Saline). B: Bar diagram shows the quantified results of dimethylated histone H3 (K-9) in the NAc (shell and core) and CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group. *P<0.001, significantly different from C57 control mice (C57+Saline) or DBA control mice (DBA+Saline) [(One-way analysis of variance (ANOVA) followed by post-hoc comparison by Tukey test].

7. Effects of Acute Ethanol Exposure on BDNF Protein Levels in

NAc and Amygdaloid Structures of C57 and DBA Mice

The effects of acute ethanol exposure on BDNF were measured in the NAc (shell and core) and amygdala (CeA, MeA, and BLA) of C57 and DBA mice using the gold-immunolabeling histochemical procedure. Figure 16A indicates the distribution patterns of BDNF in NAc structures of C57 and DBA mice injected with saline (C57/DBA + Saline) or ethanol (2g/kg) (C57/DBA + Ethanol). C57 mice express lower levels of BDNF in the shell, but not core of the NAc compared to DBA mice; there were no significant differences in levels of BDNF in amygdaloid structures between C57 and DBA mice (Figs. 16A & 16B). Acute ethanol exposure significantly ($p<0.001$) increased levels of BDNF in the shell, but not core, of the NAc in C57 mice (C57+ Ethanol) compared to controls (C57 + Saline) (Figs. 16A & 16B). Interestingly, acute ethanol exposure significantly ($p<0.001$) increased levels of BDNF in both the shell and core of the NAc in DBA mice (DBA + Ethanol) compared to controls (DBA + Saline) (Figs. 16A & 16B). Acute ethanol exposure had no significant effects on levels of BDNF in the amygdala (CeA, MeA, or BLA) of C57 or DBA mice (Fig. 16B). These data suggest the possibility that innately lower acetylation of H3 in the NAc shell may be responsible for lower levels of BDNF in C57 mice compared to DBA mice. Furthermore, acute ethanol exposure increased levels of BDNF in the shell, but not core of the NAc of C57 mice compared to control C57 mice, and also increased BDNF levels in both the shell and core of the NAc of DBA mice compared to control DBA mice.

BDNF

A.

B.

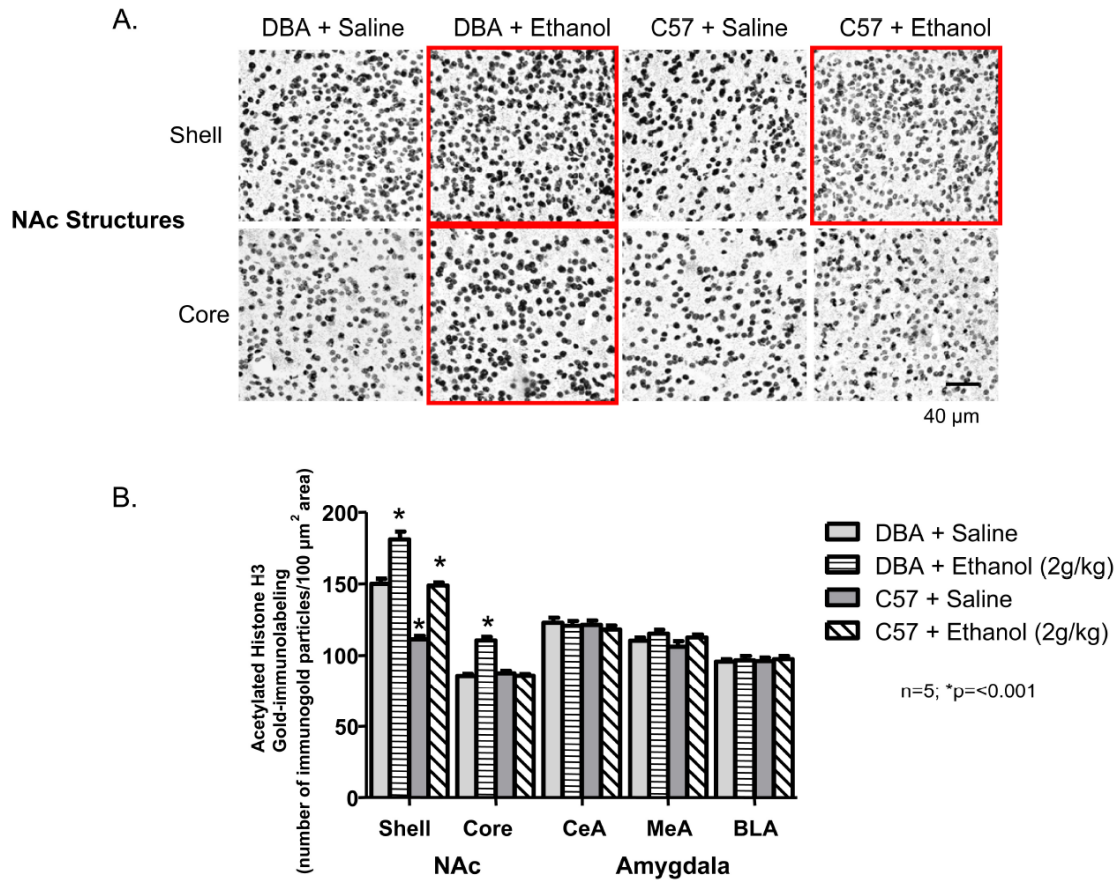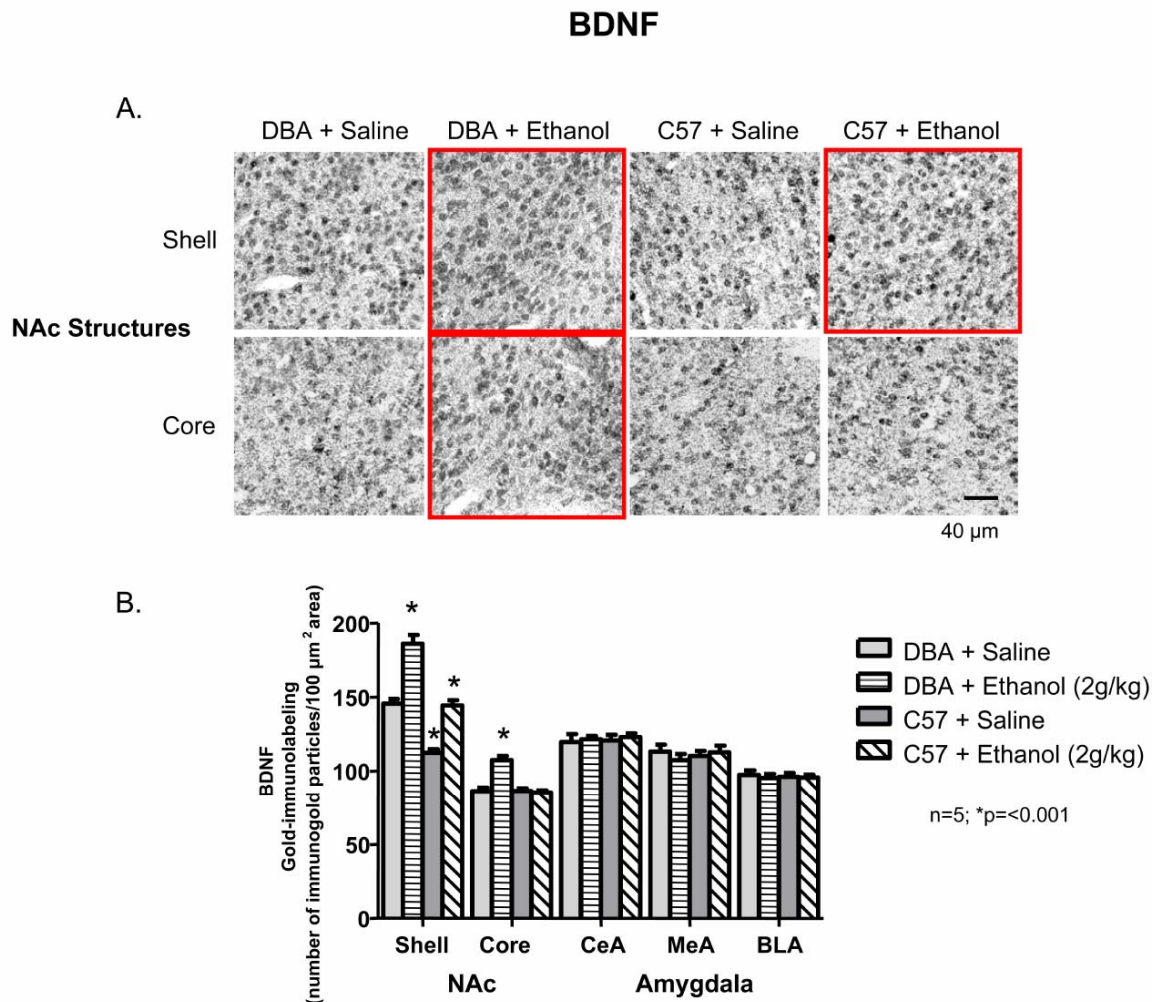**Figure 16.   Effects of acute ethanol exposure on BDNF protein levels in NAc and amygdaloid structures of C57 and DBA mice.** C57BL/6J (C57) mice innately express lower levels of brain-derived neurotrophic factor (BDNF) in the shell, but not core of the nucleus accumbens (NAc) compared to DBA/2J (DBA) mice; acute ethanol exposure (2 g/kg) significantly increased levels of BDNF in the shell, but not core of the NAc of C57 mice (C57 + Ethanol) compared to control C57 mice (C57 + Saline). Acute ethanol exposure (2 g/kg) significantly increased levels of BDNF in both the shell and core of the NAc of DBA mice (DBA + Ethanol) compared to DBA control mice (DBA + Saline). Acute ethanol exposure (2 g/kg) had no significant effects on BDNF levels in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of C57 or DBA mice. We also found that C57 mice innately express lower levels of BDNF in the shell, but not core of the NAc compared to DBA mice. A: Shows high magnification (scale bar, 40 μm) photomicrographs of BDNF gold immunolabeling in the NAc shell and core regions of C57 mice exposed to ethanol (C57 + Ethanol), C57 control mice (C57 + Saline), DBA mice exposed to ethanol (DBA + Ethanol), and DBA control mice (DBA + Saline). B: Bar diagram demonstrates the quantified results of BDNF in the NAc (shell and core) and CeA. MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group. *P<0.001, significantly different from C57 control mice (C57 + Saline) or DBA control mice (DBA + Saline) [(One-way analysis of variance (ANOVA) followed by post-hoc comparison by Tukey test].

8. <u>Effects of Acute Ethanol Exposure on Number of NeuN-Positive Neurons in NAc of C57 and DBA Mice</u>

Figure 17A indicates the distribution patterns of NeuN in NAc structures of C57 and DBA mice injected either with saline (C57/DBA + Saline) or ethanol (2 g/kg) (C57/DBA + Ethanol). There were no significant differences in baseline neuronal numbers in the NAc shell or core of C57 mice compared with DBA mice. Acute ethanol exposure had no significant effect on the number of NeuN-positive neurons in the NAc (shell or core) of C57 mice (C57+ Ethanol) compared to controls (C57 + Saline) or DBA mice (DBA + Ethanol) compared to controls (DBA + Saline) (Figs. 17A & 17B). The number of NeuN-positive neurons was similar between C57 mice compared to DBA mice in all groups. These data indicate that acute ethanol exposure had no effect on the number of NeuN-positive neurons in the shell or core of the NAc of C57 mice or DBA mice. Furthermore, these data also suggest that the observed changes in levels of CBP, acetylated histone H3 (K-9), dimethylated histone H3 (K-9), and BDNF in the NAc shell were not due to differences in neuronal numbers of C57 mice compared to DBA mice in this brain region.
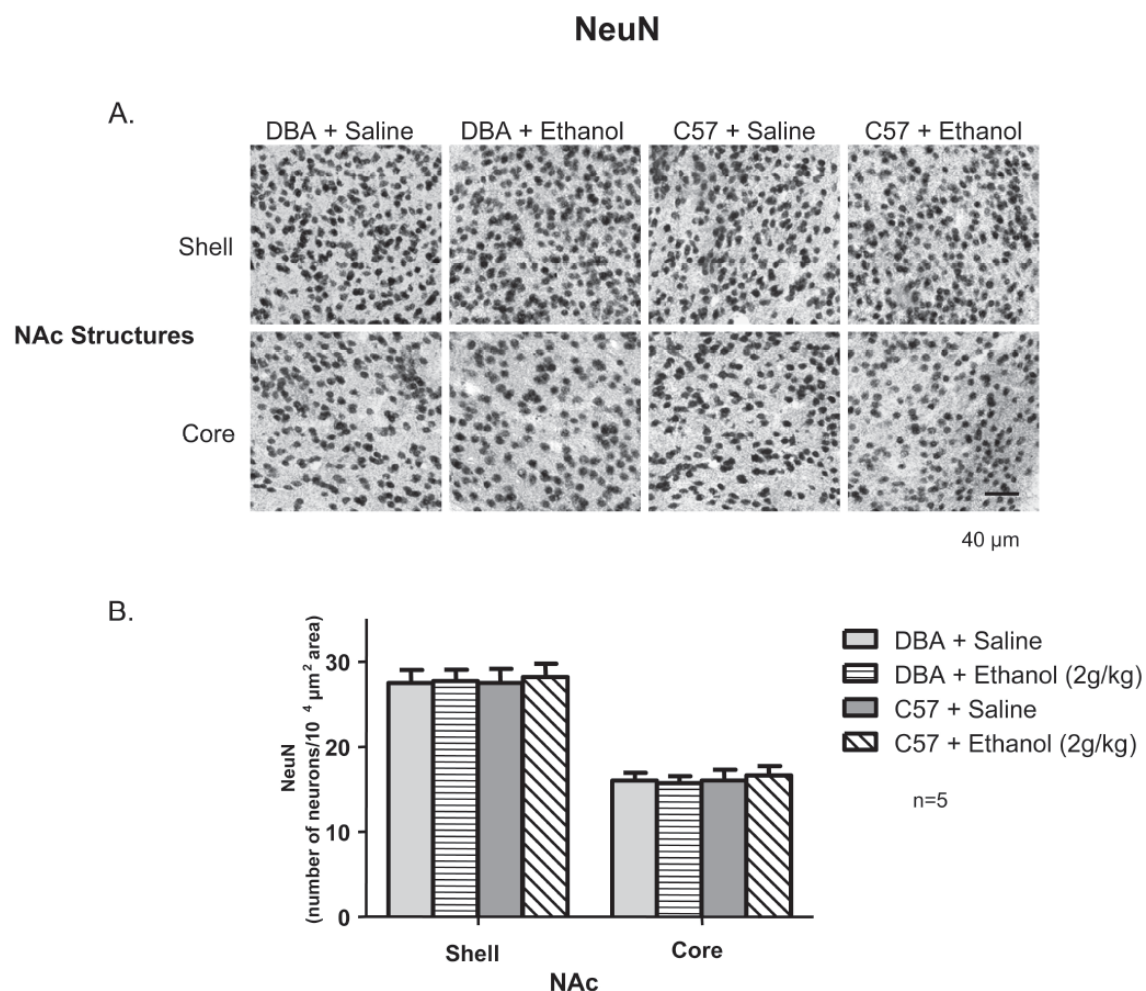
NeuN

Figure 17.   Effects of acute ethanol exposure on number of NeuN-positive neurons in NAc of C57 and DBA mice. Acute ethanol exposure (2 g/kg) had no significant effects on the number of NeuN-positive neurons in the nucleus accumbens (NAc) shell or core structures of C57BL/6J (C57) mice treated with ethanol (C57 + Ethanol) compared to C57 control mice (C57 + Saline) or DBA/2J (DBA) mice treated with ethanol (DBA + Ethanol) compared to controls (DBA + Saline). **A:** Shows high magnification (scale bar, 40 μm) photomicrographs of NeuN protein gold immunolabeling in the NAc shell or core regions of C57 mice exposed to ethanol (C57 + Ethanol), C57 control mice (C57 + Saline), DBA mice exposed to ethanol (DBA + Ethanol), or DBA control mice (DBA + Saline). **B:** Bar diagram demonstrates the number of NeuN-positive neurons in the NAc (shell and core) in terms of number of neurons/10^4 μm^2 area. Values are mean ± SEM of 5 mice in each group.

E. <u>Discussion</u>

We investigated the effects of voluntary and acute ethanol exposure on chromatin-related protein expression in regions of the limbic system (NAc and amygdala) of C57 and DBA mice, two mice models with divergent ethanol-related drinking phenotypes. Our studies identified brain-region specific epigenetic patterns that may be related to an innate preference for ethanol drinking behaviors expressed by C57 mice compared to DBA mice. Although additional experiments are required to definitively establish the epigenetic mechanisms involved in regulation of gene expression linked with alcohol drinking behaviors, the identified patterns of epigenetic markings begin to outline the differential actions of voluntary and acute ethanol exposure in specific brain regions of C57 mice compared to DBA mice.

We found that both voluntary and acute ethanol exposure increased levels of both CBP and acetylated histone H3 in the shell, but not core of the NAc of C57 mice compared to control mice. Acute ethanol exposure had no effects on levels of CBP or acetylated histone H3 in the amygdala (CeA, MeA, or BLA) of C57 mice compared to control mice. CBP and p300 are highly related transcriptional coactivators with intrinsic HAT capabilities (Chan and La Thangue, 2001). A recent study demonstrated that p300 was able to modulate gene expression in an epigenetic manner at high blood alcohol levels; p300, similar to our findings with CBP, increased acetylation of histone H3 at K-9 (Bardag-Gorce et al., 2007). Another study showed that ethanol treatment significantly increased histone H3 acetylation at K-9 in hepatocytes, which correlated with

either increased expression of or increased activation of HATs (Park et al., 2005). In addition, chronic ethanol consumption induced global hepatic protein hyperacetylation (Shepard et al., 2010). These results, along with our current findings, suggest that acute and voluntary ethanol exposure may increase histone H3 acetylation levels in the shell of the NAc of C57 mice via a CBP-HAT mediated epigenetic mechanism.

We found that both voluntary and acute ethanol exposure decreased levels of methylated histone H3 (K-9) in the shell, but not core of the NAc of C57 mice compared to control mice. Acute ethanol exposure had no effects on the level of methylated histone H3 (K-9) in the amygdala (CeA, MeA, or BLA) of C57 mice compared to C57 control mice. Interestingly, while voluntary ethanol exposure had no effect on CBP or acetylated histone H3 (K-9) levels in amygdaloid structures, we did observe decreased levels of methylated histone H3 (K-9) in the CeA, MeA, and BLA of C57 mice compared to C57 control mice. It is possible that the regulatory properties of voluntary ethanol exposure may affect methylation via a different mechanism from one that regulates acetylation in the NAc and amygdala of C57 mice. The observed decreases in methylation that occur in the shell of the NAc and amygdaloid structures may reflect differences in the neuroanatomical and/or functional characteristics associated with ethanol exposure. Several ethanol drinking experiments in alcohol-preferring (P) rats demonstrated that protein expression levels in both the NAc and amygdala were differentially affected depending on the conditions under which ethanol was consumed (Bell et al., 2006; Rodd et al., 2008). In addition,

the observed effects of ethanol exposure on the shell and not the core of the NAc or amygdala may reflect the functional importance of amygdala-NAc interactions, which has been established in a cocaine-seeking experimental rat model (Di Ciano and Everitt, 2004).

Decreased H3 K-9 methylation in the NAc shell of C57 mice or NAc shell and core of DBA mice exposed to ethanol may be involved in decreased gene expression levels in these brain regions. Methylation of histone H3 K-9 has been correlated with downregulation of gene expression by ethanol in other tissues, such as hepatocytes (Pal-Bhadra et al., 2007). In addition, histone H3 K-9 methylation was shown to be regulated by both acute and chronic stress (Hunter et al., 2009). The histone methyltransferase (HMT) G9a and the related molecule GLP are the primary enzymes responsible for the mono- and dimethylation at K-9 of histone H3, which correlates with transcriptional repression (Stewart et al., 2005; Shinkai and Tachibana, 2011). A recent study identified the *Drosophila* euchromatic HMT G9a as a key component in the epigenetic regulation of learning and memory (Kramer et al., 2011). Other current studies demonstrate an essential role for HMT G9a in cocaine-induced plasticity in the NAc and vulnerability to stress (Maze et al., 2010; Covington et al., 2011). Maze et al., identified an essential role for histone H3 K-9 dimethylation and the lysine dimethyltransferase G9a in cocaine-induced structural and behavioral plasticity (Maze et al., 2010). Similar to our findings in the NAc of C57 and DBA mice exposed to ethanol, repeated cocaine administration reduced global histone H3 dimethylation in the NAc, which was

mediated by G9a, suggesting a crucial role for histone methylation in the long-term actions of cocaine (Maze et al., 2010). In addition, Covington et al., identified repressive histone methylation mediated by decreased levels of histone H3 K-9 dimethylation in the NAc as a key mechanism linking cocaine exposure to increased stress vulnerability (Covington et al., 2011). The authors of this study found that a CRE-mediated reduction of G9a in the NAc increased susceptibility to stress, while overexpression of G9a in the NAc after repeated cocaine exposure was neuroprotective against stress, which was mediated in part by cocaine or stress-induced repression of the BDNF-TrkB-CREB signaling pathway (Covington et al., 2011). These findings, combined with our findings of decreased histone methylation due to ethanol exposure, suggest a possible role of HMT G9a in an ethanol-induced decrease in histone H3 (K-9) dimethylation in NAc brain structures. However, more studies are necessary to determine the role of HMT G9a-mediated H3 (K-9) methylation during alcoholism.

Similar to C57 mice, we observed increased levels of CBP and acetylated histone H3 and decreased levels of methylated histone H3 in the NAc and found no significant effects in the amygdala (CeA, MeA, or BLA) of DBA mice compared to control mice. However, striking neuroanatomical differences were observed between C57 and DBA mice exposed to acute ethanol, in that acute ethanol exposure targeted both the shell and core of the NAc in DBA mice whereas ethanol exposure only affected the NAc shell and not NAc core of C57 mice. The shell and core of the NAc play differential roles in behavior and addiction (Di Chiara, 2002). Ethanol has been shown to be self-administered into

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the shell, but not core of the NAc (Engleman et al., 2009). The shell of the NAc

also has a higher dopamine response compared with the core after intravenous

ethanol administration (Howard et al., 2008). While the NAc shell and core play

different roles in alcohol-seeking behavior, the core of the NAc has recently been

suggested to be involved in impulsivity linked with reward processing (Chaudhri

et al., 2010; Galtress and Kirkpatrick, 2010). Several recent studies have

implicated the core of the NAc as an important neuro-substrate involved in

ethanol-induced locomotor sensitization in DBA mice (Linsenbardt and Boehm,

2010; Hayes et al., 2011). In addition, the response of the mesolimbic dopamine

system in the NAc to ethanol exposure appears to be genetically linked with

sensitivity to ethanol-induced locomotion, suggesting that increased sensitivity to

the stimulating effects of ethanol in the NAc may be a critical factor associated

with an increased risk for developing alcoholism (Meyer et al., 2009).

Furthermore, it is possible that epigenetic changes induced by acute ethanol

exposure that we observed in the NAc core of DBA mice may be related to

ethanol-induced behavioral sensitization. Despite the observed neuroanatomical

differences in the shell and core of the NAc, both brain regions prove to be

important during the complex processes involved in the pathogenesis of alcohol

abuse disorders.

Acute ethanol exposure increased BDNF levels in the shell, but not core of

the NAc or amygdaloid structures (CeA, MeA, or BLA) in C57 mice compared to

control mice. BDNF levels were increased in both the shell and core of the NAc,

while no effects were observed in the amygdala (CeA, MeA, or BLA) of DBA

mice compared to DBA control mice. Previous studies have shown that BDNF-haplodeficient mice exhibit greater ethanol-induced place preference and psychomotor sensitization, and greater alcohol consumption after deprivation, compared to control mice (McGough et al., 2004). Inhibition of the BDNF TrkB receptor was associated with increased ethanol consumption and preference (Jeanblanc et al., 2006). Extensive exposure to ethanol leads to persistently altered BDNF signaling, which may be related to the inflexibility of addictive behaviors (Logrip et al., 2009). Voluntary ethanol intake in mice increased BDNF expression in the dorsal striatum (McGough et al., 2004). BDNF activation has been shown to control the level of ethanol self-administration in the dorsal striatum of rats, suggesting that BDNF may act as an endogenous negative regulator of ethanol intake (Jeanblanc et al., 2009). BDNF may also be regulated by changes in the chromatin structure at specific BDNF gene promoter regions (Tsankova et al., 2007). Persistent changes in H3 acetylation and methylation at the BDNF promoter have been observed in mouse models of depression and antidepressant treatment (Tsankova et al., 2006). Cocaine-induced chromatin remodeling was shown to increase BDNF expression in the rat medial prefrontal cortex, altering the efficacy of the drug (Sadri-Vakili et al., 2010). Inhibition of HDAC activity by TSA activated BDNF transcription and resulted in a compensatory change in HDAC 1 expression in neurons, possibly reflecting a genome-wide change in gene expression (Tian et al., 2010).

An interesting finding of this study was that DBA mice exhibit a predisposition to alcohol dependence. DBA mice do not innately prefer ethanol

compared to C57 mice (Misra and Pandey, 2003). However, treatment with acute ethanol exposure increased levels of CBP, acetylated histone H3 (K-9), and BDNF, while decreasing levels of methylated histone H3 (K-9) in the NAc shell and core of DBA mice compared to controls, suggesting that DBA mice can become dependent from exposure to ethanol. Similar to DBA mice, alcohol non-preferring (NP) rats also do not have an innate ethanol preference compared with alcohol-preferring (P) rats (Stewart et al., 1993; Stewart and Li, 1997). However, NP rats are less sensitive to ethanol exposure (Froehlich et al., 1988; Li, 2000; Moonat et al., 2011). Ethanol pre-exposure was shown to increase ethanol self-administration in C57 and DBA mice, despite the genetic predisposition by DBA mice to avoid alcohol, suggesting that ethanol exposure can lead to ethanol dependence in DBA mice (Camarini and Hodge, 2004). A recent study showed how intragastric self-infusion of ethanol into DBA mice increased ethanol intake and preference compared to C57 mice (Fidler et al., 2011). These studies, along with our findings, suggest that ethanol exposure can lead to epigenetic changes in NAc structures that may be related to an increased preference for and dependence to ethanol in DBA mice.

Acute ethanol exposure had no effects on the number of NeuN-positive neurons in either C57 or DBA mice. NeuN has been established as a useful neuronal marker for determining neuronal structures (Wolf et al., 1996; Korzhevshii et al., 2006). Several studies have shown how ethanol exposure altered neuronal cell numbers in the hippocampus using NeuN labeling (Noraberg and Zimmer, 1998; Zharkovsky et al., 2003; Aberg et al., 2005). While

ethanol exposure can result in loss of neuronal density in the hippocampus, our results and previous studies from our lab suggest that acute ethanol exposure does not produce any toxicity or neuronal damage in the NAc (Misra and Pandey, 2006).

In summary, the results of these studies indicate that acute and voluntary ethanol exposure affect chromatin remodeling in the NAc and amygdala of C57 and DBA mice. Further, it appears that abnormal chromatin remodeling in the NAc and amygdala plays a role in alcohol drinking behaviors through regulating the neurocircuitries involved in reward and anxiety. Future studies are necessary to fully understand these complex epigenetic mechanisms involved in the processes of alcohol addiction.

F. <u>Significance</u>

We determined how acute and voluntary ethanol exposure affects chromatin remodeling in the NAc and amygdala. We observed some similarities between the effects of acute ethanol exposure and voluntary ethanol exposure in C57 mice, in that CBP, acetylated histone H3 (K-9), and BDNF were significantly increased and methylated histone H3 (K-9) was significantly increased in the shell, but not core of the NAc compared to control mice. However, voluntary ethanol exposure did significantly decrease levels of methylated histone H3 (K-9) in the amygdala of C57 mice compared to control mice. Profound changes in histone modifications due to ethanol exposure were observed in both the shell and core of the NAc of DBA mice and NAc shell of C57 mice in these studies. Changes in chromatin remodeling in the NAc shell and core of DBA mice due to acute ethanol exposure may be related to increased sensitivity to ethanol-induced locomotion. Interestingly, it has been shown that DBA can develop an alcohol preference following experimental exposure to ethanol; our results suggest that epigenetic changes observed in the NAc shell and core of DBA mice due to ethanol exposure may be responsible for alcohol drinking behaviors observed in ethanol-exposed DBA mice. Acute ethanol exposure had no significant effects on the number of NeuN-positive neurons between of C57 and DBA mice, suggesting that our results were not due to neuronal toxicity by ethanol. Taken together, our findings suggest that ethanol exposure has a profound effect on brain chromatin remodeling in both C57 and DBA mice.

IV. CREB-INDUCED CHROMATIN REMODELING: A ROLE IN ALCOHOLISM AND ANXIETY

A. Abstract

Evidence suggests that decreased cyclic-AMP responsive element binding protein (CREB) functioning in the amygdala may be involved in anxiety and alcohol abuse disorders. CREB deficient (+/-) mice have been shown to exhibit higher anxiety-like behaviors and consume larger amounts of alcohol compared to their wild-type (+/+) littermates. We examined the possible role of CREB in chromatin remodeling in the NAc and amygdala and how epigenetic aberrations may affect anxiety-like and alcohol drinking behaviors in CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. These mice were maintained on a C57BL/6J (C57) genetic background. We also determined the effects of a histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), on chromatin remodeling and anxiety-like and alcohol drinking behaviors. We found that partial deletion of CREB led to decreased CBP and acetylation of H3 and H4 in the amygdala but not NAc of mice. We also found that treatment with TSA corrected the deficits in CBP and histone H3 and H4 acetylation in CREB deficient (+/-) mice, while there were no observable effects of TSA in wild-type (+/+) littermate mice. In addition, treatment with TSA was also able to attenuate anxiety-like and alcohol drinking behaviors of CREB deficient (+/-) mice. The results of this study suggest that partial deletion of the CREB gene produces aberrant chromatin remodeling in the amygdala, which may be operative in regulating anxiety-like and alcohol drinking behaviors.

B. <u>Introduction</u>

Alcoholism is a debilitating disease that is often comorbid with other psychiatric disorders, such as anxiety (Merikangas et al., 1996; Swendsen et al., 1998; Burns and Teesson, 2002; Petrakis et al., 2002; Grant et al., 2004). Genetic factors appear to be a crucial factor in the development of alcohol abuse disorders (Cloninger, 1987; Thome et al., 2000; Radel and Goldman, 2001). Cyclic-AMP responsive element binding protein (CREB) is a specific gene regulatory protein and the intracellular CREB signaling pathway has been shown to be a major target for the actions of ethanol in the extended amygdala (Koob et al., 1998b; Pandey, 2003; Pandey et al., 2003; 2005a; Wand, 2005). CREB belongs to the bZIP superfamily of transcription factors that contain a carboxyl (C)-terminal domain that mediates DNA binding and a leucine zipper domain that facilitates dimerization (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001). The other domains of CREB facilitate interactions with coactivators and components of the transcriptional machinery that function in the regulation of gene expression (Lonze and Ginty, 2002). Mouse and human CREB genes are comprised of 11 exons, with alternative splicing of one or more exons that give rise to the following CREB isoforms: CREBα, CREBΔ, and CREBβ (Blendy et al., 1996). The CREBα and CREBΔ isoforms contain a kinase inducible domain (KID) where an important serine-133 (Ser-133) residue is located (Chrivia et al., 1993). CREB recognizes and binds to a short DNA sequence, called the cyclic-AMP responsive element (CRE) (5'-TGACGTCA-3'), which is found in the regulatory region of genes activated by cyclic AMP (Mayr and Montminy, 2001).

In addition, CREB can heterodimerize with two other cAMP responsive activators, called cAMP responsive element modulator (CREM) and activating transcription factor- 1 (ATF-1), where CREM is mainly enriched in neuroendocrine tissues and the brain and ATF-1, like CREB, is ubiquitously expressed (Foulkes et al., 1991; Laoide et al., 1993; Bleckmann et al., 2002). All three isoforms of CREB (α, β, and Δ) are capable of activating CRE-dependent transcription in response to elevated cAMP levels in the cell (Blendy, 2006). When cyclic AMP-dependent protein kinase A (PKA) is activated, it can phosphorylate CREB on Ser-133; the activated pCREB can then recruit a transcriptional coactivator called CREB-binding protein (CBP) (Chrivia et al., 1993). CREB can also be phosphorylated by other kinases, including protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) (Shaywitz and Greenberg, 1999).

CBP contains intrinsic histone acetyltransferase (HAT) activity, which is required for chromatin remodeling of histone proteins (Silva et al., 1998; Mayr and Montminy, 2001; Hsieh and Gage, 2005). CBP adds acetyl groups to lysine (K) residues located at the amino (N)-terminal tail regions of histone proteins. Acetylation neutralizes the positive charge of K, thereby reducing the affinity of the histone tail regions for the adjacent nucleosomes. The profound effect of this type of covalent histone modification is the resulting attraction of transcriptional regulatory proteins to the loosened chromatin structure, which provides more accessible binding regions to the DNA and subsequent increases in gene expression levels (Grunstein, 1997; Verdone et al., 2005). Proteins such as CBP

can execute HAT activity and regulate gene expression by controlling the precise structure of specific chromatin domains, which in turn can dictate the function of the cell (Grunstein, 1997; Chan and La Thangue, 2001). The removal of the covalently attached acetyl groups found on N-terminal histone tails occurs by the activation of a different type of protein complex, termed histone deacetylases (HDACs) (de Ruijter et al., 2003). Since acetylation and methylation are mutually exclusive events, the displacement of the acetyl molecules on K residues allows for replacement by mono-, di-, or trimethylated molecules to the same or different lysine side chain (Rice, 2001; Zhang et al., 2009). Moreover, K methylation by histone methyltransferases (HMTs) can tighten the chromatin structure, eliminating the uncoiled DNA organization about the nucleosome, and instead replacing it with a more rigid DNA structure that prevents the binding of the transcriptional machinery, thereby resulting in an overall decrease in gene expression levels (Bannister et al., 2002; Rice et al., 2003; Trievel, 2004).

Amygdaloid neurocircuitry has also been shown to be critical in regulating anxiety and also in promoting and maintaining alcohol drinking behaviors (Petrakis et al., 2002; Pandey, 2003; Koob, 2004; Wand, 2005). In the amygdala, CREB has been shown to play a major role in learning and memory, which may be operative in contributing to changes in long-term memory associated with the development of alcoholism and anxiety (Lonze and Ginty, 2002; Pandey, 2003; Carlezon et al., 2005). Not only is CREB important in synaptic plasticity, long-term potentiation, and long-term memory, it also has been shown to play a critical role as a neuronal survival factor (Bourtchuladze et

al., 1994; Silva et al., 1998; Dawson and Ginty, 2002; Ao et al., 2006). Disruption of CREB function has also been linked with the onset of a neurodegenerative state (Mantamadiotis et al., 2002). Mice containing targeted gene mutations encoding for CREB have been developed (Bourtchuladze et al., 1994; Hummler et al., 1994; Maldonado et al., 1996). These CREB knockout mice contain mutations that comprise deletions of two isoforms of CREB (α and Δ) (Hummler et al., 1994; Blendy et al., 1996). CREB mutations in mice where CREB α and Δ isoforms were deleted (hypomorphic mutation) led to a dramatic upregulation of the CREBβ isoform while mice remained viable (Blendy et al., 1996).

CREB α and Δ are major isoforms that are highly abundant in the adult brain, whereas CREBβ expression during adulthood is usually very low, suggesting not only the importance of CREBβ during development, but also a possible compensatory mechanism by CREBβ, that results in increased CREBβ mRNA stability in order to offset loss of function due to deletion of CREB α and Δ isoforms (Blendy et al., 1996; Silva et al., 1998). Despite upregulation of the CREBβ isoform, the deletion of CREB α and Δ was found to abolish CRE-DNA binding in the amygdala, hippocampus, cerebellum, and cortex of CREB mutant mice, suggesting that CREBβ does not bind efficiently to CRE sites and does not play a major role in CREB-dependent transcription (Pandey et al., 2000). Generation of true CREB null [CREB (-/-)] mice, where all functional isoforms of CREB (CREB α, β, and Δ) were inactivated resulted in smaller mice with reduced corpus callosums and anterior commisures that died immediately after birth due to respiratory distress (Rudolph et al., 1998). Previous studies in our lab have

demonstrated that partial deletion of the CREB gene (α and Δ isoforms) promoted anxiety-like and alcohol drinking behaviors in mice (mixed genetic background of C57BL/6J and 129 SVJ mice) (Pandey et al., 2004). In addition, these CREB deficient (+/-) mice exhibited functional deficits associated with decreased levels of p-CREB in amygdaloid brain regions compared to wild-type littermate (+/+) mice (Pandey et al., 2004). Since activation of CREB and subsequent phosphorylation of CREB recruits the positive effector, CBP, it is reasonable to assume that decreased p-CREB levels in the cell may lead to decreased functional levels of CBP. Previous studies have shown that transgenic mice expressing a truncated form of CBP exhibit abnormal synaptic plasticity and memory storage in the hippocampus (Wood et al., 2005). Decreased CBP activity in the amygdala, due to reduced HAT activity, can thereby alter the levels of K acetylation on histone tail regions. These atypical alterations in amygdaloid chromatin remodeling may be the foundation for the long-term changes in synaptic plasticity that give rise to anxiety-like behaviors and increased alcohol drinking behaviors observed in CREB deficient (+/-) mice.

In this study we examined levels of CBP and acetylated histones H3 (K-9) and H4 (K-8) in the NAc (shell and core) and amygdaloid (CeA, MeA, and BLA) structures of mice lacking one allele of CREB [CREB deficient (+/-) mice] compared to wild-type (+/+) littermate mice; these mice were maintained on a pure C57 genetic background. Currently, the role that CREB may play in brain chromatin remodeling is not well understood. We found that levels of CBP, acetylated histone H3 (K-9) and acetylated histone H4 (K-8) were all decreased

in the amygdala (CeA, MeA, and BLA) but not in the NAc (shell or core) of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. We also tested the effects of the HDAC inhibitor, trichostatin A (TSA), on CBP and histone acetylation levels in the amygdala to discern the impact that HDACs inhibition had on both the levels of these chromatin remodeling proteins and on anxiety-like and alcohol drinking behaviors; since treatment with TSA has been previously shown to result in a recovery in poor learning and memory in CBP deficient (+/-) mice (Wood et al., 2005; Fischer et al., 2007).   We found that TSA treatment was able to restore diminished levels of CBP and acetylated histones H3 (K-9) and H4 (K-8) in all amygdaloid structures examined, in addition to attenuating both anxiety-like and alcohol drinking behaviors of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. Taken together, these results suggest a possible association between genetic abnormalities within the structural organization of chromatin in the amygdala and anxiety-like and alcohol drinking behaviors.

C. **Materials and Methods**

1. **Animals**

Adequate measures were taken to minimize pain and discomfort. Experiments were carried out in accordance with the National Institute of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care Committee. Adult male CREB deficient (+/-) mice and wild-type (+/+) littermates were used in this study. All mice were individually housed under a 12-hour light/dark cycle and had *ad libitum* access to food and water.

2. **Breeding and Genotyping of CREB Mice**

CREB deficient (+/-) mice used in these studies contained a pure C57 genetic background. All mice used in these studies were genotyped to confirm their genetic CREB deletion. Genotyping was performed using a PCR protocol as previously described by us (Pandey, Roy et al. 2004). DNA was isolated from mouse tail snips (Invitrogen Easy-DNA Kit, Carlsbad, CA), which was then used for PCR amplifications [93°C for 2 min; 40 cycles of 93°C for 30 sec., 47°C for 30 sec., 72°C for 1 min.; 72°C for 10 min.] to distinguish between wild-type (+/+) (150 bp band) and heterozygous (+/-) (150 +350 bp band) mice. The following primers were used as follows: CREB1: 5'-TAT TGT AGG TAA CTA AAT GA-3'; CREB2: 5'-ATG TAT TTT TAT ACC TGG GC-3'; NEO: 5'-TGA TGG ATA CTT TCT CGG CA-3'. We also determined levels of p-CREB in the brain in order to re-confirm genotypes of mice.

3. <u>Effects of HDAC Inhibition on Anxiety-Like Behaviors (EPM)</u>

The effect of HDAC inhibition using TSA on anxiety-like behaviors of CREB deficient (+/-) mice compared to wild-type (+/+) littermates was determined using the elevated plus-maze (EPM) test. TSA was dissolved in DMSO, and diluted in sterile PBS daily (1:5 dilution of TSA/DMSO:PBS) to a final concentration of 1 mg/ml. TSA, at a concentration of 2 mg/kg body weight, was injected (i.p.) into mice each morning of the experiment for three consecutive days. Anxiety-like behaviors were measured by the EPM on the third day of the experiment 2 hours after TSA injections. The EPM procedure was the same as described by us previously (Pandey et al. 1999; 2003; 2005b). Briefly, the EPM apparatus incorporated two open arms and two closed arms arranged in an alternating fashion and connected by a central platform (Lafayette Instrument, Lafayette, IN). Mice were placed in individual cages and taken into a separate room, located adjacent to the testing room, for a 5-minute habituation period prior to EPM testing. At the start of the 5-minute trial period, each mouse was placed in the center of the EPM and allowed to freely explore the apparatus for the duration of the test. The number of entries into either the open arm or closed arm of the apparatus and the amount of time spent in each arm was recorded. Results from the EPM test were expressed as the mean +/- SEM of the percent of open-arm entries and the percent of time spent in the open arms. Immediately following EPM testing, mice were anesthetized, perfused, and brains were collected for immunohistochemistry.

4. **<u>Gold-Immunolabeling of p-CREB, CBP, and Acetylated Histones</u>**

   <u>H3 (K-9) and H4 (K-8)</u>

We examined levels of CBP, and acetylated H3 (K-9) and H4 (K-8) in the

CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+)

littermate mice, treated with or without TSA, using the gold immunolabeling

histochemical procedure, previously described by us (Pandey et al., 2001;

2005b; 2006). We also measured levels of p-CREB in order to reconfirm

genotyping. The following antibodies have been well characterized in our

laboratory (Pandey et al., 2008a): p-CREB (Ser-133), acetylated H3 (K-9) and H4

(K-8) [Millipore (Billerica, MA)], and CBP (C-20) [Santa Cruz Biotechnology

(Santa Cruz, CA)]. Mice were anesthetized and then perfused intracardially with

normal saline (100 mls), followed by 100 mls of 4% ice-cold paraformaldehyde

fixative. Brains were dissected out and placed in fixative for 20 hours at $4^{o}C$.

Post-fixation, brains were soaked in a sucrose gradient (10%, 20%, followed by

30% sucrose; prepared in 0.1 M phosphate buffer, pH 7.4). Brains were frozen

and 20μm coronal sections were prepared using a cryostat then washed in 0.01

M PBS. The quantification of gold-immunolabeled proteins was performed using

an Image Analysis System (Loats Associates, Westminster, MD) connected to a

light microscope that calculated the number of gold particles/100 μm^2 area of a

defined brain area at high magnification (100x). The threshold was set up so that

each area that contained no staining would yield a count of zero. Immunogold

particles in three adjacent brain sections from three different mice specimens

were counted, and then all nine values were averaged.

5. Effects of HDAC Inhibition on Alcohol Drinking Behaviors

The effects of HDAC inhibition using TSA on alcohol drinking behaviors of CREB deficient (+/-) mice compared to wild-type (+/+) littermates were determined. Ethanol preference was measured using the two-bottle free-choice paradigm described previously by us (Misra and Pandey, 2003; Pandey et al., 2004) and others (Thiele et al., 1998; 2000). Mice were habituated to drink water equally from two bottles (approximately 2 weeks). Fresh bottles were given everyday at 5pm and drinking was measured to ensure that mice continued to drink equally from both bottles; the placement of the bottles was switched every day to prevent a positional habit from forming. Once a pattern of drinking water equally from both bottles (approximately 2 weeks) was established, mice were provided with 3% ethanol (95% ethanol diluted in tap water) in one bottle and tap water in the other bottle for 3 consecutive days, followed by 7% ethanol (95% ethanol diluted in tap water) for 3-5 days, and then 9% ethanol for the next 6 days. During the last three days of 9% ethanol drinking, mice received one injection (i.p.) daily at 5pm of either vehicle (DMSO in 0.01M PBS diluted in a 1:5 ratio) or TSA (2 mg/kg; 5 mg TSA diluted in 1 ml DMSO; solution further diluted with 0.01M PBS to a 1:5 ratio). Animals continued to drink for three more days and each day at 5pm, mice would receive their respective injections and fresh bottles. We measured consumption of ethanol and water each day prior to distribution of new bottles containing water or ethanol. The mean percentage of ethanol intake and water intake was calculated from total fluid intake over the course of the experiment.

6. __Statistical Analysis__

For baseline protein studies, we used CREB deficient (+/-) mice and wild-type (+/+) littermate mice for the gold immunolabeling studies. We applied the Student's t-test function using SigmaStat statistical software to detect significant median differences between CREB deficient (+/-) mice and wild-type (+/+) littermate mice. For TSA treatment studies (measurement of alcohol drinking and anxiety-like behaviors), we analyzed multiple groups by one-way analysis of variance (ANOVA) also using SigmaStat. All post-hoc comparison procedures were performed using Tukey's test. P-values < 0.05 were considered significant and data in the figures were expressed as mean ± SEM.

D. Results

1. Baseline Levels of p-CREB in Amygdala and NAc of CREB

Deficient (+/-) Mice and Wild-Type (+/+) Littermate Mice

Figure 18A shows p-CREB-positive staining in the amygdala, we found that p-CREB levels were significantly (p<0.001) lower in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice (Fig. 18B). Figure 19A shows p-CREB-positive staining in the NAc, we found that p-CREB levels were significantly (p<0.001) lower in the shell and core of the NAc of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice (Fig. 19B). Decreased p-CREB levels in the NAc (shell and core) and amygdaloid (CeA, MeA, and BLA) regions reconfirm the genotype of the mice.
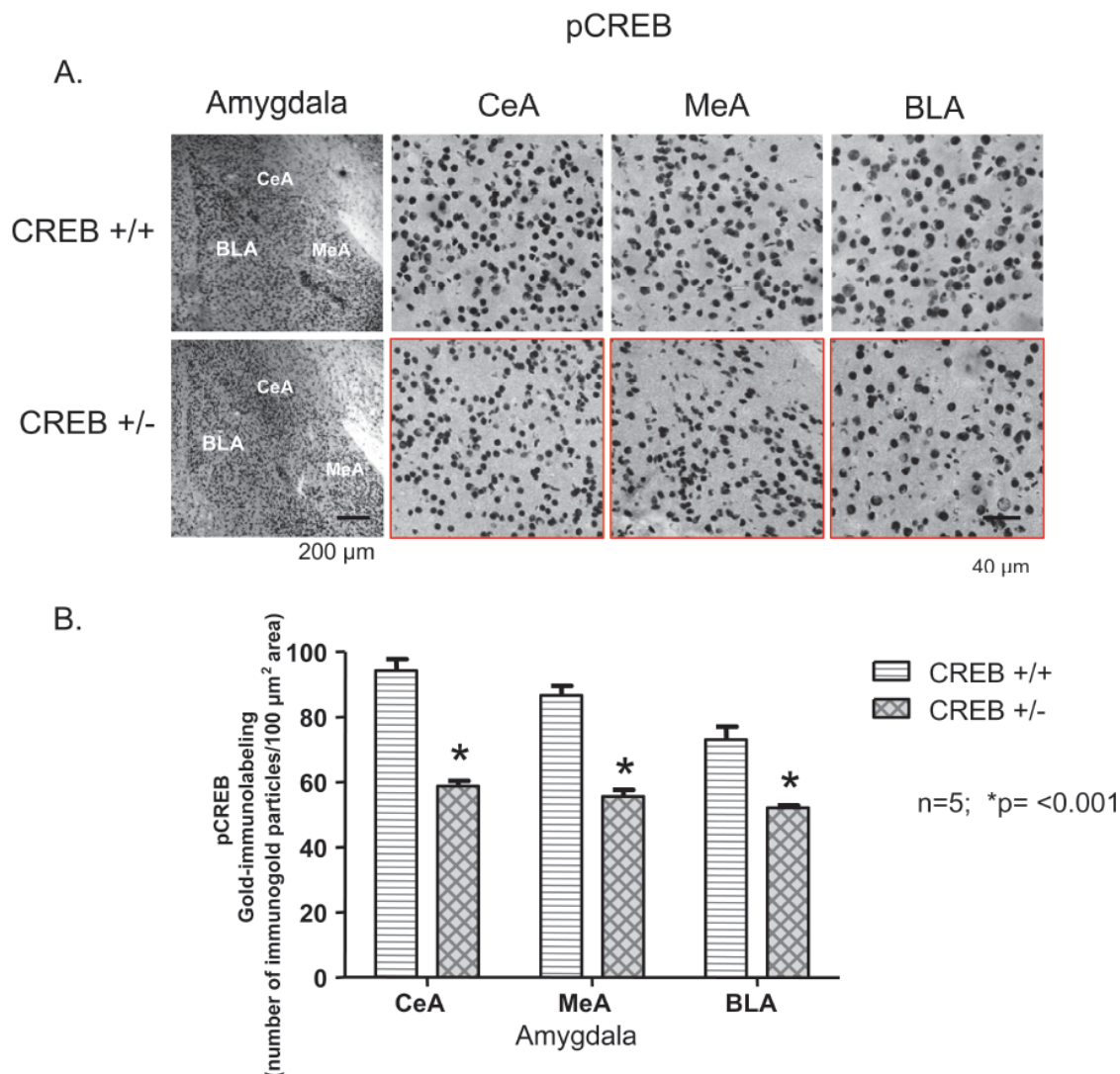
Figure 18.   Baseline levels of p-CREB in amygdala of CREB deficient (+/-) mice and wild-type (+/+) littermate mice. Levels of phosphorylated CREB (p-CREB) were significantly decreased in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of p-CREB gold immunolabeling in the CeA, MeA, and BLA of CREB (+/-) mice compared to CREB (+/+) mice. **B:** Bar diagram demonstrates the quantified results of p-CREB in the CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group. *P<0.001, significantly different from wild-type (+/+) littermate mice (Student's t-test).

pCREB

A.

B.

n=5; *p=<0.001
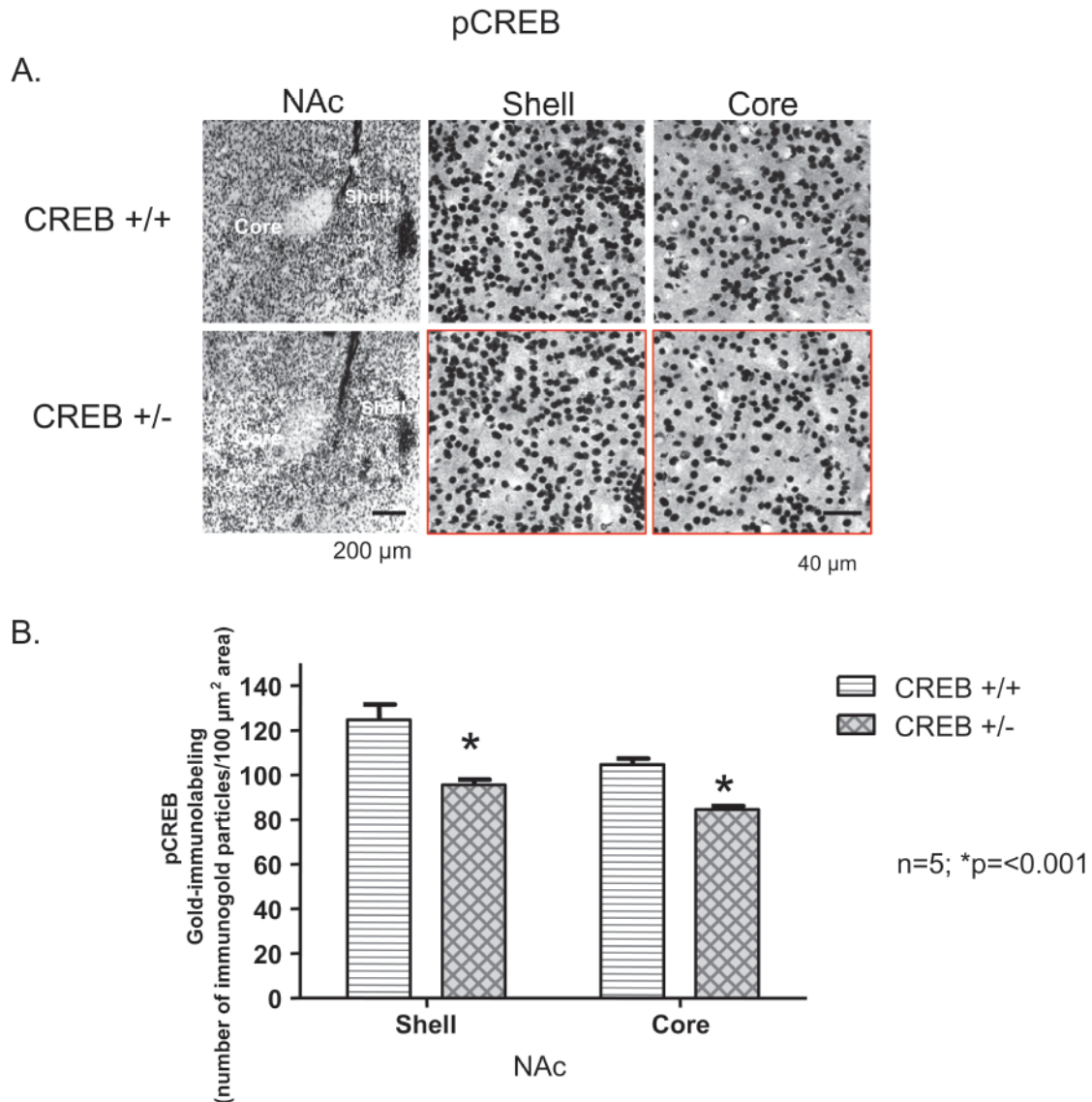
Figure 19. **Baseline levels of p-CREB in NAc of CREB deficient (+/-) mice and wild-type (+/+) littermate mice.** Levels of phosphorylated CREB (p-CREB) were significantly decreased in nucleus accumbens (NAc) shell and core of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of p-CREB gold immunolabeling in NAc shell and core of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **B:** Bar diagram demonstrates the quantified results of p-CREB in the shell and core of the NAc in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group. *P<0.001, significantly different from wild-type (+/+) littermate mice (Student's t-test).

2. **Baseline Levels of CBP in Amygdala and NAc of CREB Deficient**

   (+/-) Mice and Wild-Type (+/+) Littermate Mice

Figure 20A shows CBP-positive staining in the amygdala, we found that

CBP levels were significantly ($p < 0.001$) lower in the CeA, MeA, and BLA of

CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice (Fig. 20B).

Figure 21A shows CBP-positive staining in the NAc, we found that there were no

differences in CBP levels in the shell or core of the NAc between CREB deficient

(+/-) mice and wild-type (+/+) littermate mice (Fig. 21B). These data indicate that

CREB deficient (+/-) mice have decreased levels of CBP in CeA, MeA, and BLA

compared to wild-type (+/+) littermate mice. In addition, these data also show

that levels of CBP in the NAc (shell and core) are similar in CREB deficient (+/-)

mice compared to wild-type (+/+) littermate mice.
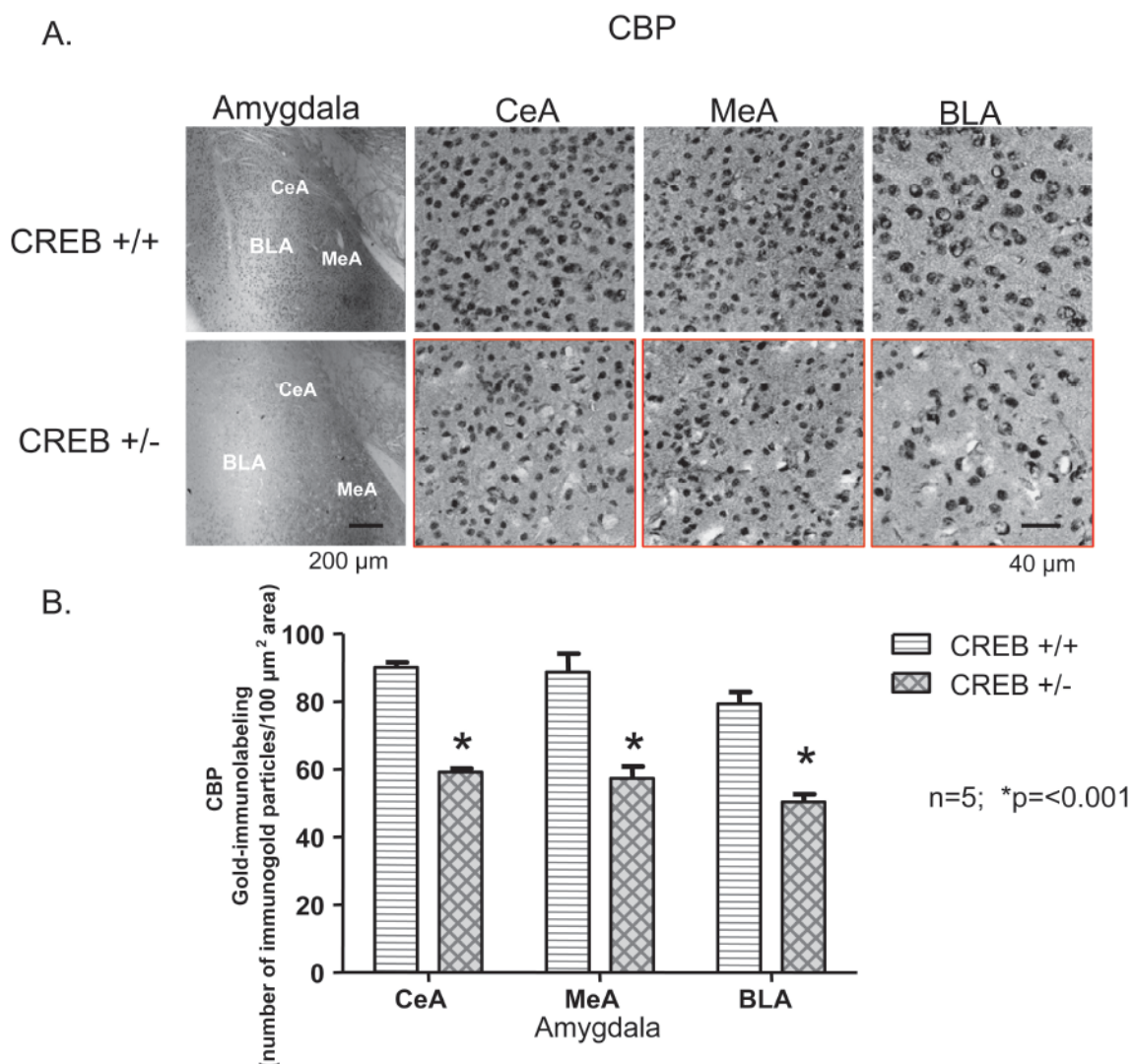
Figure 20. **Baseline levels of CBP in amygdala of CREB deficient (+/-) mice and wild-type (+/+) littermate mice.** Levels of CREB-binding protein (CBP) were significantly decreased in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of CBP gold immunolabeling in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **B:** Bar diagram demonstrates the quantified results of CBP in the CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean $\pm$ SEM of 5 mice in each group. *P<0.001, significantly different from wild-type (+/+) littermate mice (Student's t-test).

Figure 21.   Baseline levels of CBP in NAc of CREB deficient (+/-) mice and wild-type (+/+) littermate mice. There were no significant differences observed in the levels of CREB-binding protein (CBP) in the shell or core of the nucleus accumbens (NAc) of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **A:** Shows low magnification (scale bar, 200 μm) photomicrographs of CBP gold immunolabeling in shell and core of NAc and also high magnification (scale bar, 40 μm) in the shell and core of the NAc of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **B:** Bar diagram demonstrates the quantified results of CBP in the shell and core of the NAc in terms of number of immunogold particles/100 μm^2 area.   Values are mean ± SEM of 5 mice in each group.

3. <u>Baseline Levels of Acetylated Histone H3 (K-9) in Amygdala and NAc of CREB Deficient (+/-) Mice and Wild-Type (+/+) Littermate Mice</u>

Figure 22A shows acetylated histone H3-positive staining in the amygdala, we found that acetylated histone H3 levels were significantly ($p < 0.001$) lower in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice (Fig. 22B). Figure 23A shows acetylated histone H3-positive staining in the NAc, we found that there were no differences in acetylated histone H3 levels in the shell or core of the NAc between CREB deficient (+/-) mice and wild-type (+/+) littermate mice (Fig. 23B). These data indicate that CREB deficient (+/-) mice have decreased levels of acetylated histone H3 in CeA, MeA, and BLA compared to wild-type (+/+) littermate mice. In addition, these data also show that levels of acetylated histone H3 in the NAc (shell and core) are similar in CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.

Figure 22. **Baseline levels of acetylated histone H3 (K-9) in amygdala of CREB deficient (+/-) mice and wild-type (+/+) littermate mice.** Levels of acetylated histone H3 (K-9) were significantly decreased in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H3 (K-9) gold immunolabeling in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **B:** Bar diagram demonstrates the quantified results of acetylated histone H3 (K-9) in the CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group. *P<0.001, significantly different from wild-type (+/+) littermate mice (Student's t-test).

Figure 23. **Baseline levels of acetylated histone H3 (K-9) in NAc of CREB deficient (+/-) mice and wild-type (+/+) littermate mice.** There were no significant differences observed in the levels of acetylated histone H3 (K-9) in the shell or core of the nucleus accumbens (NAc) of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **A:** Shows low magnification (scale bar, 200 μm) photomicrographs of acetylated histone H3 (K-9) gold immunolabeling in shell and core of NAc and also high magnification (scale bar, 40 μm) in the shell and core of the NAc of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **B:** Bar diagram demonstrates the quantified results of acetylated histone H3 (K-9) in the shell and core of the NAc in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group.

4. Baseline Levels of Acetylated Histone H4 (K-8) in Amygdala and NAc of CREB Deficient (+/-) Mice and Wild-Type (+/+) Littermate Mice

Figure 24A shows acetylated histone H4-positive staining in the amygdala, we found that acetylated histone H4 levels were significantly ($p < 0.001$) lower in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice (Fig. 24B). Figure 25A shows acetylated histone H4-positive staining in the NAc, we found that there were no differences in acetylated histone H4 levels in the shell or core of the NAc between CREB deficient (+/-) mice and wild-type (+/+) littermate mice (Fig. 25B). These data indicate that CREB deficient (+/-) mice have decreased levels of acetylated histone H4 in CeA, MeA, and BLA compared to wild-type (+/+) littermate mice. In addition, these data also show that levels of acetylated histone H4 in the NAc (shell and core) are similar in CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.
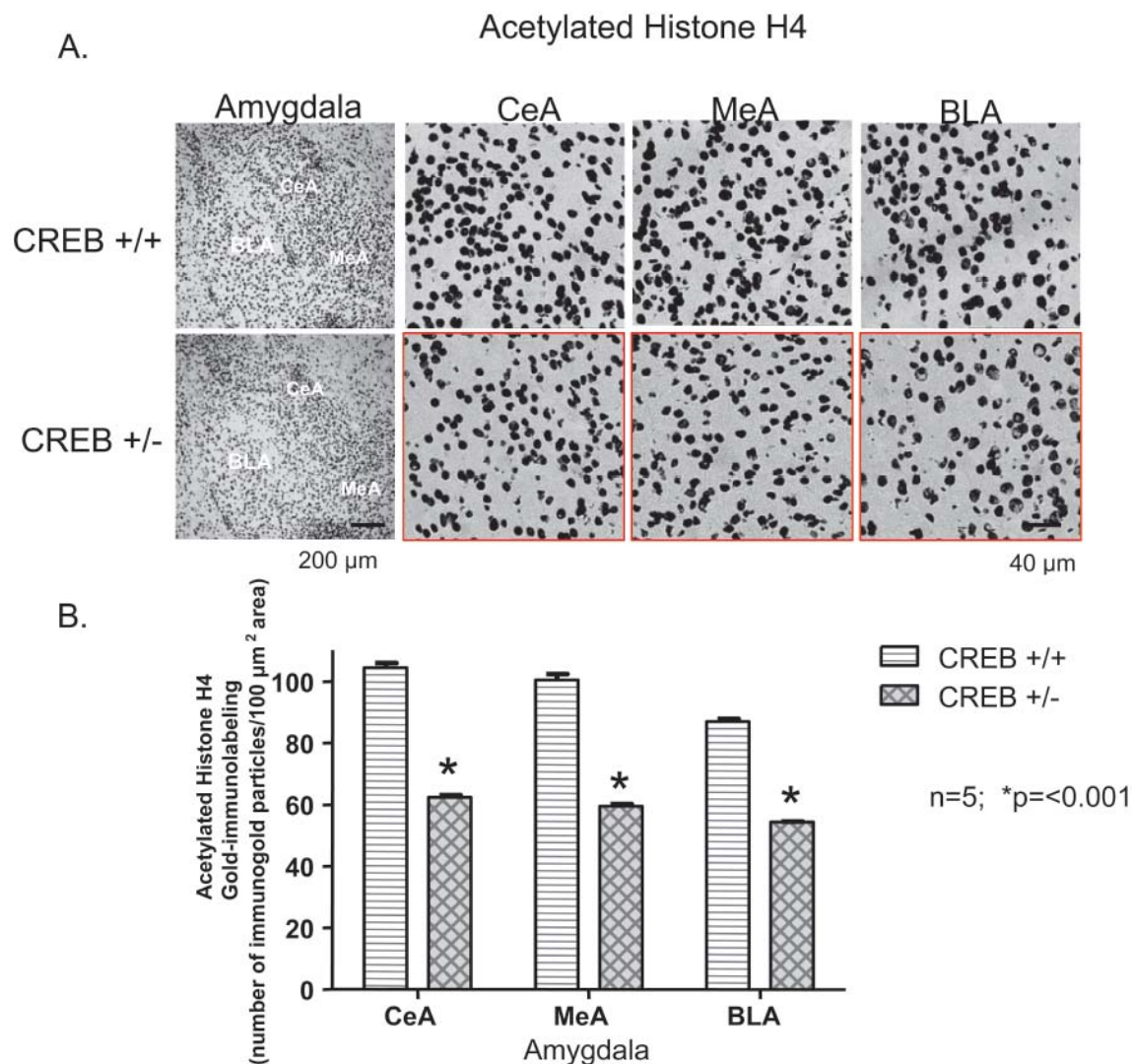
Figure 24. **Baseline levels of acetylated histone H4 (K-8) in amygdala of CREB deficient (+/-) mice and wild-type (+/+) littermate mice.** Levels of acetylated histone H4 (K-8) were significantly decreased in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **A:** Shows low magnification (scale bar, 200 μm) and also high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H4 (K-8) gold immunolabeling in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **B:** Bar diagram demonstrates the quantified results of acetylated histone H4 (K-8) in the CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group. *P<0.001, significantly different from wild-type (+/+) littermate mice (Student's t-test).

Acetylated Histone H4

A.

B.

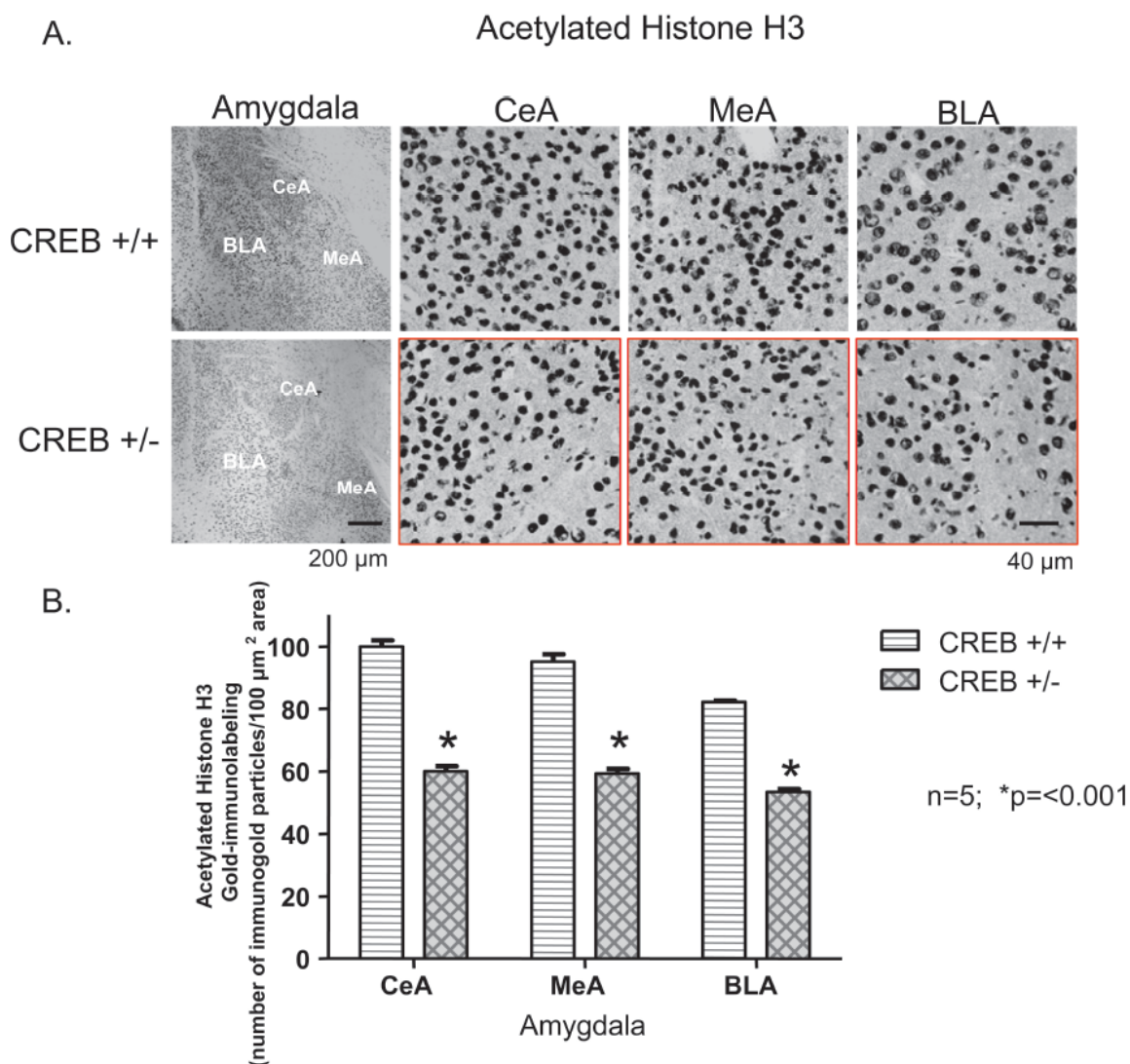**Figure 25.** **Baseline levels of acetylated histone H4 (K-8) in NAc of CREB deficient (+/-) mice and wild-type (+/+) littermate mice.** There were no significant differences observed in the levels of acetylated histone H4 (K-8) in the shell or core of the nucleus accumbens (NAc) of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **A:** Shows low magnification (scale bar, 200 μm) photomicrographs of acetylated histone H4 (K-8) gold immunolabeling in shell and core of NAc and also high magnification (scale bar, 40 μm) in the shell and core of the NAc of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. **B:** Bar diagram demonstrates the quantified results of acetylated histone H4 (K-8) in the shell and core of the NAc in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 5 mice in each group.

5. **Effects of HDAC Inhibitor Treatment on Anxiety-Like Behaviors of CREB Deficient (+/-) Mice compared to Wild-Type (+/+) Littermate Mice**

Pilot data in our laboratory suggests that HDAC activity is higher in the amygdala of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice (Pandey et al. 2011, unpublished data). Therefore, we used the HDAC inhibitor TSA to examine the effects on anxiety-like and alcohol drinking behaviors, and chromatin remodeling in CREB deficient (+/-) mice compared to wild-type (+/+) littermates (please see Figs. 26-30). Figure 26 shows data on the effects of TSA on anxiety-like behaviors of CREB deficient (+/-) mice and wild-type (+/+) littermate mice. We found that CREB deficient (+/-) mice displayed higher anxiety-like behaviors compared to wild-type (+/+) littermate mice due to lower % open-arm entries and less % time spent in the open-arm of the EPM (Fig. 26). We also found that TSA treatment attenuated anxiety-like behaviors of CREB deficient (+/-) mice by significantly increasing both the % open-arm entries and % time spent in the open arm compared to wild-type (+/+) littermate mice (Fig. 26). The total number of entries (closed and open arms) was not significantly different among the groups (Fig. 26). These results suggest that CREB deficient (+/-) mice display anxiety-like behaviors compared to wild-type (+/+) littermate mice and that TSA treatment can ameliorate these anxiety-like behaviors. Defective chromatin remodeling associated with diminished CREB functioning in the amygdala, may be responsible for anxiety-like behaviors of CREB deficient (+/-) mice, which were relieved by TSA treatment.
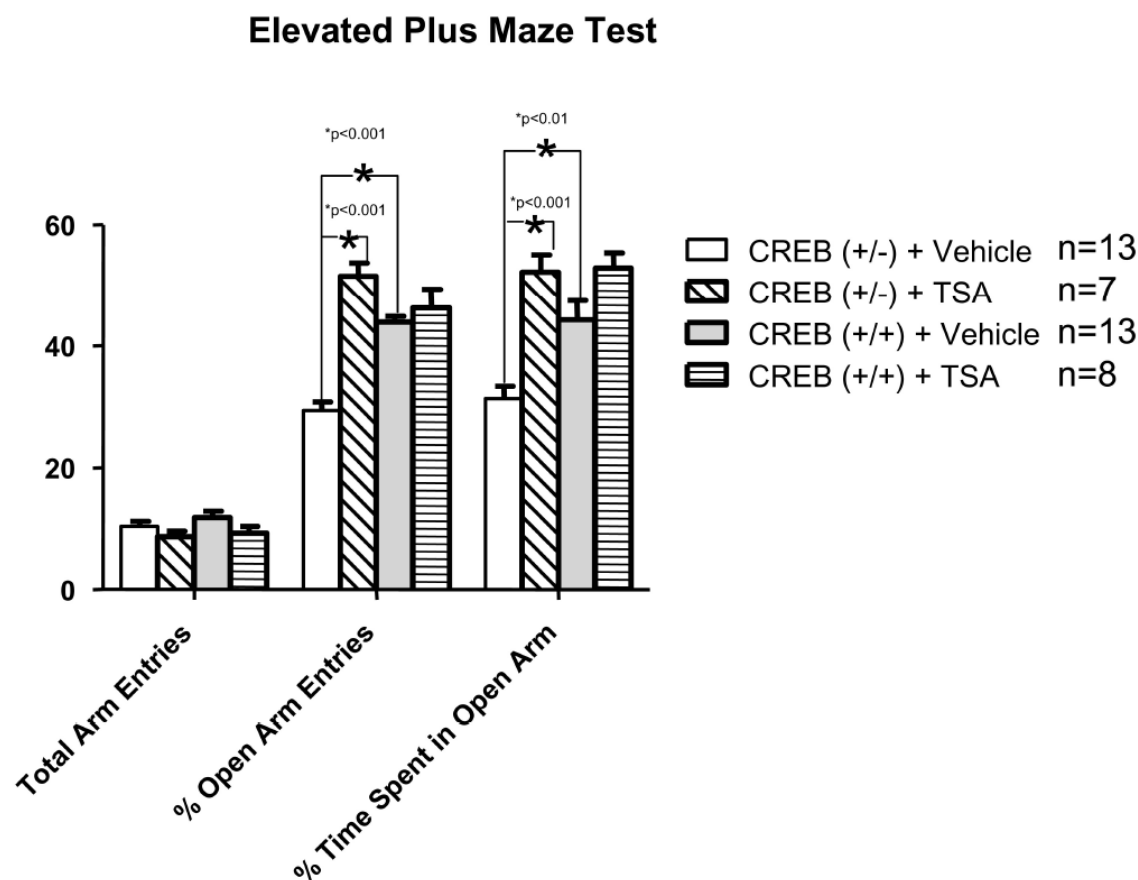
Figure 26. **Effects of HDAC inhibitor treatment on anxiety-like behaviors of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.** Cyclic-AMP responsive element binding protein (CREB) deficient (+/-) [CREB (+/-)] mice display higher anxiety-like behaviors compared to wild-type (+/+) littermate [CREB (+/+)] mice; anxiety-like behaviors of CREB deficient (+/-) mice were alleviated by treatment with the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA) (2 mg/kg dose per body weight of mouse), compared to wild-type (+/+) littermate mice. Bar diagram demonstrates the behavioral results obtained using the elevated plus maze (EPM) test for anxiety-like behaviors of CREB deficient (+/-) mice treated with vehicle [CREB (+/-) + Vehicle] or TSA [CREB (+/-) + TSA] and wild-type (+/+) littermate mice treated with vehicle [CREB (+/+) + Vehicle] or TSA [CREB (+/+) + TSA] in terms of 'Total Arm Entries', 'Percent (%) Open Arm Entries', and 'Percent (%) Time Spent in Open Arm'. Values are mean ± SEM of "n" mice [CREB (+/-) + Vehicle: n=13; CREB (+/-) + TSA: n=7; CREB (+/+) + Vehicle: n=13; and CREB (+/+) + TSA: n=8] in each group. *P<0.01-0.001, results significantly different for CREB (+/-) treated with vehicle [CREB (+/-) + Vehicle] compared to CREB (+/+) treated with vehicle [CREB (+/+) + Vehicle]; and CREB (+/-) treated with TSA [CREB (+/-) + TSA] compared to CREB (+/-) treated with vehicle [CREB (+/-) + vehicle] [One-way analysis of variance (ANOVA) followed by post-hoc comparison by Tukey test].

6. **Effects of HDAC Inhibitor Treatment on Protein Levels in Amygdala of CREB Deficient (+/-) Mice compared to Wild-Type (+/+) Littermate Mice**

   a. CBP

We reconfirmed that CBP levels were significantly ($p<0.001$) lower in the amygdala (CeA, MeA, and BLA) of CREB deficient (+/-) mice [CREB (+/-) + Vehicle] compared to wild-type (+/+) littermate mice [CREB (+/+) + Vehicle] (Figure 27A & 27B). Figure 27A shows the CBP-positive staining of the amygdala, we found that treatment with the HDAC inhibitor trichostatin A (TSA) was able to significantly ($p<0.001$) increase CBP levels in the CeA, MeA, and BLA of CREB deficient (+/-) mice [CREB (+/-) + TSA] to levels comparable to wild-type (+/+) littermate mice [CREB (+/+) + Vehicle] (Fig. 27B). We also found that TSA had no significant effects on CBP levels in wildtype (+/+) littermate mice [CREB (+/+) + TSA] (Figs. 27A & 27B). These data indicate that CBP levels increase in the CeA, MeA, and BLA of CREB deficient (+/-) mice [CREB (+/-) + TSA] after treatment with TSA. In addition, these data also show that TSA treatment does not affect CBP levels in the CeA, MeA, or BLA of wild-type (+/+) littermate mice [CREB (+/+) + TSA].
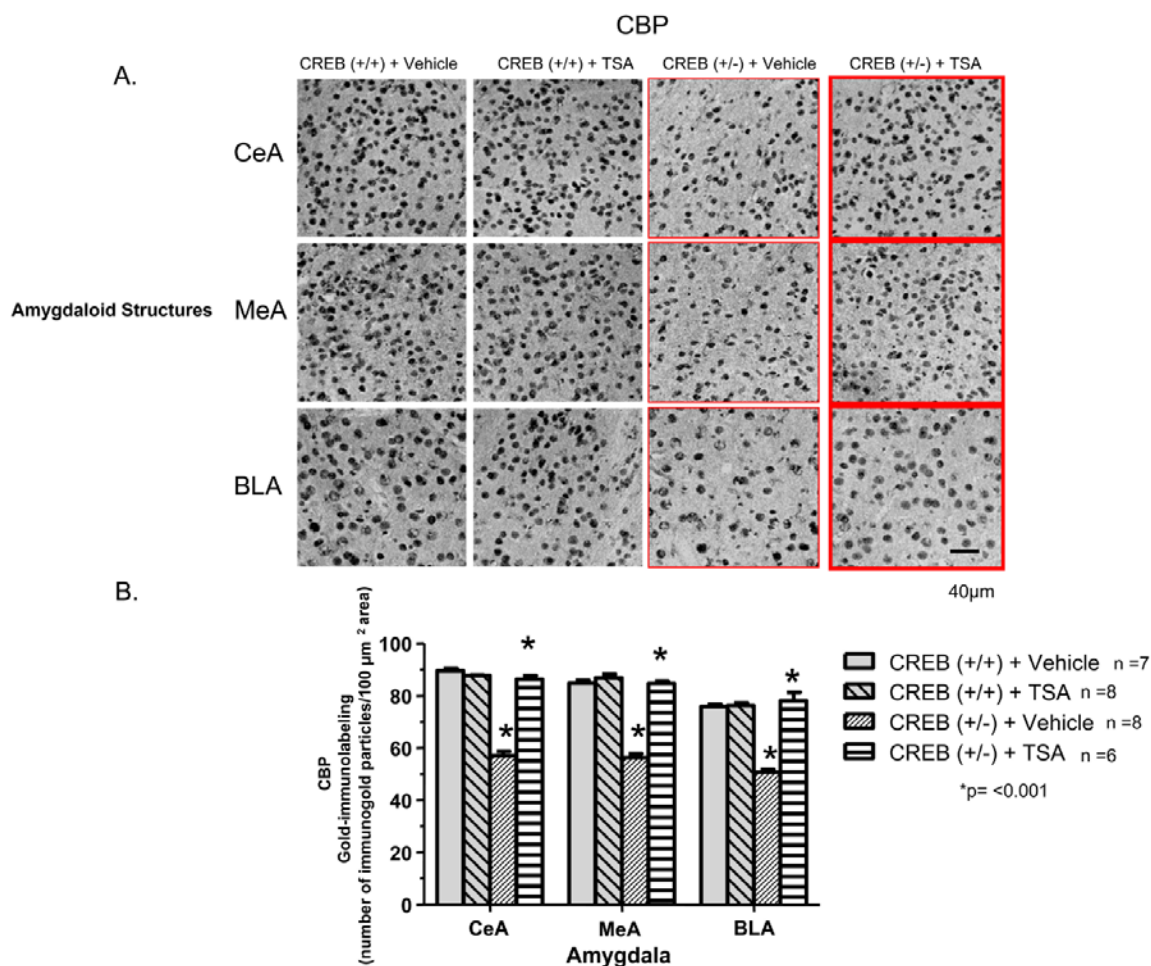
Figure 27. **Effects of HDAC inhibitor treatment on CBP levels in amygdala of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.** Trichostatin A (TSA) was able to significantly increase CREB-binding protein (CBP) levels in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of CREB deficient (+/-) mice [CREB (+/-)] treated with TSA [CREB (+/-) + TSA] compared to controls [CREB (+/-) + Vehicle]; TSA had no significant effects on CBP levels in wild-type littermate mice [CREB (+/+)] treated with TSA [CREB (+/+) + TSA] compared to controls [CREB (+/+) + Vehicle]. **A:** Shows high magnification (scale bar, 40 μm) photomicrographs of CBP gold immunolabeling in CeA, MeA, and BLA of CREB deficient (+/-) mice treated with vehicle [CREB (+/-) + Vehicle] or TSA [CREB (+/-) + TSA] and wildtype (+/+) littermate mice treated with vehicle [CREB (+/+) + Vehicle] or TSA [CREB (+/+) + TSA]. **B:** Bar diagram demonstrates the quantified results of CBP in the CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 6-8 mice in each group. *P<0.001, significantly different from CREB deficient (+/-) mice treated with vehicle [CREB (+/-) + Vehicle] [One-way analysis of variance (ANOVA) followed by post-hoc comparison by Tukey test].

b. Acetylated Histone H3 (K-9)

We reconfirmed that acetylated histone H3 (K-9) levels were significantly (p<0.001) lower in the amygdala (CeA, MeA, and BLA) of CREB deficient (+/-) mice [CREB (+/-) + Vehicle] compared to wild-type (+/+) littermate mice [CREB (+/+) + Vehicle] (Figure 28A & 28B). Figure 28A shows the acetylated histone H3 (K-9)-positive staining of the amygdala, we found that TSA treatment was able to significantly (p<0.001) increase acetylated histone H3 (K-9) levels in the CeA, MeA, and BLA of CREB deficient (+/-) mice [CREB (+/-) + TSA] to levels comparable to wild-type (+/+) littermate mice [CREB (+/+) + Vehicle] (Fig. 28B). We also found that TSA had no significant effects on acetylated histone H3 (K-9) levels in CREB deficient (+/+) mice [CREB (+/+) + TSA] (Figs. 28A & 28B). These data indicate that acetylated histone H3 (K-9) levels increase in the CeA, MeA, and BLA of CREB deficient (+/-) mice [CREB (+/-) + TSA] after treatment with TSA. In addition, these data also show that TSA treatment does not affect acetylated histone H3 (K-9) levels in the CeA, MeA, or BLA of CREB deficient (+/+) mice [CREB (+/+) + TSA].
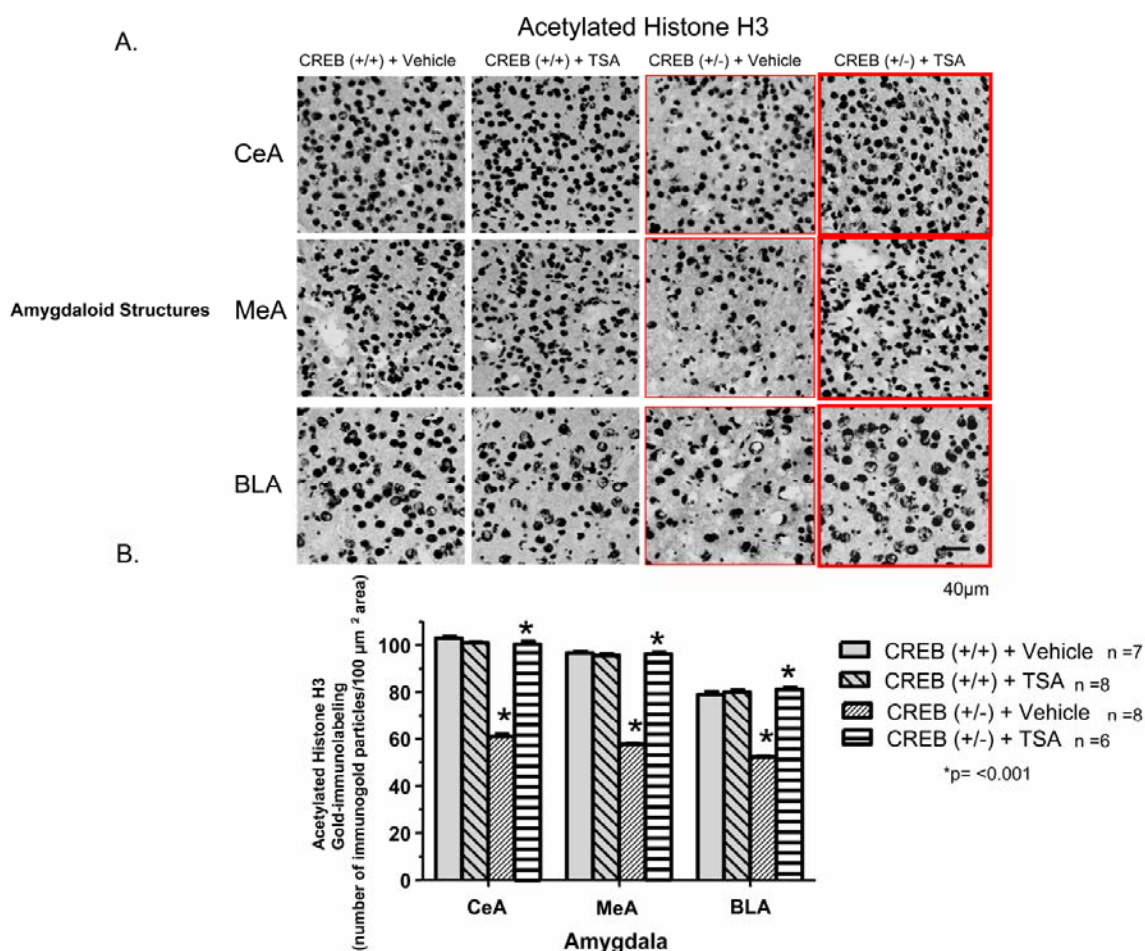
Figure 28. **Effects of HDAC inhibitor treatment on acetylated histone H3 (K-9) protein levels in amygdala of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.** TSA was able to significantly increase acetylated histone H3 (K-9) levels in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of CREB deficient (+/-) mice [CREB (+/-)] treated with TSA [CREB (+/-) + TSA] compared to controls [CREB (+/-) + Vehicle]; TSA had no significant effects on acetylated histone H3 (K-9) levels in wild-type (+/+) littermate mice [CREB (+/+)] treated with TSA [CREB (+/+) + TSA] compared to controls [CREB (+/+) + Vehicle]. **A:** Shows high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H3 (K-9) gold immunolabeling in CeA, MeA, and BLA of CREB deficient (+/-) mice treated with vehicle [CREB (+/-) + Vehicle] or TSA [CREB (+/-) + TSA] and wild-type (+/+) littermate mice treated with vehicle [CREB (+/+) + Vehicle] or TSA [CREB (+/+) + TSA]. **B:** Bar diagram demonstrates the quantified results of acetylated histone H3 (K-9) in the CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean ± SEM of 6-8 mice in each group. *P<0.001, significantly different from CREB (+/-) mice treated with vehicle [CREB (+/-) + Vehicle] [One-way analysis of variance (ANOVA) followed by post-hoc comparison by Tukey test].

c. <u>Acetylated Histone H4 (K-8)</u>

We reconfirmed that acetylated histone H4 (K-8) levels were significantly (p<0.001) lower in the amygdala (CeA, MeA, and BLA) of CREB deficient (+/-) mice [CREB (+/-) + Vehicle] compared to wild-type (+/+) littermate mice [CREB (+/+) + Vehicle] (Figure 29A & 29B). Figure 29A shows acetylated histone H4 (K-8)-positive staining of the amygdala, we found that TSA treatment was able to significantly (p<0.001) increase acetylated histone H4 (K-8) levels in the CeA, MeA, and BLA of CREB deficient (+/-) mice [CREB (+/-) + TSA] to levels comparable to that of wild-type (+/+) littermate mice [CREB (+/+) + Vehicle] (Fig. 29B). We also found that TSA had no significant effects on acetylated histone H4 (K-8) levels in wild-type (+/+) littermate mice [CREB (+/+) + TSA] (Figs. 29A & 29B). These data indicate that acetylated histone H4 (K-8) levels increase in the CeA, MeA, and BLA of CREB deficient (+/-) mice [CREB (+/-) + TSA] after treatment with TSA. In addition, these data also show that TSA treatment does not affect acetylated histone H4 (K-8) levels in the CeA, MeA, or BLA of CREB deficient (+/+) mice [CREB (+/+) + TSA].
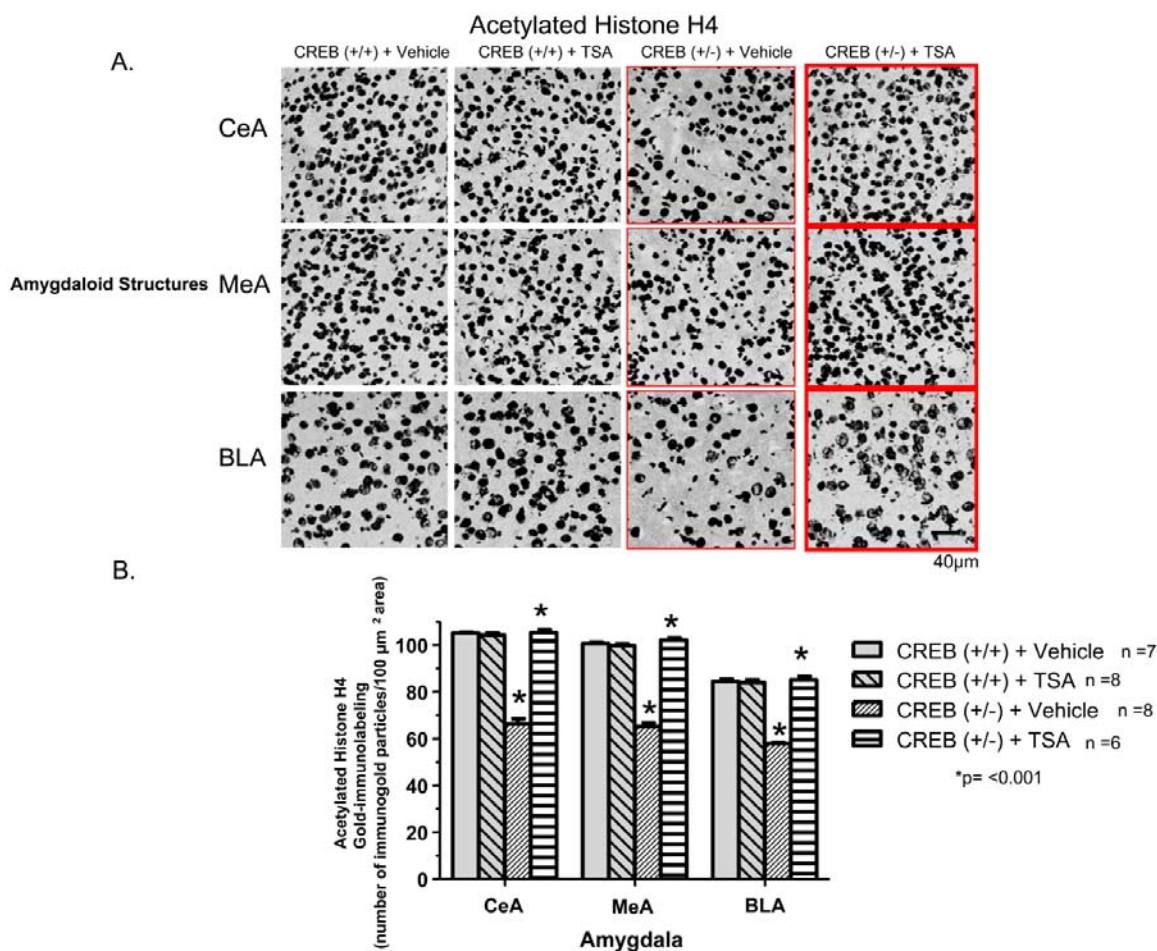
Figure 29. **Effects of HDAC inhibitor treatment on acetylated histone H4 (K-8) levels in amygdala of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.** TSA was able to significantly increase acetylated histone H4 (K-8) levels in amygdaloid structures [central nucleus of amygdala (CeA); medial nucleus of amygdala (MeA); and basolateral nucleus of amygdala (BLA)] of CREB deficient mice [CREB (+/-)] treated with TSA [CREB (+/-) + TSA] compared to controls [CREB (+/-) + Vehicle]; TSA had no significant effects on acetylated histone H4 (K-8) levels in wild-type (+/+) littermate mice [CREB (+/+)] treated with TSA [CREB (+/+) + TSA] compared to controls [CREB (+/+) + Vehicle]. **A:** Shows high magnification (scale bar, 40 μm) photomicrographs of acetylated histone H4 (K-8) gold immunolabeling in CeA, MeA, and BLA of CREB deficient (+/-) mice treated with vehicle [CREB (+/-) + Vehicle] or TSA [CREB (+/-) + TSA] and wild-type (+/+) littermate mice treated with vehicle [CREB (+/+) + Vehicle] or TSA [CREB (+/+) + TSA]. **B:** Bar diagram demonstrates the quantified results of acetylated histone H4 (K-8) in the CeA, MeA, and BLA in terms of number of immunogold particles/100 μm^2 area. Values are mean $\pm$ SEM of 6-8 mice in each group. *P<0.001, significantly different from CREB (+/-) mice treated with vehicle [CREB (+/-) + Vehicle] [One-way analysis of variance (ANOVA) followed by post-hoc comparison by Tukey test].

7. **Effects of HDAC Inhibitor Treatment on Alcohol Drinking**
   Behaviors of CREB Deficient (+/-) Mice compared to Wild-Type
   (+/+) Littermate Mice

Figure 30 shows three graphs containing drinking data of CREB deficient (+/-) mice and wild-type (+/+) littermate mice and the effects of HDAC inhibition by TSA treatment on drinking. CREB deficient (+/-) mice demonstrated a high preference for ethanol intake compared to wild-type (+/+) littermate mice (Fig. 30B). We found that TSA treatment was able to significantly decrease the levels of ethanol intake in both CREB deficient (+/-) mice and wild-type (+/+) littermate mice (Fig 30B); while at the same time increase water intake in both CREB deficient (+/-) mice and wild-type (+/+) littermate mice (Fig. 30A). Figure 30C shows the total fluid intake (water + ethanol) of all groups of mice [CREB (+/+) + Vehicle; CREB (+/+) + TSA; CREB (+/-) + Vehicle; and CREB (+/-) + TSA]. CREB deficient (+/+) mice may have developed a preference for ethanol due to chronic ethanol exposure over the duration of the experiment, which is a criterion of the two-bottle free-choice paradigm. The observed effects of TSA on alcohol drinking in wild-type (+/+) littermate mice may be related to compensatory changes in HDAC in the amygdala due to ethanol drinking, as previously reported by us in rats (Pandey, Ugale et al. 2008). These data suggest the possibility that decreased CREB functioning in the amygdala and associated abnormal amygdaloid chromatin remodeling may be operative in the increased alcohol drinking behaviors of CREB deficient (+/-) mice, and that these deficiencies can be rescued by TSA treatment.
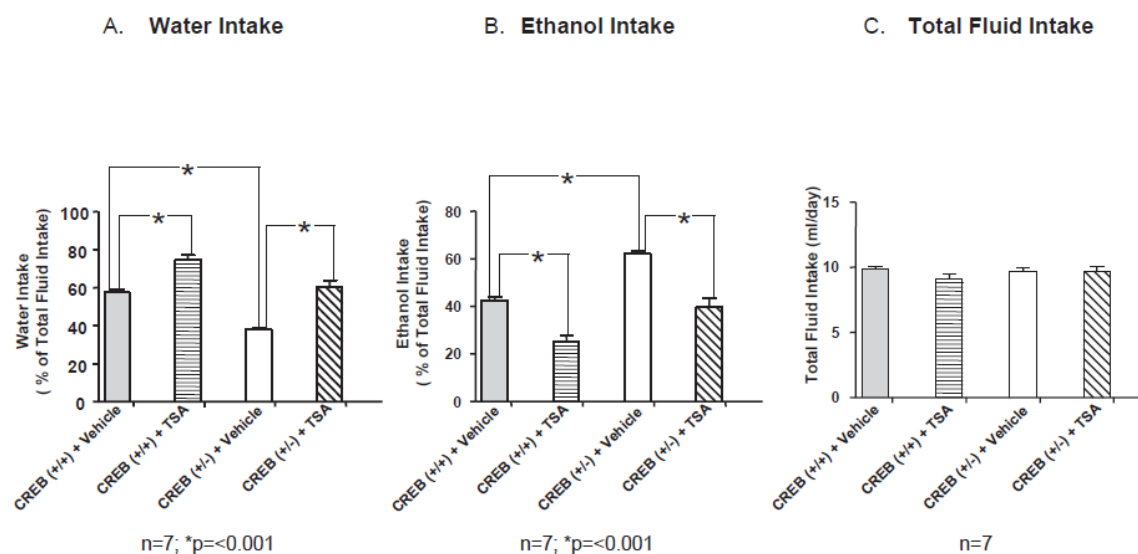
Figure 30. **Effects of HDAC inhibitor treatment on alcohol drinking behaviors of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice.** CREB deficient [CREB (+/-)] mice demonstrate a higher preference for ethanol intake compared to wild-type littermate mice [CREB (+/+)]; TSA treatment (2 mg/kg) was able to decrease ethanol preference in both CREB deficient (+/-) mice and wild-type (+/+) littermate mice; total fluid intake was similar between all groups. Bar diagrams represent 'Water Intake' (in terms of % of total fluid intake), 'Ethanol Intake' (in terms of % of total fluid intake), and 'Total Fluid Intake' (in terms of ml/day) of CREB deficient (+/-) mice treated with vehicle [CREB (+/-) + Vehicle] or TSA [CREB (+/-) + TSA] and wild-type (+/+) littermate mice treated with vehicle [CREB (+/+) + Vehicle] or TSA [CREB (+/+) + TSA]. Values are mean ± SEM of 7 mice in each group. **A:** *P<0.001, results significantly different for Water Intake: CREB (+/+) + TSA compared to CREB (+/+) + Vehicle; and CREB (+/-) + TSA compared to CREB (+/-) + Vehicle. **B:** *P<0.001, results significantly different for Ethanol Intake: CREB (+/+) + TSA compared to CREB (+/+) + Vehicle; CREB (+/-) + Vehicle compared to CREB (+/+) + Vehicle; and CREB (+/-) + TSA compared to CREB (+/-) + Vehicle [One-way analysis of variance (ANOVA followed by post-hoc comparison by Tukey Test)].

E. **Discussion**

The novel findings of Specific Aim 3 show that CREB regulates anxiety-like and alcohol drinking behaviors via modulation of the chromatin architecture within amygdaloid structures. As we reported in a previous study, the partial deletion of the CREB gene resulted in decreased levels of p-CREB in the amygdala, which was associated with high anxiety-like behaviors and increased preference for alcohol in CREB deficient (+/-) mice compared to wild-type (+/+) littermates (Pandey et al., 2004). These previous studies were performed in mice with a mixed genetic background of C57BL/6J mice and 129 SVJ mice. We extended these initial experiments to determine a possible role for CREB in the regulation of chromatin remodeling. CREB can initiate the recruitment of CBP, which contains HAT activity and targets both histones H3 and H4 (Breslin et al., 2007). Therefore, we assessed amygdaloid levels of CBP and acetylated histones H3 (K-9) and H4 (K-8) and found that CREB deficient (+/-) mice also expressed lower levels of CBP, acetylated histone H3 (K-9), and acetylated histone H4 (K-8) in the CeA, MeA, and BLA. These results suggest that decreased CREB functioning may be an important factor responsible for abnormal chromatin remodeling in the amygdala. In addition, the data presented in this study, combined with the results of our earlier studies, may provide evidence of an important link between CREB-related chromatin remodeling in the amygdala and the co-morbidity of anxiety and alcoholism.

Amygdaloid brain structures, particularly the CeA, have been implicated in anxiety and the self-medicating aspects of alcohol drinking behaviors (Pandey,

2003; Koob, 2009; Silberman et al., 2009). In addition, CREB-dependent neurotransmission in the extended amygdala has been linked with both high anxiety-like and excessive alcohol drinking behaviors (Davis et al., 1994; Pandey et al., 2003; Wand, 2005). Deficits in CREB signaling play an important role in a genetic predisposition to anxiety and alcoholism (Moonat et al., 2010). Recently, abnormal chromatin remodeling in the amygdala has been implicated in both anxiety and alcohol addiction (Pandey et al., 2008a). However, it is not clear what role CREB may play in the regulation of epigenetic modifications related to the onset of anxiety and alcoholism.

Since knockout mice of all forms of CREB are lethal in mice (Rudolph et al., 1998), we used mice lacking one allele of CREB (CREB α and Δ isoforms) in these studies. Our data show that both p-CREB and CBP levels were significantly lowered in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. Partial deletion of CREB may lead to decreased p-CREB levels, which in turn may precede the decreased recruitment of CBP to CREB, disrupting formation of the CREB/CBP complex, which is a necessary step during the formation of the apparatus required for the initiation of transcription. CBP, along with its p300 homologue, was first described as a coactivator of CREB (Mayr et al., 2001). When intracellular cyclic AMP increases, protein kinase A (PKA) translocates to the nucleus, where it can phosphorylate CREB, leading to its activation and binding to CREs (Mayr and Montminy, 2001). CBP is a large protein (~250 kD) with a bromodomain that has been shown to bind PKA-phosphorylated CREB (Chrivia et al., 1993). CBP can

then, in turn, initiate transcriptional activity at a CRE-containing promoter through the acetylation of the nucleosomal core (Ogryzyko et al., 1996).

The CREB signaling pathway has been implicated in synaptic plasticity associated with long-term memory (Bartsch et al., 1998). Loss of CBP functioning has been associated with many neurological disorders (Langley et al., 2005; Anne-Laurence et al., 2007; Kazantsev and Thompson, 2008). Knocked-down HAT activity of CBP was shown to impair long-term memory (Dekker and Haisma, 2009). In addition, heterozygous mutations in CBP are a known cause of Rubinstein-Taybi syndrome (RSTS), an autosomal dominant disease characterized by mental retardation, facial abnormalities, congenital heart defects, and increased risk of tumor formation (Tommerup et al., 1992; Petrij et al., 1995). An in vitro functional analysis study by Murata et al., demonstrated that CBP missense mutations that cause RSTS, result in abolished CBP-HAT activity (Murata et al., 2001). Another interesting study revealed that mice containing a haploinsufficiency for CBP, have impaired learning and memory, altered synaptic plasticity, and abnormal chromatin acetylation (Alarcon et al., 2004). Taken together, these data support the findings that decreased HAT activity of CBP may be a key contributor to the development of impaired neurological functioning. In addition, the chromatin-altering actions of CBP in the amygdala represent an attractive mechanism by which ensuing long-term epigenetic modifications may affect both neuronal and behavioral plasticity (Bannister and Kouzarides, 1996; Ogryzyko et al., 1996; Johannessen et al., 2004; McPherson and Lawrence, 2007; Moonat et al., 2010).

CBP is an important chromatin remodeling protein that acetylates both H3 and H4 histones (Schlitz et al., 1999; Korzus et al., 2004). In addition to reduced levels of p-CREB and CBP, our data also revealed that acetylated histones H3 (K-9) and H4 (K-8) were also significantly lowered in the CeA, MeA, and BLA of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. Several studies have outlined the relevance of a decrease in chromatin acetylation status associated with was loss of CBP function in the context of neurodegenerative diseases (Rouaux et al., 2004; Anne-Laurence et al., 2007). Acetylation of histones allows the chromatin to exist in a structure that is easily accessible to transcriptional factors, thereby increasing gene expression levels in the cell (Gorisch et al., 2005). Loss of chromatin acetylation may be deleterious to the maintenance of a homeostatic state in the cell, which can contribute to disease processes in the brain.

In addition to analyzing baseline differences in p-CREB, CBP, and acetylated histone H3 (K-9) and H4 (K-8) in amygdaloid structures, we also examined baseline levels in the NAc shell and core of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. We found that levels of p-CREB were lower in the NAc shell and core of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. Not only do these results reconfirm the genetic haplodeficiency of the CREB deficient (+/-) mice, they are also consistent with findings from a previous study done in our lab on p-CREB levels in CREB (+/-) deficient mice (Pandey et al., 2004). Interestingly, levels of CBP and acetylated histones H3 (K-9) and H4 (K-8) were not significantly different between CREB

deficient (+/-) mice compared to wild-type (+/+) littermate mice. The reasons for this are currently unknown, however, it is possible that these results arose due to a compensatory increase in CBP and histone acetylation in the NAc of CREB deficient (+/-) mice. The CREB deficient (+/-) mice were generated on a pure C57 background and from the results obtained in Specific Aim 1, we observed that C57 mice have innately lower levels of CBP and histone H3 acetylation in the NAc shell compared with DBA mice.

Histone acetylation is a reversible process, generally carried out by HDACs that work harmoniously together with HATs to either remove or add acetyl groups from N-terminal amino acid residues, respectively (Roth et al., 2001; Grozinger and Schreiber, 2002). HDAC inhibitors have recently emerged as new treatment agents in the treatment of psychiatric disorders. Many other studies have shown how HDAC inhibition can induce neuroprotective effects by restoring histone acetylation (Langley et al., 2005). In addition, HDAC inhibitors as therapeutic agents are currently a prominent theme in the treatment of neurological disease (Gottesfeld and Pandolfo, 2009; McCullough and Grant, 2010). We found that treatment with the HDAC inhibitor, TSA, was able to reinstate expression levels of CBP and acetylated histone proteins H3 (K-9) and H4 (K-8) in the amygdala of CREB deficient (+/+) mice compared to wild-type (+/+) littermate mice. These results are consistent with findings from previous studies that suggest a mechanism by which HDAC inhibition can compensate for decreased CBP-HAT activity and deficits in histone acetylation (Vecsey et al., 2007). HDAC inhibition by TSA significantly decreased anxiety-like and alcohol

drinking behaviors of CREB deficient (+/-) mice. In addition, TSA treatment

significantly decreased alcohol intake in wild-type (+/+) littermate mice. These

results are similar to other studies that have shown how chromatin modifications

by HDAC inhibition prevented behaviors associated with psychiatric disorders

such as anxiety, depression, and alcohol and drug abuse (Kumar et al., 2005;

Pandey et al., 2008a; Romieu et al., 2008; Shukla et al., 2008; Gundersen and

Blendy, 2009; Sanchis-Segura et al., 2009; Malvaez et al., 2010). Acetylated

histone H3 levels were normalized and learning and memory deficits were

restored in CBP-deficient mice treated with a HDAC inhibitor (Korzus et al.,

2004). Synaptic plasticity defects, as well as learning and memory deficits of

CBP haplodeficient mice, have been shown to be reversed by HDAC inhibitors

(Alarcon et al., 2004).   Recent studies in our lab have demonstrated how the

HDAC inhibitor TSA rescued deficits in H3 (K-9) and H4 (K-8) acetylation levels

in the amygdala, in addition to preventing both the development of alcohol-

withdrawal-related anxiety and the expression of alcohol tolerance (Pandey et al.,

2008a; Sakharkar et al., 2011). Taken together, these data highlight the

importance of CREB functioning in maintaining the integrity of the chromatin

structure and its role in the co-morbidity of anxiety and alcoholism.

Interestingly, treatment with the same dose of TSA (2 mg/kg) had no

significant effects on protein expression levels of CBP, acetylated histones H3

(K-9) or H4 (K-8) in the amygdala of wild-type (+/+) littermate mice. It may be

possible that TSA only exerts its HDAC inhibitory effects in brain regions

containing dysregulated nucleosomes. Another intriguing finding of our study

showed that over time, wild-type (+/+) littermate mice also developed a preference for ethanol intake. Wild-type (+/+) littermate mice may also display a propensity for a genetic predisposition to alcohol drinking behaviors due to their pure C57 genetic background. It is well established in the alcohol field that C57 mice innately consume higher volumes of ethanol compared to DBA mice (Gentry, 1985; Li et al., 1993). In addition, chronic ethanol treatment during the two-bottle free-choice paradigm may induce brain chromatin remodeling due to changes in HDAC activity within the amygdala, as reported by us (Pandey et al., 2008a). However, further epigenetic studies are needed to determine the effects of chronic ethanol exposure in the brains of wild-type (+/+) littermate mice.

From the innovative results of this study we were able to outline the effects of decreased CREB gene expression on chromatin remodeling in the amygdala and how aberrant regulation of CBP and histone acetylation may be associated with alcoholism and anxiety. Moreover, increased acetylation of histones resulting from HDAC inhibition prevented alcohol drinking behaviors and ameliorated anxiety in CREB deficient (+/-) mice. In conclusion, these data pinpoint the importance of pharmacological therapies that target HDACs and their ability to allay some of the pathology associated with anxiety and alcoholism.

F. <u>Significance</u>

We found that CREB deficient (+/-) mice have lower levels of p-CREB, CBP, and acetylated histones H3 (K-9) and H4 (K-8) in the CeA, MeA, and BLA compared to wild-type (+/+) littermate mice. We also found that treatment with TSA corrected the deficits in histone acetylation in CREB deficient (+/-) mice, while there were no observable effects of TSA in wild-type (+/+) littermate mice. In addition, treatment with TSA was also able to attenuate anxiety-like and alcohol drinking behaviors of CREB deficient (+/-) mice. The results of this study suggest that partial deletion of the CREB gene produced aberrant chromatin remodeling in the amygdala, which may be operative in high anxiety-like and alcohol drinking behaviors. In addition, our findings reinforce a role for the HDAC inhibitor, TSA, in the treatment of alcohol abuse disorders comorbid with anxiety.

V. CONCLUSIONS AND FUTURE DIRECTIONS

A. <u>Conclusions</u>

We addressed the hypothesis that C57 mice contain aberrant chromatin remodeling in the NAc compared to DBA mice, and that CREB plays an important role in regulation of nucleosomal chromatin remodeling of histone proteins in the NAc, which may be responsible an innate preference for alcohol consumption by C57 mice compared to DBA mice. We measured baseline levels of CBP, acetylated histones H3 (K-9) and H4 (K-8), and dimethylated histone H3 (K-9) in the NAc and amygdala using the gold-immunolabeling histochemical procedure. We selected these neuroanatomical brain regions because of their important roles in the regulation of emotion (amygdala-anxiety) and reward (NAc). We found that C57 mice expressed innately lower levels of CBP and acetylated histone H3 (K-9) and innately higher levels of methylated histone H3 (K-9) in the shell, but not core of the NAc or amygdaloid structures (CeA, MeA, or BLA) compared to DBA mice. Our findings suggest that aberrant chromatin remodeling in the shell of the NAc of C57 mice compared to DBA mice may be due to innately lower levels of CBP, which may contribute to lower acetylated histone H3 (K-9) levels. These observations are consistent with other studies that have shown decreased histone acetylation due to decreased CBP recruitment (Levine et al., 2005). Innately higher levels of methylated histone H3 (K-9) levels may also contribute to the innate preference for alcohol demonstrated by C57 mice compared to DBA mice. However, further experiments are necessary to determine the enzymatic mechanisms by which

these innate differences may occur. We stained cells for neuron-specific marker, NeuN, and found that the number of NeuN-positive neurons was similar in the shell and core of the NAc between C57 and DBA mice, suggesting that the observed changes in chromatin remodeling did not result from innate differences in the number of NeuN-positive neurons between C57 and DBA mice. In addition, we also determined that acetylated histone H3 (K-9) mainly colocalized with NeuN in the NAc shell of both C57 and DBA mice, suggesting that these epigenetic mechanisms occur mainly in neurons. These findings correlate with our previous findings that C57 mice innately express lower levels of CREB, p-CREB, and NPY in the shell of the NAc but not core of the NAc or amygdala (Misra and Pandey, 2003). These findings, in conjunction with our current findings, begin to provide a general understanding of covalent histone modifications in the NAc and amygdala of C57 mice compared to DBA mice.

The NAc has been implicated in reward, drug self-administration, and the reinforcing properties of alcohol and other drugs of abuse (Rassnick et al., 1992; Spanagel and Weiss, 1999; Olive et al., 2001). Since we found that C57 mice have innately aberrant chromatin remodeling in the shell of the NAc, we determined the effects of voluntary ethanol exposure on epigenetically relevant protein expression levels in C57 mice compared to control mice. It is well established in the field that C57 mice innately prefer to consume high amounts of ethanol compared to other mice strains (Gentry, 1985; Le et al., 1994; Rhodes et al., 2005). However, the epigenetic mechanisms involved in regulating the alcohol drinking behaviors of C57 mice are not well understood. The mesolimbic

DA pathway is activated by ethanol and has been shown to play a critical role in the rewarding aspects of ethanol and other drugs of abuse (Koob, 1992; Ortiz et al., 1995; Herz, 1997; Nestler, 2005). Excitation of DA neurons in the VTA promotes the release of dopamine in the NAc (Imperato and Di Chiara, 1986; Yoshimoto et al., 1991; Weiss et al., 1993; Brodie et al., 1999; Boileau et al., 2003). Ethanol has been shown to cause a surge of dopamine in the shell of the NAc in alcohol-preferring rats compared to alcohol-avoiding rats (Bustamante et al., 2008). The shell of the NAc is important in regulating the rewarding properties of ethanol, thus, we deemed it important to analyze the effects of ethanol exposure on epigenetic mechanisms in this particular brain region of C57 mice voluntarily exposed to ethanol (Ikemoto et al., 1997; Rodd-Henricks et al., 2002; Misra and Pandey, 2006; Engleman et al., 2009). We found that voluntary ethanol exposure had significantly increased levels of CBP and acetylated histone H3 (K-9) in the shell, but not core of the NAc or amygdala (CeA, MeA, or BLA) of C57 mice compared to control mice. Our findings here correlate with other findings that have shown how ethanol increased histone H3 acetylation in rat amygdala and hepatocytes (Park et al., 2003; Pandey et al., 2008a; Sakharkar et al., 2011). Changes in the acetylation states of histones by CBP or HDAC activity has been linked with the processes of learning and memory (Alarcon et al., 2004; Wang et al., 2010a). It may be possible that ethanol-induced increases in CBP and histone H3 (K-9) acetylation may result in a more open and accessible chromatin structure, thereby increasing gene expression levels, which may satiate innate alcohol-related craving behaviors of C57 mice.

Acetylation and methylation of histone H3 at the K-9 residue are interchangeable and reversible (Strahl and Allis, 2000; Nakayama et al., 2001; Nicolas et al., 2003; Thatcher and LaSalle, 2006). In contrast to acetylated histone H3 K-9 levels in the shell of the NAc of C57 mice, voluntary ethanol exposure was found to significantly decrease levels of methylated histone H3 K-9 in the shell, but not core of the NAc. Levels of histone H3 K-9 methylation have been shown to be affected by HDAC inhibition, suggesting the possible coupling of deacetylation with methyl group transfers at the histone H3 K-9 residue (Gupta et al., 2010). Interestingly, voluntary ethanol exposure also decreased levels of methylated histone H3 K-9 in the amygdala of C57 mice compared to controls. The amygdala is associated with the motivational characteristics of drug and alcohol abuse. Regulation of the chromatin structure in the amygdala of rats has been shown to be involved in the process of alcohol tolerance (Pandey et al., 2008a; Sakharkar et al., 2011). These data, together with our observation of decreased levels of methylated histone H3 K-9 in the shell of the NAc and amygdala of C57 mice, suggest a possible mechanism for ethanol-induced epigenetic neuroadaptations that can reverse the innately higher levels of methylation in the NAc shell of C57 mice.

Our voluntary ethanol exposure studies results provide evidence that long-term ethanol exposure can alter CBP and acetylation and methylation in the NAc shell of C57 mice compared with control mice. We also determined the effects of acute ethanol exposure on both C57 mice and DBA mice. Acute ethanol exposure significantly increased levels of CBP, acetylated histone H3 K-9, and

BDNF in the shell, but not core of the NAc or amygdala (CeA, MeA, and BLA) of C57 mice compared to controls. These findings correlate with other findings that showed how ethanol treatment significantly increased histone H3 acetylation at K-9 in hepatocytes, which was correlated with either increased expression of or increased activation of HATs (Park et al., 2005). In addition, ethanol exposure has been shown to induce global hepatic protein hyperacetylation (Shepard et al., 2010). These results, along with our current findings, suggest that acute ethanol exposure may increase histone H3 acetylation levels in the shell of the NAc of C57 mice via a CBP-HAT mediated epigenetic mechanism.

Acute ethanol exposure increased BDNF levels in the shell, but not core of the NAc or amygdaloid structures (CeA, MeA, or BLA) in C57 mice compared to control mice. Interestingly, acute ethanol exposure increased levels of CBP, acetylated histone H3 K-9, and BDNF in both the shell and core of the NAc, but had no significant effects in the amygdala (CeA, MeA, or BLA) of DBA mice compared to controls. BDNF levels were increased in both the shell and core of the NAc, while no effects were observed in the amygdala (CeA, MeA, or BLA) of DBA mice compared to control mice. Expression of BDNF is regulated by CREB, so it is possible that the observed changes in CBP-related histone acetylation may be increasing gene expression levels of BDNF, thereby providing a good explanation for increased BDNF protein levels (Tao et al., 1998). A recent study showed how CBP gene transfer increased BDNF levels and ameliorated learning and memory deficits in a mouse model for AD (Caccamo et al., 2010). BDNF-haplodeficient mice have also been shown to consume greater volumes of

ethanol after deprivation, compared to control mice (McGough et al., 2004). Our

results correlate with other studies that have shown how BDNF levels increased

after ethanol exposure (McGough et al., 2004). BDNF in the dorsal striatum was

activated by acute ethanol exposure in rodents, which correlated with decreased

sensitivity to ethanol-related behaviors (Jeanblanc et al., 2009). It has been

shown that histone acetylation in the promoter region of the BDNF gene can

regulate its BDNF expression in the hippocampus in a mouse model of

depression and antidepressant action (Tsankova et al., 2006). It is reasonable to

speculate that lower levels of BDNF in the NAc shell of C57 mice compared with

DBA mice may be related to innately lower H3 acetylation in the NAc shell and

ethanol may modulate BDNF expression by increasing H3 acetylation in NAc

structures of C57 and DBA mice. Taken together, these studies and our findings

suggest that BDNF plays an important role in the modulation of alcohol drinking

behaviors.

Acute ethanol exposure significantly decreased levels of methylated

histone H3 K-9 in the shell, but not core or amygdaloid structures (CeA, MeA, or

BLA) of C57 mice compared to controls. Acute ethanol exposure significantly

decreased levels of methylated histone H3 K-9 in both the shell and core of the

NAc, but had no significant effects in the amygdala (CeA, MeA, or BLA) of DBA

mice compared to controls. Decreased methylation in the NAc shell of C57 mice

exposed to ethanol may be related to decreased gene expression levels in this

brain region. Methylation of histone H3 at K-9 has been linked to downregulation

of gene expression by ethanol in hepatocytes (Pal-Bhadra et al., 2007). In

addition, histone H3 methylation at K-9 was shown to be regulated by acute and chronic stress (Hunter et al., 2009). Taken together, these findings suggest a crucial role for histone methylation in the NAc in the regulation of alcohol drinking behaviors of C57 and DBA mice.

Noticeable neuroanatomical differences were observed between C57 and DBA mice exposed to acute ethanol. Acute ethanol elicited effects in the shell of the NAc of C57 mice compared to control mice; in contrast, acute ethanol exposure targeted both the shell and core of the NAc in DBA mice compared to control mice. These neuroanatomical differences resulting from ethanol exposure in C57 and DBA mice may arise due to differential roles of the NAc shell and core in behavior and addiction (Di Chiara, 2002). Ethanol elicits its effects in the shell of the NAc of C57 mice, which correlate with findings that ethanol is self-administered into the shell, but not core of the NAc (Engleman et al., 2009). However, recently the core of the NAc has been suggested to be involved in impulsivity linked with reward processing (Chaudhri et al., 2010; Galtress and Kirkpatrick, 2010).

Alcohol modulates the balance between GABA, the primary inhibitory neurotransmitter in the brain, and glutamate, the major excitatory neurotransmitter (Weight et al., 1992). GABA-A receptors have been implicated in both the acute and chronic effects of ethanol including tolerance, dependence and withdrawal (Kumar et al., 2009). A recent study demonstrated how ethanol-induced locomotor sensitization, or the enhanced sensitivity to the acute locomotor stimulant actions of ethanol following repeated administrations, was

associated with changes in GABA-A gene expression levels and behavioral sensitivity to GABA-A acting drugs in the NAc but not VTA in DBA mice (Linsenbardt and Boehm, 2010). A recent study showed how the response of the mesolimbic dopamine system genetically correlated with sensitivity to ethanol- and cocaine-induced locomotion in the NAc (Meyer et al., 2009). Another recent study in mutant mice lacking normal production of the RIIβ subunit of PKA (RIIβ-/- mice) showed enhanced sensitivity to the locomotor stimulant effects of ethanol and increased behavioral sensitization, in addition to increased NPY immunoreactivity in the NAc core and ventral striatum, compared to wild-type littermate mice (Hayes et al., 2011). These studies along with our findings suggest the possibility that neuroanatomical differences in the NAc shell and core between C57 and DBA mice may be indicative of genetic differences in locomotor responses to ethanol in the mesolimbic dopamine system. Increased sensitivity to the stimulating effects of ethanol may serve as a greater risk factor for the development of alcohol abuse, therefore differences in locomotor sensitivity in the NAc between C57 and DBA mice may play a role in the observed differences in alcohol preference between these two strains of mice. Despite the observed acute ethanol-induced neuroanatomical differences in the shell and core of the NAc between C57 and DBA mice, both brain regions serve as important substrates upon which ethanol acts during the complex processes of alcoholism and addiction.

Acute ethanol exposure had no effect on the number of NeuN-positive neurons in either C57 or DBA mice, and the baseline number of NeuN-positive

neurons between C57 mice and DBA mice were similar, suggesting that acute

ethanol exposure did not produce any neuronal toxicity or neuronal damage in

the NAc. Both acute and voluntary ethanol exposure exhibited differential and

significant effects on chromatin remodeling in the NAc and amygdala of C57 and

DBA mice. Ethanol exposure was able to activate both the NAc and amygdaloid

neurocircuitries observed via epigenetic covalent modifications occurring in these

brain regions, suggesting the importance of chromatin remodeling in the NAc and

amygdala in the promotion and maintenance of alcohol drinking behaviors.

As reported earlier, partial deletion of the CREB gene resulted in

decreased levels of p-CREB in the amygdala, which was associated with high

anxiety-like behaviors and increased preference for alcohol of CREB deficient

(+/-) mice (containing a mixed genetic background of C57BL/6J mice and

126SVJ mice) compared to wild-type (+/+) littermate mice (Pandey et al., 2004).

We established a role for CREB in the regulation of chromatin remodeling using

a CREB deficient (+/-) mouse model compared to wild-type (+/+) littermate mice.

We measured levels of baseline levels of p-CREB, CBP and acetylated histones

H3 (K-9) and H4 (K-8) in the NAc and amygdala and found that CREB deficient

(+/-) mice expressed lower levels of p-CREB in the CeA, MeA, and BLA, in

addition to both the shell and core of the NAc compared to wild-type (+/+)

littermate mice, thereby reconfirming our previous findings from an earlier study

and also validating the genotype of CREB deficient (+/-) mice (Pandey et al.,

2004). We also found that levels of CBP and acetylated histones H3 (K-9) and

H4 (K-8) were significantly decreased in the CeA, MeA, and BLA of CREB

deficient (+/-) mice compared to wild-type (+/+) littermate mice. These findings

correlate with previous findings that discuss how CBP targets both histones H3

and H4 (Breslin et al., 2007). These results also suggest the possibility that

decreased CREB functioning may be an important factor responsible for

abnormal chromatin remodeling in the amygdala.

Amygdaloid brain structures have been implicated in anxiety and the self-

medicating aspects of alcohol drinking behaviors (Pandey, 2003; Koob, 2009;

Silberman et al., 2009). CREB signaling in the extended amygdala has also

been linked with high anxiety-like and excessive alcohol drinking behaviors

(Davis et al., 1994; Pandey et al., 2003; 2005b; Wand 2005). Abnormalities in

CREB-signal transduction may play an important role in a genetic predisposition

to anxiety and alcoholism (Moonat et al., 2010). Our findings may be related to

recent findings from our lab that showed how abnormal chromatin remodeling in

the amygdala was a critical factor in the development of anxiety-like behaviors of

rats during ethanol withdrawal after chronic ethanol exposure (Pandey et al.,

2008a).

CREB signaling has been linked with synaptic plasticity and long-term

memory (Bartsch et al., 1998). Activated CREB binds CBP and loss of CBP

functioning has been associated with many neurological disorders (Langley et al.,

2005; Anne-Laurence et al., 2007; Kazantsev and Thompson, 2008). CBP

haplodeficient mice have impaired learning and memory, altered synaptic

plasticity, and abnormal chromatin acetylation (Alarcon, Malleret et al. 2004).

These data, along with our findings here, suggest that deficits in CBP may lead

to impaired neurological functioning, especially in the amygdala, where these deficits can affect both neuronal and behavioral plasticity related to anxiety-like and alcohol drinking behaviors (Bannister and Kouzarides, 1996; Ogryzyko et al., 1996; Johannessen et al., 2004; McPherson and Lawrence, 2007; Moonat et al., 2010).

CBP acetylates histones H3 and H4 (Schlitz et al., 1999; Korzus et al., 2004). Our findings correlate with several other studies, which have shown how decreased acetylation was linked with loss of CBP functioning leading to a neurological disease state (Rouaux et al., 2004; Anne-Laurence et al., 2007). Histone acetylation is a readily reversible process, in that HDACs work in concert with HATs to respectively remove or add acetyl groups to histone proteins (Roth et al., 2001; Grozinger and Schreiber, 2002). HDAC inhibitors have recently emerged as promising therapeutic agents in treating neurological disorders (Anne-Laurence et al., 2007; Tsankova et al., 2007; Abel and Zukin, 2008). HDAC inhibition induces neuroprotective effects by restoring histone acetylation (Langley et al., 2005).

We found that treatment with the HDAC inhibitor, TSA, was able to restore levels of CBP and acetylated histone proteins H3 (K-9) and H4 (K-8) in the amygdala of CREB deficient (+/-) mice compared to wild-type (+/+) littermate mice. These results are consistent with findings from previous studies that suggest a mechanism by which HDAC inhibition can compensate for decreased CBP-HAT activity (Vecsey et al., 2007). HDAC inhibition by TSA also significantly decreased anxiety-like and alcohol drinking behaviors of both CREB

deficient (+/-) mice and wild-type (+/+) littermate mice. These results are similar

to other studies showing how HDAC inhibitor treatment can prevent behaviors

associated with psychiatric disorders such as anxiety, depression, and alcohol

and drug abuse (Kumar et al., 2005; Pandey et al., 2008a; Romieu et al., 2008;

Shukla et al., 2008; Gundersen and Blendy, 2009; Sanchis-Segura et al., 2009;

Malvaez et al., 2010). We recently demonstrated how TSA was able to rescue

deficits in H3 (K-9) and H4 (K-8) acetylation in the amygdala of rats, in addition to

preventing the development of alcohol-withdrawal-related anxiety (Pandey et al.,

2008a). An intriguing finding from our data revealed that TSA treatment in wild-

type (+/+) littermate mice had no significant effect on amygdaloid protein

expression levels of CBP, acetylated histones H3 (K-9) or H4 (K-8). These

findings suggest that TSA may only exert HDAC inhibitory effects in

dysfunctioning brain regions. However, further studies are necessary in order to

determine the epigenetic mechanisms by which TSA elicits its effects. Another

interesting finding of our study determined that levels of CBP and acetylated

histones H3 (K-9) and H4 (K-8) were similar in the NAc shell and core of CREB

deficient (+/-) mice compared to wild-type (+/+) littermate mice. In these studies,

we used mice lacking one allele of CREB, since CREB knockout mice

predominantly die at birth (Rudolph et al., 1998). The mice used in our studies

contained a pure C57 background. From our previous findings regarding C57

mice (see above Specific Aims 1 and 2), it is possible that NAc shell deficits may

have been compensated for resulting in normal chromatin architecture in the

NAc.

B. Future Directions

    1. Perform epigenetic studies focusing on methylation of histones and DNA and its role in the pathogenesis of alcoholism and addiction

Increasing evidence suggests that methylation of DNA is an important epigenetic mechanism emerging in the addiction field (Renthal and Nestler, 2008; Wong et al., 2010). A complex interaction between DNA methylation and histone modifications, such as deacetylation and methylation, work hand-in-hand to integrate gene-silencing networks within the cell (Bird, 2002; Fuks, 2005). DNA methylation is also an important epigenetic regulatory mechanism of gene expression that is often associated with gene silencing (Comb and Goodman, 1990). DNA methylation is more specific than histone methylation and is known to occur only at the 5-position of cytosines found at CpG dinucleotides within CpG islands (Okano et al., 1999; Bestor, 2000; Antequera, 2003). DNA methyltransferases (DNMTs) establish and maintain DNA methylation patterns and methyl-CpG binding proteins are involved in reading methylation marks (Robertson, 2005). DNMTs are abundantly expressed in postmitotic neurons and are important for normal learning and memory (Feng et al., 2010). A recent study by LaPlant et al., found that DNMT3a expression was upregulated in the NAc by chronic cocaine use and chronic social stress, suggesting an important role for DNMT3a in regulating emotional behavior and spine plasticity (LaPlant et al., 2010). Patterns of methylation are also altered in alcoholics. Lowered DNMT3b expression was associated with genomic DNA hypermethylation in

chronic alcoholics (Bonsch et al., 2006). While DNA methylation is a fascinating

subject, our studies on methylation focus mainly on histone methylation patterns

and not DNA methylation patterns; however, analysis of DNA methylation

patterns is also an important and emerging topic in the neuroscience of

alcoholism field of research. Since we found that histone methylation was

affected in our studies, it would be reasonable to assume that changes in DNA

methylation may also occur. Several recent studies have characterized the

methyl-DNA immunoprecipitation (MeDIP) technique to analyze DNA methylation

(Vucic et al., 2009; Palmke et al., 2011). We could use MeDIP to determine the

distribution of DNA methylation in functional promoter regions of the DNA.

    Methylation of histones is represented either as mono-, di-, or

trimethylated residues (Lachner and Jenuwein, 2002). We analyzed

dimethylated histone H3 K-9 in these studies. However, it would be interesting to

assay mono- and trimethylated histone H3 levels in the NAc and amygdala of our

mouse models. The levels of methylation correspond to different levels of

reversibility of these methylation states, in that trimethylated groups usually

correspond to heterochromatin and mono- and dimethylated groups correspond

to euchromatin (Nakayama et al., 2001; Dillon, 2004). A recent study found that

trimethylation of histone H3 at K-4, which represents an active mark for

transcription, was upregulated in the hippocampus 1 hour following contextual

fear conditioning (Gupta et al., 2010). We could link our findings on acetylation

with an analysis of DNA methylation, since DNA methylation and histone

acetylation work in concert to regulation memory formation and synaptic plasticity

(Miller et al., 2008). In addition to examining different levels of methylation, we could also look at different K residues. Distinct methylation patterns in histone H3 at K-4 and K-9 correlate with up and downregulation of genes by ethanol in hepatocytes (Pal-Bhadra et al., 2007).   It would be interesting to perform additional experiments on the methylation states of other K residues found on histones in relation to alcohol abuse disorders in the brains of our mice models.

2. **Perform chromatin immunoprecipitation (ChIP) experiments to determine which genes are involved in development of alcoholism and also to determine neuroanatomical targets for the treatment of alcoholism**

Another important focus of the neurobiology of alcoholism field is the determination of genes involved in the processes of alcoholism and addiction. The chromatin immunoprecipitation (ChIP) technique is a powerful procedure that allows researchers to probe specific protein-DNA interactions in vivo in a fast and simple way (Nelson et al., 2006). ChIP has proven useful in helping to better understand the molecular mechanisms of depression and antidepressant action. Several studies have utilized ChIP to analyze epigenetic mechanisms involved in neuropsychiatric disease (Colvis et al., 2005; Tsankova et al., 2006). ChIP may also prove very useful in determining candidate genes involved with alcohol abuse disorders.   Combining ChIP with sequencing (ChIP-seq) can identify genome-wide expression patterns that can provide more insight on epigenetics beyond the genome in alcoholism.

We could also perform our studies in female mice. Male and female C57 mice respond differently to diazepam (Podhorna et al., 2002). Elevated testosterone in female mice occurs during chronic ethanol withdrawal (Forquer et al., 2011). Another study in female mice indicated a fundamentally distinct neuroadaptive response by females compared to males during chronic ethanol withdrawal, suggesting an increased vulnerability of female alcoholics to ethanol-induced brain damage associated with alcohol abuse (Hashimoto and Wiren, 2008). In addition, male and female C57 and DBA mice have been shown to exhibit differing sensitivities to neurosteroid levels during acute ethanol withdrawal (Gorin-Meyer et al., 2007). It is possible that male and female C57 mice also differ in innate expression of chromatin remodeling proteins. Most of the literature contains studies using male mice models, however, it would be interesting to determine if differences exist between male and female mice models.

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