The Role of Amygdaloid Chromatin and Synaptic Remodeling

in Anxiety and Alcoholism

BY

SACHIN MOONAT
B.S., University of California at Berkeley, 2002
M.Sc., Charles University in Prague, 2005

THESIS
Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Chicago, 2014

Chicago, Illinois

Defense Committee:

Mark S. Brodie, Chair

Subhash C. Pandey, Advisor

Dennis R. Grayson

Mitchell F. Roitman

Marina Guizzetti, Psychiatry

Pradeep K. Dudeja, Medicine, Gastroenterology and Hepatology

ACKNOWLEDGEMENTS

I would like to begin by thanking my thesis committee, Dr. Mark Brodie, Dr. Pradeep Dudeja, Dr. Dennis Grayson, Dr. Marianna Guizzetti, Dr. Subhash C. Pandey and Dr. Mitchell Roitman, for their time and feedback throughout the process leading up to the defense of this thesis. Without the insight provided by each of these talented scientists, I would not have gained the knowledge and understanding I needed to develop this proposal and complete this thesis project. I would like to sincerely thank my research mentor, Dr. Subhash C. Pandey, for his support and guidance throughout the phases of my graduate student career. Dr. Pandey helped me discover my research interests and taught me to approach science with the highest standards. His devotion to my education continually pushed me to question my findings in context with emerging concepts requiring me to gain the analytical tools necessary for a career in research. On both a personal and professional level, I am truly fortunate to have had a mentor willing to spend time with me discussing where I want the next steps to take me.

I would like to acknowledge the faculty of the Graduate Program in Neuroscience, who have provided me with an invaluable education that has expanded my knowledge and understanding of neuroscience and biology as a whole. I would particularly like to mention Dr. James Unnerstall for his advice and support throughout my graduate education. I also express my gratitude to the Medical Scientist Training Program (MSTP) at UIC for giving me the opportunity to keep my mind shifting gears, allowing me to do research with one foot in the clinic.

I would also like to acknowledge the members of Dr. Pandey's Lab, Huaibo Zhang, Amul Sakharkar, Handojo Kusumo, Tara Teppen, Lei Tang, Gungbin Shi and Chang You, who helped me throughout my research project. In particular I would like to mention Dr. Zhang and Dr. Sakharkar who assisted me with data collection in the behavioral studies in this experiment and taught me the laboratory techniques that were used throughout the project. Also, I would like to thank Dr. Bela Starkman, my friend and ally in the lab. Thanks for watching my back, helping me with my science, and rounding my days out with time spent relaxing during breaks and after a stressful day of lab work.

I would like to thank my parents and family for their constant support and understanding during this endeavor. Particularly, I would like to thank my dad for instilling within me the importance of education. Finally, I would like to thank my wife, Meghan Murphy, who made sure that regardless of the amount of work I had to do and stress I experienced, I remained happy, enjoyed the journey and took time to pay attention to the important things in life. Thanks for taking care of me, always.
SM

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

CHAPTER PAGE

TABLE OF CONTENTS (continued)

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

LIST OF FIGURES (continued)

FIGURE PAGE

LIST OF ABBREVIATIONS

AB	Accessory basal nuclei of the amygdala
AC	Adenylyl cyclase
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
Arc	Activity-regulated cytoskeleton-associated (gene or protein)
AUD	Alcohol use disorder
BA	Basal amygdala
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of amygdala
CeI	Intermediate division of the central amygdala
CeL	Lateral division of the central amygdala
CeLC	Lateral capsular division of the central amygdala
CeM	Medial division of the central amygdala
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CaMKIV	Ca^{2+}/calmodulin-dependent protein kinase IV
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CRF	Corticotrophin-releasing factor
DAG	Diacylglycerol
DNMT	DNA methyltransferase
DSD	Dendritic spine density
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders Revision IV
EA	Extended amygdala
EPM	Elevated-plus maze
ERC	Entorhinal cortex
ERK1/2	Extracellular signal-regulated kinases 1/2
GABA	γ-aminobutyric acid
GPCR	G-protein coupled receptor
H3K9	H3 lysine 9
H3K9/14	H3 lysine 9 and 14
H4K8	H4 lysine 8
HAT	Histone acetyltransferase
HDAC	Histone deacetaylase
HMT	Histone methyltransferase
IEG	Immediate-early gene
IP_3	Inositol-1,4,5-triphosphate
ITC	Intercalated cell mass
LA	Lateral amygdala
lBNST	Lateral bed nucleus of the stria terminalis

LIST OF ABBREVIATIONS (continued)

LC	Locus coeruleus
LDB	Light-dark box
LH	Lateral hypothalamus
lPFC	Lateral prefrontal cortex
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MBD	Methyl-CpG binding domain
mBNST	Medial bed nucleus of the stria terminalis
MeA	Medial nucleus of amygdala
MeCP2	Methl-CpG binding protein 2
MeDIP	Methylated DNA immunoprecipitation
mGluR	Metabotropic glutamate receptors
mGN	Medial geniculate nucleus
mPFC	Medial prefrontal cortex
NAc	Nucleus accumbens
NMDA	*N*-methyl-D-aspartic acid receptor
NP	Alcohol-nonpreferring
NTS	Nucleus tractus solitarus
P	Alcohol-preferring
p-CREB	Phosphorylated cAMP-responsive element binding protein
PAG	Periaqueductal gray
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PI3K	Phosphatidyl inositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PSD-95	Post-synaptic density protein-95
PVN	Paraventricular nucleus of the hypothalamus
qPCR	Quantitative real-time polymerase chain reaction
SAHA	Suberoylanilide hydroxamic acid
siRNA	Small interfering RNA
TrkB	Tyrosine receptor kinase type B
TSA	Trichostatin A
VGCC	Voltage-gated calcium channel
VTA	Ventral tegmental area

SUMMARY

Alcoholism is a complex psychiatric disorder with severe social and health consequences that is driven by an underlying genetic basis and comorbid psychiatric disorders. Dysphoric symptoms, such as anxiety, appear to promote alcohol consumption due to the ability of alcohol to alleviate such symptoms, which may eventually lead to the development of alcohol dependence. Amygdaloid brain regions, in particular the central nucleus of amygdala (CeA), appear to play a key role in mediating the phenotypes associated with anxiety and alcoholism. The alcohol-preferring (P) and-nonpreferring (NP) rat lines have been selectively-bred for higher and lower alcohol preference, respectively. P rats also display innate anxiety-like behaviors in comparison to NP rats, thus providing a suitable animal model to investigate the comorbidity between anxiety and alcoholism.

In order to examine the relationship between anxiety and alcoholism, the effects of acute ethanol exposure on factors involved in synaptic plasticity were investigated in the amygdaloid brain regions of P and NP rats. Specifically, we examined the synaptically active proteins brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated (Arc) protein, and related changes in synaptic structure as measured by dendritic spine density (DSD). It was found that aberrantly low levels of BDNF, Arc and DSD in the CeA and medial nucleus of amygdala (MeA), but not basolateral amygdala (BLA), were associated with the anxiety-like behaviors observed in P rats. Acute ethanol exposure produced an anxiolytic effect, and resulted in increased levels of BDNF, Arc, and DSD in P rats, but not NP rats.

Recently, epigenetic mechanisms, including histone deacetylase (HDAC)-mediated chromatin remodeling, have been implicated in synaptic plasticity and psychiatric disorders, including alcoholism. Exploring the role of epigenetic mechanisms in the behavioral phenotypes of P rats, we found that in comparison to NP rats, P rats had innately higher HDAC isoform 2 (HDAC2) and lower histone acetylation levels in the CeA and MeA, but not BLA. The HDAC2-induced hypoacetylation profile of histones in the amygdaloid brain regions of P rats was found to be directly associated with regulation of *BDNF exon IV* and *Arc* genes as measured by chromatin immunoprecipitation (ChIP) assay. Exposure to voluntary and acute ethanol produced anxiolytic effects and decreased HDAC2 and increased histone acetylation in the CeA and MeA of P rats, but not NP rats. Acute ethanol exposure also increased acetylated histone H3 levels associated with the gene promoters of synaptically active genes, *BDNF* and *Arc*, suggesting a role for HDAC2-induced chromatin remodeling in the innate anxiety-like and alcohol-drinking behaviors of P rats.

To mechanistically investigate this finding, we employed siRNA-mediated knockdown of HDAC2 in the CeA of P rats and found that the reduction of HDAC2 expression resulted in anxiolytic effects associated with increased histone H3 acetylation. An increase in acetylated histone H3 levels was identified at the promoters of synaptically active genes, *BDNF exon IV* and *Arc,* which resulted in increased expression of BDNF and Arc protein, and produced an associated increase in DSD. HDAC2 siRNA infusion into CeA also reduced voluntary alcohol consumption in P rats, and this effect was sustained for several days.

Taken together, these results demonstrate for the first time that innately high expression of HDAC2 in the CeA associated with chromatin condensation plays a role in the epigenetic regulation of synaptic factors that are involved in the phenotype of anxiety and alcohol preference. Exogenous downregulation of HDAC2, as occurs during ethanol exposure, also dynamically regulates synaptic factors in the CeA and may be responsible for the anxiolytic effect of ethanol that contributes to the development of alcoholism. We anticipate that isoform-specific HDAC2 inhibitors could serve as useful therapeutic agents for the treatment of comorbid anxiety and alcohol-use disorders.

I. BACKGROUND AND REVIEW OF LITERATURE

A. Alcohol and Alcoholism

1. Historical perspectives

"…alcohol has existed longer than all human memory. It has outlived generations, nations, epochs and ages. It is a part of us, and that is fortunate indeed. For although alcohol will always be the master of some, for most of us it will continue to be the servant of man."
– Morris Chafetz, Founding Director of the NIAAA

Throughout recorded history, the use of alcohol has been prevalent in many cultures playing important roles in religious rituals, medicinal treatment, food preparation and preservation, and the encouragement of social interaction. Archeological evidence indicates that controlled fermentation of alcoholic beverages may date as far back as 10,000 B.C. (Hanson, 1995). It is believed that the production of alcohol took place in individual homes and that beverages containing alcohol were consumed on a daily basis. Writings in various cultures from antiquity to modernity have touted the beneficial aspects of alcohol use, from a perceived health benefit to an improvement in mood. Numerous civilizations, including the ancient Egyptians, Babylonians, Greeks and Chinese, seem to have respected alcohol as a "gift from the gods" that was a necessary component of certain religious events (Hanson, 1995). Despite this reverence of alcohol as beneficial and divine, the potential issues arising from inebriation were addressed in the writings of these cultures. Although few civilizations punished drunkenness, nearly all cultures discouraged excessive drinking and intoxication (Lutz, 1922). It is worth noting that due to social costs which arose from alcohol consumption, the Chinese instated and repealed laws prohibiting the use of alcohol forty-one times between 1,100 B.C. and 1,400 A.D. (Hanson, 1995).

Until the early 20th century, alcoholism was viewed as an untreatable flaw in an individual's character by most alcohol consuming cultures. Alcoholics were deemed amoral and weak, and were frequently the subject of legal prosecution or social persecution. In 1932, after the repeal of the 18th amendment which prohibited the use of alcohol in the United States, clinicians and social workers began to promote the treatment of severe alcohol use disorders. To this end, in 1935 Bill Wilson and Bob Smith founded Alcoholics Anonymous, a non-profit organization founded on the basis that alcoholism was an illness which was not under an individual's control and which was treatable (Huebner and Kantor, 2011). As the view of alcoholism as a legitimate psychiatric disorder developed, so did research into the mechanisms of alcohol and the psychopathology of alcoholism. Since the advent of the National Institute of Alcohol Abuse and Alcoholism in 1970, research in the field of alcoholism has flourished. In the following review of literature, I will focus on recent advances into the neuroscientific research underlying the effects of alcohol that help to understand the brain mechanisms by which consumption patterns may result in the development of alcoholism.

2. Clinical and epidemiological perspectives

Alcohol use disorders (AUD) have been categorized with other substance use disorders and, as in the case for other substances, separated into definitions for alcohol abuse and alcohol dependence by the Diagnostic and Statistical Manual of Mental Disorders Revision IV (DSM-IV). For the purposes of this dissertation, I will use the term alcoholism to represent both AUDs as well as alcohol consumption behaviors which may develop into defined pathologies. AUDs are characterized by the loss of control over alcohol consumption patterns resulting in excessive alcohol intake despite adverse social

or health consequences (American Psychiatric Association, 1994; Koob, 2003; Pandey, 2003; Pandey, 2004).

For a diagnosis of alcohol abuse, the DSM-IV requires the identification of an alcohol consumption pattern that results in clinically significant impairment in one or more of the following four criteria within a 12 month period: (1) recurrent alcohol consumption resulting in an inability to fulfill professional or social obligations; (2) consumption during situations that may be physically hazardous under the impairment of alcohol; (3) continued alcohol use despite legal consequences; (4) continued alcohol use despite social consequences as a result of or exacerbated by alcohol consumption (American Psychiatric Association, 1994; Andreasen and Black, 2006).

Defined as a disorder with higher severity than alcohol abuse, the DSM-IV has more stringent requirements for diagnosis of alcohol dependence that take into account the physical symptoms and signs of repetitive alcohol consumption. For a patient to be diagnosed with alcohol dependence, the patient must meet three of the following seven criteria in a 12 month period: (1) development of signs of tolerance as evidenced by (a) increased amounts of alcohol consumption to produce the desired effect, or (b) a markedly reduced effect despite continued consumption of the original amount; (2) development of signs of withdrawal as evidenced by (a) physical or psychiatric symptoms, such as seizure activity or anxiety respectively, precipitated by withdrawal from alcohol use or (b) use of alcohol or similar substances, such as benzodiazepines, to reduce or avoid withdrawal symptoms; (3) consumption of alcohol in greater amounts or longer periods of time than intended; (4) unsuccessful attempts or continued desire to reduce alcohol intake; (5) a substantial amount of time is spent in obtaining or using

alcohol, or recovering from the effects of alcohol consumption; (6) a reduction in important social, professional or recreational activities due to alcohol use; (7) continued consumption of alcohol despite knowledge of negative physical or psychological consequences related to alcohol use (American.Psychiatric.Association, 1994; Andreasen and Black, 2006). The diagnosis of alcohol dependence has been split into two subcategories defined by the presence or absence of physical signs of dependence, namely the development of withdrawal symptoms or alcohol tolerance (American.Psychiatric.Association, 1994; Andreasen and Black, 2006).

The clinical manifestations of AUDs include physical symptoms, behavioral changes attributable to excessive alcohol use or a preoccupation with obtaining alcohol and negative consequences to an individuals professional or social life (Andreasen and Black, 2006; Koob, 2003a). Recurrent consumption of alcohol has severe societal costs and widespread consequences on individuals, communities, and public health. Alcohol consumption ranks as the third leading risk factor for disease burden in industrialized nations and the fifth leading cause of death and disability worldwide (2004). In the United States, over 17.6 million people have been diagnosed with alcohol-use disorders and the societal costs of alcohol use exceed $184.6 billion annually (Grant et al., 2004a; Rehm et al., 2009).

The development of an AUD is frequently initially identified by family, friends, or colleagues who notice changes in an individual's behavioral patterns (Andreasen and Black, 2006). However, it may difficult to convince an alcoholic patient to seek medical or psychiatric treatment for the illness. Furthermore, alcoholics progressing towards alcohol dependence may alienate their social support network and lose control over their

compulsion for alcohol. Thus, the first clinical presentation of a patient may be related to admission to the emergency department or court mandate due to severe health or legal consequences related to driving under the influence, seizure activity due to severe alcohol withdrawal, alcohol poisoning, malnutrition or cognitive impairment (Andreasen and Black, 2006). The clinical course is further complicated by the fact that the majority of alcoholics relapse to alcohol use following treatment (Breese et al., 2011; Charney et al., 2009; Dawson et al., 2007). Studies indicate that the relapse rate may be as high as 70-90% depending on the use of behavioral or pharmacotherapeutic treatment and the presence of comorbid psychiatric disorders (Bradizza et al., 2006; Charney et al., 2009; Lowman et al., 1996).

Although alcoholism is a complex multifactorial disease, an important role has been clearly identified for genetics in the development of alcohol-use disorders by family studies and twin concordance rates (Cotton, 1979; Prescott and Kendler, 1999). Specifically, it has been shown that the risk of alcohol dependence is significantly higher in relatives of alcoholics, identical twins have a higher rate of concordance with regards to alcoholism than fraternal twins, and children of alcoholics adopted into low alcohol consuming families maintain a high risk for alcoholism (Cloninger, 1987; Cotton, 1979; Mayfield et al., 2008; Prescott and Kendler, 1999). Epidemiological data from various studies has estimated that the genetic heritability of alcoholism is between 40 and 60 percent, and that the risk of alcoholism is 4 to 10 times higher for offspring of alcoholics (Cloninger, 1987; Mayfield et al., 2008; Schuckit, 2000). Although genetic studies have identified some single genes that may regulate risk of alcoholism, such as the metabolic enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), it is

apparent that in most cases an individual's risk for alcoholism is dependent on a complex gene by environment interaction (Agarwal and Goedde, 1992; Crabbe et al., 2006; Spanagel, 2009). In order to emphasize the importance of environmental factors, it is worth putting forth the notion that without access and exposure to alcohol, an individual cannot become alcohol-dependent. Although this may seem trivial, it emphasizes the fact that despite the genetic risk an individual may possess, a particular environment may significantly decrease, or in many cases increase, the risk of alcoholism. Numerous studies have attempted to identify specific gene-environment interactions that have been found to impact the development of alcoholism, for example the age at first drink, exposure to stress, and family relationships (Agrawal et al., 2009; Clarke et al., 2008; Nilsson et al., 2011). The complexities underlying the gene-environment interactions suggest that mechanisms based on environmental exposure which can dynamically regulate gene expression, such as epigenetic mechanisms, may be involved in the phenotype of alcoholism (Crabbe, 2008; Hillemacher, 2011; Pandey et al., 2008a).

3. Modeling the development of alcoholism

Numerous psychiatric models have been developed to represent various aspects in the clinical course in the development of alcohol dependence (Koob, 2003a; Spanagel, 2009). The positive and negative affective states of alcoholism comprise a well-characterized behavioral and neuroanatomical model that describe the development of alcohol addicition (Koob, 2003; Moonat et al., 2010; Pandey, 2004). Reinforcement of alcohol consumption results from both the anticipation of positive effects of consumption, such as improved mood and relaxation, and the reduction of negative states, such as stress, pain, and anxiety, which may in turn result from alcohol withdrawal (Grusser et al., 2006;

Koob, 2003; Pandey, 2003). The euphoric experience following alcohol intake is associated with activation of the brain's reward circuitry, and may primarily dominate upon first exposure to ethanol (Koob, 2003; Koob et al., 1998). However, after continued alcohol intake and repetitive experience of alcohol withdrawal, the negative effects of alcohol withdrawal may take over resulting in the consumption of alcohol to self-medicate withdrawal-induced anxiety which serves to maintain excessive drinking behaviors (Koob and Le Moal, 2008; Kushner et al., 2000). These psychiatric states of alcoholism define two sides of a three-stage cycle that leads to the development of alcoholism, by promoting and maintaining alcohol consumption and eventually leading to alcohol dependence (Koob, 2003; Pandey, 2003; Pandey, 2004).

The positive affective state of alcoholism involves the mesolimbic dopaminergic system which plays a key role in regulating the experience of reward resulting in reinforcement of biologically necessary behaviors, such as food consumption and sex (Gonzales et al., 2004; Spanagel, 2009; Wise, 2004). The system consists of dopaminergic projections from the ventral tegmental area (VTA) of the midbrain to various brain regions within the limbic system, predominantly the shell of the nucleus accumbens (NAc) and prefrontal cortex (PFC), but also the extended amygdala (EA) and hippocampus (Gonzales et al., 2004; Haines, 2004; Spanagel, 2009). Dopamine release from the VTA neurons into the NAc appears to be a key mediator in the experience of reward and may result in motivated behavior to obtain further reward (Wise, 2004). In early experiments, impairment or lesion of dopaminergic function in the nigrostriatal pathway resulted in attenuated response to rewarding stimuli (Ervin et al., 1977; Wise and Schwartz, 1981; Wise et al., 1978). Further experiments utilized localized

pharmacological manipulation and intracranial self-stimulation to clearly demonstrate the tight association between dopaminergic release from VTA to NAc and behaviors reinforced by reward (Cheer et al., 2007; Fibiger et al., 1987; McBride et al., 1999; Vaccarino and Koob, 1984). The recent use of technology with more specific cellular localization and faster temporal resolution, such as optogenetic activation of dopaminergic neurons and *in vivo* fast-scan cyclic voltametry, have identified the importance of timing of phasic dopamine release in representations of reward and reinforcement of behavior (Adamantidis et al., 2011; Day et al., 2007; Owesson-White et al., 2012).

Although the dopaminergic system serves to reinforce rewards to mediate motivational aspects of normal behavior, dysregulation of the reward pathways by alcohol and other drugs of abuse appears to be a key factor in the processes leading to addiction (Koob, 2003a; Pandey, 2004; Spanagel, 2009; Wise, 2004). Early electrophysiological and microdialysis studies identified that alcohol exposure both *in vitro* and *in vivo* increased the firing rate of dopaminergic VTA neurons and resulted in the release of dopamine into the NAc (Brodie et al., 1990; Di Chiara and Imperato, 1988; Gessa et al., 1985; Weiss et al., 1993). Since these studies, various researchers have focused on the dopaminergic inputs and intracellular signaling mechanisms in the NAc. Early studies which produced chemically induced lesions of dopaminergic inputs to the NAc found a reduction in the acquisition of alcohol consumption, with no effect on maintenance of alcohol consumption behaviors (Ikemoto et al., 1997; Rassnick et al., 1993). Interestingly, this finding is in agreement with the theory that the positive affective state of alcoholism has greater significance in the initiation of alcohol

consumption, whereas the negative affective state may play a larger role in the maintenance of consumption (Koob, 2003a; Pandey, 2003; Pandey, 2004).

The negative affective state of alcohol addiction describes the development of anxiety, depression and other anhedonic psychiatric sequelae which may be caused by the cessation of chronic alcohol consumption or other factors, such as genetic predisposition (Koob, 2003a; Pandey, 2003; Pandey, 2004). The negative affective state induced by alcohol withdrawal is a robust factor in the maintenance of alcohol drinking behavior and eventual development of alcohol addiction (Pandey, 2003). The amygdala has long been implicated in fear, emotion and anxiety, and this has led to a great deal of research on the role of various amygdaloid brain regions in the negative affective state of alcohol (Davis et al., 2009; LeDoux, 2003; Pandey, 2003; Pandey, 2004). Not surprisingly, the dysphoric effects of alcohol withdrawal, particularly the promotion of anxiety-like behaviors, appear to be regulated by the EA, primarily due to the contributions of the central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), and bed nucleus of the stria terminalis (BNST) (Koob, 2003; Pandey et al., 2006; Pandey et al., 2005b; Prakash et al., 2008). Acute ethanol exposure has been shown to reduce anxiety-like behaviors in both animal models and humans, and this reduction in dysphoria may be mediated by several cell signaling pathways within the CeA (Bolton et al., 2009; Moonat et al., 2010; Pandey et al., 2006; Robinson et al., 2009). With chronic ethanol exposure, long-term changes in amygdaloid brain regions may result in the development of dysphoric psychiatric symptoms resulting from withdrawal (Moonat et al., 2010; Pandey, 2003; Pandey et al., 2006).

Epidemiological data have implicated a role for comorbid psychiatric disorders in the initiation and maintenance of alcohol-use disorders (Conway et al., 2006; Schuckit and Hesselbrock, 1994). Various studies have shown that alcohol-use disorders are commonly comorbid with innate anxiety-spectrum disorders (Grant et al., 2005; Grant et al., 2004b). Human studies and animal studies have implicated the anxiolytic effects of ethanol as a contributing factor in the consumption of ethanol, possibly through a self-medication effect on anxiety (Bolton et al., 2009; Castaneda et al., 1996; Pandey et al., 2005b; Zimmermann et al., 2003). Withdrawal from chronic alcohol use or exposure has been shown to result in anxiogenesis, and repetitive alcohol consumption and withdrawal patterns may result in the development of more severe withdrawal and anxiety symptoms (Becker, 1994; Breese et al., 2005; Duka et al., 2004; Koob, 2003; Pandey, 2003). Since studies have shown that innate anxiety can be as sensitive to the effects of alcohol intake as withdrawal-induced anxiety, the negative affective state of alcoholism appears to be an attractive neuroanatomical and behavioral framework by which a genetic predisposition to anxiety may play a role in the development of alcoholism. Common neurocircuitry in the amygdala may be responsible for the genetic predisposition to anxiety, the anxiolytic effect of ethanol and the neuroadaptations which lead to withdrawal-induced dysphoria, so it is important to identify molecular and epigenetic mechanisms which may be common to each of these phenomenon. This could lead to a greater understanding of each of the processes and may provide a therapeutic target for alcoholics that are plagued by the negative affective factors.

Various animal models have been identified to study the effects of alcohol and characteristics of alcoholism from numerous perspectives. The basic models that are

used can be separated into behavioral models, which aim to evaluate the effect of different patterns of ethanol exposure on behavioral phenotypes and alcohol consumption, and strain-based models, which use transgenic animals or selective breeding to determine innate differences in biology or behavior that may contribute to alcohol preference (Spanagel, 2000). Early discussions and critiques of the studies which had utilized animal models led to the development of criteria that would define a successful animal model for human alcoholism (Lester and Freed, 1973). The intention was to come up with a set of requirements that would emulate the definitions of human alcohol dependence. By this criteria, the characteristics that a successful animal model must display are: (1) Voluntary oral ingestion of ethanol without food deprivation and with the presence of competing fluids; (2) Consumption of ethanol yields pharmacologically relevant blood alcohol concentrations; (3) Voluntary administration of ethanol due to pharmacological effects rather than taste or caloric content; (4) Ethanol exposure should result in positive reinforcement and the performance of work to obtain ethanol access; (5) Sustained ethanol consumption results in signs of tolerance; (6) Chronic ethanol consumption results in withdrawal symptoms and signs of dependence; (7) Relapse behavior and reinstatement of alcohol dependence following prolonged abstinence (Bell et al., 2006; Lester and Freed, 1973).

The alcohol-preferring (P) and –nonpreferring (NP) rats produced and maintained by Indiana University are a well-characterized animal model that has been utilized to study the predisposition to alcoholism and alcohol preference (Spanagel, 2000). The P and NP rat lines were produced via selective breeding from an initially unselected stock of outbred Wistar rats from Walter Reed Army Institute of Research (Li et al., 1993).

The initial selection was performed by using a two-bottle free choice paradigm, and resulted in the identification of a single pair of mates with high and low alcohol preference drinking approximately 9 g/kg/day with and 1 g/kg/day, respectively (Li et al., 1993). For the production of the subsequent 15 generations, five stock rats were tested introduced into the breeding lineage using a selection criteria such that P rats consumed at least 5 g/kg/day at a ratio of at least 2:1 ethanol to water and NP rats consumed less than 1.5 g/kg/day at a ratio of at most 0.2:1 ethanol to water (Li et al., 1993). Continuing this strategy through the 30th generation yield rats that displayed stable drinking patterns, levels of genetic background indicating successful creation of an inbred rat line, and an inability to reverse the phenotype by selecting for low alcohol preference in P rats (Li et al., 1993).

Since the development of the P and NP rat lines, numerous studies have confirmed that P rats satisfy the criteria for a successful animal model of alcoholism and data can even be mapped to each the criteria for human alcohol dependence as defined by the DSM-IV (Bell et al., 2006). Interestingly, the selection for alcohol consumption patterns in P rats yielded a phenotype of anxiety-like behaviors in P rats that are absent in NP rats (Hwang et al., 2004; Pandey et al., 2005b; Stewart et al., 1993). This phenotype has been established and confirmed by several well-characterized tests of innate anxiety, including the elevated-plus maze (EPM) and the light-dark box (LDB), as well as training-based paradigms that show increased startle to conditioned stimuli in P rats than NP rats (Jones et al., 2000; McKinzie et al., 2000; Pandey et al., 2005b; Prakash et al., 2008; Stewart et al., 1993). Ethanol exposure reduced the anxiety-like behaviors in P rats, but not NP rats, suggesting a role for the anxiolytic effect of ethanol in the high

consumption patterns of P rats (Hwang et al., 2004; Jones et al., 2000; Pandey et al., 2005b; Stewart et al., 1993). Further studies identified that exposure to anxiolytic drugs, or stressors that increased anxiety-like behaviors, reduced or increased the alcohol consumption behaviors in P rats, respectively (Knapp et al., 2011; Overstreet et al., 2007; Rezvani et al., 1991; Rezvani et al., 2007). Taken together, these finding suggest an association between the anxiety-like behaviors and alcohol consumption patterns exhibited by P rats, suggesting that the reduction of dysphoria resulting from ethanol exposure may be a driving force in the high alcohol preference of P rats.

When coupled together, the positive and negative affective states of alcohol appear to provide a convincing psychiatric and neurobiological model which may underlie the promotion and maintenance of alcohol drinking and addiction. The possibility that innate anxiety may also drive the promotion of alcohol intake via the negative affective pathway suggests that common neuroanatomical substrates and molecular mechanisms may underlie the genetic predisposition to anxiety and alcoholism. Thus, study of the mechanisms within the amygdaloid brain regions in the P-NP animal model may provide useful findings regarding the comorbidity between anxiety and alcohol use disorders.

4. The pharmacology of alcohol: convergence on CREB

Unlike many drugs of abuse, identifying the direct site of action of ethanol has remained elusive for many years. Early electrophysiological studies identified that ethanol application modulated γ-aminobutyric acid (GABA) and *N*-methyl-D-aspartic acid (NMDA) receptor currents (Lovinger et al., 1989; Nestoros, 1980). Specifically, acute ethanol application was shown to potentiate GABA currents and inhibit NMDA currents

(Lovinger et al., 1989; Nestoros, 1980). Various groups hypothesized that the molecular action of ethanol was nonspecific and the effect of ethanol results from fluidization of the lipid bilayer or disruption of protein-lipid interactions (Johnson et al., 1980). More recently, however, various proteins have been identified as direct molecular targets for ethanol, including neurotransmitter receptors, ion channels and enzymes (Harris et al., 2008; Narahashi et al., 2001; Vengeliene et al., 2008). These recent findings indicate that the action of ethanol may result from specific pharmacologic interactions despite ethanol binding to a variety of protein targets. Interestingly, a number of the identified protein targets of ethanol also play a role in the regulation of cAMP-responsive element binding (CREB) protein and may mediate long-term changes associated with the positive and negative affective states of alcoholism. In this section, I will introduce a number of direct ethanol targets that could be involved in converging the effect of ethanol onto the regulation of CREB function.

NMDA Receptors

NMDA receptors were among the first identified targets of ethanol, as electrophysiological studies found that administration of ethanol resulted in inhibition of NMDA receptor-mediated currents in hippocampal cell cultures (Lovinger et al., 1989). NMDA receptor activation is also believed to be an important factor in various forms of synaptic plasticity via the induction of CREB (Alberini, 2009; Impey et al., 1999). Prolonged or repetitive activation of glutamatergic receptors has been shown to result in NMDA-mediated long term potentiation (LTP) through rapid modulation of mRNA and protein levels via activation of various signaling cascades and transcription factors, including CREB (Brown et al., 1988; Cole et al., 1989; Schulz et al., 1999). NMDA

receptors consist of a tetrameric cation channel which mediates the slow component of glutamatergic excitatory neurotransmission. In the CNS, NMDA receptors primarily exist as NR1/NR2 heteromers although the exact stoichiometry has not yet been elucidated. Four different isotypes of the NR2 subunit, NR2A-D, have been identified and the subunit composition of the NMDA receptor appears to affect function (Nagy et al., 2005; Paoletti and Neyton, 2007). NMDA receptors composed of NR1/NR2A and NR1/NR2B subunits are Mg^{2+}-sensitive, high Ca^{2+} conductance channels, thus these receptors may be particularly important in synaptic plasticity. Further, in comparison to NR2A-containing receptors, NR2B-containing receptors have a higher Ca^{2+} conductance and affinity for glutamate (Krupp et al., 1996). The role of NMDA receptors in the regulation of synaptic plasticity is underscored by the wide variety of downstream effectors of Ca^{2+} influx through NMDA receptors. Intracellular Ca^{2+} triggers a number of signaling cascades which converge on regulation of CREB function. Although complex interactions may occur between signaling pathways, increased Ca^{2+} can independently lead to the activation of CREB through Ca^{2+}/calmodulin-dependent kinase II (CaMKII), Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV), cAMP-protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) dependent signaling (Siegel, 2006). NMDA receptors regulate the organization of postsynaptic density proteins which may result in regulation of dendritic spine morphology, highlighting their importance in synaptic plasticity (O'Brien et al., 1998; Ultanir et al., 2007). Interestingly, recent studies have found a role for CREB-mediated transcription of a neuronal microRNA, miR132, that regulates morphological changes mediated by NMDA receptor interactions with

postsynaptic density proteins, possibly via the NR2B subunit (Nakazawa et al., 2008b; Wayman et al., 2008a).

Despite the early identification of ethanol modulation of NMDA receptor function, only recently have studies begun to identify a direct binding site for the action of ethanol. An early study found that increasing the carbon-length of aliphatic n-alcohols applied to hippocampal cultures resulted in a cutoff in the potency of inhibition of NMDA receptors, above which the inhibitory effect diminished (Peoples and Weight, 1995). This opposed the prevailing theory of lipid fluidization as a primary regulator of ethanol function and implied that a distinct alcohol binding pocket may exist in NMDA receptors. More recent studies utilizing site directed mutagenesis indicate that ethanol may occupy a volume in a transmembrane domain of the NMDA receptor and that binding may be regulated by specific amino acid residues (Ren et al., 2007; Ronald et al., 2001). The determination of the NMDA receptor as a direct target of ethanol has increased research into factors which may influence the sensitivity of the receptor to ethanol. For instance, recent studies have found that NR2B-containing receptors display increased sensitivity to ethanol and may provide a possible pharmacological target for the treatment of alcohol addiction (Nagy, 2004; Smothers et al., 2001). Notably, high affinity NR2B-containing receptors have high Ca^{2+} conductance channels and may be particularly important in synaptic plasticity (Paoletti and Neyton, 2007).

Modulation of NMDA receptor function and expression by acute and chronic ethanol exposure has been a topic of heavy research in the recent past. Typically, it has been shown that withdrawal from chronic ethanol shows a rebound effect from the acute inhibition of NMDA receptor-mediated currents. Early studies in cell cultures identified

that the prolonged exposure of ethanol resulted in an increase in NMDA-mediated Ca^{2+} currents and apparent NMDA-induced excitotoxicity (Chandler et al., 1993; Hu and Ticku, 1997; Smothers et al., 1997). Acute ethanol exposure in vitro has also been shown to reduce NMDA receptor-mediated currents in NAc and CeA (Nie et al., 1994; Roberto et al., 2004). Withdrawal from chronic ethanol resulted in a rebound increase in NMDA-receptor mediated currents in the CeA (Roberto et al., 2004). A follow up study found that increases in glutamate release and increases in mRNA and protein expression of NR1, NR2A and NR2B subunits correlated with the observed changes in receptor function suggesting both pre- and postsynaptic mechanisms for the functional modulation (Roberto et al., 2006). Increased NR2B subunit expression by prolonged ethanol exposure also resulted in enlargement of dendritic spines through an interaction between NMDA receptors and postsynaptic density protein-95 (PSD-95) (Carpenter-Hyland and Chandler, 2006). Taken together, these studies implicate a role for the upregulation of NMDA receptors, and particularly the NR2B subunit, in long-term changes in neuronal function and morphology associated with alcohol consumption.

Metabotropic glutamate receptors (mGluR)

The mGluR is a G-protein coupled receptor (GPCR) which responds to glutamate to produce a variety of effects depending on subtype. The mGluR family contains 8 subtypes, mGluR1-8, which are further subdivided into 3 groups based on pharmacological properties and second messenger action. Group I, mGluR1 and mGluR5, are primarily postsynaptic GPCRs coupled to $G\alpha_q$ which results in the activation of phospholipase C (PLC) and generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) which in turn result in protein kinase C (PKC) activation and an

increase in intracellular Ca^{2+}, respectively. Pre- and postsynaptic Group II, mGluR2 and mGluR3, and presynaptic group III, mGluR4 and mGluR6-8, act via $G\alpha_i$ to inhibit adenylyl cyclase (AC) (Kim et al., 2008a). Group I mGluRs are particularly important to this discussion because they serve a role in the regulation of CREB function. mGluR1 and mGluR5 have both been shown to activate the MAPK pathway through Ca^{2+} interaction with Ras protein (Wang et al., 2007). Although this is the primary CREB-activating pathway triggered by mGluR1 and mGluR5, it has also been shown that mGluR1 may also increase intracellular cAMP levels, activating PKA to result in CREB activation (Tateyama and Kubo, 2006). Previous evidence has shown that acute ethanol inhibits Ca^{2+} currents induced by mGluR5 via PKC-dependent phosphorylation (Minami et al., 1998; Netzeband and Gruol, 1995). This finding led to the study of the role of mGluR5 in the promotion of alcohol-drinking behaviors. By using a mGluR5 antagonist, 2-methyl-6-(phenylethyl)-pyridine (MPEP), various studies have indicated that reduction of mGluR5 activity results in a dose-dependent decrease of alcohol consumption in various animal models, including P rats and C57/BL6 mice (Hodge et al., 2006; McMillen et al., 2005; Olive et al., 2005; Schroeder et al., 2005). Further study showed that infusion of MPEP directly into the shell of the NAc also resulted in decreased ethanol consumption by a PKCε-dependent mechanism (Gass and Olive, 2009). These findings indicate an attractive model of ethanol action and possible pharmacotherapeutic target to explore, however a great deal more research is necessary to identify a direct binding site of ethanol or the regulator of PKC function which results in the observed effects.

Voltage-Gate Calcium Channels (VGCC)

VGCCs play an important role in the activity-dependent local increases in intracellular Ca^{2+} which may mediate synaptic plasticity. As described previously, increases in intracellular Ca^{2+} can couple to CREB activation by number signaling pathways. Seven VGCC subtypes exist and are classified by pharmacological profile and inactivation time. L-type VGCC may account for a sustained Ca^{2+} influx that plays an important role in synaptic plasticity and CREB activation (Bloodgood and Sabatini, 2008). Early studies in PC12 cell culture showed that ethanol had a significant inhibitory effect on the influx of Ca^{2+} through L-type VGCCs (Mullikin-Kilpatrick et al., 1995). Chronic ethanol administration has been shown to evoke a PKC-dependent upregulation of L-type VGCC expression and modulate the subunit composition of the channel, resulting in long-term changes in VGCC function (Katsura et al., 2006; Walter et al., 2000). Further, antagonism of L-type VGCCs has been shown to reduce alcohol consumption in both chronic ethanol treatment and genetic animal models (De Beun et al., 1996; Gardell et al., 1997; Rezvani and Janowsky, 1990). Taken together, these findings identify L-type VGCCs as an upstream mediator of the effect of ethanol which could modulate the regulation of CREB and may serve as a useful pharmacological target.

Adenylyl Cyclase (AC)

Membrane-bound AC serves as an important mechanism leading to the production of cAMP and subsequent activation of CREB, raising the possibility that direct modulation by ethanol may play a role in ethanol-mediated changes in CREB function. To date, 9 isoforms of AC, AC I-IX, have been cloned and exhibit differential tissue expression and

regulation. AC is a fairly ubiquitously expressed enzyme and each of the 9 isoforms is expressed within the CNS. The regulation of AC is complex and isoform-dependent with stimulatory and inhibitory contributions from GPCR-subunits, including $G\alpha_s$, $G\alpha_{i/o}$ and $G\beta\gamma$, Ca^{2+}-CaM, PKA and PKC (Hanoune and Defer, 2001). The structure of all 9 AC isoforms is similar and contains 2 hydrophobic domains, M_1 and M_2, with 6 transmembrane spans each, and 2 cytoplasmic domains, C_1 and C_2 (Sunahara et al., 1996). Early studies in cell cultures and rat cortex found a stimulatory effect of acute ethanol on AC resulting in increased cAMP production (Hatta et al., 1994; Mochly-Rosen et al., 1988). Further, chronic ethanol exposure has been shown to result in desensitization of AC to ethanol-induced stimulation in mouse cortex (Tabakoff et al., 1995). Previous studies utilizing transfected human embryonic kidney (HEK293) cells have shown that the sensitivity of AC to ethanol is isoform-specific. Specifically, AC VII shows a 2-3 fold higher sensitivity to ethanol than the other ethanol-sensitive ACs, and AC I and AC III are insensitive (Yoshimura and Tabakoff, 1995). This led a recent study to use AC II, III and VII chimeras to attempt to find a direct binding site for ethanol. By comparing cAMP accumulation and ethanol sensitivity of each of the chimeras, the study determined that two ethanol sensitive domains exist within each of the cytoplasmic domains (Yoshimura et al., 2006). Further study using increasing carbon length n-alkanols determined that a cutoff exists above a specific alcohol size which results in loss of the stimulatory effect of alcohol (Kou and Yoshimura, 2007). Taken together, these results strongly implicate a direct interaction between ethanol and AC. Given the important role of AC in long-term synaptic plasticity via the activation of CREB, the identification of AC as a direct upstream target of ethanol action has vast

implications on the modulation of synaptic plasticity which underlie the development of alcohol addiction.

Protein Kinases

Protein kinases occupy a central role in the regulation of CREB function that mediates synaptic plasticity. Since drugs of abuse modulate many of the triggers known to activate protein kinases, it is apparent that modulation of protein kinase function may have important implications in addiction (Lee and Messing, 2008; Ron and Jurd, 2005). Although a detailed review of various protein kinases and their targets is not possible in this review, it is worth noting the effects of acute and chronic ethanol on some kinases which regulate CREB phosphorylation, specifically CaMKII, CaMKIV, PKA and MAPK. It is important to point out that although ethanol may not have a direct modulatory effect at these protein kinases, upstream modulation of ethanol targets results in changes in protein kinase function (Machu et al., 1991).

CaMKs are regulated by rapid changes in intracellular Ca^{2+} and have an integral role in activity-dependent regulation of CREB function and synaptic strength (Wayman et al., 2008b). Various studies have implicated a role for ethanol-mediated modulation of CaMKIV on regulation of CREB function. An early study identified that withdrawal from chronic ethanol treatment resulted in a decrease in rat protein levels of CaMKIV and phosphorylated CREB (p-CREB) in rat cortical and amygdaloid structures (Pandey et al., 2001; Pandey et al., 2003b). Further studies have shown that voluntary ethanol intake results in decreased CaMKIV and p-CREB expression in the NAc, implicating decreased CaMKIV function in the NAc as a mediator of the positive affective state of alcohol addiction (Li et al., 2008; Misra et al., 2001). Ethanol has also been shown to activate

CaMKII and result in increased phosphorylation of CaMKII targets in rat cortex (Mahadev et al., 2001). Although CaMKII activation by ethanol has not been directly correlated to regulation of CREB function, CaMKII has been shown to mediate some of the effects of ethanol by directly phosphorylating channel proteins, namely $GABA_A$ receptors and voltage- and calcium-activated potassium (BK) channels (Liu et al., 2006; Machu et al., 1993).

PKA is a cAMP-activated tetrameric protein kinase consisting of two catalytic subunits and two regulatory subunits (Brandon et al., 1997). Increased intracellular cAMP, as is seen by ethanol-mediated activation of AC, results in dissociation of the PKA regulatory and catalytic subunits and subsequent phosphorylation of CREB. Cell culture studies have identified that acute ethanol application results in the translocation of the PKA catalytic subunit to the nucleus resulting in CREB phosphorylation, implicating an important role for PKA in ethanol-mediated modulation of CREB function (Constantinescu et al., 1999; Dohrman et al., 1996). In vivo, chronic ethanol treatment and withdrawal have been shown to have no effect on PKA expression or function in rat cortical and amygdaloid structures (Pandey et al., 2001; Pandey et al., 2003b). Yet, numerous studies have manipulated PKA activity to show that PKA-mediated changes in CREB function play a role in regulating alcohol consumption through euphoric and dysphoric pathways (Misra and Pandey, 2006; Pandey et al., 2003b; Yao et al., 2002; Zhang and Pandey, 2003). Inhibition of PKA function was also shown to reduce the sedative effects of acute ethanol suggesting that reduced PKA function may increase ethanol tolerance and play a role in the development of alcohol addiction (Lai et al., 2007). Transgenic mice lacking the PKA RIIβ regulatory subunit also display decreased

ethanol-induced sedation and increased alcohol consumption (Thiele et al., 2000). Further, a recent study found that CIE exposure decreased PKA activity in the NAc and EA and that this decrease may have been mediated by an upregulation of PKA inhibitor α, indicating that increased PKA-dependent transcription during acute ethanol consumption may mediate a decrease in PKA activity with repetitive withdrawal (Repunte-Canonigo et al., 2007). Taken together, these finding indicate that acute ethanol consumption results in increased PKA-mediated CREB activation which may mediate the subsequent decrease in PKA activity seen with chronic ethanol exposure.

The MAPK family of protein kinases, including extracellular signal-regulated kinases 1/2 (ERK1/2), have long been shown to be a required component of activity-regulated gene transcription mediating synaptic plasticity. ERK activation is characterized by the initial activation of Ras, a small soluble G-protein, which results in the activation of downstream proteins, Raf and MAPK/ERK kinase (MEK), and subsequent phosphorylation of ERK (Thomas and Huganir, 2004). Numerous studies have identified a relationship between ERK activation and ethanol exposure, yet the direction of modulation appears highly dependent on age, brain region, ethanol treatment regiment (Chandler and Sutton, 2005; Kalluri and Ticku, 2003; Sanna et al., 2002). In the CeA and MeA, acute ethanol has been shown to increase p-ERK, chronic ethanol treatment returns p-ERK to control levels and withdrawal from ethanol drinking results in decreased p-ERK levels (Pandey et al., 2008b). This and other studies have also found a direct correlation between ethanol-mediated modulation of ERK and CREB, implicating ERK as an important mediator of ethanol's effects on CREB (Chandler and Sutton, 2005; Pandey et al., 2008b). Recent studies have further shown that activation of ERK in the

CeA and NAc by BDNF and glial cell line-derived neurotrophic factor infusion, respectively, decreases voluntary ethanol consumption, implicating a role for ERK signaling in control of alcohol drinking behaviors (Carnicella et al., 2008; Pandey et al., 2006). Electrophysiological studies in acute slice preparations have shown that ERK modulation by acute ethanol can attenuate long-term potentiation, identifying a direct role for ERK in ethanol-induced modulation of synaptic plasticity (Roberto et al., 2003; Xie et al., 2009). Taken together, these data suggest that ERK signaling may be an important mediator of both the acute effects of ethanol, as well as the long-term changes seen in the development of alcohol addiction.

cAMP-responsive element binding protein (CREB)

The CREB gene transcription factor has long been implicated in many aspects of CNS function, including long-term memory formation, synaptic plasticity and addiction (Alberini, 2009; Carlezon et al., 2005). Regulation of CREB has been shown to be a key mediator of the increase in mRNA synthesis and protein expression that is required for long-term modification of synaptic strength (Abel and Kandel, 1998). Given that CREB protein is constitutively expressed, regulation of CREB signaling primarily involves activation by phosphorylation of CREB at serine-133 (Shaywitz and Greenberg, 1999). Numerous extracellular and intracellular stimuli trigger signal transduction pathways which converge upon the activation of CREB. The regulation of CREB activation results from complex interactions between various signal transduction cascades, including pathways involving CaMKII, CaMKIV, PKA and MAPK (Shaywitz and Greenberg, 1999). The phosphorylation of CREB results in the recruitment of CREB-binding protein

(CBP) and interaction with the cAMP-responsive element (CRE) DNA sequence in the gene promoter region, thereby regulating gene transcription (Chrivia et al., 1993).

CREB appears to occupy a central role in the mediation of long-term changes that are common in the development of addiction to various drugs and alcohol. Early studies elucidated a role for the involvement of CREB activation in the locus coeruleus (LC) and NAc in acute and chronic administration of opiates (Guitart et al., 1992; Widnell et al., 1996). By pharmacological manipulation of PKA function in the NAc of rats, increased and decreased CREB function were shown to mediate increased tolerance to cocaine reinforcement and increased cocaine preference, respectively (Self et al., 1998). Although CREB may be regulated differently by various drugs, these findings suggest that the modulation of CREB could be a common factor in cellular changes leading to the development of drug addiction (Nestler, 2001). A series of early studies regarding the role of CREB function in the effects of alcohol found that in rat cerebellar granule cells and dorsal striatum, acute ethanol resulted in a rapid increase in p-CREB and chronic ethanol treatment attenuated this ethanol-mediated increase (Yang et al., 1996; Yang et al., 1998a; Yang et al., 1998b). Another group found that in rat cortex, acute and chronic ethanol had no effect on CRE-DNA binding, yet withdrawal from chronic ethanol exposure significantly decreased CRE-DNA binding and expression of the CREB-target, BDNF (Pandey et al., 1999b). In an early study regarding the role of CREB in the reward pathway of alcoholism, voluntary ethanol intake was shown to decrease CREB phosphorylation in the rat NAc (Misra et al., 2001). Since this finding, investigators have begun to elucidate the role of CREB in the positive and negative affective states of alcohol abuse.

Although the modulation of CREB activation by acute and chronic ethanol may be regulated differently in different neurocircuitry, many findings appear to parallel one another. One of the first studies attempting to look at the role of CREB function in the amygdala utilized alcohol-preferring (P) and –nonpreferring (NP) rats that have been selectively-bred for high and low alcohol preference respectively (Bell et al., 2006; Pandey et al., 1999a). The study found that CREB levels, p-CREB levels and CRE-DNA binding activity are lower in the amygdala of P rats, in comparison to NP rats (Pandey et al., 1999a). More recently, the CREB deficit in P rats was found to be specifically in the CeA and MeA, but not the BLA, and this correlated to high alcohol consumption and high anxiety. Acute ethanol treatment was shown to increase CREB and p-CREB and decrease anxiety in P, but not NP rats (Pandey et al., 2005b). Similarly, CREB haplodeficient mice display anxiety-like behaviors and higher alcohol preference in comparison to wild-type (Pandey et al., 2004). Modulation of CREB function by manipulating PKA activity with infusions of a PKA activator (Sp-cAMP) or a PKA inhibitor (Rp-cAMP) into the CeA, mechanistically showed that increasing p-CREB resulted in decreased anxiety and alcohol consumption in P rats and decreasing p-CREB precipitated anxiety and increased alcohol consumption in NP rats (Pandey et al., 2005b). Using C57BL/6 mice, which show a higher preference for alcohol in comparison to DBA/2 mice (Belknap et al., 1993), it has been shown that innate levels of CREB and p-CREB are lower in the shell of the NAc, but not the core, in C57BL/6 mice as compared to DBA/2 mice (Misra and Pandey, 2003). Although P rats display high anxiety and alcohol consumption, in comparison to NP rats (Li et al., 1993; Pandey et al., 2005b), C57/BL6 mice display high alcohol consumption in the absence of anxiety behaviors,

when compared to DBA/2 mice (Misra and Pandey, 2003; Podhorna and Brown, 2002). Taken together, these findings appear to indicate that decreased CREB function in the NAc and CeA may mediate alcohol drinking by manipulation of the positive and negative affective states respectively, and CREB deficits may play a role in a genetic predisposition to alcoholism.

In order to study the role of CREB in withdrawal, Sprague-Dawley (SD) rats were exposed to chronic ethanol treatment and tested with and without 24-hour withdrawal. Only ethanol-withdrawn rats displayed an increase in anxiety-like behaviors which correlated to decreased p-CREB protein levels, with no change in total CREB protein levels, in the CeA and MeA. PKA manipulation of CREB function in the CeA showed that increasing CREB function in withdrawn rats decreased anxiety-like behaviors and decreasing CREB function precipitated anxiety and alcohol preference of normal rats (Pandey et al., 2003b). Another study showed that in male Sprague-Dawley rats, chronic ethanol intake resulted in a decrease in p-CREB levels in the NAc without affecting total CREB protein levels. The levels of p-CREB decreased further with 24- and 72-hour ethanol withdrawal, but were shown to increase towards control levels after 7 days (Pandey et al., 2003a). In summary, these results suggest that CREB deficits in both the NAc and CeA play a role in the promotion of alcohol-drinking due to both genetic predisposition and environmental influence, such as alcohol withdrawal, and the correction of the CREB deficit may decrease alcohol consumption. It is important to mention that a role for CREB has been identified in various aspects of alcoholism, such as the development of tolerance (Wang et al., 2009; Yang et al., 2003), ethanol-mediated changes in neuronal development (Chandler and Sutton, 2005) and mediation of ethanol

sensitivity (Acquaah-Mensah et al., 2006). As the role of CREB has been clearly identified, an important focus of current research is to identify ethanol targets which regulate CREB function and downstream CREB targets, including BDNF and the modulation of synaptic plasticity, which serve as effectors mediating neuroadaptive changes during alcoholism.

B. The Amygdaloid Complex: Neurocircuitry of Fear and Anxiety

1. Introduction

The amygdaloid complex, which lies in the medial temporal lobe, has long been described as the emotional center of the brain. Prevailing theories implicate the amygdala as a region which may modulate the functions of the central nervous system, peripheral nervous system, and endocrine system in response to emotional stimuli. Neuroanatomical and functional studies have shown that the amygdala is heavily interconnected with cortical and subcortical brain regions. Furthermore, the amygdaloid complex consists of numerous nuclei and subnuclei that are also interconnected. There exists controversy and debate with regards to the definition of various amygdaloid components, and even whether the amygdala should be regarded as a single separate entity. For example, the BNST was not originally considered a component of the amygdala, yet it is now widely accepted as an important region in the structure and function of the EA. In the following chapter, I will attempt to introduce the amygdala with regards to anatomy and physiology, with a particular focus on the CeA and the BNST. Furthermore, I will discuss studies which attempt to understand the role of the amygdala in fear conditioning and anxiety, and as a result have increased our understanding of functional interconnections with the amygdaloid complex.

2. The limbic system: an evolving view

Neurobiological research has attempted to identify and describe the neurocircuitry underlying the phenomenon of emotion for over a century. Early in the twentieth century, various researchers proposed the hypothalamus as a key structure involved in the response to emotional stimuli and the experience of emotion (LeDoux, 2003). These ideas, which gained favor through the work of Cannon and Bard, were refined by Papez, and further expanded by MacLean, resulting in the description of the limbic system, which was believed to be the neural circuit underlying emotion (Lautin, 2001; Sah et al., 2003). Papez described an anatomical circuit through which emotional stimuli entering the mammillary body of the hypothalamus could project to the cingulum via the anterior thalamic nucleus, resulting in the experience of emotion (Kandel et al., 1991; Lautin, 2001; Papez, 1995). Furthermore, cortical feedback to the cingulum could project back to the mammillary body, via the hippocampus and fornix, allowing for control of visceral responses to emotion (Kandel et al., 1991; Lautin, 2001; Papez, 1995). Through an evolutionary approach, MacLean theorized that the Papez circuit did not take into account connections between the limbic system, and the neocortical and "reptilian" levels of the triune brain, which he believed were mediated through amygdaloid and hippocampal regions (Lautin, 2001). Soon thereafter, Weiskrantz found that specific amygdaloid complex lesions could reproduce the symptoms of temporal lobe lesions produced earlier, that now define the Kluver-Bucy syndrome (LeDoux, 2003; Sah et al., 2003; Weiskrantz, 1956). Since the identification of the amygdala as a key structure in the emotional circuitry of the brain, a great deal of research has attempted to further understand the how the amygdala fits in various neurocircuitry, and the specific role of

the amygdala in emotional processing, especially with regards to fear and anxiety (Davis et al., 2009; LeDoux, 2003; LeDoux, 2000; Sah et al., 2003).

Before detailing amygdaloid anatomy and neurocircuitry, it is worth giving a brief overview of studies which increased our understanding of the amygdaloid complex. Early lesion studies in Rhesus monkeys identified a pattern of behavioral changes, now described as Kluver-Bucy syndrome, that were caused by lesions of the amygdaloid complex (Kandel et al., 1991; Weiskrantz, 1956). Monkeys with bilateral amygdaloid ablation displayed an increase in tameness, hypersexuality, and hyperorality (Kandel et al., 1991). Yet, it was also recognized by Weiskrantz that monkeys with amygdaloid ablations displayed weakened responses to previously aversive stimuli, decreased avoidance behaviors, and increased rates of extinction of conditioned avoidance behaviors (Weiskrantz, 1956). More recent studies have relied on the use of fear conditioning paradigms, primarily in rodents, to further characterize the role of the amygdala (Davis et al., 2009; LeDoux, 2003; Sah et al., 2003). Fear conditioning has been shown to result in a variety of autonomic, hormonal, and behavioral changes, including the modulation of blood pressure and heart rate, release of stress hormones, and freezing and startle responses (LeDoux, 2007; Sah et al., 2003). Anatomical and physiological studies in light of these responses have helped to identify amygdaloid output circuits to hypothalamic and brain stem nuclei, including the nucleus tractus solitarus (NTS), dorsal motor nucleus of the vagus, reticular formation, periaqueductal gray (PAG), raphe nuclei and locus coeruleus (LC), as well as other regions (Kandel et al., 1991; LeDoux, 2007; Sah et al., 2003). Furthermore, the study of contextual and specific cues which serve as conditioned stimuli in fear conditioning studies has led to the

identification of important afferent projections to the amygdala, including the thalamus, various cortical regions, and the hippocampus (LeDoux, 2000; Sah et al., 2003). Using several paradigms to model anxiety, also described as sustained fear, Davis and colleagues have identified the importance of reciprocal connections between the amygdaloid compex and the BNST (Davis et al., 2009). Various researchers have also underlined the importance of the BNST in the amygdaloid complex by including the BNST in an anatomical and functional description of the EA (Alheid, 2003; Alheid and Heimer, 1988). It is important to note that the amygdaloid complex has many other afferent and efferent projections, as well as interconnections within the amygdala, which have been identified and will be discussed in greater detail below.

Since this review primarily focuses on the amygdaloid neurocircuitry of fear and anxiety it is beyond the scope of this review to discuss mesolimbic dopaminergic connectivity in detail, yet it deserves mention. The mesolimbic dopaminergic system has been implicated in rewarding and reinforcing aspects of both physiologic goal directed behavior and pathologic formation of addictive behaviors (Gonzales et al., 2004; Koob and Swerdlow, 1988; Oades and Halliday, 1987). The neurocircuitry underlying the system consists of dopaminergic neurons that originate in the VTA and project to the NAc, amygdala, PFC, and hippocampus (Gonzales et al., 2004; Oades and Halliday, 1987). The shell of the NAc has been shown to display reciprocal connections with the amygdaloid complex and has been described as part of the EA (Alheid, 2003; Gonzales et al., 2004; Sah et al., 2003). It is important to note that the conceptual circuits described thus far, including the neurocircuitry of the limbic system, conditioned fear responses, anxiety, and the mesolimbic dopaminergic system, interact at various anatomical levels

and thus create a complex network of circuits which may be related to the function of the amygdaloid complex.

3. Overview of amygdaloid anatomy

The amygdaloid complex is made of approximately 12-13 nuclei that are structurally and functionally diverse (Kandel et al., 1991; Sah et al., 2003). The amygdala consists of heavy reciprocal connections with cortical, thalamic, hippocampal, hypothalamic and brain stem nuclei, as well as abundant internuclear and intranuclear amygdaloid connections (Haines, 2004; Kandel et al., 1991; Sah et al., 2003). The various nuclei of the amygdala have been grouped anatomically and functionally in various ways (Alheid, 2003; Davis et al., 2009; LeDoux, 2003; Paxinos and Watson, 2007; Sah et al., 2003). This has resulted in a degree of controversy regarding the grouping of amygdaloid nuclei, structures that should be included in the definition of the EA, and even whether the amygdaloid complex should be viewed as a single entity (Alheid, 2003; Lautin, 2001; LeDoux, 2003). Furthermore there are regions which contribute to the function of the amygdala, such as the intercalated cell mass (ITC) that lies between the BLA and the CeA, which have not been grouped into the classical subdivisions of the amygdaloid complex (Sah et al., 2003).

For the purposes of this review, the amygdala will be subdivided into three complexes, the basolateral complex, the cortical-like complex, and the centromedial complex (LeDoux, 2007; McDonald, 1998; Sah et al., 2003). The basolateral complex is made up of the lateral amygdala (LA), the basal amygdala (BA) and the accessory basal nuclei (AB) (LeDoux, 2007; Paxinos and Watson, 2007; Sah et al., 2003). The LA and BA nuclei are often viewed as a functional unit and may be referred to as the BLA. The

BLA has been described as the primary input center of the amygdala, and has been shown to receive projections from cortical, thalamic, and hippocampal regions, among others (LeDoux, 2000; Sah et al., 2003). Furthermore, the BLA has been shown to be involved in plasticity that underlies associative learning between sensory and emotional stimuli (Davis et al., 2009; LeDoux, 2003; LeDoux, 2007). The cortical-like complex of the amygdala lies superficially within the medial temporal lobe, and displays characteristics common to layered cortical regions (Paxinos and Watson, 2007; Sah et al., 2003). Although the cortical-like complex has been separated into various subnuclei, each of these nuclei is intimately associated with the olfactory system and may be functionally related in the processing of olfactory information (LeDoux, 2007; Sah et al., 2003). Since the cortical-like complex appears to primarily serve a role in olfactory processing with a limited contribution to the mechanisms underlying fear and anxiety, detailed discussion of this complex is outside the scope of this chapter. However it is worth noting that this complex may play a role as an input region for olfactory stimuli that may be related to appetitive or aversive responses (Brennan and Kendrick, 2006; Heimer and Van Hoesen, 2006). The centromedial complex describes the CeA, MeA, and BNST (Paxinos and Watson, 2007; Sah et al., 2003). The centromedial complex appears to serve a role as the main output center of the amygdala via two major projections, the stria terminalis and the ventral amygdalofugal pathway (Kandel et al., 1991; LeDoux, 2007; LeDoux, 2000). The CeA has been anatomically and functionally separated into four major cell groups, the medial division (CeM), the lateral division (CeL), the lateral capsular division (CeLC), and the intermediate division (CeI) (Akmaev et al., 2004; Cassell et al., 1999; Sah et al., 2003). The MeA has often been functionally associated

with the cortical-like complex as it may play a key role as an output center for emotionally significant olfactory stimuli (LeDoux, 1992). Although the MeA may have a role in certain types of fear conditioning, the CeA has been studied in greater detail and will be the primary focus of this review. It is also of note, however, that the MeA has been implicated in the regulation of sexual behaviors and appetitive responses (Cooke, 2006). Although the BNST was not originally included as a part of the amygdaloid complex, anatomical and functional studies have led to the argument that the centromedial complex extends to the BNST and the ventral pallidum, and should be termed the EA (Alheid, 2003; Alheid and Heimer, 1988). Other cell groups and nuclei may also be included in the EA, so for this review the "EA" and "centromedial" terminology will remain separate. The BNST has been subdivided into various cell groups, most notably, the lateral BNST (lBNST) and the medial BNST (mBNST) (Davis et al., 2009).

Given the controversy regarding the anatomical subdivisions of the amygdaloid complex, it is worth noting that some researchers have attempted argued that functional divisions of the complex may be more appropriate (Swanson and Petrovich, 1998). Thus, four functional divisions have been identified as the frontotemporal, autonomic, main olfactory and accessory olfactory systems (Sah et al., 2003; Swanson and Petrovich, 1998). Using this nomenclature, there exists a degree of overlap regarding the anatomical divisions described above. For instance, the centromedial complex receives afferent projections from frontotemporal and olfactory systems, and sends efferent projections to autonomic systems (Haines, 2004; LeDoux, 2007; McDonald, 1998; Sah et al., 2003). Yet, by using the primary functional descriptions of each of the subdivisions,

these functional divisions can be reconciled such that the basolateral complex is associated with the frontotemporal system, the corticomedial complex is associated with the autonomic system, and the cortical-like complex is associated with the olfactory systems (Kandel et al., 1991; LeDoux, 2007; Sah et al., 2003).

4. Connectivity of the amygdaloid complex

The amygdala is believed to receive afferent projections from brain regions involved in the consolidation of sensory information, including the cerebral cortex, the thalamus and the hippocampus (LeDoux, 2000; McDonald, 1998; Sah et al., 2003). The amygdala may then modulate hormonal, autonomic and behavioral effects via efferent projections to hypothalamic, brainstem and basal forebrain regions via the stria terminalis and ventral amygdalofugal pathway (Alheid, 2003; Kandel et al., 1991; McDonald, 1998). Thus, the amygdaloid complex appears to play a role linking brain regions that process experiential information and regions which modulate physiological and behavioral responses (Kandel et al., 1991; McDonald, 1998). Yet, it is important to note that the neurocircuitry which underlies the function of the amygdala is complex and widespread, involving redundant input pathways to various amygdaloid nuclei and interconnections between amygdaloid nuclei (McDonald, 1998; Sah et al., 2003).

Afferent connections to the amygdaloid complex can be separated into sensory inputs, polymodal inputs, and inputs involved with memory. In general, the highest density projections to the amygdala arise from association cortices carrying modality-specific and polymodal information, such as the prefrontal and insular cortices, in comparison to primary cortices or thalamic regions (Turner et al., 1980). Retrograde neuronal tracing utilizing D-aspartate injection, has identified that cortical inputs to the

amygdaloid complex are primarily from glutamatergic pyramidal neurons (Amaral and Insausti, 1992). The basolateral complex is believed to be the primary site for sensory input and plasticity during fear conditioning, yet direct cortical projections to the centromedial complex may also have a role in the consolidation of sensory information in the amygdala (Davis et al., 2009; LeDoux, 2000). It is worth noting that despite the high density of cortical inputs to the amygdaloid complex, thalamic and hippocampal inputs tend to result in more consistent excitation within the amygdala and rapidly result in associative learning (LeDoux, 2000). Efferent projections from the amygdaloid complex to the hypothalamus and brain stem appear to arise primarily from the centromedial complex (Sah et al., 2003). It has been shown that these projections from the centromedial complex may underlie various behavioral and autonomic responses that are seen in fear conditioning paradigms (Davis et al., 2009; LeDoux, 2000). Yet, cortical regions that project to the basolateral complex also receive reciprocal projections from various amygdaloid regions, and this connectivity may be involved in the focus of attention to emotionally significant stimuli, which may also have behavioral effects (McDonald, 1998).

Cortical, thalamic, and hippocampal inputs

With the exception of olfactory projections, sensory inputs to the amygdaloid complex appear to project most densely to the basolateral complex via axons which lie in the ipsilateral external capsule (Mascagni et al., 1993). Projections from primary olfactory regions, including the olfactory bulb and primary olfactory cortex, terminate on pyramidal neurons in nuclei within the cortical-like complex (McDonald, 1998). Olfactory information appears to reach the basolateral complex via projections from

cortical-like nuclei, as well as through some direct projections from the piriform cortex (McDonald, 1998). The accessory olfactory bulb also has strong projections to the MeA and mBNST (McDonald, 1998). Primary somatosensory cortex has few projections to the amygdaloid complex (Sah et al., 2003). The posterior insular cortex appears to be the primary region of somatosensory input to the BLA and CeA (McDonald, 1998; Sah et al., 2003). Projections from the pontine parabrachial nucleus and the thalamus, specifically the medial division of the medial geniculate nucleus (mGN) and posterior intralaminar nucleus (PIN) arrive primarily in the LA, with light projections to the CeA (Sah et al., 2003). It has been suggested that thalamic nuclei may be the primary afferent carrying nociceptive information that may be involved in relaying the unconditioned stimulus used in fear conditioning paradigms (LeDoux, 2000). The anterior insular cortex has been shown to be involved with the processing of gustatory and visceral sensory information (Shi and Cassell, 1998). Gustatory and visceral information from these primary cortical regions project heavily to the CeA and BLA, with minor projections to the MeA (McDonald, 1998). The ventral posteromedial thalamic nucleus (VPM) and the parabrachial nucleus receive gustatory and visceral input from the NTS (Haines, 2004; Sah et al., 2003). These subcortical regions send projections which converge with primary projections in the basolateral and centromedial complex (Sah et al., 2003). Auditory projections to the amygdala originate from secondary and tertiary auditory cortices, as well as the mGN, the auditory nucleus of the thalamus (McDonald, 1998). Projections carrying auditory information primarily terminate in the LA, with minor contributions to other basolateral and centromedial nuclei (McDonald, 1998). Since acoustic stimuli have frequently been used in fear conditioning paradigms, these

projections have been studied in depth and have been outlined as key projections in the neurocircuitry underlying fear conditioning (Davis et al., 2009; LeDoux, 2000). Visual information is not projected to the amygdala from primary or secondary cortices, or subcortical regions. Projections from higher order visual cortices in the temporal lobe primarily terminate in the LA, similar to auditory information (McDonald, 1998). Various lines of evidence suggest that the role of higher order visual input into the amygdala may relate to the recognition of emotionally relevant visual stimuli, such facial expression, body movement, and other visual stimuli which may be important for responses which underlie social behaviors (Adolphs, 2008; Kandel et al., 1991; Nakamura et al., 1992).

Cortical and subcortical regions in the temporal lobe, including the perirhinal cortex (PRC), the entorhinal cortex (ERC), the parahippocampal cortex (PHC), and the hippocampus, have been associated with the formation and recall of memories (Kandel et al., 1991; McDonald, 1998). The role of these regions has been heavily implicated in the function of the amygdala through the use context-based fear conditioning paradigms (LeDoux, 2000). Connectivity between the amygdala and these regions has been shown to be widespread and reciprocal (McDonald, 1998; Sah et al., 2003). Furthermore, numerous functional studies employing lesions, regional inactivation, and electrophysiological techniques have identified that hippocampal-amygdaloid connectivity is important for the formation of memories related to emotional stimuli, as well as the modulation of memory formation by emotional stimuli (Kandel et al., 1991; LaBar and Cabeza, 2006; LeDoux, 2000; Maren and Quirk, 2004). The PRC primarily projects to the LA, with lighter projections to the BA and CeL regions (McDonald, 1998;

Shi and Cassell, 1999). The ERC, on the other hand, projects widely to the entire amygdaloid complex (Sah et al., 2003). Hippocampal inputs to the amygdala primarily project from the ventral subiculum (McDonald, 1998; Sah et al., 2003). These projections are also widespread throughout the amygdaloid complex, however studies regarding amygdaloid function have led researchers to hypothesize that hippocampal inputs to the CeA may be particularly important in contextual fear conditioning (LeDoux, 2000; McDonald, 1998). Anatomical studies have shown that the ventral subiculum and ERC have dense projections to the medial EA, namely the MeA and the mBNST (McDonald et al., 1999). These projections may play a role in social behaviors which may have evolved from the integration of olfactory information and memory information (Luiten et al., 1985). Although amygdaloid connectivity is generally conserved from rat to monkey, it is interesting to note that the ERC and hippocampus show more dense projections to the BLA in the monkey, in comparison to the widespread connectivity seen in the rat (McDonald, 1998).

The PFC is a cortical association area that receives information from all sensory modalities and has been implicated in responses associated with reward, motivation, and social aspects of behavioral regulation (Kandel et al., 1991). The amygdaloid complex receives dense projections from various regions of the PFC, primarily the medial (mPFC) and lateral prefrontal cortex (lPFC) (McDonald, 1998). Projections from the PFC terminate primarily in the BLA, however there are some regions of the PFC that also project to the CeA and lBNST (McDonald, 1998; McDonald et al., 1999; Sah et al., 2003). Functionally, PFC projections to the amygdala have been shown to be involved in the extinction of conditioned fear responses, although various other roles for these

projections may exist (McDonald, 1998). Specifically, it has been shown that lesions of the infralimbic (IL) region of the mPFC can block extinction following fear conditioning (Morgan et al., 1993). Furthermore, antagonism of NMDA receptors in the BLA has also been shown to block extinction of conditioned response, consistent with data indicating that PFC inputs into the BLA are glutamatergic (Falls et al., 1992). Thus, it could be possible that the mPFC plays a role in the modulation of amygdaloid memory storage.

Output from the amygdaloid complex

As mentioned previously, the centromedial complex appears to have a role as the primary output center of the amygdala, and projects widely to regions in the hypothalamus and brain stem (Sah et al., 2003). Given the widespread reciprocity of cortical and subcortical connections seen in the amygdaloid complex, it is outside the scope of this review to anatomically describe all amygdaloid efferents, so focus will be given to centromedial complex efferents which may underlie hormonal, autonomic and behavioral changes to emotional stimuli. Various regions within the centromedial complex project to the output regions of the amygdala, however various lines of evidence from anatomical and functional studies indicate that the CeM and lBNST have the strongest projections to hypothalamic and brain stem nuclei (Alheid, 2003; Davis et al., 2009; LeDoux, 2000; Sah et al., 2003; Walker et al., 2003). Furthermore, both the CeM and lBNST cell groups appear to project to many of the same targets, thus both projections will be described as centromedial efferents below (Lang et al., 2000; Sah et al., 2003; Walker et al., 2003). It should be noted although these centromedial efferents may be anatomically similar, there appears to exist a functional difference between activity within the CeA and the BNST that may underlie the behavioral differences

between short-term fear and long-term anxiety (Davis et al., 2009; Lang et al., 2000; Walker et al., 2003).

Centromedial efferents to various hypothalamic regions have been shown to mediate hormonal and autonomic responses to emotional stimuli (Jankord and Herman, 2008; Lang et al., 2000; LeDoux et al., 1988; Sah et al., 2003). The paraventricular nucleus (PVN) is a hypothalamic region that contains discrete neuronal cell groups containing specific neuropeptides (Kandel et al., 1991). Projections from the PVN to the pituitary gland mediate the regulation of hormonal release throughout the body (Berne, 2004; Kandel et al., 1991). Activation of the amygdala has been shown to result in the release of corticosteroids, primarily via projections from the lBNST, which may underlie the stress response to emotional stimuli (Jankord and Herman, 2008; Lang et al., 2000; Walker et al., 2003). Centromedial efferents have also been shown to the lateral and ventromedial hypothalamus, which may result in the modulation of autonomic and behavioral responses (Kandel et al., 1991; Sah et al., 2003; Walker et al., 2003). The lateral hypothalamus (LH) receives a particularly strong projection from both the CeM and lBNST cell groups, and has been shown to project to nuclei which mediate autonomic responses in the brain stem and spinal cord (Haines, 2004; Kandel et al., 1991). Activation of the amygdaloid nuclei under certain experimental paradigms has been shown to result in increased sympathetic tone, including tachycardia, peripheral vasoconstriction and papillary dilation, which may be mediated by these projections (Sah et al., 2003; Walker et al., 2003).

The centromedial complex has also been shown to project to brain stem regions which may mediate defensive responses to emotional stimuli (Misslin, 2003; Sah et al.,

2003). Through fear conditioning experiments, various brain stem regions have been identified as primary mediators of the immediate response to a conditioned stimulus (CS), including the PAG, the parabrachial nucleus, the NTS, and the dorsal motor nucleus of the vagus (Lang et al., 2000; LeDoux, 2000; Sah et al., 2003). Both the CeM and lBNST regions have heavy projections to these brain stem nuclei (Davis et al., 2009; Sah et al., 2003). The PAG has been implicated in the promotion of freezing behaviors (Lang et al., 2000; LeDoux, 2007; Misslin, 2003). For instance, electrical and chemical stimulation of the PAG result in increased freezing responses, and PAG lesions block previously observed freezing (Misslin, 2003). Both the PAG and the parabrachial nucleus have been implicated in nociceptive transmission (Misslin, 2003; Sah et al., 2003). Centromedial projections to these nuclei may modulate the transmission of painful stimuli resulting in hypoalgesia (Lang et al., 2000; Misslin, 2003; Sah et al., 2003). The NTS and dorsal motor nucleus of the vagus play a role in the control of the parasympathetic nervous system, particularly by controlling activity within the vagus nerve (LeDoux, 2007; Saha, 2005). Projections from CeM and lBNST neurons to regions involved with vagal control may mediate autonomic modulation that is associated with defensive freezing or "fight or flight" responses (Lang et al., 2000; Saha, 2005). Projections from the centromedial complex to other brain stem nuclei, such as the LC, VTA, raphe nuclei and nucleus basalis, regulate activity of ascending modulatory systems which may affect cortical and subcortical processing (Lang et al., 2000; Sah et al., 2003). Amygdaloid modulation of ascending noradrenergic, depaminergic, serotonergic and cholinergic pathways may regulate behavioral effects such as the modulation of attention, arousal, and motivation in response to emotional stimuli (Sah et

al., 2003; Walker et al., 2003). It is important to point out that the BNST has particularly strong connections with ascending systems (Lang et al., 2000; Walker et al., 2003). Since ascending modulatory systems may produce more sustained effects than the immediate physiological and behavioral responses produced by other brain stem regions, BNST projections to these regions may play an important role in sustained fear or anxiety (Lang et al., 2000; Walker et al., 2003).

As described above, various regions of the amygdaloid complex have reciprocal connections with cortical and subcortical areas, which may play a role in the modulating attention to emotional stimuli, providing emotional feedback to the cortex, or creating the experience of emotion (LeDoux, 2000; McDonald, 1998; Sah et al., 2003). Hypothalamic and brain stem regions also have reciprocal connections to the centromedial complex, with the most dense projections reaching the CeA and lBNST (McDonald et al., 1999). Thus, it could be possible that these autonomic afferents into the amygdaloid complex provide feedback information that could in turn modulate output from the centromedial nuclei (McDonald et al., 1999). It is worth noting that various hypothalamic and brain stem regions send peptidergic, monoaminergic and cholinergic inputs into the amygdaloid complex which may also have a modulatory influence on neurotransmission.

Connectivity within the amygdaloid complex

The amygdaloid complex has heavy interconnections between nuclei and intraconnections within an individual nucleus (Krettek and Price, 1978; Sah et al., 2003). The basolateral complex has been described as the major input center of the amygdala, although the cortex-like nuclei and MeA appear to play a role in the processing of

olfactory projections (McDonald, 1998; McDonald et al., 1999; Sah et al., 2003). The cortex-like nuclei and MeA project laterally to the basolateral complex and centromedial complex, yet the heaviest amygdaloid interconnections appear to follow a lateral to medial progression (Krettek and Price, 1978; McDonald, 1998; Sah et al., 2003). For the purposes of this review, focus will be restricted to basolateral complex output to centromedial cell groups, and centromedial inter- and intraconnections. The basolateral complex has extensive glutamatergic interconnections between subnuclei which primarily progress from the LA to the BA and AB nuclei, with the heaviest projection terminating in the AB nucleus (Sah et al., 2003; Savander et al., 1997). These projections are also accompanied by reciprocal glutamatergic projections back to the LA, yet the BA has also been shown to send reciprocal GABAergic projections (Savander et al., 1997). Glutamatergic output from the basolateral complex to the centromedial complex is also extensive, with each of the LA, BA and AB nuclei sending widespread projections to the CeA, MeA and BNST (Davis et al., 2009; LeDoux, 2007; Sah et al., 2003). It should be noted that there exists some controversy regarding the role of LA projections to the CeA. Some research has indicated that no projections directly link the LA with the CeM region (Pare et al., 2004). However, it has been shown that the LA sends projections to the CeL and to ITC, which lie between the BLA and CeA (Pare et al., 2004). Thus, it could be possible that GABAergic interneurons in the ITC play an important role in certain types of fear conditioning paradigms (Pare et al., 2004). It is also important to note that basolateral complex projections to the BNST run through the CeA (Davis et al., 2009). Thus, it could be possible that studies which use lesions within the CeA could also result in decreased BLA to BNST connectivity (Davis et al., 2009).

The centromedial complex receives strong projections from all other regions of the amygdaloid complex (Sah et al., 2003). Primary centromedial input regions which receive amygdaloid projections are the CeM, lBNST, and CeLC regions (Cassell et al., 1999; Davis et al., 2009). The centromedial complex has few reciprocal connections with the basolateral complex, and primarily plays a role in amygdaloid output and processing (Sah et al., 2003). Regions within the centromedial complex display heavy GABAergic interconnections (Alheid, 2003; Cassell et al., 1999). Furthermore, excitatory input from the basolateral complex primarily appear to synapse onto GABAergic interneurons and projection neurons within the centromedial complex (Cassell et al., 1999). The CeM and lBNST cell groups appear to be the primary output centers of the amygdaloid complex and receive heavy GABAergic projections from other centromedial regions (Sah et al., 2003). Also, the CeM and lBNST regions are heavily connected via reciprocal GABAergic neurons (Alheid, 2003; Davis et al., 2009). The CeLC region primarily sends GABAergic projections to the CeM region (Cassell et al., 1999). CeL cell groups provide the greatest density of GABAergic connections to the CeM and lBNST regions (Sah et al., 2003). The CeL region also projects via corticotrophin-releasing factor (CRF) containing neurons to the lBNST (Davis et al., 2009). As mentioned previously, the CeL receives most cortical and subcortical efferents to the centromedial complex, which may indicate that the CeL is an important site of centromedial processing and plasticity (Davis et al., 2009; Sah et al., 2003). It should also be noted that the CeM region may play a role in centromedial synaptic plasticity (Samson and Pare, 2005).

5. Neuronal morphology in the amygdaloid complex

The morphology of neurons in the basolateral and cortex-like complexes have been found to resemble one another. Two major cell types have been identified and resemble the morphology of cells in cortical areas (Sah et al., 2003). The first cell type, which makes up the majority of basolateral complex neurons, appear to be homologous to cortical pyramidal neurons (Millhouse and DeOlmos, 1983). These cells may comprise glutamatergic projections from the basolateral and cortex-like complexes to the centromedial complex and reciprocal connections to cortical and subcortical input regions (McDonald, 1982). The pyramidal, or spiny, cells have three to seven spiny dendrites, with one prominent apical dendrite (McDonald, 1984; Millhouse and DeOlmos, 1983; Sah et al., 2003). Axons may originate from the cell body, or proximal regions of the dendrites, and project with seemingly random organization, unlike the parallel configuration of cortical pyramidal neurons (Sah et al., 2003). The second type of cells are aspiny stellate cells with two to six dendrites forming a concentric field of dendritic inputs (McDonald, 1982; Millhouse and DeOlmos, 1983). The cells appear to be GABAergic local interneurons which may provide tonic inhibition of excitatory projection neurons (Sah et al., 2003). It is worth noting that subpopulations of the interneurons express different calcium binding proteins, such as parvalbumin and calbindin, indicating that there may be heterogeneity among these cells (Kemppainen and Pitkanen, 2000; Pitkanen and Kemppainen, 2002).

The centromedial complex is primarily made up of GABAergic neurons which have been compared with the neuronal population of the striatum (Alheid, 2003; Cassell et al., 1999). The primary neuron in the centromedial amygdaloid complex is the

medium-sized spiny neuron, which shows morphological and physiological characteristics that are similar to medium spiny neurons found in the striatum (Sah et al., 2003). These neurons appear to have three to five dendrites that are moderately to densely spiny, and appear to send GABAergic projections to other centromedial complex regions (Akmaev et al., 2004; Cassell et al., 1999; Chieng et al., 2006). Axons of the medium-sized spiny neurons also send local collaterals within the specific centromedial subregion in which they originate (Sah et al., 2003). The second major cell type within the CeA has a single aspiny apical dendrite branching into sparsely spiny secondary dendrites (Chieng et al., 2006). These neurons have a slightly larger soma than medium spiny neurons and may extend dendrites into multiple centromedial complex subregions (Chieng et al., 2006; Sah et al., 2003). There may also exist a few other aspiny cell types, which may comprise peptide-containing centromedial neurons (Cassell and Gray, 1989). It is worth noting that these various cell types appear to be distributed homogenously throughout the centromedial complex, however there may be some distinctions (Chieng et al., 2006; Sah et al., 2003). For example, the CeL region appears to contain an higher proportion of medium-sized spiny neurons than the CeM region (Chieng et al., 2006). Furthermore, the peptide composition of neurons throughout the centromedial complex varies greatly (Cassell and Gray, 1989; Chieng et al., 2006).

6. The neurocircuitry of fear and anxiety

The study of conditioned and unconditioned fear responses have given a great deal of insight into the functional role of the amygdala and amygdaloid subregions in fear and anxiety (Davis et al., 2009; Lang et al., 2000; LeDoux, 2007; LeDoux, 2000). In order to consolidate and review some of the anatomical evidence described above, I will give an

overview of the neurocircuitry that has independently been studied by the laboratories of Davis and LeDoux (Davis et al., 2009; LeDoux, 2007). Classic pavlovian fear conditioning couples a conditioned stimulus (CS), such as a tone, to an unconditioned stimulus (US), such as an electrical shock, which may elicit a defensive response, such as freezing. After a number of repetitions of paired CS and US, the responses being monitored will be elicited by the CS alone (LeDoux, 2000). The work of LeDoux, using a tone and a foot shock, has identified the neurocircuitry which associates auditory stimuli and nociceptive transmission, resulting in amygdaloid activity and output to regions which underlie the resulting behavioral and autonomic responses (LeDoux, 2007; LeDoux, 2000). In brief, auditory inputs from the mGN of the thalamus and the auditory association cortex send glutamatergic projections to the LA (LeDoux, 2000). Subcortical and cortical somatosensory and nociceptive information are also transmitted to the LA (LeDoux, 2000). Furthermore, it has been shown that there are LA neurons that respond to both auditory and somatosensory stimulation, so simultaneous presentation of these cues may result in associative synaptic plasticity (LeDoux, 2007; Pare et al., 2004; Romanski and LeDoux, 1992). Following plasticity, the CS alone may result in the activation of output neurons from the LA to the CeA, and modulate CeA output to the PAG, LH, and PVN resulting in modification of behavioral, hormonal and autonomic states (LeDoux, 2007; LeDoux, 2000). A recent study utilizing the tools provided by optogenetic research has further identified the importance of the LA in conditioning to aversive stimuli (Johansen et al., 2010). Following transfection of LA pyramidal neurons with an activating channelrhodopsin, ChR2, researchers found that light-activation of these neurons in conjunction with auditory stimuli could be used as a training paradigm

resulting in freezing behaviors to the tone-alone (Johansen et al., 2010). This finding confirms that activation of the LA pyramidal neurons provides the aversive stimuli necessary for the associative learning involved in fear conditioning. It should be noted that the LA has only light projections to the CeM region, however the ITC, which lies between the basolateral complex and the CeA, may receive BLA output and project to the CeA (Pare et al., 2004).

Contextual fear conditioning replaces the auditory CS with the use of contextual cues, such as conditioning to the chamber in which the rats are placed (LeDoux, 2000). Interestingly, a contextual stimulus has been shown to require both the amygdaloid complex and the hippocampus (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Hippocampal projections to the BA and AB nuclei may carry contextual cue information, and nociceptive information has been shown to be transmitted to the AB nucleus (Canteras and Swanson, 1992; LeDoux et al., 1990). Thus, associative learning between a contextual CS and the US within the AB nucleus may also modulate CeA output, resulting in defensive behaviors (LeDoux, 2000). It is interesting to note that the CeA also receives nociceptive inputs, especially from the parabrachial nucleus and directly from the spinal cord (Bernard et al., 1990; Burstein and Potrebic, 1993). Thus, it could be possible, and has been shown using high frequency stimulation long-term potentiation, that the CeA may also play a role in CS-US convergence and associative learning (LeDoux, 2000; Samson and Pare, 2005).

The use of different fear conditioning paradigms has led to the identification of a varied time-course of fear responses, which Davis *et al*. describe as a difference between phasic and sustained fear, which may serve as a useful model to separate fear and anxiety

(Davis et al., 2009). The basic paradigm of fear conditioning used in this laboratory is the potentiated startle paradigm (Lang et al., 2000). In this paradigm, animals are conditioned using paired CS and US, and then tested by evaluating the response to startle-eliciting noise bursts in the absence and presence of the CS (Lang et al., 2000). In general, startle responses are larger in the presence of the CS, such that the magnitude of this difference represents the amount of CS-elicited fear (Lang et al., 2000). In order to extend this model to sustained fear, various modifications to this paradigm have been tested. In the sustained startle test, the CS-US time-course is varied by presentation of a variable duration CS, ranging from 3 s to 8 min, prior to the US (Davis et al., 2009). This paradigm results in the presentation of sustained fear, through the measurement of increased startle amplitudes, which are greater in magnitude throughout the duration of the CS than before or after the CS (Davis et al., 2009). Sustained fear can also be tested using the light-enhanced startle paradigm that compares the acoustic startle amplitude of rats that have been placed in a bright chamber, which has been shown to be anxiogenic, to rats in a dark chamber (Walker and Davis, 1997). Similarly to a long-duration CS, light exposure results in a sustained increase in the magnitude of startle amplitudes, which can be reduced via the application of various types of anxiolytic drugs (Walker and Davis, 1997; Walker and Davis, 2002).

Studies comparing these paradigms have identified a differential role for the CeA and the lBNST in the expression of phasic and sustained fear (Davis et al., 2009). Phasic fear has been shown to be blocked by lesions within the CeA, but not the BNST (Hitchcock and Davis, 1987; LeDoux et al., 1988). As previously noted fibers to the lBNST appear to travel through the CeA, thus differential inactivation of the BNST and

CeA has primarily been studied by pharmacological inactivation rather than electrolytic lesions (Davis et al., 2009; Kalin et al., 2004). Using a light-enhanced startle paradigm, it has been shown that inactivation of the lBNST, but not the CeA, results in a reduction in startle responses, implicating the BNST in both sustained and unconditioned fear responses (Kalin et al., 2004). Since this review is primarily focused on neurocircuitry rather than cell signaling mechanisms, it is outside the scope of this review to give a detailed overview of CRF. However, it should be noted that intracerebroventricular CRF infusions have been shown by various studies to result in a sustained increase in the magnitude of startle response in a manner sensitive to anxiolytic treatment (Davis et al., 2009). Furthermore, infusions of a CRF antagonist into the lBNST, but not the CeA, have been shown to block CRF-enhanced startle, further implicating the BNST in sustained and unconditioned fear (Lee and Davis, 1997). Thus, CRF release in the lBNST may be a critical component which promotes anxiety-like behaviors. The use of light- and CRF-enhanced startle paradigms, led researcher to ask whether the lBNST was involved in unconditioned fear responses or in a different time-course of fear responses. In order to investigate this question further, researchers measured startle responses during a variable duration conditioned fear paradigm in the presence of pharmacological inactivation of the lBNST or CeA (Davis et al., 2009). Interestingly, it was found that lBNST inactivation, but not CeA inactivation, resulted in a reduction in late startle responses that began 5 min following introduction of the CS (Davis et al., 2009). This study indicated that the presentation of sustained fear, or anxiety, may be dependent on the lBNST regardless of conditioning (Davis et al., 2009). It is worth noting that CRF antagonist infusion into the lBNST blocks sustained, but not phasic fear, and has no

effect in the CeA, indicating that the role of the BNST in sustained fear may be mediated by CRF transmission (Lee and Davis, 1997). It is worth noting that inactivation of the BLA has been shown to block phasic and sustained fear in various conditioned and unconditioned paradigms, indicating that basolateral complex regions may serve as the input region for the neurocircuitry of various types of fear and anxiety (Davis et al., 2009; LeDoux, 2007; LeDoux, 2000). Recently, optogenetic tools were utilized to transfect BLA projection neurons with excitatory or inhibitory light-activated channels (Tye et al., 2011). Interestingly, light-activation of the BLA directly resulted in neuronal excitation, but did not produce any changes in behavior, but light activation of BLA terminals in the CeA produced anxiolytic effects. Conversely, light-activation of inhibitory rhodopsin proteins in the BLA terminals of CeA produced anxiety-like behaviors (Tye et al., 2011). This data strongly implicates the activation of BLA-CeA projections in the precipitation of anxiety-like behaviors, and it could be possible that this effect is mediated both by CeA and also by projections to the BNST.

In order to consolidate some of the evidence presented above, I will outline a brief hypothetical model for the role of the amygdaloid complex in fear and anxiety (Fig. 1). Various inputs to the amygdala project to the basolateral complex, which may serve as an initial site for associative learning. Cortical and thalamic projections carrying specific cue information, such as an auditory tone, enter the LA, whereas hippocampal projections carrying contextual cues enter the BA and AB (LeDoux, 2000). Both the LA and AB nuclei receive nociceptive inputs and can serve as areas of synaptic plasticity which result in associative learning of conditioned and unconditioned stimuli (LeDoux, 2007; LeDoux, 2000). The basolateral complex sends glutamatergic projections to the CeA and

lBNST (Davis et al., 2009; LeDoux, 2000). Projections to the CeA may primarily be sent to the CeLC region or the ITC, which then project via GABAergic neurons to the CeM (Pare et al., 2004). Similarly, projections to the lBNST may arrive directly from the BLA, or project via the CeL or CeM regions (Davis et al., 2009). In the case of phasic fear, GABAergic projections from the CeM region to hypothalamic and brainstem nuclei result in the promotion of defensive behaviors (Davis et al., 2009; LeDoux, 2007; LeDoux, 2000). In sustained fear or anxiety, GABAergic projections from the lBNST to hypothalamic and brain stem regions mediate behavioral, hormonal and autonomic responses characteristic of anxiety-like responses (Davis et al., 2009; Lang et al., 2000). CRF transmission from the PVN to the CeL, and from the CeL to the lBNST, may also modulate sustained fear responses (Davis et al., 2009). Furthermore, it could be possible that associative learning may also occur within the CeM or lBNST (Davis et al., 2009; Lang et al., 2000; LeDoux, 2007; LeDoux, 2000; Samson and Pare, 2005). Long-term changes within these regions may result in the modification of behaviors which underlie the physiologic learning of aversive or noxious stimuli, or result in the pathologic states associated with anxiety-spectrum disorders (Davis et al., 2009; LeDoux, 2000).

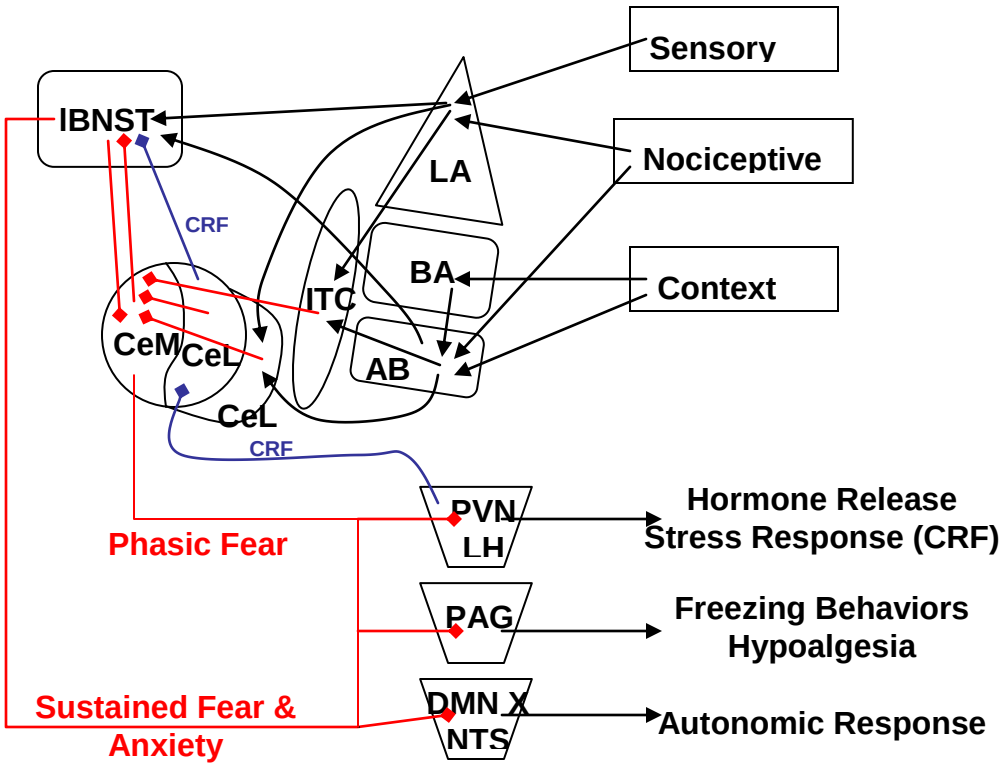


Figure 1. Schematic representation of the neurocircuitry of the extended amygdala associated with fear and anxiety behaviors.
Sensory or contextual conditioned stimuli (CS), activate neurons within the lateral amygdala (LA), or basal (BA) and accessory basal amygdala (AB). Nociceptive unconditioned stimuli (US) activate the LA or AB, and may converge with CS inputs in these regions. The LA and BA project via glutamatergic neurons to the intercalated cell mass (ITC), centrolateral capsular amygdala (CeLC), and the lateral bed nucleus of the stria terminalis (lBNST). GABAergic neurons in the ITC, CeLC, centrolateral amygdala (CeL), and lBNST project to the centromedial amygdala (CeM). GABAergic projection neurons from the CeM project to output regions and primarily appear to produce the phasic fear response. GABAergic projections from the lBNST project to output regions, and primarily appear to produce sustained fear, or anxiety. The CeM and lBNST appear to have reciprocal GABAergic connections that may also play a role in the consolidation or timing of fear responses from each of these regions. The CeL receives CRF-containing projections from the paraventricular nucleus of the hypothalamus (PVN) and projects via CRF-containing neurons to the lBNST. CRF release in the lBNST may result in the slow activation of a sustained fear response. Amygdaloid output regions include the PVN and lateral hypothalamus (LH), the periaqeductal gray (PAG), and the dorsal motor nucleus of the vagus (DMN X) and nucleus tractus solitarus (NTS). Each of these regions has different hormonal, behavioral or autonomic effects that contribute to fear and anxiety responses.
Black arrows – Glutamatergic projections
Red arrows – GABAergic projections
Blue arrows – CRF containing projections

7. Conclusion

Although a great deal of research has helped to understand the neurocircuitry within the amygdala, it is important to note that further study is required to gain a greater understanding of the many roles of this brain region. The role of the amygdala in fear, anxiety and aversive stimuli underlie much of the current knowledge that we have regarding its function. A role for the amygdala has been identified in appetitive and rewarding behaviors, social interaction, modulation of memory formation and recall, motivation, and attention. Although these topics have been studied through both human and animal research, it seems important to attempt to identify the specific neurocircuitries and mechanisms by which the amygdala may regulate these behaviors using the current models identified by fear conditioning as a guide. Greater understanding of the mechanisms underlying these behaviors may allow us to more specifically identify the role of the amygdala in human pathological conditions that include addiction, anxiety-spectrum disorders, personality disorders, autism, and other mood disorders. Furthermore, this understanding could assist in the identification of treatment paradigms and pharmacotherapeutic intervention, as well as neuropsychiatric diagnosis of specific pathologies. Furthermore, it may be important to consolidate current findings regarding the amygdala and attempt to reassemble a consolidated view of the brain region in light of the controversy that exists in the discussion of the amygdala as an anatomical and functional unit.

C. Brain-derived Neurotrophic Factor and Synaptic Plasticity

1. Introduction

Of the many aspects of brain function, the one that may be most important in the adaptive behaviors of animals, and especially humans, is the modulation of neuronal function which allows for learning. The functional and structural modification of synapses had been theorized as key factors in the ability for neurons to undergo associative learning since the histological works of Ramon y Cajal. Synaptic plasticity describes the regulation of synaptic structure and function in response to changes in neuronal activity or local neurochemistry. Long-term potentiation (LTP) is a type of increase in synaptic strength such that presynaptic neuronal firing results in the increased likelihood of firing of the postsynaptic neuron (Kandel et al., 1991). It has been found that the dynamic modulation of mRNA and protein expression in the neuron regulates synaptic plasticity and plays a role in the function of individual neurons and neuronal systems (Bailey et al., 1992; Kandel et al., 1991). Complex regulation by a vast network of intracellular signaling mechanisms and transcription factors is responsible for the downstream changes in expression that modulate synaptic strength (Poo, 2001; Thomas and Huganir, 2004; Waltereit and Weller, 2003).

The brain-derived neurotrophic factor (BDNF) signaling cascade is one such mechanism that is tightly connected to the regulation of both synaptic strength and structure (Bramham, 2008b; Messaoudi et al., 2007; Poo, 2001; Rex et al., 2007; Soule et al., 2006; Ying et al., 2002). The complex structure of the *BDNF* gene allows for tight and rapid control over the expression of BDNF and downstream effects associated with BDNF signaling which include neuronal maturation, neurite outgrown, and regulation

structural and functional synaptic plasticity (Horch, 2004; Messaoudi et al., 2002; Soule et al., 2006; West, 2008). BDNF signaling via the high affinity receptor, tyrosine receptor kinase B (TrkB), results in downstream modulation of the activity-regulated cytoskeleton-associated (Arc) immediate-early gene, also known as *Arg3.1* (Bramham et al., 2008; Pandey et al., 2008b; Ramanan et al., 2005; Ying et al., 2002). Arc mRNA has been shown to be translocated into dendrites such that local translation of Arc, also induced by BDNF, may mediate modulation of structural and functional plasticity at the dendritic spine tip (Bramham, 2008; Park et al., 2008; Soule et al., 2006; Steward and Worley, 2001). Dendritic spines are protuberances along the dendrite which make excitatory synaptic connections with presynaptic boutons. Numerous mechanisms which act within dendritic spines have been identified as regulators of dendritic morphology leading to structural plasticity (Bramham, 2008; Meng et al., 2002; Nakazawa et al., 2008a; Ultanir et al., 2007; Vickers et al., 2006). Dysregulation of BDNF signaling, Arc signaling and dendritic spine density have been shown to play a role in various neurological and psychiatric disorders, including anxiety and alcoholism (Autry and Monteggia, 2012; Davis, 2008; Pandey et al., 2006; Pandey et al., 2008b; Tsankova et al., 2006; Zuccato and Cattaneo, 2009). In the following chapter, I will review the structure and regulation of the *BDNF* gene, mechanisms of BDNF-Arc signaling in the modulation of dendritic spines and the role of these synaptic factors in anxiety and alcoholism.

2. BDNF: from gene to protein

BDNF is a neurotrophin protein family member with a complex gene structure which allows for precise control of temporal, brain region specific and subcellular expression patterns dependent upon differential regulation of mRNA variants and post-translational

events (Aid et al., 2007; Baj et al., 2011; Lu, 2003; Pattabiraman et al., 2005). *BDNF* is a complex gene coded between a common 3' exon that codes for the BDNF precursor, preproBDNF, and at least eight different 5' non-coding exons that have unique promoter regions (Aid et al., 2007). Originally, four *BDNF* exons had been identified and classified as *BDNF* exons I-IV (Timmusk et al., 1993). More recently, Aid et al. (2007) discovered novel BDNF exons and reclassified the naming convention to include non-coding *BDNF* exons I-VIII and the coding exon IX. The previous nomenclature was changed such that *BDNF* exon III became *BDNF* exon IV and *BDNF* exon IV became *BDNF* exon VI (Aid et al., 2007). Also identified were alternative splice sites in *BDNF* exon II which were classified as *BDNF* exons IIA-C and a variant of the coding exon classified as *BDNF* exon IXA (Aid et al., 2007). For the purposes of the review and research presented here, the newer classification of the *BDNF* gene will be used.

Differential promoter-specific transcription of *BDNF* followed by alternative splicing and polyadenylation produces at least 18 different BDNF mRNAs that appear to regulate the function and cellular localization of the BDNF protein (Aid et al., 2007; An et al., 2008; Greenberg et al., 2009; Timmusk et al., 1993). Activity-dependent processes which modulate BDNF expression also appear to display promoter specificity (Aid et al., 2007; Tao et al., 2002; Timmusk et al., 1993). Interestingly, translation of the various BDNF mRNAs that are produced results in a common BDNF precursor, preproBDNF, that is eventually cleaved to form a common mature BDNF (mBDNF) protein (Greenberg et al., 2009; Mowla et al., 1999). Although many of the complexities of regulation of the *BDNF* gene are not fully understood, I will introduce examples of promoter-specific

transcription, post-transcriptional modification and protein cleavage in the activity-dependent, subcellular trafficking, and cellular signaling functions of BDNF.

Activity-dependent regulation of *BDNF* transcription was initially discovered by the observation of a transient increase in BDNF mRNA following induction of epileptic seizures (Dugich-Djordjevic et al., 1992; Ernfors et al., 1991; Isackson et al., 1991). These studies specifically identified that the increase in BDNF mRNA showed temporal and regional specificity, with increases seen in hippocampal, cortical and amygdaloid brain regions (Dugich-Djordjevic et al., 1992; Ernfors et al., 1991; Isackson et al., 1991). Hippocampal BDNF mRNA expression has also been found to be increased in behavioral experiments testing learning and memory, such as the Morris water maze and fear conditioning paradigms (Falkenberg et al., 1992; Lubin et al., 2008; Ma et al., 1998; Ou and Gean, 2007). Furthermore, BDNF mRNA is increased by tetanic stimulation and it has been mechanistically shown that the upregulation of BDNF may be responsible for functional and structural changes which underlie LTP (Chen et al., 1999; Messaoudi et al., 2002; Patterson et al., 1992; Ying et al., 2002). Together, these data indicate that activity-dependent transcription of BDNF plays a role in the regulation of LTP that may underlie learning and memory.

Research into the activity-dependent regulation of BDNF has primarily focused on *BDNF* exons I and IV, however to a lesser extent the other *BDNF* exons are also induced by neuronal activity in a temporally specific manner (Aid et al., 2007). In both rat and human studies of *BDNF* gene regulation, exons I and IV are the promoters that show the greatest induction by neuronal depolarization (Aid et al., 2007; Pruunsild et al., 2011). These promoters have been found to be under the regulation of the CREB gene

transcription factor which has been tightly linked to synaptic plasticity and plays an important role in the regulation of gene expression associated with activity-dependent processes (Abel and Kandel, 1998; Alberini, 2009; Shieh et al., 1998; Tabuchi et al., 2002; Tao et al., 1998). It is worth noting that other transcription factors, including nuclear factor-kappa B (NFκB) and myocyte enhancer factor 2 (MEF2), may also play a role in the regulation of transcription at these *BDNF* promoters (Flavell et al., 2008; Jiang et al., 2008; Lubin et al., 2007; Pruunsild et al., 2011). It is also interesting to note that in the case of *BDNF* exon IV, CREB appears to play a role in activity-dependent transcription, whereas in the case of *BDNF* exon I, CREB may be specifically involved in gene transcription at basal levels (Pruunsild et al., 2011). Recently, a study by Hong et al. (2008) found that CREB-dependent regulation of the *BDNF* exon IV promoter was required for activity-dependent functions of BDNF. Specifically, a mutation was introduced into the *BDNF* exon IV gene promoter that disrupted CREB binding. The increase in hippocampal BDNF mRNA resulting from neuronal depolarization or sensory experience was significantly impaired, strongly suggesting the importance of *BDNF* exon IV in activity-dependent processes (Hong et al., 2008). Epigenetic mechanisms also play a role in the promoter-specific regulation of BDNF expression (Huang et al., 2002; Martinowich et al., 2003; Tsankova et al., 2006; Tsankova et al., 2004; Zhou et al., 2006). Although the epigenetic regulation of BDNF will be presented in greater detail in following sections, it is worth noting that behavioral conditioning paradigms, seizure activity and exposure to drugs of abuse appear to modify levels of histone acetylation, DNA methylation and methyl CpG binding protein 2 (MeCP2) binding (see below) in a promoter-specific manner (Fuchikami et al., 2010; Kumar et al., 2005; Lubin et al., 2008;

Tsankova et al., 2004). Although this is not an exhaustive review of the literature, these data suggest that various elements regulate the *BDNF* gene in a promoter-specific manner such that exons I and IV play an important role in activity-dependent processes.

Early studies into the cellular localization of BDNF identified immunoreactivity to the protein in the neuronal cell soma and fibers (Altar et al., 1997; Conner et al., 1997). In certain brain regions a lack of BDNF mRNA coupled with the presence of BDNF protein suggested anterograde transport of BDNF (Altar et al., 1997). It is worth noting that although anterograde transport of BDNF may exist, findings regarding the absence of BDNF mRNA in some brain regions, for instance the CeA, have been refuted using more sensitive techniques (Moonat et al., 2011; Pandey et al., 2006; Prakash et al., 2008; Zhou et al., 2005). Since these earlier studies, both anterograde and retrograde transport of BDNF have been identified in various brain regions (Adachi et al., 2005; Gustafsson et al., 2003; Jezierski and Sohrabji, 2003; Kohara et al., 2001; Magby et al., 2006). An emerging topic of research into the regulation of synaptic plasticity is the trafficking of mRNA into the dendrites for local translation (Bramham, 2008b; Sutton and Schuman, 2006). It has been suggested that local translation of synaptically active proteins may play an important role in the consolidation of synapse-specific changes that underlie LTP (Bramham, 2008b; Sutton and Schuman, 2006). This was first identified by the observation that treatment of synapses isolated from the cell soma with a protein synthesis inhibitor, anisomycin, resulted in a reduction in BDNF-induced LTP (Kang and Schuman, 1996). This finding suggested that BDNF-LTP could be dependent on the local translation of mRNA, and it has since been identified that BDNF- and activity-

dependent processes target both BDNF and TrkB mRNAs to the distal dendrites (Righi et al., 2000; Tongiorgi et al., 2004; Tongiorgi et al., 1997).

The dendritic targeting of BDNF mRNA has been shown to be regulated in part by polyadenylation of the BDNF transcript. The polyadenylation of BDNF can occur at either of two sites in the *BDNF* gene leading to two separate populations of BDNF mRNA, one with a short 3' untranslated region (UTR) and the other with a long 3' UTR (Timmusk et al., 1993). Recently, An et al. (2008) identified that the short and long 3' UTR of BDNF mRNA differentially regulated the subcellular localization of transcripts. Specifically, the study found that transcripts containing the long 3' UTR were preferentially targeted to the dendritic compartment of neurons (An et al., 2008). Furthermore, mutation of the polyadenylation site producing the long transcript without affecting the short transcript resulted in the impairment of dendritic trafficking of BDNF mRNA (An et al., 2008). The mutation functionally resulted in dendritic spine dysmorphogenesis and impairment of LTP induction (An et al., 2008). A follow-up study found differences in the regulation of the short and long BDNF transcripts in response to neuronal activity (Lau et al., 2010). The results indicated that the short 3' UTR transcripts were translated in greater amounts under basal conditions, whereas the long 3' UTR transcripts showed greater induction by neuronal activity (Lau et al., 2010). Furthermore, another study found that local translation of BDNF mRNA in dendrites by eukaryotic elongation factor 2 (eEF2) was associated with changes in dendritic spine morphology (Verpelli et al., 2010). These data suggest an important role for dendritic localization of BDNF mRNA and introduce another layer of complexity in the regulation of *BDNF* gene function.

Post-transcriptional modifications to the BDNF precursor protein, proBDNF, also appear to regulate the secretion of BDNF and the function of BDNF signaling. Early studies into BDNF secretion identified that neuronal activity regulated the release of BDNF from both axonal terminal and dendritic compartments (Fawcett et al., 1998; Goodman et al., 1996; Haubensak et al., 1998). Further studies utilizing GFP tagged BDNF constructs identified the protein in dense core vesicles and confirmed that secretion is primarily driven by an activity-dependent mechanism (Hartmann et al., 2001; Kojima et al., 2001; Tyler et al., 2002). BDNF exists as preproBDNF in the endoplasmic reticulum before cleavage of the signal peptide leads to trafficking of proBDNF to the golgi apparatus for packaging in secretory vesicles. The pro-region has been hypothesized to play a role in the differential sorting of neurotrophins into constitutive and regulated secretory pathways. Specifically, proBDNF has been found to bind various chaperone proteins, such as carboxypeptidase E and sortilin, which may be responsible for primary role of regulated secretion in the function of BDNF (Chen et al., 2005; Lou et al., 2005; Mowla et al., 1999). Interestingly, there exists a single nucleotide polymorphism (SNP) from G to A at nucleotide 196 which results in a valine to methionine substitution at codon 66 within the pro region (val66met) that appears to affect sorting of BDNF into secretory granules impairing activity-dependent secretion (Chen et al., 2005; Egan et al., 2003). In human and animal studies, the val66met polymorphism has been shown to be associated with psychiatric disorders such as anxiety (see below) suggesting the importance of the pro region of BDNF in behavioral phenotypes and neuronal function (Chen et al., 2006; Lang et al., 2005a).

Although BDNF was previously believed to be exclusively secreted following the cleavage of proBDNF to mBDNF by proteolytic enzymes within the secretory vesicles, more recent studies have identified a role for proBDNF secretion (Pang and Lu, 2004; Teng et al., 2005; Woo et al., 2005). *In vitro* research indicated that extracellular cleavage of proBDNF occurred via secretion of tissue plaminogen activator (tPA) which activated the protease plasmin producing mBDNF that was required for the consolidation of LTP in hippocampal cell cultures (Pang and Lu, 2004). Effects have also been seen for proBDNF signaling itself, which has been shown to bind preferentially to the $p75^{NTR}$ receptor as is the case with various other proneurotrophins (Lee et al., 2001; Teng et al., 2005; Woo et al., 2005). The effects of proBDNF signaling via $p75^{NTR}$ have been shown to include the regulation of neuronal apoptosis and long-term depression (LTD) (Teng et al., 2005; Woo et al., 2005). Although a greater deal of research is required to elucidate regulation of the divergent roles of proBDNF and mBDNF, more recent studies have provided evidence that the two forms act in conjunction to regulate neuronal development and synaptic plasticity underlying learning and memory (Barnes and Thomas, 2008; Nagappan et al., 2009; Yang et al., 2009).

The examples provided above display the complexities involved in the regulation of BDNF expression and function at transcriptional, post-transcriptional and post-translational levels. Although substantial steps have been made into the understanding of BDNF, further study is required to fully elucidate the functional roles of the gene structure, and mRNA and protein processing. The recent exploration of epigenetic regulation of BDNF (see below) adds complexity to this signaling pathway and highlights the various levels of regulation that maintain tight control of BDNF expression patterns.

3. BDNF-Arc signaling in functional and structural plasticity

Intracellular signaling by BDNF binding to the high-affinity TrkB receptor results in the downstream induction of the Arc immediate-early gene and plays a key role in the regulation of synaptic plasticity and dendritic spine structure (Bramham et al., 2008; Poo, 2001; Tyler et al., 2002). The intracellular components involved in BDNF signaling are well understood (Poo, 2001; Reichardt, 2006), however downstream events which regulate changes in synaptic structure and function are only beginning to be elucidated. As with other neurotrophins, BDNF binds to a high-affinity receptor, TrkB, and a low-affinity receptor, $p75^{NTR}$ (see above). The role of BDNF-Arc signaling in synaptic plasticity has primarily been studied with regards to TrkB binding, so particular focus will be on this pathway. The $p75^{NTR}$ receptor is tumor necrosis factor family member that consists of a transmembrane domain attached to an intracellular death domain (Reichardt, 2006). Binding of BDNF to the $p75^{NTR}$ receptor may be primarily involved in development, however recent research into the binding of proBDNF suggests a role in synaptic plasticity (see above) (Barnes and Thomas, 2008; Nagappan et al., 2009; Teng et al., 2005; Woo et al., 2005; Yang et al., 2009).

The Trk family of receptors makes up the high-affinity receptors preferential for specific neurotrophin binding such that TrkA binds nerve growth factor (NGF), TrkB binds BDNF and neurotrophin 4 (NT4), and TrkC binds neurotrophin 3 (NT3). The structure of each of the receptors consists of an extracellular binding domain, a single transmembrane domain, an intracellular catalytic domain with tyrosine kinase activity and tyrosine residues (Reichardt, 2006). Neurotrophin binding results in dimerization of the receptor and autophosphorylation of intracellular tyrosine residues which serve as

phosphorylation-dependent binding sites for adaptor proteins (Minichiello, 2009; Reichardt, 2006). The TrkB receptor exists in three distinct forms produced by alternative splicing, the full-length receptor, $TrkB^{TK+}$, and two truncated receptors, TrkB-T1 and TrkB-T2. The truncated TrkB variants lack intrinsic tyrosine kinase activity and thus do not undergo dimerization or autophophorylation following BDNF binding (Minichiello, 2009). Despite the apparent lack of activation, the truncated forms of TrkB are expressed throughout the brain in both neurons and glia, and recent research indicates that they may play a role in BDNF signaling via reduction of BDNF-$TrkB^{TK+}$ binding and differential intracellular signaling events (Davis, 2008; Luberg et al., 2010). It is also worth noting that increased expression of truncated TrkB variants has recently been associated with psychiatric disorders, including schizophrenia and depression (Razzoli et al., 2011; Wong et al., 2011). Due to the limited research available on truncated forms of TrkB, the rest of this review will focus on $TrkB^{TK+}$, which will be referred to as TrkB for simplicity.

Following BDNF binding, dimerization and autophosphorylation of TrkB leads to receptor internalization and activation of various intracellular signaling cascades (Reichardt, 2006). Three major signaling cascades are initiated by TrkB activation, the mitogen-activated protein kinase (MAPK) pathway, the phosphatidyl inositol 3-kinase (PI3K) pathway and the phospholipase Cγ (PLCγ) pathway (Minichiello, 2009). Phosphorylation of tyrosine residues on TrkB leads to binding and phosphorylation of Shc adaptor proteins via Src homology 2 (SH2) domain phosphotyrosine interactions. The presence of Shc proteins results in the recruitment of growth factor receptor-bound protein 2 (Grb2) and son of sevenless (SOS) protein which bind and activate Ras protein,

a soluble small GTPase. Ras GTPase phosphorylation results in the activation of PI3K directly, and mitogen-activated protein kinase kinase (MEK) followed by extracellular-signal regulated kinase (Erk) via Raf protein activation. PLCγ binds directly to TrkB via SH2 domain interactions which results in the production of diacylglycerol (DAG) and inositol-1,4,5 triphosphate (IP3), which in turn lead to activation of protein kinase C (PKC) and release of intracellular Ca^{2+} stores (Minichiello, 2009; Reichardt, 2006). Downstream signaling via activated protein kinases, in particular Erk, results in the activation of CREB and Elk-1 gene transcription factors that regulate Arc expression (Bramham et al., 2008; Pandey et al., 2008b; Ramanan et al., 2005; Ying et al., 2002).

Various lines of evidence indicate that there is significant interplay between BDNF and CREB. As described above, BDNF signaling results in the activation of CREB, and conversely the *BDNF* gene contains an element allowing for CREB binding resulting in upregulation of BDNF expression (Hong et al., 2008; Pandey et al., 2006; Pandey et al., 2005; Pizzorusso et al., 2000; Pruunsild et al., 2011; Tao et al., 1998; Ying et al., 2002). The TrkB receptor is also upregulated by BDNF signaling, which may also be mediated by CREB-dependent transcription (Deogracias et al., 2004; Pandey et al., 2006). Thus, BDNF signaling appears to act via activation of CREB which may in turn result in upregulation of BDNF. The regulation of synaptic plasticity occurs through various changes at the synapse that result in both functional and structural changes. Although short-term changes such as early LTP may be caused by modification of existing proteins, long-term functional and structural synaptic changes require the synthesis of new mRNA and protein (Abel and Kandel, 1998; Bailey et al., 1992; Bramham, 2008a; Citri and Malenka, 2008; Kandel et al., 1991; Malinow and Malenka,

2002). CREB and BDNF, especially through activation by Erk, have been identified as critical mediators of long-term changes in synaptic structure and function (Abel and Kandel, 1998; Bramham and Messaoudi, 2005; Pandey et al., 2008b; Ying et al., 2002).

The dendritic spine is a protrusion that makes up the postsynaptic terminal, which allows for the compartmentalization of intracellular signaling and protein synthesis resulting in functional and structural changes at a single synapse (Bramham, 2008a; Matsuzaki et al., 2004; Yuste et al., 2000). Modulation of dendritic spine morphology results from the dynamic regulation of actin that makes up the cytoskeleton of the postsynaptic density (PSD) (Cingolani and Goda, 2008; Fischer et al., 1998; Matus, 2000). Current theories implicate a role for the Rho family of small GTPases, which may provide a link between upstream regulators of dendritic morphology and downstream effectors that directly interact with filamentous actin (F-actin) and scaffolding proteins of the postsynaptic density (PSD) (Tada and Sheng, 2006). Furthermore, there is evidence that indicates that interactions between structural PSD proteins and NMDA receptors at the spine tip may also regulate spine morphology (Nakazawa et al., 2008a; Pak et al., 2001; Vickers et al., 2006). In order to regulate synapse-specific changes at the dendritic spine, proteins translated somatically must be targeted to the synapse or mRNAs must be locally translated at the synapse (Bramham, 2008a; Frey and Morris, 1997; Steward, 1997).

Although a complex network of mechanisms regulates local events at the dendritic spine, the Arc immediate-early gene (IEG) appears to play an important role in CREB- and BDNF-mediated changes in synaptic plasticity (Bramham et al., 2008; Messaoudi et al., 2007; Pandey et al., 2008b; Ying et al., 2002). The initial

characterization of *Arc* identified a unique single copy gene with mRNA inducible by neuronal activity in the hippocampus that localized within dendrites and co-precipitated with F-actin (Link et al., 1995; Lyford et al., 1995). These findings strongly suggested *Arc* as an IEG with a role in the regulation of single synapse plasticity and dendritic spine structure (Link et al., 1995; Lyford et al., 1995). Exposure to drugs of abuse also found the upregulation of Arc mRNA in striatal brain regions, and more recent studies have generalized the role of Arc to various brain regions, including the amygdala (Fosnaugh et al., 1995; Kodama et al., 1998; Kunizuka et al., 1999; Pandey et al., 2008b). Thus, it has been found that various activity-dependent processes, including the secretion of BDNF, result in the upregulation and dendritic localization of Arc mRNA throughout the brain (Bloomer et al., 2008; Pandey et al., 2008b; Steward and Worley, 2001b; Waltereit et al., 2001; Yin et al., 2002; Ying et al., 2002). Local regulation of Arc at the dendritic spine appears to play numerous roles in functional and structural changes during LTP and LTD (Bramham et al., 2010a; Bramham et al., 2008; Shepherd and Bear, 2011), however this discussion will be limited to functions of BDNF-Arc signaling in LTP.

BDNF signaling via CREB and other transcription factors plays an important role in the transcriptional regulation of Arc (Pandey et al., 2008b; Yin et al., 2002; Ying et al., 2002). The *Arc* gene contains a number of enhancer elements that link transcriptional regulation of Arc to BDNF signaling (Kawashima et al., 2009; Pintchovski et al., 2009; Waltereit et al., 2001). BDNF infusion has been shown to upregulate Arc via recruitment of serum response factor (SRF) which binds to a serum response element (SRE) and recruitment of a novel transcription factor which binds to a "zeste-like" element, which are both located upstream of the coding region (Pintchovski et al., 2009; Waltereit et al.,

2001). Elk-1, another transcription factor that is activated downstream of BDNF signaling, binds to SRF and may play a role in the regulation of Arc (Besnard et al., 2011; Pandey et al., 2008b). Another unique enhancer, termed the synaptic activity-regulated enhancer (SARE), appears to contain a binding site for CREB and SRF, and may be necessary for rapid induction of Arc by synaptic activity (Kawashima et al., 2009).

The regulation of learning and memory via study of LTP and behavioral paradigms has been found to involve BDNF signaling via the induction of Arc. Early studies into this mechanism found that exogenous infusion of BDNF into the hippocampus characterized a unique form of LTP regulated by Erk-dependent modulation of protein transcription, resulting in a persistent increase in synaptic strength (Ying et al., 2002). The induction of BDNF-induced LTP was shown to correlate with an increase in CREB phosphorylation and the upregulation of Arc mRNA (Ying et al., 2002). A follow-up study examining BDNF infusion in the presence of a transcription inhibitor suggested that the upregulation of Arc mRNA is a requirement for BDNF-induced LTP (Messaoudi et al., 2002). Knockdown of hippocampal Arc *in vivo* via antisense oligodeoxynucleotides (ODN) resulted in the impairment of LTP and learning in behavioral conditioning paradigms, and similar findings have been reported in behavioral studies involving Arc knockout mice (Guzowski et al., 2000; Messaoudi et al., 2007; Plath et al., 2006). Another study utilizing the phosphodiesterase inhibitor rolipram, found increased levels of p-CREB and Arc which correlated to improvement of fear memory consolidation, implicating CREB-dependent transcription in the role of Arc in learning and memory (Monti et al., 2006). It is important to note that the effects of BDNF-Arc signaling are very specific to the time course of LTP events. By infusing Arc

antisense ODNs at various time-points during the induction of LTP, Messaoudi et al. (2007) determined that sustained upregulation of Arc is required for late-phase LTP maintenance, whereas blockade of Arc only in the early-phase of LTP does not affect late-phase consolidation. Arc knockout mice also show temporal abnormalities in the LTP phenotype such that early LTP is actually enhanced while late LTP is impaired (Plath et al., 2006). An *in vivo* study utilizing Arc antisense ODNs infused into the lateral amygdala also found that Arc knockdown impaired the consolidation of memory during a fear conditioning paradigm, implicating Arc induction in neuronal learning in the amygdala (Ploski et al., 2008). Amygdaloid BDNF signaling has also been implicated in the consolidation of fear learning in various studies, including those which have shown exon-specific increases in *BDNF* gene transcription (Koponen et al., 2004; Lubin et al., 2008; Ou and Gean, 2007). These studies underline the importance of BDNF signaling via Arc transcription in the regulation of learning and memory at the synaptic and behavioral levels in various brain regions, including the amygdala.

The induction of LTP has been found to be accompanied by changes in dendritic spine structure that may be required for long-term modifications in synaptic plasticity (Bramham, 2008a; Matsuzaki et al., 2004). BDNF-Arc signaling has also been implicated in the regulation of dendritic morphology and expansion of dendritic spines in the regulation of synaptic plasticity. Although the specific effects of BDNF were inconsistent, early studies identified BDNF signaling as a mediator of the changes in dendritic spine morphology and DSD that accompany modulation of synaptic strength (Horch et al., 1999; Murphy et al., 1998; Shimada et al., 1998). The BDNF-mediated increase in dendritic spine density (DSD) was later found to be regulated by cAMP

signaling and Erk1/2, implicating a potential role for the activation of CREB (Alonso et al., 2004; Ji et al., 2005). More recently, Arc protein has been shown to play a role in the stabilization of F-actin, resulting in the regulation of rapid activity-dependent modulation of dendritic spine size (Huang et al., 2007; Messaoudi et al., 2007). The local translation and targeting of Arc following LTP induction appears to be dependent on Erk1/2 activation and the stabilization of actin in the dendritic spine (Huang et al., 2007). The increase in Arc expression following LTP induction also increases the phosphorylation of cofilin, which is directly involved in the stabilization of F-actin (Messaoudi et al., 2007). Conversely, the application of Arc antisense ODNs was shown to reverse LTP and result in the dephosphorylation of cofilin which was associated with actin depolymerization and dendritic spine retraction (Messaoudi et al., 2007). Interestingly, the application of the F-actin stabilizing compound jasplakinolide reversed the effect of Arc antisense on LTP (Messaoudi et al., 2007). Thus, it appears that the effects of BDNF-Arc signaling on LTP require regulation of the actin cytoskeleton by Arc, and result in the stabilization and extension of dendritic spines resulting in increased DSD.

4. BDNF and synaptic plasticity in anxiety and alcoholism

Aberrations in the regulation of BDNF signaling and synaptic plasticty have been suggested as key mediators of psychiatric and neurological disorders, including schizophrenia, neurodegenerative diseases, mood disorders and substance abuse (Agartz et al., 2006; Angelucci et al., 2005; Chao et al., 2006; Davis, 2008; Russo et al., 2009; Zuccato and Cattaneo, 2009). BDNF signaling has been shown to play a role in various aspects involved in the development of alcoholism (Davis, 2008; Lang et al., 2005a; Lubin et al., 2008; Pandey et al., 2006; Pandey et al., 2008b). Early research identified a

reduction in BDNF expression in the hippocampus and cortex following withdrawal from chronic ethanol exposure (MacLennan et al., 1995; Pandey et al., 1999b). The infusion of BDNF was also found to play a role in the maintenance of ethanol tolerance following withdrawal (Szabo and Hoffman, 1995). These early studies identified an effect of ethanol exposure on BDNF.

Further studies aimed to clarify the role of BDNF in alcoholism and alcohol consumption. BDNF haplodeficient mice have previously been found to display increased voluntary ethanol consumption patterns under certain conditions in comparison to wild-type mice (Hensler et al., 2003; Jeanblanc et al., 2006; McGough et al., 2004). Acute and voluntary ethanol exposure increased the level of BDNF in the dorsal striatum, and the BDNF haplodeficient mice displayed increased sensitivity to the effects of ethanol (Jeanblanc et al., 2006; McGough et al., 2004). These studies identified that reduced BDNF increased ethanol consumption, whereas ethanol consumption increased the levels of BDNF in the dorsal striatum (Jeanblanc et al., 2006; McGough et al., 2004). Inhibition of TrkB receptor function in the dorsal striatum also increased alcohol consumption in wild-type mice, but not BDNF haplodeficient mice (Jeanblanc et al., 2006). A following study confirmed the importance of endogenous BDNF signaling in the dorsolateral striatum in the regulation of ethanol consumption, such that reduced BDNF was associated with increased ethanol intake (Jeanblanc et al., 2009). Various studies in cell culture have also found a cytoprotective role against ethanol induced damage in neurons (Mitchell et al., 1999; Sakai et al., 2005). Taken together, these findings suggest that BDNF may be neuroprotective against changes induced by alcohol

consumption that may play a role in neuroadaptations associated with the development of alcohol dependence and the damaging effects of alcohol consumption.

Various findings also implicate BDNF as a common factor underlying the comorbidity to anxiety and alcoholism. The Val66Met polymorphism in the BDNF gene has been found to result in a reduced BDNF secretion phenotype (Chen et al., 2008; Egan et al., 2003). Human studies identified a positive association between the Met variant of the BDNF polymorphism and the development of anxiety spectrum disorders and alcoholism (Jiang et al., 2005; Lang et al., 2005b; Matsushita et al., 2004; Uhl et al., 2001). Although these previous studies measured the effects on anxiety and alcoholism separately, a more recent human study identified that presence of the Met variant was associated with both measures of higher anxiety and alcohol consumption than individuals that were homozygous for the Val variant (Colzato et al., 2011). A human imaging study also found that patients with anxiety associated with the Met-variant BDNF polymorphism showed hyperactivation of the amygdala and hippocampus when exposed to anxiety-inducing stimuli, in comparison to Val-variant patients and normal subjects, thus identifying neuroanatomical correlates of these behavioral findings (Lau et al., 2010). Generation of a mouse model homozygous for the Met variant confirmed the presence of the reduced BDNF secretion phenotype and identified increased anxiety-like behaviors (Chen et al., 2008; Chen et al., 2006). This mouse model has allowed for greater behavioral and functional study of the polymorphism and has identified a reduction in extinction of fear conditioned behavior, blockage of dendritic BDNF trafficking, and impairment of synaptic plasticity (Bath et al., 2012; Chiaruttini et al., 2009; Ninan et al., 2010; Yu et al., 2009). Taken together, these studies suggest a role for

a genetic polymorphism resulting in reduced BDNF signaling and the impairment of synaptic plasticity in the comorbidity of anxiety and alcoholism.

Numerous other strategies have been employed to further identify the specific mechanisms and brain regions that are responsible for the role of BDNF in comorbid anxiety and alcoholism. An early study found an association between the reduction in BDNF and the precipitation of withdrawal-induced anxiety-like behaviors (Pandey et al., 1999b). Conditional deletion of BDNF or TrkB, the BDNF receptor, has also been shown to result in increased anxiety-like behaviors (Bergami et al., 2009; Rios et al., 2001). In contrast, overexpression of the TrkB receptor reduced measures of anxiety-like behaviors (Koponen et al., 2004). More recently, human studies have also shown that following withdrawal from chronic alcohol use the resultant reduction in BDNF serum levels is associated with the severity of withdrawal symptoms (Heberlein et al., 2010; Huang et al., 2011).

CREB-haplodeficient mice have been also found to display higher anxiety-like behaviors and alcohol preference than wild-type mice, which was associated with lower levels of BDNF expression in various brain regions including the amygdala (Pandey et al., 2004). Acute ethanol administration had an anxiolytic effect in these mice that was associated with an increase in p-CREB and another CREB-target, neuropeptide Y (NPY), specifically in the CeA and MeA, but not BLA (Pandey et al., 2004). Interestingly, the anxiety-like behaviors and alcohol preference of P rats are also associated with lower levels of both CREB and BDNF in the CeA and MeA, but not BLA, in comparison to NP rats (Pandey et al., 2005a; Prakash et al., 2008). An antisense oligodeoxynucleotide (ODN) strategy examined the mechanistic role for amygdaloid signaling BDNF in

alcohol consumption in a study utilizing an unselected stock of rats (Pandey et al., 2006). BDNF antisense ODNs were infused into the CeA of Sprague-Dawley rats resulting in an increase in alcohol consumption and anxiety-like behavior (Pandey et al., 2006). Co-infusion of BDNF protein along with antisense ODNs restored control levels of alcohol consumption and anxiety-like behavior (Pandey et al., 2006). Taken together, these data strongly implicate a role for reduced BDNF in amygdaloid brain regions, particularly the CeA, in the association between innate anxiety and alcohol preference.

Dendritic spine morphology, a structural correlate of synaptic plasticity, shows an association with ethanol exposure such that chronic ethanol treatment reduces DSD in various areas of the brain (Lee et al., 1981; Riley and Walker, 1978). Much research following this discovery attempted to morphometrically characterize the changes in dendritic spines and it was found that the overall decrease in spines was accompanied by an increase in wide and stubby spines (Lescaudron et al., 1989). Although this may suggest an increase in immature spines, the functional implications of modulation of dendritic morphology by ethanol has not yet been explored (Chandler, 2003). Recently, more studies have used technological advances, such as two photon microscopy, to explore possible mechanisms mediating the effect of ethanol on spine morphology and dynamics. A study utilizing this approach analyzed the dendritic morphology of P rats in the NAc, and identified that chronic ethanol exposure caused dendritic dysmorphology and decreased DSD (Zhou et al., 2007). The study identified that truncated NR1 subunit, which has been implicated in the regulation of spine morphology, was upregulated within the spine tip, suggesting a role for the regulation of the NMDA receptor subunit composition in the effects of ethanol (Zhou et al., 2007). Another study found that

increased NR2B expression in hippocampal cell cultures following exposure to chronic ethanol increased NMDA clustering with the post-synaptic density protein, PSD-95, which positively correlated with an increase in dendritic spine size (Carpenter-Hyland and Chandler, 2006). These studies provide an attractive model linking ethanol-mediated changes in NMDA subunit composition to dendritic morphology. A recent study by Pandey *et al.* explored the interaction between amygdaloid BDNF-Arc signaling and DSD in the regulation of both anxiety and alcohol consumption (Pandey et al., 2008b). The study found that acute ethanol exposure in an unselected stock of Sprague-Dawley rats resulted in increased Arc expression and DSD in the CeA and MeA, but not BLA, which was associated with increased BDNF signaling. Conversely, withdrawal from chronic ethanol, which resulted in the precipitation of anxiety-like behaviors, was shown to decrease BDNF signaling, Arc expression and DSD (Pandey et al., 2008b). The study further aimed to mechanistically examine the role of BDNF via infusion into the CeA, and determined that increased BDNF signaling in alcohol withdrawn rats was anxiolytic and resulted in an increase in Arc protein (Pandey et al., 2008b). In contrast, infusion of Arc antisense ODNs into the CeA of Sprague-Dawley rats decreased Arc and DSD and resulted in increased anxiety-like behavior and alcohol consumption (Pandey et al., 2008b). These data provide a convincing role for Arc-mediated regulation of DSD in the promotion of alcohol drinking via the negative affective state of alcohol addiction. Very little is currently known about the modulation of dendritic spine morphology by ethanol, but recent studies have identified some mediators which may serve as a starting point to explore the complex regulation of structural changes. Given the vast amount of research currently looking at the regulation of dendritic spine morphology in other models of

learning and addiction, it appears important to employ similar studies and techniques within the field of alcohol research. Furthermore, various epigenetic mechanisms have been found to underlie the regulation of BDNF (see below) and may play a role in the observed interaction of synaptic plasticity, anxiety and alcoholism.

D. Epigenetic Mechanisms

1. Introduction to epigenetics

The study of genetics, initiated by Gregor Mendel in the late 19th century, aimed to identify principles that underlie the hereditary nature of biological reproduction (Berg et al., 2007). Original works identified patterns of heredity that appeared to exist across kingdoms of organisms, which eventually lead to the characterization of a molecule encoding the "code" of biology, DNA. Experiments from the work of Oswald Avery, Colin Macleod and Maclyn MacCarty in the 1930s to the groundbreaking identification of DNA structure by James Watson and Francis Crick in 1953 highlighted the undeniable importance of this molecule in defining the process of heritability (Avery et al., 1944; Berg et al., 2007; Watson and Crick, 1953). The importance of these discoveries led to a plethora of studies which aimed to identify the genetic code which underlies the transcription of DNA to RNA and RNA to amino acids, peptides and proteins (Berg et al., 2007; Crick, 1970; Crick, 1958). The decades that followed saw an increased understanding of the molecular mechanisms underlying transcription and translation, and widespread international studies were initiated to sequence the human genetic material in its entirety (IHGSC, 2004). Researchers believed that with a vast understanding of the genetic code and a complete sequencing of the genome, they could catalog and understand the genetic basis for phenotypic differences in both health and disease among

individuals (IHGSC, 2004). Although the vast amount of data uncovered by the Human Genome Project is still being analyzed and deciphered, it is now clear that there are other mechanisms that connect genotype and phenotype, including the mechanisms which underlie the field of epigenetics. The term epigenetics was initially coined by Conrad Waddington in 1942 who aimed to create a theory which could connect an individuals genes to the phenotype that they produced (Waddington, 1942). Waddington's theories developed to describe an epigenetic landscape that could modify the outcome of cellular differentiation despite the genetically inherited material (Waddington, 1957).

Since these early theories and studies, a vast array of epigenetic mechanisms have been identified which have helped define the field of epigenetics as the study of stable and dynamic changes in the regulation of gene expression that may be heritable through meiosis or mitosis, but do not result in modifications of the DNA sequence (Goldberg et al., 2007; Levenson and Sweatt, 2005). Much of the research that has been devoted to understanding epigenetic mechanisms has been focused on modifications of chromatin structure that may affect gene regulation (Black and Whetstine, 2011; Goldberg et al., 2007; Jenuwein and Allis, 2001). More recently, various studies have implicated a role for epigenetic mechanisms, including chromatin remodeling, in physiological and pathophysiological processes of brain function, including psychiatric disorders and the development of drug addiction (Borrelli et al., 2008; Guan et al., 2009; Levenson and Sweatt, 2005; Nestler, 2009; Renthal and Nestler, 2008; Robison and Nestler, 2011; Tsankova et al., 2007).

2. Chromatin structure and remodeling

Chromatin is the nuclear structure that comprises the chromosome and consists of DNA, RNA, histone proteins and other structural chromosomal proteins (Black and Whetstine, 2011). The basic component of chromatin is the nucleosome which is made up of histone core, consisting of histone H2A, H2B, H3 and H4 proteins, wrapped by 147 base pairs of DNA (Horn and Peterson, 2002; Kornberg and Klug, 1981; Kornberg and Lorch, 1999). The histone core is specifically an octamer which consists of two H2A-H2B dimers and one H3-H4 tetramer (Luger et al., 1997a; Luger et al., 1997b). Adjacent nucleosomes are connected by linker DNA giving chromatin structure a resemblance to beads of histone cores on a string of DNA. The DNA linker regions are bound to the H1 histone protein which may also bind to the histone octamer and play a role in overall chromatin structure (Jenuwein and Allis, 2001; Woodcock et al., 2006).

The structure of chromatin is complex and dynamically regulated by covalent modifications of both DNA and histone proteins (Jenuwein and Allis, 2001; Robison and Nestler, 2011). Although modifications in chromatin structure often occur locally, at the extremes of global regulation chromatin structure can be identified histologically as heterochromatin and euchromatin, which represent condensed and relaxed chromatin structure, respectively (Ross and Pawlina, 2006). Early studies identified that histone proteins could be acetylated or methylated and found that acetylation levels were positively correlated with increased transcription (Allfrey et al., 1964). As suggested by early studies, histone hyperacetylation is associated with euchromatin and increased transcription, whereas hypoacetylation is associated with heterochromatin and reduced transcription (Smith, 1991; Strahl and Allis, 2000). It has more recently been identified

that the N-terminal tails of histone proteins consist primarily of basic amino acids that interact with negatively charged DNA and play a role in the dynamic regulation of chromatin structure via a variety of covalent modifications, including acetylation, methylation, ubiquitylation, phosphorylation and ADP-ribosylation (Jenuwein and Allis, 2001; Levenson and Sweatt, 2005; Mersfelder and Parthun, 2006).

There are a variety of possible combinations of histone modifications that are each regulated by a vast network of enzymes that interact with one another and other regulatory proteins, resulting in a complex system regulating gene transcription and chromatin structure (Jenuwein and Allis, 2001; Levenson and Sweatt, 2005; MacDonald and Howe, 2009; Yang and Seto, 2008). Histone acetylation occurs at lysine residues within the N-terminus of histone proteins in a reaction catalyzed by histone acetyltransferases (HAT) which can be reversed by histone deacetylases (HDAC) (Figure 2) (Jenuwein and Allis, 2001; MacDonald and Howe, 2009; Thiagalingam et al., 2003). The complex interaction of HATs and HDACs results in the regulation of acetylation levels that regulate chromatin structure, such that it has been found that H3 and H4 acetylation are highly correlated with actively transcribed DNA (Figure 2) (Levenson and Sweatt, 2005; Vogelauer et al., 2000). Histone methylation at lysine and arginine residues is mediated by histone methyltransferases (HMT) that may have specificity for mono-, di-, or trimethylation (Black and Whetstine, 2011). Unlike acetylation, histone methylation may serve to repress or activate transcription. For instance, methylation of H3 lysine 4 is frequently associated with transcriptional activation, but methylation of H3 lysine 9 is associated with transcriptional repression (Bannister et al., 2001; Jenuwein and Allis, 2001). Epigenetic modifications at histone H3 lysine 9 appear to be tightly

associated with chromatin structure, such that acetylation of H3 lysine 9 results in relaxed chromatin while methylation at this residue results in condensed chromatin (Figure 2) (Bannister et al., 2001; Jenuwein and Allis, 2001; MacDonald and Howe, 2009). This suggests that the status of H3 lysine 9 may be an integral epigenetic mark which regulates transcriptional activity, and that monitoring of this residue during epigenetic analysis may be closely tied to chromatin structure. It is also worth noting that the level of methylation may also play a role in the functional effect on transcription. Histone H3 lysine 27 monomethylation is generally enriched in actively transcribed genes, while trimethylation at this same residue is associated with gene silencing (Rosenfeld et al., 2009). Histone phosphorylation occurs at serine and threonine residues within histone tails and appears to have a variety of cellular functions, including a significant role in processes surrounding chromosome condensation during mitotic division (Banerjee and Chakravarti, 2011). Phosphorylation can be catalyzed by numerous kinases, for instance ribosomal protein S6 kinase 2 (RSK2) which is a downstream effector of MAPK and other signal transduction pathways (Sassone-Corsi et al., 1999; Thomson et al., 1999). Ubiquitylation of histone proteins occurs at lysine residues and appears to mark entire histone proteins or particular protein regions for degradation. It has been suggested that this process may serve as a proteolytic checkpoint to reverse otherwise irreversible hypermethylation of chromatin (Jenuwein and Allis, 2001; Levenson and Sweatt, 2005).

Chromatin structure may also be modified via DNA methylation of cytosine residues, primarily those which lie within regions composed of cytosine and guanine repeats known as CpG regions (Figure 2). Throughout the genome there are areas of

CpG dinucleotides that display high levels of constitutive methylation, however CpG islands are specific clusters which show more dynamic methylation patterns (Bird, 1986). The regulation of DNA methylation occurs through DNA methyltransferases (DNMT) which have been categorized in three subtypes which appear to be differentially regulated and methylate at specific DNA sequences (Antequera, 2003; Bestor, 2000). DNMTs are the primary enzyme that serves to initiate and maintain DNA methylation patterns (Robertson, 2005). The initial attachment of a methyl group to cytosine appears to be specifically regulated by DNMT3a and DNMT3b (Okano et al., 1999). Although a specific enzyme which would serve to demethylate DNA has not yet been identified, it has been suggested that DNMT3a and DNMT3b may play a role in demethylation (Kangaspeska et al., 2008; Metivier et al., 2008; Ooi and Bestor, 2008; Sharma et al., 2010). DNA methylation has been found to directly inhibit the binding of transcriptional machinery, but is primarily thought to act through the coordinated actions of other repressor proteins, such as methyl-CpG binding domain (MBD) proteins (Figure 2) (Fan and Hutnick, 2005; Levenson and Sweatt, 2005; Wade, 2001). MBD proteins can regulate the condensation of chromatin structure and sterically hinder the binding of transcriptional machinery (Robison and Nestler, 2011). Furthermore, both DNMTs and MBD proteins have been found to recruit HDACs which can also modify chromatin components leading to chromatin condensation (Figure 2) (Fuks et al., 2000; Kimura and Shiota, 2003; Nan et al., 1998).

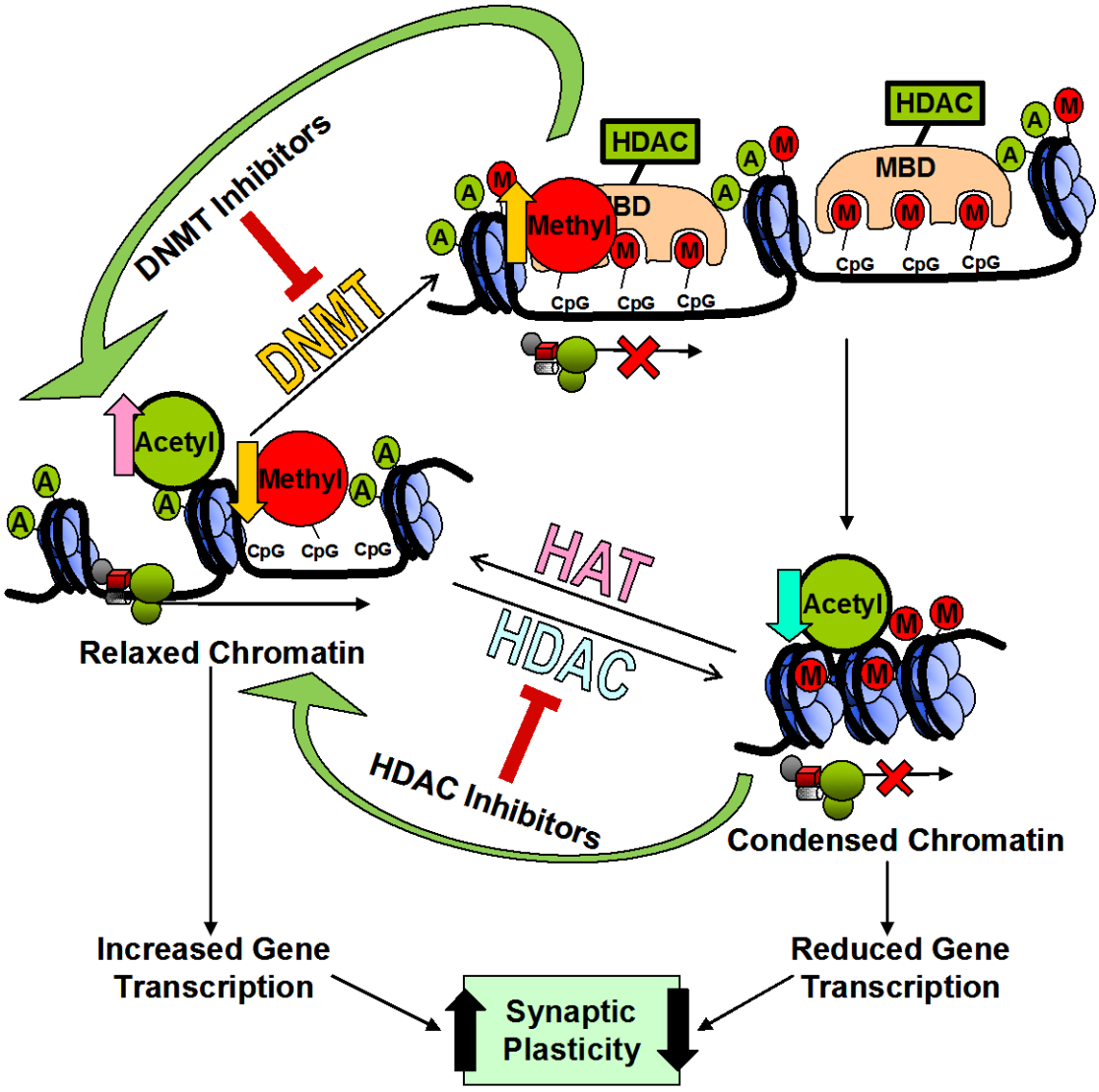

DNMT Inhibitors
DNMT
HDAC
MBD
Methyl
CpG
Acetyl
HAT
HDAC
HDAC Inhibitors
Relaxed Chromatin
Condensed Chromatin
Increased Gene Transcription
Reduced Gene Transcription
Synaptic Plasticity

Figure 2. Chromatin remodeling via histone acetylation and DNA methylation regulates gene transcription associated with changes in synaptic plasticity. During gene transcriptional processes, the chromatin structure associated with DNA to be transcribed is in a relaxed chromatin conformation due to hyperacetylation of histone proteins and hypomethylation of DNA, which allows access to transcriptional machinery. This relaxed chromatin structure results in increased gene transcription, which in neurons may cause increased expression of synaptically active proteins that result in the positive modulation of synaptic plasticity, such as increased dendritic spine density (DSD). DNA methyltransferase (DNMT) methylates DNA at CpG islands, leading to hypermethylated DNA and recruiting of methyl-CpG binding domain protein (MBD) complexes which block binding of transcriptional machinery. The MBD complex can in turn recruit histone deactylases (HDAC) which remove acetyl groups from histone proteins resulting in chromatin condensation thereby decreasing gene transcription. HDACs and histone acetyltransferases (HAT) control the histone acetylation profile, such that HDACs remove acetyl groups and HATs add acetyl groups to histone proteins. In this manner, increased HDAC expression results in hypoacetylation of histones leading to a condensed chromatin structure. Chromatin condensation resulting from HDAC-induced histone deacetylation or DNMT-induced DNA methylation causes reduced gene transcription. In neuronal cells, the reduction in gene transcription may be associated with decreased expression of synaptically active proteins and negative modulation of synaptic plasticity, such as reduced DSD. Treatment with DNMT inhibitors or HDAC inhibitors may block these enzymatic processes and return chromatin to a relaxed state, resulting in increased gene transcription and synaptic plasticity (Moonat and Pandey, 2012).

3. Histone acetylation in neuronal function

As mentioned previously, HDACs are key enzymes in the dynamic regulation of histone acetylation that underlies chromatin condensation and the repression of gene transcription (de Ruijter et al., 2003; Jenuwein and Allis, 2001; MacDonald and Howe, 2009; Thiagalingam et al., 2003). The characterization of the HDAC family of enzymes has led to the identification of 19 HDACs that have been categorized into four distinct classes based on sequence homology, cellular localization and functional regulation (de Ruijter et al., 2003; Dokmanovic et al., 2007). Class I HDACs, HDAC1, 2, 3 and 8, are found ubiquitously in cells including the brain and are localized primarily in the nucleus (de Ruijter et al., 2003). Class II HDACs have been further subcategorized into class IIA, HDAC4, 5, 7 and 9, and class IIB, HDAC6 and 10. These HDACs display variable

tissue distribution with HDAC4, 5 and 9 having been identified within the brain (Darcy et al., 2010; Sugo et al., 2010; Tsankova et al., 2004). The cellular distribution of class II HDACs defines the subcategorization such that class IIA HDACs can shuttle between the nucleus and cytoplasm, while class IIB HDACs are restricted to the cytoplasmic compartment (Thiagalingam et al., 2003). The sole member of class IV, HDAC11 shows sequence homology to both class I and II HDACs, is localized primarily in the nuclear compartment, and shows differential tissue distribution, including presence in the brain (Gao et al., 2002). Class I, II and IV HDACs bind to and are functionally dependent on the presence of zinc, whereas class III HDACs, also known as sirtuins, depend on NAD^+ as an enzymatic cofactor (Codd et al., 2009; Harting and Knoll, 2010). Each of the sirtuins, SIRT1-7, display variable tissue and subcellular localization with SIRT1 and 2 functionally present in the brain with the ability to shuttle between the nucleus and cytoplasm (Harting and Knoll, 2010).

HDAC inhibitors are a class of pharmacotherapeutic agents which either non-selectively or class specifically inhibit HDACs leading to increased histone acetylation. Suberoylanilide hydroxamic acid (SAHA) was the first, and remains the only, HDAC inhibitor approved by the Food and Drug Administration (FDA) for treatment of cutaneous T-cell lymphoma (Kavanaugh et al., 2010). Recent developments have implicated a role for HDACs in physiological and pathological neuronal processes, suggesting that HDAC inhibitors may provide useful therapeutic roles for psychiatric and neurological disorders (Abel and Zukin, 2008; Grayson et al., 2010; Levenson and Sweatt, 2005). Four major classes of HDAC inhibitors have been identified by similarities in chemical structure, including the hydroxymates, short-chain fatty acids,

cyclic peptides and benzamides (Grayson et al., 2010). The most important distinction to note regarding various HDAC inhibitors is the specificity of action. Whereas many of the currently identified drugs are pan-HDAC inhibitors, some have display activity profiles specific to certain classes of HDAC enzyme. Although the characterization of specific HDAC inhibitors is beyond the scope of this literature review, it is important to note that numerous isoform-specific HDAC inhibitors are currently under development and may allow for more selective activity of pharmacotherapeutics for particular disorders (Balasubramanian et al., 2009).

The role of histone acetylation in the dynamic modulation required for neuronal learning has highlighted the importance of epigenetic mechanisms and the robust effects of HDAC inhibitors in the brain (Guan et al., 2002; Levenson et al., 2004; Levenson and Sweatt, 2005; Molfese, 2011). The important role of CREB in gene transcription associated with long-term memory suggested that there could be a role for histone acetylation through the inherent HAT activity of CBP (Chrivia et al., 1993; Guan et al., 2002). An early study which sought to explore this role found bidirectional actions by CBP and HDAC5 in the facilitation and repression of long-term memory formation, respectively (Guan et al., 2002). The requirement for HAT activity was further confirmed by independent studies utilizing transgenic mice with impaired CBP function that displayed associated impairments in long-term memory formation (Alarcon et al., 2004; Korzus et al., 2004). Interestingly, application of trichostatin A (TSA), a class I and II HDAC inhibitor, reversed the effect of the CBP impairment on neuronal memory, suggesting that inhibition of HDACs could compensate for the reduced HAT activity (Korzus et al., 2004). In a fear conditioning task, histone H3 acetylation, but not H4

acetylation, was found to be increased following training confirming the role of chromatin remodeling in forms of learning and memory (Levenson et al., 2004). The study further identified that use of HDAC inhibitors, TSA and sodium butyrate, facilitated the induction of LTP and the behavioral expression of fear conditioning (Levenson et al., 2004). More recent studies have identified that acetylation patterns during behavioral learning or extinction are specific to genes that have been found to regulate synaptic plasticity, such as CREB and BDNF (Bredy et al., 2007; Vecsey et al., 2007). Furthermore, enhancement of memory tasks by HDAC inhibitors does not result in global changes in acetylation and follows similar gene-specific acetylation (Bredy et al., 2007; Vecsey et al., 2007). These data suggest a complex system of gene- and histone-specific acetylation patterns in the dynamic regulation of gene transcription which underlies neuronal function.

Various studies have also identified important roles for specific HDAC isoforms in neuronal function (Fischer et al., 2010). The HDAC1 isoform has been found to play a role in the regulation of cell-cycle activity in the neuron, and dysregulation of the HDAC1 enzyme may result in neuronal apoptosis and play a role in the neurodegenerative disorders (Kim et al., 2008b). The HDAC2 enzyme has been found to be specifically associated with the formation of memory, such that overexpression and knockout of HDAC2 impaired and facilitated performance in a fear learning paradigm, respectively (Guan et al., 2009). This study strongly implicated the HDAC2 isoform in the regulation of synaptic plasticity. HDAC3 has been found to have similar effects to HDAC2 as a negative regulator of memory formation, such that HDAC3 deletion or inhibition facilitates learning (McQuown et al., 2011). In contrast to HDAC2 and

HDAC3, HDAC1 has not been shown to have involvement in the synaptic events of mature neurons, however both HDAC1 and HDAC2 are important in the plasticity of developing neurons suggesting that specific HDAC isoforms may have differential roles in development and maturity (Akhtar et al., 2009; Guan et al., 2009). HDAC3 has also been identified as a positive mediator of neurotoxicity associated with polyglutamine-induced neuronal death in a Huntington's model (Bates et al., 2006). HDAC4, however, negatively regulates neuronal apoptosis (Majdzadeh et al., 2008). HDAC5 has been specifically implicated in the mechanisms underlying depression and the action of anti-depressants and will be presented in greater detail in the following section (Tsankova et al., 2006). A variety of roles have been suggested for HDAC6, including the regulation of anxiety and depression, the pathogenesis of Alzheimer's Disease, and the regulation of neurodegeneration associated with Parkinson's Disease (Ding et al., 2008; Fukada et al., 2012; Su et al., 2011). Although this review of literature regarding specific HDAC isoforms is not exhaustive, it is apparent that isoforms may have differential and contrasting roles in the brain. This suggests that further study into the unique roles of each HDAC isoform and development of isoform-specific pharmacotherapeutic agents may present useful opportunities for the treatment of psychiatric and neurological disorders. In the following section, I will present the role of specific epigenetic mechanisms and HDAC isoforms involved in the regulation of BDNF and synaptic plasticity that may play a role in psychiatric disorders, including anxiety and alcoholism.

4. Epigenetic Regulation of BDNF in Psychiatric Disorders and Therapeutics

Various epigenetic mechanisms have been shown to regulate BDNF in a promoter-specific manner that may play a role in the tight control of BDNF expression patterns. A

role for DNA methylation in the regulation of BDNF expression in neurons has been identified via the action of MeCP2 (He et al., 2010; Martinowich et al., 2003; Zhou et al., 2006). MeCP2 is an important repressor of gene transcription that binds to methylated DNA and has been found to further recruit HDACs and DNMT1 (Ballestar and Wolffe, 2001). MeCP2 has been strongly implicated as the etiological basis accounting for over 95% of cases of the neurodevelopmental disorder Rett syndrome (Samaco and Neul, 2011). The activity-regulated upregulation of BDNF exon IV following depolarization has been shown to be associated with increased histone acetylation, reduced DNA methylation and reduced MeCP2 binding (Martinowich et al., 2003). The regulation of BDNF exon IV by MeCP2 was further associated with the modulation of dendritic spine morphology (Zhou et al., 2006). Specifically, the study suggested that an activity-dependent calcium influx led to CaMKII mediated site-specific MeCP2 phosphorylation that resulted in increased BDNF and spinogenesis (Zhou et al., 2006). A more recent study utilized site-directed mutagenesis of the MeCP2 phosphorylation site and found that the increased expression of BDNF was associated in enhancements in fear learning, spatial memory and LTP (Li et al., 2011). Another study demonstrated a role for a scaffolding protein, RACK1, which was found to associate with the BDNF exon IV promoter and mediating dissociation of MeCP2 from the BDNF gene (He et al., 2010). Confirming previous results, dissociation of MeCP2 resulted in increased acetylated histone-associated BDNF exon IV promoter levels (He et al., 2010). Fear conditioning has also been associated with reduced DNA methylation in the BDNF exon IV promoter region (Lubin et al., 2008). Interestingly, it was found that BDNF exon IV was specifically associated with the consolidation of fear memory, whereas the presentation

of context along was sufficient to produce an increase in BDNF exons I and VI (Lubin et al., 2008). These results provide evidence for the overlapping epigenetic mechanisms in the regulation of BDNF exon IV associated with modulation of synaptic plasticity and learning and memory.

HDAC enzymes have also been found to play a role in the regulation of neuronal BDNF expression. An early study found that in a model of pilocarpine-induced status epilepticus, histone acetylation and the BDNF exon II promoter was increased in the hippocampus (Huang et al., 2002). This identified a unique role for chromatin remodeling via histone modifications in the regulation of BDNF expression (Huang et al., 2002). This finding was confirmed in a model of electroconvulsive shock (ECS) that found time- and promoter-dependent changes in histone acetylation levels associated with BDNF promoters (Tsankova et al., 2004). Specifically, the study identified increased H3 acetylation at the BDNF exon IV and VI promoters, and suggested a possible link between epigenetic regulation of BDNF and the activity-dependent modifications induced by ECS (Tsankova et al., 2004). As mentioned in the previous section, HDAC2-specific regulation of dendritic spines is associated with performance in a fear conditioning paradigm (Guan et al., 2009). The study found that transgenic mice overexpressing HDAC2, but not HDAC1, showed impaired memory formation in a fear conditioning paradigm that was associated with a reduction in DSD in the hippocampus (Guan et al., 2009). In contracst, knockout of HDAC2 resulted in enhanced learning that was associated with increased DSD (Guan et al., 2009). Treatment with the HDAC inhibitor, SAHA, reversed the behavioral and morphological deficits in the HDAC2 overexpressing mice, but not HDAC2 knockout mice (Guan et al., 2009). The study

further identified that in overexpressing mice, there was an association between HDAC2 and various genes involved in synaptic plasticity, including BDNF exon II, but HDAC2 knockout mice had higher levels of histone H3 acetylation at BDNF exon II and other synaptically active genes (Guan et al., 2009). Interestingly, another study identified a role for BDNF-induced nitrosylation of HDAC2 in inhibition of HDAC2 function and an increase in dendritic length (Nott et al., 2008). This could suggest a negative feedback loop that controls the activity-dependent increase in BDNF and the associated change in structural plasticity. These studies suggest a role for HDAC2 in the regulation of synaptic plasticity via BDNF and other synaptically active genes.

Acute and chronic stressors have been shown to serve as a useful animal model to study depression and the effects of antidepressants. Various studies have used the effects of chronic stress to identify the role hippocampal neuronal morphology and dendritic structure in the development of depression-like phenotypes (McEwen and Gianaros, 2010; Watanabe et al., 1992). Notably, the effects of chronic stress have also been associated with a reduced BDNF expression levels in the hippocampus (Smith et al., 1995). Various recent studies suggest that BDNF levels are modulated in a variety of brain areas, including the hippocampus, PFC and EA by acute and chronic stress (Calabrese et al., 2009; McEwen, 2008; Pizarro et al., 2004). It has been suggested that BDNF expression levels may be upregulated in response to acute stress to serve as a neuroprotective mechanism against the deleterious effects of the stressor, but repetitive and chronic stress may result in dysregulation of this mechanism leading to reduced BDNF levels (Calabrese et al., 2009; McEwen, 2008). Chronic stress models of depression have identified that the antidepressant effect of various therapeutics iis

mediated by increasing levels of hippocampal BDNF levels (Nibuya et al., 1995; Shirayama et al., 2002; Tsankova et al., 2006). As mentioned previously, low BDNF levels in amygdaloid brain regions, especially CeA , have been associated with anxiety-like behaviors (Pandey et al., 2006; Pandey et al., 2008b). Taken together, these studies suggest that dysregulated BDNF signaling may play a role in the dysphoric symptoms associated with psychiatric disorders and the pharmacotherapeutic effects of drugs which alleviate the symptoms.

In an animal model of depression and antidepressant treatment, reduced BDNF exon IV and exon VI was associated with symptoms of depression and reversed by therapeutics reducing these symptoms (Tsankova et al., 2006). Methylated histone H3 proteins at the BDNF exons IV and VI promoters was increased by chronic stress and reduced by chronic antidepressant treatment. Treated animals showed a converse increase in the acetylation of histone H3 at these BDNF exons (Tsankova et al., 2006). Antidepressant treatment was associated with a reduction in HDAC5 mRNA levels, and overexpression of HDAC5 impaired the effects of antidepressant treatment (Tsankova et al., 2006). HDAC levels in the NAc have also been implicated in the development of stress-related dysphoria. Systemic treatment with HDAC inhibitors and intracranial infusion of HDAC inhibitors has been found to reduce the depression-like phenotype (Covington et al., 2009; Tsankova et al., 2006). These results suggest a role for histone modifications in psychiatric disorders associated with the regulation of BDNF.

DNA methylation has also been implicated in the development of depression and the regulation of synaptic plasticity. Specifically, DNMT3a expression in the NAc was increased by chronic stress which was associated with an increase in depressive-like

behavior (LaPlant et al., 2010). Infusion of a DNMT inhibitor directly into the NAc reduced the phenotype of depression, whereas overexpression of DNMT3a precipitated depression and caused an increase in DSD (LaPlant et al., 2010). These results indicate a role for DNMT3a in stress-related dysphoria and control of dendritic spine structure. It would be interesting to expand upon these results and determine if a link exists between stress-associated changes in DNMT3a methylation of the BDNF gene and stress.

Recent findings have associated the anxiolytic effects of acute ethanol exposure with a reduction in HDAC activity and an increase in histone acetylation in the CeA and MeA (Pandey et al., 2008a). Anxiety following withdrawal from chronic ethanol exposure was linked with an increase in HDAC activity levels and an associated reduction in histone acetylation in these amygdaloid brain regions. Administration of TSA increased histone acetylation and reduced anxiety-like behaviors exhibited by the withdrawn rats (Pandey et al., 2008a). It is worth noting that a significant similarity exists between the effects of TSA administration and BDNF infusion (see above) in this animal model, suggesting that there may be common mechanisms at work in both pathways (Pandey et al., 2008a; Pandey et al., 2008b). More recently, HDAC inhibitors were found to elicit a significant effect on the rapid tolerance to the anxiolytic effect of ethanol that was associated with increased histone acetylation in the CeA and MeA (Sakharkar et al., 2012).

As is evident from this review of literature, there is a variety of evidence implicating epigenetic mechanisms, including the covalent modification of histones, in the regulation of psychiatric disorder such as anxiety and alcoholism. Furthermore, it is clear that epigenetic mechanisms may play a role in the modulation of synaptic plasticity

that regulate both physiological and pathological process. Yet, no studies have identified innate abnormalities in specific histone deacetylase proteins that are associated with a genetic predisposition to anxiety or alcoholism. Since it appears that common molecular pathways within the amygdala underlie both of these phenomena, it could be possible that epigenetic regulation of synaptic events in this brain region could play an important role in mediating the complex interactions by which anxiety may lead to the development of alcohol preference and alcohol consumption may reduce innate anxiety.

II. STUDY DESIGN AND METHODOLOGY

A. Rationale for Study

Alcoholism is a complex psychiatric disorder that is associated with severe personal and social consequences. The heritability of alcoholism has been indicated by various population studies leading to an increased interest in understanding the genetic basis of alcoholism (Cloninger, 1987; Mayfield et al., 2008; Schuckit, 2000). It has been suggested that comorbid anxiety-spectrum disorders may play a role in the initiation and maintenance of alcohol-use disorders (Bolton et al., 2009; Enoch, 2006; Grant et al., 2004b; Koob, 2003; Pandey, 2003; Schuckit and Hesselbrock, 1994). The selectively-bred P and NP rat lines display higher and lower voluntary ethanol consumption, respectively, and in contrast to NP rats, P rats display anxiety-like behaviors (Bell et al., 2006; Hwang et al., 2004; Pandey et al., 2005b; Stewart et al., 1993). In various animal models, amygdaloid brain regions have been implicated in both anxiety-like behaviors and ethanol preference (Davis et al., 2009; Koob, 2003; LeDoux, 2003; Pandey, 2003; Pandey, 2004; Tye et al., 2011). Thus, examination of molecular differences in the amygdaloid brain regions of P and NP rats could provide insight into the comorbidity between anxiety and alcohol-use that may underlie a genetic predisposition to alcoholism.

BDNF, a CREB target gene, is a signaling peptide associated with the regulation of synaptic plasticity and DSD, possibly via induction of the immediate-early gene, *Arc* (Bramham et al., 2008; Messaoudi et al., 2002; Pandey et al., 2008b; Pizzorusso et al., 2000; Soule et al., 2006; Ying et al., 2002). Aberrant BDNF signaling in various brain regions has been implicated in the development and symptoms of psychiatric disorders including anxiety and alcoholism (Autry and Monteggia, 2012; Berton et al., 2006;

Davis, 2008; Jeanblanc et al., 2009; Nestler et al., 2002; Pandey et al., 2006; Pandey et al., 2008b). Furthermore, human and animal studies of a BDNF polymorphism have identified an association between reduced BDNF secretion, and anxiety and alcoholism (Chen et al., 2008; Chen et al., 2006; Lang et al., 2005a; Lau et al., 2010; Matsushita et al., 2004; Uhl et al., 2001). Previously, Pandey *et al.* identified that withdrawal-induced anxiety in an unselected stock of rats was associated with reduced BDNF, Arc and DSD in the CeA and MeA (Pandey et al., 2006; Pandey et al., 2008b). Exogenous infusion of BDNF into CeA of alcohol-withdrawn rats and was anxiolytic and corrected the Arc deficit, and similarly, the anxiolytic effect associated with acute ethanol exposure increased CeA and MeA BDNF, Arc and DSD (Pandey et al., 2008b). Conversely, Arc knockdown reduced DSD in naïve rats, and resulted in increased anxiety-like behaviors and alcohol consumption (Pandey et al., 2008b). In another study, P rats were found to have lower levels of BDNF in the CeA and MeA than NP rats, which may suggest differences in Arc and DSD as well (Prakash et al., 2008). Furthermore, it could be possible that the anxiolytic effect of acute ethanol in P rats could be associated with changes in amygdaloid BDNF-Arc signaling and DSD.

Epigenetic mechanisms, including histone modifications associated with chromatin remodeling, have been implicated in the pathophysiology of psychiatric disorders, including anxiety and alcohol addiction (Moonat et al., 2011; Nestler, 2009; Pandey et al., 2008a; Renthal and Nestler, 2008; Tsankova et al., 2007b). Recently, anxiety induced by withdrawal from chronic ethanol exposure was found to be associated with condensed chromatin structure and increased HDAC activity in the CeA and MeA, and was corrected by treatment with an HDAC inhibitor, TSA, which produced

anxiolytic effects and reduced alcohol consumption (Pandey et al., 2008a). Acute ethanol exposure in naïve Sprague-Dawley rats also had an anxiolytic effect associated with reduced HDAC activity and relaxation of chromatin (Pandey et al., 2008a). Given these findings, it could be possible that innate abnormalities in chromatin structure could be associated with the anxiety-like and excessive alcohol drinking behaviors of P rats. Chromatin remodeling via changes in histone acetylation has also been found to be a regulator of the *BDNF* gene (Huang et al., 2002; Kumar et al., 2005; Tsankova et al., 2006; Tsankova et al., 2004). Thus, it could be possible that the anxiolytic effects associated with voluntary and acute ethanol exposure in P rats could be related to the effects of ethanol on HDAC-induced chromatin remodeling associated with downstream regulation of BDNF and synaptic plasticity.

Recently, various studies have begun to implicate specific HDAC isoforms in the regulation of neuronal function, neuropsychiatric disorders and psychotherapeutics (Covington et al., 2009; Ding et al., 2008; Fukada et al., 2012; Guan et al., 2009; McQuown et al., 2011; Nott et al., 2008; Su et al., 2011; Tsankova et al., 2006). The implication is that identification of specific HDAC isoforms involved may allow for design of isoform specific HDAC inhibitors that may provide therapeutics with increased selectivity. HDAC was recently identified as an important regulator of synaptic plasticity in the brain such that HDAC2 overexpression reduced DSD and impaired learning in a fear conditioning paradigm (Guan et al., 2009). In contrast, HDAC2 knockout mice displayed increased DSD and facilitated learning in a fear conditioning task, suggesting that HDAC2 may have a direct role in the regulation of synaptic plasticity associated with learning and memory (Guan et al., 2009). Thus, it could be possible that chromatin

remodeling via HDAC2-induced histone modifications in the amygdala may play a role in the regulation of synaptic plasticity that may underlie the behavioral phenotypes of P rats. Furthermore, it could be possible that exogenous manipulation of HDAC2 levels in the amygdala may have an effect on anxiety-like behaviors and alcohol preference in P rats.

B. Hypotheses and Specific Aims

The primary objective of the current proposal is to examine the role of epigenetic regulation of synaptic factors in amygdaloid brain regions involved in the comorbidity between anxiety and alcoholism. We propose to explore amygdaloid HDAC-induced chromatin remodeling in the regulation of BDNF and Arc expression, and associated changes in DSD in the genetic predisposition to anxiety and alcoholism. Our overall hypothesis states that increased HDAC2 protein and the associated deficit in chromatin and synaptic remodeling in the CeA of P rats, but not NP rats, may be responsible for the heightened anxiety-like and excessive alcohol drinking behaviors exhibited by P rats. Furthermore, the anxiolytic effects of ethanol exposure may be mediated by a reduction in HDAC2 protein and associated changes in chromatin remodeling, and synaptic proteins and structure in the amygdala. First, we studied the effects of acute ethanol exposure on anxiety-like behaviors, markers of synaptic plasticity (BDNF, Arc, and DSD), and epigenetic changes (HDAC2 and HDAC4 expression; histone acetylation and methylation; acetylated histone associated *BDNF* and *Arc* genes) in the amygdaloid brain regions of P and NP rats were investigated. We then mechanistically examined the direct role of the HDAC2 isoform in the CeA of P rats on anxiety-like and alcohol drinking behaviors. We also looked at the epigenetic regulation of *BDNF* and *Arc*, and structural

changes in DSD that were associated with the behaviors modulated by the reduction in HDAC2.

1. Specific aim 1

To examine the role of amygdaloid BDNF signaling via Arc protein on changes in synaptic plasticity that may be associated with innate anxiety-like behaviors and the anxiolytic effects of acute ethanol.

P and NP rats were tested for anxiety-like behaviors via the elevated-plus maze (EPM) or light-dark box (LDB) exploration test one hour following intraperitoneal injection of saline or acute ethanol (1 g/kg). Brains were collected for histological examination of CeA, MeA and BLA to determine BDNF and Arc protein and mRNA expression levels. Another group of brains was also be processed by Golgi-Cox staining. DSD in amygdaloid brain regions was quantified by light microscopic analysis of Golgi-Cox stained sections using neuron mapping software for quantitative analysis.

2. Specific aim 2

To examine amygdaloid histone deacetylases and associated histone modifications that may play a role in the regulation of synaptic plasticity related to innate anxiety-like behaviors as well as the anxiolytic effects of acute and voluntary ethanol exposure.

P and NP rats were injected intraperitoneally with saline or acute ethanol (1 g/kg). Rats were tested for anxiety-like behaviors via the EPM or LDB exploration test. Amygdaloid brain regions were collected and analyzed for cytosolic and nuclear HDAC activity. Brains were collected and processed for histological analysis of HDAC2 and HDAC4 protein levels, as well as histone H3K9 acetylation and dimethylation levels. Lysates of amygdaloid brain regions containing predominantly CeA and MeA of P or NP

rats treated with either saline or acute ethanol were analyzed for the association of *BDNF exon I*, *BDNF exon IV* and *Arc* genes with acetylated histone H3 via chromatin immunoprecipitation (ChIP) analysis. The levels of *BDNF* and *Arc* associated with acetylated histone H3 were determined by quantitative real-time PCR (qPCR).

Another group of P and NP rats was tested for voluntary drinking by the two-bottle free choice paradigm. Voluntary drinking was monitored by the amount of daily alcohol intake in comparison to water intake, and blood alcohol levels were calculated following alcohol exposure. Rats subjected to voluntary drinking experiments were examined for anxiety-like behaviors via the LDB exploration test. Brains were collected for analysis of HDAC2 protein, and histone H3K9 and H4K8 acetylation levels in amygdaloid brain regions.

3. Specific aim 3

To mechanistically examine the role of HDAC2-mediated histone modifications in the regulation of BDNF and Arc expression and associated changes in DSD in the CeA on anxiety-like and alcohol drinking behaviors of P rats.

P rats were anaesthetized and surgically implanted with cannulas stereotaxically targeted for infusion directly into the CeA. Using a lipid-based transfection solution (Neuromics iFect solution), HDAC2 siRNA, negative control siRNA or vehicle were infused bilaterally into the CeA. One group of P rats was tested for anxiety-like behaviors via the EPM or LDB exploration test 16 hours after infusion. The brains were collected and processed by *in situ* PCR, immunofluorescence, immunohistochemistry, Golgi-Cox staining or ChIP analysis. Using confocal microscopy, the cololocalization of fluorescence-tagged HDAC2 siRNA with neuronal marker (NeuN) was visualized to

assess successful transfection of neurons with HDAC2 siRNA. HDAC2 mRNA was quantified to confirm siRNA knockdown. The protein levels of HDAC2, acetylated histone H3K9, BDNF and Arc protein were determined. Golgi-Cox stained sections were used to analyze the DSD. ChIP was performed on the lysates of amygdaloid brain regions consisting predominantly of CeA and MeA and analyzed by qPCR to determine the levels of *Arc* and *BDNF* genes associated with acetylated histone H3. Following cannulation, another group of P rats was habituated to consume increasing concentrations of ethanol. After habituation, HDAC2 siRNA or vehicle was infused into the CeA. Voluntary ethanol consumption was measured using the two-bottle free choice drinking paradigm.

C. Materials and Methods

Animals

All experiments were conducted in accordance with the National Institute of Health's *Guidelines for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee. Adult male P and NP rats were received from the Indiana Alcohol Research Center at Indiana University, Indianapolis, IN. Animals were housed in a temperature- and humidity-controlled facility under a 12 h light/dark cycle, with *ad libitum* access to food and water. P and NP rats were age-matched and body weight was measured for confirmation that the groups did not show significant differences in the age and size of rats. Rats weighing 350 – 400 g were used.

Cannulation surgery for targeting CeA

In order to infuse siRNA directly into the CeA of P rats, the rats were surgically implanted with CMA/11 guide cannulas (CMA Microdialysis, North Chelmsford, MA) as

described previously (Pandey et al., 2008b). After at least 7 days of habituation to the animal housing facility, P rats were anaesthetized with pentobarbital (50 mg/kg) injected intraperitoneally and tested for complete anaesthesia via the tail pinch procedure. The rats were mounted into a stereotaxic apparatus and the skull was examined for the location of the bregma. The cannulas were guided using the stereotaxic apparatus to 2.5 mm posterior of bregma, 4.2 mm lateral of bregma bilaterally and 5.1 mm ventral from the surface of the skull in order to target cannula to 3 mm above the CeA. After insertion into the brain, the cannulas were secured to the skull with screws and dental cement. The cannulas were sealed with guide caps and rats were returned to clean cages for recovery. Once the rats recovered from anaesthesia, they were returned to the animal housing facility and the health status of the rats was checked daily for the duration of the experiment.

Acute ethanol injection

Acute ethanol injection was performed as previously described (Moonat et al., 2011; Pandey et al., 2008b). Rats were injected intraperitoneally with ethanol (diluted to 20% w/v in *n*-saline; 1 g/kg) or *n*-saline alone. This dose of ethanol has previously been shown to be anxiolytic in P rats, but not NP rats, and results in a blood alcohol content of approximately 80-90 mg/dL which is within the physiological range of alcohol consumption for humans (Stewart et al., 1993). One hour following acute ethanol injection, the anxiety-like behaviors of the rats were measured by using the elevated plus-maze (EPM) test or light-dark box (LDB) exploration test, as described below. Immediately following behavioral measurements, P and NP rats were anaesthetized using pentobarbital (50 mg/kg) allowing for the collection of blood for analysis of blood

alcohol content. Fresh frozen or paraformaldehyde (PFA) fixative-perfused tissue was collected from the brains for further analysis as described below.

Voluntary ethanol consumption via two-bottle free choice

Voluntary ethanol experiments were performed using the two-bottle free choice alcohol preference paradigm as previously described (Pandey et al., 2005b). Voluntary ethanol experiments were performed with both P and NP rats, or only P rats in the case of the HDAC2 siRNA infusion experiment. Rats were placed in a cage containing two identical bottles containing 50 mL of ethanol or water placed at equivalent distances from the cage edge. At the same time each day, the remaining amounts in each bottle were measured for calculation of ethanol and water consumption. The bottles were replaced with clean refilled bottles and were switched to the opposite side from the previous night to avoid the bias of preference for one bottle over the other.

The rats first received two bottles containing water in order to be habituated to the two-bottle free choice paradigm. Once the rats consistently consumed equivalent amounts of water from both bottles, the bottles were replaced with the experimental solutions. For the experiments involving both P and NP rats, rats were either given two bottles of water or one bottle of ethanol and one bottle of water. In the HDAC2 siRNA infusion experiment, all P rats were given one bottle of ethanol and one bottle of water. After habituation, 7% ethanol (w/v diluted in tap water) was given for three days, followed by 9% ethanol. HDAC2 siRNA infusion (see below) was given after three days of exposure to 9% ethanol and consumption was monitored for seven days post-infusion. Body weights of the rats were monitored throughout the experiment and data was represented as mean ± SEM of ethanol consumption in g/kg/day. In the morning

following the final day of voluntary ethanol experiments, behavioral analysis of anxiety-like behavior was performed, and brains and blood were collected for further analysis as described below.

Infusion of HDAC2 siRNA

P rats that had been previously cannulated for delivery of solutions directly into the CeA were infused with either HDAC2 siRNA, control siRNA or vehicle. The siRNAs were dissolved in iFect solution (Neuromics, Edina, MN), a cationic lipid-based transfection solution, such that the final concentration of the solution was 2 µg/µL. The sequence of the HDAC2 siRNA was as follows: 5'-CAAGUUUCUACGAUCAACATT-3'; 5'-UAUUGAUCGUAGAAACUUGAT-3'. Some of the HDAC2 siRNA (Qiagen, Valencia, CA) had been modified to include a 5' Alexa Fluor-488 fluorescent probe in order to determine the transfection efficiency and cellular localization of transfection. The control siRNA used was the AllStars Negative Control siRNA (Qiagen), which shows no homology to any known mammalian gene. To prepare the vehicle, RNase-free water was dissolved in the iFect solution in place of any siRNA. The solutions (0.5 µL) were infused bilaterally into the CeA of P rats using an automatic infusion pump which resulted in a dose of 1 µg of siRNA per side. The automatic pump was attached to a microdialysis probe which seated in the guide cannula and extended 3 mm past the tip of the cannula into the CeA.

For the experiments which looked at the anxiolytic effect of HDAC2 siRNA infusion, P rats were infused with either HDAC2 siRNA, control siRNA or vehicle at the end of the light cycle. 16 hours after the infusion, the rats were tested for anxiety-like

behaviors. Immediately following behavioral testing, rats were anaesthetized and brains were collected for further analysis.

For the voluntary drinking experiment, P rats were infused with either HDAC2 siRNA or vehicle when the bottles were changed following the third day of 9% ethanol exposure. The rats continued to be monitored for the intake of 9% ethanol for 7 days following the infusion. After the final day of voluntary drinking, the rats were anaesthetized for collection of brains and blood to confirm the cannula position and the blood alcohol levels, respectively.

Analysis of blood alcohol levels

Blood was collected into heparinized capillary tubes following behavioral testing. Following centrifugation, plasma was analyzed for blood alcohol level using an AM-1 Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA). Plasma (5 µL) was added to the alcohol analyzer in the presence of alcohol oxidase. The instrument measures changes in the oxygen content of the sample to calculate the blood alcohol levels which were represented as mean ± SEM of blood alcohol levels in mg/dL.

Measurement of anxiety-like behaviors by the EPM test

The EPM test for anxiety-like behaviors was performed as previously described (File, 1993; Pandey et al., 2005; Pandey et al., 2006; Pandey et al., 2008b). The EPM apparatus consists of two open arms and two closed arms, opposite each other, connected by a central platform. The apparatus is elevated approximately 1 m above the ground. Before testing, each rat was placed in a clean cage and habituated to the testing room for 5 min. Following habituation, each rat was placed on the central platform of the EPM apparatus facing an open arm. During the 5 min test period, exploration of the open and

closed arms of the EPM was monitored and recorded. Results were represented as the mean ± SEM of the percentage of open arm entries and the percentage of time spent on the open arms.

Measurement of anxiety-like behaviors by the LDB exploration test

The procedure for the LDB exploration test was performed as described previously by us (Pandey et al., 2008a; Zhang et al., 2010). The LDB apparatus is a modified conditioned place preference box that has a dark compartment separated from a light compartment by an opening in the separator. Each rat is placed into a clean cage in order to habituate to the testing room. Following a 5 min habituation period in the testing room, each rat was placed in the dark compartment of the LDB apparatus with its head facing away from the opening to the light compartment. During the 5 min test period, the movement of the rat was monitored via infrared sensors within the LDB apparatus and results were recorded directly to a computer system. The percentage of time spent in either the dark compartment or light compartment was calculated for each animal. The computer system also represents data regarding the total number of ambulations within the LDB apparatus. Results were represented as mean ± SEM of the percentage of time spent in each compartment.

Gold immunolabeling for analysis of protein levels

Protein levels in various experiments were determined using the gold-immunolabeling histochemical procedure as previously described by us (Pandey et al., 2008b; Prakash et al., 2008). Following behavioral testing, rats were immediately anaesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). Once anaesthetized, rats were perfused with *n*-saline followed by PFA (4% w/v in 0.1 M phosphate buffer). Following

perfusion, brains are post-fixed in PFA at 4ºC for 24 hours then cryoprotected in solutions of increasing sucrose concentration (10%-30% w/v in 0.1 M phosphate buffer). Following fixation, rat brains were frozen in 2-methylbutane at approximately -40°C and stored at -80°C until processing.

Coronal sections (20 μm) containing amygdaloid brain regions were cut on a cryostat. The sections were collected into phosphate buffered saline (PBS; 0.01 M) and washed. Slices were then incubated in RPMI 1640 (with L-glutamine) medium (Invitrogen, Grand Island, NY) for 30 min, 10% normal goat serum (Vector Labs, Burlingame, CA) in PBS containing 0.25% Triton X=100 (PBST) for 30 min and 1% bovine serum albumin (BSA) in PBST (BSA-PBST) for 30 min. Sections were then incubated overnight at room temperature in primary antibody diluted in BSA-PBST (see Table 1 below). Sections were then washed in PBS and blocked in BSA (1% in PBS) followed by incubation for 1 h in gold particle-conjugated anti-rabbit or anti-mouse secondary antibody (Nanoprobes, Yaphank, NY) [1:200 dilution in 1% BSA in PBS]. The sections are washed in BSA-PBS and distilled water followed by development in silver enhancement solution (Ted Pella, Redding, CA). The developing was stopped by washing in tap water, followed by mounting of the sections, dehydration and mounting of coverslips. The gold-immunolabeled protein levels were quantified using the Loats Image Analysis System (Loats Associates Inc., Westminster, MD) at high magnification (100x). For each brain region, nine total object fields were analyzed for the number of immunogold particles such that three fields in each of three adjacent brain sections were counted and values were averaged for each animal. Results were represented as mean ±

SEM of the number of immunogold particles/100 μm^2 area for each amygdaloid brain region, CeA, MeA and BLA.

Confocal visualization of fluorescent colocalization

In order to confirm that the HDAC2 siRNA transfected neurons, confocal imaging of sections was performed to visualize the colocalization of Alexa Fluor-488 tagged siRNA and immunofluorescent staining of neuronal marker. HDAC2 siRNA linked with a 5' Alexa Fluor-488 tag was infused into the CeA of P rats as described above. Following fixation, freezing and sectioning of slices containing amygdaloid brain regions, slices were collected in PBS. Sections were blocked in 10% NGS (Vector Labs) in PBST and incubated overnight with anti-NeuN antibody (see Table xx below) in NGS-PBST. Following incubation in primary antibody, sections were washed in PBS and blocked in PBS. Sections were incubated in Alexa Fluor-568 linked anti-mouse antibody (Invitrogen, Eugene, OR) diluted 1:200 in 10% NGS in PBS for 1 hour at 4ºC in the dark, followed by washing in PBS. The sections were mounted on slides and dried for 1 hour in the dark. Slides were coverslipped using Vectashield Mounting Medium (Vector Labs) and stored at 4ºC in the dark.

Confocal imaging was performed using the LSM710 laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY). Images were captured to a computer connected to the microscope to the image analysis program LSM 5 Image Browser (Carl Zeiss, Inc.). Confocal images of the CeA, MeA and BLA were taken with matching gain intensities. The images were processed using matching contrast and brightness settings to compare the colocalization of fluorescence between the groups.

TABLE 1. ANTIBODIES USED FOR GOLD-IMMUNOSTAINING AND IMMUNOFLUORESCENCE.

Antibody Name	Company	Dilution	Secondary Antibody
anti-Arc (H-300)	Santa Cruz Biotechnology Santa Cruz, CA	1:200	anti-rabbit
anti-BDNF (H-117)	Santa Cruz Biotechnology	1:200	anti-rabbit
anti-HDAC2	MBL International Inc., Woburn, MA	1:200	anti-rabbit
anti-HDAC4	MBL International Inc.	1:200	anti-rabbit
anti-acetyl Histone H3 (lys 9)	Millipore, Billerica, MA	1:500	anti-rabbit
anti-dimethyl Histone H3 (lys 9)	Millipore	1:500	anti-rabbit
anti-acetyl Histone H4 (lys 8)	Millipore	1:500	anti-rabbit
anti-NeuN (MAB377)	Millipore	1:200	anti-mouse

***In situ* RT-PCR for mRNA measurement**

Measurement of mRNA levels was determined using the *in situ* RT-PCR procedure as previously described by us (Pandey et al., 2008b; Prakash et al., 2008). Rats were anaesthetized with pentobarbital (50 mg/kg) and perfused with *n*-saline followed by PFA (4% w/v in 0.1 M phosphate buffer). Brains were post-fixed in PFA and cryoprotected in sucrose solutions (10%-30% w/v in phosphate buffer). Solutions used for *in situ* RT-PCR were treated with diethylpyrocarbonate (DEPC). Distilled water used to make the solutions was treated with 0.1% DEPC, stirred at 37°C for 1 hr and autoclaved. Fixed and cryoprotected rat brains were frozen and stored at -80°C until processing.

Coronal sections (40 μm) were cut in the cryostat and collected in DEPC-treated PBS. After washing, sections were treated with proteinase K (1 μg/ml in PBS with 0.05% Triton X=100) for 15 min at 37°C. A 0.1 M glycine solution in PBST was used to stop the proteinase K reaction. The glycine was washed out completely with PBST

followed by DNase digestion (Promega, Madison, WI) for 18 h. Sections were then transferred to PCR tubes containing 100 µl of the reverse transcription cocktail (Applied Biosystems, Foster City, CA) for 1 h at 42°C. Negative sections were subjected to the same conditions, although in the absence of reverse transcriptase enzyme. Sections were transferred to another tube containing the PCR cocktail (Applied Biosystems) consisting of Taq DNA polymerase, 100 pmol of each set of primers (see Table 2 below), dATP, dCTP, dGTP and digoxigenin (DIG)-11-dUTP (Roche Diagnostics, Indianapolis, IN) instead of dTTP. Following the PCR reaction, sections were washed thoroughly in PBS. The sections were mounted on slides and dried for 1 hr. Slides were washed in PBS followed by blocking in BSA. The slides were then incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics, Indianapolis, IN), and stained with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (Roche Diagnostics). The levels of mRNA in the CeA, MeA and BLA were quantified using the Loats Image Analyzer (Loats Associates) to calculate the optical density (OD) of image fields from the regions. The OD from negative sections was subtracted from the positive sections. The final OD calculation for each animal consisted of the average of three image fields from each region in each of three adjacent brain sections (9 total fields). Results were represented as mean ± SEM of the OD/100 pixels of area for each amygdaloid region.

Chromatin immunoprecipitation analysis

ChIP analysis was performed for the determination of genes associated with the acetylated histone H3. ChIP analysis was performed on P and NP rats subject to either intraperitoneal *n*-saline or acute ethanol injection, and P rats which had been infused with

either HDAC2 siRNA or vehicle. Rats were anaesthetized either 1 hour after intraperitoneal injection or 16 hours after CeA infusion. Brains were removed and the amygdaloid brain regions were dissected out, yielding tissue consisting predominantly of CeA and MeA. The brain tissue containing predominantly CeA and MeA was quickly collected bilaterally and frozen at -80ºC until processed.

Tissue samples were weighed for confirmation that the samples were consistent. The ChIP procedure was performed as described in the ChIP-IT Express kit manual (Active Motif, Carlsbad, CA). Protein and DNA in the sample were cross-linked in a 10% formaldehyde solution in PBS for 10 min followed by the addition of glycine to stop the fixation reaction. After removal of the glycine solution, the sample we homogenized in ice-cold lysis buffer in the presence of protease inhibitors. Following cell lysis, 5 cycles of ultrasonic shearing we performed with 20 s of shearing followed by 30 s on ice at an amplitude of 25% resulting in sheared DNA fragments of 200-500 base pairs.

Protein analysis of the sheared DNA sample was performed using the modified Lowry protein assay (Thermo Fisher Scientific, Rockford, IL). The amount of sheared DNA used in the ChIP reaction was normalized to 150 µg protein per sample and the reaction contained 1 µg of either anti-acetyl histone H3 (lys 9/14) antibody (Millipore) or anti-IgG antibody (Active Motif) in the presence of protease inhibitors. The ChIP reaction was carried out at 4ºC for 4 hours, followed by magnetic separation of protein G beads and solution. Following washing of beads, the DNA is eluted from the beads, DNA-protein cross-links are reversed and proteins are digested with proteinase K. Input DNA was prepared by 10 µg of protein directly form sheared DNA, reversing cross-links

and digesting proteins. DNA was purified using the QIAquick PCR purification kit (Qiagen). Purified ChIP DNA was stored at -20ºC until it was further processed.

In order to quantify the amount of DNA pulled down by ChIP, we utilized a qPCR technique using the RT^2 Sybr Green Master Mix (Super Array Biosciences, Frederick, MD). Forward and reverse primers for *Arc*, *BDNF exon I* and *BDNF exon IV* (see Table 2 below) were designed to sit in or adjacent to the promoter region of the gene of interest. Primers were prepared at a concentration of 10 µM and 0.5 µL of each was added. The qPCR procedure was run in duplicate for all samples. Analysis of qPCR results compared the difference in Ct value of input DNA samples and ChIP DNA samples between the groups, as described previously (Schmittgen and Livak, 2008). Each set of samples was normalized to the control group (the NP + n-saline group for the acute ethanol treatment experiment or the P+vehicle group for the HDAC2 siRNA infusion experiment) and data was represented as mean ± SEM of proportion of control, where each control animal was normalized to 1. Relative differences between the groups were calculated using the following formula:

$$\text{Relative Quantification} = 2^{-\Delta\Delta Ct}$$

$$\text{where } \Delta\Delta Ct = \Delta Ct(\text{Experimental}) - \Delta Ct(\text{Control})$$

$$\text{where } \Delta Ct = Ct(\text{Input}) - Ct(\text{ChIP})$$

ChIP DNA from the negative control anti-IgG antibody reaction was compared to target ChIP DNA to confirm that the effect observed in the ChIP procedure was not non-specific. Following amplification by qPCR, DNA gel electrophoresis was performed to confirm a consistent-sized amplified DNA fragment for each primer (Schmittgen and Livak, 2008).

TABLE 2. PRIMERS USED FOR *IN SITU* RT-PCR AND QPCR

Primer Name	Used for	Primer Sequence (F=forward, R=reverse)
Arc	*In situ* RT-PCR	F: 5' ACAGAGGATGAGACTGAGGCAC 3' R: 5' TATTCAGGCTGGGTCCTGTCAC 3'
BDNF	*In situ* RT-PCR	F: 5' TAACGGCGGCAGACAAAAAGACT 3' R: 5' GTGTCTATCCTTATGAATCGCCAGCCAA 3'
HDAC2	*In situ* RT-PCR	F: 5' CGGTGGCTCAGTTGCTGGGG 3' R: 5' GGCCTCTGACTTCTTGGCGTGG 3'
Arc	ChIP qPCR	F: 5' AGTGCTCTGGCGAGTAGTCC 3' R: 5' TCGGGACAGGCTAAGAACTC 3'
BDNF exon I	ChIP qPCR	F: 5' GCGCCCAAAGCCCACCTTCT 3' R: 5' GCGTCGGCTCCGTGCTTCTT 3'
BDNF exon IV	ChIP qPCR	F: 5' GTTCGCTAGGACTGGAAGTGG 3' R: 5' CTTCTGCCTCGAAATAGACAC 3'

Golgi-Cox method for measurement of DSD

The Golgi-Cox staining procedure was used to calculate the DSD of neurons in the amygdaloid brain regions. The procedure was carried out as described in the FD Rapid Golgi Stain Kit manual (FD Neuro Technologies, Baltimore, MD) and as previously described by us (Pandey et al., 2008b). Rats were anaesthetized with pentobarbital (50 mg/kg), followed by decapitation and removal of brains. Following removal, brains were immediately immersed in golgi impregnation solution for 1 week in the dark. Brains were then immersed in sucrose solution for 1 week. The initial impregnation and cryoprotection solutions were replaced with fresh solutions after 1 day. Brains were frozen in 2-methylbutane at approximately -40ºC and stored in the dark at -80ºC. Coronal sections (200 μm) containing the amygdaloid brain regions were mounted on slides and dried in the dark at room temperature. Sections were silver stained according the instruction manual, followed by dehydration in alcohol, clearing in xylene solution

and mounting of coverslips. Sections were observed via light microscopy at high magnification (100x) for the identification and analysis of dendritic spines. Spines were counted using the Neurolucida Neuroexplorer program (MicroBrightField Bioscience, Williston, VT). DSD was calculated by Sholl analysis using a 10 μm increment. Only dendrites which showed complete golgi impregnation from the soma were used for counting. For each brain region, dendrites were counted from three adjacent sections for a total of 9 dendrites which were averaged for each rat. DSD was represented as mean ± SEM of the number of dendritic spines/10 μm of dendritic length.

Statistical analyses

For the experiments with three or more groups, including the acute ethanol experiment and the HDAC2 siRNA experiments with three groups, the differences between the groups were evaluated by a one-way ANOVA test. *Post hoc* comparisons were performed using Tukey's test. For the experiments with only two groups, the differences between the groups were evaluated by Student's t-test. Two-way repeated measures ANOVA was utilized for evaluation of alcohol drinking behavior in the HDAC2 siRNA experiment. $p<0.05$ was considered to be significant.

III. RESULTS

A. Specific Aim 1: The Role of Amygdaloid BDNF, Arc and DSD in the Genetic Predisposition to Anxiety and Alcohol-Preference

1. Summary

Innate anxiety appears to be a robust factor in the promotion of alcohol intake, possibly due to the anxiolytic effects of self-medication with alcohol. Brain-derived neurotrophic factor (BDNF) and its downstream target, activity-regulated cytoskeleton-associated (Arc) protein, play a role in the regulation of synaptic function and structure. In order to examine the role of the BDNF-Arc system and associated dendritic spines in the anxiolytic effects of ethanol, we investigated the effects of acute ethanol exposure on anxiety-like behaviors of alcohol-preferring (P) and -nonpreferring (NP) rats. We also examined changes in the expression of BDNF and Arc, and dendritic spine density (DSD), in amygdaloid brain regions of P and NP rats with or without ethanol exposure. It was found that in comparison to NP rats, P rats displayed innate anxiety-like behaviors, and had lower expression of both BDNF and Arc, and also had lower DSD in the central amygdala (CeA) and medial amygdala (MeA), but not in the basolateral amygdala (BLA). Acute ethanol treatment had an anxiolytic effect in P rats, but not in NP rats, and was associated with an increase in mRNA and protein levels of BDNF and Arc, and in DSD in the CeA and MeA, but not BLA. These results suggest that innate deficits in BDNF-Arc levels, and DSD, in the CeA and MeA may be involved in the anxiety-like and excessive alcohol-drinking behaviors of P rats, as ethanol increased these amygdaloid

synaptic markers and produced anxiolytic effects in P rats, but not NP rats. The detailed results of experiments performed for this specific aim are reported below.

2. The effects of acute ethanol exposure on anxiety-like behaviors in P rats and NP rats

Using the EPM test and LDB exploration test, we examined the anxiety-like behaviors of P and NP rats one hour following intraperitoneal injection of either ethanol (1 g/kg) or *n*-saline. Acute ethanol treatment resulted in blood ethanol levels that were not significantly different between P and NP rats (P: 95.1 ± 5.3 mg/dL, NP: 93.5 ± 4.7 mg/dL; n=16). The body weights of animals across the groups also showed no significant difference (NP + n-saline: 376.7 ± 4.9 g, NP + Ethanol: 373.3 ± 4.6 g, P + n-saline: 383.9 ± 3.9 g, P + Ethanol 383.3 ± 4.4 g; n=18-19), confirming that the animals were appropriately age-matched.

In one batch of P and NP rats, we measured the anxiety-like behaviors using the EPM test (Figure 3a). The results were represented as the percentage of open arm entries and percentage of time spent in the open arms. A one-way ANOVA followed by *post hoc* analysis using Tukey's test revealed that the measures were significantly different among the groups, and had the following F- and *p*-values: percentage open arm entries, $F_{3,36}$=21.3, p<0.001; percentage time spent in open arms, $F_{3,36}$=23.0, p<0.001. At baseline, P rats displayed anxiety-like behaviors, in comparison to NP rats, as evidenced by the significantly (p<0.001) lower percentage of open arm entries and percentage of time spent on open arms by P rats. Acute ethanol exposure attenuated the anxiety-like behaviors of P rats, as shown by a significant (p<0.001) increase in the percentage of open arm entries and percentage of time spent on open arms of P rats, but not NP rats

(Figure 3a). The number of total arm entries (open plus closed arms) was significantly different among the groups ($F_{3,36}$=5.5, p<0.01). *Post hoc* analysis revealed that acute ethanol treatment significantly (p<0.05) increased total arm entries in P rats, but not NP rats (Figure 3a), and that this effect was due to a significant (p<0.001) increase in open arm entries.

In a second batch of P and NP rats, anxiety-like behaviors were measured by the LDB exploration test. The percentage of time spent in the light and dark compartments was significantly different among the groups as measured by one-way ANOVA using Tukey's test for *post hoc* analysis ($F_{3,31}$=107.9, p<0.001). At baseline, we found that P rats spent significantly (p<0.001) less time in the light compartment and more time in the dark compartment, in comparison to NP rats, indicating that P rats also express anxiety-like behaviors using this testing paradigm. Acute ethanol exposure resulted in reduced anxiety-like behaviors in P rats as demonstrated by a significant (p<0.001) increase in the time spent in the light compartment and decreased time spent in the dark compartment, whereas acute ethanol exposure did not significantly change LDB exploration in NP rats (Figure 3b). General activity was measured by the total number of ambulations in the LDB apparatus, and was similar among all groups.

Taken together, these results indicate that, in comparison to NP rats, P rats exhibit high anxiety-like behaviors at baseline, and acute ethanol exposure reduced the innate anxiety-like behaviors of P rats. Interestingly, acute ethanol exposure had no effect on EPM or LDB test performance in NP rats.

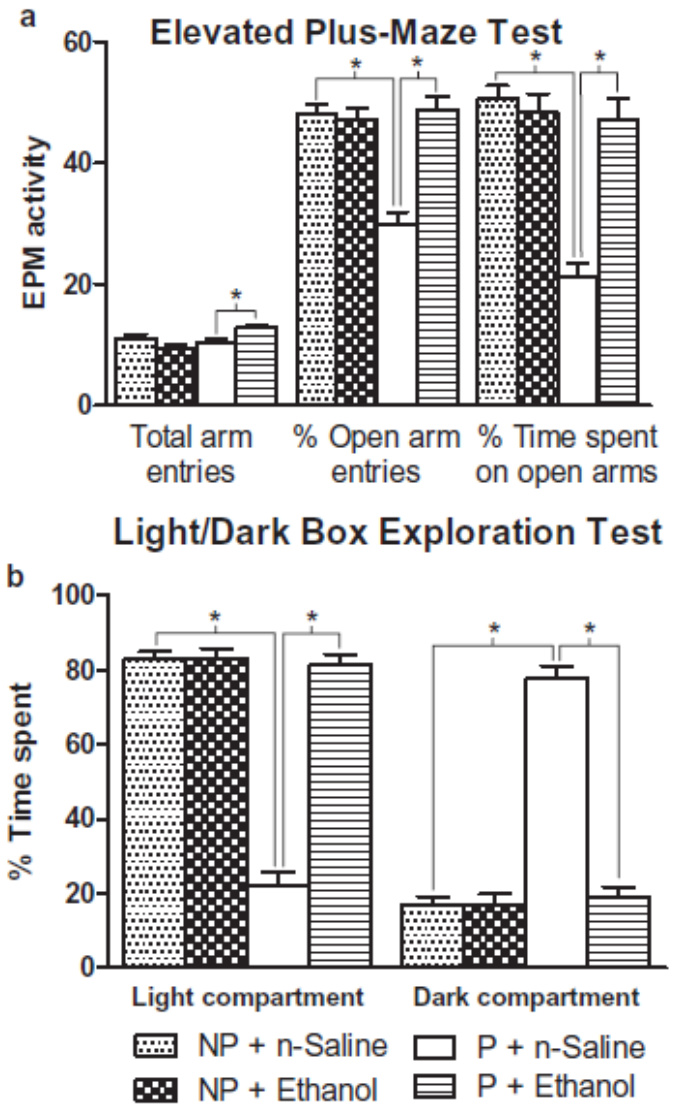


Figure 3. Innate anxiety-like behaviors and the anxiolytic effects of acute ethanol exposure in P and NP rats.

a. Alcohol-preferring (P) rats show innate anxiety-like behaviors in comparison to alcohol-nonpreferring (NP) rats as evidenced by the lower percentage of open arm entries and time spent in the open arm in the elevated-plus maze (EPM) test. Acute ethanol exposure (1 g/kg) reduces the anxiety-like behaviors in P rats, but not NP rats. Values are the mean ± SEM of 10 rats in each group. *Significantly different from their respective control groups (p<0.01-0.001; ANOVA followed by Tukey's test).

b. The light-dark box (LDB) exploration test confirmed these results as evidenced by the percentage of time spent in the light compartment and dark compartment.

Values are the mean ± SEM of 8-9 rats in each group. *Significantly different from their respective control groups (p<0.001; ANOVA followed by Tukey's test).

3. Baseline levels and the effects of acute ethanol exposure on amygdaloid BDNF expression in P and NP rats

We previously reported that innate BDNF expression levels in the CeA and MeA, but not BLA, of P rats were lower than those of NP rats (Prakash et al., 2008). Here, we have confirmed those results and extended the study to examine the effects of acute ethanol exposure on amygdaloid BDNF expression. The BDNF mRNA and protein levels in the CeA and MeA were significantly different among the groups as analyzed by one-way ANOVA using Tukey's test for *post hoc* analysis. The F- and *p*-values of the comparison between groups for BDNF mRNA measurements in the CeA were $F_{3,20}$=50.0, p<0.001, and in the MeA were $F_{3,20}$=29.0, p<0.001. These values for the BDNF protein measurements in the CeA were $F_{3,20}$=13.6, p<0.001, and in the MeA were $F_{3,20}$=8.5, p<0.001. We found that, in comparison to NP rats, P rats had significantly lower baseline levels of BDNF mRNA in the CeA (p<0.001) and MeA (p<0.01), but not BLA, and significantly lower baseline levels of BDNF protein in the CeA (p<0.001) and MeA (p<0.01), but not BLA (Figure 4). Acute ethanol treatment significantly increased BDNF mRNA and protein in the CeA (p<0.001) and MeA (p<0.01), but not BLA, of P rats, however no significant changes were observed in amygdaloid BDNF expression of NP rats (Figure 4). These results suggest the possibility that innate anxiety-like behaviors of P rats may be related to reduced levels of BDNF expression in the CeA and MeA, and that the anxiolytic effects of acute ethanol exposure may be associated with increased BDNF expression.

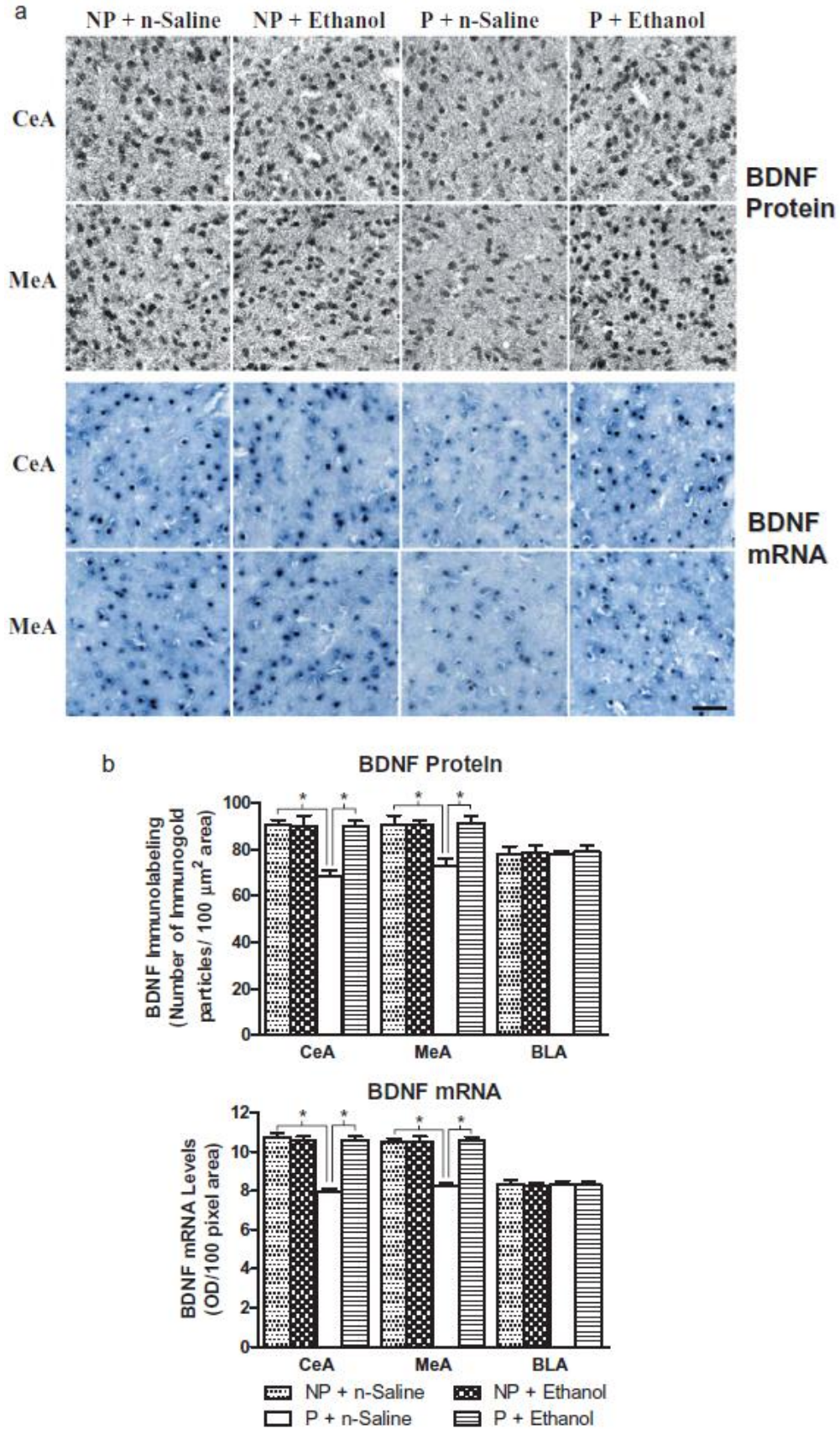

a
NP + n-Saline
NP + Ethanol
P + n-Saline
P + Ethanol
CeA
MeA
BDNF Protein
CeA
MeA
BDNF mRNA
b
BDNF Protein
BDNF Immunolabeling (Number of Immunogold particles/ 100 μm² area)
100
80
60
40
20
0
CeA
MeA
BLA
BDNF mRNA
BDNF mRNA Levels (OD/100 pixel area)
12
10
8
6
4
2
0
CeA
MeA
BLA
NP + n-Saline
NP + Ethanol
P + n-Saline
P + Ethanol

Figure 4. Baseline differences and the effects of acute ethanol exposure on BDNF mRNA and protein expression in amygdaloid brain regions.
a. Representative low-magnification photomicrographs (Scale bar = 50 μm) of brain-derived neurotrophic factor (BDNF) gold-immunolabeling (protein levels) and *in-situ* RT-PCR (mRNA levels) in central (CeA) and medial amygdala (MeA) of alcohol preferring (P) and non-preferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg).
b. Quantification of BDNF protein and mRNA were performed to analyze the differences in BDNF expression levels in the amygdaloid brain regions of P and NP rats at baseline and following acute ethanol exposure. BDNF mRNA (optical density per 100 pixel area) and protein (number of immunogold particles per 100 μm^2 area) levels are lower in the CeA and MeA , but not BLA, of P rats in comparison of NP rats. Acute ethanol exposure increases BDNF expression in the CeA and MeA of P rats, but not NP rats. Values are represented as the mean ± SEM of 6 rats in each group. *Significantly different from their respective control groups (p<0.01-0.001; ANOVA followed by Tukey's test).

4. Baseline levels and the effects of acute ethanol exposure on amygdaloid Arc expression in P and NP rats

Induction of the Arc immediate-early gene is a downstream consequence of BDNF signaling that may mediate the effects of BDNF on synaptic structure and function (Bramham et al., 2010; Messaoudi et al., 2002; Pandey et al., 2008b; Ying et al., 2002). We measured Arc mRNA and protein levels in P and NP rats following injection with either ethanol or *n*-saline. The Arc mRNA and protein levels in the CeA and MeA were significantly different among the groups (Arc mRNA in CeA: $F_{3,20}$=31.0, p<0.001; Arc mRNA in MeA: $F_{3,20}$=8.4, p<0.001; Arc protein in CeA: $F_{3,26}$=31.4, p<0.001; Arc protein in MeA: $F_{3,26}$=15.5, p<0.001). W*e* found that P rats innately expressed significantly lower levels of Arc mRNA and protein in the CeA and MeA , but not BLA, in comparison to NP rats (Figure 5). Acute ethanol injection significantly increased Arc mRNA and protein levels in the CeA (p<0.001) and MeA (Arc mRNA: p<0.01; Arc

protein: $p<0.001$), but not BLA, of P rats, without having any significant effects on Arc expression in NP rats (Figure 5). These results indicate that BDNF signaling via Arc induction in the amygdala may play a role in the anxiety-like behaviors and anxiolytic effects of ethanol observed in P rats, but not NP rats.

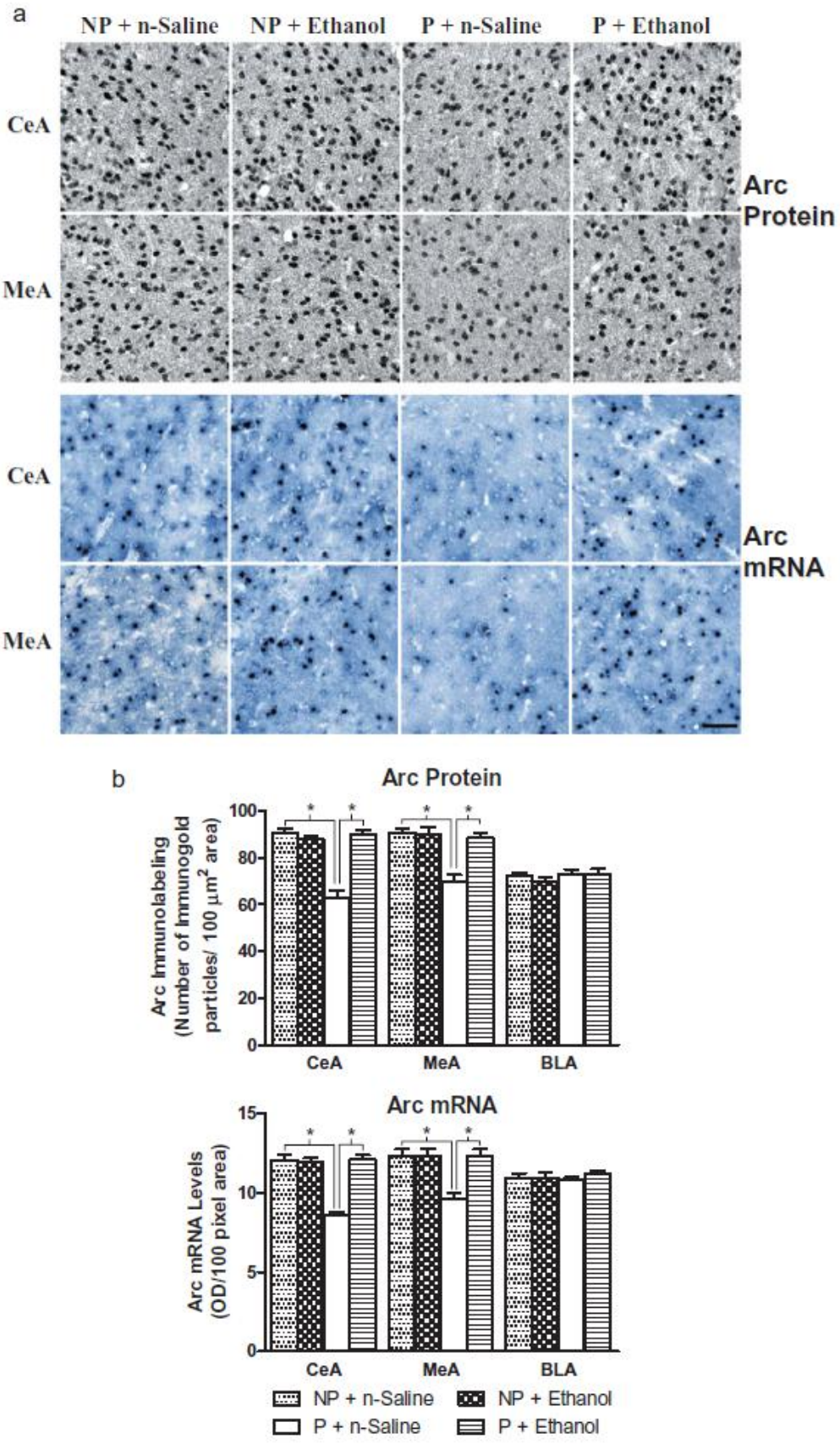

a
NP + n-Saline
NP + Ethanol
P + n-Saline
P + Ethanol
CeA
MeA
Arc Protein
CeA
MeA
Arc mRNA
b
Arc Protein
Arc Immunolabeling (Number of Immunogold particles/ 100 μm² area)
100
80
60
40
20
0
CeA
MeA
BLA
Arc mRNA
Arc mRNA Levels (OD/100 pixel area)
15
10
5
0
CeA
MeA
BLA
NP + n-Saline
NP + Ethanol
P + n-Saline
P + Ethanol

Figure 5. Baseline differences and the effects of acute ethanol exposure on Arc mRNA and protein expression in amygdaloid brain regions.
a. Representative low-magnification photomicrographs (Scale bar = 50 μm) gold-immunolabeling for activity-regulated cytoskeleton-associated (Arc) protein levels and *in-situ* RT-PCR for Arc mRNA levels in central (CeA) and medial amygdala (MeA) of alcohol preferring (P) and non-preferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg).
b. Quantification of Arc expression was performed to determine Arc mRNA and protein levels in the amygdaloid brain regions of P and NP rats at baseline and following acute ethanol treatment. Arc mRNA (optical density per 100 pixel area) and protein (number of immunogold particles per 100 μm^2 area) levels are lower in the CeA and MeA , but not BLA, of P rats in comparison of NP rats. Acute ethanol exposure increases Arc expression in the CeA and MeA of P rats, but not NP rats. Values are represented as the mean ± SEM of 6-8 rats in each group. *Significantly different from their respective control groups (p<0.01-0.001; ANOVA followed by Tukey's test).

5. Amygdaloid DSD in P and NP rats at baseline and following acute ethanol exposure

BDNF signaling via the induction of the Arc immediate-early gene has been shown to result in structural and functional synaptic changes, including the proliferation and lengthening of dendritic spines (Bramham et al., 2008; Horch, 2004; Messaoudi et al., 2007; Pandey et al., 2008b). To study the potential structural effects of amygdaloid BDNF signaling and Arc expression in P and NP rats, we quantified the DSD within amygdaloid brain regions of a group of P and NP rats at baseline and following acute ethanol administration. The DSD in the CeA and MeA was found to be significantly different among the groups (DSD in CeA: $F_{3,16}$=21.3, p<0.001; DSD in MeA: $F_{3,16}$=24.7, p<0.001). P rats were found to have significantly (p<0.001) lower levels of DSD in the CeA and MeA, but not BLA, in comparison to NP rats. Following injection of acute ethanol, DSD was significantly (p<0.001) increased in the CeA and MeA, but not BLA, of P rats. Acute ethanol did not significantly change DSD in amygdaloid regions of NP

rats (Figure 6). These findings correspond with amygdaloid BDNF and Arc expression levels suggesting that downstream BDNF-Arc signaling may result in modulation of dendritic spine morphology. Lower amygdaloid DSD may play a role in innate anxiety-like behaviors seen in P rats, as compared to NP rats, and increased DSD may be related to the anxiolytic effects of acute ethanol in P rats.

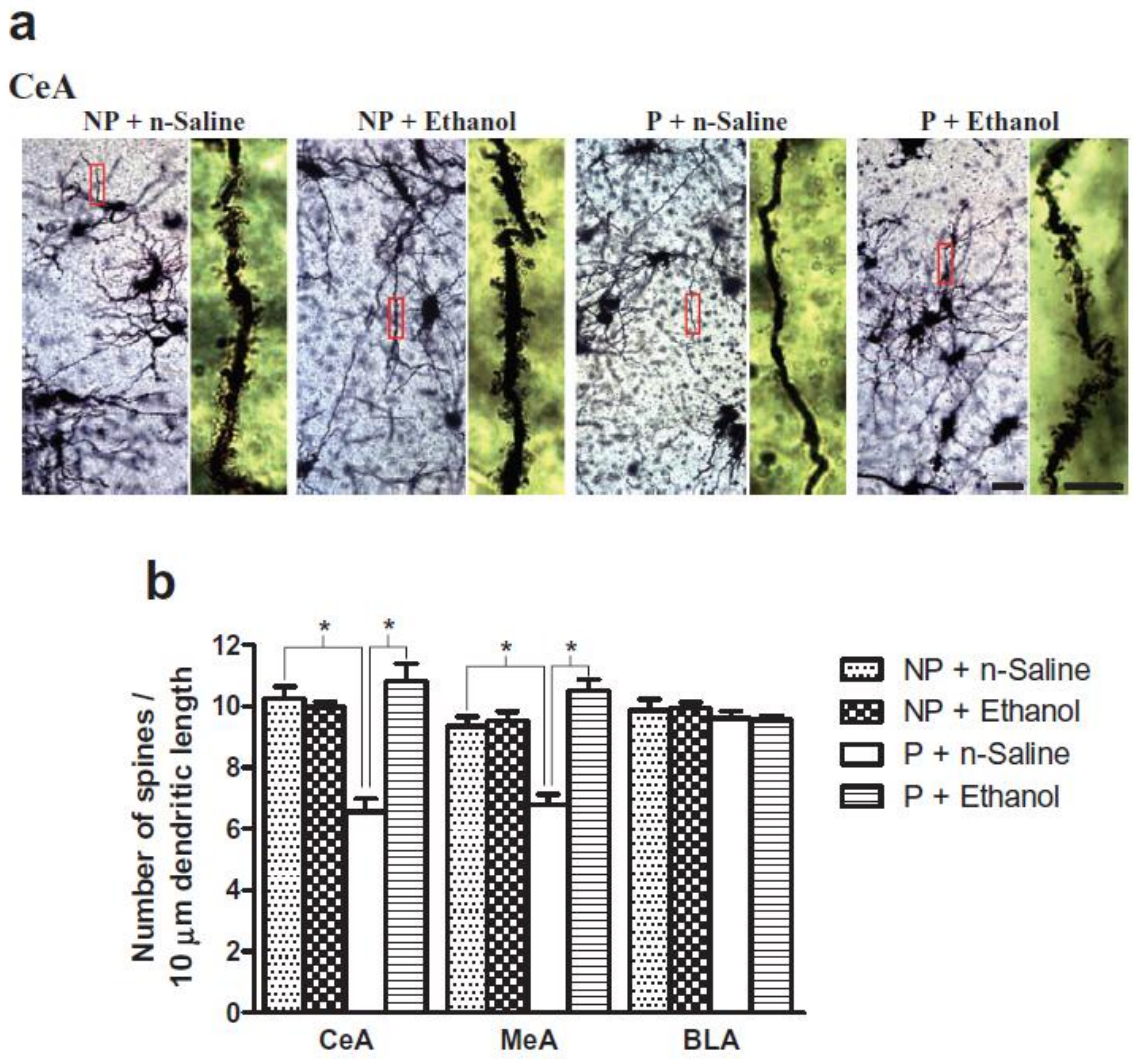


Figure 6. Baseline differences and the effect of acute ethanol exposure on the DSD of P and NP rats in amygdaloid brain regions.

a. Representative low-magnification photomicrographs (Scale bar = 50 μm) showing Golgi-impregnated neurons in the central amygdala (CeA) of alcohol preferring (P) and non-preferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg). The boxed areas of the low magnification photographs are shown at high magnification (Scale bar = 10 μm) in the adjacent photograph showing dendritic spines.

b. Dendritic spines were quantified by neuronal morphology tracking software to determine the dendritic spine density (DSD) of P and NP rats at baseline and following exposure to acute ethanol. At baseline, P rats have lower DSD in the CeA and MeA, but not BLA, than NP rats. Acute ethanol exposure results in dendritic spine proliferation in P rats, but not NP rats, as evidenced by the increase in DSD in the CeA and MeA, but not BLA. Values are represented as the mean ± SEM of the number of dendritic spines per 10 μm of dendritic length of 5 rats per group. *Significantly different from control groups ($p<0.001$; ANOVA followed by Tukey's test).

B. Specific Aim 2: Ethanol-Induced Chromatin Modifications in the Regulation of Synaptically Active Genes in the Amygdaloid Structures of P and NP Rats

1. Summary

Chromatin remodeling is an emerging field in the area of neuronal physiology and pathologies associated with psychiatric disorders including anxiety and alcoholism (Abel and Zukin, 2008; Levenson and Sweatt, 2005; Pandey et al., 2008a; Tsankova et al., 2007b). The acetylation of histone proteins, specifically histone H3 at lysine 9 and H4 at lysine 8, has been found to be a key regulator of chromatin structure that may mediate transcriptional events at specific gene promoters (Jenuwein and Allis, 2001). HDAC enzymes control the level of histone acetylation via enzymatic removal of acetyl groups from N-terminal lysine residues (Jenuwein and Allis, 2001). Recently, a role has been identified for specific HDAC isoforms in the regulation of specific behavioral events, such as memory formation, stress response, depression, and Alzheimer's disease (Covington et al., 2009; Graff et al., 2012; Guan et al., 2009; Tsankova et al., 2006). Previously, Pandey *et al.* identified that alcohol-withdrawal induced anxiety-like behaviors in an unselected stock of rats was associated with increased HDAC activity and reduced histone H3K9 and H4K8 acetylation in amygdaloid brain regions (2008a). Treatment with TSA, a HDAC inhibitor, resulted in a reduction in withdrawal-induced anxiety, and corrected the deficits in histone acetylation, suggesting an association between chromatin structure in the CeA and MeA with regulation of anxiety-behaviors. Yet, it is not known whether innate aberrations in HDAC levels and chromatin structure may play a role in genetically predisposed anxiety and alcohol preference.

To test this possibility, we examined the levels of HDAC isoform 2 and associated histone modifications, in the amygdaloid brain regions of P and NP rats allowed voluntary exposure to ethanol. In comparison to NP rats, at baseline P rats exposed had higher levels of HDAC2 in the CeA and MeA, but not BLA, which were associated with lower histone H3K9 and H4K8 acetylation levels. P rats with access to ethanol consumed greater amounts of ethanol than NP rats, and chronic ethanol exposure reduced innately high HDAC2 levels and corrected deficits in histone H3K9 and H4K8 acetylation in the CeA and MeA. In order to test differences between P and NP rats exposed to a given dose of ethanol, we treated rats with an acute ethanol and examined HDAC2 and HDAC4, and histone H3K9 acetylation and methylation. These data identified that in comparison to NP rats, P rats have higher levels of HDAC2, but not HDAC4, associated with deficits in histone H3K9 acetylation in the CeA and MeA, but not BLA. Treatment with acute ethanol produced anxiolytic effects associated with decreased HDAC2, increased histone H3K9 acetylation and reduced histone H3K9 methylation in the CeA and MeA of P rats, but not NP rats. ChIP analysis of specific genes identified that P rats have lower levels of histone acetylation associated with the promoters of *Arc* and *BDNF exon IV*, but not *BDNF exon I*, in the amygdala than NP rats. The acetylation levels of histone H3 associated with *Arc* and *BDNF exon IV* were increased by acute ethanol treatment, suggesting a role for epigenetic regulation of synaptically active proteins in amygdaloid brain regions in the regulation of anxiety and the anxiolytic effect of ethanol exposure that may be associated with a genetic predisposition to alcoholism.

2. Voluntary ethanol consumption and the anxiolytic effects of chronic exposure in P rats and NP rats

In order to determine the effects of voluntary ethanol consumption on chromatin structure in amygdaloid brain regions, we utilized a two-bottle free choice paradigm which examined alcohol preference in P and NP rats. Following habituation to the paradigm, rats were given either two bottle of water or one bottle of water and one bottle of ethanol, beginning with 7% ethanol for three days and increasing to 9% ethanol for the subsequent 7 days. We found that P rats consumed significantly greater amounts of alcohol in comparison to NP rats during periods of 7% and 9% ethanol exposure (Figure 7a; 7%: t_{15} = 22.9, $p<0.001$; 9%: t_{15} = 25.7, $p<0.001$; by Student's t-test). Analysis of blood ethanol levels on the final day of ethanol exposure confirmed the difference in ethanol intake such that P rats consumed ethanol to significantly higher blood ethanol concentrations than NP rats (t_{15} = 19.7, $p<0.001$; P: 166.9 ± 5.8 mg/dL, NP: 35.2 ± 2.7 mg/dL; n=8-9). This finding confirmed that the increased ethanol consumption of P rats effectively resulted in higher blood ethanol levels than NP rats. Importantly, the total fluid intake between P and NP rats exposed to voluntary ethanol was not significantly different among the groups (Figure 7a). The similar total fluid consumption suggests that the higher ethanol intake of P rats is associated with the preference for ethanol as opposed to increased levels of general fluid consumption.

In order to determine the effects of chronic ethanol exposure on anxiety in P and NP rats, we tested the rats via the LDB exploration test on the final day of ethanol exposure. One-way ANOVA analysis of LDB testing indicated that there were significant differences in the percent of time spent in the light and dark compartment

between P and NP rats exposed either to water/water or to water/ethanol ($F_{3,28} = 82.5$, $p<0.001$; by one-way ANOVA). Post-hoc analysis by Tukey's test confirmed that in P and NP rats exposed to only water, P rats spent significantly ($p<0.001$) more time in the dark compartment and less time in the light compartment than NP rats, confirming the baseline anxiety-like behaviors present in P rats (Figure 7b). Chronic ethanol exposure significantly ($p<0.001$) increased the percentage of time spent in the light compartment and reduced the percentage of time spent in the dark compartment by P rats, but had no effect in NP rats (Figure 7b). Measurement of total ambulations did not differ among the groups (Figure 7b), suggesting that ethanol exposure did not produce changes in the general activity of P and NP rats. These data indicate that chronic ethanol exposure produces anxiolytic effects in P rats, but not NP rats.

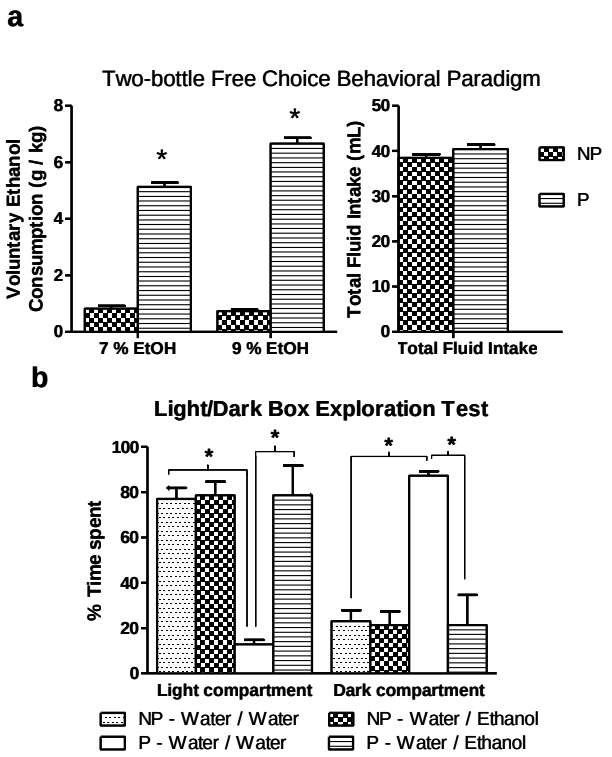


Figure 7. Differences in voluntary ethanol consumption and the anxiolytic effects of chronic ethanol exposure between P and NP rats.

a. P rats voluntarily consume more ethanol than NP rats during the two-bottle free choice paradigm when given access to water, and 7 % or 9 % ethanol. The total fluid intake does not differ between the two groups suggesting that the higher ethanol consumption of P rats is not due to an overall increase in fluid consumption. Values for ethanol drinking represent mean ± SEM of g ethanol consumed per kg body weight per day for n=8-9 rats. Total fluid intake is represented by the mean ± SEM of mL fluid consumed daily by P and NP rats throughout the behavioral monitoring period. *Significantly different from control group (p<0.001 by Student's t-test).

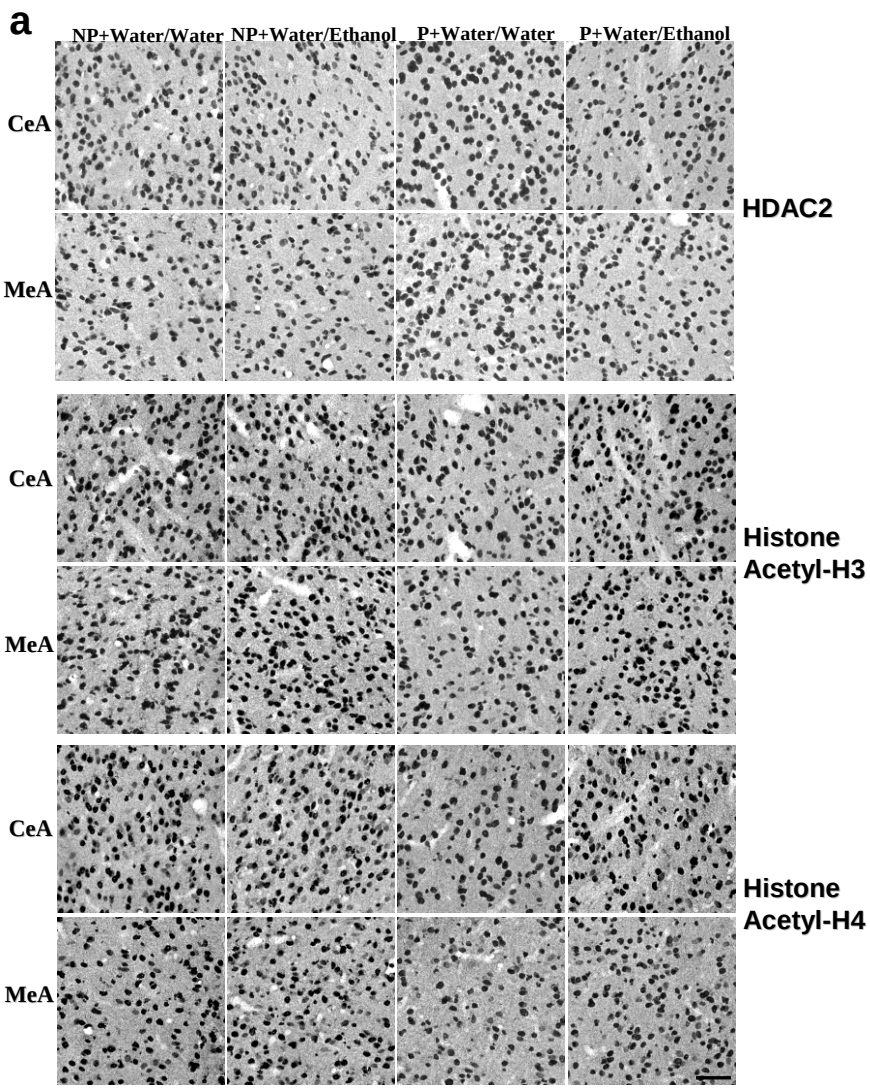
b. Light-dark box (LDB) exploration test data was recorded by sensors within the apparatus and analyzed to determine the percentage of time spent in each compartment. P rats exposed to only water (P + Water / Water) spend less time in the light compartment and more time in the dark compartment of the LDB than similarly treated NP rats (NP + Water / Water), confirming the innate anxiety-like behaviors displayed by P rats, but not NP rats. The anxiety-like behaviors of P rats are sensitive to the anxiolytic effects of chronic ethanol consumption, as evidenced by an increase in the amount of time spent in the light compartment and a decrease in the amount of time spent in the dark compartment of the LDB apparatus in P rats with free access to water and ethanol (P + Water / Ethanol) as opposed to P rats with only access to water. Ethanol-exposed NP rats (NP + Water / Ethanol) do not show differences in anxiety-like behaviors in comparison to the NP + Water / Water group. Values are represented as mean ± SEM of the percentage of time spent in the light and dark compartments for n=7-9 rats. *Significantly different from control groups (p<0.001 by ANOVA followed by Tukey's test).

3. The effects of chronic ethanol exposure on chromatin remodeling and HDAC2 protein in the amygdaloid brain regions of P and NP rats

To study the effects of chronic ethanol exposure on amygdaloid chromatin remodeling in P and NP rats, we explored the levels of H3K9 and H4K8 acetylation by utilizing immunohistochemical analysis in amygdaloid brain regions after exposing the groups to the two-bottle free choice paradigm of voluntary drinking for 10 days. Following the final day of ethanol exposure, P and NP rats allowed free access to only water or water and 9% ethanol were sacrificed and analyzed for histone acetylation levels. We found that the levels of acetylated histone H3K9 and H4K8 were significantly different between the groups in the CeA and MeA, but not BLA (H3K9 acetylation in CeA: $F_{3,22} = 35.8$, $p<0.001$; H3K9 acetylation in MeA: $F_{3,22} = 8.8$, $p<0.001$; H3K9 acetylation in BLA: $F_{3,22} = 0.736$, $p=0.542$; H4K8 acetylation in CeA: $F_{3,22} = 92.9$, $p<0.001$; H4K8 acetylation in MeA: $F_{3,22} = 40.6$, $p<0.001$; H4K8 acetylation in BLA: $F_{3,22} = 1.3$, $p=0.312$). *Post hoc* analysis by Tukey's test revealed that acetylation levels of histones were significantly lower in the CeA (H3K9: $p<0.001$; H4K8: $p<0.001$) and MeA (H3K9: $p<0.001$; H4K8: $p<0.001$), but not BLA, of P rats with water only in comparison to NP rats with water only (Figure 8). Exposure to chronic ethanol via voluntary free access to ethanol or water resulted in a significant increase in H3K9 and H4K8 acetylation levels in the CeA (H3K9: $p<0.001$; H4K8: $p<0.001$) and MeA (H3K9: $p<0.05$; H4K8: $p<0.001$) of P rats (Figure 8). Chronic ethanol exposure has no significant effects on histone acetylation levels in NP rats (Figure 8).

In order to study a potential regulator of the observed changes in histone acetylation levels, we examined the levels of HDAC2 protein following the two-bottle

free choice paradigm. Corresponding with the histone acetylation data, we found that differences existed among the groups in the CeA and MeA, but not BLA (CeA: $F_{3,22}$ = 21.2, p<0.001; MeA: $F_{3,22}$ = 24.5, p<0.001; BLA: $F_{3,22}$ = 1.0, p=0.392; by one-way ANOVA followed by Tukey's test), such that P rats had significantly higher levels of HDAC2 in the CeA (p<0.001) and MeA (p<0.001) than NP rats at baseline. Chronic ethanol exposure significantly reduced the levels of HDAC2 in the CeA (p<0.001) and MeA (p<0.001), but not BLA, in P rats, but not NP rats (Figure 8). Taken together, these data indicate that chronic voluntary ethanol exposure in P rats produces changes in histone acetylation in CeA and MeA that are representative of chromatin relaxation and may be related to a reduction in HDAC2 protein. Given that these effects are not present in NP rats, this may suggest that the higher ethanol preference observed in P rats could be related to the ethanol effect on HDAC2-mediated chromatin relaxation on the innately condensed chromatin structure in the CeA and MeA of P rats.


a
NP+Water/Water
NP+Water/Ethanol
P+Water/Water
P+Water/Ethanol
CeA
MeA
HDAC2
CeA
MeA
Histone
Acetyl-H3
CeA
MeA
Histone
Acetyl-H4

b

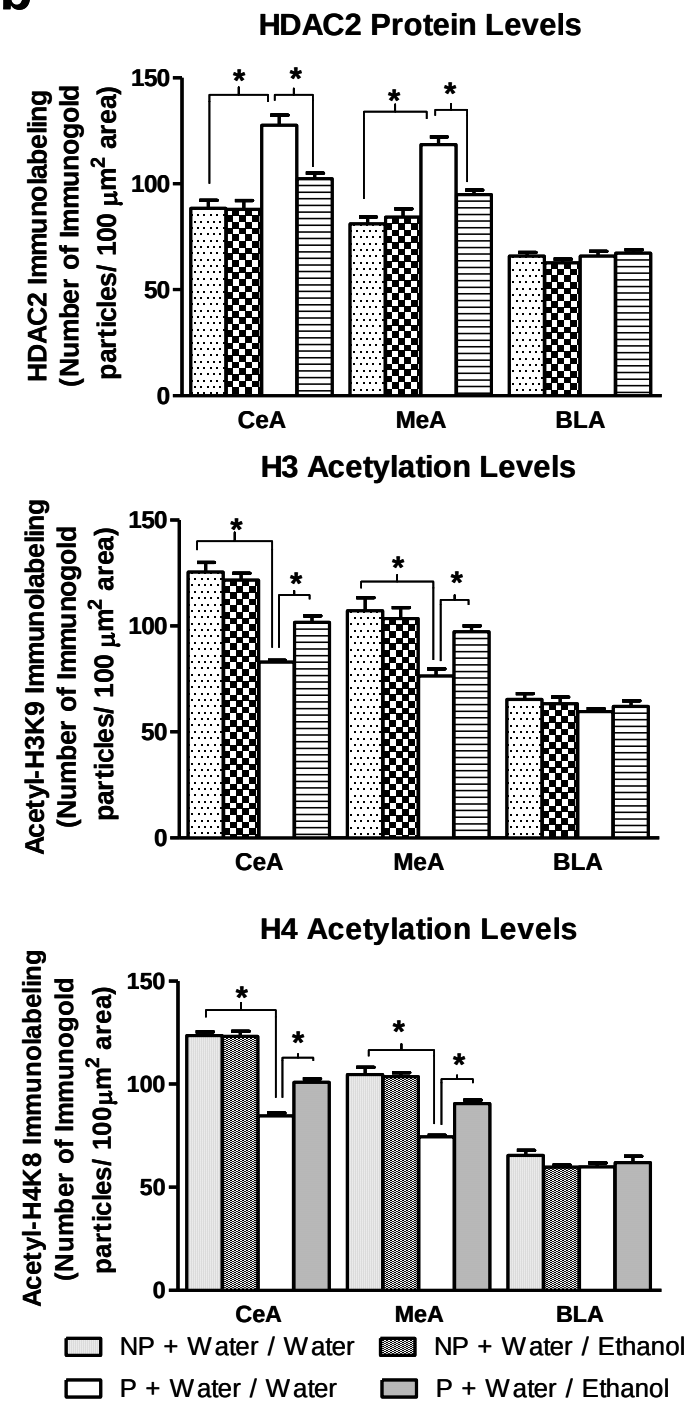

HDAC2 Protein Levels
HDAC2 Immunolabeling (Number of Immunogold particles/ 100 μm² area)
150
100
50
0
CeA
MeA
BLA
H3 Acetylation Levels
Acetyl-H3K9 Immunolabeling (Number of Immunogold particles/ 100 μm² area)
H4 Acetylation Levels
Acetyl-H4K8 Immunolabeling (Number of Immunogold particles/ 100μm² area)
NP + Water / Water
NP + Water / Ethanol
P + Water / Water
P + Water / Ethanol

Figure 8. Histone acetylation and HDAC2 levels in the amygdaloid brain regions of P and NP rats following exposure to chronic ethanol.
a. Representative low-magnification photomicrographs (Scale bar = 50 μm) of histone H3 lysine 9 (H3K9) and H4 lysine 8 (H4K8) acetylation, and histone deacetylase isoform 2 (HDAC2) protein levels as measured by gold-immunolabeling in central amygdala (CeA) and medial amygdala (MeA) of alcohol-preferring (P) and –nonpreferring (NP) rats after 10 days of free access to either water only (Water/Water) or water and ethanol (Water/Ethanol).
b. Quantification of acetylated histones H3K9 and H4K8, and HDAC2 protein levels was performed in the amygdaloid brain regions of P and NP rats following exposure to the two-bottle free choice paradigm. Histone acetylation levels were lower and HDAC2 protein levels were higher in the CeA and MeA, but not BLA, of P rats than NP rats exposed to only water. Exposure to chronic ethanol increased levels of H3K9 and H4K8 acetylation and decreased HDAC2 protein levels in the CeA and MeA of P rats, but not NP rats. These results suggest a role for condensed chromatin and chromatin remodeling in the alcohol preference of P rats. Values are represented as the mean ± SEM of the number of immunogold particles per 100 μm^2 area of 6-7 rats per group. *Significantly different from control groups (p<0.001-0.05; ANOVA followed by Tukey's test).

4. The effects of acute ethanol exposure on anxiety-like behaviors and expression of HDAC isoforms in the amygdaloid brain regions of P and NP rats

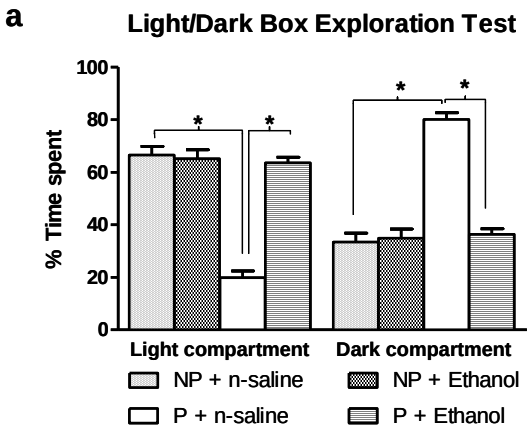
Following intraperitoneal acute ethanol injection (1 g/kg), we assessed the anxiety-like behaviors of P and NP rats using the LDB exploration test. The percentage of time spent in the light and dark compartment were significantly different among the groups, as measure by one-way ANOVA followed by *post hoc* analysis using Tukey's test ($F_{3,48}$ = 58.8, p<0.001). In agreement with our previous experiment (see above), we found that at baseline, P rats spent significantly (p<0.001) less time in the light compartment than NP rats, and that acute ethanol treatment produced an anxiolytic effect in P rats, but not NP rats, as evidenced by a significant (p<0.001) increase in the percentage of time spent in the light compartment (Figure 9a). Consistently with the previous data (see above), the total number of ambulations did not differ significantly between the groups, indicating

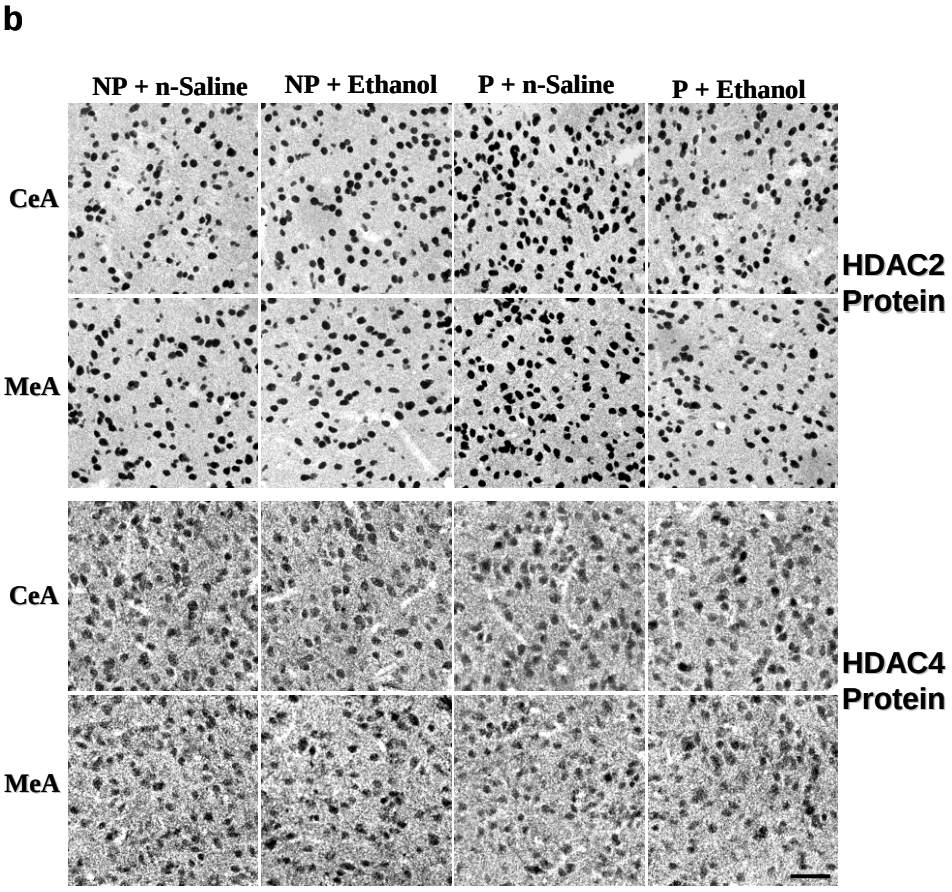
that the findings were not related to difference or changes in overall activity (Figure 9a). Acute ethanol treatment produced blood ethanol concentrations that did not differ between the P and NP rats exposed to ethanol (P: 100.8 ± 5.0 mg/dL, NP: 101.1 ± 5.2 mg/dL; n=13). The body weights of the rats did not differ significantly among the groups (NP + n-saline: 373.3 ± 4.9 g, NP + Ethanol: 374.0 ± 4.5 g, P + n-saline: 382.5 ± 6.3 g, P + Ethanol: 378.5 ± 6.6 g; n=13), suggesting appropriately age- and body weight-matched animals.

Unpublished observations in our lab demonstrated that P rats have higher levels of amygdaloid HDAC activity than NP rats, and that acute ethanol treatment reduced HDAC activity in P rats, but not NP rats. Therefore, we explored the possibility that these findings were related to differences in the expression of specific HDAC isoforms in the amygdala. HDAC proteins have been classified into four separate groups based upon homology, regulation and cellular localization (de Ruijter et al., 2003; Dokmanovic et al., 2007). The findings regarding HDAC activity specifically implicated class I and II HDAC proteins which are localized in the nucleus, but not the cytosol. HDAC2, a nuclear class I HDAC, has recently been identified as a modulator of synaptic plasticity and dendritic spines (Guan et al., 2009). Given our findings regarding the role of amygdaloid synaptic plasticity and DSD in the anxiolytic effects of ethanol (Moonat et al., 2011), we sought to explore the expression levels of amygdaloid HDAC2 protein in P and NP rats following acute ethanol exposure. For comparison we also studied the expression levels of HDAC4, a cytosolic class II isoform.

We treated P and NP rats with either *n*-saline or acute ethanol and analyzed the levels of HDAC2 and HDAC4 protein in amygdaloid brain regions. One-way ANOVA

followed by *post hoc* analysis by Tukey's test revealed that HDAC2 protein levels in the CeA and MeA were significantly different among the groups (HDAC2 protein in CeA: $F_{3,20} = 57.3$, $p<0.001$; HDAC2 protein in MeA: $F_{3,20} = 68.8$, $p<0.001$). We found that at baseline, P rats had significantly ($p<0.001$) higher levels of HDAC2 protein, but not HDAC4 protein, in the CeA and MeA, but not BLA, in comparison to NP rats (Figure 9b,c). Acute ethanol treatment resulted in a significant ($p<0.001$) reduction in HDAC2 levels, but not HDAC4 levels, in P rats, but not NP rats (Figure 9b,c). These data suggest that HDAC2 expression levels may play a role in the observed differences in amygdaloid HDAC activity between P and NP rats.


a
Light/Dark Box Exploration Test
% Time spent
100
80
60
40
20
0
*
*
*
*
Light compartment
Dark compartment
NP + n-saline
P + n-saline
NP + Ethanol
P + Ethanol


b
NP + n-Saline
NP + Ethanol
P + n-Saline
P + Ethanol
CeA
MeA
HDAC2 Protein
CeA
MeA
HDAC4 Protein

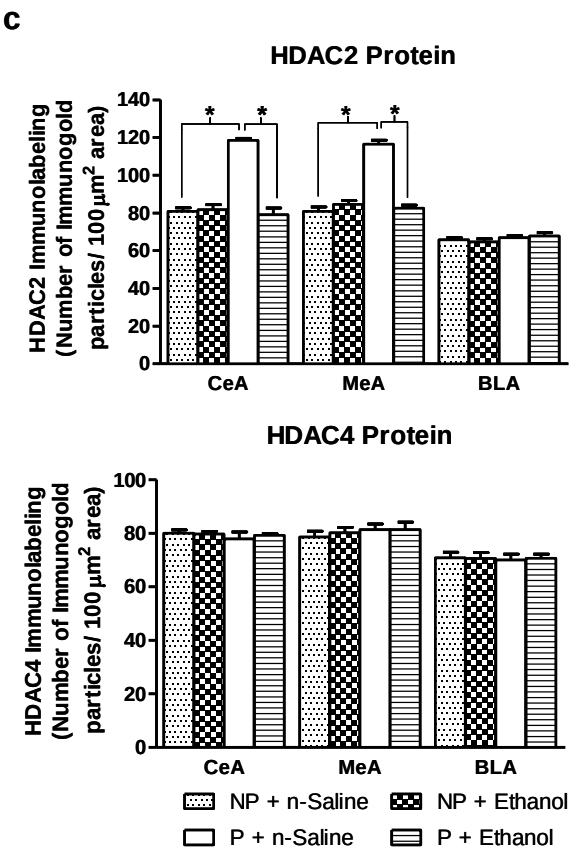


Figure 9. The effect of acute ethanol on the anxiety-like behaviors and amygdaloid expression of HDAC2 and HDAC4 in P and NP rats.

a. The light-dark box (LDB) exploration test confirmed that alcohol-preferring (P) rats display innate anxiety-like behaviors in comparison to alcohol-nonpreferring (NP) rats. Acute ethanol treatment (1 g/kg) reduced the anxiety-like behaviors of P rats, but not NP rats. Values are represented as the mean ± SEM of the percentage of time spent in each compartment of 13 rats per group. *Significantly different from control groups ($p<0.001$; ANOVA followed by Tukey's test).

b. Representative low-magnification photomicrographs (Scale bar = 50 μm) of histone deacetylase isoform 2 (HDAC2) and isoform 4 (HDAC4) gold-immunolabeling in central (CeA) and medial amygdala (MeA) of alcohol-preferring (P) and -nonpreferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg).

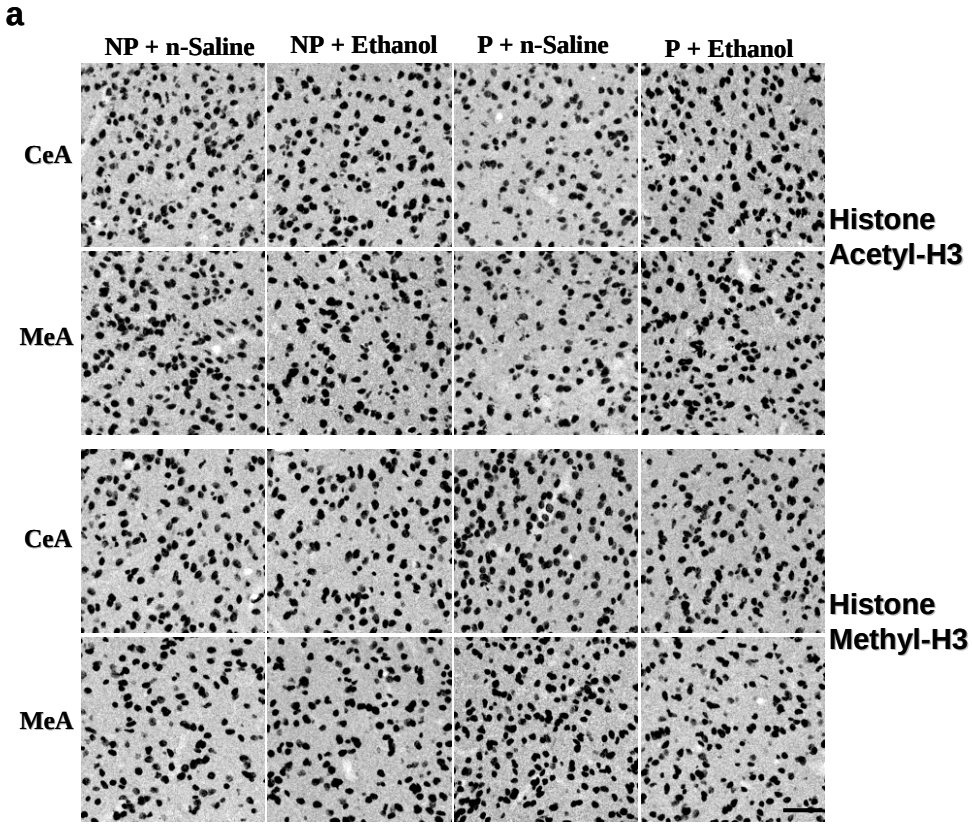
c. Quantification of HDAC2 and HDAC4 gold-immunolabeling was performed to analyze the protein levels of these HDAC enzymes in the amygdaloid brain regions of P and NP rats at baseline and following treatment with acute ethanol. HDAC2 protein levels, but not HDAC4 protein levels, were higher in the CeA and MeA , but not BLA, of P rats in comparison of NP rats. Acute ethanol exposure reduced HDAC2 expression, but did not modulate HDAC4 expression, in the CeA and MeA of P rats, but not NP rats. Values are represented as the mean ± SEM of the number of immunogold particles per 100 μm^2 area of 6 rats per group. *Significantly different from their respective control groups ($p<0.001$; ANOVA followed by Tukey's test).

5. The effects of acute ethanol exposure on chromatin remodeling in the amygdaloid brain regions of P and NP rats.

Since HDAC proteins are key regulators of histone acetylation levels, we aimed to analyze the histone acetylation and methylation status in the amygdaloid brain regions of P and NP rats. The lysine residue at N-terminal position 9 of histone H3 (H3K9) appears to be a consistent marker for the functional effect of acetylation and methylation on transcription (Jenuwein and Allis, 2001). Specifically, H3K9 acetylation is associated with transcriptional activation which is believed to correspond with chromatin relaxation, whereas methylation at this residue is associated with transcriptional repression and chromatin condensation (Jenuwein and Allis, 2001). Thus, the acetylation and methylation status of H3K9 serve as markers for the level of relaxation or condensation of the chromatin structure and in turn increased or decreased gene transcription, respectively.

In order to study the role of amygdaloid chromatin remodeling in the anxiolytic effects of acute ethanol in P rats, we explored the levels of H3K9 acetylation and dimethylation using immunohistochemical analysis in the CeA and MeA of P and NP rats injected with either *n*-saline or acute ethanol. We found that the H3K9 acetylation and methylation levels were significantly different between the groups by analysis by one-way ANOVA followed by Tukey's test (H3K9 acetylation in CeA: $F_{3,20} = 101.3$, $p<0.001$; H3K9 acetylation in MeA: $F_{3,20} = 112.7$, $p<0.001$; H3K9 methylation in CeA: $F_{3,20} = 68.1$, $p<0.001$; H3K9 methylation in MeA: $F_{3,20} = 45.0$, $p<0.001$). We found that at baseline, H3K9 acetylation levels were significantly ($p<0.001$) lower and H3K9 methylation levels were significantly ($p<0.001$) higher in the CeA and MeA, but not

BLA, of P rats in comparison to NP rats (Figure 10). Acute ethanol exposure resulted in a significant (p<0.001) increase in H3K9 acetylation levels and a significant (p<0.001) reduction in H3K9 methylation levels in the CeA and MeA of P rats (Figure 10). No significant effects of acute ethanol on the status of H3K9 were observed in NP rats (Figure 10). These data suggest that the innately condensed chromatin structure of P rats may be associated with the higher anxiety-like behaviors of these rats as compared to NP rats. Also, acute ethanol may affect chromatin remodeling via increased histone acetylation in the CeA and MeA of P rats, but not NP rats, such that chromatin relaxation may be associated with the anxiolytic effect of ethanol.



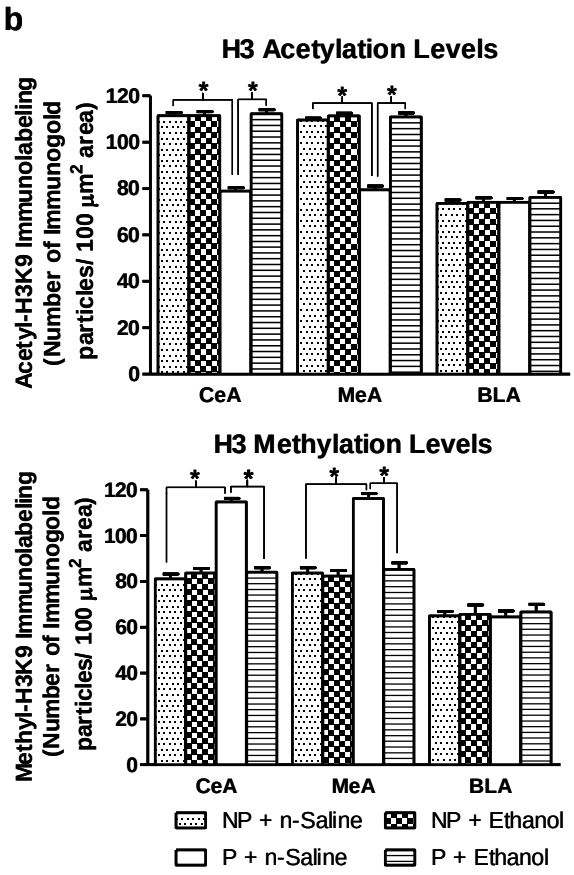


Figure 10. Histone acetylation and methylation levels in the amygdaloid brain regions of P and NP rats at baseline and following acute ethanol exposure.
a. Representative low-magnification photomicrographs (Scale bar = 50 μm) of histone H3 lysine 9 (H3K9) acetylation and methylation as measured by gold-immunolabeling in central (CeA) and medial amygdala (MeA) of alcohol-preferring (P) and -nonpreferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg).
b. Gold-immunolabeled acetylated histone H3K9 and methylated histone H3K9 was quantified for analysis of these markers of chromatin remodeling in P and NP rats with and without exposure to acute ethanol. Acetylated H3K9 levels were lower and methylated H3K9 levels were higher in the CeA and MeA , but not BLA, of P rats in comparison of NP rats at baseline. Treatment with acute ethanol (1 g/kg) increased levels of H3K9 acetylation and decreased H3K9 methylation in the CeA and MeA of P rats, but not NP rats. These results suggest a role for condensed chromatin in the innate anxiety-like behaviors and chromatin relaxation in the anxiolytic effects of ethanol in P rats. Values are represented as the mean ± SEM of the number of immunogold particles per 100 μm^2 area of 6 rats per group. *Significantly different from their respective control groups ($p<0.001$; ANOVA followed by Tukey's test).

6. Levels of acetylated histone H3 in the gene promoter regions of *BDNF* and *Arc* in the amygdala of P and NP rats following acute ethanol exposure

Since we previously observed that the innate anxiety-like behaviors of P rats were associated with lower amygdaloid levels of the synaptically active proteins, BDNF and Arc, and that acute ethanol exposure increased the expression of these proteins, we wanted to explore the possibility that the *BDNF* and *Arc* genes were regulated by chromatin remodeling in P and NP rats subject to acute ethanol exposure. HDAC2 isoform and BDNF-Arc signaling have both previously been shown to play a role in the regulation of DSD (Guan et al., 2009; Messaoudi et al., 2007; Pandey et al., 2008b; Soule et al., 2006), further suggesting involvement of chromatin remodeling in the synaptic plasticity in the amygdaloid brain regions of P rats.

To test this hypothethesis, we performed ChIP analysis by immunoprecipitating and quantifying levels of the *BDNF exon I, BDNF exon IV* and *Arc* genes associated with acetylated histone H3K9/14 in P and NP rats injected with either *n*-saline or acute ethanol. The tissue samples collected for analysis consisted primarily of CeA and MeA. Quantification of DNA was performed by qPCR analysis and the fold changes were calculated using the ΔΔCt method (Schmittgen and Livak, 2008). The NP + n-saline group was normalized to a value of 1 and the other P and NP rats were compared to this group. Analysis of our data by one-way ANOVA followed by execution of a *post hoc* Tukey's test, indicated that there were significant differences between the groups for acetylated H3K9/14-associated *BDNF exon IV* and *Arc* genes (BDNF exon IV: $F_{3,20}$ = 7.0, p = 0.002; Arc: $F_{3,20}$ = 15.1, p<0.001). We found that at baseline, P rats had significantly lower levels of *BDNF exon IV* (p<0.01) and *Arc* (p<0.001) genes associated

with acetylated H3K9/14 as compared to NP rats (Figure 11). Acute ethanol treatment increased the levels of acetylated H3K9/14 associated *BDNF exon IV* ($p<0.05$) and *Arc* ($p<0.001$) genes in P rats, but not NP rats (Figure 11). No significant differences or changes were observed in the levels of *BDNF exon I* associated with acetylated H3K9/14 (Figure 11). These results suggest that the chromatin condensation or relaxation status associated with innate anxiety-like behaviors and the anxiolytic effects of acute ethanol, respectively, may play a role in the regulation of genes involved in the control of synaptic plasticity, namely *BDNF exon IV* and *Arc*, in P rats. Furthermore, given the data regarding HDAC2 reported above, it could be possible that HDAC2-mediated regulation of histone acetylation is involved in synaptic remodeling associated with the anxiety-like behaviors of P and the anxiolytic effects of acute ethanol.

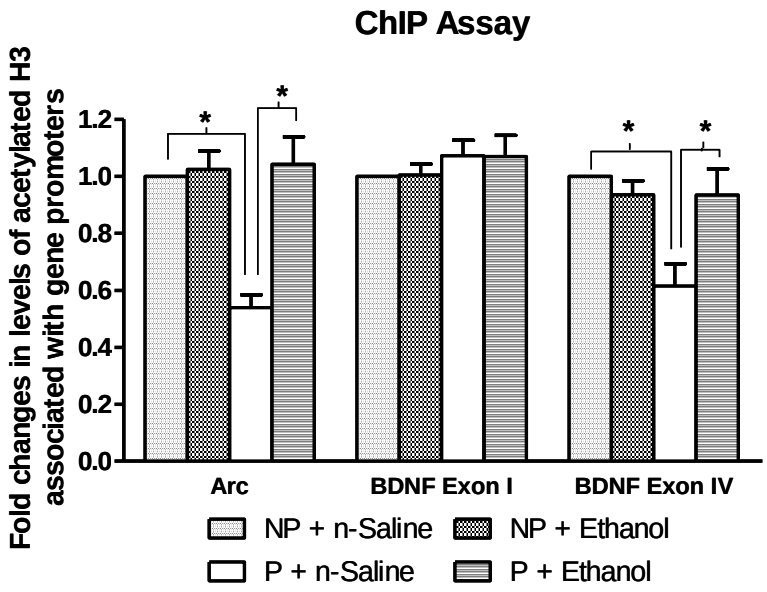


Figure 11. The effects of acute ethanol treatment on the levels of acetylated histone H3-associated promoters of synaptically active genes *BDNF* and *Arc* in the amygdaloid brain regions of P and NP rats.

a. Levels of acetylated histone H3 lysine 9/14 (H3K9/14)-associated genes, *brain-derived neurotrophic factor (BDNF) exon IV* and *activity-regulated cytoskeleton-associated gene (Arc)*, but not *BDNF exon I*, were lower in tissue homogenates composed primarily of central (CeA) and medial amygdala (MeA) in alcohol-preferring (P) rats than –nonpreferring (NP) rats at baseline. Treatment with acute ethanol (1 g/kg) increased amygdaloid levels of acetylated H3K9/14-associated *BDNF exon IV* and *Arc*, but not *BDNF exon I*, in P rats, but not NP rats. These results suggest a role for amygdaloid chromatin structure and remodeling in the regulation of synaptically active genes, *BDNF exon IV* and *Arc*, in the innate anxiety-like behaviors and anxiolytic effects of ethanol in P rats. Values are represented as the mean ± SEM of the fold change of acetylated histone H3K9/14-associated genes normalized to the NP + n-saline group with n=6 rats per group. *Significantly different from their respective control groups (p<0.05-0.001; ANOVA followed by Tukey's test).

C. Specific Aim 3: The Role of Amygdaloid HDAC2-Induced Histone Modifications on Synaptic Plasticity in the Anxiety-Like and Alcohol Drinking Behaviors of P Rats

1. Summary

Epigenetic mechanisms, including HDAC isoform-specific modulation of chromatin structure, have been implicated in the regulation of gene expression that is associated with synaptic plasticity and psychiatric disorders (Guan et al., 2009; Lubin et al., 2008; Tsankova et al., 2006). It has been suggested that transgenerational epigenetic inheritance may play a role in the phenotypes associated with psychopathologies and may play a role in the genetic predisposition to psychiatric illnesses (Franklin et al., 2010; Roth et al., 2009; Tsankova et al., 2007b). Studies evaluating at the pharmacological manipulation of HDAC enzymes via HDAC inhibitors indicate the importance of epigenetic modifications via histone acetylation in the symptoms and treatment of brain disorders (Covington et al., 2009; Pandey et al., 2008a; Renthal and Nestler, 2008). Taken together this recent research has made a case for the identification of specific HDAC isoforms involved in psychiatric illnesses and the development of isoform-specific HDAC inhibitors for the treatment of these illnesses. Yet, the role of innate differences in HDAC isoforms in the genetic predisposition to anxiety and alcoholism has not yet been studied. Recently, the HDAC2 isoform was specifically identified to play a key role in the regulation of synaptic plasticity and memory formation (Guan et al., 2009). Given the data presented thus far which implicates chromatin remodeling and synaptic plasticity in the anxiety-like behaviors and anxiolytic effects of ethanol, we aimed to mechanistically examine the role of amygdaloid HDAC2 in the regulation of the synaptic factors that may play a role in the behaviors of P rats.

In order to further examine the role of HDAC2, we utilized a siRNA strategy to specifically knockdown HDAC2 in the CeA. Initially, we infused vehicle, HDAC2 siRNA, or negative control siRNA directly into the CeA of P rats and measured anxiety-like behaviors via LDB and EPM 16 hours post-infusion. We found that HDAC2 siRNA resulted in a reduction in anxiety-like behaviors in the P rats. We confirmed the adequate transfection of neurons via confocal microscopy which showed colocalization of fluorescent tagged HDAC2 siRNA and neuronal marker in the CeA, but not MeA or BLA. We also analyzed of HDAC2 mRNA and protein levels within the amygdaloid brain regions and determined that levels of HDAC2 in CeA, but not MeA or BLA, were reduced in HDAC2 siRNA-infused rats, but not those infused with vehicle or negative control siRNA. We also found that CeA histone acetylation levels were increased in the HDAC2 siRNA group, suggesting that the reduction in HDAC2 functionally affected chromatin structure. In order to analyze the effect of HDAC2 knockdown on synaptic factors, we examined the amygdaloid levels of BDNF and Arc protein, and DSD, which were each increased in the CeA, but not MeA or BLA. To mechanistically explore the effect of chromatin remodeling in these changes, we utilized ChIP analysis and found an increase in histone acetylation at the BDNF exon IV, but not exon I, and Arc gene promoters. Finally, we examined the effect of HDAC2 siRNA infusion in the two-bottle free choice paradigm of voluntary ethanol consumption. We found that HDAC2-infused P rats consumed less ethanol than vehicle-infused controls, and that this effect lasted for several days before returning to baseline. Taken together, these data suggest a mechanistic role for HDAC2 in the regulation of synaptic plasticity that is associated with the genetic predisposition to anxiety-like behaviors and alcohol preference in P rats.

2. The effects of HDAC2 siRNA infusion into CeA on the innate anxiety-like behaviors of P rats

In order to determine the effect of HDAC2 siRNA infusion on anxiety, we utilized the EPM test and LDB exploration test to examine the anxiety-like behaviors of P rats 16 hours following following infusion of vehicle, HDAC2 siRNA, and negative control siRNA. The rats utilized for these experiments were age- and body weight matched such that the body weight did not differ significantly among the treatment groups (Vehicle: 372.9 ± 2.8 g, HDAC2 siRNA: 369.3 ± 3.2 g, Control siRNA: 367.2 ± 5.7 g; n=13-25).

We first measured the anxiety-like behaviors using the EPM test, which showed significant differences among the groups via one-way ANOVA on the percentage of open arm entries ($F_{2,21} = 46.7$, $p < 0.001$) and the percentage of time spent in the open arms ($F_{2,21} = 52.2$, $p < 0.001$). The number of total arm entries did not differ between groups ($F_{2,21} = 1.2$, $P = 0.323$), suggesting that the treatment had no effect on the general activity level of the rats (Figure 12a). *Post hoc* analysis by Tukey's test indicated that the HDAC2 siRNA group showed a significantly increased percentage of open arm entries ($p<0.001$) and time spent in the open arms ($p<0.001$) in comparison to the control groups (Figure 12a). This suggests that infusion of HDAC2 siRNA into the CeA reduced anxiety-like behaviors as measured by the EPM test.

In a second batch of P rats, anxiety-like behaviors were measured by the LDB exploration test. The percentage of time spent in the light and dark compartments was significantly different among the groups as measured by one-way ANOVA using Tukey's test for *post hoc* analysis ($F_{2,36} = 36.3$, $p<0.001$). P rats treated with HDAC2 siRNA infusion spent significantly ($p<0.001$) more time in the light compartment and

less time in the dark compartment, in comparison rats infused with either vehicle or control siRNA, confirming that anxiety-like behaviors were reduced by HDAC2 siRNA infusion (Figure 12b). General activity was measured by the total number of ambulations in the LDB apparatus, which was not significantly different among the groups ($F_{2,36}$ = 2.5, p=0.094). Taken together, these data show that HDAC2 siRNA infusion into the CeA of P rats reduced anxiety-like behaviors in two independent behavioral paradigms. This strongly suggests that HDAC2 in the CeA plays a mechanistic role in the regulation of anxiety in P rats.

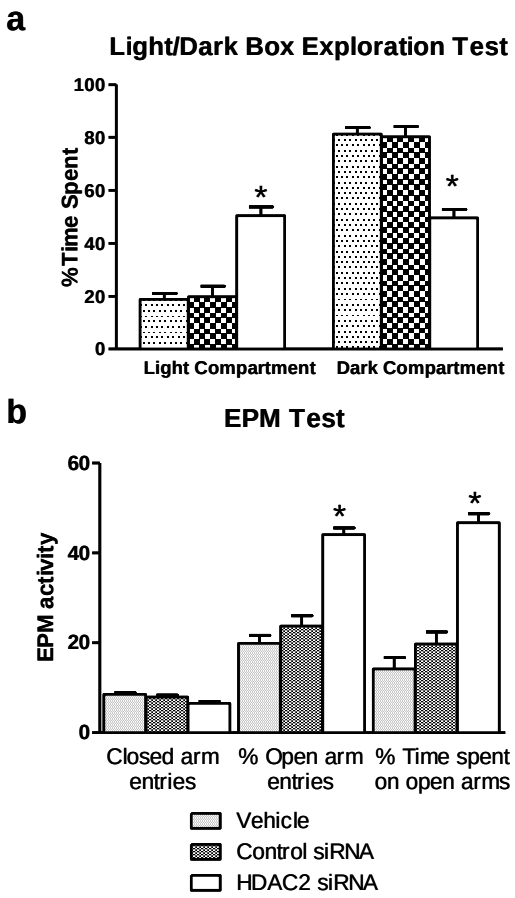


Figure 12. The effect of HDAC2 siRNA infusion into the CeA on the innate anxiety-like behaviors of P rats as measured by the EPM and LDB exploration test.
a. The elevated-plus maze (EPM) behavioral test of anxiety-like behaviors demonstrated that histone deacetylase isoform 2 (HDAC2) siRNA infusion into the central amygdala (CeA) resulted in a reduction in the innate anxiety-like behaviors of alcohol-preferring (P) rats 16 hours after infusion. P rats infused bilaterally with HDAC2 siRNA showed a higher percentage of open arm entries and time spent in the open arm than those infused with vehicle or negative control siRNA. The number of closed arm entries does not differ significantly between the groups, suggesting that there are no effects of HDAC2 siRNA infusion on general activity level. Values represent the mean ± SEM of the percentage of open arm entries, percentage of time spent in the open arm, and number of total arm entries of n=8 rats per group. *Significantly different from control groups ($p<0.001$; ANOVA followed by Tukey's test).
b. The light-dark box (LDB) exploration test confirmed that HDAC2 siRNA infusion reduced the innate anxiety-like behaviors of P rats in comparison to vehicle- or control siRNA-infused P rats. HDAC2 siRNA infused P rats spent more time in the light compartment and less time in the dark compartment than vehicle- and control siRNA-infused P rats. Values are represented as the mean ± SEM of the percentage of time spent in each compartment of n=5-17 rats per group. *Significantly different from control groups ($p<0.001$; ANOVA followed by Tukey's test).

3. Confocal microscopic analysis of neuronal transfection following HDAC2 siRNA infusion into the CeA of P rats

In order to confirm that the infusion of HDAC2 siRNA was resulting in effective penetration of neurons by siRNA, we infused a fluorescent probe-tagged HDAC2 siRNA into the CeA and examined the amygdaloid brain regions using confocal microscopy. Colocalization of the fluorescent probe and neurons was visualized via immunofluorescent labeling of neuronal marker (NeuN). Qualitative analysis of the confocal images revealed that HDAC2 siRNA-linked fluorescence was restricted to the CeA and did not enter the MeA or BLA (Figure 13). Furthermore, the siRNA-linked fluorescent tag primarily colocalized with cells strongly expressing NeuN, suggesting that siRNA effectively transfected the neuronal population in the CeA. It is worth noting that although fluorescent staining appears predominantly in neurons, there is some fluorescent staining that does not colocalize with neurons, suggesting the presence of some HDAC2 siRNA transfection in the glial population. Qualitative comparison of NeuN staining in the CeA of HDAC2 siRNA-infused rats with negative control siRNA- and vehicle-infused rats indicated that NeuN staining was similar among the groups (data not shown). The qualitative analysis performed suggests that i-Fect mediated transfection of neurons was successful and localized to the CeA.

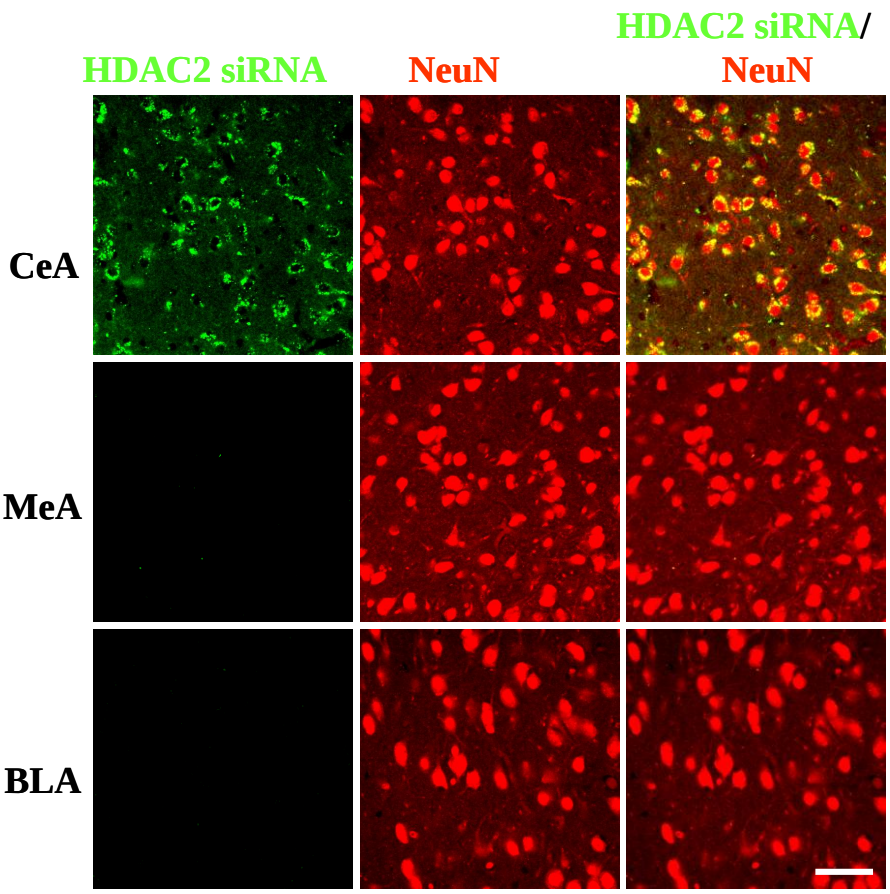


Figure 13. Confocal analysis of neuronal transfection following HDAC2 siRNA infusion into the CeA of P rats via colocalization of fluorescence-linked siRNA and neuronal marker

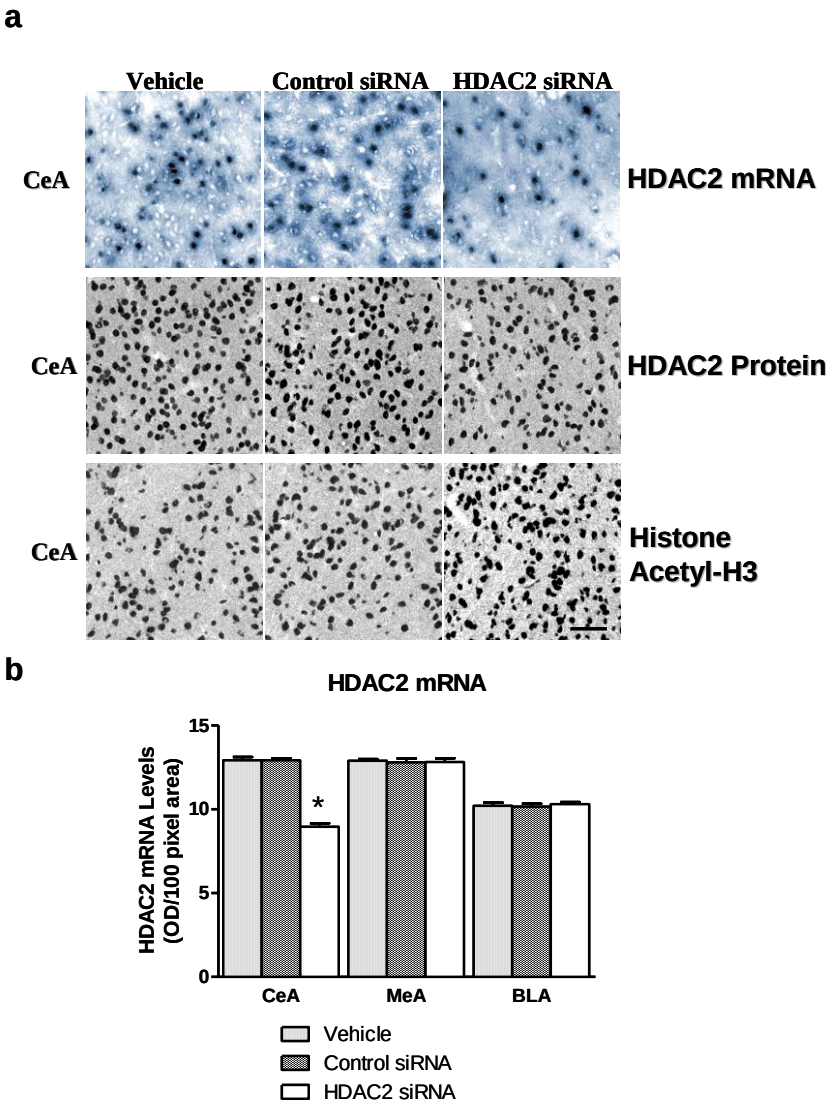
Representative confocal photomicrographs reveal that histone deacetylase isoform 2 (HDAC2) siRNA penetrates neurons in the central amygdala (CeA), but not medial (MeA) or basolateral amygdala (BLA). 16 hours following infusion of HDAC2 siRNA into the CeA, brain sections were processed for analysis of fluorescence-tagged HDAC2 siRNA (left panel) and immunofluorescent staining for neuronal marker (NeuN, middle panel). Qualitative analysis of the colocalization of HDAC2 siRNA with NeuN (right panel) indicated that a majority of the neuronal population has been transfected by HDAC2 siRNA in the CeA, but not MeA or BLA. Scale bar = 50 µm.

4. The effects of HDAC2 siRNA infusion on HDAC2 mRNA and protein, and histone H3K9 acetylation in amygdaloid brain regions of P rats

Further confirmation of successful tranfection and activity of HDAC2 siRNA was performed via the measurement of HDAC2 mRNA by *in situ* RT-PCR analysis of the amygdaloid brain regions. In brains processed 16 hours post-infusion, analysis of the optical density of amygdaloid brain regions revealed that significant differences in HDAC2 mRNA levels existed among the groups in the CeA, but not MeA or BLA (CeA, $F_{2,12} = 161.0$, $p<0.001$; MeA, $F_{2,12} = 0.1$, $p= 0.914$; BLA, $F_{2,12} = 0.2$, $p = 0.800$; by one-way ANOVA). *Post hoc* analysis by Tukey's test revealed that HDAC2 siRNA infusion significantly ($p<0.001$) reduced HDAC2 mRNA in the CeA, but not MeA or BLA, of P rats in comparison to vehicle or control siRNA infusion (Figure 14a,b). Thus, HDAC2 siRNA was effective in the reduction of HDAC2 mRNA in the CeA, but not MeA or BLA.

In order to examine the functional effects of HDAC2 siRNA infusion, we utilized immunohistochemical analysis to determine the levels of HDAC2 protein and histone H3 acetylation in the amygdaloid brain regions. Results were analyzed to determine the average number of immunogold particles per 100 μm^2 area and analysis by one-way ANOVA indicated that significant differences existed between the groups in the CeA, but not MeA or BLA (HDAC2 protein: CeA, $F_{2,17} = 127.0$, $p<0.001$; MeA, $F_{2,17} = 0.9$, $p=0.436$; BLA, $F_{2,17} = 1.2$, $p=0.334$; Acetylated H3K9: CeA, $F_{2,17} = 70.0$, $p<0.001$; MeA, $F_{2,17} = 0.2$, $p=0.783$; BLA, $F_{2,17} = 0.03$, $p=0.964$). In comparison to P rats infused with vehicle or negative control siRNA, HDAC2 siRNA infusion resulted in a significant reduction in HDAC2 protein ($p<0.001$) and a significant increase in levels of acetylated

H3K9 (p<0.001) in the CeA, but not MeA or BLA (Figure 14a,c). These data indicate that HDAC2 siRNA infusion resulted in functional changes in HDAC2 protein and histone H3K9 acetylation, suggesting that the anxiolytic effect of HDAC2 knockdown could be mediated by HDAC2-induced chromatin relaxation due to increased histone acetylation.



c

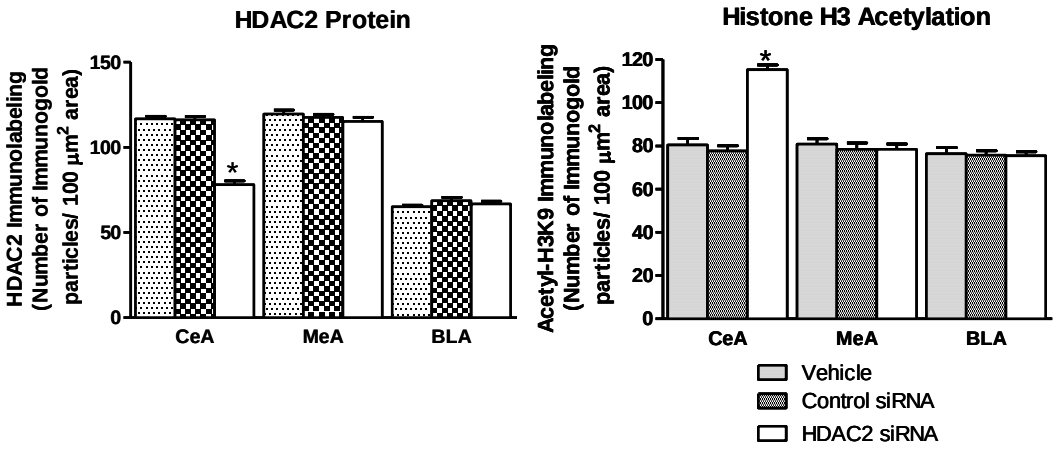


Figure 14. The effect of HDAC2 siRNA infusion into the CeA of P rats results on HDAC2 mRNA and protein, and associated effects on histone H3K9 acetylation.
a. Representative low-magnification photomicrographs illustrating *in situ* RT-PCR for histone deacetylase isoform 2 (HDAC2) mRNA, and gold-immunolabeling of HDAC2 protein and acetylated histone H3 lysine 9 (H3K9) in the central (CeA) of alcohol-preferring (P) rats 16 hours following infusion with vehicle, negative control siRNA or HDAC2 siRNA. Scale bar = 50 μm.
b. Quantification of the optical density of positive staining for HDAC2 mRNA was performed in P rats 16 hours following infusion of vehicle, negative control siRNA and HDAC2 siRNA. HDAC2 mRNA levels were lower in the CeA, but not MeA or BLA, of P rats infused with HDAC2 siRNA than those infused with vehicle or control siRNA. These results confirm that HDAC2 siRNA infusion causes a significant reduction in HDAC2 mRNA in the CeA. Values are represented as the mean ± SEM of the optical density of HDAC2 mRNA positive labeling per 100 pixel area of 5 rats per group. *Significantly different from control groups ($p<0.001$; by one-way ANOVA followed by Tukey's test).
c. Quantification of gold-immunolabeling was performed to determine the effect of HDAC2 siRNA infusion into the CeA of P rats on HDAC2 protein and acetylated histone H3K9 levels. HDAC2 siRNA infusion in to the CeA of P rats results in a reduction in HDAC2 protein in the CeA, but not medial amygdala (MeA) or basolateral amygdala (BLA), in comparison to those infused with vehicle or control siRNA. These results identify the functional reduction in HDAC2 protein and increase in histone H3K9 acetylation by HDAC2 siRNA infusion and suggest that knockdown of HDAC2 levels is associated with relaxation of chromatin structure in the CeA. Values are represented as the mean ± SEM of the number of the immunogold positive particles per 100 μm^2 area of 6-7 rats per group. *Significantly different from control groups ($p<0.001$; by one-way ANOVA followed by Tukey's test).

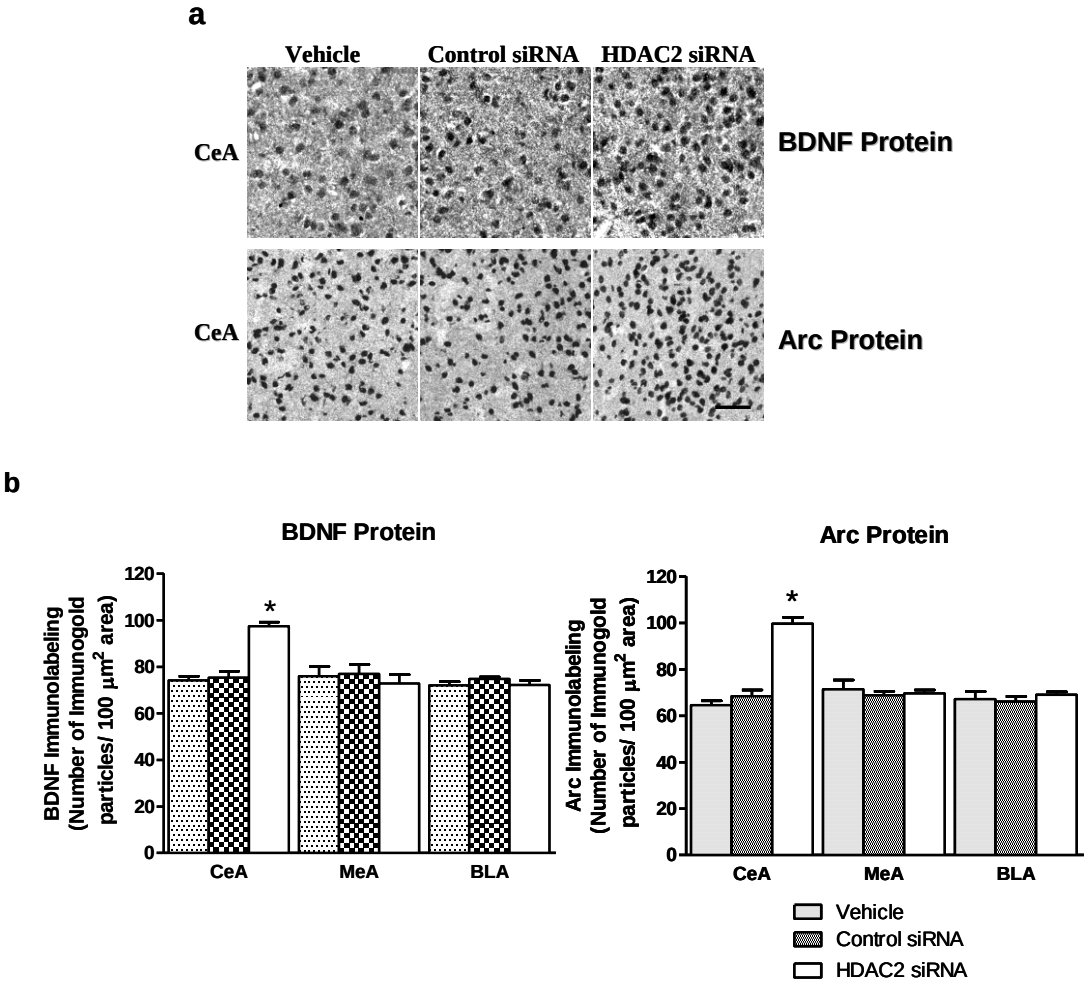
5. The effects of HDAC2 siRNA on BDNF and Arc protein expression and on levels of acetylated histone H3 associated with synaptically active genes, *BDNF* and *Arc*, in the amygdaloid brain regions of P rats

Having identified the successful knockdown of HDAC2 protein and associated changes in chromatin structure, we sought to examine whether HDAC2-related chromatin relaxation could play a role in the regulation of BDNF and Arc protein. The experiments performed for the previous specific aims identified that increased HDAC2 protein in the CeA of P rats was associated with lower levels of BDNF, Arc and DSD (see above), however this finding was correlative in nature. In order to mechanistically determine whether or not a link exists between HDAC2 levels and these synaptically active genes, we performed immunogold staining for analysis of BDNF and Arc protein levels in the amygdaloid brain regions of P rats following the infusion of HDAC2 siRNA, vehicle or negative control siRNA into the CeA. Analysis of BDNF and Arc protein levels identified a significant difference between the vehicle-, HDAC2 siRNA-, and control siRNA-infused P rat groups in the CeA, but not MeA or BLA (BDNF: CeA, $F_{2,11} = 34.5$, $p<0.001$; MeA, $F_{2,11} = 0.3$, $p=0.743$; BLA, $F_{2,11} = 1.0$, $p=0.394$; Arc: CeA, $F_{2,14} = 57.8$, $p<0.001$; MeA, $F_{2,14} = 0.3$, $p=0.761$; BLA, $F_{1,14} = 0.4$, $p=0.649$; by one-way ANOVA followed by Tukey's test). HDAC2 siRNA infused P rats showed significantly ($p<0.001$) higher levels of BDNF and Arc protein in the CeA than P rats infused with vehicle or negative control siRNA (Figure 15a,b). These data identify a mechanistic link between HDAC2 expression and increased levels of BDNF and Arc protein.

Expression levels of BDNF are under tight regulation by the unique gene structure which allows for transcriptional events at individual promoters in the *BDNF* gene (Aid et

al., 2007). *BDNF exon IV* appears to play a significant role in the regulation of synaptic plasticity and has been shown to be under the regulation of epigenetic mechanisms (Lubin et al., 2008; Sakata et al., 2009; Tsankova et al., 2006). In order to mechanistically examine the association between HDAC2 knockdown and synaptically active genes, we utilized ChIP analysis to determine whether there was a change in the acetylation level of histone H3K9/14 associated with the gene promoters for *BDNF* and *Arc*. To further determine if there was an exon-specific effect, we looked at both *BDNF exon I* and *exon IV*. ChIP results were evaluated by qPCR analysis comparing ChIP DNA to input DNA and calculating the fold change in acetylated H3K9/14-associated genes in the amygdala, primarily CeA and MeA, of HDAC2 siRNA-treated P rats as normalized to the vehicle treated group. Analysis of the normalized results revealed that HDAC2 siRNA infusion increased acetylated histone H3K9/14-associated *BDNF exon IV* and *Arc*, but not *BDNF exon I* (Figure 15c; exon I: $t_{10} = -0.1$, $p=0.514$; exon IV: $t_{10} = 5.0$, $p< 0.001$; Arc: $t_{10} = 7.2$, $p< 0.001$; by Student's t-test). These results suggest that the increases in BDNF and Arc protein associated with HDAC2 knockdown in the CeA are specifically related to increased gene transcription associated with increased histone acetylation at the *BDNF exon IV* and *Arc* genes. Taken together, these data mechanistically implicate HDAC2-mediated regulation of histone acetylation with the regulation of these synaptically active genes. In the previous specific aims, we found that the anxiolytic effects of acute ethanol were associated with reduced HDAC2 levels and an increase in BDNF and Arc levels in the CeA and MeA of P rats (see above). Exogenous reduction in HDAC2 levels in the CeA produced anxiolytic effects and also resulted in an increase BDNF and Arc suggesting that the anxiolytic effects of ethanol

may be acting in part through the downstream effects of HDAC2 which may be mediated through changes in synaptically active proteins.



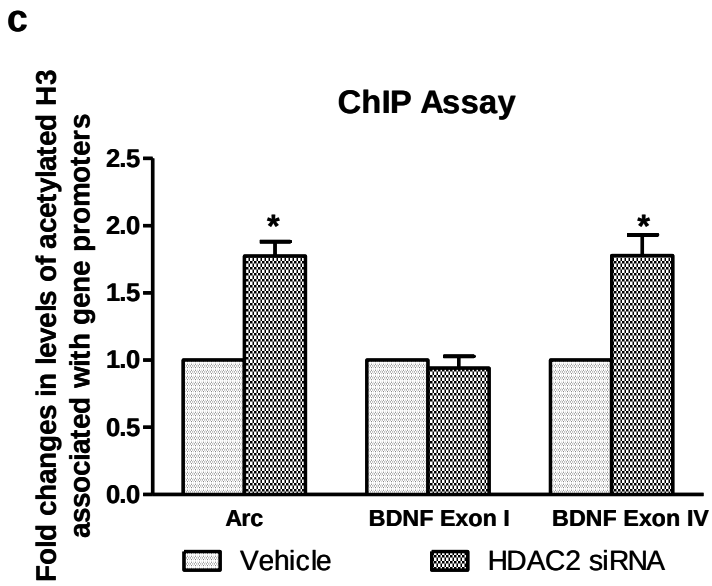


Figure 15. The effects of HDAC2 siRNA infusion into the CeA of P rats on BDNF and Arc protein expression, and levels of acetylated histone H3-associated *BDNF* and *Arc* genes.

a. Representative low-magnification photomicrographs illustrating gold-immunolabeling of brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated (Arc) protein in the central (CeA) of alcohol-preferring (P) rats 16 hours following infusion with histone deacetylase isoform 2 (HDAC2) siRNA, negative control siRNA or vehicle. Scale bar = 50 µm.

b. Quantification of gold-immunolabeling for BDNF and Arc protein in the amygdaloid brain regions of P rats was performed to determine the effect of CeA infusion of HDAC2 siRNA, negative control siRNA and vehicle on these synaptically active proteins. HDAC2 siRNA infusion into the CeA of P rats results in an increase of BDNF and Arc protein in the CeA, but not medial amygdala (MeA) or basolateral amygdala (BLA), in comparison to P rats infused with vehicle or control siRNA. Values are represented as the mean ± SEM of the number of the immunogold positive particles per 100 μm^2 area of 4-6 rats per group. *Significantly different from control groups (p<0.001; by one-way ANOVA followed by Tukey's test).

c. ChIP analysis reveals that HDAC2 siRNA infusion increases the association of acetylated histone H3 with the gene promoters of *BDNF exon IV* and *Arc*, but not *BDNF exon I*, in tissue consisting predominantly of CeA and MeA. These data suggest that the HDAC2-mediated regulation of histone H3 acetylation may play a direct role in the modulation of genes involved in the control of synaptic plasticity. Values represent mean ± SEM of the fold change versus vehicle of acetylated histone H3-associated genes *BDNF exon I, BDNF exon IV* and *Arc* for n=6 rats per group. *Significantly different from vehicle-infused P rats (p<0.001; by one-way ANOVA followed by Tukey's test).

6. Measurement of amygdaloid DSD following HDAC2 siRNA infusion into the CeA of P rats via analysis of Golgi-Cox staining

An important downstream effector of BDNF-Arc signaling is the modulation of synaptic plasticity and dendritic spine morphology (Bramham et al., 2008; Messaoudi et al., 2007; Pandey et al., 2008b; Soule et al., 2006). The regulation of HDAC2 levels appears to play a role in the control of DSD, such that increased HDAC2 results in decreased DSD and reduced HDAC2 increases DSD (Guan et al., 2009). In order to see if the HDAC2 siRNA-infusion paradigm we utilized had a downstream effect on synaptic morphology, we utilized Golgi-Cox staining to quantify the DSD of P rats following infusion. We found that in comparison to vehicle-infused rats, HDAC2 siRNA infusion significantly increased the DSD of P rats in the CeA, but not MeA or BLA (Figure 16; CeA: $t_{10} = -11.7$, $p < 0.001$; MeA: $t_{10} = -0.9$, $p = 0.369$; BLA: $t_{10} = -0.4$, $p = 0.683$; by Student's t-test). These data suggest that the HDAC2-mediated regulation of chromatin structure may affect DSD. Furthermore, given the association between BDNF-Arc signaling and dendritic spine morphology, this effect may be mediated by the increase in BDNF and Arc proteins that is related to the increase in histone acetylation the gene promoters of synaptically active genes. This data also implicates the modulation of synaptic structure in the CeA in the effects of HDAC2 regulation on the anxiety-like behaviors of P rats.

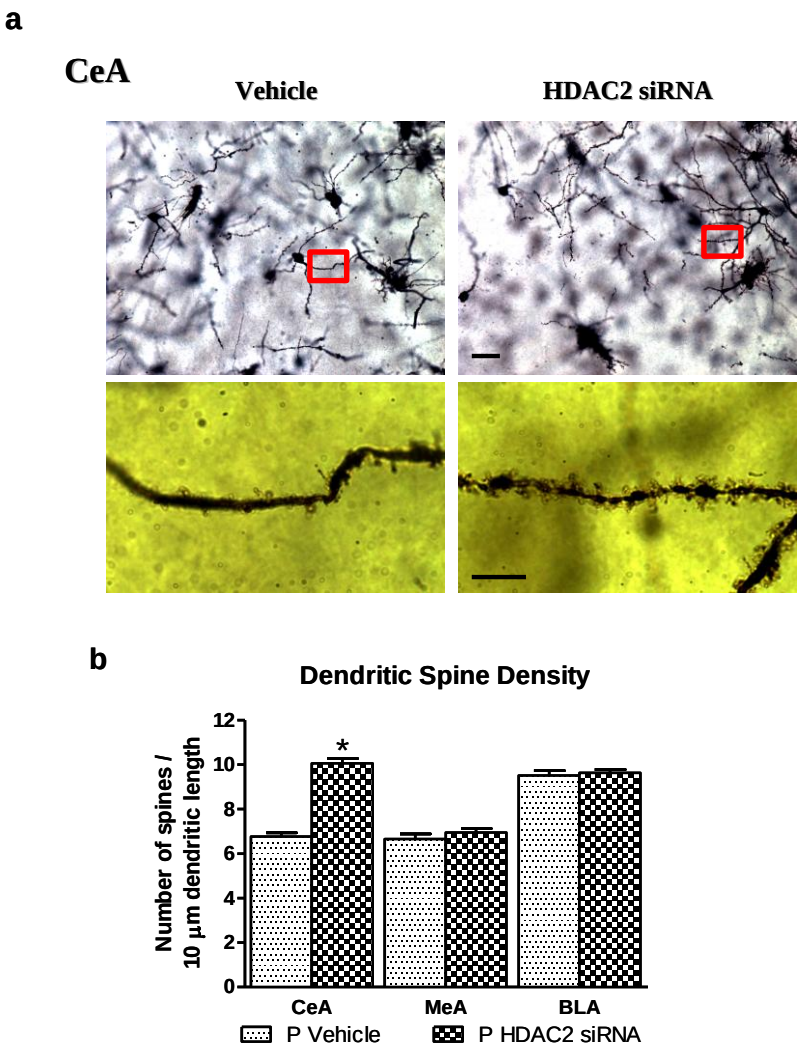


Figure 16. The effects of HDAC2 siRNA infusion into the CeA of P rats on amygdaloid DSD in amygdaloid brain regions

a. Representative low-magnification photomicrographs (Scale bar = 50 µm) showing Golgi-Cox impregnated neurons in the central amygdala (CeA) of alcohol-preferring (P) rats 16 hours after infusion with histone deacetylase isoform 2 (HDAC2) siRNA or vehicle. Neuron selected for quantification of dendritic spine density (DSD) showed complete impregnation from the cell body to the dendritic tip. The boxed areas of the low magnification photographs are shown at high magnification (Scale bar = 10 µm) in the adjacent photomicrograph allowing for visualization of dendritic spines.

b. Quantification of DSD was performed via neuronal morphology tracking software in the amygdaloid brain regions of P rats following infusion with HDAC2 siRNA or vehicle in the CeA. HDAC2 siRNA infusion into the CeA of P rats results in increased DSD in the CeA, but not medial (MeA) or basolateral amygdala (BLA) in comparison to vehicle-infused rats. Values are represented as the mean ± SEM of the number of dendritic spines per 10 µm of dendritic length of n=5 rats per group. *Significantly different from control groups ($p<0.001$; Student's t-test).

7. The effect of HDAC2 siRNA infusion into the CeA on the voluntary ethanol consumption of P rats

In order to determine the effects of HDAC2 knockdown and associated changes in chromatin structure and synaptic plasticity on the alcohol-drinking behaviors of P rats, we utilized the two-bottle free choice paradigm of voluntary ethanol consumption. Following habituation to the apparatus, P rats were exposed to three days of free access to water and 7% ethanol followed by three days of free access to water and 9% ethanol. On the sixth day, P rats were infused with HDAC2 siRNA or vehicle into the CeA and consumption of water and 9% ethanol were monitored for several days. Analysis by two-way ANOVA with repeated measures indicated a significant difference in ethanol consumed between groups ($F_{1,120} = 71.9$, $p<0.001$), and also revealed a significant group by day interaction ($F_{12,120} = 13.8$, $p<0.001$). Further, *post hoc* analysis identified that HDAC2 siRNA-infused P rats consumed significantly ($p<0.01$ - 0.001) less ethanol than vehicle-infused P rats for six days following the infusion, specifically from days 7 to 12 during 9% ethanol exposure (Figure 17). Total fluid intake did not significantly differ between the groups (Figure 17). These data suggest a mechanistic role for HDAC2-mediated regulation of chromatin structure via histone acetylation in the CeA in the modulation of alcohol preference in P rats. Taken together, anxiety-like behavior and alcohol consumption may indicate that HDAC2 levels in the CeA serve as a common mechanism in the interaction between anxiety and alcohol preference that play a role in these comorbid phenotypes. Furthermore, the effects of HDAC2 on synaptic plasticity may play a role in both anxiety-like behaviors and alcohol drinking behaviors of P rats. This may suggest that innate aberrations of amygdaloid HDAC2 protein and associated

changes in synaptic signaling and structure may be factors in the genetic predisposition to anxiety and alcoholism. Furthermore, inhibition of the specific HDAC2 isoform may be a potential target in the pharmacotherapeutic treatment of comorbid anxiety-spectrum and alcohol-use disorders.

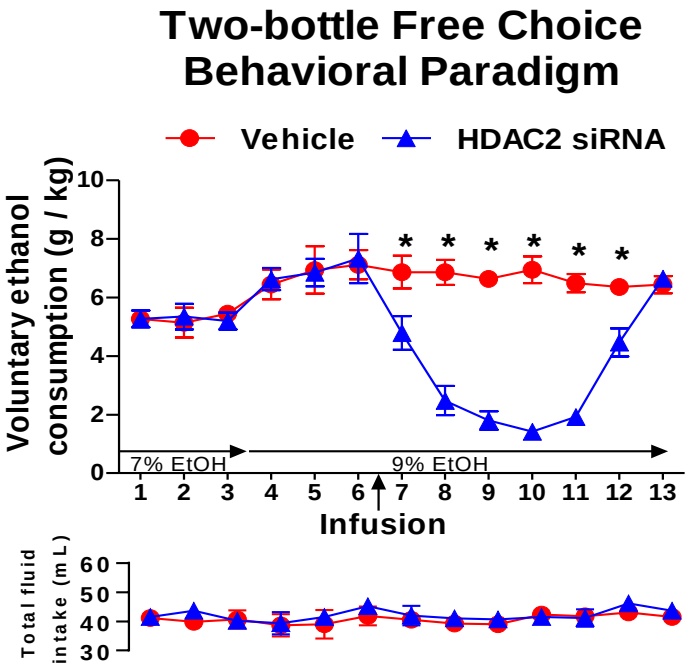


Figure 17. The effects of HDAC2 siRNA Infusion into the CeA of P rats on voluntary ethanol consumption as measured by the two-bottle free choice paradigm. Monitoring the voluntary ethanol consumption of alcohol-preferring (P) rats via the two-bottle free choice paradigm following infusion of vehicle or histone deacetylase isoform 2 (HDAC2) siRNA into the central amygdala (CeA) demonstrates that high HDAC2 levels may mediate the high alcohol drinking behaviors of P rats. P rats were given access to water and 7% ethanol followed by water and 9% ethanol. On the sixth day of ethanol access P rats received infusion of vehicle or HDAC2 siRNA and consumption of water and 9% ethanol were monitored for several days. The ethanol consumption of P rats infused with HDAC2 siRNA, but not vehicle, were reduced for 6 days following the infusion. Total fluid intake did not significantly differ between the groups. Values are represented as the mean ± SEM of the ethanol consumption (g / kg / day) and total fluid intake (mL) plotted daily for n=6 rats per treatment group. *Significantly different between the groups (p<0.01 - 0.001; by two-way ANOVA with repeated measures followed by *post hoc* analysis of the group by day interaction).

IV. DISCUSSION

A. The Comorbidity of Anxiety and Alcoholism

In order to explore the comorbidity between anxiety and alcoholism, we utilized the P and NP rat lines that have been selectively bred for high and low alcohol preference, respectively (Bell et al., 2006; Li et al., 1993). Although no intentional selection was performed for anxiety, P rats were found to display high innate anxiety-like behaviors in addition to alcohol preference, suggesting that the rat line could serve as a useful animal model for comorbid anxiety and alcoholism (Pandey et al., 2005b; Stewart et al., 1993). Previous studies have also determined that the innate anxiety-like behaviors of P rats may be reduced by acute ethanol administration, and this effect is not observed in NP rats (Hwang et al., 2004; Pandey et al., 2005a; Stewart et al., 1993). These studies have primarily utilized the EPM test, the results of which were confirmed in our study. The findings were further generalized by utilizing the LDB exploration test, confirming the effects of ethanol with another paradigm to test for anxiety-like behaviors. Furthermore, acute ethanol treatment and chronic ethanol exposure via voluntary self-administration were both found to produce anxiolytic effects in P rats, with no effect in NP rats.

In human studies, acute alcohol exposure has been shown to have an anxiolytic effect, and various epidemiological findings have identified a correlation between the presence of independent anxiety-spectrum disorders and alcohol-use disorders (Grant et al., 2004b; Lipscomb et al., 1980; Novak et al., 2003; Schuckit and Hesselbrock, 1994). It has been suggested that individuals with innate anxiety disorders may consume alcohol as an attempt to self medicate the dysphoric symptoms that are associated with anxiety

disorders (Bolton et al., 2009; Robinson et al., 2009; Schuckit and Hesselbrock, 1994; Valentiner et al., 2004; Wilson, 1988). Various forms of dysphoria may contribute to alcohol consumption patterns, although anxiety appears to have a particularly robust interaction with alcoholism (Robinson et al., 2009; Zimmermann et al., 2003).

Taken together, the concept of self-medication and the anxiolytic effects of ethanol exposure observed in P rats may suggest that the high voluntary ethanol intake of P rats, in comparison to NP rats, could be due to relief from dysphoria provided by ethanol consumption. Our results regarding the acute effects of ethanol on the anxiety-like behaviors of P rats clearly suggest that P rats are highly sensitive to the anxiolytic effect of ethanol. The anxiolytic effect of ethanol exposure that is observed following 10 days of voluntary ethanol intake in the second specific aim of this study may also suggest that the continued reduction of dysphoria in these rats is responsible for the high levels of drinking. This is further evidenced in the third specific aim by the finding that the anxiolytic effect of exogenous HDAC2 infusion is associated with a reduction in alcohol consumption. These findings provide evidence that alleviation of the high innate anxiety-like behaviors by ethanol exposure may be a key factor in the alcohol preference of P rats.

Alcoholism and anxiety have a complex relationship such that prolonged exposure to chronic ethanol and repetitive ethanol withdrawal may lead to neuroadaptations that make dysphoric symptoms more severe or result in the development of tolerance to the anxiolytic effects of ethanol (Becker, 1994; Breese et al., 2005; Duka et al., 2004; Koob, 2003; Pandey, 2003; Sakharkar et al., 2012). It could be possible that continued exposure of the P rats to chronic ethanol may result in

neuroadaptations leading to increased anxiogenesis due to withdrawal from chronic ethanol that may eventually result in alcohol dependence. This effect has been illustrated by studies that have utilized various models of chronic ethanol exposure and ethanol withdrawal to elicit signs of dependence and increased alcohol consumption in P rats (Gilpin et al., 2008; Kampov-Polevoy et al., 2000; Knapp and Breese, 2012; Rodd-Henricks et al., 2000). Along with the findings of our study, these data may indicate that the innate anxiety-like behaviors of P rats promote both the initiation and maintenance of alcohol-drinking that may lead to development of alcohol dependence.

B. The Role of Amygdaloid BDNF, Arc and DSD in Anxiety Associated with Alcoholism and the Anxiolytic Effects of Acute Ethanol

The experiments performed for the first specific aim of the present investigation provide evidence that reduced BDNF and Arc expression levels, and DSD, in the CeA and MeA may be involved in the anxiety-like behaviors of P rats, in comparison to NP rats, and that increased amygdaloid BDNF-Arc signaling and DSD may be related to the anxiolytic effects of ethanol in P rats (Figure 18). Studies in humans and animal models have implicated a role for BDNF signaling in both anxiety and alcoholism (Chen et al., 2008; Davis, 2008; Hashimoto, 2007). The methionine variant of the BDNF single nucleotide polymorphism Val66Met has been found to result in a reduced BDNF secretion phenotype in both heterozygous and homozygous individuals and animal models (Chen et al., 2008; Egan et al., 2003). This genotype has been found to have an association with the development of anxiety spectrum disorders (Chen et al., 2008; Chen et al., 2006; Jiang et al., 2005; Lang et al., 2005b). Furthermore, a connection has been suggested between presence of the variant BDNF phenotype and a predisposition for

alcoholism (Matsushita et al., 2004; Uhl et al., 2001). In animal models, BDNF haplodeficiency increases the voluntary consumption of ethanol as compared to wild-type mice (Hensler et al., 2003; McGough et al., 2004). Conditional deletion of BDNF has also been shown to have an anxiogenic effect, further confirming the connection between BDNF and anxiety (Rios et al., 2001). Furthermore, the deletion of the TrkB receptor results in an increase in anxiety-like behaviors, which was associated with a reduction in DSD, potentially suggesting that the effect of BDNF signaling on anxiety is mediated via downstream effect on synaptic function and structure (Bergami et al., 2009; Bergami et al., 2008). Overexpressing TrkB results in anxiolytic effects, further suggesting a role for BDNF-TrkB signaling in the regulation of anxiety-like behaviors (Koponen et al., 2004).

Taken together these data suggest that reductions in BDNF signaling play a role in both anxiety-like behaviors and alcohol consumption. Our results confirm this notion and suggest that the CeA brain region is particularly important for this effect. The importance of the CeA in mediating the behaviors associated with anxiety were confirmed by a recent optogenetic study which identified that in the CeA, but not BLA, light-activation and -inhibition of BLA projection neurons resulted in anxiolytic and anxiogenic effects, respectively (Tye et al., 2011). Previously, Pandey *et al.* have identified a role for amygdaloid BDNF signaling in anxiety-like and alcohol-drinking behaviors in an unselected stock of rats via exogenous manipulation of amygdaloid BDNF (Pandey et al., 2006). The study identified that BDNF knockdown via infusion of antisense oligodeoxynucleotides (ODNs) into the CeA and MeA, but not BLA, provoked anxiety-like behaviors and increased ethanol consumption. These effects could be alleviated by the co-infusion of BDNF itself (Pandey et al., 2006). These studies

demonstrated a mechanistic role for amygdaloid BDNF signaling in the regulation of anxiety-like behaviors and ethanol consumption. BDNF signaling in the hippocampus and striatum have also been implicated as a potential pathway that may play a role in alcohol preference and neuroprotection from the development of alcohol dependence (Jeanblanc et al., 2006; Logrip et al., 2008; McGough et al., 2004). Recently a study identified endogenous BDNF signaling in the dorsolateral striatum as a key regulator of alcohol-drinking behaviors (Jeanblanc et al., 2009).

BDNF signaling has been implicated in the regulation of synaptic function and structure, which may be mediated by the induction of the downstream effector immediate-early gene *Arc* (Bramham et al., 2008; Messaoudi et al., 2002; Pandey et al., 2008b; Ying et al., 2002). Binding of BDNF to the high-affinity TrkB receptor results in the activation of extracellular-signal regulated kinase (Erk1/2), which subsequently phosphorylates and activates transcription factors CREB and Elk-1, resulting in the downstream upregulation of Arc (Pandey et al., 2008b; Pizzorusso et al., 2000; Poo, 2001; Reichardt, 2006; Tao et al., 1998). The *Arc* gene contains response elements with unique binding sites for CREB and Elk-1, such that regulation of *Arc* gene transcription occurs through BDNF signaling and synaptic activity (Bramham et al., 2010; Kawashima et al., 2009; Pintchovski et al., 2009; Waltereit et al., 2001). Following transcription, Arc mRNA is transported to distal dendrites where local translation occurs within synaptically active dendritic spines, resulting in spine proliferation (Huang et al., 2007; Messaoudi et al., 2007; Steward et al., 1998; Steward and Worley, 2001).

Recently, it was identified that anxiety-like behaviors induced in Sprague-Dawley rats by alcohol withdrawal following chronic ethanol exposure showed reduced BDNF

and Arc expression and signaling in the CeA and MeA, but not BLA, and this effect was associated with a reduction in DSD (Pandey et al., 2008b). Exogenous BDNF infusion into the CeA of withdrawn rats had an anxiolytic effect associated with increased BDNF-Arc signaling and expression (Pandey et al., 2008b). It was also determined that acute ethanol exposure resulted in increased BDNF-Arc signaling and which correlated with proliferation of dendritic spines, represent by an increase in DSD (Pandey et al., 2008b). Taken together along with previous evidence, these data suggest that reduced amygdaloid BDNF expression in the CeA may be responsible for the reduced levels of Arc and DSD that may be involved in anxiety-like and excessive alcohol drinking behaviors of P rats.

Arc protein has several functions at the level of the dendritic spine, which may underlie the effects of BDNF signaling on synaptic function and structure (Bramham et al., 2010; Bramham et al., 2008; Soule et al., 2006). Examination of learning tasks in Arc knockout mice and rats subjected to intra-hippocampal infusion of Arc antisense ODNs suggests that Arc protein is critical to the processes underlying learning and memory, including long-term potentiation (LTP) (Guzowski et al., 2000; McIntyre et al., 2005; Plath et al., 2006). However, the mechanisms underlying the regulatory processes governing spine morphology via Arc have been less explored. It has been shown that blockade of LTP induction via the infusion of Arc antisense ODNs results in decreased F-actin that can be reversed by the actin stabilizing drug, jasplakinolide (Messaoudi et al., 2007). The stabilization of F-actin has been suggested as a key downstream mediator of dendritic spine enlargement and an increase in DSD (Bramham, 2008; Fischer et al., 1998). Recently, our lab examined the role of Arc and associated changes in DSD in anxiety-like and alcohol drinking behaviors (Pandey et al., 2008b). The infusion of Arc

antisense ODNs into the CeA of Sprague-Dawleys rats produced anxiogenic effects and resulted in a reduction in DSD (Pandey et al., 2008b). This reduction in Arc expression and DSD was also associated with increased alcohol intake (Pandey et al., 2008b). These data suggest that reduced Arc signaling and DSD in the CeA may be responsible for anxiety-like behaviors and increased alcohol consumption. In light of these findings, it could be possible that the reduced BDNF-Arc signaling and DSD in the CeA and MeA of P rats may play a critical role in the observed anxiety-like and alcohol drinking behaviors of P rats. Furthermore, the anxiolytic effects of acute ethanol exposure may be mediated by increased amygdaloid BDNF-Arc signaling associated with spine proliferation (Figure 18).

The effects of chronic ethanol exposure on dendritic spine number and morphology have been studied by various researchers (Chandler, 2003). Chronic ethanol exposure has been found to result in the modification of dendritic spine morphology and DSD in various brain regions of both mice and rats (Lescaudron et al., 1989; Riley and Walker, 1978; Zhou et al., 2007). A reduction in DSD has also been shown in the cortical pyramidal neurons of human alcoholics (Ferrer et al., 1986). These studies suggest that changes in DSD and spine morphology due to ethanol exposure may play a role in the altered synaptic plasticity associated with alcoholism. Thus, it could be possible that innate and environmental abnormalities of DSD due to BDNF-Arc signaling could be involved in the comorbidity of anxiety and alcoholism.

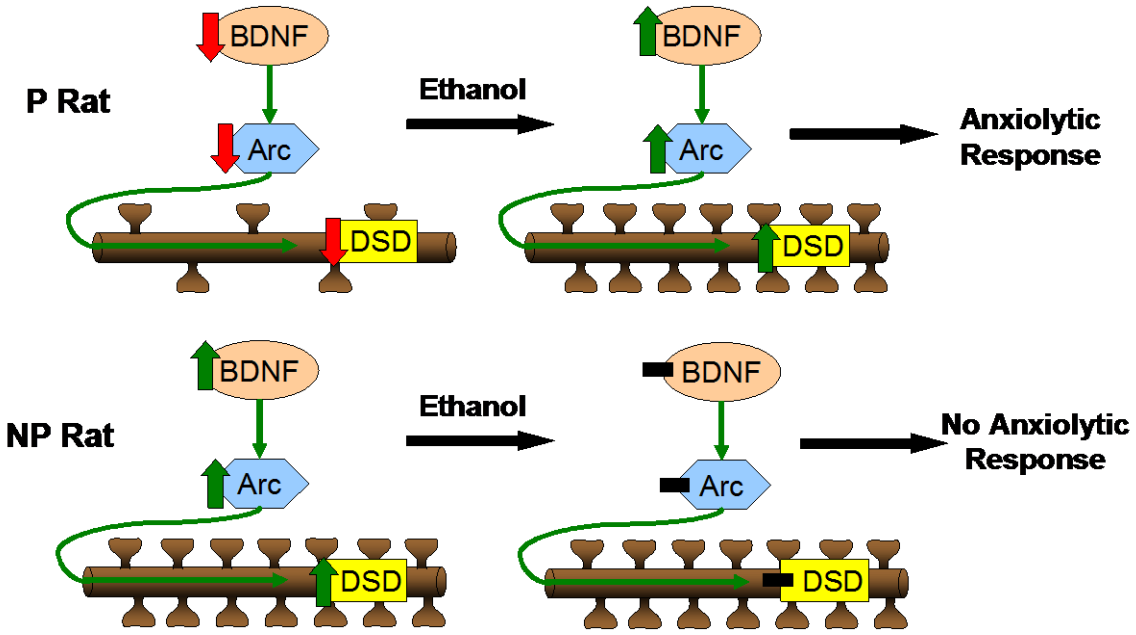


Figure 18. A hypothetical model of the anxiolytic response of acute ethanol and BDNF-induced changes in synaptic plasticity in the amygdaloid circuitry.
In comparison to alcohol-nonpreferring (NP) rats, alcohol-preferring (P) rats have lower levels of brain-derived neurotrophic factor (BDNF), activity-regulated cytoskeleton-associated protein (Arc) and dendritic spine density (DSD) in the central (CeA) and medial amygdala (MeA), which is associated with innate anxiety in P rats, but not NP rats. Acute ethanol administration results in anxiolytic effects in P rats, but not NP rats, that are associated with increased levels of BDNF, Arc and DSD in the CeA and MeA. This suggests that innately low levels of BDNF and Arc, and associated DSD, in the CeA and MeA, may play a role in the anxiety-like behaviors of P rats, and that the anxiolytic effects of acute ethanol may be related increased BDNF-Arc signaling and associated changes in DSD (Moonat et al.. 2011).

C. The Regulation of Chromatin Structure via Histone Acetylation Mediates Changes in *BDNF* and *Arc* Associated with Anxiety and Alcoholism

In the findings related to the second specific aim of this study, we identified that reduced histone acetylation in the CeA and MeA of P rats was associated with high innate anxiety. Acute and chronic ethanol exposure increased these deficits in histone acetylation levels and reduced anxiety-like behaviors in P rats, suggesting a role for

chromatin remodeling in the anxiolytic effects of ethanol. Previously, in an unselected stock of rats, we found that withdrawal from chronic ethanol exposure resulted in increased anxiety-like behaviors that were associated with a reduction in histone acetylation in the central (CeA) and medial amygdala (MeA) (Pandey et al., 2008a). Treatment of alcohol-withdrawn animals with trichostatin A (TSA), a class I and II HDAC inhibitor, reduced anxiety-like behaviors and normalized the levels of histone acetylation (Pandey et al., 2008a). Furthermore, treatment of alcohol-naïve rats with acute ethanol produced anxiolytic effects and reduced histone acetylation levels in the CeA and MeA (Pandey et al., 2008a). Taken together, these results strongly suggest that chromatin condensation associated with reduced histone acetylation may play a role in both environmental and genetic factors underlying the anxiety associated with alcoholism, and increased histone acetylation may mediate the anxiolytic effects of acute ethanol exposure.

Recently, it has been identified that various epigenetic mechanisms, including histone acetylation, play an important role in the regulation of BDNF expression and signaling. Increased histone acetylation has been found to be a factor in the upregulation of BDNF expression, suggesting a role for epigenetic regulation of transcriptional events in the control of the *BDNF* gene (Huang et al., 2002). The authors of this study found that levels of histone H4 acetylation associated with the gene promoter of *BDNF exon II* were increased by neuronal activity in the hippocampus, suggesting a role for chromatin remodeling in the regulation of BDNF (Huang et al., 2002). Tsankova *et al.* also demonstrated a role for histone acetylation in the regulation of hippocampal BDNF in a model of electroconvulsive shock therapy where they demonstrated time- and promoter-

dependent changes in histone acetylation levels associated with BDNF promoters (Tsankova et al., 2004). Subsequent studies identified a role for histone modifications in the regulation of BDNF expression in the striatum during chronic cocaine exposure and in the hippocampus in a model of depression induced by chronic social defeat stress (Kumar et al., 2005; Tsankova et al., 2006). Importantly, these studies identified a role for specific HDAC isoforms in the complex mechanisms of chromatin remodeling, suggesting a therapeutic role for isoform-specific HDAC inhibitors in substance abuse and depression (Kumar et al., 2005; Tsankova et al., 2006).

The second specific aim of this study identified the role of histone modifications in the effects of acute and chronic ethanol in P and NP rats. Specifically, the innate anxiety behaviors of P rats were found to be associated with reduced histone acetylation indicating condensed chromatin in the CeA and MeA. Furthermore, the anxiolytic effects of acute and chronic ethanol in P rats were related to an increase in histone acetylation associated with chromatin relaxation. We also identified that the changes in chromatin structure were directly associated with a specific BDNF promoter region, *BDNF exon IV*. *BDNF exon IV* has been found to be regulated various epigenetic mechanisms, including increased histone acetylation, reduced DNA methylation and reduced MeCP2 binding at *BDNF exon IV* (Fuchikami et al., 2010; He et al., 2010; Lubin et al., 2008; Martinowich et al., 2003; Sadri-Vakili et al., 2010). The reduction of DNA methylation levels in the *BDNF exon IV* promoter region have been shown to increase BDNF expression during a fear conditioning experiment (Lubin et al., 2008). Interestingly, *BDNF exon IV* was specifically associated with the consolidation of fear memory, whereas an increase in *BDNF exons I* and *VI* was seen with the presentation of context alone (Lubin et al.,

2008). This could suggest that epigenetic regulation of *BDNF exon IV* is specifically important for the changes associated with associative learning and synaptic plasticity. These finding correspond with the results from the second specific aim of this study, which may suggest that the epigenetic regulation of *BDNF exon IV* plays a role in the dynamic regulation of synaptic plasticity associated with the innate anxiety-like behaviors and alcohol preference of P rats.

Histone acetylation associated with *BDNF exon IV* has been found to play a role in the response to stress and associated stress-related disorders, such as depression (Fuchikami et al., 2010; Tsankova et al., 2006). This suggests that aberrant regulation of histone acetylation at the *BDNF exon IV* gene could be a mediator of dysphoria associated with psychiatric disorders. Thus, it could be possible that chromatin remodeling via histone acetylation at the *BDNF exon IV* gene is a mediator of changes in synaptic plasticity that are associated with dysphoric states related to the anxiety-like behaviors of P rats. It has previously been identified that antidepressant treatment in a stress-induced model of depression may act via increased histone acetylation associated with the *BDNF* gene (Tsankova et al., 2006). This may suggest that correcting deficits in histone acetylation associated with the regulation of *BDNF* can reverse dysphoric symptoms. Since the anxiolytic effects of acute and voluntary ethanol exposure produced increases in histone acetylation associated with *BDNF exon IV*, it could be possible that the effect of amygdaloid histone acetylation on BDNF expression play a role in anxiety-like and alcohol drinking behaviors of P rats. It is worth noting that we also identified an interaction between the changes in histone acetylation and the *Arc* gene promoter. Recent studies have also identified that epigenetic mechanisms, including MeCP2

binding and histone acetylation, play a role in the regulation of *Arc* that appears to play a role in cocaine sensitivity and Huntington's disease (Giralt et al., 2012; Su et al., 2012). Given that histone acetylation regulates both *BDNF* and *Arc*, it seems likely that the effect of reduced amygdaloid histone acetylation in P rats, in comparison to NP rats, plays a role in the low levels of BDNF, Arc and DSD, and that increased histone acetylation following ethanol exposure increases these markers of synaptic plasticity. Thus, the regulation of amygdaloid chromatin structure and synaptic plasticity by histone acetylation may play a key role in comorbid anxiety and alcoholism.

D. Amygdaloid HDAC2-Induced Chromatin and Synaptic Remodeling Mediate Anxiety and Alcohol Consumption

In our acute and chronic experiments, we specifically identified the involvement of HDAC2 in the CeA and MeA in the innate anxiety-like behaviors and anxiolytic effects of ethanol exposure in P rats. P rats were found to have innately high levels of HDAC2 associated with reduced histone acetylation in the CeA and MeA, such that ethanol exposure reduced HDAC2 levels and increased histone acetylation. The final specific aim of this study mechanistically identified that exogenous reduction of HDAC2 levels in the CeA of P rats resulted in increased histone acetylation, and reduced anxiety-like behaviors and alcohol preference. These findings strongly suggest that HDAC2-induced regulation of chromatin structure via histone acetylation in the CeA may mediate the innate anxiety-like behaviors and alcohol preference of P rats.

Various studies have recently identified that regulation of specific HDAC isoforms may play a role in brain function and disease (Covington et al., 2009; Ding et al., 2008; Fukada et al., 2012; Guan et al., 2009; McQuown et al., 2011; Nott et al., 2008;

Su et al., 2011; Tsankova et al., 2006). Aberrant regulation of HDAC2 has recently been linked to psychiatric and neurological disorders incluing depression, Alzheimer's disease and Huntington's disease (Covington et al., 2009; Graff et al., 2012; Mielcarek et al., 2011). A recent study identified that a transient reduction in histone acetylation levels in the NAc following social defeat stress was followed by a persistent reduction in histone acetylation associated with increased HDAC2 levels and depression-like behaviors (Covington et al., 2009). Interestingly, infusion of an HDAC inhibitor into the NAc increased histone acetylation and produced antidepressant-like effects, suggesting that the depression-like behaviors may have been associated with the transient reduction in histone acetylation (Covington et al., 2009). The authors suggest that the persistent reduction in HDAC2 served as an adaptative mechanism to restore normal function. As mentioned previously, upregulation of BDNF has also been suggested as a homeostatic pathway involved in the neuroprotection against alcohol dependence (Jeanblanc et al., 2006). Taking these findings into consideration, our findings regarding HDAC2-induced regulation of the *BDNF* gene may suggest that upregulation of BDNF via HDAC2-mediated changes in histone acetylation regulate neuroadaptation to environmental stressors. Furthermore, it could be possible that the aberrant regulation of HDAC2 in P rats results in an inability for adaptive responses which may be associated with vulnerability to alcohol dependence and dysphoria associated with anxiety.

In a recent study, it was found that HDAC2 played a key role in the regulation of dendritic spines and memory formation. Using HDAC2 overexpressing mice, Guan *et al.* found that increased HDAC2 levels negatively regulated memory formation in a fear conditioning paradigm, which was associated with a reduction in dendritic spine density

in the hippocampus (Guan et al., 2009). Furthermore, treatment of HDAC2 overexpressing mice with HDAC inhibitors reversed these deficits (Guan et al., 2009). Conversely, HDAC2 knockout facilitated learning and increased dendritic spine density (Guan et al., 2009). These data are in agreement with the results of the third specific aim of this study, which indicate that exogenous reduction of HDAC2 levels in the CeA leads to reduced DSD associated with a reduction in anxiety-like behaviors and alcohol preference in P rats.

Abnormally high HDAC2 levels were recently identified in the hippocampus of brain samples from Alzheimer's patients and a mouse model for neurodegeneration associated with Alzheimer's disease (Graff et al., 2012). In the mouse model, exogenous reduction of HDAC2 resulted in better performance in tests of memory function (Graff et al., 2012). Interestingly, neurodegeneration resulted in high HDAC2 and low histone acetylation associated with the *BDNF exon IV* and *Arc* genes, while exogenous reduction of HDAC2 increased histone acetylation at these gene promoters, and increased BDNF and Arc expression (Graff et al., 2012). This finding is consistent with our data in P rats, such that innately high HDAC2 induced low levels of histone acetylation associated with *BDNF exon IV* and *Arc*, and exogenous reduction of HDAC2 via siRNA or acute ethanol increased histone acetylation at these gene promoters. Given the association between BDNF-Arc signaling and synaptic plasticity, this strongly implicates a role for HDAC2-induced chromatin remodeling via histone acetylation associated with the *BDNF exon IV* and *Arc* genes in the downstream regulation of DSD in the CeA of P rats that is associated with anxiety-like behaviors and alcoholism. The use of HDAC inhibitors and exogenous HDAC2 knockdown in the aforementioned studies strongly suggests that

HDAC2 may serve as an important pharmacotherapeutic target for various psychiatric and neurological disorders. Given the profound effects of exogenous HDAC2 knockdown on anxiety-like behaviors and alcohol consumption in P rats, it could be possible that HDAC2-specific inhibitors would provide effective treatment for patients with comorbid anxiety and alcoholism.

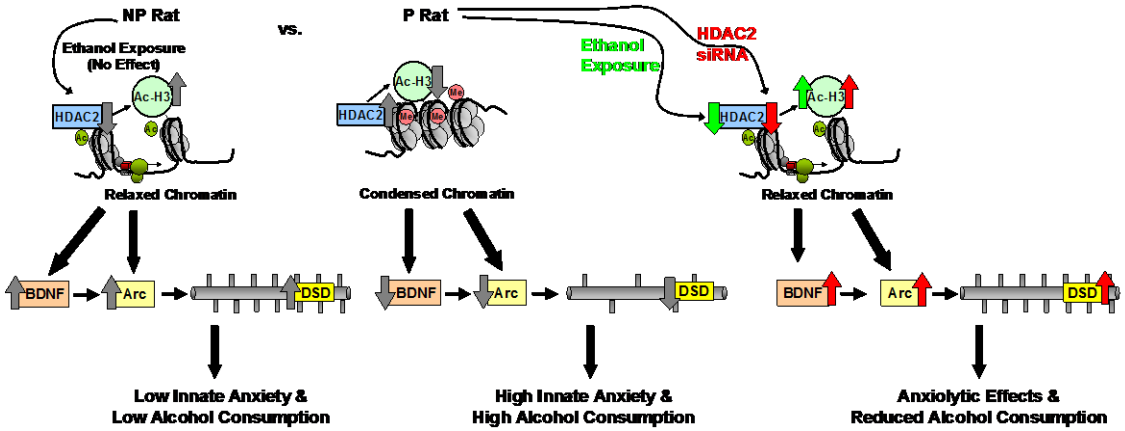


Figure 19. A hypothetical model for the role of HDAC2-mediated changes on chromatin structure and synaptic plasticity in the central amygdala in the regulation of anxiety and alcohol consumption.
In comparison to alcohol-preferring (NP) rats, alcohol-preferring (P) rats have higher levels of histone deacetylase isoform 2 (HDAC2) in the central (CeA) and medial amygdala (MeA) resulting in deficits in histone acetylation. The innately high HDAC2 levels are associated with reduced histone H3 acetylation and a reduced association of histone H3 acetylation with the promoters of the *brain-derived neurotrophic factor* (*BDNF*) and *activity-regulated cytoskeleton-associated* (*Arc*) genes in the amygdala. This suggests that HDAC2-induced chromatin condensation due to low histone acetylation levels result in reduced BDNF-Arc signaling, and low dendritic spine density (DSD) in the CeA and MeA which may play a role in the observed high innate anxiety-like behaviors and alcohol preference of P rats as compared to NP rats. In P rats, the anxiolytic effects of ethanol exposure are associated with a reduction in HDAC2 levels in the CeA and MeA which result in relaxation of chromatin due to increased histone H3 acetylation which is directly associated with the *BDNF* and *Arc* genes. The resulting upregulation of BDNF and Arc protein is associated with increased DSD in the CeA and MeA. Exogenous reduction of HDAC2 via infusion of HDAC2 siRNA in the CeA results in similar effects on chromatin and synaptic remodeling, and decreases the anxiety-like behaviors and alcohol preference of P rats. These results implicate a clear role for HDAC2-induced chromatin and synaptic remodeling in the CeA in the comorbidity to anxiety and alcoholism.

V. CONCLUSION

A. Summary of Findings and Conclusion

To briefly summarize the findings of the studies performed herein, we initially identified that the innate anxiety-like behaviors of P rats were sensitive to the anxiolytic effects of ethanol, whereas these effects were not seen in NP rats. The innate anxiety-like behaviors of P rats were associated with reduced levels of synaptically active proteins, BDNF and Arc, and DSD in the CeA and MeA in comparison to NP rats. Furthermore, the anxiety-like behaviors observed at baseline were associated with findings indicating the condensation of chromatin structure due to low levels of histone acetylation and high levels of HDAC2 in the CeA and MeA. The anxiolytic effects of acute ethanol corrected the deficits in both synaptic factors and chromatin remodeling in P rats, but not NP rats. Furthermore, acute ethanol exposure resulted in increased association of *BDNF exon IV* and *Arc* genes with acetylated histone H3 in P rats, but not NP rats, suggesting a role for epigenetic modulation of these synaptically active genes. Chronic ethanol exposure through voluntary ethanol consumption also produced anxiolytic effects that were associated with the HDAC2-mediated relaxation of chromatin due to increased histone acetylation. These data suggest that HDAC2-mediated changes in chromatin structure at specific genes which modulate synaptic plasticity may play a role in both the anxiety-like behaviors and alcohol-drinking behaviors of P rats.

To mechanistically examine the role of amygdaloid HDAC2, we specifically knocked down HDAC2 in the CeA of P rats. HDAC2 knockdown produced both anxiolytic effects and reduced the voluntary ethanol consumption of P rats, further identifying a clear role for HDAC2 in the CeA in the regulation of comorbid anxiety and

alcoholism. The reduction in HDAC2 was associated with increased histone H3 acetylation representative of chromatin relaxation. Furthermore, increased acetylation of histone H3 was directly associated with the *BDNF exon IV* and *Arc* genes, and resulted in increased levels of BDNF and Arc proteins, and an associated increase in DSD. Taken together, these data suggest that in the CeA, HDAC2 regulates chromatin structure via histone acetylation which modulation *BDNF* and *Arc*, and synaptic plasticity that is associated with anxiety-like behaviors and alcohol preference of P rats. It could be possible that the exogenous manipulation of HDAC2 via isoform-specific HDAC inhibitors may serve as a potential pharmacotherapy in the treatment of comorbid anxiety and alcoholism.

The overall conclusions of this study are as follows:

1. Innate abnormalities in HDAC2 regulation of chromatin structure and synaptic plasticity in the CeA play a role in the genetic predisposition to comorbid anxiety and alcoholism.

2. The effect of ethanol exposure on HDAC2-induced histone modifications associated with synaptic plasticity in the CeA mediates the anxiolytic effect that promotes alcohol consumption associated with the genetic predisposition to anxiety and alcoholism.

3. HDAC2-induced chromatin and synaptic remodeling in the CeA mediate the behavioral phenotypes associated with anxiety and alcohol preference, suggesting that HDAC2 may serve as a useful pharmacotherapeutic target for the treatment of comorbid anxiety and alcoholism

B. Future Directions

1. Examination of the role of DNA methylation in the regulation of BDNF in the genetic predisposition to anxiety and alcoholism.

The novel findings of this study identified a specific histone isoform associated with changes in histone acetylation that were associated with BDNF regulation in the genetic predisposition to anxiety and alcoholism. A significant addition to the findings presented in this study would take into account DNA methylation status that play a role in epigenetic regulation of BDNF and synaptic plasticity associated with anxiety and alcoholism.

Although the current study focused primarily on the role of HDAC-induced changes in histone acetylation, the role of DNA methylation in the regulation of gene transcription is becoming increasingly important in psychiatric disorders, including addiction (Levenson and Sweatt, 2005; Nestler, 2009; Renthal and Nestler, 2008; Tsankova et al., 2007a). Beyond an increased understanding of the mechanisms involved in anxiety and alcoholism, research into the role of DNA methylation may also have clinical applications due to the existence and continued design of DNA methyltransferase (DNMT) inhibitors (Marquez et al., 2005; Yoo et al., 2004; Yoo and Medina-Franco, 2012). DNA methylation has also been identified as a mechanism involved in the regulation of BDNF and synaptic plasticity (Lubin et al., 2008; Roth et al., 2011). A recent study even identified an investigational DNMT inhibitor that produced an antidepressant effect associated with increased BDNF expression (Sales et al., 2011). Given the role of BDNF in the phenotype of P rats, it would be interesting to and

pertinent to determine the DNA methylation status of the *BDNF* gene and potential effect of DNMT inhibitors in the anxiety and alcohol preference of P rats.

2. Identification of amygdaloid gene networks associated with histone acetylation and DNA methylation in the genetic predisposition to anxiety and alcoholism.

The current study utilized ChIP analysis of individual genes to identify the role of HDAC2-induced chromatin remodeling in the regulation of synaptic plasticity. Recent technologies allow for sequencing based analysis of genes associated with histone modifications and DNA methylation allowing for the identification of gene networks regulated by epigenetic modifications associated with anxiety and alcoholism (Bardet et al., 2011; Taiwo et al., 2012).

The complex interactions between histone acetylation and DNA methylation may provide clues into the regulation of gene networks associated with behavioral phenotypes and potential pharmacotherapeutic targets (Bird, 2002; Fuks, 2005). Chromatin immunoprecipitation sequencing (ChIP-Seq) and methylated DNA immunoprecipitation sequencing (MeDIP-Seq) allow for genome-wide analysis of histone acetylation and DNA methylation, respectively (Bardet et al., 2011; Taiwo et al., 2012). The development of these high throughput technologies to sequence immunoprecipitated DNA allows for analysis at the epigenomic level (Bernstein et al., 2007). Thus, it would be possible to obtain a greater understanding of gene interactions and clusters of genes which may be regulated by epigenetic mechanisms that play a role in anxiety and alcoholism.

From a clinical perspective, the analysis of epigenetic patterns of genes may serve as useful biomarkers for risk of disease or responsiveness to treatment. For instance, a recent study attempted to create a predictive model for identification of patients diagnosed with major depressive disorder by looking at the methylation status of the *BDNF* gene (Fuchikami et al., 2011). Although the study only examined two BDNF promoters, the high predictive power of the model suggested that further development of epigenetic biomarkers for psychiatric disease could be useful (Fuchikami et al., 2011). Thus, via these technologies, it could be possible to create epigenetic patterns within genetic networks that would allow the identification for patients at risk for comorbid anxiety and alcoholism. Furthermore, findings regarding the histone acetylation and DNA methylation status at certain genes, such as *BDNF*, could allow for the development of a model to predict the therapeutic potential of HDAC inhibitors and DNMT inhibitors.

3. Examination of functional changes in synaptic plasticity in the CeA that are related to the effect of ethanol on HDAC2-induced regulation of BDNF-Arc signaling and DSD

In this study, we identified that ethanol exposure produced HDAC2-induced changes in synaptic plasticity via regulation of the *BDNF* and *Arc* genes by histone acetylation. It is well known that BDNF-Arc signaling produces a variety of functional and structural synaptic changes (Bramham et al., 2008; Messaoudi et al., 2007; Ying et al., 2002). Previously, it has been shown that BDNF signaling via Erk phosphorylation is required for consolidation of LTP via induction of Arc protein (Ying et al., 2002). Thus, downstream blockade of BDNF signaling via pharmacological inhibition of MEK with U0126 results in inhibition of Arc induction and LTP consolidation (Ying et al., 2002).

The study of LTP consolidation in Arc knockout mice has shown that innate deficits in Arc signaling can lead to disruption of late-phase LTP maintenance, such that the time-course of LTP maintenance is reduced in Arc knockout mice in comparison to wild type mice (Plath et al., 2006).

Acute and chronic ethanol exposure, as well as ethanol withdrawal, have also been shown to modulate the magnitude and time-course of LTP in various brain regions (Fujii et al., 2008; Roberto et al., 2003; Xie et al., 2009). However, the role of amygdaloid HDAC2-induced changes in BDNF signaling and Arc protein in the modulation of LTP by ethanol exposure is not well understood. Furthermore, the role of differences in LTP consolidation in the CeA has not been studied with regards to the genetic predisposition to anxiety and alcohol preference. A great deal of insight into the functional changes in synaptic plasticity could be gained by electrophysiological study of changes in the CeA associated with ethanol exposure. The use of pharmacological agents, such as HDAC inhibitors and U0126, could help to determine the role of histone acetylation and BDNF signaling in downstream changes in synaptic function. These studies could help to further understand the mechanisms at work in the HDAC2-induced changes in synaptic plasticity, and associate our structural findings with functional changes at the synapse.

CITED LITERATURE

Abel T, Kandel E (1998) Positive and negative regulatory mechanisms that mediate long-term memory storage. Brain Res Brain Res Rev 26(2-3):360-78.

Abel T, Zukin RS (2008) Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders. Curr Opin Pharmacol 8(1):57-64.

Acquaah-Mensah GK, Misra V, Biswal S (2006) Ethanol sensitivity: a central role for CREB transcription regulation in the cerebellum. BMC Genomics 7:308.

Adolphs R (2008) Fear, faces, and the human amygdala. Curr Opin Neurobiol 18(2):166-72.

Agarwal DP, Goedde HW (1992) Pharmacogenetics of alcohol metabolism and alcoholism. Pharmacogenetics 2(2):48-62.

Agrawal A, Sartor CE, Lynskey MT, Grant JD, Pergadia ML, Grucza R, Bucholz KK, Nelson EC, Madden PA, Martin NG, Heath AC (2009) Evidence for an interaction between age at first drink and genetic influences on DSM-IV alcohol dependence symptoms. Alcohol Clin Exp Res 33(12):2047-56.

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res 85(3):525-35.

Akhtar MW, Raingo J, Nelson ED, Montgomery RL, Olson EN, Kavalali ET, Monteggia LM (2009) Histone deacetylases 1 and 2 form a developmental switch that controls excitatory synapse maturation and function. J Neurosci 29(25):8288-97.

Akmaev IG, Kalimullina LB, Sharipova LA (2004) The central nucleus of the amygdaloid body of the brain: cytoarchitectonics, neuronal organization, connections. Neurosci Behav Physiol 34(6):603-10.

Alarcon JM, Malleret G, Touzani K, Vronskaya S, Ishii S, Kandel ER, Barco A (2004) Chromatin acetylation, memory, and LTP are impaired in CBP+/- mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. Neuron 42(6):947-59.

Alberini CM (2009) Transcription factors in long-term memory and synaptic plasticity. Physiol Rev 89(1):121-45.

Alheid GF (2003) Extended amygdala and basal forebrain. Ann N Y Acad Sci 985:185-205.

Alheid GF, Heimer L (1988) New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata. Neuroscience 27(1):1-39.

Allfrey VG, Faulkner R, Mirsky AE (1964) Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. Proc Natl Acad Sci U S A 51:786-94.

Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature 389(6653):856-60.

Amaral DG, Insausti R (1992) Retrograde transport of D-[3H]-aspartate injected into the monkey amygdaloid complex. Exp Brain Res 88(2):375-88.

American Psychiatric Association (1994) Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV), Washington, D.C.

An JJ, Gharami K, Liao GY, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B, Xu B (2008) Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. Cell 134(1):175-87.

Andreasen NC, Black DW (2006) *Introductory textbook of psychiatry.* 4th ed. American Psychiatric Pub., Washington, DC.

Antequera F (2003) Structure, function and evolution of CpG island promoters. Cell Mol Life Sci 60(8):1647-58.

Autry AE, Monteggia LM (2012) Brain-derived neurotrophic factor and neuropsychiatric disorders. Pharmacol Rev 64(2):238-58.

Balasubramanian S, Verner E, Buggy JJ (2009) Isoform-specific histone deacetylase inhibitors: the next step? Cancer Lett 280(2):211-21.

Ballestar E, Wolffe AP (2001) Methyl-CpG-binding proteins. Targeting specific gene repression. Eur J Biochem 268(1):1-6.

Banerjee T, Chakravarti D (2011) A peek into the complex realm of histone phosphorylation. Mol Cell Biol 31(24):4858-73.

Bardet AF, He Q, Zeitlinger J, Stark A (2011) A computational pipeline for comparative ChIP-seq analyses. Nat Protoc 7(1):45-61.

Bates EA, Victor M, Jones AK, Shi Y, Hart AC (2006) Differential contributions of Caenorhabditis elegans histone deacetylases to huntingtin polyglutamine toxicity. J Neurosci 26(10):2830-8.

Bath KG, Jing DQ, Dincheva I, Neeb CC, Pattwell SS, Chao MV, Lee FS, Ninan I (2012) BDNF Val66Met Impairs Fluoxetine-Induced Enhancement of Adult Hippocampus Plasticity. Neuropsychopharmacology.

Becker HC (1994) Positive relationship between the number of prior ethanol withdrawal episodes and the severity of subsequent withdrawal seizures. Psychopharmacology (Berl) 116(1):26-32.

Belknap JK, Crabbe JC, Young ER (1993) Voluntary consumption of ethanol in 15 inbred mouse strains. Psychopharmacology (Berl) 112(4):503-10.

Bell RL, Rodd ZA, Lumeng L, Murphy JM, McBride WJ (2006) The alcohol-preferring P rat and animal models of excessive alcohol drinking. Addict Biol 11(3-4):270-88.

Berg JM, Tymoczko JL, Stryer L (2007) *Biochemistry*. 6th ed. W.H. Freeman, New York.

Bergami M, Berninger B, Canossa M (2009) Conditional deletion of TrkB alters adult hippocampal neurogenesis and anxiety-related behavior. Commun Integr Biol 2(1):14-6.

Bergami M, Rimondini R, Santi S, Blum R, Gotz M, Canossa M (2008) Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. Proc Natl Acad Sci U S A 105(40):15570-5.

Bernard JF, Huang GF, Besson JM (1990) Effect of noxious somesthetic stimulation on the activity of neurons of the nucleus centralis of the amygdala. Brain Res 523(2):347-50.

Berne RM (2004) *Physiology.* 5th ed. Mosby, St. Louis.

Bernstein BE, Meissner A, Lander ES (2007) The mammalian epigenome. Cell 128(4):669-81.

Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 311(5762):864-8.

Bestor TH (2000) The DNA methyltransferases of mammals. Hum Mol Genet 9(16):2395-402.

Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16(1):6-21.

Bird AP (1986) CpG-rich islands and the function of DNA methylation. Nature 321(6067):209-13.

Black JC, Whetstine JR (2011) Chromatin landscape: methylation beyond transcription. Epigenetics 6(1):9-15.

Bloodgood BL, Sabatini BL (2008) Regulation of synaptic signalling by postsynaptic, non-glutamate receptor ion channels. J Physiol 586(6):1475-80.

Bolton JM, Robinson J, Sareen J (2009) Self-medication of mood disorders with alcohol and drugs in the National Epidemiologic Survey on Alcohol and Related Conditions. J Affect Disord 115(3):367-75.

Bradizza CM, Stasiewicz PR, Paas ND (2006) Relapse to alcohol and drug use among individuals diagnosed with co-occurring mental health and substance use disorders: a review. Clin Psychol Rev 26(2):162-78.

Bramham CR (2008) Local protein synthesis, actin dynamics, and LTP consolidation. Curr Opin Neurobiol 18(5):524-31.

Bramham CR, Alme MN, Bittins M, Kuipers SD, Nair RR, Pai B, Panja D, Schubert M, Soule J, Tiron A, Wibrand K (2010) The Arc of synaptic memory. Exp Brain Res 200:125-140.

Bramham CR, Worley PF, Moore MJ, Guzowski JF (2008) The immediate early gene arc/arg3.1: regulation, mechanisms, and function. J Neurosci 28(46):11760-7.

Brandon EP, Idzerda RL, McKnight GS (1997) PKA isoforms, neural pathways, and behaviour: making the connection. Curr Opin Neurobiol 7(3):397-403.

Bredy TW, Wu H, Crego C, Zellhoefer J, Sun YE, Barad M (2007) Histone modifications around individual BDNF gene promoters in prefrontal cortex are associated with extinction of conditioned fear. Learn Mem 14(4):268-76.

Breese GR, Overstreet DH, Knapp DJ (2005) Conceptual framework for the etiology of alcoholism: a "kindling"/stress hypothesis. Psychopharmacology (Berl) 178(4):367-80.

Breese GR, Sinha R, Heilig M (2011) Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse. Pharmacol Ther 129(2):149-71.

Brennan PA, Kendrick KM (2006) Mammalian social odours: attraction and individual recognition. Philos Trans R Soc Lond B Biol Sci 361(1476):2061-78.

Burstein R, Potrebic S (1993) Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. J Comp Neurol 335(4):469-85.

Calabrese F, Molteni R, Racagni G, Riva MA (2009) Neuronal plasticity: a link between stress and mood disorders. Psychoneuroendocrinology 34 S1:S208-16.

Canteras NS, Swanson LW (1992) Projections of the ventral subiculum to the amygdala, septum, and hypothalamus: a PHAL anterograde tract-tracing study in the rat. J Comp Neurol 324(2):180-94.

Carlezon WA, Jr., Duman RS, Nestler EJ (2005) The many faces of CREB. Trends Neurosci 28(8):436-45.

Carnicella S, Kharazia V, Jeanblanc J, Janak PH, Ron D (2008) GDNF is a fast-acting potent inhibitor of alcohol consumption and relapse. Proc Natl Acad Sci U S A 105(23):8114-9.

Carpenter-Hyland EP, Chandler LJ (2006) Homeostatic plasticity during alcohol exposure promotes enlargement of dendritic spines. Eur J Neurosci 24(12):3496-506.

Cassell MD, Freedman LJ, Shi C (1999) The intrinsic organization of the central extended amygdala. Ann N Y Acad Sci 877:217-41.

Cassell MD, Gray TS (1989) Morphology of peptide-immunoreactive neurons in the rat central nucleus of the amygdala. J Comp Neurol 281(2):320-33.

Castaneda R, Sussman N, Westreich L, Levy R, O'Malley M (1996) A review of the effects of moderate alcohol intake on the treatment of anxiety and mood disorders. J Clin Psychiatry 57(5):207-12.

Chandler LJ (2003) Ethanol and brain plasticity: receptors and molecular networks of the postsynaptic density as targets of ethanol. Pharmacol Ther 99(3):311-26.

Chandler LJ, Newsom H, Sumners C, Crews F (1993) Chronic ethanol exposure potentiates NMDA excitotoxicity in cerebral cortical neurons. J Neurochem 60(4):1578-81.

Chandler LJ, Sutton G (2005) Acute ethanol inhibits extracellular signal-regulated kinase, protein kinase B, and adenosine 3':5'-cyclic monophosphate response element binding protein activity in an age- and brain region-specific manner. Alcohol Clin Exp Res 29(4):672-82.

Charney DA, Zikos E, Gill KJ (2009) Early recovery from alcohol dependence: factors that promote or impede abstinence. J Subst Abuse Treat 38(1):42-50.

Chen ZY, Bath K, McEwen B, Hempstead B, Lee F (2008) Impact of genetic variant BDNF (Val66Met) on brain structure and function. Novartis Found Symp 289:180-8; discussion 188-95.

Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science 314(5796):140-3.

Chiaruttini C, Vicario A, Li Z, Baj G, Braiuca P, Wu Y, Lee FS, Gardossi L, Baraban JM, Tongiorgi E (2009) Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation. Proc Natl Acad Sci U S A 106(38):16481-6.

Chieng BC, Christie MJ, Osborne PB (2006) Characterization of neurons in the rat central nucleus of the amygdala: cellular physiology, morphology, and opioid sensitivity. J Comp Neurol 497(6):910-27.

Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365(6449):855-9.

Clarke TK, Treutlein J, Zimmermann US, Kiefer F, Skowronek MH, Rietschel M, Mann K, Schumann G (2008) HPA-axis activity in alcoholism: examples for a gene-environment interaction. Addict Biol 13(1):1-14.

Cloninger CR (1987) Neurogenetic adaptive mechanisms in alcoholism. Science 236(4800):410-6.

Colzato LS, Van der Does AJ, Kouwenhoven C, Elzinga BM, Hommel B (2011) BDNF Val66Met polymorphism is associated with higher anticipatory cortisol stress response, anxiety, and alcohol consumption in healthy adults. Psychoneuroendocrinology 36(10):1562-9.

Constantinescu A, Diamond I, Gordon AS (1999) Ethanol-induced translocation of cAMP-dependent protein kinase to the nucleus. Mechanism and functional consequences. J Biol Chem 274(38):26985-91.

Conway KP, Compton W, Stinson FS, Grant BF (2006) Lifetime comorbidity of DSM-IV mood and anxiety disorders and specific drug use disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. J Clin Psychiatry 67(2):247-57.

Cooke BM (2006) Steroid-dependent plasticity in the medial amygdala. Neuroscience 138(3):997-1005.

Cotton NS (1979) The familial incidence of alcoholism: a review. J Stud Alcohol 40(1):89-116.

Covington HE, 3rd, Maze I, LaPlant QC, Vialou VF, Ohnishi YN, Berton O, Fass DM, Renthal W, Rush AJ, 3rd, Wu EY, Ghose S, Krishnan V, Russo SJ, Tamminga C, Haggarty SJ, Nestler EJ (2009) Antidepressant actions of histone deacetylase inhibitors. J Neurosci 29(37):11451-60.

Crabbe JC (2008) Review. Neurogenetic studies of alcohol addiction. Philos Trans R Soc Lond B Biol Sci 363(1507):3201-11.

Crabbe JC, Phillips TJ, Harris RA, Arends MA, Koob GF (2006) Alcohol-related genes: contributions from studies with genetically engineered mice. Addict Biol 11(3-4):195-269.

Davis M, Walker DL, Miles L, Grillon C (2009) Phasic vs Sustained Fear in Rats and Humans: Role of the Extended Amygdala in Fear vs Anxiety. Neuropsychopharmacology.

Davis MI (2008) Ethanol-BDNF interactions: still more questions than answers. Pharmacol Ther 118(1):36-57.

Dawson DA, Goldstein RB, Grant BF (2007) Rates and correlates of relapse among individuals in remission from DSM-IV alcohol dependence: a 3-year follow-up. Alcohol Clin Exp Res 31(12):2036-45.

De Beun R, Schneider R, Klein A, Lohmann A, De Vry J (1996) Effects of nimodipine and other calcium channel antagonists in alcohol-preferring AA rats. Alcohol 13(3):263-71.

de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochemical Journal 370(Pt 3):737-49.

Ding H, Dolan PJ, Johnson GV (2008) Histone deacetylase 6 interacts with the microtubule-associated protein tau. J Neurochem 106(5):2119-30.

Dohrman DP, Diamond I, Gordon AS (1996) Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus. Proc Natl Acad Sci U S A 93(19):10217-21.

Dokmanovic M, Clarke C, Marks PA (2007) Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 5(10):981-9.

Duka T, Gentry J, Malcolm R, Ripley TL, Borlikova G, Stephens DN, Veatch LM, Becker HC, Crews FT (2004) Consequences of multiple withdrawals from alcohol. Alcohol Clin Exp Res 28(2):233-46.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 112(2):257-69.

Enoch MA (2006) Genetic and environmental influences on the development of alcoholism: resilience vs. risk. Ann N Y Acad Sci 1094:193-201.

Falls WA, Miserendino MJ, Davis M (1992) Extinction of fear-potentiated startle: blockade by infusion of an NMDA antagonist into the amygdala. J Neurosci 12(3):854-63.

Ferrer I, Fabregues I, Rairiz J, Galofre E (1986) Decreased numbers of dendritic spines on cortical pyramidal neurons in human chronic alcoholism. Neurosci Lett 69(1):115-9.

Fischer A, Sananbenesi F, Mungenast A, Tsai LH (2010) Targeting the correct HDAC(s) to treat cognitive disorders. Trends Pharmacol Sci 31(12):605-17.

Fischer M, Kaech S, Knutti D, Matus A (1998) Rapid actin-based plasticity in dendritic spines. Neuron 20(5):847-54.

Franklin TB, Russig H, Weiss IC, Graff J, Linder N, Michalon A, Vizi S, Mansuy IM (2010) Epigenetic transmission of the impact of early stress across generations. Biol Psychiatry 68(5):408-15.

Fuchikami M, Morinobu S, Segawa M, Okamoto Y, Yamawaki S, Ozaki N, Inoue T, Kusumi I, Koyama T, Tsuchiyama K, Terao T (2011) DNA methylation profiles of the brain-derived neurotrophic factor (BDNF) gene as a potent diagnostic biomarker in major depression. PLoS One 6(8):e23881.

Fuchikami M, Yamamoto S, Morinobu S, Takei S, Yamawaki S (2010) Epigenetic regulation of BDNF gene in response to stress. Psychiatry Investig 7(4):251-6.

Fujii S, Yamazaki Y, Sugihara T, Wakabayashi I (2008) Acute and chronic ethanol exposure differentially affect induction of hippocampal LTP. Brain Res 1211:13-21.

Fukada M, Hanai A, Nakayama A, Suzuki T, Miyata N, Rodriguiz RM, Wetsel WC, Yao TP, Kawaguchi Y (2012) Loss of deacetylation activity of hdac6 affects emotional behavior in mice. PLoS One 7(2):e30924.

Fuks F (2005) DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev 15:490-495.

Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T (2000) DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat Genet 24(1):88-91.

Gao L, Cueto MA, Asselbergs F, Atadja P (2002) Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J Biol Chem 277(28):25748-55.

Gardell LR, Reid LD, Boedeker KL, Liakos TM, Hubbell CL (1997) Isradipine and naltrexone in combination with isradipine interact with a period of abstinence to reduce rats' intakes of an alcoholic beverage. Alcohol Clin Exp Res 21(9):1592-8.

Gass JT, Olive MF (2009) Role of protein kinase C epsilon (PKCvarepsilon) in the reduction of ethanol reinforcement due to mGluR5 antagonism in the nucleus accumbens shell. Psychopharmacology (Berl).

Gilpin NW, Richardson HN, Lumeng L, Koob GF (2008) Dependence-induced alcohol drinking by alcohol-preferring (P) rats and outbred Wistar rats. Alcohol Clin Exp Res 32(9):1688-96.

Giralt A, Puigdellivol M, Carreton O, Paoletti P, Valero J, Parra-Damas A, Saura CA, Alberch J, Gines S (2012) Long-term memory deficits in Huntington's disease are associated with reduced CBP histone acetylase activity. Hum Mol Genet 21(6):1203-16.

Gonzales RA, Job MO, Doyon WM (2004) The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. Pharmacol Ther 103(2):121-46.

Graff J, Rei D, Guan JS, Wang WY, Seo J, Hennig KM, Nieland TJ, Fass DM, Kao PF, Kahn M, Su SC, Samiei A, Joseph N, Haggarty SJ, Delalle I, Tsai LH (2012) An epigenetic blockade of cognitive functions in the neurodegenerating brain. Nature 483(7388):222-6.

Grant BF, Dawson DA, Stinson FS, Chou SP, Dufour MC, Pickering RP (2004a) The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991-1992 and 2001-2002. Drug Alcohol Depend 74(3):223-34.

Grant BF, Stinson FS, Dawson DA, Chou SP, Dufour MC, Compton W, Pickering RP, Kaplan K (2004b) Prevalence and co-occurrence of substance use disorders and independent mood and anxiety disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. Arch Gen Psychiatry 61(8):807-16.

Grayson DR, Kundakovic M, Sharma RP (2010) Is there a future for histone deacetylase inhibitors in the pharmacotherapy of psychiatric disorders? Mol Pharmacol 77(2):126-35.

Grusser SM, Morsen CP, Flor H (2006) Alcohol craving in problem and occasional alcohol drinkers. Alcohol Alcohol 41(4):421-5.

Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J, Nieland TJ, Zhou Y, Wang X, Mazitschek R, Bradner JE, DePinho RA, Jaenisch R, Tsai LH (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459(7243):55-60.

Guan Z, Giustetto M, Lomvardas S, Kim JH, Miniaci MC, Schwartz JH, Thanos D, Kandel ER (2002) Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. Cell 111(4):483-93.

Guitart X, Thompson MA, Mirante CK, Greenberg ME, Nestler EJ (1992) Regulation of cyclic AMP response element-binding protein (CREB) phosphorylation by acute and chronic morphine in the rat locus coeruleus. J Neurochem 58(3):1168-71.

Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF, Barnes CA (2000) Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J Neurosci 20(11):3993-4001.

Hanoune J, Defer N (2001) Regulation and role of adenylyl cyclase isoforms. Annu Rev Pharmacol Toxicol 41:145-74.

Hanson DJ (1995) *Preventing alcohol abuse : alcohol, culture, and control.* Praeger, Westport, Conn.

Harris RA, Trudell JR, Mihic SJ (2008) Ethanol's molecular targets. Sci Signal 1(28):re7.

Hashimoto K (2007) BDNF variant linked to anxiety-related behaviors. Bioessays 29(2):116-9.

Hatta S, Saito T, Ohshika H (1994) Effects of ethanol on the function of G proteins in rat cerebral cortex membranes. Alcohol Alcohol Suppl 29(1):45-51.

He DY, Neasta J, Ron D (2010) Epigenetic regulation of BDNF expression via the scaffolding protein RACK1. J Biol Chem 285(25):19043-50.

Heberlein A, Muschler M, Wilhelm J, Frieling H, Lenz B, Groschl M, Kornhuber J, Bleich S, Hillemacher T (2010) BDNF and GDNF serum levels in alcohol-

dependent patients during withdrawal. Prog Neuropsychopharmacol Biol Psychiatry 34(6):1060-4.

Heimer L, Van Hoesen GW (2006) The limbic lobe and its output channels: implications for emotional functions and adaptive behavior. Neurosci Biobehav Rev 30(2):126-47.

Hensler JG, Ladenheim EE, Lyons WE (2003) Ethanol consumption and serotonin-1A (5-HT1A) receptor function in heterozygous BDNF (+/-) mice. J Neurochem 85(5):1139-47.

Hillemacher T (2011) Biological mechanisms in alcohol dependence--new perspectives. Alcohol Alcohol 46(3):224-30.

Hitchcock JM, Davis M (1987) Fear-potentiated startle using an auditory conditioned stimulus: effect of lesions of the amygdala. Physiol Behav 39(3):403-8.

Hodge CW, Miles MF, Sharko AC, Stevenson RA, Hillmann JR, Lepoutre V, Besheer J, Schroeder JP (2006) The mGluR5 antagonist MPEP selectively inhibits the onset and maintenance of ethanol self-administration in C57BL/6J mice. Psychopharmacology (Berl) 183(4):429-38.

Horch HW (2004) Local effects of BDNF on dendritic growth. Rev Neurosci 15(2):117-29.

Hu XJ, Ticku MK (1997) Functional characterization of a kindling-like model of ethanol withdrawal in cortical cultured neurons after chronic intermittent ethanol exposure. Brain Res 767(2):228-34.

Huang F, Chotiner JK, Steward O (2007) Actin polymerization and ERK phosphorylation are required for Arc/Arg3.1 mRNA targeting to activated synaptic sites on dendrites. J Neurosci 27(34):9054-67.

Huang MC, Chen CH, Liu HC, Chen CC, Ho CC, Leu SJ (2011) Differential patterns of serum brain-derived neurotrophic factor levels in alcoholic patients with and without delirium tremens during acute withdrawal. Alcohol Clin Exp Res 35(1):126-31.

Huang Y, Doherty JJ, Dingledine R (2002) Altered histone acetylation at glutamate receptor 2 and brain-derived neurotrophic factor genes is an early event triggered by status epilepticus. J Neurosci 22(19):8422-8.

Huebner RB, Kantor LW (2011) Advances in Alcoholism Treatment. Alcohol Res Health 33(4):295-99.

Hwang BH, Stewart R, Zhang JK, Lumeng L, Li TK (2004) Corticotropin-releasing factor gene expression is down-regulated in the central nucleus of the amygdala of alcohol-preferring rats which exhibit high anxiety: a comparison between rat lines selectively bred for high and low alcohol preference. Brain Res 1026(1):143-50.

Jankord R, Herman JP (2008) Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. Ann N Y Acad Sci 1148:64-73.

Jeanblanc J, He DY, Carnicella S, Kharazia V, Janak PH, Ron D (2009) Endogenous BDNF in the dorsolateral striatum gates alcohol drinking. J Neurosci 29(43):13494-502.

Jeanblanc J, He DY, McGough NN, Logrip ML, Phamluong K, Janak PH, Ron D (2006) The dopamine D3 receptor is part of a homeostatic pathway regulating ethanol consumption. J Neurosci 26(5):1457-64.

Jenuwein T, Allis CD (2001) Translating the histone code. Science 293(5532):1074-80.

Jiang X, Xu K, Hoberman J, Tian F, Marko AJ, Waheed JF, Harris CR, Marini AM, Enoch MA, Lipsky RH (2005) BDNF variation and mood disorders: a novel functional promoter polymorphism and Val66Met are associated with anxiety but have opposing effects. Neuropsychopharmacology 30(7):1353-61.

Johansen JP, Hamanaka H, Monfils MH, Behnia R, Deisseroth K, Blair HT, LeDoux JE (2010) Optical activation of lateral amygdala pyramidal cells instructs associative fear learning. Proc Natl Acad Sci U S A 107(28):12692-7.

Johnson DA, Cooke R, Loh HH (1980) Involvement of lipids in the action of ethanol and other anesthetics. Adv Exp Med Biol 126:65-8.

Jones AE, McBride WJ, Murphy JM, Lumeng L, Li T, Shekhar A, McKinzie DL (2000) Effects of ethanol on startle responding in alcohol-preferring and -non-preferring rats. Pharmacol Biochem Behav 67(2):313-8.

Kalin NH, Shelton SE, Davidson RJ (2004) The role of the central nucleus of the amygdala in mediating fear and anxiety in the primate. J Neurosci 24(24):5506-15.

Kalluri HS, Ticku MK (2003) Regulation of ERK phosphorylation by ethanol in fetal cortical neurons. Neurochem Res 28(5):765-9.

Kampov-Polevoy AB, Matthews DB, Gause L, Morrow AL, Overstreet DH (2000) P rats develop physical dependence on alcohol via voluntary drinking: changes in seizure thresholds, anxiety, and patterns of alcohol drinking. Alcohol Clin Exp Res 24(3):278-84.

Kandel ER, Schwartz JH, Jessell TM (1991) *Principles of neural science.* 3rd ed. Appleton & Lange, Norwalk, Conn.

Kang H, Schuman EM (1996) A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science 273(5280):1402-6.

Kangaspeska S, Stride B, Metivier R, Polycarpou-Schwartz M, Ibberson D, Carmouche RP, Benes V, Gannon F, Reid G (2008) Transient cyclical methylation of promoter DNA. Nature 452(7183):112-115.

Katsura M, Shibasaki M, Hayashida S, Torigoe F, Tsujimura A, Ohkuma S (2006) Increase in expression of alpha1 and alpha2/delta1 subunits of L-type high voltage-gated calcium channels after sustained ethanol exposure in cerebral cortical neurons. J Pharmacol Sci 102(2):221-30.

Kavanaugh SM, White LA, Kolesar JM (2010) Vorinostat: A novel therapy for the treatment of cutaneous T-cell lymphoma. Am J Health Syst Pharm 67(10):793-7.

Kawashima T, Okuno H, Nonaka M, Adachi-Morishima A, Kyo N, Okamura M, Takemoto-Kimura S, Worley PF, Bito H (2009) Synaptic activity-responsive element in the Arc/Arg3.1 promoter essential for synapse-to-nucleus signaling in activated neurons. Proc Natl Acad Sci U S A 106(1):316-21.

Kemppainen S, Pitkanen A (2000) Distribution of parvalbumin, calretinin, and calbindin-D(28k) immunoreactivity in the rat amygdaloid complex and colocalization with gamma-aminobutyric acid. J Comp Neurol 426(3):441-67.

Kim CH, Lee J, Lee JY, Roche KW (2008a) Metabotropic glutamate receptors: phosphorylation and receptor signaling. J Neurosci Res 86(1):1-10.

Kim D, Frank CL, Dobbin MM, Tsunemoto RK, Tu W, Peng PL, Guan JS, Lee BH, Moy LY, Giusti P, Broodie N, Mazitschek R, Delalle I, Haggarty SJ, Neve RL, Lu Y, Tsai LH (2008b) Deregulation of HDAC1 by p25/Cdk5 in neurotoxicity. Neuron 60(5):803-17.

Kim JJ, Fanselow MS (1992) Modality-specific retrograde amnesia of fear. Science 256(5057):675-7.

Kimura H, Shiota K (2003) Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J Biol Chem 278(7):4806-12.

Knapp DJ, Breese GR (2012) Models of chronic alcohol exposure and dependence. Methods Mol Biol 829:205-30.

Knapp DJ, Overstreet DH, Huang M, Wills TA, Whitman BA, Angel RA, Sinnett SE, Breese GR (2011) Effects of a stressor and corticotrophin releasing factor on

ethanol deprivation-induced ethanol intake and anxiety-like behavior in alcohol-preferring P rats. Psychopharmacology (Berl) 218(1):179-89.

Koob GF (2003) Alcoholism: allostasis and beyond. Alcohol Clin Exp Res 27(2):232-43.

Koob GF, Le Moal M (2008) Addiction and the brain antireward system. Annu Rev Psychology 59:29-53.

Koob GF, Roberts AJ, Schulteis G, Parsons LH, Heyser CJ, Hyytia P, Merlo-Pich E, Weiss F (1998) Neurocircuitry targets in ethanol reward and dependence. Alcohol Clin Exp Res 22(1):3-9.

Koob GF, Swerdlow NR (1988) The functional output of the mesolimbic dopamine system. Ann N Y Acad Sci 537:216-27.

Koponen E, Voikar V, Riekki R, Saarelainen T, Rauramaa T, Rauvala H, Taira T, Castren E (2004) Transgenic mice overexpressing the full-length neurotrophin receptor trkB exhibit increased activation of the trkB-PLCgamma pathway, reduced anxiety, and facilitated learning. Mol Cell Neurosci 26(1):166-81.

Korzus E, Rosenfeld MG, Mayford M (2004) CBP histone acetyltransferase activity is a critical component of memory consolidation. Neuron 42(6):961-72.

Kou J, Yoshimura M (2007) Isoform-specific enhancement of adenylyl cyclase activity by n-alkanols. Alcohol Clin Exp Res 31(9):1467-72.

Krettek JE, Price JL (1978) A description of the amygdaloid complex in the rat and cat with observations on intra-amygdaloid axonal connections. J Comp Neurol 178(2):255-80.

Krupp JJ, Vissel B, Heinemann SF, Westbrook GL (1996) Calcium-dependent inactivation of recombinant N-methyl-D-aspartate receptors is NR2 subunit specific. Mol Pharmacol 50(6):1680-8.

Kumar A, Choi KH, Renthal W, Tsankova NM, Theobald DE, Truong HT, Russo SJ, Laplant Q, Sasaki TS, Whistler KN, Neve RL, Self DW, Nestler EJ (2005) Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. Neuron 48(2):303-14.

Kushner MG, Abrams K, Thuras P, Hanson KL (2000) Individual differences predictive of drinking to manage anxiety among non-problem drinkers with panic disorder. Alcohol Clin Exp Res 24(4):448-458.

LaBar KS, Cabeza R (2006) Cognitive neuroscience of emotional memory. Nat Rev Neurosci 7(1):54-64.

Lai CC, Kuo TI, Lin HH (2007) The role of protein kinase A in acute ethanol-induced neurobehavioral actions in rats. Anesth Analg 105(1):89-96.

Lang PJ, Davis M, Ohman A (2000) Fear and anxiety: animal models and human cognitive psychophysiology. J Affect Disord 61(3):137-59.

Lang UE, Hellweg R, Kalus P, Bajbouj M, Lenzen KP, Sander T, Kunz D, Gallinat J (2005a) Association of a functional BDNF polymorphism and anxiety-related personality traits. Psychopharmacology 180(1):95-9.

Lang UE, Hellweg R, Kalus P, Bajbouj M, Lenzen KP, Sander T, Kunz D, Gallinat J (2005b) Association of a functional BDNF polymorphism and anxiety-related personality traits. Psychopharmacology (Berl) 180(1):95-9.

LaPlant Q, Vialou V, Covington HE, 3rd, Dumitriu D, Feng J, Warren BL, Maze I, Dietz DM, Watts EL, Iniguez SD, Koo JW, Mouzon E, Renthal W, Hollis F, Wang H, Noonan MA, Ren Y, Eisch AJ, Bolanos CA, Kabbaj M, Xiao G, Neve RL, Hurd YL, Oosting RS, Fan G, Morrison JH, Nestler EJ (2010) Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. Nat Neurosci 13(9):1137-43.

Lau JY, Goldman D, Buzas B, Hodgkinson C, Leibenluft E, Nelson E, Sankin L, Pine DS, Ernst M (2010) BDNF gene polymorphism (Val66Met) predicts amygdala and anterior hippocampus responses to emotional faces in anxious and depressed adolescents. Neuroimage 53(3):952-61.

Lautin A (2001) *The limbic brain.* Kluwer Academic / Plenum Publishers, New York.

LeDoux J (2003) The emotional brain, fear, and the amygdala. Cell Mol Neurobiol 23(4-5):727-38.

LeDoux J (2007) The amygdala. Curr Biol 17(20):R868-74.

LeDoux JE (1992) Brain mechanisms of emotion and emotional learning. Curr Opin Neurobiol 2(2):191-7.

LeDoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23:155-84.

LeDoux JE, Cicchetti P, Xagoraris A, Romanski LM (1990) The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. J Neurosci 10(4):1062-9.

LeDoux JE, Iwata J, Cicchetti P, Reis DJ (1988) Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. J Neurosci 8(7):2517-29.

Lee AM, Messing RO (2008) Protein kinases and addiction. Ann N Y Acad Sci 1141:22-57.

Lee K, Dunwiddie T, Deitrich R, Lynch G, Hoffer B (1981) Chronic ethanol consumption and hippocampal neuron dendritic spines: a morphometric and physiological analysis. Exp Neurol 71(3):541-9.

Lee Y, Davis M (1997) Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex. J Neurosci 17(16):6434-46.

Lescaudron L, Jaffard R, Verna A (1989) Modifications in number and morphology of dendritic spines resulting from chronic ethanol consumption and withdrawal: a Golgi study in the mouse anterior and posterior hippocampus. Exp Neurol 106(2):156-63.

Lester D, Freed EX (1973) Criteria for an animal model of alcoholism. Pharmacol Biochem Behav 1(1):103-7.

Levenson JM, O'Riordan KJ, Brown KD, Trinh MA, Molfese DL, Sweatt JD (2004) Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem 279(39):40545-59.

Levenson JM, Sweatt JD (2005) Epigenetic mechanisms in memory formation. Nat Rev Neurosci 6(2):108-18.

Li H, Zhong X, Chau KF, Williams EC, Chang Q (2011) Loss of activity-induced phosphorylation of MeCP2 enhances synaptogenesis, LTP and spatial memory. Nat Neurosci 14(8):1001-8.

Li J, Bian WL, Xie GQ, Cui SZ, Wu ML, Li YH, Que LL, Yuan XR (2008) Chronic ethanol intake-induced changes in open-field behavior and calcium/calmodulin-dependent protein kinase IV expression in nucleus accumbens of rats: naloxone reversal. Acta Pharmacol Sin 29(6):646-52.

Li TK, Lumeng L, Doolittle DP (1993) Selective breeding for alcohol preference and associated responses. Behav Genet 23(2):163-70.

Lipscomb TR, Nathan PE, Wilson GT, Abrams DB (1980) Effects of tolerance on the anxiety-reducing function of alcohol. Arch Gen Psychiatry 37(5):577-82.

Liu J, Asuncion-Chin M, Liu P, Dopico AM (2006) CaM kinase II phosphorylation of slo Thr107 regulates activity and ethanol responses of BK channels. Nat Neurosci 9(1):41-9.

Logrip ML, Janak PH, Ron D (2008) Dynorphin is a downstream effector of striatal BDNF regulation of ethanol intake. FASEB J 22(7):2393-404.

Lovinger DM, White G, Weight FF (1989) Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science 243(4899):1721-4.

Lowman C, Allen J, Stout RL (1996) Replication and extension of Marlatt's taxonomy of relapse precipitants: overview of procedures and results. The Relapse Research Group. Addiction 91 Suppl:S51-71.

Lubin FD, Roth TL, Sweatt JD (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J Neurosci 28(42):10576-86.

Luiten PG, Koolhaas JM, de Boer S, Koopmans SJ (1985) The cortico-medial amygdala in the central nervous system organization of agonistic behavior. Brain Res 332(2):283-97.

Lutz HF (1922) *Viticulture and brewing in the ancient Orient.* J. C. Hinrichs; New York city, G. E. Stechert & co., Leipzig,.

Machu TK, Firestone JA, Browning MD (1993) Ca2+/calmodulin-dependent protein kinase II and protein kinase C phosphorylate a synthetic peptide corresponding to a sequence that is specific for the gamma 2L subunit of the GABAA receptor. J Neurochem 61(1):375-7.

Machu TK, Olsen RW, Browning MD (1991) Ethanol has no effect on cAMP-dependent protein kinase-, protein kinase C-, or Ca(2+)-calmodulin-dependent protein kinase II-stimulated phosphorylation of highly purified substrates in vitro. Alcohol Clin Exp Res 15(6):1040-4.

MacLennan AJ, Lee N, Walker DW (1995) Chronic ethanol administration decreases brain-derived neurotrophic factor gene expression in the rat hippocampus. Neurosci Lett 197(2):105-8.

Mahadev K, Chetty CS, Vemuri MC (2001) Effect of prenatal and postnatal ethanol exposure on Ca2+ /calmodulin-dependent protein kinase II in rat cerebral cortex. Alcohol 23(3):183-8.

Majdzadeh N, Wang L, Morrison BE, Bassel-Duby R, Olson EN, D'Mello SR (2008) HDAC4 inhibits cell-cycle progression and protects neurons from cell death. Dev Neurobiol 68(8):1076-92.

Maren S, Quirk GJ (2004) Neuronal signalling of fear memory. Nat Rev Neurosci 5(11):844-52.

Marquez VE, Kelley JA, Agbaria R, Ben-Kasus T, Cheng JC, Yoo CB, Jones PA (2005) Zebularine: a unique molecule for an epigenetically based strategy in cancer chemotherapy. Ann N Y Acad Sci 1058:246-54.

Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y, Fan G, Sun YE (2003) DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. Science 302(5646):890-3.

Mascagni F, McDonald AJ, Coleman JR (1993) Corticoamygdaloid and corticocortical projections of the rat temporal cortex: a Phaseolus vulgaris leucoagglutinin study. Neuroscience 57(3):697-715.

Matsushita S, Kimura M, Miyakawa T, Yoshino A, Murayama M, Masaki T, Higuchi S (2004) Association study of brain-derived neurotrophic factor gene polymorphism and alcoholism. Alcohol Clin Exp Res 28(11):1609-12.

Mayfield RD, Harris RA, Schuckit MA (2008) Genetic factors influencing alcohol dependence. Br J Pharmacol 154(2):275-87.

McDonald AJ (1982) Neurons of the lateral and basolateral amygdaloid nuclei: a Golgi study in the rat. J Comp Neurol 212(3):293-312.

McDonald AJ (1984) Neuronal organization of the lateral and basolateral amygdaloid nuclei in the rat. J Comp Neurol 222(4):589-606.

McDonald AJ (1998) Cortical pathways to the mammalian amygdala. Prog Neurobiol 55(3):257-332.

McDonald AJ, Shammah-Lagnado SJ, Shi C, Davis M (1999) Cortical afferents to the extended amygdala. Ann N Y Acad Sci 877:309-38.

McEwen BS (2008) Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. Eur J Pharmacol 583(2-3):174-85.

McEwen BS, Gianaros P (2010) Plasticity of the Brain in Relationship to Stress. Annu Rev Med.

McGough NN, He DY, Logrip ML, Jeanblanc J, Phamluong K, Luong K, Kharazia V, Janak PH, Ron D (2004) RACK1 and brain-derived neurotrophic factor: a homeostatic pathway that regulates alcohol addiction. J Neurosci 24(46):10542-52.

McIntyre CK, Miyashita T, Setlow B, Marjon KD, Steward O, Guzowski JF, McGaugh JL (2005) Memory-influencing intra-basolateral amygdala drug infusions

modulate expression of Arc protein in the hippocampus. Proc Natl Acad Sci U S A 102(30):10718-23.

McKinzie DL, Sajdyk TJ, McBride WJ, Murphy JM, Lumeng L, Li TK, Shekhar A (2000) Acoustic startle and fear-potentiated startle in alcohol-preferring (P) and -nonpreferring (NP) lines of rats. Pharmacol Biochem Behav 65(4):691-6.

McMillen BA, Crawford MS, Kulers CM, Williams HL (2005) Effects of a metabotropic, mglu5, glutamate receptor antagonist on ethanol consumption by genetic drinking rats. Alcohol Alcohol 40(6):494-7.

McQuown SC, Barrett RM, Matheos DP, Post RJ, Rogge GA, Alenghat T, Mullican SE, Jones S, Rusche JR, Lazar MA, Wood MA (2011) HDAC3 is a critical negative regulator of long-term memory formation. J Neurosci 31(2):764-74.

Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z (2002) Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron 35(1):121-33.

Messaoudi E, Kanhema T, Soule J, Tiron A, Dagyte G, da Silva B, Bramham CR (2007) Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. J Neurosci 27(39):10445-55.

Messaoudi E, Ying SW, Kanhema T, Croll SD, Bramham CR (2002) Brain-derived neurotrophic factor triggers transcription-dependent, late phase long-term potentiation in vivo. J Neurosci 22(17):7453-61.

Metivier R, Gallais C, Tiffoche C, Le Peron C, Jurkowska RZ, Carmouche RP, Ibberson D, Barath P, Demay F, Reid G, Benes V, Jeltsch A, Gannon F, Salbert G (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature 452(7183):45-50.

Mielcarek M, Benn CL, Franklin SA, Smith DL, Woodman B, Marks PA, Bates GP (2011) SAHA decreases HDAC 2 and 4 levels in vivo and improves molecular phenotypes in the R6/2 mouse model of Huntington's disease. PLoS One 6(11):e27746.

Millhouse OE, DeOlmos J (1983) Neuronal configurations in lateral and basolateral amygdala. Neuroscience 10(4):1269-300.

Minami K, Gereau RWt, Minami M, Heinemann SF, Harris RA (1998) Effects of ethanol and anesthetics on type 1 and 5 metabotropic glutamate receptors expressed in Xenopus laevis oocytes. Mol Pharmacol 53(1):148-56.

Minichiello L (2009) TrkB signalling pathways in LTP and learning. Nat Rev Neurosci 10(12):850-60.

Misra K, Pandey SC (2003) Differences in basal levels of CREB and NPY in nucleus accumbens regions between C57BL/6 and DBA/2 mice differing in inborn alcohol drinking behavior. J Neurosci Res 74(6):967-75.

Misra K, Pandey SC (2006) The decreased cyclic-AMP dependent-protein kinase A function in the nucleus accumbens: a role in alcohol drinking but not in anxiety-like behaviors in rats. Neuropsychopharmacology 31(7):1406-19.

Misra K, Roy A, Pandey SC (2001) Effects of voluntary ethanol intake on the expression of Ca(2+) /calmodulin-dependent protein kinase IV and on CREB expression and phosphorylation in the rat nucleus accumbens. Neuroreport 12(18):4133-7.

Misslin R (2003) The defense system of fear: behavior and neurocircuitry. Neurophysiol Clin 33(2):55-66.

Mitchell JJ, Paiva M, Walker DW, Heaton MB (1999) BDNF and NGF afford in vitro neuroprotection against ethanol combined with acute ischemia and chronic hypoglycemia. Dev Neurosci 21(1):68-75.

Mochly-Rosen D, Chang FH, Cheever L, Kim M, Diamond I, Gordon AS (1988) Chronic ethanol causes heterologous desensitization of receptors by reducing alpha s messenger RNA. Nature 333(6176):848-50.

Molfese DL (2011) Advancing neuroscience through epigenetics: molecular mechanisms of learning and memory. Dev Neuropsychol 36(7):810-27.

Monti B, Berteotti C, Contestabile A (2006) Subchronic rolipram delivery activates hippocampal CREB and arc, enhances retention and slows down extinction of conditioned fear. Neuropsychopharmacology 31(2):278-86.

Moonat S, Pandey SC (2012) Stress, Epigenetics, and Alcoholism. Alcohol Res 34(4):495-505.

Moonat S, Sakharkar AJ, Zhang H, Pandey SC (2011) The role of amygdaloid brain-derived neurotrophic factor, activity-regulated cytoskeleton-associated protein and dendritic spines in anxiety and alcoholism. Addict Biol 16(2):238-50.

Moonat S, Starkman BG, Sakharkar A, Pandey SC (2010) Neuroscience of alcoholism: molecular and cellular mechanisms. Cell Mol Life Sci 67(1):73-88.

Morgan MA, Romanski LM, LeDoux JE (1993) Extinction of emotional learning: contribution of medial prefrontal cortex. Neurosci Lett 163(1):109-13.

Mullikin-Kilpatrick D, Mehta ND, Hildebrandt JD, Treistman SN (1995) Gi is involved in ethanol inhibition of L-type calcium channels in undifferentiated but not differentiated PC-12 cells. Mol Pharmacol 47(5):997-1005.

Nagy J (2004) The NR2B subtype of NMDA receptor: a potential target for the treatment of alcohol dependence. Curr Drug Targets CNS Neurol Disord 3(3):169-79.

Nakamura K, Mikami A, Kubota K (1992) Activity of single neurons in the monkey amygdala during performance of a visual discrimination task. J Neurophysiol 67(6):1447-63.

Nakazawa T, Kuriu T, Tezuka T, Umemori H, Okabe S, Yamamoto T (2008a) Regulation of dendritic spine morphology by an NMDA receptor-associated Rho GTPase-activating protein, p250GAP. J Neurochem 105(4):1384-93.

Nakazawa T, Kuriu T, Tezuka T, Umemori H, Okabe S, Yamamoto T (2008b) Regulation of dendritic spine morphology by an NMDA receptor-associated Rho GTPase-activating protein, p250GAP. J Neurochem 105(4):1384-93.

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393(6683):386-9.

Narahashi T, Kuriyama K, Illes P, Wirkner K, Fischer W, Muhlberg K, Scheibler P, Allgaier C, Minami K, Lovinger D, Lallemand F, Ward RJ, DeWitte P, Itatsu T, Takei Y, Oide H, Hirose M, Wang XE, Watanabe S, Tateyama M, Ochi R, Sato N (2001) Neuroreceptors and ion channels as targets of alcohol. Alcohol Clin Exp Res 25(5 Suppl ISBRA):182S-188S.

Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci 2(2):119-28.

Nestler EJ (2009) Epigenetic mechanisms in psychiatry. Biol Psychiatry 65(3):189-90.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002) Neurobiology of depression. Neuron 34(1):13-25.

Nestoros JN (1980) Ethanol specifically potentiates GABA-mediated neurotransmission in feline cerebral cortex. Science 209(4457):708-10.

Netzeband JG, Gruol DL (1995) Modulatory effects of acute ethanol on metabotropic glutamate responses in cultured Purkinje neurons. Brain Res 688(1-2):105-13.

Nibuya M, Morinobu S, Duman RS (1995) Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. J Neurosci 15(11):7539-47.

Nie Z, Madamba SG, Siggins GR (1994) Ethanol inhibits glutamatergic neurotransmission in nucleus accumbens neurons by multiple mechanisms. J Pharmacol Exp Ther 271(3):1566-73.

Nilsson KW, Comasco E, Aslund C, Nordquist N, Leppert J, Oreland L (2011) MAOA genotype, family relations and sexual abuse in relation to adolescent alcohol consumption. Addict Biol 16(2):347-55.

Ninan I, Bath KG, Dagar K, Perez-Castro R, Plummer MR, Lee FS, Chao MV (2010) The BDNF Val66Met polymorphism impairs NMDA receptor-dependent synaptic plasticity in the hippocampus. J Neurosci 30(26):8866-70.

Nott A, Watson PM, Robinson JD, Crepaldi L, Riccio A (2008) S-Nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. Nature 455(7211):411-5.

Novak A, Burgess ES, Clark M, Zvolensky MJ, Brown RA (2003) Anxiety sensitivity, self-reported motives for alcohol and nicotine use, and level of consumption. J Anxiety Disord 17(2):165-80.

O'Brien RJ, Lau LF, Huganir RL (1998) Molecular mechanisms of glutamate receptor clustering at excitatory synapses. Curr Opin Neurobiol 8(3):364-9.

Oades RD, Halliday GM (1987) Ventral tegmental (A10) system: neurobiology. 1. Anatomy and connectivity. Brain Res 434(2):117-65.

Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99(3):247-257.

Olive MF, McGeehan AJ, Kinder JR, McMahon T, Hodge CW, Janak PH, Messing RO (2005) The mGluR5 antagonist 6-methyl-2-(phenylethynyl)pyridine decreases ethanol consumption via a protein kinase C epsilon-dependent mechanism. Mol Pharmacol 67(2):349-55.

Ooi SKT, Bestor TH (2008) The colorful history of active DNA demethylation. Cell 133(7):1145-1148.

Overstreet DH, Knapp DJ, Breese GR (2007) Drug challenges reveal differences in mediation of stress facilitation of voluntary alcohol drinking and withdrawal-induced anxiety in alcohol-preferring P rats. Alcohol Clin Exp Res 31(9):1473-81.

Pak DT, Yang S, Rudolph-Correia S, Kim E, Sheng M (2001) Regulation of dendritic spine morphology by SPAR, a PSD-95-associated RapGAP. Neuron 31(2):289-303.

Pandey SC (2003) Anxiety and alcohol abuse disorders: a common role for CREB and its target, the neuropeptide Y gene. Trends Pharmacol Sci 24(9):456-60.

Pandey SC (2004) The gene transcription factor cyclic AMP-responsive element binding protein: role in positive and negative affective states of alcohol addiction. Pharmacol Ther 104(1):47-58.

Pandey SC, Carr LG, Heilig M, Ilveskoski E, Thiele TE (2003a) Neuropeptide y and alcoholism: genetic, molecular, and pharmacological evidence. Alcohol Clin Exp Res 27(2):149-54.

Pandey SC, Mittal N, Lumeng L, Li TK (1999a) Involvement of the cyclic AMP-responsive element binding protein gene transcription factor in genetic preference for alcohol drinking behavior. Alcohol Clin Exp Res 23(9):1425-34.

Pandey SC, Pandey RK, Bhatnagar SK, Pradhan KL, Pradhan R, Chandra S (2005a) Archform in cleft palate--a computerized tomographic classification. J Clin Pediatr Dent 30(2):131-3.

Pandey SC, Roy A, Mittal N (2001) Effects of chronic ethanol intake and its withdrawal on the expression and phosphorylation of the creb gene transcription factor in rat cortex. J Pharmacol Exp Ther 296(3):857-68.

Pandey SC, Roy A, Zhang H (2003b) The decreased phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein in the central amygdala acts as a molecular substrate for anxiety related to ethanol withdrawal in rats. Alcohol Clin Exp Res 27(3):396-409.

Pandey SC, Roy A, Zhang H, Xu T (2004) Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors. J Neurosci 24(21):5022-30.

Pandey SC, Ugale R, Zhang H, Tang L, Prakash A (2008a) Brain chromatin remodeling: a novel mechanism of alcoholism. J Neurosci 28(14):3729-37.

Pandey SC, Zhang D, Mittal N, Nayyar D (1999b) Potential role of the gene transcription factor cyclic AMP-responsive element binding protein in ethanol withdrawal-related anxiety. J Pharmacol Exp Ther 288(2):866-78.

Pandey SC, Zhang H, Roy A, Misra K (2006) Central and medial amygdaloid brain-derived neurotrophic factor signaling plays a critical role in alcohol-drinking and anxiety-like behaviors. J Neurosci 26(32):8320-31.

Pandey SC, Zhang H, Roy A, Xu T (2005b) Deficits in amygdaloid cAMP-responsive element-binding protein signaling play a role in genetic predisposition to anxiety and alcoholism. J Clin Invest 115(10):2762-73.

Pandey SC, Zhang H, Ugale R, Prakash A, Xu T, Misra K (2008b) Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. J Neurosci 28(10):2589-600.

Pang PT, Lu B (2004) Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. Ageing Res Rev 3(4):407-30.

Paoletti P, Neyton J (2007) NMDA receptor subunits: function and pharmacology. Curr Opin Pharmacol 7(1):39-47.

Papez JW (1995) A proposed mechanism of emotion. 1937. J Neuropsychiatry Clin Neurosci 7(1):103-12.

Pare D, Quirk GJ, Ledoux JE (2004) New vistas on amygdala networks in conditioned fear. J Neurophysiol 92(1):1-9.

Park S, Park JM, Kim S, Kim JA, Shepherd JD, Smith-Hicks CL, Chowdhury S, Kaufmann W, Kuhl D, Ryazanov AG, Huganir RL, Linden DJ, Worley PF (2008) Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3.1 essential for mGluR-LTD. Neuron 59(1):70-83.

Paxinos G, Watson C (2007) *The rat brain in stereotaxic coordinates.* 6th ed. Academic Press/Elsevier, Amsterdam ; Boston ;.

Peoples RW, Weight FF (1995) Cutoff in potency implicates alcohol inhibition of N-methyl-D-aspartate receptors in alcohol intoxication. Proc Natl Acad Sci U S A 92(7):2825-9.

Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci 106(2):274-85.

Pintchovski SA, Peebles CL, Kim HJ, Verdin E, Finkbeiner S (2009) The serum response factor and a putative novel transcription factor regulate expression of the immediate-early gene Arc/Arg3.1 in neurons. J Neurosci 29(5):1525-37.

Pitkanen A, Kemppainen S (2002) Comparison of the distribution of calcium-binding proteins and intrinsic connectivity in the lateral nucleus of the rat, monkey, and human amygdala. Pharmacol Biochem Behav 71(3):369-77.

Pizarro JM, Lumley LA, Medina W, Robison CL, Chang WE, Alagappan A, Bah MJ, Dawood MY, Shah JD, Mark B, Kendall N, Smith MA, Saviolakis GA,

Meyerhoff JL (2004) Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. Brain Res 1025(1-2):10-20.

Pizzorusso T, Ratto GM, Putignano E, Maffei L (2000) Brain-derived neurotrophic factor causes cAMP response element-binding protein phosphorylation in absence of calcium increases in slices and cultured neurons from rat visual cortex. J Neurosci 20(8):2809-16.

Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, Mao X, Engelsberg A, Mahlke C, Welzl H, Kobalz U, Stawrakakis A, Fernandez E, Waltereit R, Bick-Sander A, Therstappen E, Cooke SF, Blanquet V, Wurst W, Salmen B, Bosl MR, Lipp HP, Grant SG, Bliss TV, Wolfer DP, Kuhl D (2006) Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. Neuron 52(3):437-44.

Podhorna J, Brown RE (2002) Strain differences in activity and emotionality do not account for differences in learning and memory performance between C57BL/6 and DBA/2 mice. Genes Brain Behav 1(2):96-110.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2(1):24-32.

Prakash A, Zhang H, Pandey SC (2008) Innate differences in the expression of brain-derived neurotrophic factor in the regions within the extended amygdala between alcohol preferring and nonpreferring rats. Alcohol Clin Exp Res 32(6):909-20.

Prescott CA, Kendler KS (1999) Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. Am J Psychiatry 156(1):34-40.

Ramanan N, Shen Y, Sarsfield S, Lemberger T, Schutz G, Linden DJ, Ginty DD (2005) SRF mediates activity-induced gene expression and synaptic plasticity but not neuronal viability. Nat Neurosci 8(6):759-67.

Rehm J, Mathers C, Popova S, Thavorncharoensap M, Teerawattananon Y, Patra J (2009) Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. Lancet 373(9682):2223-33.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361(1473):1545-64.

Ren H, Salous AK, Paul JM, Lipsky RH, Peoples RW (2007) Mutations at F637 in the NMDA receptor NR2A subunit M3 domain influence agonist potency, ion channel gating and alcohol action. Br J Pharmacol 151(6):749-57.

Renthal W, Nestler EJ (2008) Epigenetic mechanisms in drug addiction. Trends Mol Med 14(8):341-50.

Repunte-Canonigo V, Lutjens R, van der Stap LD, Sanna PP (2007) Increased expression of protein kinase A inhibitor alpha (PKI-alpha) and decreased PKA-regulated genes in chronic intermittent alcohol exposure. Brain Res 1138:48-56.

Rezvani AH, Janowsky DS (1990) Decreased alcohol consumption by verapamil in alcohol preferring rats. Prog Neuropsychopharmacol Biol Psychiatry 14(4):623-31.

Rezvani AH, Overstreet DH, Janowsky DS (1991) Drug-induced reductions in ethanol intake in alcohol preferring and Fawn-Hooded rats. Alcohol Alcohol Suppl 1:433-7.

Rezvani AH, Overstreet DH, Levin ED, Rosenthal DI, Kordik CP, Reitz AB, Vaidya AH (2007) Effects of atypical anxiolytic N-phenyl-2-[1-[3-(2-pyridinylethynyl)benzoyl]-4-piperidine]acetamide (JNJ-5234801) on alcohol intake in alcohol-preferring P rats. Alcohol Clin Exp Res 31(1):57-63.

Riley JN, Walker DW (1978) Morphological alterations in hippocampus after long-term alcohol consumption in mice. Science 201(4356):646-8.

Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, Lechan RM, Jaenisch R (2001) Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. Mol Endocrinol 15(10):1748-57.

Roberto M, Bajo M, Crawford E, Madamba SG, Siggins GR (2006) Chronic ethanol exposure and protracted abstinence alter NMDA receptors in central amygdala. Neuropsychopharmacology 31(5):988-96.

Roberto M, Nelson TE, Ur CL, Brunelli M, Sanna PP, Gruol DL (2003) The transient depression of hippocampal CA1 LTP induced by chronic intermittent ethanol exposure is associated with an inhibition of the MAP kinase pathway. Eur J Neurosci 17(8):1646-54.

Roberto M, Schweitzer P, Madamba SG, Stouffer DG, Parsons LH, Siggins GR (2004) Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an in vitro and in vivo analysis. J Neurosci 24(7):1594-603.

Robertson KD (2005) DNA methylation and human disease. Nat Rev Genet 6:597-610.

Robinson J, Sareen J, Cox BJ, Bolton J (2009) Self-medication of anxiety disorders with alcohol and drugs: Results from a nationally representative sample. J Anxiety Disord 23(1):38-45.

Robison AJ, Nestler EJ (2011) Transcriptional and epigenetic mechanisms of addiction. Nat Rev Neurosci 12(11):623-37.

Rodd-Henricks ZA, McKinzie DL, Shaikh SR, Murphy JM, McBride WJ, Lumeng L, Li TK (2000) Alcohol deprivation effect is prolonged in the alcohol preferring (P) rat after repeated deprivations. Alcohol Clin Exp Res 24(1):8-16.

Romanski LM, LeDoux JE (1992) Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. J Neurosci 12(11):4501-9.

Ron D, Jurd R (2005) The "ups and downs" of signaling cascades in addiction. Sci STKE 2005(309):re14.

Ronald KM, Mirshahi T, Woodward JJ (2001) Ethanol inhibition of N-methyl-D-aspartate receptors is reduced by site-directed mutagenesis of a transmembrane domain phenylalanine residue. J Biol Chem 276(48):44729-35.

Rosenfeld JA, Wang Z, Schones DE, Zhao K, DeSalle R, Zhang MQ (2009) Determination of enriched histone modifications in non-genic portions of the human genome. BMC Genomics 10:143.

Ross MH, Pawlina W (2006) *Histology : a text and atlas : with correlated cell and molecular biology.* 5th ed. Lippincott Wiliams & Wilkins, Baltimore, MD.

Roth TL, Lubin FD, Funk AJ, Sweatt JD (2009) Lasting epigenetic influence of early-life adversity on the BDNF gene. Biol Psychiatry 65(9):760-9.

Roth TL, Zoladz PR, Sweatt JD, Diamond DM (2011) Epigenetic modification of hippocampal Bdnf DNA in adult rats in an animal model of post-traumatic stress disorder. J Psychiatr Res 45(7):919-26.

Sadri-Vakili G, Kumaresan V, Schmidt HD, Famous KR, Chawla P, Vassoler FM, Overland RP, Xia E, Bass CE, Terwilliger EF, Pierce RC, Cha JH (2010) Cocaine-induced chromatin remodeling increases brain-derived neurotrophic factor transcription in the rat medial prefrontal cortex, which alters the reinforcing efficacy of cocaine. J Neurosci 30(35):11735-44.

Sah P, Faber ES, Lopez De Armentia M, Power J (2003) The amygdaloid complex: anatomy and physiology. Physiol Rev 83(3):803-34.

Saha S (2005) Role of the central nucleus of the amygdala in the control of blood pressure: descending pathways to medullary cardiovascular nuclei. Clin Exp Pharmacol Physiol 32(5-6):450-6.

Sakai R, Ukai W, Sohma H, Hashimoto E, Yamamoto M, Ikeda H, Saito T (2005) Attenuation of brain derived neurotrophic factor (BDNF) by ethanol and cytoprotective effect of exogenous BDNF against ethanol damage in neuronal cells. J Neural Transm 112(8):1005-13.

Sakata K, Woo NH, Martinowich K, Greene JS, Schloesser RJ, Shen L, Lu B (2009) Critical role of promoter IV-driven BDNF transcription in GABAergic transmission and synaptic plasticity in the prefrontal cortex. Proc Natl Acad Sci U S A 106(14):5942-7.

Sakharkar AJ, Zhang H, Tang L, Shi G, Pandey SC (2012) Histone deacetylases (HDAC)-induced histone modifications in the amygdala: a role in rapid tolerance to the anxiolytic effects of ethanol. Alcohol Clin Exp Res 36(1):61-71.

Sales AJ, Biojone C, Terceti MS, Guimaraes FS, Gomes MV, Joca SR (2011) Antidepressant-like effect induced by systemic and intra-hippocampal administration of DNA methylation inhibitors. Br J Pharmacol 164(6):1711-21.

Samaco RC, Neul JL (2011) Complexities of Rett syndrome and MeCP2. J Neurosci 31(22):7951-9.

Samson RD, Pare D (2005) Activity-dependent synaptic plasticity in the central nucleus of the amygdala. J Neurosci 25(7):1847-55.

Sanna PP, Simpson C, Lutjens R, Koob G (2002) ERK regulation in chronic ethanol exposure and withdrawal. Brain Res 948(1-2):186-91.

Savander V, Miettinen R, Ledoux JE, Pitkanen A (1997) Lateral nucleus of the rat amygdala is reciprocally connected with basal and accessory basal nuclei: a light and electron microscopic study. Neuroscience 77(3):767-81.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3(6):1101-8.

Schroeder JP, Overstreet DH, Hodge CW (2005) The mGluR5 antagonist MPEP decreases operant ethanol self-administration during maintenance and after repeated alcohol deprivations in alcohol-preferring (P) rats. Psychopharmacology (Berl) 179(1):262-70.

Schuckit MA (2000) Genetics of the risk for alcoholism. Am J Addict 9(2):103-12.

Schuckit MA, Hesselbrock V (1994) Alcohol dependence and anxiety disorders: what is the relationship? Am J Psychiatry 151(12):1723-34.

Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, Nestler EJ (1998) Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. J Neurosci 18(5):1848-59.

Sharma RP, Gavin DP, Grayson DR (2010) CpG methylation in neurons: message, memory, or mask? Neuropsychopharmacology 35:2009-2020.

Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821-61.

Shi CJ, Cassell MD (1998) Cortical, thalamic, and amygdaloid connections of the anterior and posterior insular cortices. J Comp Neurol 399(4):440-68.

Shi CJ, Cassell MD (1999) Perirhinal cortex projections to the amygdaloid complex and hippocampal formation in the rat. J Comp Neurol 406(3):299-328.

Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J Neurosci 22(8):3251-61.

Siegel GJ (2006) *Basic neurochemistry : molecular, cellular, and medical aspects.* 7th ed. Elsevier, Amsterdam ; Boston.

Smith MA, Makino S, Kvetnansky R, Post RM (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J Neurosci 15(3 Pt 1):1768-77.

Smith MM (1991) Histone structure and function. Curr Opin Cell Biol 3(3):429-37.

Smothers CT, Clayton R, Blevins T, Woodward JJ (2001) Ethanol sensitivity of recombinant human N-methyl-D-aspartate receptors. Neurochem Int 38(4):333-40.

Smothers CT, Mrotek JJ, Lovinger DM (1997) Chronic ethanol exposure leads to a selective enhancement of N-methyl-D-aspartate receptor function in cultured hippocampal neurons. J Pharmacol Exp Ther 283(3):1214-22.

Soule J, Messaoudi E, Bramham CR (2006) Brain-derived neurotrophic factor and control of synaptic consolidation in the adult brain. Biochem Soc Trans 34(Pt 4):600-4.

Spanagel R (2000) Recent animal models of alcoholism. Alcohol Res Health 24(2):124-31.

Spanagel R (2009) Alcoholism: a systems approach from molecular physiology to addictive behavior. Physiol Rev 89(2):649-705.

Steward O, Wallace CS, Lyford GL, Worley PF (1998) Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. Neuron 21(4):741-51.

Steward O, Worley PF (2001) A cellular mechanism for targeting newly synthesized mRNAs to synaptic sites on dendrites. Proc Natl Acad Sci U S A 98(13):7062-8.

Stewart RB, Gatto GJ, Lumeng L, Li TK, Murphy JM (1993) Comparison of alcohol-preferring (P) and nonpreferring (NP) rats on tests of anxiety and for the anxiolytic effects of ethanol. Alcohol 10(1):1-10.

Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403(6765):41-5.

Su D, Cha YM, West AE (2012) Mutation of MeCP2 alters transcriptional regulation of select immediate-early genes. Epigenetics 7(2):146-54.

Su M, Shi JJ, Yang YP, Li J, Zhang YL, Chen J, Hu LF, Liu CF (2011) HDAC6 regulates aggresome-autophagy degradation pathway of alpha-synuclein in response to MPP+-induced stress. J Neurochem 117(1):112-20.

Sunahara RK, Dessauer CW, Gilman AG (1996) Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol 36:461-80.

Swanson LW, Petrovich GD (1998) What is the amygdala? Trends Neurosci 21(8):323-31.

Szabo G, Hoffman PL (1995) Brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5 maintain functional tolerance to ethanol. Eur J Pharmacol 287(1):35-41.

Tabakoff B, Whelan JP, Ovchinnikova L, Nhamburo P, Yoshimura M, Hoffman PL (1995) Quantitative changes in G proteins do not mediate ethanol-induced downregulation of adenylyl cyclase in mouse cerebral cortex. Alcohol Clin Exp Res 19(1):187-94.

Tada T, Sheng M (2006) Molecular mechanisms of dendritic spine morphogenesis. Curr Opin Neurobiol 16(1):95-101.

Taiwo O, Wilson GA, Morris T, Seisenberger S, Reik W, Pearce D, Beck S, Butcher LM (2012) Methylome analysis using MeDIP-seq with low DNA concentrations. Nat Protoc 7(4):617-36.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20(4):709-26.

Tateyama M, Kubo Y (2006) Dual signaling is differentially activated by different active states of the metabotropic glutamate receptor 1alpha. Proc Natl Acad Sci U S A 103(4):1124-8.

Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci 983:84-100.

Thiele TE, Willis B, Stadler J, Reynolds JG, Bernstein IL, McKnight GS (2000) High ethanol consumption and low sensitivity to ethanol-induced sedation in protein kinase A-mutant mice. J Neurosci 20(10):RC75.

Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5(3):173-83.

Tsankova N, Renthal W, Kumar A, Nestler EJ (2007a) Epigenetic regulation in psychiatric disorders. Nat Rev Neurosci 8(5):355-67.

Tsankova N, Renthal W, Kumar A, Nestler EJ (2007b) Epigenetic regulation in psychiatric disorders. Nat Rev Neurosci 8(5):355-67.

Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci 9(4):519-25.

Tsankova NM, Kumar A, Nestler EJ (2004) Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. J Neurosci 24(24):5603-10.

Turner BH, Mishkin M, Knapp M (1980) Organization of the amygdalopetal projections from modality-specific cortical association areas in the monkey. J Comp Neurol 191(4):515-43.

Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature 471(7338):358-62.

Uhl GR, Liu QR, Walther D, Hess J, Naiman D (2001) Polysubstance abuse-vulnerability genes: genome scans for association, using 1,004 subjects and 1,494 single-nucleotide polymorphisms. Am J Hum Genet 69(6):1290-300.

Ultanir SK, Kim JE, Hall BJ, Deerinck T, Ellisman M, Ghosh A (2007) Regulation of spine morphology and spine density by NMDA receptor signaling in vivo. Proc Natl Acad Sci U S A 104(49):19553-8.

Valentiner DP, Mounts NS, Deacon BJ (2004) Panic attacks, depression and anxiety symptoms, and substance use behaviors during late adolescence. J Anxiety Disord 18(5):573-85.

Vecsey CG, Hawk JD, Lattal KM, Stein JM, Fabian SA, Attner MA, Cabrera SM, McDonough CB, Brindle PK, Abel T, Wood MA (2007) Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. J Neurosci 27(23):6128-40.

Vengeliene V, Bilbao A, Molander A, Spanagel R (2008) Neuropharmacology of alcohol addiction. Br J Pharmacol 154(2):299-315.

Vickers CA, Stephens B, Bowen J, Arbuthnott GW, Grant SG, Ingham CA (2006) Neurone specific regulation of dendritic spines in vivo by post synaptic density 95 protein (PSD-95). Brain Res 1090(1):89-98.

Waddington CH (1942) The Epigenotype. International Journal of Epidemiology.

Waddington CH (1957) *The strategy of the genes; a discussion of some aspects of theoretical biology.* Allen & Unwin, London,.

Walker DL, Davis M (1997) Anxiogenic effects of high illumination levels assessed with the acoustic startle response in rats. Biol Psychiatry 42(6):461-71.

Walker DL, Davis M (2002) Light-enhanced startle: further pharmacological and behavioral characterization. Psychopharmacology (Berl) 159(3):304-10.

Walker DL, Toufexis DJ, Davis M (2003) Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. Eur J Pharmacol 463(1-3):199-216.

Walter HJ, McMahon T, Dadgar J, Wang D, Messing RO (2000) Ethanol regulates calcium channel subunits by protein kinase C delta -dependent and -independent mechanisms. J Biol Chem 275(33):25717-22.

Waltereit R, Dammermann B, Wulff P, Scafidi J, Staubli U, Kauselmann G, Bundman M, Kuhl D (2001) Arg3.1/Arc mRNA induction by Ca2+ and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. J Neurosci 21(15):5484-93.

Wang JQ, Fibuch EE, Mao L (2007) Regulation of mitogen-activated protein kinases by glutamate receptors. J Neurochem 100(1):1-11.

Wang Y, Ghezzi A, Yin JC, Atkinson NS (2009) CREB regulation of BK channel gene expression underlies rapid drug tolerance. Genes Brain Behav.

Watanabe Y, Gould E, McEwen BS (1992) Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. Brain Res 588(2):341-5.

Wayman GA, Davare M, Ando H, Fortin D, Varlamova O, Cheng HY, Marks D, Obrietan K, Soderling TR, Goodman RH, Impey S (2008a) An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. Proc Natl Acad Sci U S A 105(26):9093-8.

Wayman GA, Lee YS, Tokumitsu H, Silva A, Soderling TR (2008b) Calmodulin-kinases: modulators of neuronal development and plasticity. Neuron 59(6):914-31.

Weiskrantz L (1956) Behavioral changes associated with ablation of the amygdaloid complex in monkeys. J Comp Physiol Psychol 49(4):381-91.

West AE (2008) Biological functions of activity-dependent transcription revealed. Neuron 60(4):523-5.

Widnell KL, Self DW, Lane SB, Russell DS, Vaidya VA, Miserendino MJ, Rubin CS, Duman RS, Nestler EJ (1996) Regulation of CREB expression: in vivo evidence for a functional role in morphine action in the nucleus accumbens. J Pharmacol Exp Ther 276(1):306-15.

Wilson GT (1988) Alcohol and anxiety. Behav Res Ther 26(5):369-81.

Wise RA (2004) Dopamine, learning and motivation. Nat Rev Neurosci 5(6):483-94.

Xie GQ, Wang SJ, Li J, Cui SZ, Zhou R, Chen L, Yuan XR (2009) Ethanol attenuates the HFS-induced, ERK-mediated LTP in a dose-dependent manner in rat striatum. Alcohol Clin Exp Res 33(1):121-8.

Yang X, Diehl AM, Wand GS (1996) Ethanol exposure alters the phosphorylation of cyclic AMP responsive element binding protein and cyclic AMP responsive element binding activity in rat cerebellum. J Pharmacol Exp Ther 278(1):338-46.

Yang X, Horn K, Baraban JM, Wand GS (1998a) Chronic ethanol administration decreases phosphorylation of cyclic AMP response element-binding protein in granule cells of rat cerebellum. J Neurochem 70(1):224-32.

Yang X, Horn K, Wand GS (1998b) Chronic ethanol exposure impairs phosphorylation of CREB and CRE-binding activity in rat striatum. Alcohol Clin Exp Res 22(2):382-90.

Yang X, Oswald L, Wand G (2003) The cyclic AMP/protein kinase A signal transduction pathway modulates tolerance to sedative and hypothermic effects of ethanol. Alcohol Clin Exp Res 27(8):1220-5.

Yao L, Arolfo MP, Dohrman DP, Jiang Z, Fan P, Fuchs S, Janak PH, Gordon AS, Diamond I (2002) betagamma Dimers mediate synergy of dopamine D2 and adenosine A2 receptor-stimulated PKA signaling and regulate ethanol consumption. Cell 109(6):733-43.

Ying SW, Futter M, Rosenblum K, Webber MJ, Hunt SP, Bliss TV, Bramham CR (2002) Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. J Neurosci 22(5):1532-40.

Yoo CB, Cheng JC, Jones PA (2004) Zebularine: a new drug for epigenetic therapy. Biochem Soc Trans 32(Pt 6):910-2.

Yoo J, Medina-Franco JL (2012) Trimethylaurintricarboxylic acid inhibits human DNA methyltransferase 1: insights from enzymatic and molecular modeling studies. J Mol Model 18(4):1583-9.

Yoshimura M, Pearson S, Kadota Y, Gonzalez CE (2006) Identification of ethanol responsive domains of adenylyl cyclase. Alcohol Clin Exp Res 30(11):1824-32.

Yoshimura M, Tabakoff B (1995) Selective effects of ethanol on the generation of cAMP by particular members of the adenylyl cyclase family. Alcohol Clin Exp Res 19(6):1435-40.

Yu H, Wang Y, Pattwell S, Jing D, Liu T, Zhang Y, Bath KG, Lee FS, Chen ZY (2009) Variant BDNF Val66Met polymorphism affects extinction of conditioned aversive memory. J Neurosci 29(13):4056-64.

Zhang H, Pandey SC (2003) Effects of PKA modulation on the expression of neuropeptide Y in rat amygdaloid structures during ethanol withdrawal. Peptides 24(9):1397-402.

Zhang H, Sakharkar AJ, Shi G, Ugale R, Prakash A, Pandey SC (2010) Neuropeptide Y Signaling in the Central Nucleus of Amygdala Regulates Alcohol-Drinking and Anxiety-Like Behaviors of Alcohol-Preferring Rats. Alcohol Clin Exp Res 34:451-461.

Zhou FC, Anthony B, Dunn KW, Lindquist WB, Xu ZC, Deng P (2007) Chronic alcohol drinking alters neuronal dendritic spines in the brain reward center nucleus accumbens. Brain Res 1134(1):148-61.

Zhou Z, Hong EJ, Cohen S, Zhao WN, Ho HY, Schmidt L, Chen WG, Lin Y, Savner E, Griffith EC, Hu L, Steen JA, Weitz CJ, Greenberg ME (2006) Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. Neuron 52(2):255-69.

Zimmermann P, Wittchen HU, Hofler M, Pfister H, Kessler RC, Lieb R (2003) Primary anxiety disorders and the development of subsequent alcohol use disorders: a 4-year community study of adolescents and young adults. Psychol Med 33(7):1211-22.

Zuccato C, Cattaneo E (2009) Brain-derived neurotrophic factor in neurodegenerative diseases. Nat Rev Neurol 5(6):311-22.

VITA

Name Sachin Moonat

Education B.A., Molecular and Cell Biology emphasis Neurobiology, University of California at Berkeley, Berkeley, California, 2002

M.Sc., Biology and Animal Physiology emphasis Neurobiology, Charles University in Prague, Department of Physiology, Faculty of Natural Sciences, Prague, Czech Republic, 2005

M.D., University of Illinois at Chicago, College of Medicine, Chicago, Illinois, 2014

Ph.D., Neuroscience, University of Illinois at Chicago, Deprtment of Psychiatry and Graduate Program in Neuroscience, Chicago, Illinois, 2014

Teaching Course Director, LA3519: Neuroscience and the Mind, School of the Art Institute of Chicago, Chicago, Illinois, Jan – May 2013

Lecturer, PHYB310: Prematriculation Physiology Course: Neurophysiology, University of Illinois at Chicago College of Medicine, Chicago, Illinois, July 2010

Lecturer, PHYB310: Prematriculation Physiology Course: Muscle Physiology, University of Illinois at Chicago College of Medicine, Chicago, Illinois, July 2009

Lecturer, BMS660: Physiology for Medical Students: Neurophysiology Review, University of Illinois at Chicago College of Medicine, Chicago, Illinois, Mar 2008

Honors Alpha Omega Alpha Honors Society, 2013
AUPN / NINDS / ANA Career Development Symposium Travel Award, 2010

Publications Sakharkar A, Zhang H, Tang L, Baxstrom K, Shi G, **Moonat S**, Pandey SC (2014) Effects of Histone Deacetylase Inhibitors on Amygdaloid Histone Acetylation and Neuropeptide Y Expression: A Role in Anxiety-like and Alcohol Drinking Behaviors. Int J Neuropsych, 2014 Feb 17: 1-14. PMID: 24528596

Moonat S, Sakharkar A, Zhang H, Tang L, Pandey SC (2013) Abberant histone deacetylase 2-mediated histone modifications and synaptic

plasticity in the amygdala predisposes to anxiety and alcoholism. Biol Psychiatry, 2013 Apr 15, 73(8): 763-73. PMID 23485013

Moonat S, and Pandey SC (2012) Stress, Epigenetics and Alcoholism. Alcohol Res, 34(4): 495-505. PMID 23584115

Moonat S, Sakharkar A, Zhang H, Pandey SC (2011) The role of amygdaloid brain-derived neurotrophic factor, activity-regulated cytoskeleton-associated protein and dendritic spines in anxiety and alcoholism. Add Biol, 2011, Apr, 16(2): 238-50. PMID 21182574

Moonat S, Starkman BG, Sakharkar A, Pandey SC (2010) Neuroscience of alcoholism: molecular and cellular mechanisms. Cell Mol Life Sci, Jan, 67(1): 73-88. PMID 19756388

Jelinkova I, Yan Z, Liang Z, **Moonat S**, Teisinger J, Stojilkovic SS, and Zemkova H (2006) Identification of P2X4 receptor-specific residues contributing to the ivermectin effects on channel deactivation. Biochem Biophys Res Comm, 2006, Oct, 349(2): 619-25. PMID 16949036

Abstracts ***Invited Speaker/Oral Presentations***

Moonat S, The role of amygdaloid chromatin and synaptic remodeling in anxiety and alcoholism. Doctoral Dissertation Defense, Department of Psychiatry, UIC, Chicago, IL, 2012.

Moonat S, and Floreani C, Alcohol Abuse and Comorbid Psychiatric Disorders: Biology and Future Treatment Options. Center for Clinical and Translational Sciences Grand Rounds, UIC, Chicago, IL, 2011.

Moonat S, To Alcohol: The Cause Of and Solution To All Life's Problems. NerdNite Chicago, Chicago, IL, 2011.

Moonat S, Amygdaloid brain-derived neurotrophic factor signaling and dendritic spines: A role in anxiety and alcoholism. Symposium Presentation, 32nd Annual Scientific Meeting of the Research Society on Alcoholism, San Diego, CA, 2009.

Moonat S, Animal Models for Anxiety and Alcoholism. Biology Colloquium Meets MSTP, Chicago, IL, 2009.

Moonat S, Alcoholic Rats and their Anxious Amygdalas. Biology Colloquium Meets MSTP, Chicago, IL, 2008.

Moonat S, The independence of ivermectin action at the P2X4 ectodomain. Master Diploma Defense, Department of Physiology, Faculty of Natural Science, Charles University in Prague, Czech Republic, 2005.

Poster Presentations

Moonat S, Sakharkar A, Zhang H, and Pandey SC. The role of histone deacetylase isoform 2 in anxiety and alcoholism. Psychiatry Research Extravaganza, Chicago, IL, 2011.

Moonat S, Sakharkar A, Zhang H, and Pandey SC. The role of amygdaloid HDAC2 in the regulation of anxiety associated with alcohol preference. Brain Research Foundation, University of Chicago, Chicago, IL, 2011.

Moonat S, Sakharkar A, Zhang H, and Pandey SC. The specific role of histone deacetylase isoform 2 in amygdaloid epigenetic modifications associated with anxiety. UIC College of Medicine Research Forum, Chicago, IL, 2010.

Moonat S, Zhang H, Sakharkar A, and Pandey SC. Deficits in BDNF signaling in the amygdala: A role in the genetic predisposition to anxiety and alcoholism. Psychiatry Research Extravaganza, Chicago, IL, 2010.

Moonat S, Zhang H, Sakharkar A, and Pandey SC. The role of amygdaloid BDNF, Arc and dendritic spine density in the interaction between ethanol preference and anxiety. UIC College of Medicine Research Forum, Chicago, IL, 2009.

Moonat S, Zhang H, Sakharkar A, and Pandey SC. The role of amygdaloid dendritic spine density on the interaction between ethanol exposure and anxiety-like behavior. The American Society for Clinical Investigation and American Association of Physicians Joint Meeting, Chicago, IL, 2009.

Moonat S, Zhang H, Sakharkar A, and Pandey SC. Effects of acute ethanol exposure on amygdaloid dendritic morphology and anxiety-like behaviors in P and NP rats. American Society for Neurochemistry 40th Annual Meeting, Charleston, SC, 2009.

Moonat S, Teisinger J, and Zemkova H. Localization of ivermectin binding site outside purinergic P2X4 receptor ectodomain. Forum of European Neuroscience Societies, Lisbon, Portugal, 2004.