The Role of Histone and DNA Methylation Mechanisms in Alcohol Drinking and Anxiety-like Behaviors

BY
TIFFANI DIANE MARIA BERKEL
B.S., University of Illinois at Urbana-Champaign, IL, 2011

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
2020

Chicago, Illinois

Defense Committee:

Subhash Pandey, Advisor, Psychiatry
Mark Brodie, Chair, Physiology and Biophysics
Amy Lasek, Psychiatry
Dennis Grayson, Psychiatry
Mariann Piano, College of Nursing

Dedicated to my mother,

Suzanne Marie Berkel, née Lee

“Boys have germs,

Study study study,

And always do your extra credit.”

ACKNOWLEDGEMENTS

First, I would like to thank my thesis advisor and mentor, Dr. Subhash C. Pandey, for his superb combination of persistence, patience, and guidance. Despite his explosion of responsibilities, he always strived to be present in the development of my project and never failed to provide a personal touch to every meeting to help ensure my sanity. I will forever be grateful for his contribution to my career. Additionally, thank you to the numerous lab members who have aided me every step of the way and answered my innumerable questions: Tara Teppen, Harish Krishnan, Amul Sakharkar, Huaibo Zhang, Evan Kyzar, Svetlana Dzitoyeva, Peyton Bohnsack, and beyond.

My committee also deserves significant recognition. Each of them, both members of the preliminary and dissertation committees, provided critical contributions to the progress of my project. They respectfully addressed my shortfalls and professionally guided me to the finish line with expertise. I would especially like to thank Dr. Brodie for his impactful commitment, both directly and indirectly, to every aspect of my education, from medical school curriculum to his outstanding support as my Chair.

I would also like to thank Dr. Edward J. Roy at the University of Illinois at Urbana-Champaign for teaching me to always remember to take care of myself and know what is truly important in life. I would also like to recognize the amazing commitment of the Medical Scientist Training Program to its students and excellence at UIC. Thank you also to the college of medicine for providing ample opportunities for leadership and growth throughout the curriculum thus far.

To my family and friends, thank you for not only enduring me during this adventure but backing me from beginning to end, despite my long departures from home. I could not imagine having been able to come close to achieving my goals if not for the life-changing

friendships I found at UIC and for those I have managed to hold onto since childhood. Thank you, Fawn Hooben, for always being my strongest emotional safety net and source of support—prepared for even the most radical of my decisions—despite being so far away. Thank you to Elisa Hildebrand for allowing me to share the insanity of this experience and for undoubtedly being the single largest influence on my daily life throughout. Special thanks absolutely must go to Dan and Rufus for filling every minute they could muster with much needed love and laughs. I cannot fathom the emptiness of this chapter of my life had they not been in it. And finally, thank you to the many animals who have silently and nobly sacrificed their lives in the name of science and the progress of our miraculous corner of the cosmos.

--TDMB

Contribution of Authors

Chapters III represents a significant body of published and unpublished work serving to answer questions pertaining to my hypotheses, detailed in Chapter II. Portions of this chapter were completed with the collaborative assistance of Tara Teppen, Amul Sakharkar, Huaibo Zhang, and Evan Kyzar. This includes verifying/duplicating data and providing pilot data. This includes data from figures 5 and 8-10. My role was principal in the works presented here, and all work thus far has been synthesized and written solely by me. Some of this work has been published, as noted in chapters where relevant, and some or all of the presently unpublished work will likely be published in the near future as part of a co-authored manuscript with myself as first author. Chapters IV and V represent my synthesis of this body of work.

TABLE OF CONTENTS

CHAPTER **PAGE**

TABLE OF CONTENTS (continued)

CHAPTER **PAGE**

TABLE OF CONTENTS (continued)

CHAPTER **PAGE**

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF FIGURES (continued)

FIGURE **PAGE**

LIST OF ABBREVIATIONS

5-aza	5-azacytidine
Ac	Acetylation
ACTH	Adrenocorticotropic hormone
ALDH	Aldehyde dehydrogenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arc	Activity-regulated cytoskeleton-associated protein
AUD	Alcohol use disorder
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of amygdala
CBP	CREB-binding protein
CET	Chronic ethanol tolerance
ChIP	Chromatin immunoprecipitation
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
DNMT	DNA methyltransferase
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
EA	Extended amygdala
EPM	Elevated plus maze
GABA	γ-aminobutyric acid
GLP	G9a-like protein
GRIN2A	Glutamate receptor ionotropic, NMDA type subunit 2A; NR2A
GRIN2B	Glutamate receptor ionotropic, NMDA type subunit 2B; NR2B
H3K4	H3 lysine 4

LIST OF ABBREVIATIONS (continued)

H3K9	H3 lysine 9
HAD	High alcohol-drinking rat line
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HMT	Histone methyltransferase
HT	Hypothalamus
ICV	Intracerebroventricular
IP	Intraperitoneal
LAD	Low alcohol-drinking rat line
LDB	Light/dark box
MC	Melanocortin
MC3r	Melanocortin 3 receptor
MC4r	Melanocortin 4 receptor
Me	Monomethylation
Me2	Dimethylation
Me3	Trimethylation
MeA	Medial nucleus of amygdala
MeCP2	Methyl-CpG binding protein 2
MSH	Melanocyte-stimulating hormone
NAc	Nucleus accumbens
NMDA	*N*-methyl-D-aspartic acid receptor
NP	Alcohol non-preferring
NPY	Neuropeptide Y
P	Alcohol-preferring

LIST OF ABBREVIATIONS (continued)

PDYN	Prodynorphin
PFA	Paraformaldehyde
PFC	Prefrontal cortex
POMC	Pro-opiomelanocortin
PRDM2	PR domain containing 2, with ZNF domain
qPCR	Quantitative real-time polymerase chain reaction
RET	Rapid Ethanol Tolerance
SAHA	Suberoylanilide hydroxamic acid
SAM	S-adenosyl methionine
SET	Su(var)3-9—enhancer of *zeste*-trithorax domain
siRNA	Small interfering RNA
TF	Transcription factor
TSA	Trichostatin A
VTA	Ventral Tegmental Area

SUMMARY

Alcohol Use Disorder (AUD) is a multifactorial psychiatric illness and significant public health concern associated with millions of annual deaths worldwide and costing the United States hundreds of billions of dollars annually. Comorbid anxiety is common in AUD, exhibiting a reciprocal contributory relationship. This complex relationship is believed to be facilitated by the dominant anxiolytic effect of alcohol mediated by the amygdala, which has been extensively implicated in regulation of negative affective states associated with drug use, such as anxiety. To study this critical association, animal models of both intrinsic and acquired alcohol and anxiety-related phenotypes have been developed. One such model comprises the high-anxiety alcohol-preferring (P) versus non-preferring (NP) rats, which are selectively bred from Wistar rats for alcohol preference and non-preference, respectively. Additionally, because acquired tolerance to the anxiolytic effect of alcohol is believed to substantially influence alcohol intake, a model of rapid ethanol tolerance (RET) to the anxiolytic effects of alcohol is used and serves as an important index for chronic tolerance.

Epigenetic mechanisms have recently emerged as strong candidates for treatment targets in psychiatric disorders, including AUD and anxiety disorders. Therefore, we investigated the role of histone and DNA methylation-based epigenetic mechanisms in anxiety and alcohol preference phenotypes, using the P/NP and RET models. The histone methyltransferase (HMT) known as G9a (KMT1C/EHMT2) and its downstream dimethylated histone H3 lysine 9 (H3K9me2) marker are both known to downregulate gene expression via inhibitory chromatin remodeling and have recently been implicated

in regulating addiction and anxiety. Here, we determined that P rats exhibit innately higher mRNA and protein expression of G9a and H3K9me2, relative to NP rats in the central and medial amygdala (CeA and MeA) but not the basolateral amygdala (BLA).

To determine gene-specific changes in G9a levels and function in P and NP rats, we utilized chromatin immunoprecipitation (ChIP) to evaluate the occupancy of G9a and H3K9me2 at the promoter regions of various genes of interest that were found to be differentially expressed between the amygdala of P and NP rats. Expression of the anxiolytic neuropeptide Y (*Npy*) is known to be lower in P rats relative to NP rats. The *N*-methyl-D-aspartic acid (NMDA) receptor subtypes 2a and 2b (*Grin2a/2b*), which are involved in synaptic plasticity, were respectively expressed higher and lower in P rats relative to NP rats. The mRNA expression of anxiogenic amygdalar pro-opiomelanocortin (*Pomc*) and melanocortin 4 receptor (*Mc4r*) were also higher in the P rat relative to NP rats. Additionally, higher protein expression of MC4r and the downstream cleavage product of POMC, α-MSH, was found in the CeA and MeA, but not the BLA of P rats. Despite globally higher G9a and H3K9me2 levels in the amygdala of P rats, ChIP results suggested that G9a influenced expression of both upregulated and downregulated genes, as the inhibitory G9a occupancy was higher at promoters of *Npy* and *Grin2b* and lower at the promoters of *Pomc*, *Mc4r*, and *Grin2a* in the P rat amygdala. Similarly, ChIP analysis revealed H3K9me2 occupancy echoed G9a occupancy at *Pomc*, *Mc4r*, and *Grin2a* promoters; however, H3K9me2 occupancy remained undifferentiated at the *Grin2b* and *Npy* promoters, where P rat G9a occupancy was higher.

In recent years, the dynamic relationship between HMTs and DNA methyltransferases (DNMTs) has been increasingly elucidated, revealing reciprocal cooperation in downregulating gene expression. As such, we investigated DNMT status in the P and NP rat amygdala and found DNMT activity to be higher in P rats. Additionally, *Dnmt1* and *Dnmt3b*, but not *Dnmt3a*, mRNA expression was higher in P rats, accompanied by higher DNMT3B, but not DNMT1, protein in P rat CeA and MeA. DNA methylation measurement using the Methylminer approach revealed higher methylation at the *Grin2b* and *Npy* gene promoter sites where G9a occupancy was higher in P rats and H3K9me2 was undifferentiated. We then investigated the effects of an intraperitoneal DNMT inhibitor treatment and reported decreased alcohol drinking behaviors in P rats and a concurrent increase in G9a occupancy at the promoters for *Pomc, Mc4r*, and *Grin2b* genes.

G9a's dynamic influence on biological pathways regulating P rat drinking and G9a's documented involvement in anxiety inspired inquiry into its potential role in acquired behaviors. Utilizing the RET model, we determined that acute ethanol exposure reduces G9a and H3K9me2 in the CeA and MeA, but not the BLA, relative to saline-exposed controls. Two identical doses of acute ethanol 24 hours apart produced tolerance to ethanol's anxiolytic effects, despite equivalent blood alcohol concentration to the acutely exposed group. Furthermore, two consecutive doses induced no changes in G9a or H3K9me2 protein levels in the CeA and MeA relative to controls, suggesting the tolerant phenotype is related to the hindrance of ethanol-induced attenuation of G9a and H3K9me2 expression and/or activity. Specifically, acute ethanol exposure reduced

H3K9me2 and G9a occupancy at the *Npy* gene near a putative cAMP response element (CRE) site, whereas two consecutive doses 24 hours apart induced no such change. Notably, the transcription factor, CRE-binding protein (CREB), can potentially bind this site and has been heavily implicated in anxiety and addiction mechanisms via expression regulation of multiple genes, including *Npy*. This suggests that G9a-mediated chromatin remodeling may contribute to the expression regulation of the anxiolytic *Npy* in the amygdala. We then tried to determine if inhibition of G9a, before the second ethanol exposure, could recover sensitivity to ethanol-induced anxiolysis. Subacute IP administration of a G9a inhibitor not only reversed tolerance to anxiolysis but also reduced anxiety in controls. Treatment with the G9a inhibitor was also associated with reduced H3K9me2 and increased NPY protein levels in the CeA and MeA, further implicating G9a-mediated expression regulation of anxiolytic amygdalar NPY in this effect.

Cumulatively, these findings suggest that G9a likely influences multiple anxiety and AUD-related phenotypes, including innate and alcohol-induced traits, via its complex chromatin remodeling mechanisms. Overall, G9a-mediated chromatin compression in the CeA and MeA is associated with both innate AUD behaviors and acquired tolerance to the anxiolytic effects of alcohol. We have shown that G9a modulates phenotypes through a diverse set of anxiolytic and anxiogenic pathways. G9a may be responsible for critical regulation of crucial genes in these pivotal pathways, thus ultimately contributing to the development of AUD. We believe this evidence suggests that targeting G9a-mediated mechanisms has significant merit as potential treatment for anxiety and AUD.

I. Introduction

(Portions of this chapter were adapted with permission from work previously published as: Berkel, TDM and Pandey, SC. (2017) Emerging Role of Epigenetic Mechanisms in Alcohol Addiction. Alcohol Clin Exp Res*,* 41*(4), 666-680.)*

A. Alcohol Use Disorder

1. History and Epidemiology of Alcohol Use Disorder

Across the globe, alcohol has been and continues to be one of the single most widely used and abused drugs. With traces of deliberate alcohol consumption in humans dating back as far as six thousand years (1), modern use and abuse of alcohol is now known to significantly contribute to over 200 diseases and injury-related health conditions and to facilitate nearly 6% of all deaths worldwide (2). In the United States alone, over 85% of adults report consuming alcohol, and as of 2015, alcohol use disorder (AUD) afflicted an estimated 17 million Americans and cost the United States well over a trillion dollars in the previous decade, predominantly due to excessive drinking (3).

Over millennia, numerous societal guidelines and regulations of varying severities have been established in attempt to curb the astronomical burden of AUD and excessive drinking on societies while balancing the professed positive aspects of light to moderate consumption by the populous. In recent U.S. history, for instance, the 18th amendment ushered in prohibition due to widespread concern over damaging alcohol use, only to be repealed less than 15 years later. By 1984, significantly less extreme legislation was created to encourage a minimum alcohol-purchasing age of 21. Even more recently, however, increased individual liberties were secured in the U.S., as the final remaining states legalized homebrewing and cemented the surging craft beer culture of modern

America, perhaps reflecting the current positioning of the societal pendulum seeking equilibrium of shared success and alcohol access. Today, though not strictly universal, alcohol consumption is interlaced with normal life throughout many populations in the US, ranging from adolescent rebellion to celebrating life milestones. Ultimately, modern culture nurtures a widespread familiarity and comfort with alcohol use in America but also a contemporary support for recognition of AUD as a valid and concerning psychiatric ailment. As such, a plethora of developments in AUD and addiction research have surfaced in the last generation. In this review, I will highlight many of these developments as key components of our current understanding of the neurobiological basis of AUD.

2. Defining Alcohol Use Disorder

Individuals suffering from AUD often present clinically with a variety of symptoms, ranging from the inability to control their drinking, seizures, abdominal distention, and epigastric pain, to jaundice—among many others (4, 5). Due to the heavy societal burden of excessive drinking, it is important to consider screening in a clinical setting, where care providers can utilize questionnaires to reveal risk of AUD. Notably, many patients exhibit strong avoidance or denial regarding these concerns, potentially rendering the questionnaire or primary care intervention ineffective. Consequently, patients can qualify as moderate or severe at even the first clinical presentation, which may occur due to medical or personal emergencies. Upon positive screening via the CAGE questionnaire or emergent presentation at the emergency department, evaluation for AUD diagnosis should be considered.

Classifications of alcoholics have also been described, with Cloninger's Type I and Type II definitions serving as a foundation for many studies for decades. Cloninger Type I patients are characterized with atypically high anxiety, genetic predisposition, adult-onset dependence, and rapid onset of tolerance—particularly to the anxiolytic effects (6). On the contrary, Cloninger Type II patients, consisting almost exclusively of men, are characterized by anti-social personality traits, high criminality, dependence on euphoric side effects of alcohol, and increased likelihood of adolescent or young adulthood onset (6). Similarly, Babor *et al.* categorized patient populations into two groups. Babor Type A patients exhibit adult-onset mild dependence with minimal psychological dysfunction, while Type B patients exhibited familial predisposition, early life risk factors, significant dependence, and greater psychopathological dysfunction (7). Lesch expanded upon this concept and more precisely distinguished multiple groups of alcoholics based on characteristics of addiction and motivational influences on intake (8). Accordingly, Lesch Type I patients typically initiate alcohol intake in an abusive fashion and progress to a severe physical addiction, where consumption ultimately serves primarily to mitigate withdrawal symptoms. Lesch Type II patients, similarly to Cloninger's Type I, self-medicate innate or acquired anxiety, such as to overcome social anxiety, attenuate personal or interpersonal inhibitions, or suspend overly analytical behavior. Lesch Type III patients primarily utilize alcohol as an antidepressant and unsurprisingly tend to have comorbid depression. Specifically, the alcohol is used to mitigate depressive symptoms such as sleep disturbances, anhedonia, and anxiety; however, these symptoms are ultimately exacerbated with prolonged use. Type IV patients, reminiscent of Cloninger's Type II, comprise cases with developmental etiology, such as adolescent-onset

impulsivity, behavioral disorders, and exceptional susceptibility to the negative effects of alcohol on the brain that results in very early onset consumption and escalation ultimately leading to cognitive impairment, persistent behavioral disruptions, and dependence (8).

In formal diagnosis, psychiatric symptoms of AUD were divided into two clusters, supporting the diagnosis of either “alcohol dependence” or “alcohol abuse” up until very recently. However, with the advent of the 5th edition Diagnostic and Statistical Manual of Mental Disorders (DSM-5) in 2013, the American Psychiatric Association’s globally recognized tool for psychiatric classification and diagnostics combined these symptoms into a single list of 11 criteria for a broad and encompassing “alcohol use disorder”. While meeting criteria for AUD has been simplified into meeting at least 2 of the 11 distinct conditions, the severity of AUD in patients now includes sub-classifications of mild, moderate, or severe based on the extent of conditions met. Briefly, diagnosis of AUD mandates 2 of the following 11 criteria from the DSM-5:

[1] drinking more or longer than intended

[2] drinking despite repeated efforts to reduce or stop intake

[3] spending excessive time drinking or recovering from drinking

[4] craving alcohol and becoming preoccupied with the thought of drinking

[5] suffering home, occupation, or academic consequences due to drinking or recovery from drinking

[6] continuing to drink despite interpersonal consequences

[7] prioritizing drinking over otherwise important or interesting activities

[8] repeatedly entering dangerous situations such as driving or swimming while drinking or intoxicated

[9] continuing to drink despite related depressiveness, anxiety, or health consequences

[10] experiencing tolerance

[11] experiencing withdrawal symptoms upon discontinued consumption.

Furthermore, severity sub-classification is as follows: “mild” for 2-3 criteria met, “moderate” for 4-5 criteria met, or “severe” for 6 or more (5).

3. The Complex Contributions of Anxiety to Alcohol Use Disorder

The alarming rates of AUD is further accentuated by the high prevalence of comorbid disorders that have been consistently chronicled for decades. AUD is a particularly complex psychiatric dysfunction known to be exacerbated by other psychiatric disturbances, especially stress and anxiety. Indeed, the relationship between AUD and anxiety is continually being elucidated across the stages of AUD. Currently, it is believed that anxiety encourages initial consumption, facilitates cyclical patterns of abuse, prominently aggravates withdrawal and craving symptoms, and worsens relapse patterns (9, 10). It is likely that over time alcohol shifts from a promoter of positive effects during the graduation of early consumption to an inhibitor of negative effects during dependence.

Theories regarding initiation and maintenance of comorbidity patterns in AUD and anxiety include the “self-medication” and “tension reduction” theories. These theories emphasize the perceived value of alcohol and other substances in alleviating psychiatric distress, such as anxiety (11, 12). Notably, many adolescents and adults willingly report the

alleviation of stress or negative emotional avoidance as their primary motivator to drink (13, 14), and individuals who acknowledge their drinking serves as a coping mechanism—as opposed to a social lubricant, for example—consume significantly more alcohol than their peers (14). Unsurprisingly, this self-reported finding is consistently supported by analyses of the National Epidemiological Survey on Alcohol and Related Conditions. These studies also frequently suggest that self-medication of mood disorders increase likelihood of alcohol intake and AUD burden (15-17). Additionally, adolescents with an anxiety disorder or heightened sensitivity to stress are significantly more likely to develop AUD in adulthood (18, 19). This trend persists, such that more stress and higher coping motivation in adults results in significantly increased consequential drinking and overall intake in multiple studies tracking individuals from 6 months to 5 years (20, 21).

Various forms of stress—including early life stress, PTSD, acute stress, and chronic stress—are known to significantly increase the likelihood of future alcohol consumption and subsequent development of AUD (22-26). As such, individuals with innate anxiety may have intrinsically altered neurocircuitry and an associated predisposition for alcohol use, tolerance, dependence, severe withdrawal, and/or relapse. Studies have shown that an individual with AUD is approximately 2-3 times more likely to exhibit one or more comorbid anxiety disorder and nearly 5 times more likely than a healthy individual to specifically meet criteria for generalized anxiety disorder (27). Indeed, over 1/3rd of AUD patients qualify for an anxiety disorder diagnosis in the previous year alone (28) and having a comorbid anxiety disorder worsens recovery prognoses in patients attempting to quit (21, 28, 29).

In addition to anxiety increasing the likelihood of AUD, studies suggest a reciprocal relationship between the two disorders where AUD also induces persistent neurobiochemical alterations that dysregulate anxiety mechanisms in the brain, ultimately increasing psychiatric comorbidities, tolerance, and withdrawal symptoms (23, 30, 31). Indeed, adolescent-onset AUD is exceptionally predictive of severe AUD and mood disturbances in adulthood (19). While anxiety disorders are strongly predictive of future AUD diagnosis and often precede alcohol abuse, mood disorders such as anxiety can begin after alcohol abuse patterns have been established in adults, particularly in men (27, 28). Prolonged or escalated drinking, such as via a cycle of anxiety production and alleviation through alcohol consumption, results in tolerance to the positive effects of alcohol, including euphoria and anxiolysis (32, 33). Tolerance is a previously described diagnostic criterion of AUD and is also believed to increase alcohol intake, thereby facilitating subsequent dependence (5, 32-34). The aforementioned “tension reduction” and “self-medication” theories support this concept, as individuals consume more alcohol to attain the previously achieved and desired positive effects, resulting in increased pathophysiological insults to the brain. Alcohol withdrawal during abstinence is also a criterion for AUD diagnosis and a consequence of persistent neurobiochemical changes that manifest as increased sensitivity to negative affective withdrawal symptoms (5). Symptoms such as anxiety can provide significant motivation for individuals with AUD to consume alcohol and attenuate the negative repercussions of their abstinence. Though anxiety generally decreases over time in abstaining patients, persistent anxiety is both a strong predictor and significant active trigger of patient relapse and negative outcomes during AUD treatment (31, 35-37). Unfortunately, relapse resulting in physical

dependence can occur in a matter of days even after years of abstinence (38). Ultimately, these cumulative findings highlight the complex role of anxiety in alcohol-consumption, such that self-medication of innate anxiety, attempts at stress reduction at different stages of life, acquired tolerance to the anxiolytic effects of alcohol, physical dependence on alcohol to stabilize anxiety levels, and associated withdrawal-induced anxiety all significantly contribute to alcohol consumption and subsequent complications (Figure 1).

4. Current Treatment Options

Currently, patients with AUD have limited therapeutic options beyond psychosocial intervention and “12-step programs”. Three FDA-approved pharmacotherapies are available: acamprosate (Campral), disulfiram (Antabuse), and naltrexone (Revia/Vivitrol). Notably, none of these medications are FDA-approved for individuals under 18, pregnant women, or breastfeeding women (39). Despite evidence suggesting that these medications reduce drinking, relapse rates (40), and health care costs (41-43), these pharmacotherapies are scarcely employed in AUD treatment (44). This is partially due to low patient regimen adherence, high burden of use (45), and contraindications and side effects. For instance, the most efficacious option, naltrexone, is contraindicated in patients with liver disease--a medical condition commonly associated with AUD (46, 47).

Disulfiram was approved by the FDA in 1951 and remains a pharmaceutical option for patients wishing to stop drinking (48). Disulfiram inhibits mitochondrial aldehyde dehydrogenase (ALDH), an enzyme that converts the toxic metabolite, acetaldehyde, into acetic acid. Inhibition of this enzyme results in accumulation of acetaldehyde and rapid onset of nausea, vomiting, and other negative side effects, thus overshadowing the

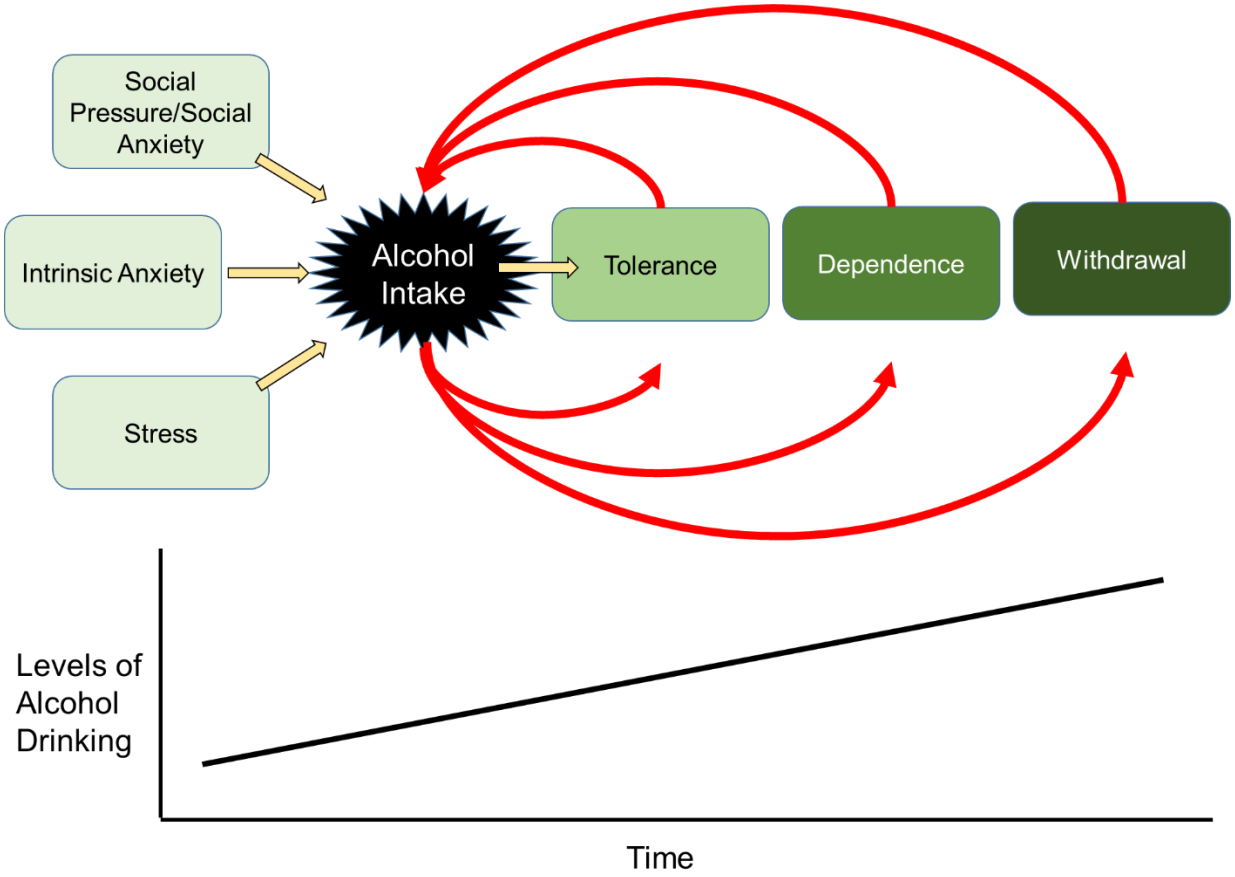


Figure 1. Visualization of the complex reciprocal nature of anxiety-related influences on alcohol drinking.
Initial alcohol consumption is due to a variety of reasons, but often tied to anxiety. These reasons include social norm-driven pressure, self-medication of intrinsic anxiety, and tension reduction during stress. Intake then leads to tolerance to the effects, including anxiolysis. This, in turn, increases alcohol intake over time and likelihood of developing physical or emotional dependence. This, in turn, requires alcohol consumption to stabilize the patient, including their negative affective states. Once dependence is established, cessation will result in withdrawal-induced anxiety, and neurobiochemical changes that make dysregulation stress and anxiety neurocircuitry often trigger relapse long after overt withdrawal symptoms have subsided. This complex, reciprocal nature of alcoholism and anxiety is believed to strongly fuel increased alcohol intake over time.

positively reinforcing effects of alcohol and theoretically deterring future intake (40). While there is evidence that disulfiram can reduce drinking if under significant supervision and open-label provision (49), studies repeatedly bring into the question the efficacy of the drug, with patient compliance as a significant limitation (50). Recently, the safety of disulfiram has also been under intense scrutiny as it has been found to potentially result in psychosis, cranial neuropathy, and hepatotoxicity in addition to the intentional effects of the drug (48).

Acamprosate (calcium N-acetylhomotaurinate) was approved by the FDA in 2006 is often prescribed alongside psychosocial support to help recovering alcoholics abstain and prevent relapse (48), and clinical trials cumulatively reporting on thousands of patients show substantial efficacy in safe reduction of alcohol intake and abstinence maintenance (51). Though the exact method remains elusive, studies suggest acamprosate reduces craving and relapse by restoring normal excitability of glutamatergic neurons, which can become excessively excitable due to neuroadaptations from chronic alcohol exposure that compensate for the suppressive effects of alcohol (52, 53).

Naltrexone was approved by the FDA in 1994 (and 2006 in the case of extended release formulations) for the treatment of alcohol dependence. Studies repeatedly report attenuation of drinking and cravings in addition to increased rates of abstinence in AUD patients treated with naltrexone, and systematic reviews of available literature confirm the consensus (51, 54). Like acamprosate, the mechanism by which naltrexone alleviates alcohol craving is not fully understood; however, evidence suggests that naltrexone and

its active metabolite bind opioid receptors, thereby attenuating some of the positively reinforcing effects of alcohol consumption, such as euphoria (48). Naltrexone also appears to be particularly effective when coupled with behavioral psychotherapeutic interventions that target abstinence and medication compliance (55, 56). Because acamprosate and naltrexone have both been found variably effective with reducing intake and cravings, combined therapies have been explored via randomized controlled trials (RCTs) with mixed, yet promising, results (56, 57).

Numerous off-label medications have been studied for AUD treatment, mainly anticonvulsants and antidepressants. Two separate Cochrane reviews investigating either broad criteria RCTs or RCTs and controlled clinical trials in male patients both concluded there was currently insufficient evidence to support treatment for AUD or withdrawal with anticonvulsants; however, several preliminary clinical trials individually support treatment with topiramate or gabapentin, specifically (39, 58, 59). Both drugs mediate gamma-aminobutyric acid (GABA)-associated mechanisms, theoretically accounting for alleviation of withdrawal symptoms, where GABA receptors are reduced in sensitivity and/or number (48). Importantly, topiramate is FDA-approved for use in children with epilepsy. Antidepressants often treat anxiety, but surprisingly are ineffective in alleviating AUD in patients without comorbid disorders (39).

Given the high degree of comorbidity and intricate relationship of AUD and anxiety disorders, the importance of mechanistic exploration of their relationship and how to treat them in a unified fashion cannot be overstated. It is critical to consider pharmacological

care in alcoholic patients with anxiety, as commonly used interpersonal methods such as cognitive behavioral and group therapy are not generally well-tolerated due to the social impairments of the population, aggravating the population's already worse outcome profile (29). Recent studies have shown that earlier intervention in anxiety has protective effects for future AUD (60). Furthermore, waiting to treat anxiety until after the AUD has been addressed likely provides no benefit for patients if they are not severely physically dependent upon alcohol (18). While there are many pharmacological options for anxiety disorders and AUD being treated independently, certain anti-anxiety medications can be contraindicated, or controversial at the least, in comorbid alcoholics due to potentially problematic pharmacological properties. Benzodiazepines have high efficacy for treating various forms of acute and long-term anxiety (61) but possess addictive potential in addition to significantly elevated medical emergency risk when used in combination with alcohol (62). Monoamine oxidase inhibitor medication is also a treatment option for certain anxiety disorders but poses significant risk with intake of various alcoholic beverages (63). Despite these limitations, several clinical studies have revealed potentially efficacious pharmaceutical interventions for patients suffering with comorbid anxiety and AUD. An RCT of the complex SSRI, nefazodone, resulted in significant reduction in anxiety symptoms, alcohol consumption, and days spent drinking heavily in patients with comorbid AUD and major depression (64). Similarly, buspirone is a serotonergic drug that was found to significantly reduce alcohol craving and anxiety measures in alcoholic patients and those with comorbid anxiety in double blind RCTs in patients motivated to quit (65, 66). There is reasonable, albeit limited, evidence to suggest that the anticonvulsant, topiramate, may decrease drinking-behavior and mood disorder

symptoms such as anxiety in recovering AUD patients, resulting in reduced relapse rate; however, this effect may be limited to targeted populations based on specific polymorphisms (39). Notably, recent animal studies have shown that chronic topiramate treatment in rats alleviate withdrawal-induced anxiety (67).

The broad range of mechanisms of the three FDA-approved and off-label prescriptions still undergoing scientific scrutiny highlights the complex mechanisms involved in AUD, the current lack of in depth understanding of these mechanisms, and the necessity of further investigation. As the exploration of pharmacological and behavioral studies in AUD treatment of humans and animal models continues (68, 69), the expansion of study in more complex individuals and animal models—specifically those with comorbidities—must become a priority (31).

5. Modeling Alcohol Use Disorder and Capturing the Impact of Anxiety

Several animal models have been established to investigate the diverse characteristics of AUD, particularly in attempt to mimic and better understand the behavioral and neurobiological findings in humans (4). Rodents have emerged as a prime subject for several reasons. In addition to the plethora of reasons that rodents have been paramount to studies throughout biological research, they are known to consume ethanol willingly and naturally in the wild, via rotten fruit for example, to the point of presumed intoxication. Also, the subcortical brain structures that mediate alcohol-related behaviors are strongly conserved in rodents and humans (70). Though high ethanol content (over approximately 6%) is predominantly aversive to most animals due to its unpalatable nature, the variability present in scientifically studied strains of rodents has allowed for preference

and ethanol-drinking behaviors to be distinguished, isolated, and selected for through breeding (70). Guidelines for creating an appropriate and more comprehensive animal model for neurobehavioral study of AUD and AUD-associated phenotypes suggest that the model exhibit the following behaviors:

[1] oral self-administration of ethanol under free-choice conditions

[2] self-administration of ethanol for pharmacological effects

[3] …at clinically relevant blood alcohol concentrations (BACs)

[4] exerting effort for ethanol access

[5] tolerance to the effects of ethanol

[6] withdrawal symptoms upon cessation of chronic ethanol access

[7] relapse-like escalation in consumption after prolonged abstinence (71, 72).

The alcohol-preferring (P) rat, alongside its non-preferring (NP) rat counterpart, is a model maintained at Indiana University and initially produced from dichotomous selective breeding of a closed-colony Wistar foundation stock from the Walter Reed Hospital based on high or low alcohol preference of 10% ethanol solutions (73, 74). Breeding selection mandated that the P and NP rats consume at least 5 g/kg ethanol per day and less than 1 g/kg ethanol per day, respectively, with food and water available *ad libitum* (74) and the P rats choose an ethanol solution over both water and palatable solutions (75). Several other models of AUD have been used to investigate the neurobiological basis of AUD—such as the high and low-alcohol drinking (HAD/LAD) rats, ALKO alcohol and non-alcohol (AA/ANA) rats, and Sardinian alcohol-preferring and non-preferring rats. These models are beyond the scope of this review but are described elsewhere (4, 70, 72, 74, 76-80).

P rats meet the aforementioned recommendations for an animal model of AUD (71, 72, 78) and serve as genetic models of AUD that exhibit innate ethanol preference and anxiety-like behaviors (81, 82). As stated, P rats [1] freely choose to orally consume ethanol without environmental manipulation (78, 79). It is understood that this consumption is [2] for the pharmacological effects as opposed to flavor or calorie accumulation because they will self-administer intragastrically or directly to the reward circuitry of the brain (83-85). P rats will [3] consume enough ethanol by free-choice to attain a BAC of approximately 100 mg%, which is equivalent to a moderate drunken state in humans, for whom the legal limit to drive in the United States is 80 mg% (78), and [4] they will put forth exceptional effort, up to 30 or even hundreds of lever presses in some cases, to attain these levels (86-89). P rats also [5] exhibit metabolic and functional tolerance to the anxiolytic, motor, and aversive effects of ethanol exposure (90, 91). P rats [6] rapidly develop physical dependence with free access to ethanol, resulting in withdrawal symptoms upon cessation (82, 92), and they will [7] resume intake at an escalated level after deprivation, mimicking human relapse behaviors (93). This pattern has been successfully stabilized in the inbred strain such that even selection of low ethanol preference (relatively) in P rats maintains the phenotypes (74).

Importantly, many of the P rat characteristics are directly correlative to DSM-5 diagnostic measures of AUD in humans. Though we cannot always make firm conjectures as to the motivation and desires of animal models, some critical diagnostic criteria are indeed met, excluding elements such as ‘drinking more or longer than intended,’ ‘drinking despite repeated efforts to reduce or stop intake,’ etc., as they require insight far more

sophisticated than is possible with an animal model. However, akin to several of the DSM-5 diagnostic requirements for human AUD, P rats do drink to the point of impairment despite their innately elevated tolerance; spend excessive time drinking, particularly when reintroduced to ethanol after a period of abstinence; exhibit behaviors highly suggestive of craving; prioritize ethanol consumption over water and highly palatable substances; and experience significant tolerance and withdrawal (75, 76, 78, 79, 82, 87, 88, 90-92, 94, 95). As previously mentioned, P rats exhibit innate anxiety relative to NP rats, as determined by a variety of tests that measure anxiety-like behavior, including but not limited to the elevated plus maze (EPM) and light/dark box (LDB), and this comorbidity is a natural biproduct of bidirectional selection based on ethanol preference (81, 96, 97). Interestingly, several rodent models of innately high ethanol intake exhibit anxiety as a result of ethanol preference-based selection, reinforcing the relationship between anxiety and alcohol consumption we see in humans (77, 80). Another typical characteristic of AUD patients is tendency for relapse during recovery, though it is not a diagnostic requirement for AUD (98). Only recently have relapse behaviors been acknowledged as an expectation of AUD animal models, which is met by P rats (78). Notably, this behavior has clinical importance, as relapse is an overwhelming obstacle for patients seeking care and wishing to ultimately abstain from alcohol, and the connection of stress and anxiety to relapse-like behaviors has been established in rodents for decades (99).

Other models can progress investigation of more specific aspects of AUD, as opposed to the P rat's generalized model of genetically-driven innate behavior. For instance, understanding the mechanisms involved with tolerance production and maintenance is of

particular interest to AUD research (5, 33). Tolerance can occur innately or be acquired, both of which require increased doses of alcohol to produce a desired response. This phenomenon, noted as a DSM-5 diagnostic criterion of AUD, is understood to encourage increased drinking, thus facilitating dependence and pathophysiological changes associated with increased exposure to ethanol (32, 100, 101). Interestingly, studies of men with genetic predisposition for AUD were found to have reduced tolerance to the positive affective effects of alcohol consumption and increased tolerance to the negative impairments, potentially increasing intake and risk of subsequent dependence (16).

There are three characterized and modeled forms of tolerance: acute ethanol tolerance, rapid ethanol tolerance (RET), and chronic ethanol tolerance (CET). Acute functional tolerance represents tolerance developed from a single session of alcohol consumption within the first hours of exposure. RET represents tolerance developed between 8 and 24 hours after exposure, while CET is that of extensive long-term exposure to ethanol (33, 102-105). Notably, rodent models of innately high alcohol intake, such as P rats, can exhibit exceedingly abnormal metabolic and functional tolerance, making them unacceptable for studying specific and controlled mechanisms of tolerance outside of the context of innate predisposition (74, 77, 90, 91). For example, P rats express a more robust metabolic tolerance with rapidly decreasing BAC (91), an innate tolerance to effects on locomotor activity (90), and a sustained functional tolerance to multiple effects of ethanol that can last up to 14 days after even a single acute dose (91).

Still, rodent models have thus far elucidated molecular and functional patterns of ethanol-induced tolerance to sedation, body temperature regulation, motor control, and anxiety, for example (105-111). Tolerance to the anxiolytic effects of alcohol is of interest due to the complex relationship between AUD and anxiety. RET has been recognized as a potential molecular index of CET, which is a typical characteristic and diagnostic criterion of AUD (5, 103, 112); however, RET remains relatively unexplored (100, 104, 105). Due to the contributory role of tolerance in escalating intake and forming alcohol dependence, it is imperative to identify the molecular mechanisms responsible for RET development in order to advance understanding of AUD pathophysiology. Mammalian RET has been produced and studied in rats and mice (100, 103, 105, 113), but studies in invertebrates are beyond the scope of this review. Reviews pertaining to invertebrate ethanol-associated genomics in tolerance models are available (114-116).

Mammalian studies consistently suggest that pharmacokinetics and metabolic changes cannot solely account for ethanol tolerance. Rather, genetic predisposition, functional tolerance, cellular adaptations, and mechanisms generally upstream of protein synthesis regulate mammalian rapid and chronic ethanol tolerance to metrics such as hypothermia, motor control, and anxiety (105, 117-119). Specifically, cellular and epigenetic changes in brain regions responsible for reward, stress, and addiction, such as the amygdala and nucleus accumbens (NAc) have emerged as significant contributors to psychiatric disorder pathophysiology, thereby mediating genetic predisposition, tolerance, and associated behavioral phenotypes (113, 120, 121).

B. The Amygdala, Anxiety, and Alcohol Use Disorder

1. Anatomy of the Amygdala

The amygdala is a brain structure originally derived from the telencephalon and located in the rostral portion of the temporal lobe of a fully developed brain. Originally named for the almond-shaped nuclei first discovered, the amygdala comprises a complex neuronal arrangement that is known to be fundamental to emotional processing and appropriate behavioral reactivity. Most investigations of the amygdala neurocircuitry and associated behavioral modulation has been executed in rats, with a moderate but growing number of studies in humans and non-human primates. Notably, the famous Kluver-Bucy study was conducted in rhesus monkeys, wherein bilateral lesions of the amygdalar region induced a variety of marked symptoms, including hyperphagia, hyperorality, and blunted affect and reactivity. Furthermore, these findings have been replicated in humans, secondary to lesions caused by tumors, for example (122, 123).

The amygdala is a small but critical structure, comprised of a variety of nuclei and divisions detailed below (124, 125). There are slight variations between rats and humans, which will be noted (124). First, there is the basolateral nuclear group, which has 3 main divisions—the lateral nucleus, basolateral nucleus, and accessory basal nucleus. Sometimes, the paralaminar nucleus is also included within this basolateral nuclear group. Frequently, the basolateral nuclear group is simply referred to as the basolateral amygdala (BLA) (125). The lateral nucleus (LA) is further divided to the lateral and medial divisions in humans or the dorsolateral, ventrolateral, and medial in rats (124). The basal portion is further divided into the rostral magnocellular, intermediate, and parvicellular

portions, and the accessory basal nucleus is further divided into the magnocellular, intermediate, parvicellular, and ventromedial division (the latter of which is not present in rats) (124). Second, there is the centromedial amygdala nuclear group, which has recently widely been identified as a significant portion of the “extended amygdala” (EA), and this consists of several nuclei particularly critical to the attached report. The EA comprises the central nucleus of the amygdala (CeA), medial nucleus of the amygdala (MeA), and more recently the amygdalar portion of the bed nucleus of the stria terminalis (BNST) and portions of the NAc (125). The CeA is further divided into the lateral and medial subdivisions, in addition to the capsular and intermediate subdivisions in rats (124). In humans, the MeA is undivided, but in rats the rostral, central, and caudal subdivisions are recognized. In some recent human studies, the medial and central nucleus have been divided based on anatomic localization, but the delineation exists independent of their related functionality (126). Third is the corticomedial nuclear region of the amygdala. Within this region are the nucleus of the lateral olfactory tract, bed nucleus of the accessory olfactory tract, anterior cortical nucleus, posterior cortical nucleus, and the periamygdaloid cortex—further divided into the medial and sulcal divisions (124). Finally, the remaining nuclei are grouped due to their independence anatomically, physiologically, or histochemically from other documented groups, and these are the anterior amygdala area, the amygdalo-hippocampal area, and the intercalated nuclei (124, 127, 128).

2. Neuroconnectivity of the Amygdala

The amygdala functions as a neuroconnectivity nexus regulating a range of emotionality and related function—including but not limited to fear, anxiety, and memory—via input

and output connections with a variety of brain regions. Afferent connections largely originate from cortical brain structures, thalamic and hypothalamic nuclei, and the brain stem (125). More specifically, the cerebral cortex sources much of sensory inputs to the amygdala via glutamatergic neurons originating in primary sensory cortical tissue, allowing for communication of stimuli information—olfactory, visual, auditory, pain, taste, and memories, for example (129). Indeed, most afferents to the amygdala possess excitatory glutamatergic properties (127). In general, these sensory inputs are believed to predominantly project to the BLA, oftentimes the LA specifically (127). Notably, projections to the CeA are more likely to exhibit inhibitory properties (130).

For instance, virtually all olfactory components project to the amygdaloid complex in some manner, such as via direct projections to the BLA or minor projections to the corticomedial structures (125). The somatosensory afferents largely pass through insular cortex before projecting to the BLA or CeA, similar to taste and visceral projections that extend from insular cortex to the BLA and CeA (131), though some originate from the thalamus (125). Auditory and visual impact on the amygdala often originate from thalamic nuclei, resulting in significant BLA termination and minor CeA termination (127, 129). Given that all sensory stimuli result in information pathways merging in the prefrontal cortex, it is no surprise that the prefrontal cortex also provides substantial projections to the amygdala. Similar to previously described sensory afferents, most prefrontal cortex projections to the amygdala terminate in the BLA while minor afferents terminate in the corticomedial nuclei (129, 132). Beyond sensory information, memory-associated circuitry is also strongly tied to the amygdala, resulting in robust reciprocal neuronal connections between

the amygdala and regions such as the hippocampus and entorhinal cortex (129). The role of the amygdala in memory (and subsequently anxiety), is highlighted by the nearly ubiquitous nature of these reciprocal connections among amygdalar nuclei, though it's notable that the hippocampal afferents do predominately terminate in the BLA (125). Unlike the aforementioned afferents, the hypothalamus (HT) and brain stem information to the amygdala is largely projected onto the CeA and MeA with minimal projections elsewhere (125). Despite these numerous afferent pathways, the amygdala is generally resistant to spontaneous excitation due to a collection of powerful inhibitory mechanisms that maintain low response to a stimulus that isn't novel, for instance (127).

If information has been effectively communicated via amygdalar afferents, most occurring in the BLA, intra-amygdalar communication allows for this information to ultimately reach output amygdalar centers, such as the CeA (127). Thus, the considerable network of intra- and inter-nuclear connections within the amygdala complex are believed to exhibit a largely medial flow, culminating in termination at output nuclei (133). Interestingly, while most sensory afferents terminate in the lateral BLA, the memory system afferents tend to project to the medial BLA, and the overall lateral to medial flow of intra-amygdalar connections suggest that the medial BLA may function to merge this information and allow critical comparisons of new information with stored information (125). Similarly, lateral to medial projections of sensory information converge with the hypothalamic and brain stem afferents at the corticomedial structures, and this convergence is believed to be cumulatively processed and thereby influence the amygdalar outputs to autonomic regulators (125). Despite the net projection of intra-amygdalar communication, weak

reciprocal communication in the medial to lateral direction does exist throughout the network, though they predominantly terminate on more medial subdivisions of intended target nuclei (125). Indeed, the CeA collects projections from all amygdalar regions at varying strengths but with few minor reciprocal projections; however, the CeA possesses an extensive network of connections among its divisions, allowing for complex regulation of the ultimate output (134). Thus, the CeA is understood to contribute greatly to the synthesis of information sent to the amygdala (130).

Corticomedial structures are responsible for significant efferent networks emanating from the amygdala, and components of the EA are specifically believed to be critical for regulating innate emotional reactivity and related physiological processes (127). The CeA, a critical component of the EA, largely consists of GABAergic neurons that serve as inhibitory interneurons within the CeA or projection efferent neurons (130). CeA sourced projections often extend to the brain stem, HT, and vagal system nuclei to regulate freezing behaviors, vocalization, cardiovascular system responses and other anxiety-associated responses (125, 127). Furthermore, the CeA communicates strongly with the BNST, largely regarded as part of the EA, which together innervate the HT. Unlike the CeA, the BLA sources glutamatergic efferent connections to numerous brain regions, including reciprocal connections to various cortical regions and more substantial projections to the memory neurocircuitry, hippocampus, and nucleus accumbens (NAc) (125). Projections from the BLA can regulate physical activity, memory formation, and complex thought processes in fear responses (135). Ultimately, a healthy amygdala with

lateromedial flow allows for synthesis of a multitude of inputs from a variety of brain regions to allow for more complete regulation and effective efferent control (Figure 2).

3. The Amygdala in Alcohol Use and Anxiety Disorders

As previously discussed, there is a significant reciprocal relationship between AUD and anxiety, and this is believed to significantly depend on the EA—a critical anatomical substrate that integrates a variety of sensory, stress, and memory signals (Figure 2). Within the amygdala, ethanol is believed to modulate a variety of molecular mechanisms, such as increasing GABAergic transmission and inhibiting glutamatergic transmission (136). Specific and relevant molecular mechanisms are discussed below, but the effects of these altered mechanisms within the amygdala are entrenched in the observation that regulation of negative affective states associated with addiction, such as anxiety, is largely orchestrated by components of the EA, and this role contributes greatly to the longitudinal disturbances of addiction and AUD (120). Indeed, the CeA has been shown to be one of the most sensitive regions of ethanol consumption regulation, and the previously described communication between the amygdala and other brain regions through its efferent GABAergic neurons result in dysregulated function of the ventral tegmental area (VTA), NAc, and more (34). As such, this is one means by which the EA is critical to the dysregulation of brain networks controlling negative affective states and therefore reinforcement to perpetuate the disorder.

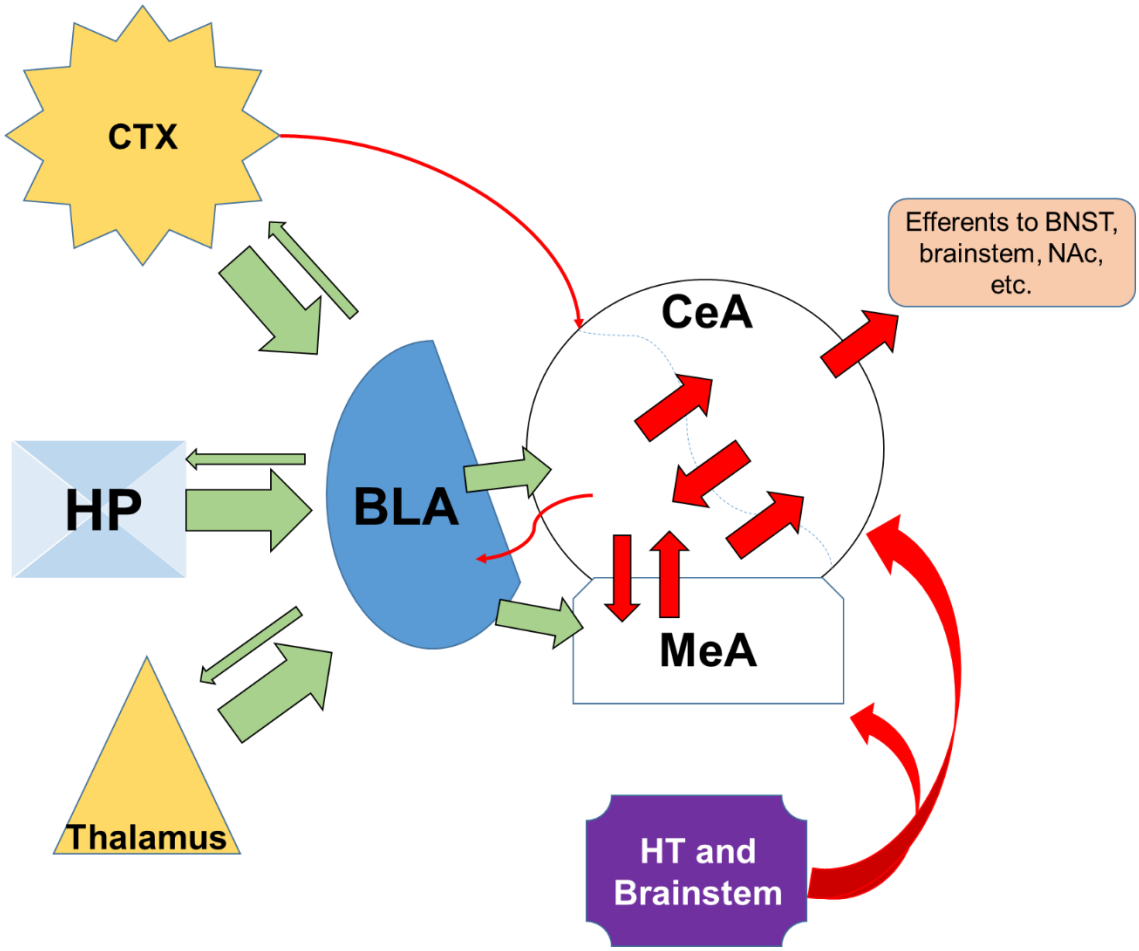


Figure 2. Representation of fundamental afferent and efferent connections of the amygdala, including intra-amygdalar lateromedial flow of information.
The cortex (CTX) and thalamus send sensory information-based projections and the hippocampus (HP) sends memory-based projections, primarily via glutamatergic excitatory neurons, to the basolateral amygdala (BLA), where afferent information converges and is processed. Additionally, small reciprocal connections extend from the BLA. The BLA then projects processed information to the centromedial output center of the extended amygdala, including the central nucleus of the amygdala (CeA) and medial nucleus of the amygdala (MeA). Minor reciprocal inhibitory connections between the CeA and BLA exist. Minimal sensory information as well as substantial projections from the hypothalamus (HT) and brainstem are projected directly to the (CeA) as well. Complex and often reciprocal connections within the CeA itself and between the CeA and MeA are believed to be crucial to culminative processing within the amygdala. Predominantly inhibitory projections then leave the CeA output region of the amygdala after final processing to effect behavior via a variety of brain regions such as the bed nucleus of the stria terminalis (BNST) and brainstem. Green and red arrows correspond to glutamatergic and GABAergic projections, respectively.

C. Epigenetics

1. Introduction to Epigenetics in Alcohol Use Disorder

AUD is a multifaceted disorder, with a range of significantly contributing etiologies. The genetic component of AUD has been investigated since at least the 19th century and was more recently scientifically verified through a multitude of twin and adoption studies, including a 2015 quantitative meta-analysis of 17 such studies, confirming approximately 50% heritability (137). This suggests the development and regulation of AUD is a product of both genetic and environmental influences on brain function (34, 138). Fittingly, epigenetic mechanisms are chemical modifications of the genome—such as DNA methylation, histone acetylation and methylation, and other less understood mechanisms—influenced by genetic and environmental variables to stably or transiently alter gene expression (139-141). These modifications can occur concurrently to varying degrees at different loci within the epigenome, allowing for an extremely diverse array of modification "signatures" that uniquely regulate gene expression based on the specific signature (142-145). A recent surge in epigenetics research has implicated epigenetic mechanisms in various psychiatric disorders (138, 146-148). Specifically, epigenetic mechanisms have emerged as important molecular components of AUD pathophysiology, anxiety regulation, and addictive behaviors (10, 149-151). This review of information will focus on the available epigenetic studies in AUD phenotypes, limited to histone acetylation, histone methylation, and DNA methylation mechanisms.

2. Epigenetic Mechanisms Involved in Regulation of Gene Expression

Chromatin consists of repeated units of DNA wound around an octamer of histone proteins that dynamically organize nuclear genetic material (152, 153). Histone proteins

possess amino-terminal tail domains that can be post-translationally altered via stable or transient acetylation, methylation, and other modifications (154, 155). Depending on the modification location and type, associated chromatin is remodeled to become more or less condensed, thereby altering access of DNA-binding proteins such as transcription factors (TFs) and regulating transcription (153, 156-159).

Histone acetylation is a permissive mark established by histone acetyltransferase (HAT) transfer of an acetyl group from acetyl-coA to lysine residues on the N-terminal tails of histone proteins and generates open chromatin in a transcriptionally competent state (154, 160). Addition of acetyl groups to positively charged lysine residues is believed to negate the molecular charge and associated electrostatic interactions with the negatively charged phosphate groups present in the backbone of DNA (154). Conversely, histone deacetylases (HDACs) remove acetyl groups from histone tails (154). There are 5 families of HATs [including p300/CREB-binding protein (CBP) transcription factors] and 4 classes of HDACs (I-IV), which have been linked to neuronal differentiation, synaptic plasticity, cognitive function, and psychiatric disorders (161-164).

Recently, histone methylation has gained traction in epigenetic research due to its complex and precise potential to regulate gene expression and so far, has been linked to cancer, development, neuroplasticity, learning, addiction, and anxiety (165-175). Histone methylation occurs when a histone methyltransferase (HMT) transfers one or more methyl groups from the methyl donor, s-adenosyl methionine (SAM), to histone N-terminal tail lysine or arginine residues, and histone demethylases (HDMs) function to remove methyl

groups from histones (139). Arginine residues can maintain one or two methyl groups, and lysine residues can be mono-, di-, or tri-methylated (175). Unlike histone acetylation, histone methylation has variable effects on transcription depending on the modified residues, interactions with other epigenetic factors, and valence of methylation (150).

DNA methylation is a predominantly repressive mark characterized by methylation of the cytosine pyrimidine ring at carbon-5. Dense regions of CpG dinucleotides (CpG islands) are often found near promoter transcription start sites and their methylation is generally recognized as an inhibitor of TF access and silencer of associated genes (176). Furthermore, some repressor complexes and TFs gravitate toward methyl-binding-proteins like Methyl-CpG binding protein 2 (MeCP2) and subsequently modify histones, thus further regulating gene expression (177-179). Recently, DNA methylation in the gene body has been found to induce variable gene expression regulation, but it is currently poorly understood (180). To establish DNA methylation, DNA methyltransferases (DNMT1, 3a, and 3b) relocate a methyl group from SAM to the target cytosine (176). Importantly, these DNMTs are abundant in fully differentiated adult neurons and are believed to play a critical role in gene regulation (181, 182). Methylation removal from DNA also occurs, presumably via removal of the methyl group or secondary to inhibition or loss of DNMT activity (183). Notably, multiple base-excision and nucleotide-excision repair mechanisms result in demethylation (183-185). Furthermore, recent investigations into the ten-eleven translocation (TET) family of enzymes that hydrolyze 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) suggest 5hmC may alter transcription regulation by

inhibiting identification by DNA-binding enzymes such as DNMT1 or by activating the base-excision repair pathway (186, 187).

3. Histone Acetylation in Alcohol Use Disorder

Abundant evidence over the last decade suggests that acute and chronic alcohol exposure modulate histone acetylation in addiction, stress, and/or reward neurocircuitry, leading to various attributes of alcohol intoxication, tolerance, and dependence (141, 147, 150). Such acetylation has reportedly occurred at a variety of histone 3 and histone 4 lysine residues (97, 188-193). Investigations into AUD have repeatedly implicated widespread histone acetylation that alter gene expression and synaptic plasticity, for instance via pathway activation or attenuation of cAMP response element-binding protein (CREB) and its cofactor and intrinsic HAT, CBP (194-197).

In the central and medial nuclei of the amygdala (CeA and MeA)—a complex brain region that participates in numerous pathways associated with stress, addiction, and anxiety (see above) (34, 198)—acute ethanol exposure induces CREB activation and CBP expression while inhibiting HDAC activity (199, 200). Accordingly, acute ethanol exposure increases H3K9 acetylation (H3K9ac) and H4K8ac within the amygdala and upregulates notable CREB target genes, including the synaptic plasticity-associated brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc), neuropeptide Y (NPY), and prodynorphin (PDYN) (147, 199, 201, 202). These findings correlate with increased dendritic spine density within the CeA and MeA and a reduction in anxiety-like behaviors (105, 199, 203, 204).

Also in the amygdala, P rats possess innately increased HDAC2 and decreased CREB and NPY levels in the amygdala compared to NP rats. Elevated HDAC2 expression in the CeA and MeA is correlated with decreased H3K9ac both globally and at promoters of *Bdnf* and *Arc* genes, which are innately less expressed in P rats compared with NP rats. Upon alcohol exposure, P rats exhibit reduced anxiety-like behaviors and increased CREB activation, BDNF and Arc expression, and dendritic spine density in the CeA and MeA (203, 205). Central amygdalar infusion of HDAC2 small interfering RNA (siRNA) or systemic administration of the HDAC inhibitor, Trichostatin A (TSA), in P rats reduces anxiety-like and alcohol drinking behaviors and normalizes the deficits in H3K9ac, NPY, BDNF, and Arc in the CeA and MeA (97, 206).

Several labs have also explored histone acetylation signatures in other brain regions. The cerebellum is a brain region responsible for motor coordination that is negatively affected by alcohol (207), and cerebellar expression of CBP and associated H3ac and H4ac is reduced after chronic ethanol exposure (195, 208). Furthermore, acute ethanol exposure downregulates HDAC2 in rodent cortex, possibly due to a reduction in H3K9ac at the HDAC2 promoter (189). And a study of rodent NAc in response to chronic ethanol shows decreased H4ac (209).

These findings highlight the role of swift and dynamic epigenetic changes in modulating short and long-term molecular processes secondary to ethanol exposure. Short-term repeated exposure, even a singular additional dose, can produce behavioral and molecular profiles akin to that of chronic exposure and associated tolerance (103, 112,

121, 210). The few studies available report that both RET and chronic tolerance are characterized by similar tolerance to the effects of ethanol on amygdalar histone acetylation mechanisms—such as the reduction in amygdalar HDAC activity and anxiety (105, 147, 211, 212). Notably, TSA administration in RET model rats attenuates development of tolerance to the anxiolytic effect of alcohol while rescuing CeA and MeA expression of both anxiolytic NPY and levels of the activating epigenetic markers H3K9ac and H4K8ac (105). Alcohol withdrawal occurs after acute and/or chronic exposure and is characterized in part by the development of anxiety (204, 213). Contrary to acute ethanol exposure, withdrawal after chronic ethanol exposure reduces H3K9ac, CREB activation, CBP expression, NPY levels, and expression of synaptic plasticity-associated genes in the EA (147, 197, 199, 204, 211). TSA treatment during withdrawal in animals reduces anxiety-like behaviors and rescues the deficits in amygdalar H3K9Ac, BDNF, Arc, and NPY expression (147, 211).

Indeed, studies have consistently demonstrated the molecular and behavioral impact of HDAC inhibitor treatment in the context of AUD. TSA and vorinostat (SAHA) were also found to significantly reduce voluntary alcohol consumption in a variety of animal models of ethanol intake (97, 209, 214). Somewhat contrary to these findings, Qiang et al. (2014) investigated chronic ethanol exposure in mice and reported that systemic TSA treatment accelerated drinking; however, the authors note effective studies provide HDAC inhibitor treatment when chromatin architecture is either innately dysregulated or perturbed secondary to ethanol exposure as opposed to before. Sodium butyrate (NaB) and MS-275 are also HDAC inhibitors (215). Intraperitoneal (IP) and intracerebroventricular (ICV)

administration of NaB and MS-275, respectively, were found to independently decrease operant self-administration of ethanol, preference for ethanol, and relapse-like behaviors in ethanol-dependent rats (214, 216). Additionally, both treatments variably altered histone acetylation in brain regions associated with addiction and reward networks (214, 216). Finally, valproic acid has been shown to selectively induce HDAC2 degradation (217) and to dose-dependently decrease ethanol intake and preference in rats (218).

It is evident from these cumulative works that histone acetylation and regulatory mechanisms involved in its production or removal are crucial contributors to the pathophysiological establishment and maintenance of AUD—ranging from initial alcohol exposure response to withdrawal (Figure 3).

4. Histone Methylation in Alcohol Use Disorder

Limited studies are available regarding histone methylation mechanisms in AUD relative to histone acetylation and DNA methylation, but the field has recently expanded. A study of acute ethanol in mice showed that cortex expresses significantly higher levels of activating trimethylated H3K4 (H3K4me3) (189). Similarly, binge ethanol-drinking in rats induces activating H3K4me2 production in the prefrontal cortex (PFC) at the promoters of TFs known to regulate synaptic plasticity (219). Repressive histone methylation marks in the cortical structures have also emerged in epigenetic studies of AUD. For instance, Qiang et al. (2011) investigated cell cultures of mouse cortical neurons and showed that binge-like ethanol exposure decreased the suppressive H3K9me2 and H3K9me3 marks at the NMDA receptor subtype 2b gene (*Grin2b*) (171).

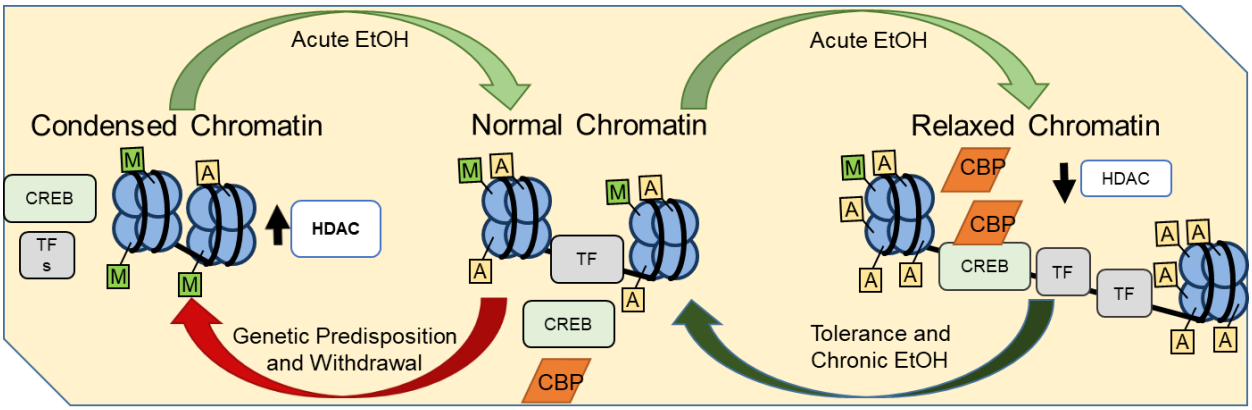


Figure 3. Alcohol-induced histone modifications (acetylation and methylation) in the amygdala.
Within the amygdala, acute ethanol (EtOH) induces histone H3 and H4 acetylation secondary to inhibited histone deacetylase (HDAC) activity and increased expression of the histone acetyltransferase [CREB-binding protein (CBP)]. These changes remodel chromatin to a less condensed form that allows for transcription factors (TFs) such as activated CREB and CBP to access chromatin, resulting in increased expression of CREB target genes, such as *Npy*. These modifications and related gene products in the amygdala are known to reduce anxiety-like and alcohol drinking behaviors. Tolerance and chronic exposure to EtOH aberrantly normalize molecular signatures of the epigenome upon EtOH exposure. Upon withdrawal or in the case of genetic predisposition to alcoholism, HDAC activity is increased, and the subsequent histone deacetylation condenses the chromatin and downregulates the CREB pathway. This results in increased anxiety-like and alcohol drinking behaviors. EtOH will then recover normal chromatin from these states. [Image and legend adapted and modified with permission from Berkel TD, Pandey SC. Emerging role of epigenetic mechanisms in alcohol addiction. *Alcoholism, clinical and experimental research*. 2017;41(4):666-680].

Furthermore, the decrease in repressive H3K9 methylation markers correlated with a likely causative decline in HMT expression, including that of G9a, Setdb1, and Suv39h1 (171). Recently, the HMT known as PRDM2 (PR domain containing 2, with ZNF domain) that monomethylates H3K9 was found to mediate post-dependent AUD phenotypes, including ethanol intake and stress-induced relapse. Interestingly, knockdown of *Prdm2* in non-dependent rat PFC initiated post-dependent behaviors, revealing the future treatment potential of HMT manipulation (220).

In the amygdala, acute ethanol in rats significantly reduces H3K27me3 at the promoters of *Pdyn* and *Pnoc* (prepronociceptin) (201), which have been implicated in the regulation of alcohol dependence (221, 222). We have determined that adolescent alcohol exposure in rats alters histone methylation in the amygdala, increases anxiety-like behaviors, and increases alcohol intake in adulthood (149, 223, 224). Specifically, the H3K4 and H3K9 demethylase, LSD1, and its neuron-specific isoform, *Lsd1+8a*, were both persistently downregulated in the amygdala (223). Furthermore, an associated increase in global and *Bdnf* exon IV-specific H3K9me2 but not H3K4me2 was observed in rats exposed to alcohol in adolescence compared to controls (223). In human studies, investigation of post-mortem frontal cortex tissue of alcoholics reveals multiple upregulated H3K4-centric HMTs alongside a concurrent elevation of activating H3K4me3 (225).

Secondary to advancements in chromatin immunoprecipitation sequencing and other techniques, labs have recently begun to escalate studies to broader investigations of epigenetic signatures. For instance, an exploration of genome-wide H3K4me3 activation

marks in post-mortem hippocampus samples of AUD individuals revealed a distinct pattern of H3K4me3 at gene networks known to be functionally interconnected and either potentially or definitively linked to addiction regulation (226). Another study specifically reported on genome-wide associations of alcohol withdrawal symptoms and described multiple significant polymorphisms in the gene for an HDM known as KDM4C (227), implicating histone methylation as a regulator of AUD phenotypes that warrants further investigation. Ultimately, data regarding histone methylation in AUD, specifically, is intriguing but inadequate, as current studies are predominantly restricted to the PFC and the amygdala.

5. The Histone Methyltransferase, G9a, and Associated Histone Methylation

In the last 15 years, the field of histone methylation has rapidly grown. These studies have clarified a role for HMTs and HDMs in gene regulation via substrate-selective modifications (228). G9a and G9a-like protein (GLP) are well-characterized and critical HMTs that predominantly convert H3K9 to H3K9me or H3K9me2 via homomeric or heteromeric complexes, subsequently silencing genes via methylation-regulated chromatin architecture reorganization (229, 230). This catalytic methyl transfer is orchestrated via the Su(var)3-9—Enhancer of *zeste*-Trithorax domain, also referred to as the SET domain, which is present among virtually all HMTs (231). G9a's importance, however, is highlighted by the *in vivo* embryonic lethality of homozygous G9a deficiency (169). *In vitro* and *in vivo* analysis of G9a knockout embryos and cell culture demonstrate that G9a is particularly crucial for differentiated cell development and the widespread production of methylated H3K9, which persists at approximately 12% of wild type levels in knockouts and occurs primarily in euchromatin regions. Interestingly, loss of H3K9me2

via G9a knockout is also correlated with increases of activating epigenetic marks—such as H3K9ac, H3K4me, and reduced DNA methylation—highlighting the importance of G9a in the communication and epigenetic homeostasis required for appropriate epigenetic signatures in healthy cells and its likely role in dynamic inhibition of active gene expression (169, 232).

Recent studies on the relationship between HMTs and DNA methylation mechanisms have increased (233-235), revealing reciprocal cooperation in gene silencing involving direct and indirect interactions between G9a and DNMTs (236-240). This relationship is believed to be multi-faceted and not completely understood. Some studies conclude that DNA methylation production and/or maintenance can be dependent on the presence of G9a (241-243). Furthermore, evidence suggests that the ankyrin domain within G9a can generate and read H3K9me2 in addition to interacting with DNMTs; however, specific residues within the domain remain independent to their respective function (244, 245).

Recently, epigenetic regulation of gene expression in specific reward and stress neurocircuitry has been compellingly implicated in various psychiatric disorders, including addiction, AUD, and anxiety (150, 151, 246). Specifically, G9a has emerged as a key contributor to mediation of addictive phenotypes, anxiety, and fetal alcohol spectrum disorders (FASD). While the details of development and FASD epigenetics are beyond the scope of this review, a recent review by Basavarajappa and Subbana superbly details the known role of G9a to date (247). Exploration of the role in G9a in adult AUD mechanisms has yet to be fully explored.

Drug exposure is believed to epigenetically alter gene expression and ultimately induce persistent neuronal changes that perpetuate addictive behaviors. Interestingly, G9a is believed to modulate dendritic spine density and synapses within the NAc via inhibitory epigenetic regulation of brain-derived neurotrophic factor (BDNF) signaling and subsequently the cAMP response element binding protein (CREB) (165, 248), both of which our lab and others have extensively linked to AUD and anxiety in preclinical and human studies (150, 165, 195, 197, 203-206, 211, 224, 249-257). In mice, morphine exposure inhibits G9a activity, reduces H3K9me2, and increases BDNF expression in the CeA (258). In both a mouse model of cocaine abuse with comorbid depression and another of chronic opiate exposure, *G9a* mRNA and H3K9me2 are downregulated in the NAc, and this trend is confirmed in the NAc of depressed humans as well (165, 259). The G9a and histone methylation decreases seen in the NAc after chronic cocaine exposure are also associated with increased expression of FosB, ultimately resulting in increased dendritic spine density and synaptic plasticity of the NAc alongside increased cocaine preference (168). Interestingly, G9a overexpression and knockdown in the NAc with concomitant changes in H3K9me2 respectively attenuates and enhances cocaine-conditioned place preference. However, induced overexpression of G9a in the NAc also increases motivation to self-administer cocaine and exacerbates stress-induced relapse, suggesting G9a-mediated gene expression can promote addictive behaviors and anxiogenesis (248).

Unsurprisingly, other studies involving G9a/GLP have revealed a complex role in anxiety regulation. For example, *Glp* heterozygous knockout mice have increased anxiety, as

determined by several anxiety measurements, including the EPM, LDB, and open field exploration (260). However, conditional neuronal *G9a* and *Glp* knockouts (deficiencies confirmed in the cortex, hippocampus, and striatum, etc.) with confirmed reductions in H3K9me2 both independently presented with robust reductions in anxiety, determined via EPM (167). While these results appear contradictory, the global heterozygous *Glp* knockouts also experienced several other severe developmental and social behavior alterations likely because GLP reduction is known to affect G9a function, which is critical for development (230, 261, 262).

Given the explosion of research on the role of epigenetics in addiction, epigenetic manipulation of gene expression has become a strong candidate for therapeutic targeting (150, 151, 162). Several of the aforementioned G9a-centric studies used conditional G9a knockout mice in which G9a was deleted in neurons. Chemical interventions are also available, but many others are analogs of S-adenosyl-methionine (SAM), thereby preventing G9a-mediated methyl transfer from SAM. However, SAM is a very broad acting methylation cofactor of several methyltransferases (such as DNA and arginine methyltransferases), making this method of G9a inhibition rather nonspecific. Since 2007, several inhibitors (BIX-01294, UNC0638, A-366, and UNC0642) specific to G9a/GLP have been discovered that instead block the substrate-binding cleft or the lysine channel of the HMTs in mouse and human cell lines (263-267). Unfortunately, several of these drugs are severely restricted by cytotoxicity at low micromolar concentrations or by poor pharmacokinetics, forcing researchers to largely depend upon intracranial injections, subcutaneous injections, or *in vitro* investigation (174, 264, 267).

Recently, a potent selective inhibitor of G9a was discovered (UNC0642) that is non-competitive with SAM, has an extended half-life of over 90 minutes, has low toxicity, and can penetrate the brain with a brain/plasma concentration ratio of 0.33 (263). This drug is an appealing option for *in vivo* G9a inhibition in brain tissue. To date, the few published studies involving *in vivo* UNC0642 reported that a modest IP dose of 2.5 mg/kg for 5 days in neonatal mouse pups was well-tolerated and significantly reduces morbidity and mortality in a model of Prader-Willi Syndrome (268). Chronic IP provision in mice significantly reduces anxiety in a dose-dependent manner (269). Thus, this compound has significant therapeutic potential in treating neurological and psychiatric disorders where aberrant epigenetic mechanisms are occurring due to increased expression or activity of G9a.

6. DNA Methylation in Alcohol Use Disorder

DNA methylation mechanisms have been extensively implicated in regulation of psychiatric disorders, including AUD (138, 162). However, studies on the effects of alcohol exposure on DNA methylation remain conflicting. An early study of chronic ethanol exposure in rats reported more relaxed chromatin, specifically in neurons, and implicated non-histone protein-DNA interactions (270). Since then, multiple debatable theories and contrary findings have emerged in attempt to explain this phenomenon.

Firstly, AUD patients often present with significant deficiencies in folate and vitamin B, theoretically boosting homocysteine levels and downregulating SAM production indirectly (271). Conversely, increased homocysteine has been elsewhere linked to global hypermethylation, which contradicts a downregulation of the methyl donor, SAM (272).

These findings generally correlate with condensed chromatin as opposed to the original determination of less constricted chromatin (270). Secondly, extensive alcohol consumption insults genomic material, thereby triggering base-excision repair and associated DNA hypomethylation (273). And thirdly, evidence suggests DNMT3b expression is generally reduced in AUD patients, possibly accounting for less restricted neuronal chromatin; however, DNA hypermethylation was paradoxically present in these patients (274). Ultimately, there are no conclusive theories to explain global neuronal hypomethylation after chronic alcohol exposure. In fact, recent evidence is increasingly contrary to this dogma. The few available studies, both *in vitro* and *in vivo*, suggest acute alcohol exposure may attenuate DNMT expression and activity in the brain (150, 275). More specifically, acute ethanol in rodents decreases DNMT3a expression in astrocytes (276) and reduces DNMT activity in adolescent BNST and amygdala (191). Contrary to acute exposure, chronic exposure in mice induces increased DNMT1 expression within the NAc (209). Similarly, another study which examined the NAc and PFC of ethanol-dependent rats after weeks of abstinence showed a persistent global DNA hypermethylation as well as a persistent increase in DNMT1 expression that was associated with the downregulation of a cluster of synaptic genes specifically within PFC neurons (277). A very recent study in rats also showed that chronic ethanol exposure resulted in increased cellular methylation capacity within the cerebellum (278). These studies cumulatively suggest that chronic ethanol exposure may induce global DNA hypermethylation in various brain regions associated with addiction and AUD while acute exposure may attenuate DNMT activity (Figure 4).

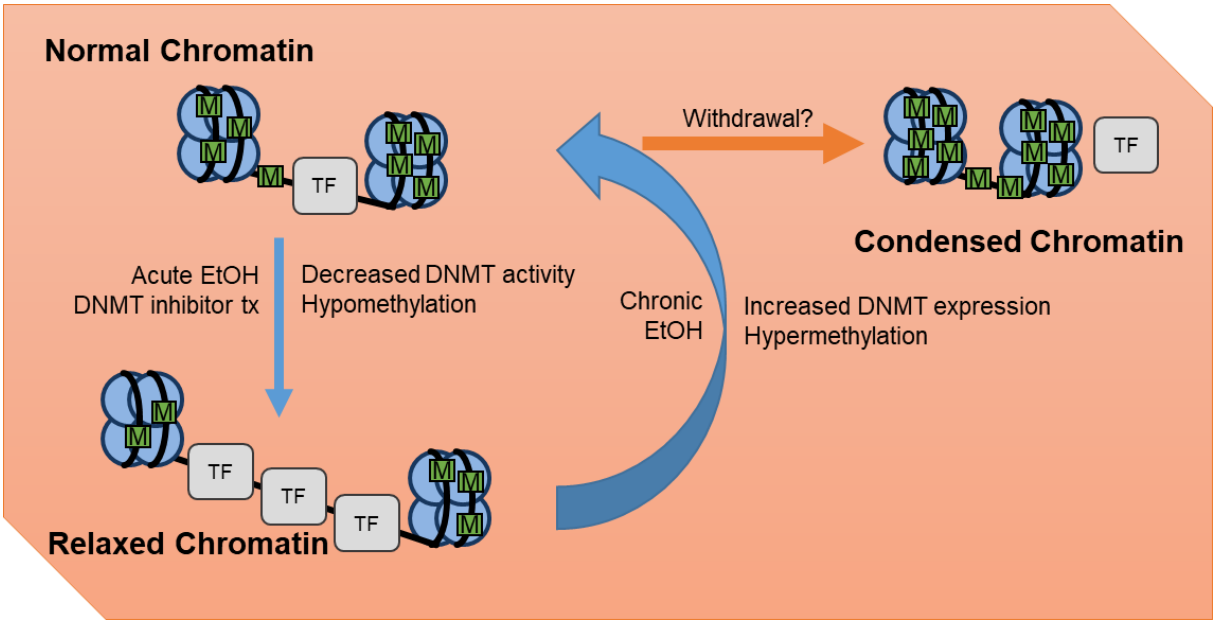


Figure 4. Hypothetical model indicating possible changes in DNA methylation after acute ethanol, chronic ethanol, and withdrawal.
Animal studies consistently report that acute ethanol (EtOH) exposure induces global DNA demethylation and presumably relaxed chromatin within various brain nuclei, altering transcription factors' (TFs) access and associated gene expression. Chronic exposure in animal and human samples reportedly increases global DNA methylation in several brain regions and peripheral blood samples. Based on these findings and the role chromatin remodeling plays in anxiety and drinking behavior, it is hypothesized that alcohol withdrawal results in especially hypermethylated DNA and presumably condensed chromatin as a consequence of dysregulated epigenetic mechanisms. It is possible that DNMT inhibitor treatment (tx) or acute EtOH exposure may lead to relaxed chromatin architecture due to DNMT inhibition. [Image and legend modified and adapted with permission from Berkel TD, Pandey SC. Emerging role of epigenetic mechanisms in alcohol addiction. *Alcoholism, clinical and experimental research*. 2017;41(4):666-680].

The effect of acute and chronic ethanol exposure on DNA methylation signatures at individual promoter sequences has also been investigated, and they continue to support the growing hypothesis that alcohol-induced epigenetic mechanisms regulate synaptic plasticity. In a study examining astrocytic tissue plasminogen activator (tPA)—a contributor to neuroplasticity regulation—astrocytes exposed to acute ethanol *in vitro* exhibited reduced DNMT activity and DNMT3a expression, decreased DNA methylation at the tPA promoter, and increased tPA expression, suggesting a potential indirect role of DNA methylation in regulating neuroplasticity upon alcohol exposure (276). An *in vitro* study of chronic ethanol in murine cortical neurons presented a reduction in DNA methylation at the *Grin2b* promoter, correlating with increased expression of GRIN2B (279). Similarly, chronic ethanol in fetal rats induces hypermethylation of *Bdnf* and associated reduction in BDNF protein expression within the olfactory bulb (280). Recently, a study showed that the NAc of mice exhibiting higher alcohol drinking behaviors expressed less *Bdnf*, which possessed more DNA methylation at the promoter site, and less GADD45B, which participates in demethylation of neurotrophic factor gene promoters such as *Bdnf*. Acute ethanol exposure in these mice increased GADD45b levels, *Bdnf* expression, and histone acetylation while decreasing DNA methylation (281). Like HDAC inhibition, DNMT inhibition is a promising therapeutic option in AUD, potentially altering global and promoter-specific DNA methylation signatures in brain regions critical to addiction neurocircuitry. Administering DNMT inhibitors, such as systemic 5-azacytidine (5-aza) and ICV RG108, in rodents significantly decreases alcohol intake and binge-like behaviors while reducing global NAc DNA methylation and increasing expression of genes involved in synaptic plasticity (209, 277). ICV RG108

administration also reverses ethanol-induced hypermethylation and expression changes in PFC synaptotagmin 2 (*Syt2*), which regulates ethanol-drinking behaviors (277). These combined studies emphasize the importance of DNA methylation intervention in regulating chromatin remodeling and gene expression in alcoholism and show promise for DNMT inhibition as a treatment for AUD.

In human studies, genome-wide and promoter-specific DNA methylation assays on peripheral blood cells is a newly developing approach to isolating genes of interest and potential biomarkers in humans. At a time when biomarkers for acute and chronic substance abuse are likely to become important for diagnosis, study, and treatment (282, 283), DNA methylation is a potentially reliable signature of environmental exposures for future clinical testing (284). In AUD research, many gene networks and epigenetic signatures have emerged in human studies revealing changes in DNA methylation signatures within addiction-related pathways. During withdrawal, patients exhibit significant hypermethylation or no change at the dopamine transporter gene (*DAT*), with a duplicated report of negatively correlated craving symptoms (285, 286). A similar study investigating DNA methylation of the monoamine oxidase A gene (*MAOA*) reported a significant association between the degree of alcohol dependence in female patients and the level of *MAOA* methylation (287) A very recent study detected methylation changes of extremely heavy daily drinkers at GABA receptor genes (*GABRD* and *GABBR1*), providing a potential future biomarker of current or recent heavy drinking (288). Furthermore, the aforementioned hypomethylation of *GRIN2B* in ethanol-dependent rodents is also present in human alcoholics, and the gene is increasingly hypomethylated

depending on disease severity (289). Notably, many of these genes direct synaptic exchanges or reception of neurotransmitters in the brain, which is detailed later in this review.

Other gene-specific investigations have reported significant changes in pathways tied to alcohol craving, such as pro-opiomelanocortin (*POMC*) and alpha-synuclein (*SNCA*) (290-292). Specifically, a cluster of DNA methylation alterations within the *POMC* promoter was found to correlate with alcohol craving (293), and certain *POMC* CpG islands were reportedly hypermethylated in certain AUD patient populations (294). At the *SNCA* promoter, hypermethylation was reported in alcoholics both during withdrawal and after acute exposure relative to controls (295). A very recent study of postmortem cerebellar tissue in chronic alcoholics also revealed abnormally high DNA methylation signatures at the promoter for a specific GABA receptor subunit, presumed to be secondary to decreased demethylation and *Tet1* mRNA expression (296).

Genome-wide studies have also emphasized a variety of genes involved in alcohol metabolism and related reward circuitry. For instance, comparing AUD patients to their nondependent siblings reveals several genes of interest with differential methylation, including that of an aldehyde dehydrogenase (*ALDH1L2*) involved in eliminating alcohol metabolites, a GABA receptor (*GABRP*) suggestive of decreased GABA binding in alcoholics, a glutamate decarboxylase (*GAD1*) involved in production of GABA, and a dopamine beta-hydroxylase (*DBH*) that is strongly linked to alcohol tolerance (297). A separate preliminary genome-wide investigation also reported epigenetically modified

gene clusters in the ALDH family, stress regulation pathways, and inflammatory responses (294).

Recent studies have begun to combine genome-wide DNA methylation analysis with intriguing phenotypic metrics. For instance, ganglioside-induced differentiation associated protein 1 (*GDAP1*) gene is hypomethylated in AUD patients, and the degree of methylation is significantly associated with symptom severity (298). Another recent study examined DNA methylation in monozygotic twins with discordant AUD status and complemented this work with personality evaluation and MRI imaging during an impulsiveness task. They found a significant association among hypermethylation at the 3'-proteinphosphatase-1G gene (*PPM1G*) and two established risk factors of AUD—adolescent escalation of alcohol intake and impulsivity (299). Given the complex and multifaceted nature of AUD, multidimensional approaches such as these garner invaluable insight.

Post-mortem tissues provide an important opportunity to investigate brain region-specific changes in AUD. Unfortunately, such studies are significantly limited in breadth of tissue and sample size due to restrictions in tissue availability. Thus far, post-mortem studies have elicited contradictory results, likely due to inadequate knowledge of patient alcohol status at death (150). In the precuneus and putamen brain regions of AUD post-mortem tissue, significantly altered DNA methylation status was identified in gene networks involved in the immune and inflammatory response, lipid metabolism, and gastrointestinal disease (300). In the amygdala and PFC of alcoholics, studies are more extensive, though

results vary greatly. One study reports global DNA hypomethylation and decreased transcription of *DNMT1*, contrary to several animal studies (225). Others report extensive DNA hypermethylation in males but not females (301) or no change (302). Interestingly, the Ponomarev study also suggested that GC content altered the dysregulation pattern of genes. Specifically, GC-rich and GC-poor gene networks were respectively upregulated and downregulated in alcoholics (225). Post-mortem studies have also revealed epigenetic signature shifts in a myriad of gene networks, including synaptic transmission genes in PFC neurons of alcoholics, which is consistent with epigenetic animal studies (141, 225). Similarly, multiple genes involved in chromatin remodeling, histone deacetylation, and transcription repression were significantly upregulated in the amygdala and PFC of alcoholics (225), and several histone gene promoters were found to be heavily methylated (302), echoing the growing relevance of histone modifications in AUD.

Upon investigation of specific genetic loci in post-mortem tissue, Taqi et al. (2011) investigated known *PDYN* CpG polymorphisms associated with increased risk of alcoholism and discovered differential DNA methylation in PFC. This study has specifically emphasized the value of combining genome-wide population studies with epigenomic studies to effectively isolate risk-associated epigenomic shifts in certain populations. Other studies have begun investigating comorbidities and revealed altered TET1 expression in PFC of comorbid psychotic and alcoholic post-mortem brains, broadening the scope of alcohol-induced chromatin remodeling (303). Though the study of post-mortem epigenomic signatures in alcoholism is in its infancy, investigations of

various brain regions will likely continue to increase, hopefully elucidating the significance of these findings and guiding animal model investigations as well.

D. Molecular Hypotheses in Alcohol Use Disorder and Anxiety

1. Molecular and Neuronal Effects of Alcohol—Narrowing the Scope

Despite decades of dedicated investigation, the list of detected molecular targets of ethanol remains incomplete. However, general changes in behavior, synaptic connectivity, and brain circuitry induced by acute and/or chronic ethanol exposure are well documented. Indeed, ethanol-induced changes within the brain, secondary to direct and indirect effects of ethanol, are believed to coordinate associated behavior, from depressed central nervous system activity to euphoria. The broad range of effects elicited by ethanol is believed to be due in part to the simple structure of the molecule and the nonspecific nature of its interactions (304). To date, several molecular targets of ethanol have been determined within the brain, including a variety of neurotransmitter receptors and cell membrane channels. Though these mechanisms are not fully understood, ethanol is believed to be particularly well-poised to interfere with transmembrane components of proteins, thus potentially allowing for structural influence of the aforementioned channels and their function (304, 305).

2. Introduction to Pro-opiomelanocortin and Melanocortin 4 Receptor

The central nervous melanocortin (MC) pathways are comprised of a variety of neurons that release endogenous ligands for G protein-coupled MC receptors. Pro-opiomelanocortin (POMC) is a large precursor polypeptide produced primarily within the arcuate nucleus of the HT; however, many studies have suggested that POMC-transcribing neurons stem from numerous brain regions, including the amygdala (306-

309). POMC is processed through a series of complex posttranslational mechanisms to form several penultimate and subsequent proteolytic products that function as MC receptor ligands, including but not limited to adrenocorticotropic hormone (ACTH), β-melanocyte stimulating hormone (β-MSH), and α-MSH (310). Their influence at the level of MC receptors and associated pathways critically regulate multiple important physiological mechanisms, including those of blood pressure, energy homeostasis, mammalian metabolism and sympathetic nervous responses (311). ACTH is a hormone often principally associated with stress regulation via the hypothalamic-pituitary-adrenal axis (HPA axis), by which it modulates adrenal glucocorticoid release after stimulation of highly specific MC receptors of cortisol-producing cells. β-MSH is not present in rodents. Notably, β-MSH and ACTH remain outside of the scope of this review. The predominant POMC-derived MC peptide, known as α-MSH, is peptide that significantly regulates energy homeostasis, calorie consumption, and liquid intake (312, 313). Early studies showed that varying forms of α-MSH administration could induce or inhibit anorexic responses, even overriding previously established pharmacological interventions that would otherwise manipulate feeding behavior (314, 315).

Multiple receptors receive and react to POMC proteolytic products, but the two principal receptors of α-MSH are melanocortin 3 and 4 receptors (MC3R and MC4R). MC3R is bound predominantly by γ-MSH within the HT (316); conversely, MC4R is rather ubiquitous throughout the brain and predominantly bound by α-MSH and β-MSH (317). Early studies of MC4r and α-MSH showed that α-MSH-releasing POMC neurons exhibit their anorexigenic effects vis MC4R target neurons (318, 319) and that MC4R knockout

mice innately develop obesity (320). Extensive studies since the original discoveries of POMC neurons and α-MSH have led to a significantly more comprehensive understanding of the physiology and neurocircuitry involved in MC-mediated energy homeostasis, and these roles have recently expanded to attract investigations into the contribution of these mechanisms in anxiety, alcohol consumption, and associated addiction development.

3. The Role of α-melanocyte Stimulating Hormone and Melanocortin 4 Receptor in Alcohol Use Disorder: Amygdala-mediated Anxiogenesis

In addition to feeding and energy homeostasis regulation, the MC pathway, particularly via α-MSH, has also been found to regulate anxiety-like behavior in rodents, such that even the anorexigenic effect of α-MSH is believed to possibly be stress-related (321). Both central and systemic administration of α-MSH induce anxiety in a dose dependent manner in addition to the anorexigenic effects via inhibitory influence on the GABAergic system (322, 323). Evidence suggests that these effects are regulated via the MC4R receptor (324). Studies have shown that MC4R-specific agonists mimic the anxiogenic effect of α-MSH and that antagonists attenuate or prevent various anxiety-like behavior responses, including those induced by physical and social stress (321, 325-328). Clearly the role of the MC system is important to anxiety-related mechanisms in the brain, but until recently the effects were presumed to be solely mediated via the HT. Notably, the previously detailed studies on α-MSH and MC4R utilized systemic and central interventions that would potentially affect multiple brain regions, and—while controversial—studies do suggest that POMC can be initially produced in regions outside the HT (312). Interestingly, the amygdala is a critical site in anxiety regulation that has

recently emerged as a suspected component of the α-MSH-MC4R pathway, as mounting studies suggest that POMC and MC4R are transcribed and processed in the amygdala (310). Indeed, the amygdala exhibits a dense collection of α-MSH neuronal fibers and plasma membrane MC4R receptors (329, 330), and intra-amygdalar injection of α-MSH and MC4R agonists, particularly the CeA, has been shown to induce an anxiogenic response in rats and mice (329, 331). Additionally, both *Pomc* and *Mc4r* gene expression are significantly increased in rodent amygdala after acute stress (332), and MC4R loss-of-function rats are known to display significantly reduced stress responses (333). More studies are needed to elucidate the degree of contribution that POMC and MC4R-associated mechanisms make to amygdalar regulation of anxiety.

In addition to regulating energy homeostasis and anxiety, it was first postulated that hypothalamic mechanisms may influence alcohol consumption in 1970 (334). Soon after, studies had shown that both electrical stimulation of the lateral hypothalamus (LHT) and lesions to the medial hypothalamus (MHT) induced alcohol intake, likely due to the role of the HT, and specifically the MC system, in energy acquisition and reward (334, 335). It was not long before studies revealed the potential of the MC system in mediating AUD phenotypes via anxiety and alcohol consumption regulation. As described above, α-MSH is a downstream MC proteolytic product of POMC posttranslational processing, and in conjunction with its dominant receptor, MC4R, α-MSH is believed to regulate energy acquisition and anxiety through pathways that extend far beyond simply the HT (312). Indeed, *Pomc* mRNA, *Mc4r* mRNA, α-MSH protein, and MC4R protein have all been reported by numerous labs in the amygdala (307-309).

Recently, POMC and MC4R mechanisms have been reported as potentially robust regulators of AUD, particularly via amygdalar mechanisms, and the role of MC mechanisms in AUD remains an active discussion in the scientific community. Human studies have shown that genetic variability in the *POMC* gene are significantly associated with addiction risk, including that of AUD (336). Interestingly, epigenetic mechanisms have also been linked to the dysregulation of the MC mechanisms believed to be present in AUD, such that DNA methylation at the *POMC* promoter has been found to be significantly correlated to craving reported in AUD patients (293). Furthermore, our recent study of adolescent alcohol exposure found that adult rats who were intermittently exposed to ethanol in adolescence exhibited increased *Pomc* mRNA in the HT and increased *Mc4r* mRNA in the amygdala that was correlated with histone acetylation patterns indicative of their influence on transcription levels (308). While these studies suggest that epigenetic mechanisms may play a crucial role in the mechanistic dysregulation of the MC system in the context of AUD, the details of pathophysiological contribution of the POMC/α-MSH/MC4R system to AUD remains poorly understood.

Despite studies showing that α-MSH/MC4R pathway activation in the HT and amygdala confer increased anxiety, several studies have shown that central and systemic agonists of MC receptors can paradoxically reduce drinking while antagonists of MC4R or MC3R/MC4R have minimal effect (337, 338). Notably, fundamental errors in these studies bring results into question—such as improperly variable dosing, inadequate control of diet (given the importance of the MC system in energy homeostasis and feeding), and use of nonselective pharmacological interventions. Additionally, some of

these effects were reportedly particularly short-lived and transient despite chronic manipulation of the MC mechanism (339). Conversely, several studies have suggested that the MC4R-mediated MC pathways in the brain can instigate anxiety, alcohol consumption, and several other pathophysiological components of AUD phenotypes. For instance, centrally administered α-MSH can attenuate the anxiolytic effects of ethanol while MC4R-specfic inhibition can augment the anxiolytic effects of ethanol (31, 323). In agreement with this, the high-anxiety Sardinian alcohol-preferring rats exhibit higher levels of baseline *Pomc* mRNA in the HT, and these levels are influenced by alcohol consumption (80, 306). In the VTA, exogenous α-MSH promotes the reward response to ethanol, evident by increased lever presses by rodents to induce ethanol self-administration, while VTA-specific MC4R inhibition blocks this effect (340). Centrally administered MC4R-specific inhibition can also prevent the typical development of anxiety-like behaviors during ethanol withdrawal (323). In the alcohol-preferring P rat, MC4R-specific inhibition substantially reduces ethanol consumption, but only if the associated hyperphagia and MC-regulated energy homeostasis is controlled (341). Additionally, centrally and intranasally administered MC4R antagonists attenuate ethanol withdrawal-induced hyperalgesia (342). Clearly, dysregulated MC mechanisms play a complex role in the development of AUD and associated phenotypes, and work regarding these mechanisms in brain regions including the amygdala require more investigation.

4. Introduction to Neuropeptide Y

NPY is a complex and highly conserved 36-amino acid peptide abundantly expressed and influential in several brain regions, including but not limited to the amygdala, NAc, hippocampus, and hypothalamic nuclei (343) in order to pivotally regulate physiological

functions and behaviors associated with appetite, stress and anxiety, energy balance, alcohol intake, and more (130, 344). Given the broad spectrum of NPY regulation in the brain, many NPY-mediated mechanisms are beyond the scope of this review; rather, we will focus on the role of NPY in stress and anxiety to preface its role in AUD.

NPY acts through a collection of G-protein coupled receptors, known as Y receptors, of which humans have four among the five recognized mammalian receptors (345). Y_1 and Y_2 are the most widely expressed and abundant of the Y receptors and possess the strongest affinities to NPY. To date, it is generally believed that Y_1 and Y_2 respectively function to regulate the postsynaptic and presynaptic neuronal physiology of NPY via their G_i/G_o coupled reactivity, which subsequently inhibit activation of adenylyl cyclase and accumulation of cAMP. In the context of this introduction, the roles of Y_1 and Y_2 in anxiety are currently believed to be opposing one another, such that activation of postsynaptic Y_1 is believed to be predominantly anxiolytic, particularly in the central nucleus of the amygdala, while activation of the Y_2 presynaptic autoreceptor is believed to have differing effects dependent on brain region and neuronal cell type, partly due to their opposite effects on neuronal calcium channel activity (346, 347). Neuronal NPY receptors within brain regions that are particularly relevant to stress responses, such as the amygdala and HT, predominantly regulate neuronal components of GABAergic, glutamatergic, corticotropin-releasing factor-mediated, and norepinephrine-mediated systems (346). Notably, both are generally recognized as pro-stress neurotransmitters, and NPY is believed to function as a counterbalance of these systems as it is ultimately an anxiolytic neuropeptide (348-350).

5. The role of NPY in Alcohol Use Disorder: Amygdala-mediated Anxiolysis

Multidirectional NPY-mediated communications between the amygdala and other brain regions, including the HT, are understood to facilitate the anxiolytic effects of NPY (130). Several studies have shown that both exogenously administered NPY and vector-mediated overexpression within the ICV space or amygdala produces robust anxiolytic effects in rodents via amygdalar Y_1 receptors (349, 351-353). This is particularly true in animal models of high anxiety (354, 355). Similarly, acute stress has been shown to rapidly reduce NPY expression in the amygdala of rats (356).

Studies highlighting the anxiolytic effects of amygdalar NPY have been instrumental in establishing the role of anxiety-regulating mechanisms in alcohol drinking behaviors, with amygdalar NPY recognized as a key component (348), and studies by multiple labs in a variety of animal models have shown that higher amygdalar NPY expression is associated with anxiolysis and attenuated ethanol intake while amygdalar NPY deficiencies are associated with anxiety-like and AUD behaviors (130, 197, 355, 357, 358). For instance, in addition to the demonstrated anxiolytic effects of ICV NPY in certain anxious rodent models, ICV administration of NPY has also reportedly attenuated ethanol intake in both P rats (359) and the HAD rodent models (360). In fact, both CeA-specific overexpression and exogenous administration of NPY in the amygdala produce increased CREB activation, of which *Npy* is a known target, and reduced anxiety-like behaviors in addition to reduced ethanol consumption in anxious and alcohol-preferring models (348, 355, 357, 361, 362). Direct infusion of NPY or a vector-induced overexpression within the CeA also respectively attenuate post-dependence ethanol intake and escalation of

ethanol intake (363, 364), and P rats innately express lower levels of anxiolytic NPY in the CeA and MeA relative to their non-preferring NP rat counterpart (96, 205, 358), and both ethanol consumption and NPY administration directly into the CeA can significantly reduce the high anxiety phenotype seen in P rats (205, 355). Furthermore, the expression levels of Y_2 was found to be decreased in the MeA of AA rats, relative to their non-preferring counterparts (365).

Genetic information regarding NPY has also provided evidence further clarifying the anxiolytic role of amygdalar NPY. For instance, NPY mutant animals exhibit higher ethanol preference as well as increased sensitivity to ethanol withdrawal-induced anxiety (366), and heterozygous *Npy* knockout rats from NP strains exhibit increased ethanol consumption relative to NP wildtype controls (367). Furthermore, human studies of *NPY* polymorphisms have strongly implicated *NPY* in both AUD and anxiety mechanisms, including levels of drinking and severity of withdrawal symptoms (23, 368-373).

Intricate mechanisms dictate the anxiolytic properties of NPY in the brain (130, 344). Interestingly, a moderately undefined relationship among NPY, the MC system components, and other stress neuropeptides has been recognized in modulating feeding, depression, and anxiety (120, 318, 319, 374, 375). Recently, an amygdalar mechanism involving NPY and the MC system has evolved in the literature (308, 345). *Pomc* and *Mc4r* mRNA is increased while *Npy* mRNA is decreased in the amygdala of rodents who are acutely stressed (332, 376). And as described previously, direct exogenous administration of α-MSH into the amygdala evokes anxiety in rodents while exogenous

administration of NPY or NPY Y_1 agonists reduce anxiety (329). Interestingly, the anxiolytic effects of exogenous administration of NPY or NPY Y_1 agonists into the amygdala can be significantly attenuated with pre-administration of α-MSH into the amygdala (329). Additionally, simultaneous administration of exogenous NPY or NPY Y_1 agonists with an MC4R antagonist produces a synergistic anxiolysis (329). Furthermore, heterozygous *Npy* knockout rats that exhibited increased anxiety and alcohol consumption, also expressed more *Pomc* throughout the brain while homozygous knockouts expressed significantly more *Mc4r* (367). Clearly, a relationship between the MC system and NPY exists, especially in the amygdala, but it has yet to be fully elucidated. Understanding this mechanism in full has lately garnered interest in the field of AUD research. For instance, a new study of adolescent alcohol exposure in rats showed that adults given intermittent ethanol access during adolescence expressed reduced NPY, increased MC4R, and increased α-MSH protein in the central and medial amygdala in addition to increased *Mc4r* mRNA in whole amygdala samples (308). These works indicate a need for exploration of amygdalar NPY in other molecular pathways in the context of AUD as more relevant mechanisms become elucidated.

6. Introduction to Glutamate Receptor Ionotropic, NMDA Type Subunits 2A and 2B

The NMDA receptor is a transmembrane ionotropic glutamate receptor that is activated by the binding of glutamate and glycine. Glutamatergic synapses mediate a significant portion of excitatory neurotransmission in humans, making postsynaptic NMDA receptors particularly important. The NMDA receptor in humans is constructed by the assembly of subunits known as GRIN1 (NR1), GRIN2A-D (GRIN2A-D), and GRIN3A-B (NR3A-B)

(377). In the amygdala, functional NMDA receptors exist as heteromeric complexes of the GRIN1 subunit with either GRIN2A or GRIN2B. Presently, NMDA receptors are believed to mediate persistent synaptic changes that confer long term shifts in behavior, such as through learning, memory, and fear (378). Though not fully understood, the prevailing theory suggests that synaptic activity that activates NMDA receptors initiates a neuronal calcium influx and therefore calcium-dependent downstream signaling, particularly those that coordinate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor phosphorylation, activation, and deposition onto dendritic membranes (378). AMPA receptors are another glutamate receptor, and these fluctuations in synaptic activity through the presence of NMDA and AMPA receptors facilitate the persistent changes in neuronal activity, downstream cascades, and gene transcription known (379). Notably, one such NMDA-mediated signaling cascade activates the transcription factor, CREB, which was described above and has a crucial role in synaptogenesis, dendritic spine density, and synaptic connectivity throughout the brain, including the amygdala (121, 196). However, the properties of GRIN2A and GRIN2B-containing receptor complexes exhibit very different molecular and phenotypic properties (380). These specific pathways are far from fully understood, but there are studies regarding the GRIN2B subunit in rodents that show overexpression of the GRIN2B subunit can facilitate heightened memory (381) and that neurons differing rations of GRIN2B to GRIN2A subunit-containing receptors have been shown to modulate CREB-dependent gene expression and synapses strength (382, 383).

7. The Role of Glutamate Receptor Ionotropic, NMDA Type Subunits 2A and 2B in Alcohol Use Disorder: Synaptic Connectivity

A prevailing theory regarding AUD posits that tenacious disruptions in learning and memory processes facilitate development and maintenance of AUD (384). Amygdala circuitry is believed to coordinate the relationship between learning/memory and anxiety (130, 385) and control negative affective traits that contribute to addiction (120, 196). Thus, synaptic mechanisms, particularly those within the mesolimbic system including the amygdala, are key molecular processes underlying the aberrant emotional circuitry of AUD (101, 149, 192, 199, 203, 206, 211, 249, 251, 253, 254). As described above, GRIN2A and GRIN2B are components of excitatory cellular regulators (NMDA receptors) that alter synaptic plasticity and dendritic spine formation (386-388), and the FDA-approved pharmacological treatment for AUD known as acamprosate functions as a mild NMDA receptor antagonist that has been shown to alter the NMDA receptor protein content within various brain regions (39, 52). As such, NMDA-mediated synaptic plasticity, including within the amygdala, is believed to regulate addiction (136, 384).

Preclinical studies support the rising theory that GRIN2A and GRIN2B play a particularly broad and important role in AUD development and associated phenotypes (279, 378, 389-391). In general, acute ethanol exposure inhibits NMDA receptor activity, including its activity-induced strengthening of synapses in neurons, while chronic exposure is presently believed to increase functionality and possibly the overall presence of GRIN2B-containing NMDA receptors (136). The specific nature of the direct or indirect interaction between ethanol and the receptors are not clear (392), though transmembrane domains

have been implicated in such a relationship based on studies where mutations altered NMDA receptor ethanol-sensitivity (304) and the general body of evidence suggesting ethanol targets transmembrane domains (304). NMDA receptors are believed to be particularly sensitive to the chronic effects of alcohol, resulting in increased excitation sensitivity secondary to activation-induced calcium influx (393). Alcohol-preferring P rats in a withdrawal state exhibit a significantly prolonged upregulation of GRIN2B protein expression in the CeA, likely due to compensatory upregulation secondary to the inhibitory function of ethanol on the receptors (394). Interestingly, the *GRIN2B* gene possesses an ethanol-responsive CREB binding site, and the chronic exposure and withdrawal-induced upregulation of GRIN2B is believed to be epigenetically regulated through histone and DNA methylation mechanisms (171, 395). Within the rodent amygdala, infusion of GRIN2A/B antagonists will disrupt development of stress-induced fear (396, 397), and amygdalar administration of downstream target inhibitors reduce self-administration of ethanol (398). Systemic NMDA receptor inhibitors also reduce relapse-like behaviors induced by the ethanol deprivation effect (399). Ultimately, preclinical studies implicate NMDA receptor mechanisms in a variety of AUD-associated maladaptive cellular processes. Such maladaptive processes can be apparent in recovering AUD patients long after abstinence and the conclusion of obvious withdrawal symptoms (400, 401). Much like NPY and MC system studies in humans, clinical investigations of NMDA receptor subunit genetic variation, expression, and dysregulation in the brain have highlighted intriguing associations with alcoholism (289, 402-405). Specifically, polymorphisms in the gene bodies and promoters of both the *GRIN2A* and *GRIN2B* genes have been strongly linked to AUD diagnosis as well as fear conditioning

(403, 406-409). Notably, multiple studies have been able to replicate these findings in independent populations, indicating a significant role across genetic populations, and some have specifically isolated a contribution to familial and early-onset pattern of alcohol intake and dependence (406-408).

Interestingly, epigenetic regulation of NMDA receptor expression has repeatedly been implicated in the mediation of effects of ethanol. For instance, an *in vitro* study of chronic ethanol exposure in cortical neurons of mice showed a significant reduction in inhibitory DNA methylation at the promoter of the *Grin2b* gene, which predictably correlated with upregulated GRIN2B protein expression (279). This protein and associated methylation pattern were later confirmed in a human study of AUD patients, such that GRIN2B expression was found to be higher in peripheral blood cells of humans during withdrawal after chronic ethanol exposure and correlated with DNA hypomethylation of the gene promoter. Furthermore, the degree of hypomethylation correlated with AUD phenotype severity (289). More recently, histone methylation has been implicated in ethanol-induced *Grin2b* gene regulation, such that *in vitro* neurons exposure to binge-like ethanol patterns exhibited altered H3K9me2 levels (171).

II. Study Design

(Portions of this chapter were adapted with permission from work previously published as: Berkel, T.D.M., Zhang, H., Teppen, T., Sakharkar, A.J., Pandey, S.C. (2019) Essential Role of Histone Methyltransferase G9a in Rapid Tolerance to the Anxiolytic Effects of Ethanol. Int J Neuropsychopharmacol*, 22(4):292-302.)*

A. Hypotheses and Specific Aims

1. Hypothesis 1: Alcohol-preferring P and alcohol non-preferring NP rats exhibit baseline differences in the expression of epigenetic methylomic components within the amygdala that may be operative in the alcohol drinking behaviors of P rats.

Specific Aim 1A: Explore the role of G9a in regulating gene expression of various anxiogenic, anxiolytic, and synaptic structure proteins within P and NP rat amygdala by determining mRNA and protein levels of G9a and H3K9me2, their occupancy at the promoters of specific genes of interest, as well as the mRNA expression and select protein levels of these genes in the amygdaloid structures.

Adult, male P and NP rat brains were collected for histological analysis within the CeA, MeA, and BLA. The mRNA expression of *G9a* in these regions was determined via *in situ* RT-qPCR. Protein levels of G9a, H3K9me2, α-MSH, and MC4r were determined via a histochemical gold immunolabeling procedure. A separate cohort of adult, male P and N rat brains were also collected, and the amygdala was dissected out for biochemical analysis. Chromatin Immunoprecipitation (ChIP) assay followed by real time qPCR in these tissues determined the occupancy of G9a and H3K9me2 at the promoter regions

of *Pomc*, *Mc4r*, *Grin2a*, *Grin2b*, and *Npy*. The mRNA expression of *Pomc*, *Mc4r*, *Grin2a*, and *Grin2b* was also determined in these tissues via RT-qPCR.

Specific Aim 1B: Determine DNA methylation at promoters of anxiogenic, anxiolytic, and synaptic structure-associated genes of interest; DNMT activity; DNMT gene expression; and DNMT protein expression in the amygdaloid structures of P and NP rats.

Adult, male P and NP rat brains were collected for histological analysis of the CeA, MeA, and BLA. Protein levels of DNMT1 and DNMT3b were determined via a histochemical gold immunolabeling procedure. A separate cohort of adult, male P and NP rat brains were collected, and the amygdala was dissected out for biochemical analysis of DNMT activity via a DNMT activity kit, mRNA expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* via RT-qPCR, and DNA methylation at the promoter regions of various genes of interest via a methylated double-stranded DNA enrichment kit (MBD-based) followed by RT-qPCR.

2. **Hypothesis 2: G9a, a histone methyltransferase that possibly interacts with DNA methyltransferases and facilitates methylation of both histone H3 lysine 9 and DNA, regulates the high alcohol drinking behaviors of P rats, and treatment with a DNA methyltransferase inhibitor will reduce ethanol intake and preference in P rats by altering the G9a occupancy levels on anxiety-related genes in the amygdala.**

Specific Aim 2: Investigate the effects of treatment with a DNMT inhibitor on the alcohol drinking behaviors of P rats as well as the P rat G9a occupancy at promoters of anxiogenic, anxiolytic, and synaptic structure-related genes of interest in the amygdala.

A group of adult, male P rats were IP injected with either vehicle (DMSO:n-saline) or 5-azacytidine (5-aza; 1mg/kg) once daily for four days after acclimation to increasing ethanol percentage via a two-bottle free-choice paradigm in their home cage. Ethanol and water intake were monitored daily before, during, and after the four days of treatment, and fresh solutions were provided daily between 5:00 and 6:00 PM. A separate cohort of adult, male P rats received IP administration of 5-aza once daily for four days, and the amygdala was dissected out two hours after the final injections for G9a ChIP assay at various genes.

3. **<u>Hypothesis 3: G9a-mediated epigenetic regulation within the amygdala modulates the rapid tolerance to the anxiolytic effect of alcohol and is thereby involved in the development of alcohol use disorder.</u>**

Specific Aim 3A: Determine G9a and H3K9me2 levels in the amygdala, both globally and specifically along *Npy*, in a model of rapid tolerance to the anxiolytic effects of alcohol.

Adult, male Sprague-Dawley rats were used to establish the RET model (105). The Saline group was administered two n-saline IP injections 24 hours apart. The Ethanol group was administered one IP n-saline injection followed by an acute 20% (w/v) ethanol (1g/kg) injection in n-saline 24 hours later. The Tolerance group received two IP injections of ethanol (1g/kg) in n-saline 24 hours apart. Anxiety-like behavior was assessed via light/dark box (LDB) exploration one hour after the final ethanol or saline injections, as previously described (105). Atrial blood was sampled for BAC determination at time of sacrifice after behavioral experiments were completed. A subset of brains was extracted

for immunohistochemical review of global G9a and H3K9me2 protein levels in the CeA, MeA, and BLA. The amygdala was dissected out of a separate subset for ChIP analysis of G9a and H3K9me2 occupancy along *Npy*.

Specific Aim 3B: Examine the effect of G9a inhibition on rapid tolerance to the anxiolytic effects of alcohol and global amygdalar expression of G9a, H3K9me2, and NPY.

Adult, male Sprague-Dawley rats were used to establish the RET model (105) and were additionally administered two IP injections (DMSO:PBS) of vehicle control (Saline + Vehicle, Ethanol + Vehicle, and Tolerance + Vehicle). The G9a inhibitor, UNC0642, was administered twice via IP injection (2.5mg/kg each) to a control group and a tolerance group (Saline + UNC0642 and Tolerance + UNC0642). The inhibitor was administered 6 hours and 23 hours after the first saline or ethanol injections. Anxiety-like behavior was measured via LDB one hour after the final saline or ethanol injection, and brains were collected for histological analysis of the CeA, MeA, and BLA. Protein levels of G9a, H3K9me2, and NPY were determined via histochemical gold immunolabeling procedure.

B. Methods and Materials

1. Experiments with Alcohol-preferring P and Alcohol Non-preferring NP Rats

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 from generations 79-81 were

received from the Indiana Alcohol Research Center at Indiana University, Indianapolis, IN and housed within a 12 h light/dark cycle, with *ad libitum* access to food and water unless otherwise noted. Rats were age and weight-matched to ensure consistency between experiment groups. Rats weighing approximately 300g were used. All animals were anesthetized and sacrificed with a combination of isoflurane and either decapitation or perfusion followed by decapitation. Extracted brain tissues were stored at -80^{o}C.

Two-bottle free choice paradigm with or without 5-azacytidine treatment

Voluntary ethanol intake behaviors were evaluated using the two-bottle free choice paradigm as described previously (205, 355). Adult, male P rats were administered either 5-aza or vehicle IP injections to test the effect of 5-aza on P rat alcohol preference. First, P rats were habituated to drink water equally from two identical bottles at symmetrical locations. Once they consumed water equally from both bottles, rats were familiarized with ethanol (w/v) consumption by simultaneously being given one 50mL bottle of water and one 50mL bottle of ethanol daily. Furthermore, ethanol versus water bottle positioning was alternated every 24 hours to prevent preference for bottle location. For this, the ethanol was 3% for 3 days, 7% for 3 days, and 9% for 3 days. 9% ethanol was provided thereafter. After the third day of 9% ethanol drinking, adult male P rats were administered 1mg/kg IP 5-aza or equivalent volume of control vehicle every 24 hours for four days. Notably, 1mg/kg dose of systemic 5-aza has been reported to effectively reduce binge drinking and alcohol preference in post-dependent mice (410). The drug was dissolved in DMSO to 5mg/mL and subsequently diluted to 1mg/mL in sterilized n-saline. After the

fourth day of injections, animals received no further treatments and maintained access to 9% ethanol and water for three days post-treatment to examine effects.

Animals were sacrificed via isoflurane and subsequent decapitation three days after the experimental injections were completed. Throughout habituation, experimentation, and recovery, the consumption of alcohol and water was recorded at the same time each day before replacing with fresh contents and alternating the bottle positions. Body weight of rats was also recorded throughout and used to measure alcohol intake (g EtOH/kg body weight). Alcohol preference was also recorded (mL EtOH intake/mL total fluid intake).

Gold Immunolabeling for Protein Measurements

In multiple experiments, gold-labelling immunohistochemistry was utilized to determine protein expression levels, as described previously (97, 206). Animals were anesthetized with isoflurane and then perfused with n-saline followed by paraformaldehyde (4% w/v in 0.1M phosphate buffer). Perfused brains were then set in PFA for 24 hours, followed by graduating sucrose solutions (10%, 20%, and 30% w/v in 0.1M phosphate buffer). The brains were then frozen in -20^{o}C to -40^{o}C 2-methylbutane, prepared to divide regions of interest, and subsequently stored at -80^{o}C until the immunolabeling process was initiated.

To initiate brain processing, tissues were brought to -20^{o}C, cut into 20 µm thick bregma-matched coronal slices that contained amygdala and collected in 0.01M Phosphate buffered saline (PBS). At room temperature, samples were then rinsed in PBS briefly, in RPMI 1640 with L-glutamine medium (Invitrogen, Grand Island, NY) for 30 minutes, in

10% normal goat serum (Vector Labs, Burlingame, CA) in 0.25% Triton-X 100 in PBS (PBST) for 30 minutes, and in 1% bovine serum albumin (BSA) in PBST (BSA/PBST) for 30 minutes. Samples were then set in appropriate primary antibody diluted in BSA/PBST (Table I) overnight. The following day, samples were rinsed in PBS and blocked in 1% BSA in 0.01M PBS (BSA/PBS) for 30 minutes. Samples were then incubated in 1:200 gold particle-conjugated anti-rabbit secondary antibody (Nanoprobes, Yaphank, NY) in BSA/PBS. After incubation, samples were rinsed briefly in BSA/PBS then distilled water. Samples were then developed in 1:1 silver enhancement solution (Ted Pella, Redding, CA) and rinsed in tap water. Finally, samples were mounted on slides, dehydrated, and covered with glass coverslips.

Table I. Primary Antibodies used for gold immunolabeling

<table>
<tr><th>Antibody</th><th>Source</th><th>Dilution</th><th>Secondary</th></tr>
<tr><td>Anti-Di-Methyl-Histone H3 (Lys 9) (9753S)</td><td>Cell Signaling, Beverly, MA, USA</td><td>1:500</td><td rowspan="8">Anti-rabbit</td></tr>
<tr><td>Anti-DNMT1 (210513)</td><td rowspan="2">US Biological, Salem, MA, USA</td><td rowspan="5">1:200</td></tr>
<tr><td>Anti-DNMT3B (222209)</td></tr>
<tr><td>Anti-EHMT2/G9a (ab31874)</td><td>Abcam, Cambridge, UK</td></tr>
<tr><td>Anti-MC4R (orb214232)</td><td rowspan="2">Biorbyt, San Francisco, CA, USA</td></tr>
<tr><td>Anti-MSH alpha (orb13589)</td></tr>
<tr><td>Anti-NPY (22940)</td><td>Immunostar, Hudson, WI, USA</td><td>1:500</td></tr>
</table>

Protein levels in the samples were calculated using the Image Analyzer software in conjunction with a light microscope. After determining the appropriate threshold for non-immunostained amygdalar regions to read negatively, the number of immunogold-labeled particles per 100μm^2 of amygdala area was determined at high magnification (100x). The CeA, MeA, and BLA labeling was independently calculated for each animal via 9 total object fields per region (three fields in 3 separate slices) and then averaged and reported as mean ± SEM of gold articles/100 μm^2 for each experimental group.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was used to determine the relative occupancy of proteins at certain genes between experimental groups, including baseline alcohol-naïve P versus NP rats or P rats that had been treated with IP 5-aza or vehicle. As described above, rats were decapitated after anesthetization with isoflurane. In the case of the 5-aza treatment experiments, rats received a daily injection of 5-aza (described above) for four days and were sacrificed two hours after the last injection. For both the baseline and 5-aza experiments, the amygdala was immediately dissected out after sacrifice and stored at -80^oC.

Weighed amygdala samples were processed via a previously described ChIP protocol (223, 411). After homogenization, sample DNA and associated proteins were cross-linked in a 1% final concentration formaldehyde solution at 37^oC for 5 minutes. The reaction was quenched with 1.0M glycine, which was removed after centrifugation. Cells were then lysed, and components were separated via centrifugation. The resulting DNA-chromatin

complexes were sheared via 5 minutes of sonication at 5% duty factor in a Covaris M220 Focused-ultrsonicator Instrument (Covaris, Woburn, MA), resulting in fragments of approximately 500 base pairs. The chromatin fragments were then immunoprecipitated using antibodies to various DNA-binding proteins or IgG negative control (Table II) with total sample input controls. The resulting immunoprecipitates were collected via protein A/G plus-agarose beads (Santa Cruz, Santa Crus, CA, USA) while the input controls were isolated via ethanol solutions. Both immunoprecipitates and controls were then boiled for 10 minutes in 10% final Chelex solutions (Bio-rad Berkeley, CA, USA). The resulting contents were then centrifuged, allowing for isolation of DNA fragments for real-time quantitative PCR analysis in a CFX Connect PCR instrument (Bio-rad, Berkeley, CA, USA) with appropriate primers (Table III) and SYBR Green master mix (Bio-rad, Berkeley, CA, USA). Data was analyzed via the ΔΔCt method (412). Values were reported as mean fold change ± SEM, relative to control groups, normalized to the sample input.

Table II. Antibodies used for chromatin immunoprecipitation assay

Antibody	Source	µg Utilized
Anti-EHMT2/G9a – ChIP Grade (ab40542)	Abcam, Cambridge, UK	4
Anti-Histone H3 (di methyl K9) antibody – ChIP Grade (ab1220)	Abcam, Cambridge, UK	4
Anti-IgG	Abcam, Cambridge, UK	1

***In situ* RT-PCR**

In situ RT-PCR was utilized to measure mRNA expression levels in amygdala regions of adult P and NP rat brains, as described previously by our lab (97, 355). The brains were

perfused, extracted, protected, and then stored at -80ºC identically to the protein immunolabelling procedure (see above) except that solutions were treated with 0.1% diethylpyrocarbonate (DEPC) in distilled water at 37ºC to inactivate RNase enzymes and later autoclaved to inactivate DEPC. To begin processing, brains were warmed to -20ºC, cut into 40µm coronal sections, collected in DEPC/PBS, and treated with proteinase K (1µg/mL) in 0.05% PBST at 37ºC for 15 minutes. To quench the reaction, 0.1M glycine in PBST was used, followed by an overnight DNase digestion (Promega, Madison, WI) at 37ºC. Samples were then individually placed in PCR tubes containing 100uL reverse transcription solutions (Applied Biosystems, Foster City, CA, USA) at 42ºC for 1 hour. Samples were then relocated to fresh PCR tubes containing 100pmol of *G9a* mRNA primers (Table III) and digoxigenin-11-dUTP (Roche Diagnostics, Indianapolis, IN, USA), and cycled appropriately. After washing the samples in DEPC/PBS, the sections were mounted on gelatin-coated slides and dried. They were washed in DEPC/PBS and blocked in a BSA-based buffer for 10 minutes. The slides were incubated in anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics, Indianapolis, IN, USA) at 4ºC overnight. The slides were stained via nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (Roche Diagnostics, Indianapolis, IN, USA). Optical density (OD) per 100-pixel area was determined at 20x magnification for the CeA, MeA, and BLA separately via analysis of three fields in three sections (9 fields total) per amygdala brain region. Each animal's OD/100-pixel area for each region was reported as the mean ± SEM for each experimental group and brain region.

Table III. Primers used for qPCR and *in situ*, ChIP, and DNA methylation RT-PCR

Primer	Utilization	Sequence (F=forward, R=reverse)
G9a	In situ	F: 5' GCAGACGTGAGAGAGGATGA 3' R: 5' TGTCCCTGGAGCTGAAGAAG 3'
Grin2a	ChIP and DNA methylation	F: 5' CTGTCCGGAGTGGAACAGAAA 3' R: 5' CCGCGACTCTCAGACCTCAT 3'
Grin2b		F: 5' GGGCTTGGCTCAATGGAGAA 3' R: 5' TCAGTCTCTCGGGTTGGGAA3'
Mc4r loc 1		F: 5' GTTGGTCAGCTCAAGACGGA 3' R: 5' TACACATTGGGCCACCTTCC 3'
Mc4r loc 2		F: 5' GTTCCCCACAGCATACCCAT 3' R: 5' AAAAGCACTCTGTCCTGGCT 3'
Npy loc a		F: 5' ACGCACGCACCTCATTTA3' R: 5' GGCTCTGTGATTAGGGCTTTAT 3'
Npy loc b		F: 5' GTTAGAGGAGGGTTGCTTCTATG 3' R: 5' ACATGCTGTCATTCTCCGATAC 3'
Npy loc c		F: 5' AGTAGGTCCAGTAGGTCCAGTAGGT 3' R: 5' GAAGCAGTCGAGCAAGGTTTT 3'
Npy loc d		F: 5' CCAAGTCTGAGCCTTCTGTATC 3' R: 5' AAACACACGAGCAGGGATAG 3'
Npy loc e		F: 5' CTCTTAACCACTGAGCCATCTT 3' R: 5' CAACAACCAACGAGCCAATC 3'
Pomc		F: 5' GGTGCTCTGAAGCAAGACCA 3' R: 5' CCACGTACCAGGAAGGAACC 3'
Grin2a	mRNA	F: 5' CCCATTGCATCCTCCACCTTCTC 3' R: 5' GGCCCCACAGATTTCGAAGTTCG 3'
Grin2b		F: 5' GCGAGAAGAGGACCCTGGATATTCC 3' R: 5' GGAACGAGCTTTGCTGCCTGATAC 3'
Hprt1		F: 5' TCCTCAGACCGCTTTTCCCGC 3' R: 5' TCATCATCACTAATCACGACGCTGG 3'
Mc4r		F: 5' TGTCATCATCTGCCTCATTACC 3' R: 5' GAGGACAGCGATCCTCTTAATG 3'
Pomc		F: 5' TCCTCAGAGAGCTGCCTTTC 3' R: 5' AGCGACTGTAGCAGAATCTCG 3'
Dnmt1		F: 5' AAGCCAGCTATGCGACTTGGAAAC 3' R: 5' ACAACCGTTGGCTTTCTGAGTGAG 3'
Dnmt3a		F: 5' CACCTACAACAAGCAGCCCATGTA 3' R: 5' AGCCTTGCCAGTGTCACTTTCATC 3'
Dnmt3b		F: 5' TGTGCAGAGTCCATTGCTGTAGGA 3' R: 5' GCTTCCGCCAATCACCAAGTCAAA 3'

Quantitative RT-PCR

PCR was utilized to determine gene expression levels. Total RNA was isolated from amygdala tissue (extracted and stored as described above) with Trizol (Life Technologies, Grand Island, NY, USA). RNA purification was then performed with an RNeasy mini kit (Qiagen, Valencia, CA, USA) along with subsequent product quantification. Aliquots of purified RNA were then reverse transcribed with random primers and MulV reverse transcriptase (Life Technologies) in 20uL reaction solutions. Using SYBR green master mix (Fermentas, Glen Burnie, MD, USA) and appropriate primers (Table III), RT-PCR and associated analysis was performed in a Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA) with MxPro software. In all RT-PCR analyses, *Hprt1* (hypoxanthine-guanine phospho-ribosyltransferase) was used as the reference gene, and the reaction conditions were as follows: 10 minutes at 95^{o}C, followed by 40 cycles of 30 seconds at 95^{o}C, 1 minute at the appropriate annealing temperature (determined by melting temperature of primer pair, approximately 58^{o}C), and 1 minute at 72^{o}C. Relative mRNA expression levels were calculated via the ΔΔCt method (412) using *Hprt1* as the control gene. Values were reported as mean fold change ± SEM, relative to control groups.

DNA methylation

Amygdalar tissue was extracted from rats immediately after sacrifice (see above), and DNA was further isolated using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). DNA was then fragmented via sonication with five 20 second pulses at 25% duty factor with 30 second intervals via the Covaris M220 Focused-ultrasonicator Instrument

(Covaris, Woburn, MA), resulting in DNA fragments of approximately 200-500 base pairs. Utilizing the Methylminer Methylated DNA Enrichment Kit (Life Technology, Carlsbad, CA, USA), methylated double-stranded DNA was precipitated, opposed to unprecipitated input controls. This was accomplished by facilitating the binding of methylated double-stranded DNA to methyl-CpG binding domains of human MBD2 protein coupled to magnetic Dynabeads M-280 Streptavidin, followed by sodium chloride elution and ethanol purification. The resulting methylated double-stranded DNA was then assayed via RT-qPCR analysis in a CFX Connect PCR instrument (Bio-rad, Berkeley, CA, USA) with appropriate primers (Table III) and SYBR Green master mix (Bio-rad, Berkeley, CA, USA). Data was analyzed via the ΔΔCt method (412). Values were reported as mean fold change ± SEM, relative to control groups, normalized to the sample input.

DNA methyltransferase enzyme activity

Amygdalar tissues were extracted from rat brains immediately after sacrifice (see above) and stored at -80^{o}C. For processing, the tissues were homogenized in lysis buffer containing protease inhibitors. The resulting homogenate was centrifuged to isolate the nuclear fraction, and the protein yield was determined via the Modified Lowry Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). DNMT activity in the resulting nuclear protein was then evaluated as O.D. following the use of the EpiQuik DNA Methyltransferases Activity/Inhibition Assay Kit (Epigentek, Brooklyn, NY, USA). We analyzed 10µg of each sample and measured O.D. with the Spectra MR plate reader (Dynex Technologies, Chantilly, VA, USA) at a wavelength of 450nm. The results were calculated and reported as mean ± SEM O.D./mg protein/hour of reaction.

Statistical analyses

For experiments consisting of two groups, the statistical difference between the groups was determined via Student's t-test. For evaluating the effect of IP injections of 5-azacytidine on ethanol preference in P rats (before, during, and after injections), the statistical differences between the groups and treatment status were evaluated by a two-way ANOVA test and subsequent *post hoc* comparisons using Tukey's test. For the evaluation of alcohol drinking behavior with the two-bottle free choice paradigm, two-way repeated measures ANOVA was used, followed by *post hoc* Tukey's test analysis. Throughout, $p<0.05$ was considered statistically significant.

2. Experiments with the Rapid Ethanol Tolerance Model

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 Sprague-Dawley rats weighing approximately 300g were used. Animals were housed under a 12 h light/dark cycle, with *ad libitum* access to food and water unless otherwise noted. Rats were age-matched or weight-matched to ensure consistency between groups. All animals were anesthetized and sacrificed with a combination of isoflurane and either decapitation or perfusion followed by decapitation. Extracted brain tissues were ultimately stored at -80^{o}C.

Establishment of rapid ethanol tolerance model

To develop the model for rapid ethanol tolerance (RET), we executed a previously reported paradigm (100, 105), wherein a sequence of two n-saline, ethanol, or

combinatorial IP injections is provided over the course of two days. On the first day, animals in the Saline control group and Ethanol group received n-saline (5ul/g of body weight) while the Tolerance group received 1g/kg of ethanol (20% w/v in n-saline solution). On the second day, 24 hours after the first injection, control Saline group animals received another n-saline injection while the Ethanol and Tolerance groups received ethanol injections (1g/kg using 20% w/v solution in n-saline) identical to the ethanol provided on the first day to the tolerance group.

Blood alcohol concentration determination

Right atrial blood from RET model rats was collected into microcentrifuge tubes and stored on ice at time of sacrifice. Tubes were centrifuged before 5uL of plasma was added to an AM-1 Analox Alcohol Analyzer (Analox Instruments, Lunenberg, MA). The device reported BAC (mg/dL) based on oxygen levels produced by alcohol oxidase.

Light/dark box exploration test

The light/dark box (LDB) exploration test consists of a compartmentalized box, with a brightly lit space connected to a smaller darker space (413). Individual rats are habituated for 5 minutes to the testing facility before being set in the dark space apart from the light space connection. Once inside, the movement of the rats is recorded via infrared light sensors for 5 minutes. In the RET model, animals are tested in the LDB one hour after the second administration of n-saline/ethanol. Final data is reported as mean ± SEM of the percentage of time spent in each compartment, where time spent in the dark compartment correlates with relatively lower anxiety. Total ambulations recorded by the

device suggest general activity of the rat and are reported as mean ± SEM of total ambulations.

Light/dark box paradigm with or without UNC0642 treatment

To test the effects of UNC0642 (Sigma Aldrich, St. Louis, MO, USA) treatment on the anxiety-like behaviors in the RET model, we intraperitoneally administered 2.5mg/kg of UNC0642 (dissolved in DMSO at 5mg/mL and diluted in PBS to 1:7) both 6 and 23 hours after the first day of n-saline/ethanol provision to a Saline group and a Tolerance group. A Saline group, Ethanol group, and Tolerance group received vehicle injections (DMSO:PBS, 1:7 dilution). Finally, LDB was utilized 1 hour after the final administration of n-saline/ethanol to test anxiety-like behaviors, as described above.

Chromatin immunoprecipitation and protein immunolabeling

ChIP and protein immunolabeling were completed as described above. Relevant antibodies and primers are listed in Tables I-III.

Statistical analyses

Behavioral and immunohistochemistry data were analyzed with one-way ANOVA followed by *post-hoc* comparisons using Tukey's test. ChIP fold change was analyzed via the nonparametric Kruskal-Wallis test followed by *post hoc* Dunn's test. Significance for all experiments was set at $p<0.05$.

III. RESULTS

(Portions of this chapter were adapted with permission from work previously published as: Berkel, T.D.M., Zhang, H., Teppen, T., Sakharkar, A.J., Pandey, S.C. (2019) Essential Role of Histone Methyltransferase G9a in Rapid Tolerance to the Anxiolytic Effects of Ethanol. Int J Neuropsychopharmacol*, 22(4):292-302.)*

A. Hypothesis 1: Alcohol-preferring P and alcohol non-preferring NP rats exhibit baseline differences in the expression of epigenetic methylomic components within the amygdala that may be operative in the alcohol drinking behaviors of P rats.

1. Overview

Alcohol-preferring (P) and non-preferring (NP) rats are bidirectionally bred from Wistar stock for alcohol preference or non-preference, respectively. P rats exhibit innate anxiety-like behaviors, which in humans is known to predispose individuals to alcohol use disorder (AUD) (11, 16, 31). Several molecular mechanisms involving synaptic plasticity and stress pathways in the amygdala have been shown to alter anxiety or addiction phenotypes (30, 101). The histone methyltransferase (HMT), G9a, has recently emerged as a potentially crucial regulator of anxiety and addiction by altering gene expression in the nucleus accumbens (NAc) and forebrain via histone H3 lysine 9 (H3K9) methylation (170, 171, 248, 259, 269). Interestingly, DNA methylation via DNA methyltransferases (DNMTs) is a separate but related epigenetic mechanism that is believed to possibly function in concert with G9a to downregulate gene expression (233, 235, 414).

To determine the baseline differences in methylomic epigenetic components within the amygdala of P and NP rats, we analyzed the mRNA expression of *G9a*, DNA

methyltransferase 1 (*Dnmt1*), *Dnmt3a*, and *Dnmt3b* in alcohol-naïve adult, male P and NP rats. We then confirmed protein level differences for the genes that showed differential mRNA expression. Global levels of dimethylated H3K9 (H3K9me2) and DNMT activity in the amygdala were also investigated. We determined mRNA expression of several genes known to regulate AUD—pro-opiomelanocortin (*Pomc*), melanocortin 4 receptor (*Mc4r*), glutamate receptor ionotropic, NMDA type subunits 2A and 2B (*Grin2a* and *Grin2b*), and neuropeptide Y (*Npy*). To determine if changes in *G9a*, *Dnmt1*, and *Dnmt3b* might regulate these genes in the amygdala of P and NP rats, chromatin immunoprecipitation (ChIP) with G9a and H3K9me2 antibodies as well as DNA methylation assays were performed at their promoter regions. We also investigated select protein expression [the POMC cleavage product, alpha-melanocyte-stimulating hormone (α-MSH), and MC4r] in the amygdala of P and NP rats. We found P rat amygdala to exhibit higher *G9a*, *Dnmt1*, and *Dnmt3b* mRNA expression in the amygdala relative to NP rats. We also found higher G9a, DNMT3b, and H3K9me2 protein levels in the central and medial nuclei of the amygdala (CeA and MeA) as well as higher DNMT activity in the amygdala of P rats. Furthermore, we found differential occupancy of G9a, H3K9me2, and/or DNA methylation at the promoters for *Pomc*, *Mc4r*, *Grin2a*, *Grin2b*, and *Npy* in P versus NP rats. Notably, gene expression and associated protein levels of these target genes within the amygdala was accurately predicted by the epigenetic differences determined by ChIP and DNA methylation assays. These findings suggest innate methylomic epigenetic components may lead to aberrant gene expression patterns that may be critical to regulation of P rat anxiety and alcohol drinking behaviors.

2. Innate differences in G9a and H3K9me2 levels between P and NP rat amygdala.

Our previous studies comparing the amygdala of P and NP rats have reported an innate difference in acetylated H3K9 (H3K9ac) secondary to aberrant histone deacetylase (HDAC) levels, particularly within the CeA and MeA, that significantly contribute to the P rat phenotype (97, 206). Similarly, other labs have elucidated mechanisms involving G9a-induced histone methylation in addiction and anxiety phenotypes in separate, but related brain regions (167, 168, 248, 269). We extended these studies and examined the innate differences in expression of G9a and associated H3K9me2 in the amygdala of P and NP rats. It was found that mRNA and protein expression of G9a are higher in the CeA and MeA (*G9a* CeA and MeA mRNA, $p<0.01$, $t_{(8)}=-4.6$; G9a CeA protein, $p<0.05$, $t_{(8)}=-2.4$; G9a MeA protein, $p<0.01$, $t_{(8)}=-4.2$), but not the basolateral amygdala (BLA), as compared with NP rats (Figure 5). Similarly, H3K9me2 protein was higher in the CeA and MeA (CeA, $p<0.001$, $t_{(10)}=-6.4$; MeA, $p<0.001$, $t_{(10)}=-5.5$) but not the BLA (Figure 5) of P rats when compared to NP rats. These findings suggest G9a may be dysregulated in the P rat CeA and MeA, thereby altering the inhibitory H3K9 dimethylation signature and associated gene expression.

3. G9a and H3K9me2 occupancy at promoters of *Pomc*, *Mc4r*, and *Npy* and their associated expression in the amygdala of P and NP rats

Diverse mechanisms involving the amygdala can regulate anxiety and addiction. For instance, melanocortin (MC) mechanisms involving POMC and its downstream cleavage product receptor, MC4r, can facilitate anxiogenic mechanisms via pathways within the amygdala (332). Conversely, higher amygdaloid NPY is anxiolytic (349, 354) and possibly

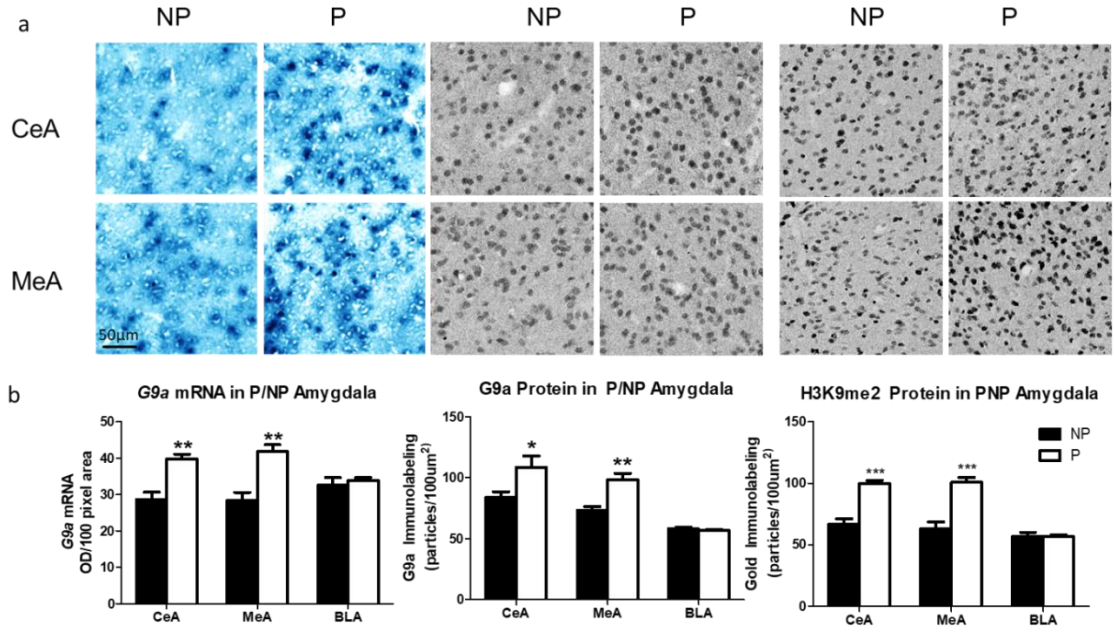


Figure 5. P and NP rats exhibit baseline differences in G9a mRNA and protein expression as well as H3K9me2 levels in amygdaloid structures.

a. Low-magnification micrographs of G9a and H3K9me2 protein gold immunolabeling and in-situ PCR (G9a positive cells) mRNA in the central (CeA) and medial (MeA), nuclei of the amygdala in alcohol preferring (P) and non-preferring (NP) rats.

b. Quantitative analysis of *G9a* mRNA and G9a and H3K9me2 protein in P and NP amygdaloid structures. *G9a* mRNA (optical density per 100pixel area) and protein levels (number of immunogold particles/100µm^2 area) are higher in the CeA and MeA, but not the BLA, of the P rats compared to NP rats. Values are mean ± SEM of 5-6 rats in each group. (*p<0.05, **p<0.01, ***p<0.001; Student's t-test).

interacts with the MC mechanisms (367). Notably, mechanisms involving NPY, MC4r, POMC, and the predominant cleavage product of POMC, alpha-melanocyte stimulating hormone (α-MSH), in the amygdala have been shown to modulate alcohol intake, likely due to documented relationships with anxiety regulation (341, 367). Here, we show that P rats innately express higher mRNA levels of *Pomc* (p<0.05, $t_{(8)}$=-3.0) and *Mc4r* (p<0.05, $t_{(8)}$=-2.6) in the amygdala relative to NP rats (Figure 6). Our lab and others have previously revealed lower levels of *Npy* expression in the CeA and MeA of P rats relative to NP rats

(96, 97, 205, 358). We also measured protein expression of α-MSH and MC4r and found both to be higher in CeA and MeA, but not the BLA (α-MSH in CeA, $p<0.001$, $t_{(8)}=-5.5$; α-MSH in MeA, $p<0.01$, $t_{(8)}=-4.5$; MC4r in CeA, $p<0.01$, $t_{(10)}=-4.6$; MC4r in MeA, $p<0.05$, $t_{(10)}=-3.1$), of P rats relative to NP rats (Figure 6). These findings suggest a potential dysregulation of amygdalar MC and NPY pathways may be involved in P rat phenotypes.

G9a-mediated H3K9me2 occupancy at a gene promoter represses gene expression (415). Because of the innately differential expression of amygdalar G9a, H3K9me2, *Pomc*, *MC4r*, and *Npy* between P and NP rats, we used chromatin immunoprecipitation (ChIP) to quantify G9a and H3K9me2 occupancy at the promoters of *Pomc* and *Mc4r* and verify the potential epigenetic influence of G9a-mediated H3K9me2 on their expression. The results showed that P rats exhibit higher G9a occupancy at the *Npy* promoter ($p<0.05$, $t_{(16)}=-2.7$) and lower G9a occupancy at the *Pomc* and *Mc4r* promoters [*Pomc,* $p<0.05$, $t_{(9)}=2.3$; *Mc4r loc 1* (-488 base pairs upstream of transcription start site), $p<0.05$, $t_{(10)}=3.1$; *Mc4r loc 2* (-1317 base pairs upstream of transcription start site), $p<0.05$, $t_{(9)}=2.4$] (Figure 7). H3K9me2 occupancy was also lower at *Pomc* and *Mc4r* promoters (*Pomc* $p<0.05$, $t_{(8)}=2.5$; *Mc4r loc 1*, $p<0.01$, $t_{(8)}=4.7$; *Mc4r loc 2, p*<0.05, $t_{(10)}=3.0$), but unchanged at *Npy* (Figure 7). The dissimilar inhibitory G9a occupancy at these loci in P and NP rat amygdala appropriately correlates with respective gene expression and suggests that G9a inhibitory epigenetic mechanisms, including H3K9me2, may contribute to the dysregulation of anxiolytic and anxiogenic molecular pathways within the amygdala that influence the anxiety-like and alcohol-drinking P rat phenotypes.

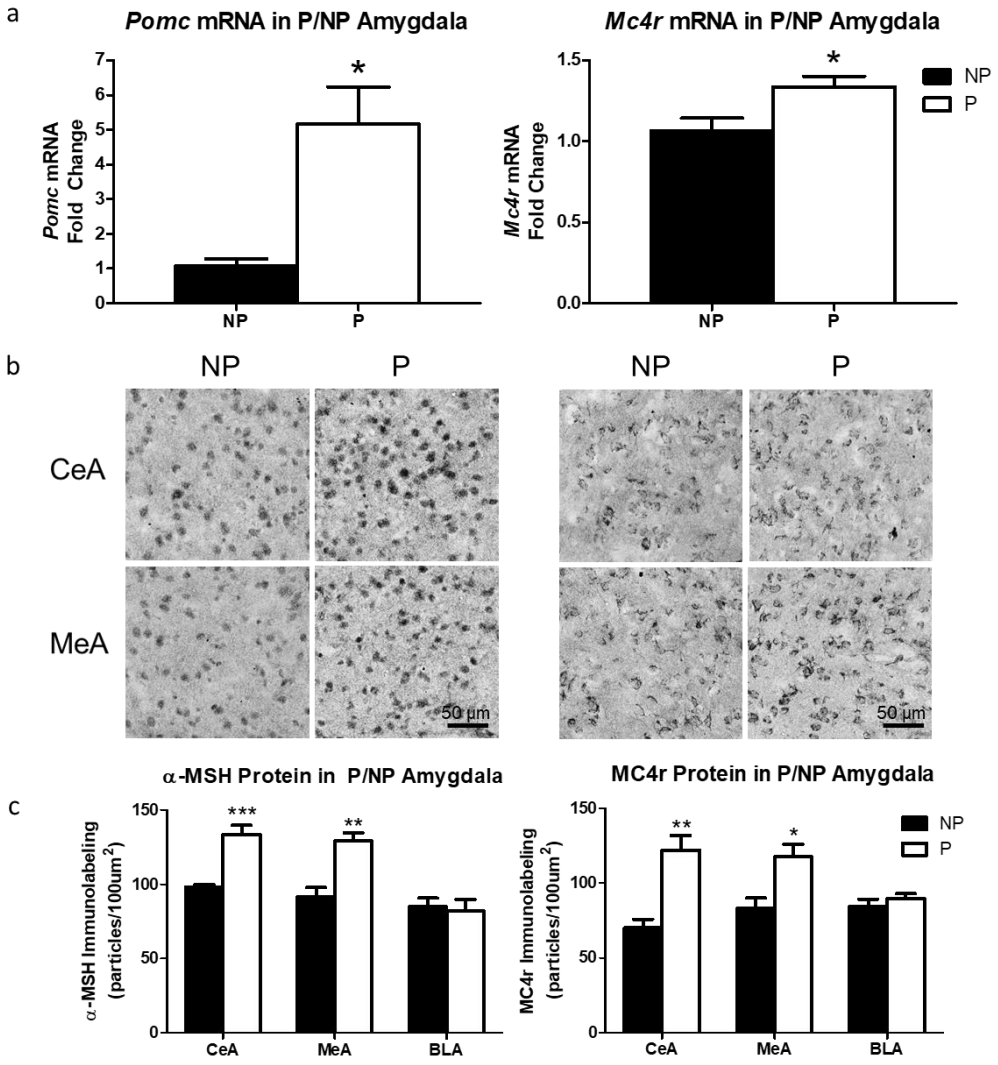


Figure 6. P rats exhibit higher amygdaloid pro-opiomelanocortin (*Pomc*) and melanocortin 4 receptor (*Mc4r)* mRNA and higher protein levels of MC4r and the downstream POMC cleavage product, alpha-melanocyte stimulating hormone (α-MSH) than NP rats.

a. Quantitative analysis of RT-PCR in alcohol-preferring (P) and alcohol non-preferring (NP) rat amygdala. P rat amygdala express a significantly higher expression of *Pomc* and *Mc4r* mRNA (fold change of mRNA) relative to NP rats. Values are mean ± SEM of 4-6 rats in each group. (**p*<0.05, via Student's t-test)
b. Low-magnification microphotographs of gold immunolabeling of MC4r and the POMC cleavage product, alpha-msh (α-MSH) within the central (CeA) and medial (MeA) nuclei of the amygdala of P and NP rats.
c. Quantitative analysis of α-MSH and MC4r protein in the amygdaloid structures of P and NP rats. These findings show that α-MSH and MC4r protein expression (number of immunogold particles/100µm^2 area) is higher in the CeA and MeA, but not the basolateral amygdala (BLA) of P rats compared to NP rats. Values are mean ± SEM of 5-6 rats in each group. (**p*<0.05, ***p*<0.01, ****p*<0.001; Student's t-test)

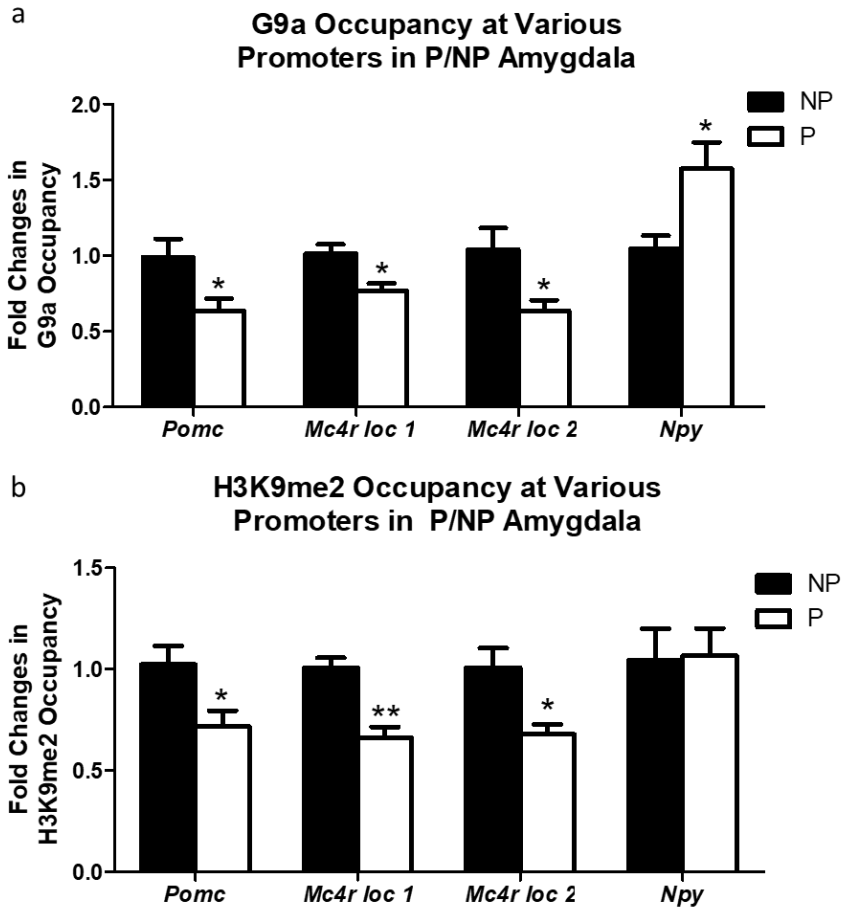


Figure 7. Repressive G9a and H3K9me2 protein occupancy at the promoters of *Pomc*, *Mc4r*, and *Npy* genes in the amygdala differs between P and NP rats.

a. Chromatin immunoprecipitation (ChIP) assay of repressive G9a occupancy at promoter regions of *Pomc*, *Mc4r*, and *Npy* genes in P and NP amygdala. G9a occupancy was found to be significantly lower at the *Pomc* and *Mc4r* promoters and significantly higher at the *Npy* promoter in P rats compared to NP rats. This accurately correlates to the observed higher expression of *Pomc* and *Mc4r* mRNA in P rats versus NP rats presented in Figure 6. Additionally, higher G9a occupancy at the *Npy* promoter correlates with previously published lower *Npy* mRNA expression in the amygdala of P rats versus NP rats (205). Values are mean ± SEM of 5-9 rats in each group. (*p<0.05; via Student's t-test)

b. ChIP assay of repressive H3K9me2 occupancy at promoter regions of the *Pomc*, *Mc4r*, and *Npy* genes in P and NP amygdala. H3K9me2 occupancy was found to be significantly lower at the *Pomc* and *Mc4r* promoters with no change in occupancy at the *Npy* promoter. This accurately correlates with observed *Pomc* and *Mc4r* mRNA expression as well as the reported G9a occupancy at these promoters. However, higher G9a occupancy at the *Npy* promoter does not appear to mediate H3K9me2 at this location. Values are mean ± SEM of 5-6 rats in each group. (*p<0.05, **p<0.01; via Student's t-test)

4. Occupancy of G9a and H3K9me2 at promoters of *Grin2a* and *Grin2b* and their associated expression in P and NP rats.

There are several well-studied mechanisms that modulate synaptic strength and plasticity in the brain that are believed to facilitate anxiety and AUD phenotypes. For instance, our lab has previously detailed the importance of the brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc) pathway in anxiety and AUD phenotype regulation, including that of P and NP rats (199, 203). A separate neuroplasticity mechanism of interest is that which is facilitated by the excitatory cellular mechanism regulators, GRIN2A and GRIN2B. Indeed, GRIN2A and GRIN2B are strongly linked to general synaptic plasticity and dendritic spine formation (386-388). This influence, particularly within the amygdala, is believed to alter phenotypes related to addiction and is highlighted by human studies of genetic variations associated with dysregulated fear, anxiety, and addiction-related behaviors (403, 406, 416). Here, we show that P rats innately express higher and lower levels of *Grin2a* ($p<0.01$, $t_{(10)}=-3.8$) and *Grin2b* ($p<0.05$, $t_{(9)}=2.4$) mRNA, respectively, relative to NP rats (Figure 8).

Knowing G9a and H3K9me2 expression differed between the amygdala of P and NP rats, we again utilized ChIP to determine G9a and H3K9me2 promoter occupancy at *Grin2a* and *Grin2b* genes. The findings showed that G9a occupancy is lower at *Grin2a* ($p<0.05$, $t_{(9)}=2.3$) and higher at *Grin2b* ($p<0.05$, $t_{(8)}=-2.7$) in the amygdala of P rats relative to NP rats (Figure 8). H3K9me2 occupancy was also found to be lower at *Grin2a* ($p<0.05$, $t_{(9)}=2.4$) in the amygdala of P rats relative to NP rats; however, H3K9me2 occupancy at *Grin2b* was not different between the two groups (Figure 8). These findings suggest that

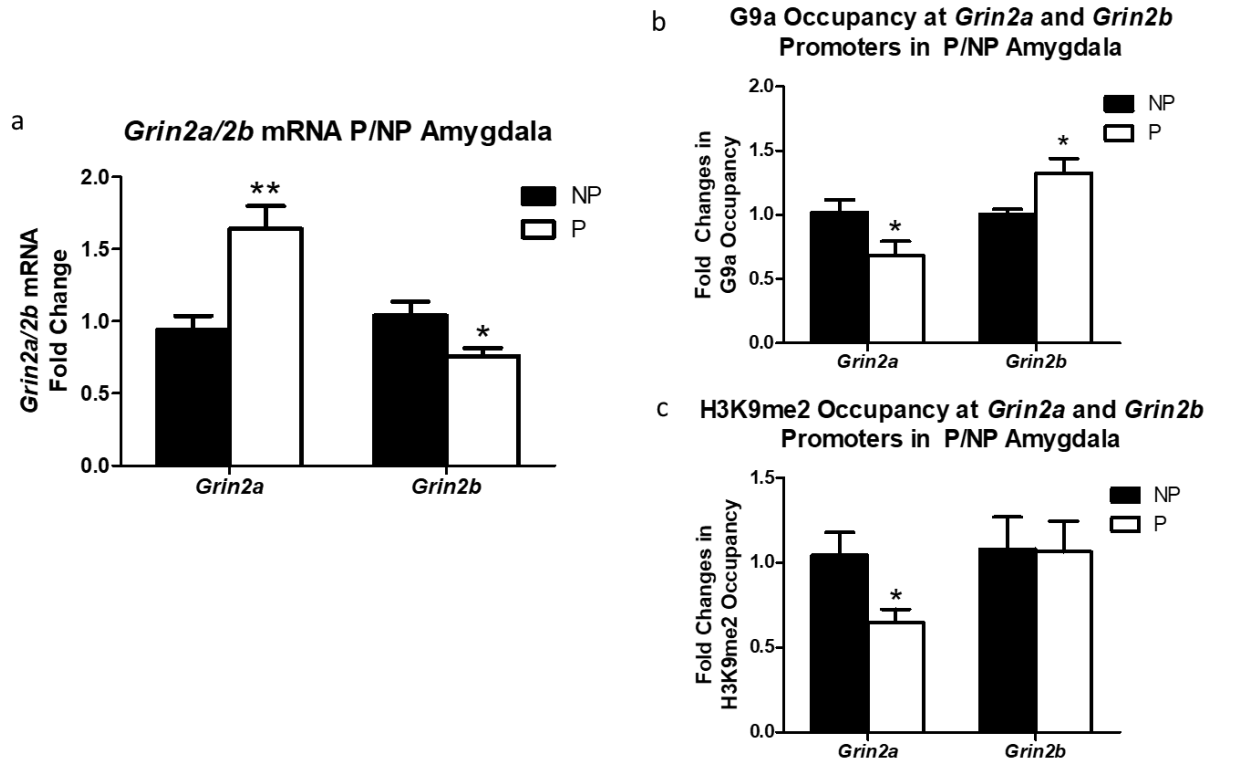


Figure 8. P rats express higher glutamate receptor ionotropic, NMDA type subunit 2A (*Grin2a)* and lower *Grin2b* mRNA than NP rats in the amygdala, which may be due to repressive G9a-associated epigenetic mechanisms.

a. Quantitative analysis of mRNA levels in the amygdala using RT-PCR in P and NP rats. This analysis reveals respectively higher and lower expression of *Grin2a* and *Grin2b* mRNA (fold change of mRNA levels) in P rat amygdala relative to NP rats. Values are mean ± SEM of 5-6 rats in each group. (**p*<0.05. ***p*<0.01; Student's t-test)

b. ChIP assay of repressive G9a occupancy at promoter regions of the *Grin2a* and *Grin2b* genes in the amygdala of P and NP rats. G9a occupancy was found to be significantly lower in the P rat amygdala at the *Grin2a* promoter and significantly higher at the *Grin2b* promoter relative to NP rats. This accurately correlates with observed mRNA expression, suggesting G9a occupancy may be regulating *Grin2a* and *Grin2b* gene expression. Representations are mean ± SEM of 5-6 rats in each group. (**p*<0.05; via Student's t-test)

c. ChIP assay of repressive G9a-associated H3K9me2 occupancy at *Grin2a* and *Grin2b* promoters in P and NP rats. H3K9me2 occupancy was found to be significantly lower at the *Grin2a* promoter with no change at the *Grin2b* promoter. This accurately correlates to the observed *Grin2a* mRNA expression but not *Grin2b.* Additionally, the lowered H3K9me2 occupancy in P rats at *Grin2a* correlates with the reportedly lower G9a occupancy, thereby suggesting the H3K9me2 may be G9a-mediated and responsible the discrepancy in *Grin2a*, but not *Grin2b,* mRNA expression in the amygdala of P and NP rats. Values are mean ± SEM of 5-6 rats in each group. (**p*<0.05; via Student's t-test)

the expression of synaptic plasticity modulator genes known to be associated with anxiety and addiction are dysregulated in the amygdala of P rats. Furthermore, the G9a-affiliated repressive epigenetic mechanisms may be contributing to the differential expression of these synaptic plasticity modulators, thereby influencing the P rat phenotype.

5. Innate differences in DNA methylation of *Grin2a*, *Grin2b*, and *Npy* gene promoters in the amygdala of P and NP rats.

As shown above, repressive G9a occupancy was found to be elevated at *Grin2b* and *Npy* promoters in P rats relative to NP rats within the amygdala, but H3K9me2 occupancy was unchanged at these sites (Figures 7 and 8). As such, we sought to explore the possibility that a separate G9a-mediated mechanism may be dysregulating *Grin2b* and *Npy* gene expression in P rats. DNMTs have recently been shown to possibly coordinate methylation mechanisms with G9a through direct and indirect interactions (234, 236, 237, 239, 244), and DNA methylation at promoter CpG islands are predominantly recognized as repressive epigenetic marks akin to G9a and H3K9me2 (178, 417). This suggests that the differential G9a occupancy may be associated with differential DNA methylation at these sites, as opposed to H3K9me2, thereby facilitating inhibitory epigenetic mechanisms that may regulate the lower *Grin2b* and *Npy* gene expression of P rats.

To examine this possibility, we utilized the Methylminer Methylated DNA Enrichment Kit followed by qPCR to quantify relative DNA methylation levels at the same *Grin2b* and *Npy* promoter sites as described above with regards to G9a occupancy in the P and NP rat whole amygdala. We found DNA methylation levels at the promoters of *Grin2b* and *Npy* to be significantly higher (*Grin2b, p*<0.01, $t_{(12)}$=-3.6; *Npy, p*<0.05, t(9)=-3.2) in the

amygdala of P rats when compared to NP rats (Figure 9). DNA methylation levels at the *Grin2a* promoter are also shown to represent a location where gene expression was upregulated, G9a occupancy was lower, and H3K9me2 was lower in P rats relative to NP rats but there was no difference in DNA methylation (Figure 8 and 9). These findings suggest that G9a and DNA methylation mechanisms may work in concert to specifically downregulate gene expression critical to the P rat phenotype.

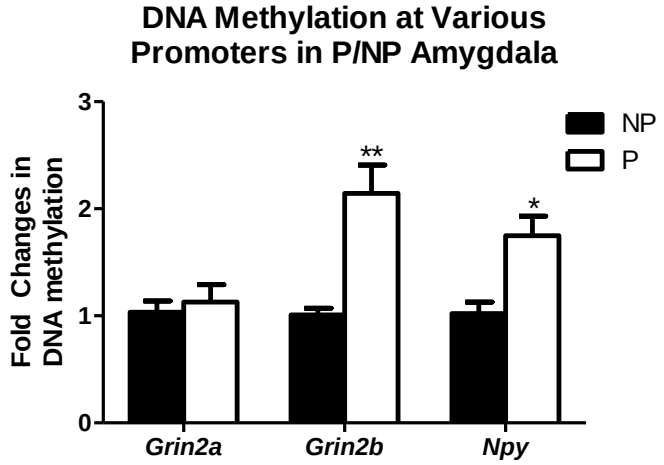


Figure 9. The P rat amygdala exhibits higher DNA methylation at *Grin2b* and *Npy* promoters, where G9a occupancy is also higher but H3K9me2 is not, when compared to NP rats

Quantitative representation of DNA methylation levels in P and NP rat amygdala (fold change in DNA methylation). *Grin2b* and *Npy* promoters possess higher DNA methylation in the amygdala of P rats relative to NP rats. Notably, these promoters were found to exhibit higher G9a occupancy, but not H3K9me2 occupancy (Figure 7 and 8). Because G9a is known to interact with DNA methyltransferases (DNMTs) and possibly attenuate gene expression, these findings suggest G9a and DNMTs may be downregulating gene expression in concert in P rats. *Grin2a* is shown to represent the DNA methylation difference in P versus NP rats at a location where G9a and H3K9me2 occupancy is different but DNA methylation is not. Values are mean ± SEM of 5-8 rats in each group. (*p<0.05, **p<0.01; via Student's t-test)

6. DNMT activity, gene expression, and protein expression in the P and NP rat amygdala.

We have found that the P rats exhibit higher levels of G9a and H3K9me2 in the amygdala compared to NP rats (Figure 5) and that the occupancy of these inhibitory epigenetic markers is dissimilar between P and NP rats at several genes of interest (Figure 7 and 8). These findings, in conjunction with previous studies, suggest that P rats possess overall more condensed chromatin in the amygdala than their NP rat counterparts (97, 206). We also found that DNA methylation is higher in the P rat amygdala relative to NP rats specifically at the downregulated *Npy* and *Grin2b* promoters (Figure 9). Notably, DNA methylation is known to contribute to chromatin remodeling, and these mechanisms, perhaps in concert with G9a mechanisms, may facilitate the condensed chromatin architecture in the amygdaloid structures of P rats and ultimately regulate pivotal gene expression. Three individual DNMTs—DNMT1, DNMT3A, and DNMT3b—catalyze CpG-based cytosine methylation. Variable or dysregulated DNMT expression, DNMT activity, and DNA methylation patterns have been linked to psychiatric disorders, addiction, development, and tumorigenesis (418-422). DNMT1 is largely viewed as the predominant enzyme responsible for DNA methylation maintenance and as a minor contributor to *de novo* methylation. DNMT3A and 3B, however, are critical for *de novo* methylation (250, 423). In general, increased promoter DNA methylation is associated with a decrease in gene expression. Because of the condensed chromatin architecture, increased G9a expression, and increased DNA methylation at the *Grin2b* and *Npy* promoters of P rats relative to NP rats, we also examined DNMT activity and DNMT expression in the P and NP rat amygdala.

The data revealed significantly higher DNMT activity in the nuclear fraction of the amygdala of P rats relative to NP rats ($p<0.05$; $t_{(14)}=-2.5$), suggesting P rats likely exhibit more global DNA methylation (Figure 10). We then sought to determine whether increased expression of DNMTs could possibly account for this difference in DNMT activity. *Dnmt1* and *Dnmt3b*, but not *Dnmt3a*, mRNA expression in the amygdala was found to be significantly higher in P rats relative to NP rats (*Dnmt1*, $p<0.05$, $t_{(8)}=-2.4$; *Dnmt3b*, $p<0.01$, $t_{(10)}=-4.1$) (Figure 10). When protein levels were examined in the P and NP amygdala, DNMT1 protein levels were the same while the P rat amygdala was found to have more DNMT3b protein in the CeA and MeA, but not the BLA, relative to NP rats (CeA, $p<0.01$, $t_{(10)}=4.4$; MeA, $p<0.001$, $t_{(10)}=-9.7$) (Figure 10). Cumulatively, the higher DNMT activity and expression levels of DNMT3b in the P rat amygdala indicates a potential role of DNA methylation mechanisms in dysregulated P rat chromatin architecture and subsequent gene expression, specifically at genes critical to anxiety-like and alcohol-drinking behavior.

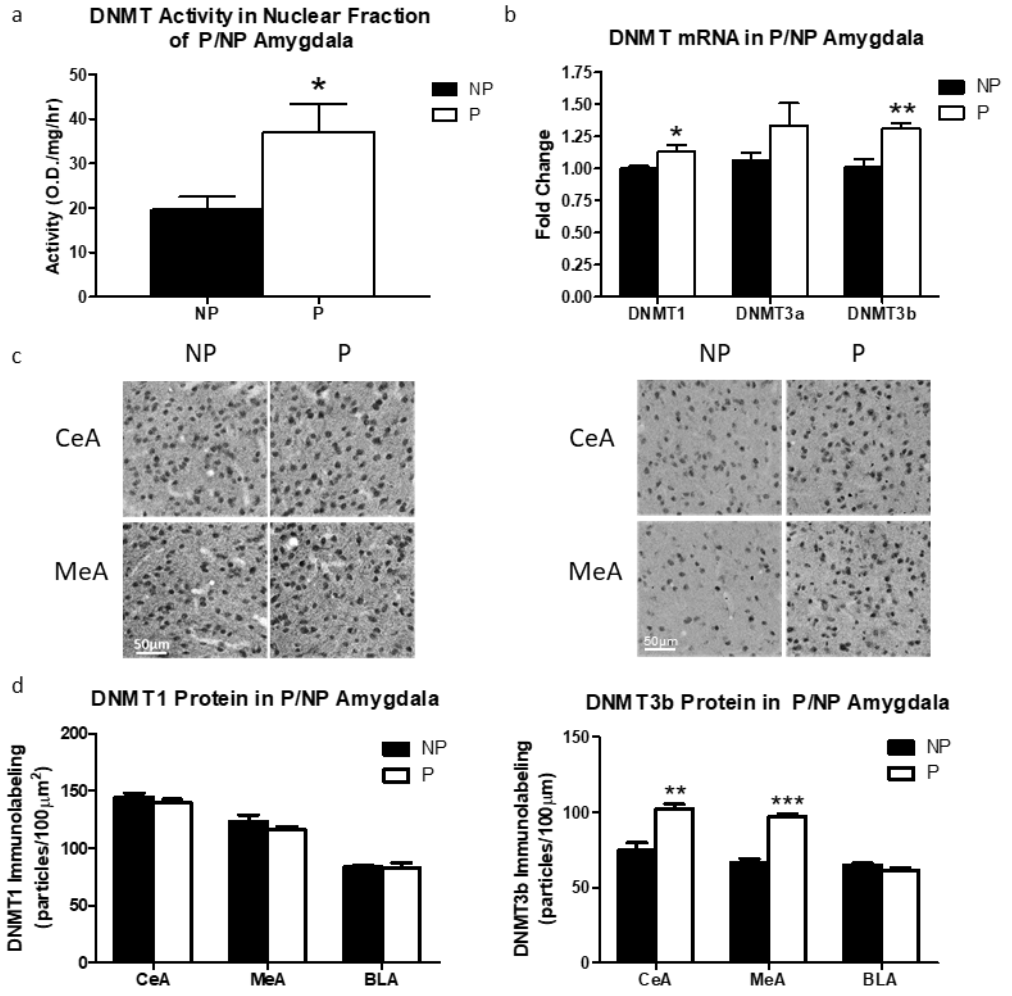


Figure 10. P rats exhibit higher innate DNMT activity in the amygdala than NP rats, possibly due to increased *Dnmt3b* expression and subsequent protein production.

a. Quantitative analysis of DNMT activity (presented as optical density, or O.D., per mg of protein per hour of reaction) within the nuclear fraction of amygdala lysates. The P rat amygdala exhibits higher DNMT activity compared to NP rats. Values are the mean ± SEM of 8 rats in each group. (**p*<0.05, via Student's t-test)

b. Quantitative analysis of RT-PCR in P and NP rat amygdala. This analysis reveals higher mRNA expression of *Dnmt1* and *Dnmt3b* (fold change of mRNA expression) in P rat amygdala when compared to NP rats. Values are mean ± SEM of 5 rats in each group. (**p*<0.05; ***p*<0.01, via Student's t-test)

c. Low-magnification micrographs of DNMT3b gold immunolabeling in the CeA and MeA of P and NP rats.

d. Quantitative representation of DNMT1 and DNMT3b protein (immunogold particles per 100µm^2 area) in amygdaloid structures of P and NP rats. Relative to NP rats, P rats exhibit more DNMT3b protein, but not DNMT1, in the CeA and MeA. Values are mean ± SEM of 6-8 rats in each group. (***p*<0.01; ***p*<0.001 via Student's t-test)

B. Hypothesis 2: G9a, a histone methyltransferase that possibly interacts with DNA methyltransferases and facilitates methylation of both histone H3 lysine 9 and DNA, regulates the high alcohol drinking behaviors of P rats, and treatment with a DNA methyltransferase inhibitor will reduce ethanol intake and preference in P rats by altering the G9a occupancy levels on anxiety-related genes in the amygdala.

1. Overview

DNA methylation and histone methylation are highly correlated with one another in mammalian epigenomes (424) and are associated with gene silencing (425, 426). In fact, certain DNA methylation can be dependent on the presence of G9a (242, 243), and G9a deficiencies generate DNA hypomethylation (232). Other studies have confirmed direct and indirect interactions between G9a and DNMTs, suggesting potentially mutual facilitation of histone and DNA methylation (236, 237, 240). Both histone and DNA methylation mechanisms emerged in recent years as pivotal epigenetic mechanisms related to addictive and anxiety-like behaviors (150). Though few studies have investigated the relationship between DNMTs/G9a and anxiety, available studies suggest inhibition of neuronal DNMTs and G9a may significantly reduce anxiety-like or alcohol-drinking behaviors (167, 427). Specifically, mice and rats given DNMT inhibitor treatment, such as 5-azacytidine (5-aza) and RG108, have shown reduced alcohol intake in several models of alcohol consumption, including post-dependent consumption and binge drinking (209, 277, 428). Furthermore, both chronic G9a inhibition and G9a neuronal knockouts exhibit decreased anxiety (167, 269). However, it is not currently known whether the inhibition of the DNA methylation mechanisms via DNMT inhibitors will alter

behavior in a genetic model of comorbid AUD and innate anxiety, nor is it known whether systemic DNMT inhibition in P rats will alter G9a occupancy patterns in the amygdala. Notably, there is some precedence in this downstream mechanistic targeting, as treatment with certain DNMT inhibitors have manipulated H3K9 epigenetic signatures in dysregulated *in vitro* cancer models (429).

To explore the possibility that treatment with a DNMT inhibitor would attenuate innate alcohol-drinking behaviors of P rats, we measured voluntary ethanol consumption in P rats who had been administered two separate IP injections of the DNMT inhibitor, 5-aza, at 2.5 mg/kg each and found that P rats treated with 5-aza displayed reduced alcohol preference despite no change in total fluid consumption, relative to saline-treated controls. To test whether this behavior could be explained by DNMT inhibitor-induced fluctuations in G9a occupancy at promoter regions of genes known to regulate alcohol-drinking and anxiety-like behaviors, we utilized ChIP analysis to examine the levels of G9a occupancy at the previously investigated gene promoters—*Pomc*, *Mc4r*, *Npy*, *Grin2a*, and *Grin2b*—in the amygdala. This analysis revealed a distinct difference in repressive G9a occupancy at both the *Pomc* and *Mc4r* promoter regions within the amygdala, in that P rats treated with the inhibitor had significantly higher G9a occupancy than vehicle controls. Notably, our previous findings showed that P rats have inherently lower G9a occupancy at these promoters relative to NP rats (Figure 7). Additionally, the promoter region for *Grin2b* also had increased G9a occupancy in the amygdala of P rats treated with 5-aza, while occupancy at *Grin2a* and *Npy* promoters remained unchanged between groups. These findings implicate a possible interaction between DNMTs and

G9a in epigenetic regulation of critical gene expression within the amygdala that is possibly contributing to anxiety-like and alcohol drinking behaviors, particularly via *Pomc* and *Mc4r* gene expression dysregulation.

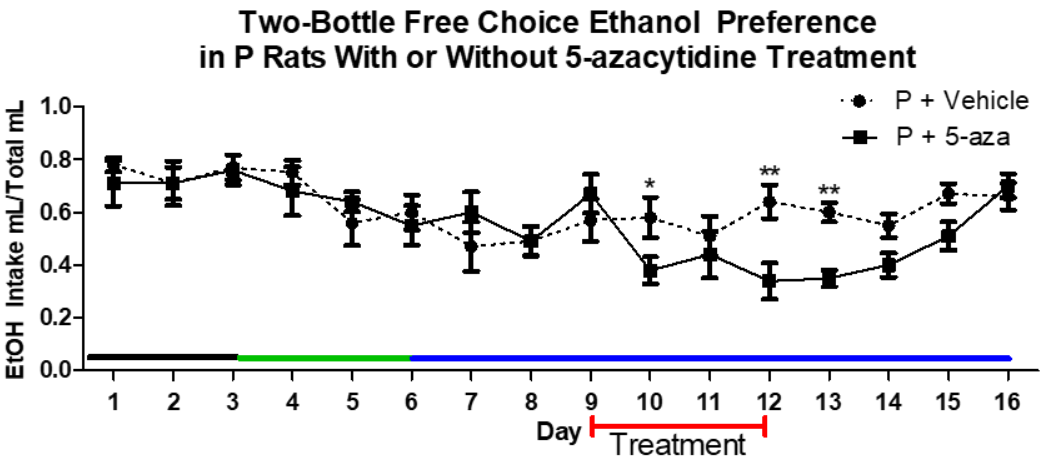
2. <u>The effects of systemic DNA methyltransferase inhibitor treatment on voluntary alcohol intake in alcohol-preferring P rats.</u>

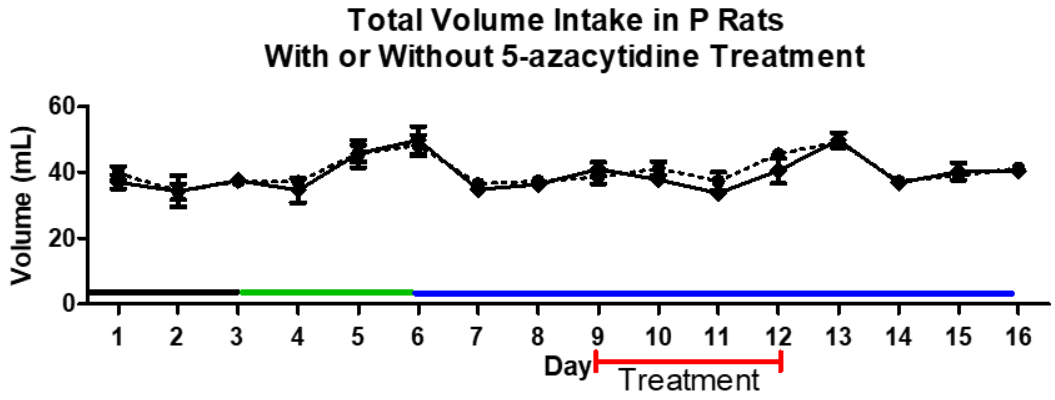
We have demonstrated the dissimilar methylomic epigenetic signature between the P and NP rat amygdala and the promising potential of DNMT inhibition as both a regulator of G9a and an effective intervention for anxiety and AUD. Given this, we explored the prospect that systemic treatment with the well-characterized DNMT inhibitor, 5-aza, could attenuate voluntary alcohol consumption in P rats. We utilized the two-bottle free choice paradigm to monitor voluntary alcohol intake. After initial habituation to two water bottles, P rats were provided water in one bottle and 3% ethanol in another bottle for three days, followed by water and 7% ethanol for 3 days, followed by water and 9% ethanol for the remainder of the experiment. After 3 days of 9% ethanol consumption, we began administering daily IP injections of 5-aza (1mg/kg) or vehicle for 4 days. The alcohol and water intake measurement was continued for an additional 3 days following treatment cessation to examine the post-treatment effects.

Using two-way ANOVA with repeated measure analysis followed by *post hoc* analysis, we found a significant difference in alcohol preference between treatment groups on three of the four days that the DNMT inhibitor was administered (p<0.01-0.05) that was normalized soon after treatment was stopped (Figure 11). Analysis revealed a significant interaction between group and day of treatment ($F_{9,90}$= 4.9, p<0.001), showing the

difference between treated and untreated rats was dependent on the day (treatment day or not) (Figure 11). Importantly, total volume intake was unchanged between the groups throughout (Figure 11). We performed a separate analysis by grouping the days by treatment status—pre-treatment, during treatment, or post-treatment days—and reporting alcohol preference. Two-way ANOVA followed by *post hoc* analysis revealed a significant interaction between treatment group and treatment status ($F_{2.30} = 3.6$, $p<0.05$) and that alcohol preference is significantly reduced in P rats during 5-aza treatment ($p<0.05$) but not after. These results clearly indicate a significant impact of systemic DNMT inhibitors on alcohol-preferring phenotype of P rats while also implicating DNA methylation mechanisms in dysregulation of alcohol-drinking behaviors. These findings join a growing body of evidence that validates DNMTs and associated mechanisms as a treatment target for future studies of AUD using a genetic animal model of alcoholism.

a





b

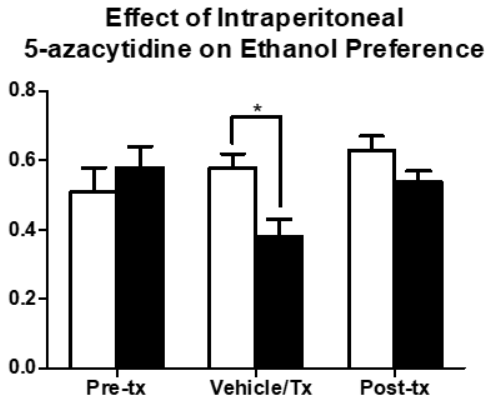


Figure 11. P rats exhibit reduced alcohol (EtOH) preference with no change in total fluid intake when treated with IP DNMT inhibitor, 5-azacytidine (5-aza).

a. Effect of 5-aza on daily P rat EtOH preference (EtOH consumption mL/Total fluid intake mL) in a two-bottle free choice paradigm before, during, and after exposure to a daily IP injection of either 1mg/kg 5-azacytidine or vehicle for four days. P rats had access to both EtOH and water throughout the experiment, with 3% EtOH for 3 days,

7% EtOH for 3 days, and 9% EtOH for the remainder of the experiment. After 3 days of 9% EtOH exposure, IP injections began. The EtOH preference of treated P rats (P + 5-aza) was significantly lower than that of vehicle-treated P rats (P + Vehicle). These findings suggest that methylomic epigenetic mechanisms may regulate the P rat phenotype. Values are the mean ± SEM of 6 rats per group. (*p<0.05, **p<0.01 for treatment group by day interaction, overall p<0.001 difference between treatments; all analysis was performed using repeated measure two-way ANOVA followed by *post hoc* Tukey's test)

b. Effect of 5-azacytidine on daily P rat EtOH preference (EtOH Intake mL/Total fluid intake mL), presented as the averages of 3-4 days based on treatment status. These statuses are reported as the first 3 days of 9% EtOH exposure before 5-aza administration (Pre-tx), the four days of 9% EtOH during treatment (Vehicle/Tx), and the last 3 days of 9% EtOH after treatment cessation (Post-tx). When averaged based on treatment status, EtOH preference is significantly reduced during treatment, relative to vehicle-treated controls. Values are the mean ± SEM for 6 rats per group. (*p<0.05; via two-way ANOVA followed by *post hoc* Tukey's test)

3. The effects of systemic DNA methyltransferase inhibitor treatment on G9a occupancy of various genes of interest in the amygdala of P rats.

We showed that G9a-associated methylation-based epigenetic mechanisms in P rats were innately aberrant (Figure 7-10), and treatment of P rats with the DNMT inhibitor, 5-aza, attenuated P rat alcohol preference (Figure 11). We aimed to examine whether treatment with 5-aza was altering the repressive G9a occupancy at these promoter regions. We treated a separate cohort of adult, male P rats with 4 daily IP injections of 1mg/kg 5-aza or vehicle control and performed ChIP analysis on the amygdala tissue extracted 2 hours after the last injection. G9a occupancy was evaluated at the promoters for the previously described anxiogenic, anxiolytic, and synaptic plasticity-modulating genes—*Pomc*, *Mc4r*, *Grin2a*, *Grin2b*, *Npy*. Results indicated an increase in G9a occupancy at the promoters for *Pomc*, *Mc4r*, and *Grin2b* (Figure 12) when P rats were treated with 5-aza relative to vehicle control (*Pomc*, p<0.05, $t_{(9)}$=-3.1; *Mc4r loc 1*, p<0.05, $t_{(9)}$=-2.6; *Mc4r loc 2*, p<0.05, $t_{(9)}$=-2.5; *Grin2b*, p<0.05, $t_{(9)}$=-2.5). In previous experiments,

we have reported here found P rats to innately exhibit aberrantly decreased G9a occupancy at amygdalar *Pomc* and *Mc4r* promoters (Figure 7), correlating with higher expression of the anxiogenic and AUD-associated *Pomc* and *MC4r* genes in the P rat amygdala and higher α-MSH and MC4r protein levels specifically within the P rat CeA and MeA (Figure 6). Here, we showed that 5-aza treatment in P rats increased inhibitory G9a occupancy at these promoters. This suggests that treatment with 5-azacytidine may attenuate alcohol-preferring behaviors of P rats in part by normalizing inhibitory G9a occupancy at the *Pomc* and *Mc4r* promoters, thereby altering the expression patterns within the amygdala and subsequent behavior.

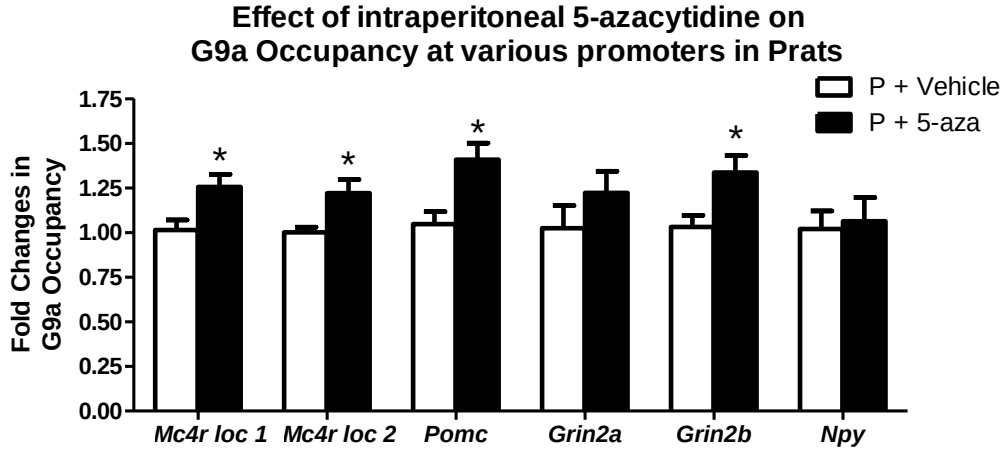


Figure 12. Treatment of P rats with the DNMT inhibitor, 5-azacytidine (5-aza), increases G9a occupancy at *Pomc*, *Mc4r*, and *Grin2b* promoters.
ChIP assay of repressive G9a occupancy at *Pomc*, *Mc4r*, *Grin2a, Grin2b*, and *Npy* promoters in the amygdala of P rats treated with 4 daily IP injections of the DNMT inhibitor, 5-aza (1mg/kg), or vehicle. In treated P rat amygdala (P + 5-aza), G9a occupancy was found to be significantly higher at the *Pomc, Mc4r*, and *Grin2b* promoters but unaltered at *Grin2a* and *Npy*, relative to vehicle-treated controls (P + Vehicle). We previously reported that G9a occupancy was lower at *Pomc* and *Mc4r* in P rats relative to non-preferring NP rats (Figure 7). This suggests that treatment with 5-aza may reduce the alcohol preference of P rats via epigenetic regulation of *Pomc* and *Mc4r* expression, which is dysregulated in P rats (Figure 6). Values are mean ± SEM of 4-6 in each group. (*p<0.05; via Student's t-test)

C. Hypothesis 3: G9a-mediated epigenetic regulation within the amygdala modulates the rapid tolerance to the anxiolytic effect of alcohol and is thereby involved in the development of alcohol use disorder.

1. Overview

In addition to innate anxiety, tolerance to the anxiolytic effects of alcohol has been implicated in increased alcohol consumption and subsequent AUD (32-34, 117). As previously explained, rapid ethanol tolerance (RET) is developed within 24 hours of exposure to ethanol and, importantly, has been recognized as a reliable molecular index of chronic tolerance (103, 112, 210). Building off previous work in mice (100), we established a RET model in adult, male Sprague-Dawley rats consisting of three groups (105). A Saline group receives two n-saline IP injections, an Ethanol group receives one n-saline injection followed by acute ethanol 24 hours later, and a third Tolerance group receives two acute ethanol injections 24 hours apart. Anxiety-like behaviors of animals are then tested one hour after the final injection, followed by sacrifice. Ethanol group displays significant anxiolytic-like behavior in response to acute ethanol exposure, while the Tolerance group fails to exhibit anxiolytic behaviors after the second day of alcohol exposure. This is despite metabolic tolerance equivalent to that of the Ethanol group, implicating a functional tolerance as opposed to metabolic. Previously, our lab determined that the anxiolytic peptide, NPY, is upregulated in the Ethanol group, but normalized in the Tolerance group (105), suggesting dysregulation of NPY production contributes to tolerance to ethanol-induced anxiolysis. Furthermore, this study confirmed that treating rats with a systemic HDAC inhibitor altered H3K9ac-associated *Npy* regulation, permitted NPY production in the Tolerance group, and attenuated tolerance to the anxiolytic effects

of ethanol (105). We showed in previous specific aims that G9a-associated mechanisms are possibly responsible for behavioral phenotypes in genetic models of comorbid anxiety and AUD, specifically via *Npy* expression regulation, among others; however, the role of G9a-associated mechanisms in tolerance is not understood. Furthermore, as previously mentioned, recent studies in the field of methylomic regulation have supported the concept that G9a-associated epigenetic mechanisms may facilitate addiction and anxiety (165, 167, 168, 248).

Cumulatively, this information led us to test the hypothesis that G9a-associated mechanisms regulate rapid tolerance to the anxiolytic effects of ethanol. First, we determined whether G9a, and its downstream effector, H3K9me2, were present at varying levels within the RET model amygdala. We found G9a and H3K9me2 protein expression levels to be decreased in the CeA and MeA, but not the BLA, of Ethanol group rats. In the Tolerance group, G9a and H3K9me2 protein expression was equivalent to that of the Saline group, suggesting G9a may interact with HDACs and facilitate anxiety-like behavior reported in the RET model. Knowing that *Npy* expression has proven critical to phenotypic regulation in RET, we utilized ChIP to map H3K9me2 levels at several regions of interest along the *Npy* gene—including three promoter sites and two gene body sites (*loc a*-*e*). One promoter site (*loc c*) and one gene body site (*loc d*) were near CpG islands, and one gene body site (*loc e*) was near a putative binding site for the transcription factor, CREB (cAMP response element-binding protein). Notably, CREB has been extensively linked to AUD as a biomarker and crucial regulator of cellular mechanisms and gene expression within humans and animal models of AUD and anxiety

(150, 197, 204, 205, 257). ChIP analysis revealed that H3K9me2 and G9a occupancy near the putative CREB-binding site mimicked the global patterning in CeA and MeA, with less H3K9me2 and G9a at this locus in the Ethanol group relative to the Saline group and no discernable change in the Tolerance group. Given that *Npy* was previously shown to be upregulated in the Ethanol group and recovered after RET establishment (105), these findings suggest inhibitory G9a-associated epigenetic mechanisms facilitate anxiolytic *Npy* regulation and contribute to the RET phenotypes. We then tested the theory that systemically administering a G9a inhibitor would reverse tolerance to the anxiolytic effects of ethanol. Therefore, we treated a Saline group and a Tolerance group with two IP doses of the G9a inhibitor, UNC0642. Additionally, a Saline group, Ethanol group, and Tolerance group received IP vehicle injections. We then evaluated their anxiety-like behaviors via LDB analysis and found UNC0642 treatment in both the Saline and Tolerance groups resulted in reduced anxiety-like behaviors, with levels similar to those in the vehicle-treated Ethanol group. Thus, G9a inhibition significantly decreased anxiety in saline controls while also preventing tolerance to the anxiolytic effects of ethanol. We then investigated G9a, H3K9me2 and NPY protein expression and found that systemic G9a inhibition had no effect on amygdalar G9a protein expression, though it reduced H3K9me2 production and increased NPY protein production in the CeA and MeA of both the UNC0642-treated Saline and Tolerance groups. Ultimately, our findings highlight the potential role G9a-mediated mechanisms play in modulating NPY protein production and rapid tolerance to ethanol-associated anxiolysis.

2. Blood alcohol content and anxiety-like behaviors in the rapid ethanol tolerance model

By measuring anxiety-like behaviors via the LDB exploration test, we confirmed production of the RET phenotype in adult, male Sprague-Dawley rats following two IP injections of either n-saline or ethanol (1g/kg at 20% w/v in saline), as described previously by us (105). As detailed in the methods section, the Saline control group received two IP n-saline injections administered 24 hours apart, the Ethanol group received a n-saline injection followed by an ethanol injection 24 hours later, and the Tolerance group received two ethanol injections administered 24 hours apart. These groups were then evaluated for anxiety-like behaviors via LDB one hour after the second injections, and the findings from the LDB exploration test were represented as the percentage of time spent in the dark or light components (Figure 13). We analyzed the findings with a one-way ANOVA and subsequent *post hoc* Tukey's test and successfully confirmed an overall difference among the groups ($p<0.001$, $F_{2,20}=15.2$). Specifically, the Ethanol group displayed significantly lower anxiety-like behaviors by spending less time in the dark compartment than the Saline and Tolerance groups (% time spent in dark compartment: saline x ethanol group, $p<0.001$; ethanol x tolerance group, $p=0.001$) (Figure 13), thus confirming our previous publication about development of rapid tolerance to anxiolytic effects of ethanol in rats (105). To verify that the tolerance group phenotype was not caused by metabolic tolerance, we verified that the blood alcohol content of rats in both the ethanol and tolerance groups were the same via Student's *t*-test analysis (Ethanol Group, 99.10 mg/dl ± 5.202 SEM; Tolerance Group, 101.3 mg/dl ± 8.432 SEM), suggesting a functional tolerance (Figure 13).

3. Global amygdalar G9a and histone H3 lysine 9 dimethylation protein expression in the rapid ethanol tolerance model

Previous work from our lab has revealed a reduction in HDAC activity and increase in amygdalar NPY expression after acute ethanol exposure. Furthermore, tolerance to the anxiolytic effects induced by a second ethanol exposure 24 hours later (RET) normalized NPY expression and HDAC activity. HDAC inhibitor treatment inhibited HDAC activity, increased H3K9ac, increased NPY in the amygdala, and RET reversal. This suggests that rapid tolerance to the anxiolytic effects of alcohol is at least partially controlled by epigenetic manipulation of NPY expression via the H3K9 locus in the amygdala (105).

Because of the increasingly documented role of G9a and histone methylation mechanisms in addiction and anxiety (165, 167, 168, 171, 174, 189, 259, 430) as well as the reciprocal relationship of histone acetylation and methylation mechanisms at H3K9 in gene regulation (231, 431, 432), we set out to examine the differences in amygdalar expression of H3K9me2 and G9a protein in the RET model. Overall, one-way ANOVA revealed H3Kme2 and G9a protein levels were different among groups in the CeA (G9a, $p<0.01$, $F_{2,12}=11.1$; H3K9me2, $p<0.001$, $F_{2,18}=15.5$) and MeA (G9a, $p<0.01$, $F_{2,12}=12.0$; H3K9me2, $p<0.05$, $F_{2,18}=5.7$), but not in the BLA, with acute ethanol significantly reducing both H3K9me2 and G9a in the Ethanol group (Figure 14). In the Tolerance group, however, there was no significant difference relative to the control group, suggesting the functional tolerance to the anxiolytic influence of ethanol may be related to these cellular differences, as G9a may be dysregulated in the tolerant rat CeA and MeA, thereby altering the inhibitory H3K9me2 signature and associated gene expression in the amygdala.

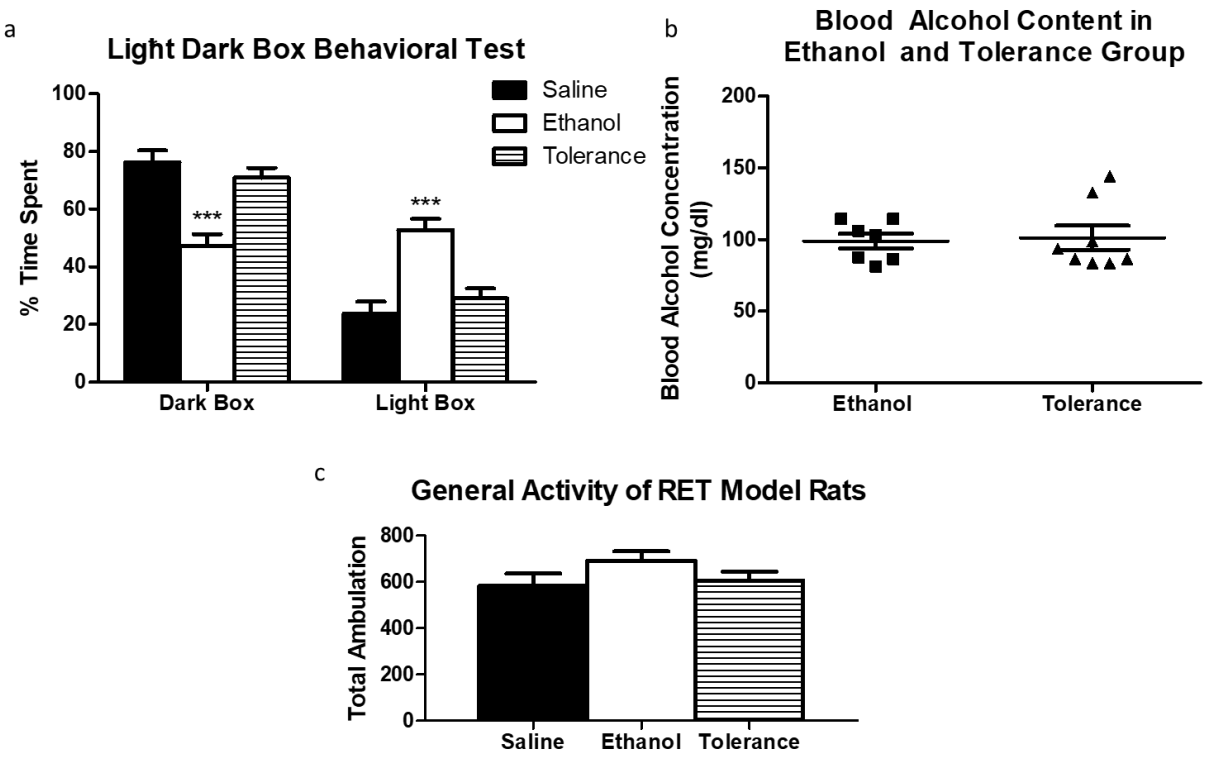


Figure 13. Rapid Ethanol Tolerance (RET) is induced in adult, male Sprague-Dawley rats after two IP injections of ethanol are administered 24 hours apart.

a. Light/dark Box (LDB) exploration test of adult RET model rats. Adult male Sprague-Dawley rats were administered either two IP n-saline injections 24 hours apart (Saline group), a single IP n-saline injection followed by a single IP injection of 20% (w/v) ethanol (1g/kg) 24 hours later (Ethanol group), or two IP injections of 20% (w/v) ethanol (1g/kg) (Tolerance group). Behavior was measured one hour after the second injection of saline or ethanol. The Saline group serves as a control, revealing a significant anxiolytic effect in the Ethanol group and a tolerance to this anxiolytic effect in the Tolerance group. Values are the mean ± SEM of 7-8 rats per group. (**p<0.01, ***p<0.001, via one-way ANOVA followed by *post hoc* Tukey's test for significance when compared to controls)

b. Blood alcohol concentration in the Ethanol group and Tolerance group 1 hour after last injection. Both the Ethanol group and the Tolerance group have equivalent mg/dl blood alcohol concentration following the second day of injections despite contrasting anxiety-like behaviors. This analysis suggests RET is functional rather than metabolic in nature. Values are the individual specimen blood alcohol concentration along with the mean ± SEM of the 7-8 rats in each group. (p=0.8346, Student's t-test)

c. General activity of adult RET model rats. Ambulation among all groups had no significant difference, indicating the experimental groups had similar general activity. Values are the mean ± SEM of 7-8 rats per group. Evaluated via one-way ANOVA. (Figure adapted from Berkel, T.D.M. et al. (2019) *Int J Neuropsychopharmacol*)

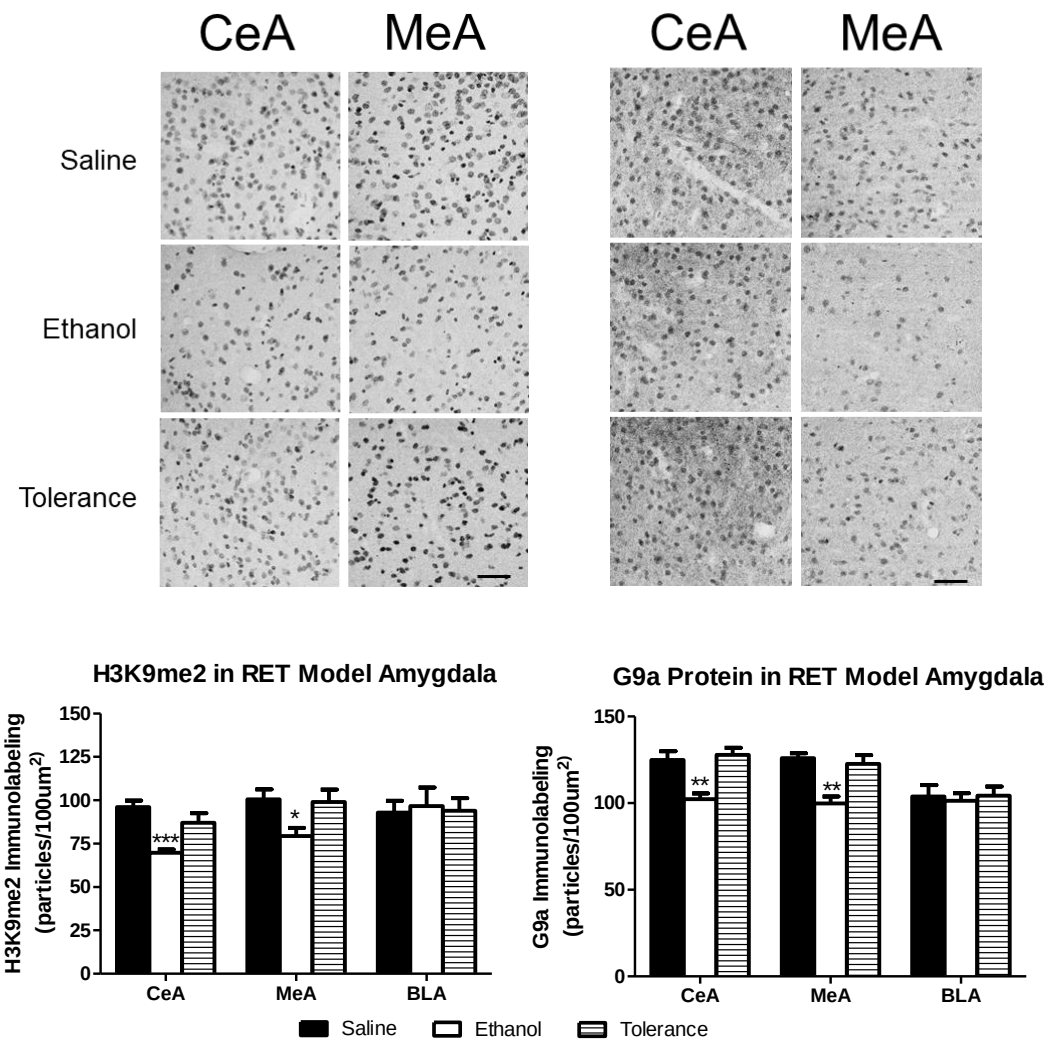


Figure 14. Acute ethanol is associated with a reduction in G9a and H3K9me2 protein in the CeA and MeA, two equivalent doses 24 hours apart is not.

a. Low-magnification representative micrographs of amygdaloid structures in the Saline group, Ethanol group, and Tolerance group of the RET model (Scale bar 50μM)

b. Quantitative analysis of the G9a and H3K9me2 protein levels in RET amygdaloid structures. Protein (immunogold particles per 100μm^2 area) expression was found to be lower in CeA and MeA, but not the BLA, in the Ethanol group relative to the Saline group controls. The Tolerance group expressed equivalent levels relative to controls. Values are mean ± SEM of 5-7 rats in each group. (*p<0.05, **p<0.01, ***p<0.001; via one-way ANOVA followed by *post hoc* Tukey's test for significance when compared to controls).
(Figure adapted from Berkel, T.D.M. et al. (2019) *Int J Neuropsychopharmacol*)

4. Dimethylated histone H3 lysine 9 and G9a promoter occupancy of the *Npy* gene in the amygdala during rapid ethanol tolerance

NPY is an anxiolytic neuropeptide in the amygdala (350, 354). Previous studies have extensively linked NPY to AUD and anxiety (96, 197, 348, 353-355, 357, 362, 363, 369). Our lab previously implicated NPY dysregulation in RET pathophysiology (105). This study also confirmed that treating rats with a systemic HDAC inhibitor altered H3K9ac-associated *Npy* regulation, permitted NPY production in the Tolerance group amygdala, and partially attenuated RET to the anxiolytic effects of ethanol (105), encouraging further exploration of epigenetic mechanisms in this model.

To examine the potential role of H3K9me2 epigenetic mechanisms in amygdalar NPY expression in RET, we used ChIP to investigate H3K9me2 occupancy at multiple locations (*loc a*-*e*) along the promoter and gene body of *Npy* (Figure 15). Nonparametric Kruskal-Wallis one-way ANOVA analysis followed by *post hoc* Dunn's test revealed the Ethanol group exhibited significantly ($p<0.05$) decreased H3K9me2 levels at one site (*loc e*), which lies adjacent to a putative CREB-binding site, but the Tolerance group exhibited no such change (Figure 15). At other locations (*loc a*-*d*), neither the Ethanol group nor the Tolerance group exhibited significant changes in H3K9me2 occupancy (Figure 15). To affirm the role of G9a in the H3K9me2 changes, we examined G9a occupancy at *loc e* and found the Ethanol group exhibited reduced G9a occupancy ($p<0.05$) with no change in the Tolerance group relative to the Saline group (Figure 15). The dissimilar H3K9me2 and G9a occupancy between the Ethanol and Tolerance groups at a potential CREB-binding site in the *Npy* gene implicates epigenetic control of *Npy* transcription by G9a-mediated H3K9me2 in RET.

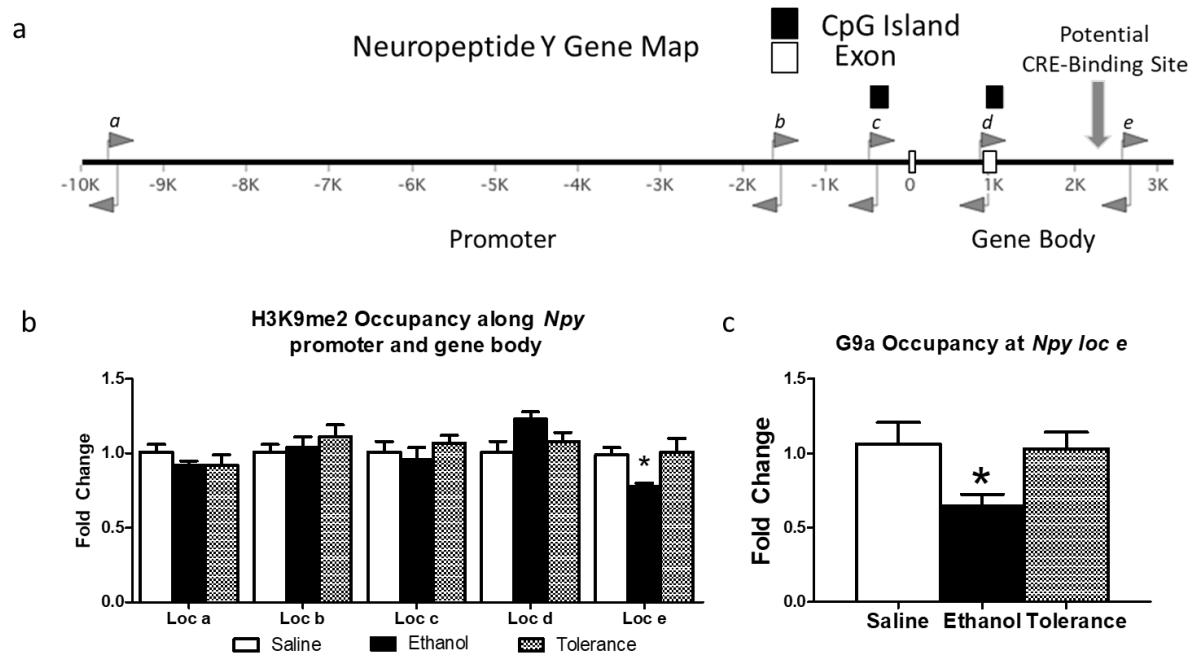


Figure 15. Acute ethanol, but not two doses 24 hours apart, is associated with reduced occupancy of H3K9me2 and G9a adjacent to a putative CREB-binding site within the *Npy* gene.

a. Gene map of *Npy* promoter and gene body. Five sites of interest are noted on the map (loci *a*-*e*), chosen based on their positioning along the promoter (*a*-*b*), proximity to CpG islands (*c*-*d*) or proximity to a putative CRE-binding site (*e*).

b. ChIP assay of H3K9me2 occupancy at loci along *Npy* promoter and gene body in the amygdala of RET model rats. H3K9me2 occupancy was significantly lower at a putative CRE-binding sequence (*loc e*) in the Ethanol group but not the Tolerance group. No significant change in H3K9me2 occupancy was detected at the other explored loci (*loc a*-*d*), suggesting amygdalar occupancy of H3K9me2 at *loc e* may contribute to *Npy* gene regulation. Values are mean ± SEM of 5-6 rats in each group. (*p<0.05; via nonparametric Kruskal-Wallis one-way ANOVA followed by *post-hoc* Dunn's test for significance when compared to controls).

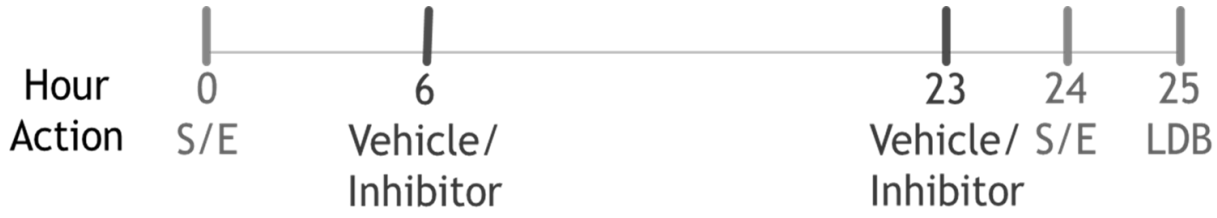
c. ChIP assay of G9a occupancy in a RET model at *loc e* within the *Npy* gene. The Ethanol Group exhibited significantly lower G9a occupancy at *loc e* while the Tolerance Group exhibited no such change. This implicates the enzymatic activity of G9a in regulating amygdalar H3K9me2 occupancy at this site and correlated expression of *Npy* in the RET model. This suggests that the concerted influence of inhibitory H3K9me2 and G9a may facilitate the amygdalar increase in *Npy* expression induced by acute ethanol and also may control rapid tolerance to this effect. Values are mean ± SEM of 7-8 rats in each group. (*p<0.05; via nonparametric Kruskal-Wallis one-way ANOVA followed by *post-hoc* Dunn's test for significance when compared to controls).

(Figure adapted from Berkel, T.D.M. et al. (2019) *Int J Neuropsychopharmacol*)

5. The effect of systemic G9a inhibition via UNC0642 on anxiety-like behaviors in the rapid ethanol tolerance model.

In order to investigate the potential role of G9a-mediated epigenetic mechanisms in the tolerance to the anxiolytic effects of alcohol, we utilized the LDB exploration paradigm to monitor anxiety-like behaviors of RET model rats after administration of a recently developed G9a inhibitor known as UNC0642 (263). As previously described, a Saline group, an Ethanol group, and a Tolerance group received two saline injections 24 hours apart, a saline injection followed by an ethanol injection 24 hours later, or two ethanol injections 24 hours apart—respectively. In this experiment, these rats were also administered two IP injections of vehicle control solution at 6 and 23 hours after the first day of RET establishment (Saline + Vehicle, Ethanol + Vehicle, and Tolerance + Vehicle, respectively). Additional Saline and Tolerance groups were administered the G9a inhibitor, UNC0642, via two IP doses of 2.5mg/kg—once at 6 hours and once at 23 hours after the first day of RET establishment (Saline + UNC0642 and Tolerance + UNC0642). One hour after the second and final exposure of either saline or ethanol, anxiety-like behavior was tested (Figure 16).

One-way ANOVA analysis revealed a significant difference in percentage of time spent in light and dark boxes among experimental groups ($p<0.001$; $F_{4,29} = 9.9$). Subsequent *post hoc* Tukey's test confirmed successful production of RET in the vehicle-treated rats (Figure 17) that mimicked baseline RET described above (Figure 13). The vehicle-treated ethanol group exhibited significantly decreased anxiety-like behaviors, reported as an increased percentage of time spent in the light box. The vehicle-treated tolerance group



Experimental Group	Day 1 Exposure	Treatments	Day 2 Exposure
Saline	S	Vehicle	S
Saline + UNC0642	S	Inhibitor	S
Ethanol	S	Vehicle	E
Tolerance	E	Vehicle	E
Tolerance + UNC0642	E	Inhibitor	E

Figure 16. Experimental timeline for testing the effect of G9a inhibitor, UNC0642, on rapid functional tolerance to the anxiolytic effects of ethanol.

a. Hourly timeline for investigating the effect of UNC0642 (Inhibitor) or vehicle control (Vehicle) on the rapid ethanol tolerance (RET) to the anxiolytic effects of alcohol. Animals were administered vehicle or inhibitor 6 hours and 23 hours after the first day of either ethanol (E) or N-saline (S). Ethanol or N-saline was again administered, as appropriate, 24 hours after the first exposure (1 hour after the final dose of inhibitor or vehicle). Light/dark Box (LDB) test was administered 1 hour after the second exposure to ethanol or saline.

b. Daily representation of experimental design to test the effect of UNC0642 or vehicle control on RET to the anxiolytic effects of alcohol. Five groups were established. The three original core groups (Saline, Ethanol, and Tolerance) were established, as previously described, in addition to two intraperitoneal (IP) injections of vehicle control solution between day 1 and day 2 of ethanol or saline exposure. Two separate groups (Saline + UNC0642 and Tolerance + UN0642) were included, receiving two IP injections of 2.5mg/kg UNC0642 between day 1 and day 2 of ethanol or saline exposure.

S=n-saline; E=1mg/kg ethanol

exhibited spent time in the light box equivalent to the vehicle-treated saline group (Saline + Vehicle x Ethanol + Vehicle, $p<.01$; Ethanol + Vehicle x Tolerance + Vehicle, $p<.001$). UNC0642 treatment after the first day's exposure to saline or ethanol resulted in significant decreases in time spent in the light box for both the treated saline group ($p<0.05$) and the treated tolerance group ($p<0.05$) relative to vehicle-treated counterparts (Figure 17). General activity was evaluated by the total number of ambulation recorded during the LDB exploration test, and there were no differences between any groups (Figure 17). These findings indicate that treatment with a G9a inhibitor can both reduce baseline anxiety levels and reverse RET establishment without altering general activity levels. Thus, these results implicate G9a-mediated epigenetic mechanisms in regulating anxiety and alcohol tolerance to the anxiolytic effects of ethanol and fuel the novel potential of G9a as a therapeutic target in AUD.

6. The effect of systemic G9a inhibition via UNC0642 on expression of G9a, dimethylated histone H3 lysine 9, and neuropeptide Y in the amygdala

Previously detailed experiments showed that acute anxiolytic ethanol exposure reduced G9a and H3K9me2 protein levels in the CeA and MeA relative to n-saline-exposed controls while a second equivalent dose of ethanol administered 24 hours later resulted in RET to these behavioral (Figure 13) and cellular effects (Figure 14). Studies have shown that acute anxiolytic ethanol upregulates amygdalar *Npy* while animals exhibiting RET to anxiolysis also express tolerance to ethanol-induced *Npy* upregulation (105, 355). Given that subacute treatment with the G9a inhibitor, UNC0642, resulted in reversal of the RET to the anxiolytic effects of alcohol (Figure 17), we sought to determine whether

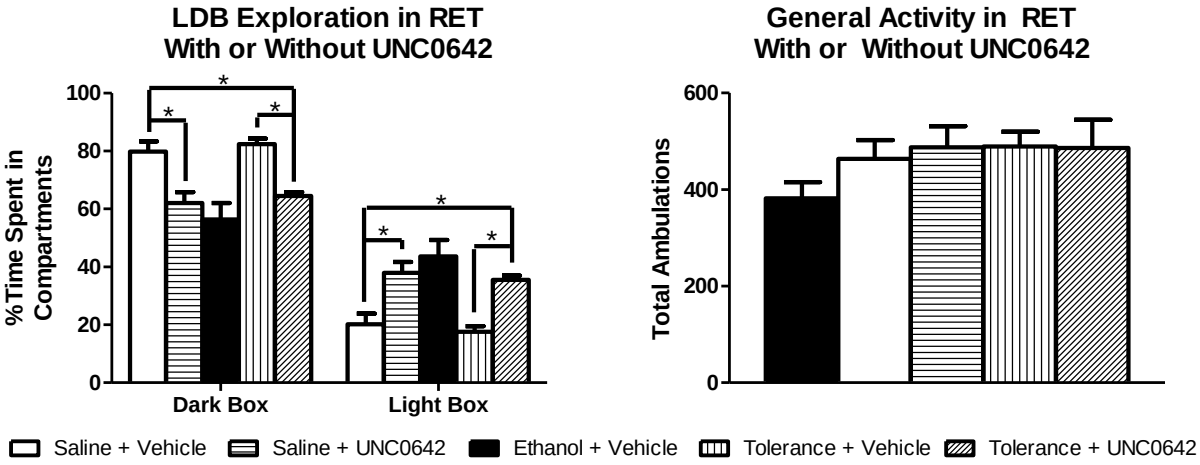


Figure 17. Systemic treatment with the G9a inhibitor, UNC0642 reduces anxiety-like behaviors and reverses the tolerance to anxiolytic effects of ethanol typically produced in RET.

In the rapid ethanol tolerance (RET) model, treatment with the G9a inhibitor UNC0642 reduces anxiety-like behaviors in the saline group and tolerance group, as evidenced by decreased percentage of time spent in the dark compartment during the light/dark box (LDB) exploration test. Both the tolerance group rats exposed to ethanol twice, 24 hours apart, and treated with intraperitoneal (IP) G9a inhibitor UNC0642 (Tolerance + UNC0642) as well as saline group rats exposed to n-saline twice, 24 hours apart, and treated with IP UNC0642 (Saline + UNC0642) exhibit decreased anxiety-like behaviors relative to the tolerance and saline groups treated with vehicle control solution (Tolerance + Vehicle and Saline + Vehicle, respectively). Total ambulations of all groups measured during the LDB exploration test showed no difference. Values are the mean ± SEM of 6-7 rats per group. (*p<0.05, using one-way ANOVA followed by *post hoc* Tukey's test for significance when compared to controls).

(Figure adapted from Berkel, T.D.M. et al. (2019) *Int J Neuropsychopharmacol*)

these UNC0642-induced phenotypic changes were due to fluctuations in G9a-mediated H3K9me2 and NPY protein production. Therefore, we employed gold immunolabelling to quantify protein expression of H3K9me2, G9a, and NPY within the amygdala of rats from Figure 17. Overall, we found significantly different protein expression among groups in the CeA and MeA (G9a in CeA, $p<0.001$; $F_{4,24} = 15.7$**;** G9a in MeA, $p<0.001$; $F_{4,24} = 7.8$; H3K9me2 in CeA, $p<0.001$, $F_{4,20}=17.7$; H3K9me2 in MeA, $p<0.001$, $F_{4,20}=22.6$; NPY in CeA, $p<0.001$, $F_{4,24}=20.0$; NPY in MeA, $p<0.001$, $F_{4,24}=7.2$). As expected, acute ethanol (1g/kg) exposure (Ethanol + Vehicle) significantly reduced H3K9me2 and G9a and increased NPY protein levels in the CeA, but ethanol-tolerant rats (Tolerance + Vehicle) exhibited no such changes (Figure 18). As described above, subacute UNC0642 IP injections were administered between the first and second day of n-saline (Saline + UNC0642) or ethanol (Tolerance + UN0642) provision. Per one-way ANOVA analysis followed by *post hoc* Tukey's Test, the Tolerance + UNC0642 group exhibited significantly reduced levels of H3K9me2 as well significantly increased NPY in the CeA (Tolerance + Vehicle x Tolerance + UNC0642; H3K9me2 $p<0.001$; NPY $p<0.001$) and MeA (Tolerance + Vehicle x Tolerance + UNC0642; H3K9me2 $p<0.001$; NPY $p<0.05$) relative to controls (Figure 18). Interestingly, the Saline + UNC0642 rats also exhibited significantly reduced H3K9me2 and increased NPY expression in the CeA (Saline + Vehicle x Saline + UNC0642; H3K9me2, $p<0.001$; NPY, $p<0.001$) and MeA (Saline + Vehicle x Saline + UNC0642; H3K9me2, $p<0.001$; NPY, $p<0.05$) relative to controls (Figure 18). No such changes were found in the BLA of any groups. These findings suggest that inhibition of G9a possibly reversed the development of anxiolysis tolerance via alteration of the H3K9me2 and subsequent expression of anxiolytic NPY in the CeA and MeA.

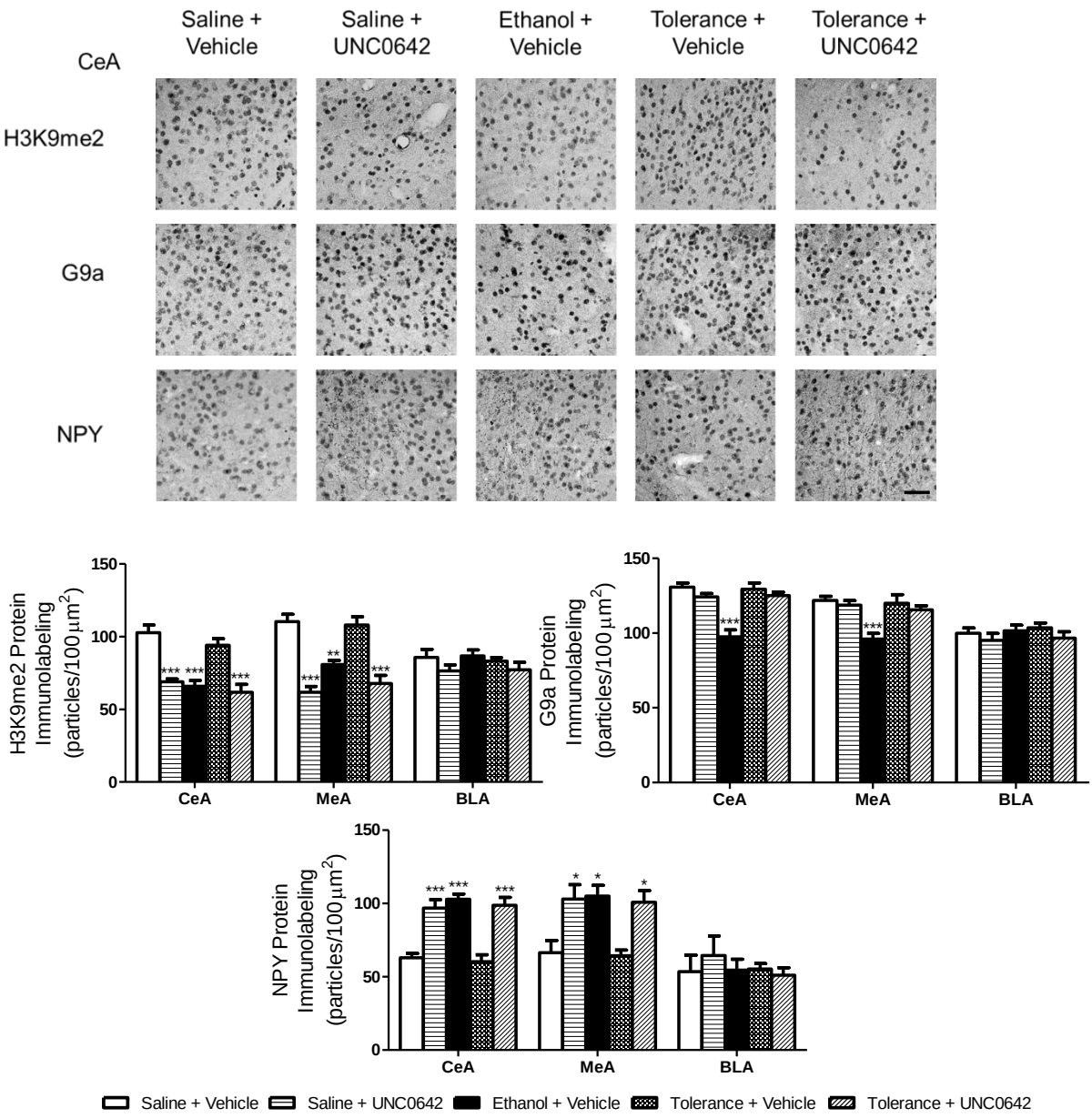


Figure 18. Systemic treatment with UNC0642 alters amygdalar H3K9me2 and NPY protein expression in a model of rapid ethanol tolerance (RET) treated with G9a inhibitor, UNC042.

a. Low-magnification micrographs of CeA from the RET model when treated with G9a inhibitor, UNC0642, or vehicle control. Gold immunolabeling of G9a, H3K9me2, and NPY protein.

b. Quantitative analysis of G9a, H3K9me2, and NPY protein in the RET model administered either G9a inhibitor, UNC0642, or vehicle control. Treatment with UNC0642 significantly reduced H3K9me2 protein (count of immunogold labeled particles per 100μm^2 area) and significantly increased NPY protein in the CeA and MeA of the Saline + UNC0642 group and Tolerance + UNC0642 group. As expected, G9a protein and H3K9me2 expression is significantly reduced and NPY is significantly

increased in the CeA and MeA of the Ethanol + Vehicle group relative to controls (Saline + Vehicle). Tolerance + Vehicle group animals expressed protein levels of G9a, H3K9me2, and NPY that were equivalent to those of controls throughout the amygdala. No changes were detected among the groups in the BLA. Values are mean ± SEM of 5-6 rats in each group. (*$p<0.05$, ***$p<0.001$; using one-way ANOVA followed by *post-hoc* Tukey's test for significance when compared to controls).
(Figure adapted from Berkel, T.D.M. et al. (2019) *Int J Neuropsychopharmacol*)

IV. Discussion

(Portions of this chapter were adapted with permission from work previously published as: Berkel, T.D.M., Zhang, H., Teppen, T., Sakharkar, A.J., Pandey, S.C. (2019) Essential Role of Histone Methyltransferase G9a in Rapid Tolerance to the Anxiolytic Effects of Ethanol. Int J Neuropsychopharmacol*, 22(4):292-302)*

A. The Reciprocal Facilitation Between Anxiety and Alcoholism

1. G9a-mediated DNA and Histone Methylation Regulates Alcohol-Drinking Behavior in Comorbid Anxiety and Alcohol Use Disorder

AUD is a multifaceted neurobiological disorder with significant and complex psychiatric dysfunction known to be exacerbated by stress and anxiety. The relationship between AUD and anxiety, though far from fully understood, has long been recognized and examined (11). Thus far, investigators have determined an intimate reciprocation of negative affect and behaviors present in comorbid anxiety and alcohol use that facilitates cyclical patterns of abuse and interferes with abstinence and even long-term recovery from AUD (9, 10). Intrinsic anxiety and genetic predisposition for AUD robustly coexists in the patient population and presents challenging obstacles for recovery (17). A high-quality model for studying comorbid genetic predisposition to AUD and innate anxiety is the P and non-preferring NP rat, which are selectively bred for alcohol preference or non-preference, respectively. In studying the P rat model, in addition to a plethora of other animal models of AUD-associated behaviors, molecular mechanisms involving synaptic plasticity and stress pathways in the amygdala repeatedly emerge as critical regulators (30, 101), and more recently research into the epigenetic regulation of these mechanisms has elucidated significant mechanistic pathophysiology behind addiction and reward pathways and emphasized the therapeutic potential of targeting epigenetic mechanisms (150).

G9a is a critical HMT that can establish H3K9me2, subsequently reducing gene expression via chromatin architecture reorganization (229, 230). G9a knockout-induced reduction of H3K9me2 is also correlated with increased activating epigenetic marks, such as H3K9ac and significant DNA hypomethylation. This underscores the epigenetic homeostasis required for transcription and the likely role of G9a in dynamic organization of active gene expression (169, 232). Recent studies have suggested the collaborative potential of G9a with other epigenetic mechanisms, suggesting that epigenetic enzyme recruitment and activity can involve both G9a and HDACs (433-435) or G9a and DNMTs (236-240), for example. Our lab and several others have repeatedly distinguished a significant role of HDACs in regulation of the alcohol-drinking and high anxiety phenotypes of P rats, but the role of G9a and DNA methylation remains less clear (150).

Previous studies in our lab have shown that P rats exhibit decreased amygdalar histone acetylation secondary to dysregulated HDAC expression, and more specifically HDAC2 isoform expression, relative to NP rats, suggesting the P rat amygdala possess generally more condensed chromatin relative to NP rats (97, 206). Supporting this theory, our data is the first to demonstrate that, relative to NP rats, P rats exhibit overall higher levels of G9a, H3K9me2, DNMT activity, and DNMT3B than NP rats within the amygdala. Expression of several genes known to influence AUD phenotypes were also found to be dissimilar in the amygdaloid structures between P and NP rats; specifically, *Pomc*, *Mc4r*, and *Grin2a* were upregulated, *Grin2b* was downregulated, and *Npy* was elsewhere shown to be downregulated (96, 97, 355) in P rats relative to NP rats. We further confirmed

higher protein expression of the POMC protein product, α-MSH, and its receptor, MC4R. Interestingly, the partial inhibitory epigenetic signature comprising G9a, H3K9me2, and DNA methylation at the promoters for these genes was distinct between P and NP rat amygdala and correlated with altered gene expression. We determined that G9a occupancy and H3K9me2 were paradoxically lower in the amygdala of P rats than NP rats at specific gene promoters—*Pomc*, *Mc4r*, and *Grin2a*—aligning with their higher mRNA expression in P rats (Figure 20). Additionally, we found higher G9a occupancy and DNA methylation at the promoter regions for the downregulated *Npy* and *Grin2b* genes, despite no difference in H3K9me2 at the same sites (Figure 20).

We then sought to determine the effect of systemic DNMT inhibition on alcohol-preference in P rats using 5-aza treatment in a two-bottle choice paradigm. Treatment with DNMT inhibitor attenuated alcohol preference in P rats and modulated G9a occupancy at the promoters of *Pomc*, *Mc4r*, and *Grin2b*. Recently, a diverse array of molecular mechanisms in the amygdala have emerged as robust regulators of AUD, including involvement of *Pomc* and its downstream pathway receptor *Mc4r* (171, 293, 308, 323, 336, 340). In P rats, administration of a MC4R antagonist was found to substantially reduce alcohol intake (341), and in humans, multiple single nucleotide polymorphisms within *POMC* significantly correlate with various forms of substance dependence, including AUD (336). Our lab recently reported increased *MC4r* mRNA and protein in the amygdala after intermittent adolescent alcohol exposure in adulthood (308). Here, we have expanded these studies and showed that P rats innately express more α-

MSH and MC4R in the amygdala relative to NP rats. Notably, P rats innately exhibit aberrantly decreased G9a occupancy at amygdaloid *Pomc* and *Mc4r* promoters,

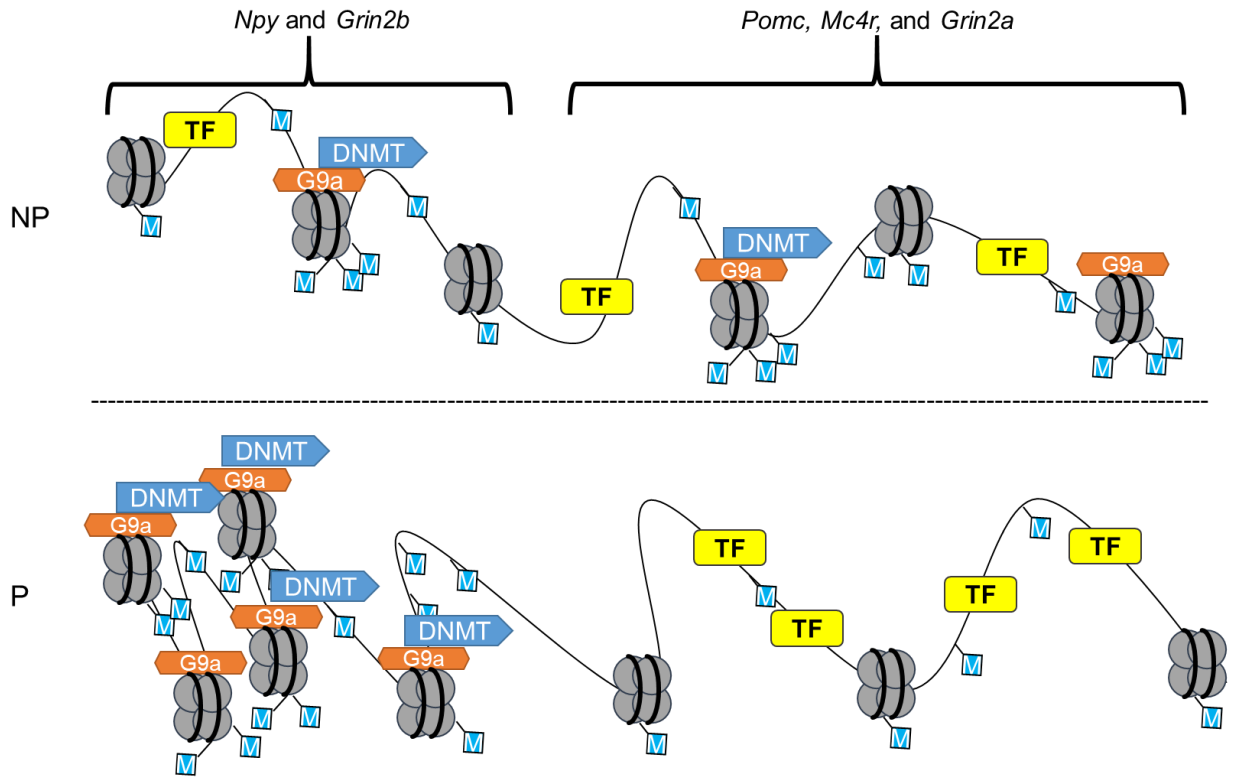


Figure 20. Visualization of general and selective expression and occupancy patterns of G9a, DNMTs, and histone and DNA methylation at certain genes of interest in NP versus P rat amygdala.
Though P rats express significantly more G9a, DNA methyltransferase (DNMT) activity, and H3K9me2 overall in the amygdala relative to NP rats, suggesting an overall condensed chromatin structure, the expression of *Pomc*, *Mc4r*, and *Grin2a* remain elevated in P rats compared to NP rats. This suggests differential chromatin dynamics at promoters leading to aberrant gene expression. Upon closer investigations on epigenetic dynamics, we found that G9a and H3K9me2 occupancy was indeed lower at the promoters for *Pomc*, *Mc4r*, and *Grin2a*, allowing for open chromatin structure and transcription factor (TF) binding. Conversely, G9a occupancy and DNA methylation was higher at the promoters for *Npy* and *Grin2b,* while H3K9me2 was unchanged.

correlating with higher expression of the anxiogenic and AUD-associated *Pomc* and *Mc4r* genes, relative to NP rats. This suggests that treatment with 5-aza may attenuate alcohol-

preferring behaviors of P rats in part by normalizing G9a occupancy at the *Pomc* and *Mc4r* promoters in the amygdala, thereby altering the expression patterns and subsequent behavior. These results clearly indicate a significant impact of systemic DNMT inhibitors on ethanol intake in P rats while also implicating G9a-mediated methylation mechanisms in modulation of *Pomc* and *Mc4r* gene expression.

In recent years, the dynamic relationship between HMTs and DNMTs has been increasingly elucidated (233-235, 414), revealing reciprocal cooperation in gene silencing involving direct and indirect interactions between G9a and DNMTs, which potentially facilitates targeting of histone and/or DNA methylation (236-240). The importance of the synchronous relationship of these mechanisms has only just now begun to be recognized, as is evidenced by the very recent production of dual inhibitors of G9a and HDACs (433) as well as G9a and DNMTs (436). Here, we presented findings that bolster the developing understanding that G9a and DNMTs interact and coordinate gene expression in a pivotal manner and indicate a previously unreported role of G9a-mediated mechanisms in the context of comorbid AUD and anxiety. Our findings also add to the mounting evidence suggesting that DNMT and G9a possess a dynamic relationship and that G9a's catalytic activities act independently in dysregulation of these specific genes in P rats. Notably, production of H3K9me2 and recruitment of DNMTs by G9a are believed to occur at independent sites on the G9a enzyme (239).

In conclusion, our data further provide novel evidence that supports our previous reports suggesting P rats possess condensed chromatin in the amygdala (147, 150), believed to

be largely responsible for negative affective states, such as anxiety, in the context of substance use (120). We also further propose that G9a-mediated histone and DNA methylation mechanisms may work in concert within the CeA and MeA to manipulate gene expression affiliated with a variety of pathways critical to the P rat phenotype. Specifically, the interaction between innate histone and DNA methylation machinery may lead to changes in gene expression patterns in the amygdala that are partially responsible for the alcohol-preferring phenotype of P rats via activation of the α-MSH and MC4r-mediated anxiogenic pathway. We have also demonstrated the dissimilar epigenetic signatures between the P and NP rat amygdala as well as the promising potential of DNMT inhibition as both a regulator of G9a occupancy and an effective intervention for alcohol-drinking behaviors. Our novel findings ultimately reinforce the cooperative role of G9a and DNMT activity in regulating expression of several genes in the amygdala, possibly in concert with HDAC2 (206), and highlight critical dysregulation of these epigenetic mechanisms in states of comorbid anxiety and alcoholism (121).

2. G9a-Mediated *Npy* expression Modulates Tolerance to the Anxiolytic Effects of Alcohol

Anxiety and AUD are believed to reciprocally facilitate one another through a variety of mechanisms, as described above, including acquired dysfunction of anxiety-regulation neurocircuitry (30, 437). The anxiolytic properties of alcohol are believed to encourage alcohol intake, and tolerance to these effects further increases alcohol consumption and likelihood of sustained neuroconnectivity dysfunction and alcohol dependence (32, 34, 105). In RET, rats given equivalent doses of ethanol 24 hours apart develop tolerance to the anxiolytic effects of alcohol when compared to animals exposed to one acute dose

(100, 105). Previous work from our lab reported that acute ethanol exposure decreases HDAC activity and increases H3K9ac and NPY expression in the CeA and MeA of rats while an identical second ethanol exposure 24 hours later resulted in no difference in these measures compared to saline group controls (105). Here, we extended these studies and found that a single ethanol exposure attenuated global and *Npy*-specific H3K9me2 and G9a levels and increased NPY levels in the CeA and MeA while animals administered an identical dose 24 hours later exhibited no such differences. These results further suggest that acute ethanol opens chromatin via increased histone acetylation and decreased histone methylation due to inhibition of both HDACs and G9a activity or expression in the amygdala and that these epigenetic marks appears to play an important role in RET.

Novel data presented here also suggest that systemic administration of the G9a inhibitor, UNC0642, reverses rapid tolerance to the anxiolytic effects of ethanol. We believe UNC0642 altered chromatin architecture via inhibition of G9a-mediated H3K9me2 production and thereby permitted production of anxiolytic NPY in the CeA and MeA. Furthermore, our findings suggest UNC0642 produced anxiolytic effects in alcohol-naive controls through a similar or identical mechanism. Ultimately, G9a-mediated regulation of histone methylation within the CeA and MeA is a potential therapeutic target that plays a significant role in anxiety and AUD development. In non-alcohol addiction studies, morphine and cocaine exposure have been found to reduce G9a levels in the NAc—a theorized partial extension of the EA— (168, 259), and overexpression of G9a and G9a-mediated H3K9me2 in the NAc increases sensitivity to drug reinforcement and drug-

seeking behaviors (168, 248, 259). Furthermore, G9a is also believed to modulate activation of CREB, via control of upstream BDNF signaling (165, 248), both of which our lab and others have extensively linked to AUD and anxiety in preclinical and human studies (150, 249, 257). Notably, the *Npy* gene is a recognized CREB target (197, 256, 438), and our study showed that G9a-mediated H3K9me2 occupancy is reduced on the *Npy* gene near a putative CREB-binding site in response to acute ethanol exposure, suggesting acute ethanol exposure is associated with altered chromatin architecture at this site that would promote transcriptional competence.

Though few studies have investigated the relationship of H3K9me2 and G9a with anxiety, conditional neuronal knockdown and chronic pharmacological inhibition of G9a each reduce H3K9me2 in the brain and produce anxiolytic effects (167, 269). Here, we have expanded on these studies and systemically administered the G9a inhibitor, UNC0642, and confirmed amygdalar H3K9me2, G9a, and NPY protein expression changes associated with significant reductions in anxiety-like behaviors, including the reversal of tolerance to alcohol-induced anxiolysis. Interestingly, a recent study reported that while chronic UNC0642 administration via intraperitoneal injection in mice reduced anxiety in a dose-dependent manner, a single injection failed to produce significant reductions in anxiety 30 minutes later (269). However, our findings show that subacute provision of UNC0642 (two doses of 2.5mg/kg 17 hours apart) is sufficient for an anxiolytic effect recorded 2 hours after last drug treatment. In conclusion, our findings highlight that G9a-mediated H3K9me2 in the emotional amygdala neurocircuitry may orchestrate the chromatin remodeling of the *Npy* gene, resulting in increased NPY expression in

response to acute ethanol and normalized expression during RET. Furthermore, these novel findings established the therapeutic potential of G9a inhibition in reducing anxiety and reversing tolerance to the anxiolytic effects of ethanol. This study of G9a-mediated H3K9me2 in conjunction with our previous findings regarding HDAC-mediated NPY expression regulation in the context of AUD and anxiety (97, 105, 147) further solidify the emerging role of epigenetic regulation in AUD pathophysiology [Figure 20; (150)].

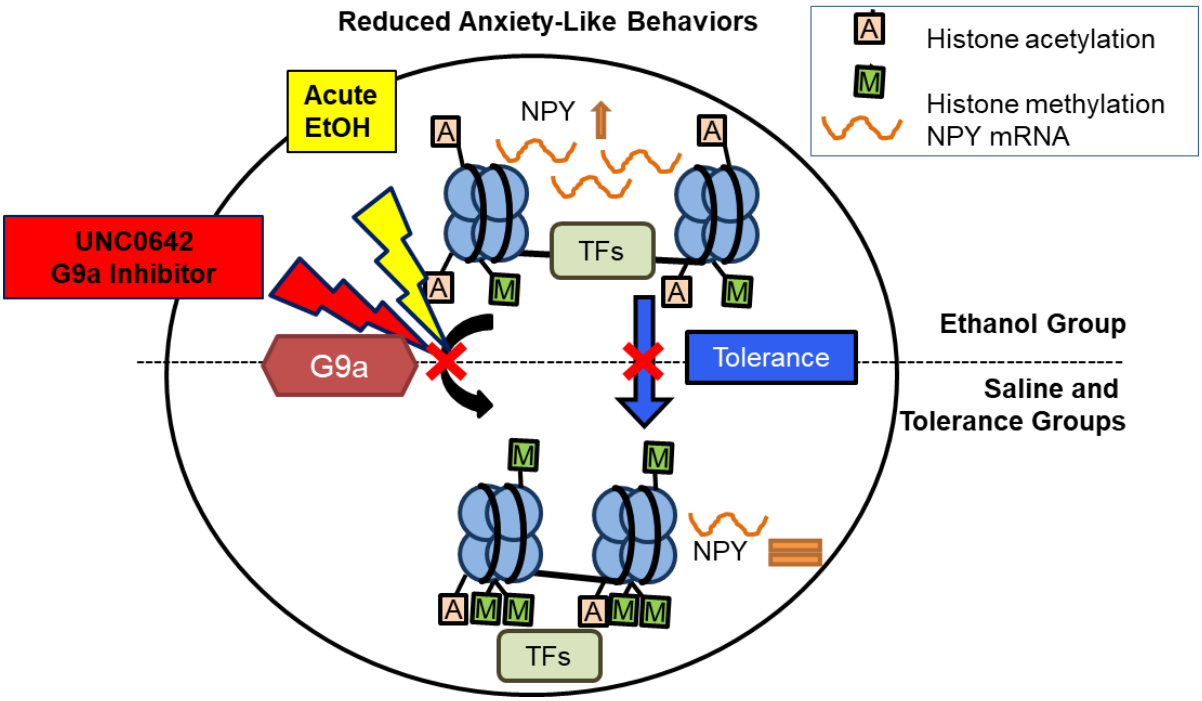


Figure 20. Model of proposed epigenetic mechanisms in the amygdala implicated in development of rapid ethanol tolerance to the anxiolytic effects of ethanol via neuropeptide Y regulation.
This model depicts that acute ethanol reduces anxiety via inhibition of G9a-mediated dimethylation of histone H3 lysine 9 (H3K9me2), in addition to the previously established role of H3K9 acetylation, thereby altering chromatin architecture and permitting expression of anxiolytic NPY in the amygdala. Furthermore, while an identical dose of ethanol 24 hours later results in tolerance to these cellular and behavioral effects, we propose that systemic inhibition of G9a via UNC0642 reverses rapid tolerance to the anxiolytic effects of alcohol via a similar or identical mechanism as that of acute ethanol.

V. Conclusion

A. Review

Throughout this work, we have studied the multifaceted role of G9a-mediated mechanisms in AUD and anxiety. We accomplished this by using two separate models highlighting crucial contributions to clinical diagnosis, development, and maintenance of AUD. These models, the alcohol-preferring P rat and the model of RET, allowed us to investigate the role of G9a-mediated methylation in both innate and acquired molecular signatures of G9a and associated pathways in the context of AUD and how these regulate relevant phenotypes and the molecular organizers of these behaviors from within the amygdala. With these studies, we have implicated G9a-mediated epigenetic mechanisms in both innate comorbid anxiety and AUD as well as in regulating acquired tolerance to the anxiolytic effects of alcohol. Our findings suggest G9a-mediated mechanisms of histone and DNA methylation within these models aberrantly regulate gene expression of pivotal genes—such as *Pomc*, *Mc4r*, *Grin2a*, *Grin2b*, and *Npy*—as elucidated through both molecular and behavioral studies. This evidence joins an ever-growing body of work that implicates histone acetylation and methylation as well as DNA methylation in complex control of gene expression in the context of anxiety and alcoholism [Figure 20 (121, 150)]. Ultimately, our findings affirm the importance of epigenetic mechanisms in the development of AUD and tolerance, and our novel reductions in anxiety-like behaviors and alcohol preference through G9a and DNMT inhibition highlight the therapeutic potential of targeting histone methylation pathways in AUD.

B. Future Work

1. Investigating the potential of G9a manipulation and dual inhibition in comorbid anxiety and alcoholism

Recent studies have indicated a poorly understood relationship between G9a and DNMTs regulates gene expression via histone and DNA methylation mechanisms. Here, we have determined that multiple days of systemic DNMT inhibition attenuates alcohol intake and alcohol preference in P rats, known for exhibiting comorbid high alcohol preference and anxiety. Systemic DNMT inhibition also modifies G9a and H3K9me2 occupancy within the amygdala along promoters of several genes known to regulate AUD, presumably controlling respective gene expression. This study and others have begun to elucidate the mechanistic nature of the G9a-DNMT relationship; however, the reciprocal nature of the relationship needs to be explored. Though we have determined the impact of DNMT inhibition on G9a signatures and behavior, the impact of G9a manipulation on comorbid alcohol preference and anxiety seen in P rats should be pursued to further elucidate the reach of the G9a and DNMT cooperation in this model's amygdalar gene expression and associated phenotype. Specifically, future investigation of G9a inhibition and/or amplification in this model should be pursued to determine the effects on alcohol preference, anxiety-like behaviors, and the impact on DNA methylation signatures at genes of interest. Furthermore, this work joins novel studies in the field of epigenetics in psychiatric disorders and continuation of this project should utilize the contemporary emergence of dual inhibitor molecules to provide a more comprehensive investigation into the effects of epigenetic dysregulation in comorbid anxiety and alcoholism. It is

possible that dual inhibition of G9a and DNMTs, for instance, may provide a synergistic or additive effect on behavioral modification. Furthermore, anxiety is understood to have a significant influence on the alcohol-drinking behaviors of P rats, and anxiety in studies presented here heavily implicate anxiety via the altered gene expression of genes such as *Pomc* and *Mc4r*; however, the role of DNMT inhibition in anxiety-like behaviors of P rats has yet to be elucidated beyond the altered epigenetic signature of *Pomc* and *Mc4r.* Future work should aim to determine if reduced alcohol preference of P rats secondary to systemic inhibition of DNMT and subsequently increased G9a occupancy of *Pomc* and *Mc4r* is associated with decreased anxiety-like behaviors.

VI. CITED LITERATURE

1. Tanasi D, Greco E, Di Tullio V, Capitani D, Gullì D, Ciliberto E. 1H-1H NMR 2D-TOCSY, ATR FT-IR and SEM-EDX for the identification of organic residues on Sicilian prehistoric pottery. Microchemical Journal. 2017 November 2017;135(Supplement C):140-7.

2. World Health Organization. Global status report on alcohol and health 2014.

3. Sacks JJ, Gonzales KR, Bouchery EE, Tomedi LE, Brewer RD. 2010 National and State Costs of Excessive Alcohol Consumption. Am J Prev Med. 2015 Nov;49(5):e73-9.

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

5. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. 5th ed. Arlington, VA: American Psychiatric Publishing; 2013.

6. Cloninger CR, Sigvardsson S, Gilligan SB, von Knorring AL, Reich T, Bohman M. Genetic heterogeneity and the classification of alcoholism. Adv Alcohol Subst Abuse. 1988;7(3-4):3-16.

7. Babor TF, Hofmann M, DelBoca FK, Hesselbrock V, Meyer RE, Dolinsky ZS, et al. Types of alcoholics, I. Evidence for an empirically derived typology based on indicators of vulnerability and severity. Arch Gen Psychiatry. 1992 Aug;49(8):599-608.

8. Lesch OM, Walter H. Subtypes of alcoholism and their role in therapy. Alcohol Alcohol. 1996 Mar;31 Suppl 1:63-7.

9. Koob GF. A Role for Brain Stress Systems in Addiction. Neuron. 2008 7/10;59(1):11-34.

10. Moonat S, Pandey SC. Stress, epigenetics, and alcoholism. Alcohol Res Curr Rev. 2012 2012/;34(4):495-505.

11. Kushner MG, Sher KJ, Beitman BD. The relation between alcohol problems and the anxiety disorders. Am J Psychiatry. 1990 1990/;147(6):685-95.

12. Khantzian EJ. The self-medication hypothesis of substance use disorders: a reconsideration and recent applications. Harv Rev Psychiatry. 1997 Jan-Feb;4(5):231-44.

13. Goldstein AL, Flett GL. Personality, alcohol use, and drinking motives: a comparison of independent and combined internal drinking motives groups. Behav Modif. 2009 Mar;33(2):182-98.

14. Kuntsche E, Knibbe R, Gmel G, Engels R. 'I drink spirits to get drunk and block out my problems...' beverage preference, drinking motives and alcohol use in adolescence. Alcohol Alcohol. 2006 Sep-Oct;41(5):566-73.

15. Robinson JA, Sareen J, Cox BJ, Bolton JM. Correlates of self-medication for anxiety disorders: results from the National Epidemiolgic Survey on Alcohol and Related Conditions. J Nerv Ment Dis. 2009 Dec;197(12):873-8.

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

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

18. Wolitzky-Taylor K, Guillot CR, Pang RD, Kirkpatrick MG, Zvolensky MJ, Buckner JD, et al. Examination of anxiety sensitivity and distress tolerance as transdiagnostic mechanisms linking multiple anxiety pathologies to alcohol use problems in adolescents. Alcohol Clin Exp Res. 2015 Mar;39(3):532-9.

19. Wolitzky-Taylor K, Bobova L, Zinbarg RE, Mineka S, Craske MG. Longitudinal investigation of the impact of anxiety and mood disorders in adolescence on subsequent substance use disorder onset and vice versa. Addict Behav. 2012 Aug;37(8):982-5.

20. Windle M, Windle RC. A prospective study of stressful events, coping motives for drinking, and alcohol use among middle-aged adults. J Stud Alcohol Drugs. 2015 May;76(3):465-73.

21. Farris SG, Epstein EE, McCrady BS, Hunter-reel D. Do co-morbid anxiety disorders predict drinking outcomes in women with alcohol use disorders? Alcohol Alcohol. 2012 /;47(2):143-8.

22. Gordon HW. Early environmental stress and biological vulnerability to drug abuse. Psychoneuroendocrinology. 2002 Jan-Feb;27(1-2):115-26.

23. Uhart M, Wand GS. Stress, alcohol and drug interaction: an update of human research. Addict Biol. 2009 Jan;14(1):43-64.

24. Sinha R. Chronic stress, drug use, and vulnerability to addiction. Ann N Y Acad Sci. 2008 Oct;1141:105-30.

25. Pandey SC, Sakharkar AJ, Tang L, Zhang H. Adolescent intermittent ethanol-induced alcohol drinking and anxiety like behaviors at adulthood: A role for histone deacetylases. Alcohol Clin Exp Res. 2012 /;36:359A.

26. Pohorecky LA. Stress and alcohol interaction: An update of human research. Alcohol Clin Exp Res. 1991 1991/;15(3):438-59.

27. Kessler RC, Crum RM, Warner LA, Nelson CB, Schulenberg J, Anthony JC. Lifetime co-occurrence of DSM-III-R alcohol abuse and dependence with other psychiatric disorders in the national comorbidity survey. Arch Gen Psychiatry. 1997 /;54(4):313-21.

28. Comorbidity of Alcoholism and Psychiatric Disorders [Internet]; 2002. Available from: http://pubs.niaaa.nih.gov/publications/arh26-2/81-89.htm.

29. Ipser JC, Wilson D, Akindipe TO, Sager C, Stein DJ. Pharmacotherapy for anxiety and comorbid alcohol use disorders. Cochrane Database Syst Rev. 2015 Jan 20;1:CD007505.

30. Koob GF. Brain stress systems in the amygdala and addiction. Brain Res. 2009 10/1;1293(0):61-75.

31. Gimeno C, Dorado ML, Roncero C, Szerman N, Vega P, Balanza-Martinez V, et al. Treatment of Comorbid Alcohol Dependence and Anxiety Disorder: Review of the Scientific Evidence and Recommendations for Treatment. Front Psychiatry. 2017 Sep 22;8:173.

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

33. Tabakoff B, Cornell N, Hoffman PL. Alcohol tolerance. Ann Emerg Med. 1986 1986/;15(9):1005-12.

34. Koob GF, Volkow ND. Neurocircuitry of addiction. Neuropsychopharmacology. 2010 /;35(1):217-38.

35. Brown SA, Vik PW, McQuaid JR, Patterson TL, Irwin MR, Grant I. Severity of psychosocial stress and outcome of alcoholism treatment. J Abnorm Psychol. 1990 Nov;99(4):344-8.

36. Kushner MG, Abrams K, Thuras P, Hanson KL, Brekke M, Sletten S. Follow-up study of anxiety disorder and alcohol dependence in comorbid alcoholism treatment patients. Alcohol Clin Exp Res. 2005 Aug;29(8):1432-43.

37. Noone M, Dua J, Markham R. Stress, cognitive factors, and coping resources as predictors of relapse in alcoholics. Addict Behav. 1999 Sep-Oct;24(5):687-93.

38. Besancon F. Time to alcohol dependence after abstinence and first drink. Addiction. 1993 Dec;88(12):1647-50.

39. Winslow BT, Onysko M, Hebert M. Medications for Alcohol Use Disorder. Am Fam Physician. 2016 Mar 15;93(6):457-65.

40. Chick J, Gough K, Falkowski W, Kershaw P, Hore B, Mehta B, et al. Disulfiram treatment of alcoholism. Br J Psychiatry. 1992 1992/;161(JULY):84-9.

41. Baser O, Chalk M, Rawson R, Gastfriend DR. Alcohol dependence treatments: comprehensive healthcare costs, utilization outcomes, and pharmacotherapy persistence. Am J Manag Care. 2011 /;17 Suppl 8:S222; 234.

42. Bryson WC, McConnell J, Krothuis T, McCarty D. Extended-release naltrexone for alcohol dependence: persistence and healthcare costs and utilization. Am J Manag Care. 2011 /;17 Suppl 8:S222; 234.

43. Mark TL, Montejano LB, Kranzler HR, Chalk M, Gastfriend DR. Comparison of healthcare utilization among patients treated with alcoholism medications. Am J Managed Care. 2010 /;16(12):879-88.

44. Knudsen HK, Roman PM. Financial factors and the implementation of medications for treating opioid use disorders. J Addict Med. 2012 /;6(4):280-6.

45. Knudsen HK, Abraham AJ, Roman PM. Adoption and implementation of medications in addiction treatment programs. J Addict Med. 2011 /;5(1):21-7.

46. Mandayam S, Jamal MM, Morgan TR. Epidemiology of alcoholic liver disease. Semin Liver Dis. 2004 /;24(3):217-32.

47. Mann RE, Smart RG, Govoni R. The epidemiology of alcoholic liver disease. Alcohol Res Health. 2003 /;27(3):209-19.

48. Akbar M, Egli M, Cho YE, Song BJ, Noronha A. Medications for alcohol use disorders: An overview. Pharmacol Ther. 2018. May;185:64-85.

49. Laaksonen E, Koski-Jannes A, Salaspuro M, Ahtinen H, Alho H. A randomized, multicentre, open-label, comparative trial of disulfiram, naltrexone and acamprosate in the treatment of alcohol dependence. Alcohol Alcohol. 2008 Jan-Feb;43(1):53-61.

50. Skinner MD, Lahmek P, Pham H, Aubin HJ. Disulfiram efficacy in the treatment of alcohol dependence: a meta-analysis. PLoS One. 2014 Feb 10;9(2):e87366.

51. Jonas DE, Amick HR, Feltner C, Bobashev G, Thomas K, Wines R, et al. Pharmacotherapy for adults with alcohol use disorders in outpatient settings: a systematic review and meta-analysis. JAMA. 2014 May 14;311(18):1889-900.

52. Mason BJ. Treatment of alcohol-dependent outpatients with acamprosate: a clinical review. J Clin Psychiatry. 2001;62 Suppl 20:42-8.

53. Reilly MT, Lobo IA, McCracken LM, Borghese CM, Gong D, Horishita T, et al. Effects of acamprosate on neuronal receptors and ion channels expressed in Xenopus oocytes. Alcohol Clin Exp Res. 2008 /;32(2):188-96.

54. Rosner S, Hackl-Herrwerth A, Leucht S, Vecchi S, Srisurapanont M, Soyka M. Opioid antagonists for alcohol dependence. Cochrane Database Syst Rev. 2010 Dec 8;(12):CD001867.

55. Donovan DM, Anton RF, Miller WR, Longabaugh R, Hosking JD, Youngblood M, et al. Combined pharmacotherapies and behavioral interventions for alcohol dependence (The COMBINE Study): examination of posttreatment drinking outcomes. J Stud Alcohol Drugs. 2008 Jan;69(1):5-13.

56. Anton RF, O'Malley SS, Ciraulo DA, Cisler RA, Couper D, Donovan DM, et al. Combined pharmacotherapies and behavioral interventions for alcohol dependence: the COMBINE study: a randomized controlled trial. JAMA. 2006 May 3;295(17):2003-17.

57. Kiefer F, Jahn H, Tarnaske T, Helwig H, Briken P, Holzbach R, et al. Comparing and combining naltrexone and acamprosate in relapse prevention of alcoholism: a double-blind, placebo-controlled study. Arch Gen Psychiatry. 2003 Jan;60(1):92-9.

58. Pani PP, Trogu E, Pacini M, Maremmani I. Anticonvulsants for alcohol dependence. Cochrane Database Syst Rev. 2014 Feb 13;(2):CD008544.

59. Minozzi S, Amato L, Vecchi S, Davoli M. Anticonvulsants for alcohol withdrawal. Cochrane Database Syst Rev. 2010 Mar 17;(3):CD005064..

60. Kendall PC, Safford S, Flannery-Schroeder E, Webb A. Child anxiety treatment: outcomes in adolescence and impact on substance use and depression at 7.4-year follow-up. J Consult Clin Psychol. 2004 Apr;72(2):276-87.

61. Offidani E, Guidi J, Tomba E, Fava GA. Efficacy and tolerability of benzodiazepines versus antidepressants in anxiety disorders: a systematic review and meta-analysis. Psychother Psychosom. 2013;82(6):355-62.

62. Day C. Benzodiazepines in Combination with Opioid Pain Relievers or Alcohol: Greater Risk of More Serious ED Visit Outcomes. In: The CBHSQ Report. Rockville (MD): ; 2013.1-9.

63. Wimbiscus M, Kostenko O, Malone D. MAO inhibitors: risks, benefits, and lore. Cleve Clin J Med. 2010 Dec;77(12):859-82.

64. Hernandez-Avila CA, Modesto-Lowe V, Feinn R, Kranzler HR. Nefazodone treatment of comorbid alcohol dependence and major depression. Alcohol Clin Exp Res. 2004 Mar;28(3):433-40.

65. Bruno F. Buspirone in the treatment of alcoholic patients. Psychopathology. 1989;22 Suppl 1:49-59.

66. Tollefson GD, Montague-Clouse J, Tollefson SL. Treatment of comorbid generalized anxiety in a recently detoxified alcoholic population with a selective serotonergic drug (buspirone). J Clin Psychopharmacol. 1992 Feb;12(1):19-26.

67. Junqueira-Ayres DD, Asth L, Ayres AS, Lobao-Soares B, Soares-Rachetti VP, Gavioli EC. Topiramate reduces basal anxiety and relieves ethanol withdrawal-induced anxious behaviors in male rats. Exp Clin Psychopharmacol. 2017 Apr;25(2):105-13.

68. Azevedo CA, Mammis A. Neuromodulation Therapies for Alcohol Addiction: A Literature Review. Neuromodulation. Feb;21(2):144-148.

69. Baker AL, Thornton LK, Hiles S, Hides L, Lubman DI. Psychological interventions for alcohol misuse among people with co-occurring depression or anxiety disorders: a systematic review. J Affect Disord. 2012 Aug;139(3):217-29.

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

71. Lester D, Freed EX. Criteria for an animal model of alcoholism. PHARM BIOCHEM BEHAV. 1973 1973/;1(1):103-7.

72. McBride WJ, Li T-. Animal models of alcoholism: Neurobiology of high alcohol-drinking behavior in rodents. Crit Rev Neurobiol. 1998 1998/;12(4):339-69.

73. Lumeng L, Hawkins TD, Li T-K. New strains of rats with alcohol preference and nonpreference. In: Thurman RG, Williamson JR, Drott HR, Chance B, editor. Alcohol and Aldehyde Metabolizing Systems. New York: Academic Press; 1977. p. 537.

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

75. Lankford MF, Roscoe AK, Pennington SN, Myers RD. Drinking of high concentrations of ethanol versus palatable fluids in alcohol-preferring (P) rats: valid animal model of alcoholism. Alcohol. 1991 Jul-Aug;8(4):293-9.

76. McBride WJ, Rodd ZA, Bell RL, Lumeng L, Li T-. The alcohol-preferring (P) and high-alcohol-drinking (HAD) rats - Animal models of alcoholism. Alcohol. 2014 /;48(3):209-15.

77. Colombo G, Lobina C, Carai MAM, Gessa GL. Phenotypic characterization of genetically selected Sardinian alcohol-preferring (sP) and -non-preferring (sNP) rats. Addict Biol. 2006 /;11(3-4):324-38.

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

79. Murphy JM, Stewart RB, Bell RL, Badia-Elder NE, Carr LG, McBride WJ, et al. Phenotypic and genotypic characterization of the Indiana university rat lines selectively bred for high and low alcohol preference. Behav Genet. 2002 /;32(5):363-88.

80. Colombo G, Agabio R, Lobina C, Reali R, Zocchi A, Fadda F, et al. Sardinian alcohol-preferring rats: A genetic animal model of anxiety. Physiol Behav. 1995 1995/;57(6):1181-5.

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

82. Kampov-Polevoy AB, Matthews DB, Gause L, Morrow AL, Overstreet DH. P Rats Develop Physical Dependence on Alcohol Via Voluntary Drinking: Changes in Seizure Thresholds, Anxiety, and Patterns of Alcohol Drinking. Alcoholism: Clinical and Experimental Research. 2000;24(3):278-84.

83. McBride WJ, Murphy JM, Gatto GJ, Levy AD, Yoshimoto K, Lumeng L, et al. CNS mechanisms of alcohol self-administration. Alcohol Alcohol Suppl. 1993;2:463-7.

84. Waller MB, McBride WJ, Gatto GJ, Lumeng L, Li TK. Intragastric self-infusion of ethanol by ethanol-preferring and -nonpreferring lines of rats. Science. 1984 Jul 6;225(4657):78-80.

85. Gatto GJ, McBride WJ, Murphy JM, Lumeng L, Li TK. Ethanol self-infusion into the ventral tegmental area by alcohol-preferring rats. Alcohol. 1994 Nov-Dec;11(6):557-64.

86. Ciccocioppo R, Angeletti S, Weiss F. Long-Lasting Resistance to Extinction of Response Reinstatement Induced by Ethanol-Related Stimuli: Role of Genetic Ethanol Preference. Alcoholism: Clinical and Experimental Research. 2001;25(10):1414-9.

87. Murphy JM, Gatto GJ, McBride WJ, Lumeng L, Li TK. Operant responding for oral ethanol in the alcohol-preferring P and alcohol-nonpreferring NP lines of rats. Alcohol. 1989 Mar-Apr;6(2):127-31.

88. Rodd ZA, Bell RL, Kuc KA, Murphy JM, Lumeng L, Li TK, et al. Effects of repeated alcohol deprivations on operant ethanol self-administration by alcohol-preferring (P) rats. Neuropsychopharmacology. 2003 Sep;28(9):1614-21.

89. Penn PE, McBride WJ, Lumeng L, Gaff TM, Li TK. Neurochemical and operant behavioral studies of a strain of alcohol-preferring rats. Pharmacol Biochem Behav. 1978 Apr;8(4):475-81.

90. Lumeng L, Li TK. The development of metabolic tolerance in the alcohol-preferring P rats: comparison of forced and free-choice drinking of ethanol. Pharmacol Biochem Behav. 1986 Nov;25(5):1013-20.

91. Gatto GJ, Murphy JM, Waller MB, McBride WJ, Lumeng L, Li TK. Persistence of tolerance to a single dose of ethanol in the selectively-bred alcohol-preferring P rat. Pharmacol Biochem Behav. 1987 Sep;28(1):105-10.

92. Waller MB, McBride WJ, Lumeng L, Li TK. Induction of dependence on ethanol by free-choice drinking in alcohol-preferring rats. Pharmacol Biochem Behav. 1982 Mar;16(3):501-7.

93. Rodd-Henricks ZA, McKinzie DL, Shaikh SR, Murphy JM, McBride WJ, Lumeng L, et al. Alcohol Deprivation Effect Is Prolonged in the Alcohol Preferring (P) Rat After Repeated Deprivations. Alcoholism: Clinical and Experimental Research. 2000;24(1):8-16.

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

95. Chiamulera C, Valerio E, Tessari M. Resumption of ethanol-seeking behaviour in rats. Behav Pharmacol. 1995 Jan;6(1):32-9.

96. Hwang BH, Zhang JK, Ehlers CL, Lumeng L, Li TK. Innate differences of neuropeptide Y (NPY) in hypothalamic nuclei and central nucleus of the amygdala between selectively bred rats with high and low alcohol preference. Alcohol Clin Exp Res. 1999 Jun;23(6):1023-30.

97. Sakharkar AJ, Zhang H, Tang L, Baxstrom K, Shi G, Moonat S, et al. Effects of histone deacetylase inhibitors on amygdaloid histone acetylation and neuropeptide Y expression: a role in anxiety-like and alcohol-drinking behaviours. Int J Neuropsychopharmacol. 2014 Aug;17(8):1207-20.

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

99. Le AD, Quan B, Juzytch W, Fletcher PJ, Joharchi N, Shaham Y. Reinstatement of alcohol-seeking by priming injections of alcohol and exposure to stress in rats. Psychopharmacology (Berl). 1998 Jan;135(2):169-74.

100. Debatin T, Barbosa ADE. Effect of isopregnanolone on rapid tolerance to the anxiolytic effect of ethanol. Rev Bras Psiquiatr. 2006 /;28(1):18-23.

101. Koob GF, Le Moal M. Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. Nat Neurosci. 2005 Nov;8(11):1442-4.

102. Kalant H. Research on Tolerance: What Can We Learn From History? Alcoholism: Clinical and Experimental Research. 1998;22(1):67-76.

103. Khanna JM, Chau A, Shah G. Characterization of the Phenomenon of rapid tolerance to ethanol. Alcohol. 1996 Nov-Dec;13(6):621-8.

104. Koob GF, Wall TL, Schafer J. Rapid induction of tolerance to the antipunishment effects of ethanol. Alcohol. 1987 1987/;4(6):481-4.

105. Sakharkar AJ, Zhang H, Tang L, Shi G, Pandey SC. Histone Deacetylases (HDAC)-Induced Histone Modifications in the Amygdala: A Role in Rapid Tolerance to the Anxiolytic Effects of Ethanol. Alcohol Clin Exp Res. 2012 /;36(1):61-71.

106. Chandler LJ, Harris RA, Crews FT. Ethanol tolerance and synaptic plasticity. Trends Pharmacol Sci. 1998 Dec;19(12):491-5.

107. Crabbe JC, Rigter H, Uijlen J, Strijbos C. Rapid development of tolerance to the hypothermic effect of ethanol in mice. J Pharmacol Exp Ther. 1979 Jan;208(1):128-33.

108. Gill K, Deitrich RA. Acute tolerance to the ataxic effects of ethanol in short-sleep (SS) and long-sleep (LS) mice. Psychopharmacology (Berl). 1998 Mar;136(1):91-8.

109. Bitran M, Kalant H. Development of rapid tolerance to pentobarbital and cross-tolerance to ethanol on a motor performance test with intoxicated practice. Pharmacol Biochem Behav. 1993 Apr;44(4):981-3.

110. Bitran M, Kalant H. Learning factor in rapid tolerance to ethanol-induced motor impairment. Pharmacol Biochem Behav. 1991 Aug;39(4):917-22.

111. Kurtz DL, Stewart RB, Zweifel M, Li TK, Froehlich JC. Genetic differences in tolerance and sensitization to the sedative/hypnotic effects of alcohol. Pharmacol Biochem Behav. 1996 Mar;53(3):585-91.

112. Khanna JM, Kalant H, Shah G, Weiner J. Rapid tolerance as an index of chronic tolerance. Pharmacology Biochemistry and Behavior. 1991 2;38(2):427-32.

113. Pandey SC. Anxiety and Alcohol Use Disorders: A Perspective from Molecular and Epigenetic Studies
. In: *Neurobiology of Alcohol Dependence*. Elsevier Inc.; 2014. p. 451,-466.

114. Park A, Ghezzi A, Wijesekera TP, Atkinson NS. Genetics and genomics of alcohol responses in Drosophila. Neuropharmacology. 2017 Aug 1;122:22-35.

115. Berger KH, Heberlein U, Moore MS. Rapid and chronic: two distinct forms of ethanol tolerance in Drosophila. Alcohol Clin Exp Res. 2004 Oct;28(10):1469-80.

116. Atkinson NS. Tolerance in Drosophila. J Neurogenet. 2009;23(3):293-302.

117. Pietrzykowski AZ, Treistman SN. The molecular basis of tolerance. Alcohol Res Health. 2008;31(4):298-309.

118. Khanna JM, Shah G, Weiner J, Wu PH, Kalant H. Effect of NMDA receptor antagonists on rapid tolerance to ethanol. Eur J Pharmacol. 1993 Jan 5;230(1):23-31.

119. Rustay NR, Crabbe JC. Genetic analysis of rapid tolerance to ethanol's incoordinating effects in mice: inbred strains and artificial selection. Behav Genet. 2004 Jul;34(4):441-51.

120. Koob GF, Volkow ND. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry. 2016 Aug;3(8):760-73.

121. Pandey SC, Kyzar EJ, Zhang H. Epigenetic basis of the dark side of alcohol addiction. Neuropharmacology. 2017 Aug 1;122:74-84.

122. AKERT K, GRUESEN RA, WOOLSEY CN, MEYER DR. Kluver-Bucy syndrome in monkeys with neocortical ablations of temporal lobe. Brain. 1961 Sep;84:480-98.

123. Pilleri G. The Kluver-Bucy Syndrome in man. A clinico-anatomical contribution to the function of the medial temporal lobe structures. Psychiatr Neurol (Basel). 1966;152(2):65-103.

124. Chareyron LJ, Banta Lavenex P, Amaral DG, Lavenex P. Stereological analysis of the rat and monkey amygdala. J Comp Neurol. 2011 Nov 1;519(16):3218-39.

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

126. Tyszka JM, Pauli WM. In vivo delineation of subdivisions of the human amygdaloid complex in a high-resolution group template. Hum Brain Mapp. 2016 Nov;37(11):3979-98.

127. LeDoux J. The amygdala. Curr Biol. 2007 Oct 23;17(20):R868-74.

128. Alheid GF. Extended amygdala and basal forebrain. Ann New York Acad Sci. 2003 2003/;985:185-205.

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

130. Gilpin NW, Herman MA, Roberto M. The central amygdala as an integrative hub for anxiety and alcohol use disorders. Biol Psychiatry. 2015 May 15;77(10):859-69.

131. Shi CJ, Cassell MD. Cortical, thalamic, and amygdaloid connections of the anterior and posterior insular cortices. J Comp Neurol. 1998 Oct 5;399(4):440-68.

132. Mcdonald AJ, Mascagni F, Guo L. Projections of the medial and lateral prefrontal cortices to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat. Neuroscience. 1996 Mar;71(1):55-75.

133. Pitkanen A, Savander V, LeDoux JE. Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. Trends Neurosci. 1997 Nov;20(11):517-23.

134. Jolkkonen E, Pitkanen A. Intrinsic connections of the rat amygdaloid complex: projections originating in the central nucleus. J Comp Neurol. 1998 May 25;395(1):53-72.

135. Sah P. Fear, Anxiety, and the Amygdala. Neuron. 2017 Sep 27;96(1):1-2.

136. Lovinger DM, Roberto M. Synaptic effects induced by alcohol. Curr Top Behav Neurosci. 2013;13:31-86.

137. Verhulst B, Neale MC, Kendler KS. The heritability of alcohol use disorders: a meta-analysis of twin and adoption studies. . 2015;45(5):1061-72.

138. Starkman BG, Sakharkar AJ, Pandey SC. Epigenetics-beyond the genome in alcoholism. Alcohol Res. 2012;34(3):293-305.

139. Holliday R. Epigenetics: a historical overview. Epigenetics. 2006 /;1(2):76-80.

140. Kouzarides T. Chromatin Modifications and Their Function. Cell. 2007 2007/02;128(4):693-705.

141. Krishnan HR, Sakharkar AJ, Teppen TL, Berkel TD, Pandey SC. The epigenetic landscape of alcoholism. Int Rev Neurobiol. 2014;115:75-116.

142. Henkels CH, Khorasanizadeh S. Implications of a histone code mimic in epigenetic signaling. Mol Cell. 2007 Aug 17;27(4):521-2.

143. Jenuwein T, Allis CD. Translating the histone code. Science. 2001 Aug 10;293(5532):1074-80.

144. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. Nat Rev Genet. 2016 Aug;17(8):487-500.

145. Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, et al. Combinatorial patterns of histone acetylations and methylations in the human genome. Nat Genet. 2008 Jul;40(7):897-903.

146. Feng J, Nestler EJ. Epigenetic mechanisms of drug addiction. Curr Opin Neurobiol. 2013 /;23(4):521-8.

147. Pandey SC, Ugale R, Zhang H, Tang L, Prakash A. Brain chromatin remodeling: A novel mechanism of alcoholism. J Neurosci. 2008 2008/04;28(14):3729-37.

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

149. Kyzar EJ, Floreani C, Teppen TL, Pandey SC. Adolescent Alcohol Exposure: Burden of Epigenetic Reprogramming, Synaptic Remodeling, and Adult Psychopathology. Front Neurosci. 2016 May 31;10:222.

150. Berkel TD, Pandey SC. Emerging Role of Epigenetic Mechanisms in Alcohol Addiction. Alcohol Clin Exp Res. 2017 Apr;41(4):666-80.

151. Palmisano M, Pandey SC. Epigenetic mechanisms of alcoholism and stress-related disorders. Alcohol. 2017 May;60:7-18.

152. Bednar J, Horowitz RA, Grigoryev SA, Carruthers LM, Hansen JC, Koster AJ, et al. Nucleosomes, linker DNA, and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin. Proc Natl Acad Sci U S A. 1998 Nov 24;95(24):14173-8.

153. Li XY, Thomas S, Sabo PJ, Eisen MB, Stamatoyannopoulos JA, Biggin MD. The role of chromatin accessibility in directing the widespread, overlapping patterns of Drosophila transcription factor binding. Genome Biol. 2011;12(4):R34.

154. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. Cell Res. 2011 Mar;21(3):381-95.

155. Smolle M, Workman JL. Transcription-associated histone modifications and cryptic transcription. Biochim Biophys Acta. 2013 Jan;1829(1):84-97.

156. Guccione E, Martinato F, Finocchiaro G, Luzi L, Tizzoni L, Dall'Olio V, et al. Myc-binding-site recognition in the human genome is determined by chromatin context. Nat Cell Biol. 2006 Jul;8(7):764-70.

157. Guertin MJ, Lis JT. Chromatin landscape dictates HSF binding to target DNA elements. PLoS Genet. 2010 Sep 9;6(9):e1001114.

158. Kan PY, Lu X, Hansen JC, Hayes JJ. The H3 tail domain participates in multiple interactions during folding and self-association of nucleosome arrays. Mol Cell Biol. 2007 Mar;27(6):2084-91.

159. Sperling AS, Grunstein M. Histone H3 N-terminus regulates higher order structure of yeast heterochromatin. Proc Natl Acad Sci U S A. 2009 Aug 11;106(32):13153-9.

160. Marmorstein R, Roth SY. Histone acetyltransferases: function, structure, and catalysis. Curr Opin Genet Dev. 2001 Apr;11(2):155-61.

161. Cho Y, Cavalli V. HDAC signaling in neuronal development and axon regeneration. Curr Opin Neurobiol. 2014 Aug;27:118-26.

162. Nestler EJ, Pena CJ, Kundakovic M, Mitchell A, Akbarian S. Epigenetic Basis of Mental Illness. Neuroscientist. 2016;22(5):447-63 .

163. Pirooznia SK, Elefant F. Targeting specific HATs for neurodegenerative disease treatment: translating basic biology to therapeutic possibilities. Front Cell Neurosci. 2013 Mar 28;7:30.

164. Yeh SH, Lin CH, Gean PW. Acetylation of nuclear factor-kappaB in rat amygdala improves long-term but not short-term retention of fear memory. Mol Pharmacol. 2004 May;65(5):1286-92.

165. Covington HE,3rd, Maze I, Sun H, Bomze HM, DeMaio KD, Wu EY, et al. A role for repressive histone methylation in cocaine-induced vulnerability to stress. Neuron. 2011 Aug 25;71(4):656-70.

166. Kim JT, Li J, Jang ER, Napier DL, Weiss HL, Lee E, et al. Chemical inhibitors of the Wnt pathway or G9a showed anti-tumor effects in human neuroendocrine tumor cells with deregulated Wnt/(beta)-catenin signaling. Gastroenterology. 2013 /;144(5):S805.

167. Schaefer A, Sampath SC, Intrator A, Min A, Gertler TS, Surmeier DJ, et al. Control of Cognition and Adaptive Behavior by the GLP/G9a Epigenetic Suppressor Complex. Neuron. 2009 12/10;64(5):678-91.

168. Maze I, Covingtoni III HE, Dietz DM, Laplant Q, Renthal W, Russo SJ, et al. Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. Science. 2010 2010/;327(5962):213-6.

169. Tachibana M, Sugimoto K, Nozaki M, Ueda J, Ohta T, Ohki M, et al. G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. Genes Dev. 2002 2002/07;16(14):1779-91.

170. Subbanna S, Shivakumar M, Umapathy NS, Saito M, Mohan PS, Kumar A, et al. G9a-mediated histone methylation regulates ethanol-induced neurodegeneration in the neonatal mouse brain. Neurobiol Dis. 2013 Jun;54:475-85.

171. Qiang M, Denny A, Lieu M, Carreon S, Li J. Histone H3K9 modifications are a local chromatin event involved in ethanol-induced neuroadaptation of the NR2B gene. Epigenetics. 2011 /;6(9):1095-104.

172. Liu C-, Cha S-, Tan C-, Kuo M-. Histone methyltransferase G9a promotes the oral cancer cells recovery from drug-induced DNA damage. Cancer Res. 2013 2013/04;73(8).

173. Ding D, Qu X, Li L, Zhou X, Liu S, Lin S, et al. Involvement of histone methyltransferase GLP in HIV-1 latency through catalysis of H3K9 dimethylation. Virology. 2013 2013/06;440(2):182-9.

174. Subbanna S, Basavarajappa BS. Pre-administration of G9a/GLP inhibitor during synaptogenesis prevents postnatal ethanol-induced LTP deficits and neurobehavioral abnormalities in adult mice. Exp Neurol. 2014 Nov;261:34-43.

175. Pattaroni C, Jacob C. Histone methylation in the nervous system: functions and dysfunctions. Mol Neurobiol. 2013 Apr;47(2):740-56.

176. Li E, Zhang Y. DNA methylation in mammals. Cold Spring Harb Perspect Biol. 2014 May 1;6(5):a019133.

177. Chahrour M, Jung SY, Shaw C, Zhou X, Wong ST, Qin J, et al. MeCP2, a key contributor to neurological disease, activates and represses transcription. Science. 2008 May 30;320(5880):1224-9.

178. Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet. 1998 Jun;19(2):187-91.

179. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature. 1998 May 28;393(6683):386-9.

180. Yang X, Han H, De Carvalho DD, Lay FD, Jones PA, Liang G. Gene body methylation can alter gene expression and is a therapeutic target in cancer. Cancer Cell. 2014 Oct 13;26(4):577-90.

181. Feng J, Chang H, Li E, Fan G. Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. J Neurosci Res. 2005 Mar 15;79(6):734-46.

182. Szulwach KE, Li X, Li Y, Song CX, Wu H, Dai Q, et al. 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. Nat Neurosci. 2011 Oct 30;14(12):1607-16.

183. Wu SC, Zhang Y. Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol. 2010 Sep;11(9):607-20.

184. Bhutani N, Burns DM, Blau HM. DNA demethylation dynamics. Cell. 2011 Sep 16;146(6):866-72.

185. Ma DK, Guo JU, Ming GL, Song H. DNA excision repair proteins and Gadd45 as molecular players for active DNA demethylation. Cell Cycle. 2009 May 15;8(10):1526-31.

186. Guo JU, Su Y, Zhong C, Ming GL, Song H. Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. Cell. 2011 Apr 29;145(3):423-34.

187. Valinluck V, Sowers LC. Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res. 2007 Feb 1;67(3):946-50.

188. Bardag-Gorce F, French BA, Joyce M, Baires M, Montgomery RO, Li J, et al. Histone acetyltransferase p300 modulates gene expression in an epigenetic manner at high blood alcohol levels. Exp Mol Pathol. 2007 Apr;82(2):197-202.

189. Finegersh A, Homanics GE. Acute ethanol alters multiple histone modifications at model gene promoters in the cerebral cortex. Alcohol Clin Exp Res. 2014 Jul;38(7):1865-73.

190. Finegersh A, Ferguson C, Maxwell S, Mazariegos D, Farrell D, Homanics GE. Repeated vapor ethanol exposure induces transient histone modifications in the brain that are modified by genotype and brain region. Front Mol Neurosci. 2015 Aug 5;8:39.

191. Sakharkar AJ, Tang L, Zhang H, Chen Y, Grayson DR, Pandey SC. Effects of acute ethanol exposure on anxiety measures and epigenetic modifiers in the extended amygdala of adolescent rats. Int J Neuropsychopharmacol. 2014 17(12):2057-67.

192. Sakharkar AJ, Vetreno RP, Zhang H, Kokare DM, Crews FT, Pandey SC. A role for histone acetylation mechanisms in adolescent alcohol exposure-induced deficits in hippocampal brain-derived neurotrophic factor expression and neurogenesis markers in adulthood. Brain Struct Funct. 2016; ;221(9):4691-4703.

193. Ghezzi A, Krishnan HR, Lew L, Prado FJ,3rd, Ong DS, Atkinson NS. Alcohol-induced histone acetylation reveals a gene network involved in alcohol tolerance. PLoS Genet. 2013;9(12):e1003986.

194. Correa F, De Laurentiis A, Franchi AM. Ethanol downregulates N-acyl phosphatidylethanolamine-phospholipase D expression in BV2 microglial cells via epigenetic mechanisms. Eur J Pharmacol. 2016 Jun 5;786:224-33.

195. Guo W, Crossey EL, Zhang L, Zucca S, George OL, Valenzuela CF, et al. Alcohol exposure decreases CREB binding protein expression and histone acetylation in the developing cerebellum. PLoS One. 2011;6(5):e19351.

196. Kyzar EJ, Pandey SC. Molecular mechanisms of synaptic remodeling in alcoholism. Neurosci Lett. 2015 Aug 5;601:11-9.

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

198. Janak PH, Tye KM. From circuits to behaviour in the amygdala. Nature. 2015 Jan 15;517(7534):284-92.

199. Pandey SC, Zhang H, Ugale R, Prakash A, Xu T, Misra K. Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. J Neurosci. 2008 2008/03;28(10):2589-600.

200. Sakharkar AJ, Tang L, Zhang H, Pandey SC. Effects of various doses of ethanol exposure on HDAC and dnmt activities in the extended amygdala of adolescent rats. Alcohol Clin Exp Res. 2012 /;36:181A.

201. D'Addario C, Caputi FF, Ekstrom TJ, Di Benedetto M, Maccarrone M, Romualdi P, et al. Ethanol induces epigenetic modulation of prodynorphin and pronociceptin gene expression in the rat amygdala complex. J Mol Neurosci. 2013 Feb;49(2):312-9.

202. McCarthy MJ, Duchemin AM, Neff NH, Hadjiconstantinou M. CREB involvement in the regulation of striatal prodynorphin by nicotine. Psychopharmacology (Berl). 2012 May;221(1):143-53.

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

204. Pandey SC, Roy A, Zhang H. 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. 2003 Mar;27(3):396-409.

205. Pandey SC, Zhang H, Roy A, Xu T. Deficits in amygdaloid cAMP-responsive element–binding protein signaling play a role in genetic predisposition to anxiety and alcoholism. - The Journal of Clinical Investigation. 2005(- 10):- 2762.

206. Moonat S, Sakharkar AJ, Zhang H, Tang L, Pandey SC. Aberrant histone deacetylase2-mediated histone modifications and synaptic plasticity in the amygdala predisposes to anxiety and alcoholism. Biol Psychiatry. 2013 2013/04;73(8):763-73.

207. Harper C. The neuropathology of alcohol-related brain damage. Alcohol Alcohol. 2009 Mar-Apr;44(2):136-40.

208. Kalkhoven E. CBP and p300: HATs for different occasions. Biochem Pharmacol. 2004 Sep 15;68(6):1145-55.

209. Warnault V, Darcq E, Levine A, Barak S, Ron D. Chromatin remodeling-a novel strategy to control excessive alcohol drinking. Transl Psychiatry. 2013 2013/;3.

210. Khanna JM, Kalant H, Weiner J, Shah G. Rapid tolerance and cross-tolerance as predictors of chronic tolerance and cross-tolerance. Pharmacology Biochemistry and Behavior. 1992 2;41(2):355-60.

211. You C, Zhang H, Sakharkar AJ, Teppen T, Pandey SC. Reversal of deficits in dendritic spines, BDNF and Arc expression in the amygdala during alcohol dependence by HDAC inhibitor treatment. Int J Neuropsychopharmacol. 2014 /;17(2):313-22.

212. Lopez-Moreno JA, Marcos M, Calleja-Conde J, Echeverry-Alzate V, Buhler KM, Costa-Alba P, et al. Histone Deacetylase Gene Expression Following Binge Alcohol Consumption in Rats and Humans. Alcohol Clin Exp Res. 2015 Oct;39(10):1939-50.

213. Driessen M, Meier S, Hill A, Wetterling T, Lange W, Junghanns K. The course of anxiety, depression and drinking behaviours after completed detoxification in alcoholics with and without comorbid anxiety and depressive disorders. Alcohol Alcohol. 2001 2001/;36(3):249-55.

214. Jeanblanc J, Lemoine S, Jeanblanc V, Alaux-Cantin S, Naassila M. The Class I-Specific HDAC Inhibitor MS-275 Decreases Motivation to Consume Alcohol and Relapse in Heavy Drinking Rats. Int J Neuropsychopharmacol. 2015 Mar 11;18(9):10.

215. Qiang M, Li JG, Denny AD, Yao JM, Lieu M, Zhang K, et al. Epigenetic mechanisms are involved in the regulation of ethanol consumption in mice. Int J Neuropsychopharmacol. 2014 Oct 31;18(2):10.

216. Simon-O'Brien E, Alaux-Cantin S, Warnault V, Buttolo R, Naassila M, Vilpoux C. The histone deacetylase inhibitor sodium butyrate decreases excessive ethanol intake in dependent animals. Addict Biol. 2015 Jul;20(4):676-89.

217. Kramer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA, et al. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. EMBO J. 2003 Jul 1;22(13):3411-20.

218. Al Ameri M, Al Mansouri S, Al Maamari A, Bahi A. The histone deacetylase (HDAC) inhibitor valproic acid reduces ethanol consumption and ethanol-conditioned place preference in rats. Brain Res. 2014 Oct 2;1583:122-31.

219. Pascual M, Do Couto BR, Alfonso-Loeches S, Aguilar MA, Rodriguez-Arias M, Guerri C. Changes in histone acetylation in the prefrontal cortex of ethanol-exposed adolescent rats are associated with ethanol-induced place conditioning. Neuropharmacology. 2012 Jun;62(7):2309-19.

220. Barbier E, Johnstone AL, Khomtchouk BB, Tapocik JD, Pitcairn C, Rehman F, et al. Dependence-induced increase of alcohol self-administration and compulsive drinking mediated by the histone methyltransferase PRDM2. Mol Psychiatry. 2017 Dec;22(12):1746-58.

221. Karpyak VM, Winham SJ, Preuss UW, Zill P, Cunningham JM, Walker DL, et al. Association of the PDYN gene with alcohol dependence and the propensity to drink in negative emotional states. Int J Neuropsychopharmacol. 2013 Jun;16(5):975-85.

222. Xuei X, Dick D, Flury-Wetherill L, Tian HJ, Agrawal A, Bierut L, et al. Association of the kappa-opioid system with alcohol dependence. Mol Psychiatry. 2006 Nov;11(11):1016-24.

223. Kyzar EJ, Zhang H, Sakharkar AJ, Pandey SC. Adolescent alcohol exposure alters lysine demethylase 1 (LSD1) expression and histone methylation in the amygdala during adulthood. Addict Biol. 2017 Sep;22(5):1191-1204.

224. Pandey SC, Sakharkar AJ, Tang L, Zhang H. Potential role of adolescent alcohol exposure-induced amygdaloid histone modifications in anxiety and alcohol intake during adulthood. Neurobiol Dis. 2015 Oct;82:607-19.

225. Ponomarev I, Wang S, Zhang L, Harris RA, Mayfield RD. Gene coexpression networks in human brain identify epigenetic modifications in alcohol dependence. J Neurosci. 2012 Feb 1;32(5):1884-97.

226. Farris SP, Harris RA, Ponomarev I. Epigenetic modulation of brain gene networks for cocaine and alcohol abuse. Front Neurosci. 2015 May 20;9:176.

227. Wang KS, Liu X, Zhang Q, Wu LY, Zeng M. Genome-wide association study identifies 5q21 and 9p24.1 (KDM4C) loci associated with alcohol withdrawal symptoms. J Neural Transm (Vienna). 2012 Apr;119(4):425-33.

228. Klose RJ, Zhang Y. Regulation of histone methylation by demethylimination and demethylation. Nat Rev Mol Cell Biol. 2007 Apr;8(4):307-18.

229. Tachibana M, Sugimoto K, Fukushima T, Shinkai Y. SET Domain-containing Protein, G9a, is a Novel Lysine-preferring Mammalian Histone Methyltransferase with Hyperactivity and Specific Selectivity to Lysines 9 and 27 of Histone H3. J Biol Chem. 2001 2001/07;276(27):25309-17.

230. Shinkai Y, Tachibana M. H3K9 methyltransferase G9a and the related molecule GLP. Genes Dev. 2011 Apr 15;25(8):781-8.

231. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, et al. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature. 2000 Aug 10;406(6796):593-9.

232. Ikegami K, Iwatani M, Suzuki M, Tachibana M, Shinkai Y, Tanaka S, et al. Genome-wide and locus-specific DNA hypomethylation in G9a deficient mouse embryonic stem cells. Genes Cells. 2007 Jan;12(1):1-11.

233. Du J, Johnson LM, Jacobsen SE, Patel DJ. DNA methylation pathways and their crosstalk with histone methylation. Nat Rev Mol Cell Biol. 2015 Sep;16(9):519-32.

234. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet. 2009 May;10(5):295-304.

235. Zhao Q, Zhang J, Chen R, Wang L, Li B, Cheng H, et al. Dissecting the precise role of H3K9 methylation in crosstalk with DNA maintenance methylation in mammals. Nat Commun. 2016 Aug 24;7:12464.

236. Epsztejn-Litman S, Feldman N, Abu-Remaileh M, Shufaro Y, Gerson A, Ueda J, et al. De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. Nat Struct Mol Biol. 2008 Nov;15(11):1176-83.

237. Esteve PO, Chin HG, Smallwood A, Feehery GR, Gangisetty O, Karpf AR, et al. Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. Genes Dev. 2006 Nov 15;20(22):3089-103.

238. Rai K, Jafri IF, Chidester S, James SR, Karpf AR, Cairns BR, et al. Dnmt3 and G9a cooperate for tissue-specific development in zebrafish. J Biol Chem. 2010 Feb 5;285(6):4110-21.

239. Tachibana M, Matsumura Y, Fukuda M, Kimura H, Shinkai Y. G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. EMBO J. 2008 EMBO Press;27(20):2681-90.

240. Chang Y, Sun L, Kokura K, Horton JR, Fukuda M, Espejo A, et al. MPP8 mediates the interactions between DNA methyltransferase Dnmt3a and H3K9 methyltransferase GLP/G9a. Nat Commun. 2011 Nov 15;2:533.

241. Zhang T, Termanis A, Ozkan B, Bao XX, Culley J, de Lima Alves F, et al. G9a/GLP Complex Maintains Imprinted DNA Methylation in Embryonic Stem Cells. Cell Rep. 2016 Apr 5;15(1):77-85.

242. Smith ZD, Meissner A. DNA methylation: roles in mammalian development. Nat Rev Genet. 2013 Mar;14(3):204-20.

243. Dong KB, Maksakova IA, Mohn F, Leung D, Appanah R, Lee S, et al. DNA methylation in ES cells requires the lysine methyltransferase G9a but not its catalytic activity. EMBO J. 2008 Oct 22;27(20):2691-701.

244. Bittencourt D, Lee BH, Gao L, Gerke DS, Stallcup MR. Role of distinct surfaces of the G9a ankyrin repeat domain in histone and DNA methylation during embryonic stem cell self-renewal and differentiation. Epigenetics Chromatin. 2014 Oct 22;7:27,8935-7-27.

245. Collins RE, Northrop JP, Horton JR, Lee DY, Zhang X, Stallcup MR, et al. The ankyrin repeats of G9a and GLP histone methyltransferases are mono- and dimethyllysine binding modules. Nat Struct Mol Biol. 2008 Mar;15(3):245-50.

246. Nestler EJ. Epigenetic mechanisms of drug addiction. Neuropharmacology. 2014 Jan;76 Pt B:259-68.

247. Basavarajappa BS, Subbanna S. Epigenetic Mechanisms in Developmental Alcohol-Induced Neurobehavioral Deficits. Brain Sci. 2016 Apr 8;6(2).

248. Anderson EM, Larson EB, Guzman D, Marie Wissman A, Neve RL, Nestler EJ, et al. Overexpression of the histone dimethyltransferase G9a in nucleus accumbens shell increases cocaine self-administration, stress-induced reinstatement, and anxiety. J Neurosci. 2018 Jan 24;38(4):803-813.

249. Warnault V, Darcq E, Morisot N, Phamluong K, Wilbrecht L, Massa SM, et al. The BDNF Valine 68 to Methionine Polymorphism Increases Compulsive Alcohol Drinking in Mice That Is Reversed by Tropomyosin Receptor Kinase B Activation. Biol Psychiatry. 2016 Mar 15;79(6):463-73.

250. Krishnan HR, Zhang H, Sakharkar AJ, Pandey SC. Role of DNA methylation in amygdaloid circuitrymediated anxiolytic behaviors following acute ethanol exposure. Alcohol Clin Exp Res. 2013 /;37:62A.

251. Colzato LS, Van der Does AJW, Kouwenhoven C, Elzinga BM, Hommel B. BDNF Val66Met polymorphism is associated with higher anticipatory cortisol stress response, anxiety, and alcohol consumption in healthy adults. Psychoneuroendocrinology. 2011 11;36(10):1562-9.

252. Moonat S, Zhang H, Prakash A, Ugale R, Pandey SC. Amygdaloid brain-derived neurotrophic factor signaling and dendritic spines: A role in anxiety and alcoholism. Alcohol Clin Exp Res. 2009 /;33:321A.

253. Prakash A, Zhang H, Pandey SC. 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. 2008 /;32(6):909-20.

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

255. Chen Z-, Jing D, Bath KG, Ieraci A, Khan T, Siao C-, et al. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science. 2006 2006/10;314(5796):140-3.

256. Pandey SC, Roy A, Zhang H, Xu T. Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors. J Neurosci. 2004 May 26;24(21):5022-30.

257. Wand G. The anxious amygdala: CREB signaling and predisposition to anxiety and alcoholism. J Clin Invest. 2005 Oct;115(10):2697-9.

258. Zhang Z, Tao W, Hou YY, Wang W, Kenny PJ, Pan ZZ. MeCP2 repression of G9a in regulation of pain and morphine reward. J Neurosci. 2014 Jul 2;34(27):9076-87.

259. Sun H, Maze I, Dietz DM, Scobie KN, Kennedy PJ, Damez-Werno D, et al. Morphine epigenomically regulates behavior through alterations in histone H3 lysine 9 dimethylation in the nucleus accumbens. J Neurosci. 2012 2012/11;32(48):17454-64.

260. Balemans MCM, Huibers MMH, Eikelenboom NWD, Kuipers AJ, van Summeren RCJ, Pijpers MMCA, et al. Reduced exploration, increased anxiety, and altered social behavior: Autistic-like features of euchromatin histone methyltransferase 1 heterozygous knockout mice. Behav Brain Res. 2010 3/17;208(1):47-55.

261. Liu N, Zhang Z, Wu H, Jiang Y, Meng L, Xiong J, et al. Recognition of H3K9 methylation by GLP is required for efficient establishment of H3K9 methylation, rapid target gene repression, and mouse viability. Genes Dev. 2015 2015/;29(4):379-93.

262. Audergon PN, Catania S, Kagansky A, Tong P, Shukla M, Pidoux AL, et al. Epigenetics. Restricted epigenetic inheritance of H3K9 methylation. Science. 2015 Apr 3;348(6230):132-5.

263. Liu F, Barsyte-Lovejoy D, Li F, Xiong Y, Korboukh V, Huang X-, et al. Discovery of an in vivo chemical probe of the lysine methyltransferases G9a and GLP. J Med Chem. 2013 2013/11;56(21):8931-42.

264. Liu F, Chen X, Allali-Hassani A, Quinn AM, Wigle TJ, Wasney GA, et al. Protein lysine methyltransferase g9a inhibitors: Design, synthesis, and structure activity relationships of 2,4-diamino-7-aminoalkoxy-quinazolines. J Med Chem. 2010 2010/08;53(15):5844-57.

265. Kubicek S, O'Sullivan RJ, August EM, Hickey ER, Zhang Q, Teodoro M, et al. Reversal of H3K9me2 by a Small-Molecule Inhibitor for the G9a Histone Methyltransferase. Mol Cell. 2007 2/9;25(3):473-81.

266. Sweis RF, Michaelides MR. Chapter Thirteen - Recent Advances in Small-Molecule Modulation of Epigenetic Targets: Discovery and Development of Histone Methyltransferase and Bromodomain Inhibitors. Annual Reports in Medicinal Chemistry. 2013;48(0):185-203.

267. Vedadi M, Barsyte-Lovejoy D, Liu F, Rival-Gervier S, Allali-Hassani A, Labrie V, et al. A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. Nat Chem Biol. 2011 /;7(8):566-74.

268. Kim Y, Lee HM, Xiong Y, Sciaky N, Hulbert SW, Cao X, et al. Targeting the histone methyltransferase G9a activates imprinted genes and improves survival of a mouse model of Prader-Willi syndrome. Nat Med. 2017 Feb;23(2):213-22.

269. Wang DY, Kosowan J, Samsom J, Leung L, Zhang KL, Li YX, et al. Inhibition of the G9a/GLP histone methyltransferase complex modulates anxiety-related behavior in mice. Acta Pharmacol Sin2018 May;39(5):866-874.

270. Mahadev K, Vemuri MC. Effect of ethanol on chromatin and nonhistone nuclear proteins in rat brain. Neurochem Res. 1998 Sep;23(9):1179-84.

271. Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr. 2002 Aug;132(8 Suppl):2333S-5S.

272. Bonsch D, Lenz B, Reulbach U, Kornhuber J, Bleich S. Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism. J Neural Transm (Vienna). 2004 Dec;111(12):1611-6.

273. Chen CH, Pan CH, Chen CC, Huang MC. Increased oxidative DNA damage in patients with alcohol dependence and its correlation with alcohol withdrawal severity. Alcohol Clin Exp Res. 2011 Feb;35(2):338-44.

274. Bonsch D, Lenz B, Fiszer R, Frieling H, Kornhuber J, Bleich S. Lowered DNA methyltransferase (DNMT-3b) mRNA expression is associated with genomic DNA hypermethylation in patients with chronic alcoholism. J Neural Transm (Vienna). 2006 Sep;113(9):1299-304.

275. Garro AJ, McBeth DL, Lima V, Lieber CS. Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. Alcohol Clin Exp Res. 1991 Jun;15(3):395-8.

276. Zhang X, Kusumo H, Sakharkar AJ, Pandey SC, Guizzetti M. Regulation of DNA methylation by ethanol induces tissue plasminogen activator expression in astrocytes. J Neurochem. 2014 Feb;128(3):344-9.

277. Barbier E, Tapocik JD, Juergens N, Pitcairn C, Borich A, Schank JR, et al. DNA methylation in the medial prefrontal cortex regulates alcohol-induced behavior and plasticity. J Neurosci. 2015 Apr 15;35(15):6153-64.

278. Auta J, Zhang H, Pandey SC, Guidotti A. Chronic Alcohol Exposure Differentially Alters One-Carbon Metabolism in Rat Liver and Brain. Alcohol Clin Exp Res. 2017 Jun;41(6):1105-11.

279. Marutha Ravindran CR, Ticku MK. Changes in methylation pattern of NMDA receptor NR2B gene in cortical neurons after chronic ethanol treatment in mice. Brain Res Mol Brain Res. 2004 Feb 5;121(1-2):19-27.

280. Maier SE, Cramer JA, West JR, Sohrabji F. Alcohol exposure during the first two trimesters equivalent alters granule cell number and neurotrophin expression in the developing rat olfactory bulb. J Neurobiol. 1999 Nov 15;41(3):414-23.

281. Gavin DP, Kusumo H, Zhang H, Guidotti A, Pandey SC. Role of Growth Arrest and DNA Damage-Inducible, Beta in Alcohol-Drinking Behaviors. Alcohol Clin Exp Res. 2016 Feb;40(2):263-72.

282. Andersen AM, Dogan MV, Beach SR, Philibert RA. Current and Future Prospects for Epigenetic Biomarkers of Substance Use Disorders. Genes (Basel). 2015 Oct 14;6(4):991-1022.

283. Volkow ND, Koob G, Baler R. Biomarkers in substance use disorders. ACS Chem Neurosci. 2015 Apr 15;6(4):522-5.

284. Ladd-Acosta C. Epigenetic Signatures as Biomarkers of Exposure. Curr Environ Health Rep. 2015 Jun;2(2):117-25.

285. Hillemacher T, Frieling H, Hartl T, Wilhelm J, Kornhuber J, Bleich S. Promoter specific methylation of the dopamine transporter gene is altered in alcohol dependence and associated with craving. J Psychiatr Res. 2009 Jan;43(4):388-92.

286. Nieratschker V, Grosshans M, Frank J, Strohmaier J, von der Goltz C, El-Maarri O, et al. Epigenetic alteration of the dopamine transporter gene in alcohol-dependent patients is associated with age. Addict Biol. 2014 Mar;19(2):305-11.

287. Philibert RA, Gunter TD, Beach SR, Brody GH, Madan A. MAOA methylation is associated with nicotine and alcohol dependence in women. Am J Med Genet B Neuropsychiatr Genet. 2008 Jul 5;147B(5):565-70.

288. Liu C, Marioni RE, Hedman AK, Pfeiffer L, Tsai PC, Reynolds LM, et al. A DNA methylation biomarker of alcohol consumption. Mol Psychiatry. 2018 Feb;23(2):422-433.

289. Biermann T, Reulbach U, Lenz B, Frieling H, Muschler M, Hillemacher T, et al. N-methyl-D-aspartate 2b receptor subtype (NR2B) promoter methylation in patients during alcohol withdrawal. J Neural Transm (Vienna). 2009 May;116(5):615-22.

290. Bonsch D, Reulbach U, Bayerlein K, Hillemacher T, Kornhuber J, Bleich S. Elevated alpha synuclein mRNA levels are associated with craving in patients with alcoholism. Biol Psychiatry. 2004 Dec 15;56(12):984-6.

291. Bonsch D, Greifenberg V, Bayerlein K, Biermann T, Reulbach U, Hillemacher T, et al. Alpha-synuclein protein levels are increased in alcoholic patients and are linked to craving. Alcohol Clin Exp Res. 2005 May;29(5):763-5.

292. Foroud T, Wetherill LF, Liang T, Dick DM, Hesselbrock V, Kramer J, et al. Association of alcohol craving with alpha-synuclein (SNCA). Alcohol Clin Exp Res. 2007 Apr;31(4):537-45.

293. Muschler MA, Hillemacher T, Kraus C, Kornhuber J, Bleich S, Frieling H. DNA methylation of the POMC gene promoter is associated with craving in alcohol dependence. J Neural Transm (Vienna). 2010 Apr;117(4):513-9.

294. Zhang H, Herman AI, Kranzler HR, Anton RF, Zhao H, Zheng W, et al. Array-based profiling of DNA methylation changes associated with alcohol dependence. Alcohol Clin Exp Res. 2013 Jan;37 Suppl 1:E108-15.

295. Bonsch D, Lenz B, Kornhuber J, Bleich S. DNA hypermethylation of the alpha synuclein promoter in patients with alcoholism. Neuroreport. 2005 Feb 8;16(2):167-70.

296. Gatta E, Auta J, Gavin DP, Bhaumik DK, Grayson DR, Pandey SC, et al. Emerging Role of One-Carbon Metabolism and DNA Methylation Enrichment on delta-Containing GABAA Receptor Expression in the Cerebellum of Subjects with Alcohol Use Disorders (AUD). Int J Neuropsychopharmacol. 2017 Dec 1;20(12):1013-26.

297. Zhao R, Zhang R, Li W, Liao Y, Tang J, Miao Q, et al. Genome-wide DNA methylation patterns in discordant sib pairs with alcohol dependence. Asia Pac Psychiatry. 2013 Mar;5(1):39-50.

298. Bruckmann C, Di Santo A, Karle KN, Batra A, Nieratschker V. Validation of differential GDAP1 DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome. Epigenetics. 2016 Jun 2;11(6):456-63.

299. Ruggeri B, Nymberg C, Vuoksimaa E, Lourdusamy A, Wong CP, Carvalho FM, et al. Association of Protein Phosphatase PPM1G With Alcohol Use Disorder and Brain Activity During Behavioral Control in a Genome-Wide Methylation Analysis. Am J Psychiatry. 2015 Jun;172(6):543-52.

300. Hagerty SL, Bidwell LC, Harlaar N, Hutchison KE. An Exploratory Association Study of Alcohol Use Disorder and DNA Methylation. Alcohol Clin Exp Res. 2016 Aug;40(8):1633-40.

301. Wang F, Xu H, Zhao H, Gelernter J, Zhang H. DNA co-methylation modules in postmortem prefrontal cortex tissues of European Australians with alcohol use disorders. Sci Rep. 2016 Jan 14;6:19430.

302. Manzardo AM, Henkhaus RS, Butler MG. Global DNA promoter methylation in frontal cortex of alcoholics and controls. Gene. 2012 Apr 25;498(1):5-12.

303. Guidotti A, Dong E, Gavin DP, Veldic M, Zhao W, Bhaumik DK, et al. DNA methylation/demethylation network expression in psychotic patients with a history of alcohol abuse. Alcohol Clin Exp Res. 2013 Mar;37(3):417-24.

304. Abrahao KP, Salinas AG, Lovinger DM. Alcohol and the Brain: Neuronal Molecular Targets, Synapses, and Circuits. Neuron. 2017 Dec 20;96(6):1223-38.

305. Howard RJ, Trudell JR, Harris RA. Seeking structural specificity: direct modulation of pentameric ligand-gated ion channels by alcohols and general anesthetics. Pharmacol Rev. 2014 Feb 10;66(2):396-412.

306. Zhou Y, Colombo G, Niikura K, Carai MA, Femenia T, Garcia-Gutierrez MS, et al. Voluntary alcohol drinking enhances proopiomelanocortin gene expression in nucleus accumbens shell and hypothalamus of Sardinian alcohol-preferring rats. Alcohol Clin Exp Res. 2013 Jan;37 Suppl 1:E131-40.

307. Niikura K, Zhou Y, Ho A, Kreek MJ. Proopiomelanocortin (POMC) expression and conditioned place aversion during protracted withdrawal from chronic intermittent escalating-dose heroin in POMC-EGFP promoter transgenic mice. Neuroscience. 2013 Apr 16;236:220-32.

308. Kokare DM, Kyzar EJ, Zhang H, Sakharkar AJ, Pandey SC. Adolescent Alcohol Exposure-Induced Changes in Alpha-Melanocyte Stimulating Hormone and Neuropeptide Y Pathways via Histone Acetylation in the Brain During Adulthood. Int J Neuropsychopharmacol. 2017 Sep 1;20(9):758-68.

309. Civelli O, Birnberg N, Herbert E. Detection and quantitation of pro-opiomelanocortin mRNA in pituitary and brain tissues from different species. J Biol Chem. 1982 Jun 25;257(12):6783-7.

310. Toda C, Santoro A, Kim JD, Diano S. POMC Neurons: From Birth to Death. Annu Rev Physiol. 2017 Feb 10;79:209-36.

311. do Carmo JM, da Silva AA, Wang Z, Fang T, Aberdein N, Perez de Lara CE, et al. Role of the brain melanocortins in blood pressure regulation. Biochim Biophys Acta. 2017 Oct;1863(10 Pt A):2508-14.

312. Anderson EJ, Cakir I, Carrington SJ, Cone RD, Ghamari-Langroudi M, Gillyard T, et al. 60 YEARS OF POMC: Regulation of feeding and energy homeostasis by alpha-MSH. J Mol Endocrinol. 2016 May;56(4):T157-74.

313. Cone RD, Lu D, Koppula S, Vage DI, Klungland H, Boston B, et al. The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. Recent Prog Horm Res. 1996;51:287,317.

314. Poggioli R, Vergoni AV, Bertolini A. ACTH-(1-24) and alpha-MSH antagonize feeding behavior stimulated by kappa opiate agonists. Peptides. 1986 Sep-Oct;7(5):843-8.

315. Shimizu H, Shargill NS, Bray GA, Yen TT, Gesellchen PD. Effects of MSH on food intake, body weight and coat color of the yellow obese mouse. Life Sci. 1989;45(6):543-52.

316. Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, et al. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. Proc Natl Acad Sci U S A. 1993 Oct 1;90(19):8856-60.

317. Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol. 1994 Oct;8(10):1298-308.

318. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature. 1997 Jan 9;385(6612):165-8.

319. Marsh DJ, Hollopeter G, Huszar D, Laufer R, Yagaloff KA, Fisher SL, et al. Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. Nat Genet. 1999 Jan;21(1):119-22.

320. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell. 1997 Jan 10;88(1):131-41.

321. Liu J, Garza JC, Truong HV, Henschel J, Zhang W, Lu XY. The melanocortinergic pathway is rapidly recruited by emotional stress and contributes to stress-

induced anorexia and anxiety-like behavior. Endocrinology. 2007 Nov;148(11):5531-40.

322. Rao TL, Kokare DM, Sarkar S, Khisti RT, Chopde CT, Subhedar N. GABAergic agents prevent alpha-melanocyte stimulating hormone induced anxiety and anorexia in rats. Pharmacol Biochem Behav. 2003 Dec;76(3-4):417-23.

323. Kokare DM, Chopde CT, Subhedar NK. Participation of alpha-melanocyte stimulating hormone in ethanol-induced anxiolysis and withdrawal anxiety in rats. Neuropharmacology. 2006 Sep;51(3):536-45.

324. Cragnolini AB, Schioth HB, Scimonelli TN. Anxiety-like behavior induced by IL-1beta is modulated by alpha-MSH through central melanocortin-4 receptors. Peptides. 2006 Jun;27(6):1451-6.

325. Chaki S, Ogawa S, Toda Y, Funakoshi T, Okuyama S. Involvement of the melanocortin MC4 receptor in stress-related behavior in rodents. Eur J Pharmacol. 2003 Aug 1;474(1):95-101.

326. Shimazaki T, Chaki S. Anxiolytic-like effect of a selective and non-peptidergic melanocortin 4 receptor antagonist, MCL0129, in a social interaction test. Pharmacol Biochem Behav. 2005 Mar;80(3):395-400.

327. Kokare DM, Dandekar MP, Singru PS, Gupta GL, Subhedar NK. Involvement of alpha-MSH in the social isolation induced anxiety- and depression-like behaviors in rat. Neuropharmacology. 2010 Jun;58(7):1009-18.

328. Serova LI, Laukova M, Alaluf LG, Sabban EL. Intranasal infusion of melanocortin receptor four (MC4R) antagonist to rats ameliorates development of depression and anxiety related symptoms induced by single prolonged stress. Behav Brain Res. 2013 Aug 1;250:139-47.

329. Kokare DM, Dandekar MP, Chopde CT, Subhedar N. Interaction between neuropeptide Y and alpha-melanocyte stimulating hormone in amygdala regulates anxiety in rats. Brain Res. 2005 May 10;1043(1-2):107-14.

330. Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB, Elmquist JK. Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. J Comp Neurol. 2003 Mar 10;457(3):213-35.

331. Bhorkar AA, Dandekar MP, Nakhate KT, Subhedar NK, Kokare DM. Involvement of the central melanocortin system in the effects of caffeine on anxiety-like behavior in mice. Life Sci. 2014 Jan 30;95(2):72-80.

332. Yamano Y, Yoshioka M, Toda Y, Oshida Y, Chaki S, Hamamoto K, et al. Regulation of CRF, POMC and MC4R gene expression after electrical foot shock

stress in the rat amygdala and hypothalamus. J Vet Med Sci. 2004 Nov;66(11):1323-7.

333. Ryan KK, Mul JD, Clemmensen C, Egan AE, Begg DP, Halcomb K, et al. Loss of melanocortin-4 receptor function attenuates HPA responses to psychological stress. Psychoneuroendocrinology. 2014 Apr;42:98-105.

334. Marfaing-Jallat P, Larue C, Le Magnen J. Alcohol intake in hypothalamic hyperphagic rats. Physiol Behav. 1970 Mar;5(3):345-51.

335. Wayner MJ, Greenberg I, Carey RJ, Nolley D. Ethanol drinking elicited during electrical stimulation of the lateral hypothalamus. Physiol Behav. 1971 Nov;7(5):793-5.

336. Zhang H, Kranzler HR, Weiss RD, Luo X, Brady KT, Anton RF, et al. Pro-opiomelanocortin gene variation related to alcohol or drug dependence: evidence and replications across family- and population-based studies. Biol Psychiatry. 2009 Jul 15;66(2):128-36.

337. Ploj K, Roman E, Kask A, Hyytia P, Schioth HB, Wikberg JE, et al. Effects of melanocortin receptor ligands on ethanol intake and opioid peptide levels in alcohol-preferring AA rats. Brain Res Bull. 2002 Oct 30;59(2):97-104.

338. Olney JJ, Navarro M, Thiele TE. Targeting central melanocortin receptors: a promising novel approach for treating alcohol abuse disorders. Front Neurosci. 2014 Jun 3;8:128.

339. Polidori C, Geary N, Massi M. Effect of the melanocortin receptor stimulation or inhibition on ethanol intake in alcohol-preferring rats. Peptides. 2006 Jan;27(1):144-9.

340. Shelkar GP, Kale AD, Singh U, Singru PS, Subhedar NK, Kokare DM. Alpha-melanocyte stimulating hormone modulates ethanol self-administration in posterior ventral tegmental area through melanocortin-4 receptors. Addict Biol. 2015 Mar;20(2):302-15.

341. York DA, Boghossian S, Park-York M. Melanocortin activity in the amygdala influences alcohol intake. Pharmacol Biochem Behav. 2011 Mar;98(1):112-9.

342. Roltsch Hellard EA, Impastato RA, Gilpin NW. Intra-cerebral and intra-nasal melanocortin-4 receptor antagonist blocks withdrawal hyperalgesia in alcohol-dependent rats. Addict Biol. 2017 May;22(3):692-701.

343. Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR, et al. Neuropeptide Y distribution in the rat brain. Science. 1983 Aug 26;221(4613):877-9.

344. Adam TC, Epel ES. Stress, eating and the reward system. Physiol Behav. 2007 Jul 24;91(4):449-58.

345. Barson JR, Leibowitz SF. Hypothalamic neuropeptide signaling in alcohol addiction. Prog Neuropsychopharmacol Biol Psychiatry. 2016 Feb 4;65:321-9.

346. Enman NM, Sabban EL, McGonigle P, Van Bockstaele EJ. Targeting the Neuropeptide Y System in Stress-related Psychiatric Disorders. Neurobiol Stress. 2015 Jan 1;1:33-43.

347. Wiley JW, Gross RA, MacDonald RL. Agonists for neuropeptide Y receptor subtypes NPY-1 and NPY-2 have opposite actions on rat nodose neuron calcium currents. J Neurophysiol. 1993 Jul;70(1):324-30.

348. Gilpin NW. Corticotropin-releasing factor (CRF) and neuropeptide Y (NPY): Effects on inhibitory transmission in central amygdala, and anxiety- & alcohol-related behaviors. Alcohol. 2012 6;46(4):329-37.

349. Heilig M, McLeod S, Brot M, Heinrichs SC, Menzaghi F, Koob GF, et al. Anxiolytic-like action of neuropeptide Y: mediation by Y1 receptors in amygdala, and dissociation from food intake effects. Neuropsychopharmacology. 1993 Jun;8(4):357-63.

350. Primeaux SD, Wilson SP, Cusick MC, York DA, Wilson MA. Effects of altered amygdalar neuropeptide Y expression on anxiety-related behaviors. Neuropsychopharmacology. 2005 Sep;30(9):1589-97.

351. Heilig M. Antisense inhibition of neuropeptide Y (NPY)-Y1 receptor expression blocks the anxiolytic-like action of NPY in amygdala and paradoxically increases feeding. Regul Pept. 1995 Oct 20;59(2):201-5.

352. Sajdyk TJ, Vandergriff MG, Gehlert DR. Amygdalar neuropeptide Y Y1 receptors mediate the anxiolytic-like actions of neuropeptide Y in the social interaction test. Eur J Pharmacol. 1999 Mar 5;368(2-3):143-7.

353. Christiansen SH, Olesen MV, Gotzsche CR, Woldbye DP. Anxiolytic-like effects after vector-mediated overexpression of neuropeptide Y in the amygdala and hippocampus of mice. Neuropeptides. 2014 Dec;48(6):335-44.

354. Heilig M, Soderpalm B, Engel JA, Widerlov E. Centrally administered neuropeptide Y (NPY) produces anxiolytic-like effects in animal anxiety models. Psychopharmacology (Berl). 1989;98(4):524-9.

355. Zhang H, Sakharkar AJ, Shi G, Ugale R, Prakash A, Pandey SC. Neuropeptide y Signaling in the central nucleus of amygdala regulates alcohol-drinking and

anxiety-like behaviors of alcohol-preferring rats. Alcohol Clin Exp Res. 2010 /;34(3):451-61.

356. Thorsell A, Carlsson K, Ekman R, Heilig M. Behavioral and endocrine adaptation, and up-regulation of NPY expression in rat amygdala following repeated restraint stress. Neuroreport. 1999 Sep 29;10(14):3003-7.

357. Primeaux SD, Wilson SP, Bray GA, York DA, Wilson MA. Overexpression of neuropeptide Y in the central nucleus of the amygdala decreases ethanol self-administration in "anxious" rats. Alcohol Clin Exp Res. 2006 May;30(5):791-801.

358. Suzuki R, Lumeng L, McBride WJ, Li T, Hwang BH. Reduced neuropeptide Y mRNA expression in the central nucleus of amygdala of alcohol preferring (P) rats: its potential involvement in alcohol preference and anxiety. Brain Res. 2004 7/16;1014(1–2):251-4.

359. Badia-Elder NE, Stewart RB, Powrozek TA, Roy KF, Murphy JM, Li TK. Effect of neuropeptide Y (NPY) on oral ethanol intake in Wistar, alcohol-preferring (P), and -nonpreferring (NP) rats. Alcohol Clin Exp Res. 2001 Mar;25(3):386-90.

360. Badia-Elder NE, Stewart RB, Powrozek TA, Murphy JM, Li TK. Effects of neuropeptide Y on sucrose and ethanol intake and on anxiety-like behavior in high alcohol drinking (HAD) and low alcohol drinking (LAD) rats. Alcohol Clin Exp Res. 2003 Jun;27(6):894-9.

361. Thiele TE, Marsh DJ, Ste Marie L, Bernstein IL, Palmiter RD. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. Nature. 1998 Nov 26;396(6709):366-9.

362. Gilpin NW, Stewart RB, Murphy JM, Li TK, Badia-Elder NE. Neuropeptide Y reduces oral ethanol intake in alcohol-preferring (P) rats following a period of imposed ethanol abstinence. Alcohol Clin Exp Res. 2003 May;27(5):787-94.

363. Gilpin NW, Misra K, Koob GF. Neuropeptide Y in the central nucleus of the amygdala suppresses dependence-induced increases in alcohol drinking. Pharmacol Biochem Behav. 2008 Sep;90(3):475-80.

364. Thorsell A, Repunte-Canonigo V, O'Dell LE, Chen SA, King AR, Lekic D, et al. Viral vector-induced amygdala NPY overexpression reverses increased alcohol intake caused by repeated deprivations in Wistar rats. Brain. 2007 May;130(Pt 5):1330-7.

365. Caberlotto L, Thorsell A, Rimondini R, Sommer W, Hyytia P, Heilig M. Differential expression of NPY and its receptors in alcohol-preferring AA and alcohol-avoiding ANA rats. Alcohol Clin Exp Res. 2001 Nov;25(11):1564-9.

366. Sparta DR, Fee JR, Knapp DJ, Breese GR, Thiele TE. Elevated anxiety-like behavior following ethanol exposure in mutant mice lacking neuropeptide Y (NPY). Drug Alcohol Depend. 2007 Oct 8;90(2-3):297-300.

367. Qiu B, Bell RL, Cao Y, Zhang L, Stewart RB, Graves T, et al. Npy deletion in an alcohol non-preferring rat model elicits differential effects on alcohol consumption and body weight. J Genet Genomics. 2016 Jul 20;43(7):421-30.

368. Zhou Z, Zhu G, Hariri AR, Enoch MA, Scott D, Sinha R, et al. Genetic variation in human NPY expression affects stress response and emotion. Nature. 2008 Apr 24;452(7190):997-1001.

369. Lappalainen J, Kranzler HR, Malison R,et al. A functional neuropeptide y leu7pro polymorphism associated with alcohol dependence in a large population sample from the united states. Archives of General Psychiatry. 2002 September 1;59(9):825-31.

370. Kauhanen J, Karvonen MK, Pesonen U, Koulu M, Tuomainen TP, Uusitupa MI, et al. Neuropeptide Y polymorphism and alcohol consumption in middle-aged men. Am J Med Genet. 2000 Jul 17;93(2):117-21.

371. Koehnke MD, Schick S, Lutz U, Willecke M, Koehnke AM, Kolb W, et al. Severity of alcohol withdrawal symptoms and the T1128C polymorphism of the neuropeptide Y gene. J Neural Transm (Vienna). 2002 Nov;109(11):1423-9.

372. Okubo T, Harada S. Polymorphism of the neuropeptide Y gene: an association study with alcohol withdrawal. Alcohol Clin Exp Res. 2001 Jun;25(6 Suppl):59S-62S.

373. Mottagui-Tabar S, Prince JA, Wahlestedt C, Zhu G, Goldman D, Heilig M. A novel single nucleotide polymorphism of the neuropeptide Y (NPY) gene associated with alcohol dependence. Alcohol Clin Exp Res. 2005 May;29(5):702-7.

374. Hansen MJ, Morris MJ. Evidence for an interaction between neuropeptide Y and the melanocortin-4 receptor on feeding in the rat. Neuropharmacology. 2002 May;42(6):792-7.

375. Goyal SN, Kokare DM, Chopde CT, Subhedar NK. Alpha-melanocyte stimulating hormone antagonizes antidepressant-like effect of neuropeptide Y in Porsolt's test in rats. Pharmacol Biochem Behav. 2006 Oct;85(2):369-77.

376. Thorsell A, Svensson P, Wiklund L, Sommer W, Ekman R, Heilig M. Suppressed neuropeptide Y (NPY) mRNA in rat amygdala following restraint stress. Regul Pept. 1998 Sep 25;75-76:247-54.

377. Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, et al. Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev. 2010 Sep;62(3):405-96.

378. McCool BA, Christian DT, Diaz MR, Lack AK. Glutamate plasticity in the drunken amygdala: the making of an anxious synapse. Int Rev Neurobiol. 2010;91:205-33.

379. Rao VR, Finkbeiner S. NMDA and AMPA receptors: old channels, new tricks. Trends Neurosci. 2007 Jun;30(6):284-91.

380. Brimecombe JC, Boeckman FA, Aizenman E. Functional consequences of NR2 subunit composition in single recombinant N-methyl-D-aspartate receptors. Proc Natl Acad Sci U S A. 1997 Sep 30;94(20):11019-24.

381. Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M, et al. Genetic enhancement of learning and memory in mice. Nature. 1999 Sep 2;401(6748):63-9.

382. Bendova Z, Sladek M, Svobodova I. The expression of NR2B subunit of NMDA receptor in the suprachiasmatic nucleus of Wistar rats and its role in glutamate-induced CREB and ERK1/2 phosphorylation. Neurochem Int. 2012 Jul;61(1):43-7.

383. Shipton OA, Paulsen O. GluN2A and GluN2B subunit-containing NMDA receptors in hippocampal plasticity. Philos Trans R Soc Lond B Biol Sci. 2013 Dec 2;369(1633):20130163.

384. Morisot N, Ron D. Alcohol-dependent molecular adaptations of the NMDA receptor system. Genes Brain Behav. 2017 Jan;16(1):139-48.

385. Kalueff A, Nutt DJ. Role of GABA in memory and anxiety. Depress Anxiety. 1996 - 1997;4(3):100-10.

386. Ali F, Meier R. Primate home range and GRIN2A, a receptor gene involved in neuronal plasticity: implications for the evolution of spatial memory. Genes Brain Behav. 2009 Jun;8(4):435-41.

387. Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, et al. Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science. 2004 May 14;304(5673):1021-4.

388. Akashi K, Kakizaki T, Kamiya H, Fukaya M, Yamasaki M, Abe M, et al. NMDA receptor GluN2B (GluR epsilon 2/NR2B) subunit is crucial for channel function, postsynaptic macromolecular organization, and actin cytoskeleton at hippocampal CA3 synapses. J Neurosci. 2009 Sep 2;29(35):10869-82.

389. Daut RA, Busch EF, Ihne J, Fisher D, Mishina M, Grant SG, et al. Tolerance to ethanol intoxication after chronic ethanol: role of GluN2A and PSD-95. Addict Biol. 2015 Mar;20(2):259-62.

390. Inta D, Vogt MA, Pfeiffer N, Kohr G, Gass P. Dichotomy in the anxiolytic versus antidepressant effect of C-terminal truncation of the GluN2A subunit of NMDA receptors. Behav Brain Res. 2013 Jun 15;247:227-31.

391. Roberto M, Schweitzer P, Madamba SG, Stouffer DG, Parsons LH, Siggins GR. Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an in vitro and in vivo analysis. J Neurosci. 2004 Feb 18;24(7):1594-603.

392. Bell RL, Hauser SR, McClintick J, Rahman S, Edenberg HJ, Szumlinski KK, et al. Ethanol-Associated Changes in Glutamate Reward Neurocircuitry: A Minireview of Clinical and Preclinical Genetic Findings. Prog Mol Biol Transl Sci. 2016;137:41-85.

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

394. Obara I, Bell RL, Goulding SP, Reyes CM, Larson LA, Ary AW, et al. Differential effects of chronic ethanol consumption and withdrawal on homer/glutamate receptor expression in subregions of the accumbens and amygdala of P rats. Alcohol Clin Exp Res. 2009 Nov;33(11):1924-34.

395. Rani CS, Qiang M, Ticku MK. Potential role of cAMP response element-binding protein in ethanol-induced N-methyl-D-aspartate receptor 2B subunit gene transcription in fetal mouse cortical cells. Mol Pharmacol. 2005 Jun;67(6):2126-36.

396. Walker DL, Davis M. Amygdala infusions of an NR2B-selective or an NR2A-preferring NMDA receptor antagonist differentially influence fear conditioning and expression in the fear-potentiated startle test. Learn Mem. 2008 Jan 28;15(2):67-74.

397. Rodrigues SM, Schafe GE, LeDoux JE. Intra-amygdala blockade of the NR2B subunit of the NMDA receptor disrupts the acquisition but not the expression of fear conditioning. J Neurosci. 2001 Sep 1;21(17):6889-96.

398. Salling MC, Faccidomo SP, Li C, Psilos K, Galunas C, Spanos M, et al. Moderate Alcohol Drinking and the Amygdala Proteome: Identification and Validation of Calcium/Calmodulin Dependent Kinase II and AMPA Receptor Activity as Novel Molecular Mechanisms of the Positive Reinforcing Effects of Alcohol. Biol Psychiatry. 2016 Mar 15;79(6):430-42.

399. Vengeliene V, Bachteler D, Danysz W, Spanagel R. The role of the NMDA receptor in alcohol relapse: a pharmacological mapping study using the alcohol deprivation effect. Neuropharmacology. 2005 May;48(6):822-9.

400. Santucci AC, Cortes C, Bettica A, Cortes F. Chronic ethanol consumption in rats produces residual increases in anxiety 4 months after withdrawal. Behav Brain Res. 2008 Mar 17;188(1):24-31.

401. Roberts MC, Emsley RA, Pienaar WP, Stein DJ. Anxiety disorders among abstinent alcohol-dependent patients. Psychiatr Serv. 1999 Oct;50(10):1359-61.

402. Bhandage AK, Jin Z, Bazov I, Kononenko O, Bakalkin G, Korpi ER, et al. GABA-A and NMDA receptor subunit mRNA expression is altered in the caudate but not the putamen of the postmortem brains of alcoholics. Front Cell Neurosci. 2014 Dec 5;8:415.

403. Cacciaglia R, Nees F, Pohlack ST, Ruttorf M, Winkelmann T, Witt SH, et al. A risk variant for alcoholism in the NMDA receptor affects amygdala activity during fear conditioning in humans. Biol Psychol. 2013 Sep;94(1):74-81.

404. Krystal JH, Petrakis IL, Krupitsky E, Schutz C, Trevisan L, D'Souza DC. NMDA receptor antagonism and the ethanol intoxication signal: from alcoholism risk to pharmacotherapy. Ann N Y Acad Sci. 2003 Nov;1003:176-84.

405. Petrakis IL, Limoncelli D, Gueorguieva R, Jatlow P, Boutros NN, Trevisan L, et al. Altered NMDA glutamate receptor antagonist response in individuals with a family vulnerability to alcoholism. Am J Psychiatry. 2004 Oct;161(10):1776-82.

406. Domart MC, Benyamina A, Lemoine A, Bourgain C, Blecha L, Debuire B, et al. Association between a polymorphism in the promoter of a glutamate receptor subunit gene (GRIN2A) and alcoholism. Addict Biol. 2012 Jul;17(4):783-5.

407. Schumann G, Johann M, Frank J, Preuss U, Dahmen N, Laucht M, et al. Systematic analysis of glutamatergic neurotransmission genes in alcohol dependence and adolescent risky drinking behavior. Arch Gen Psychiatry. 2008 Jul;65(7):826-38.

408. Wernicke C, Samochowiec J, Schmidt LG, Winterer G, Smolka M, Kucharska-Mazur J, et al. Polymorphisms in the N-methyl-D-aspartate receptor 1 and 2B subunits are associated with alcoholism-related traits. Biol Psychiatry. 2003 Nov 1;54(9):922-8.

409. Kim JH, Park M, Yang SY, Jeong BS, Yoo HJ, Kim JW, et al. Association study of polymorphisms in N-methyl-D-aspartate receptor 2B subunits (GRIN2B) gene with Korean alcoholism. Neurosci Res. 2006 Oct;56(2):220-3.

410. Warnault V, Ron D. Chromatin remodeling: a new landscape to treat harmful alcohol-use disorders. Future Med Chem. 2013 Nov;5(17):2011-3.

411. Nelson JD, Denisenko O, Sova P, Bomsztyk K. Fast chromatin immunoprecipitation assay. Nucleic Acids Res. 2006 Jan 5;34(1):e2.

412. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.

413. Crawley J, Goodwin FK. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav. 1980 Aug;13(2):167-170.

414. Castillo-Aguilera O, Depreux P, Halby L, Arimondo PB, Goossens L. DNA Methylation Targeting: The DNMT/HMT Crosstalk Challenge. Biomolecules. 2017 Jan 5;7(1):10.3390/biom7010003.

415. Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, et al. Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. Mol Cell. 2003 Dec;12(6):1591-8.

416. Zhao B, Zhu Y, Wang W, Cui HM, Wang YP, Lai JH. Analysis of variations in the glutamate receptor, N-methyl D-aspartate 2A (GRIN2A) gene reveals their relative importance as genetic susceptibility factors for heroin addiction. PLoS One. 2013 Aug 5;8(8):e70817.

417. Razin A, Cedar H. DNA methylation and gene expression. Microbiol Rev. 1991 Sep;55(3):451-8.

418. Anier K, Malinovskaja K, Aonurm-Helm A, Zharkovsky A, Kalda A. DNA methylation regulates cocaine-induced behavioral sensitization in mice. Neuropsychopharmacology. 2010 Nov;35(12):2450-61.

419. Hedrich CM, Mabert K, Rauen T, Tsokos GC. DNA methylation in systemic lupus erythematosus. Epigenomics. 2017 Apr;9(4):505-25.

420. Pan Y, Liu G, Zhou F, Su B, Li Y. DNA methylation profiles in cancer diagnosis and therapeutics. Clin Exp Med. 2017 Jul 27.

421. Takashima S, Takehashi M, Lee J, Chuma S, Okano M, Hata K, et al. Abnormal DNA methyltransferase expression in mouse germline stem cells results in spermatogenic defects. Biol Reprod. 2009 Jul;81(1):155-64.

422. Zhubi A, Cook EH, Guidotti A, Grayson DR. Epigenetic mechanisms in autism spectrum disorder. Int Rev Neurobiol. 2014;115:203-44.

423. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999 Oct 29;99(3):247-57.

424. Meissner A. Epigenetic modifications in pluripotent and differentiated cells. Nat Biotechnol. 2010 Oct;28(10):1079-88.

425. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, et al. High-resolution profiling of histone methylations in the human genome. Cell. 2007 May 18;129(4):823-37.

426. Law JA, Jacobsen SE. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet. 2010 Mar;11(3):204-20.

427. Morris MJ, Na ES, Autry AE, Monteggia LM. Impact of DNMT1 and DNMT3a forebrain knockout on depressive- and anxiety like behavior in mice. Neurobiol Learn Mem. 2016 Nov;135:139-45.

428. Ponomarev I, Stelly CE, Morikawa H, Blednov YA, Mayfield RD, Harris RA. Mechanistic insights into epigenetic modulation of ethanol consumption. Alcohol. 2017 May;60:95-101.

429. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, et al. Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. Cancer Res. 2002 Nov 15;62(22):6456-61.

430. Kim JS, Shukla SD. Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. Alcohol Alcohol. 2006 Mar-Apr;41(2):126-32.

431. Vaute O, Nicolas E, Vandel L, Trouche D. Functional and physical interaction between the histone methyl transferase Suv39H1 and histone deacetylases. Nucleic Acids Res. 2002 Jan 15;30(2):475-81.

432. Wu J, Wang SH, Potter D, Liu JC, Smith LT, Wu YZ, et al. Diverse histone modifications on histone 3 lysine 9 and their relation to DNA methylation in specifying gene silencing. BMC Genomics. 2007 May 24;8:131.

433. Zang L, Kondengaden SM, Zhang Q, Li X, Sigalapalli DK, Kondengadan SM, et al. Structure based design, synthesis and activity studies of small hybrid molecules as HDAC and G9a dual inhibitors. Oncotarget. 2017 Jun 28;8(38):63187-207.

434. Fang S, Miao J, Xiang L, Ponugoti B, Treuter E, Kemper JK. Coordinated recruitment of histone methyltransferase G9a and other chromatin-modifying enzymes in SHP-mediated regulation of hepatic bile acid metabolism. Mol Cell Biol. 2007 Feb;27(4):1407-24.

435. Duan Z, Zarebski A, Montoya-Durango D, Grimes HL, Horwitz M. Gfi1 coordinates epigenetic repression of p21Cip/WAF1 by recruitment of histone lysine methyltransferase G9a and histone deacetylase 1. Mol Cell Biol. 2005 Dec;25(23):10338-51.

436. Rodriguez-Madoz JR, San Jose-Eneriz E, Rabal O, Zapata-Linares N, Miranda E, Rodriguez S, et al. Reversible dual inhibitor against G9a and DNMT1 improves human iPSC derivation enhancing MET and facilitating transcription factor engagement to the genome. PLoS One. 2017 Dec 27;12(12):e0190275.

437. Smith JP, Randall CL. Anxiety and alcohol use disorders: comorbidity and treatment considerations. Alcohol Res. 2012;34(4):414-31.

438. McClung CA, Nestler EJ. Regulation of gene expression and cocaine reward by CREB and DeltaFosB. Nat Neurosci. 2003 Nov;6(11):1208-15.

VII. Appendix I

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

May 10, 2018

This Agreement between Miss. Tiffani Berkel ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4345410468242
License date	May 10, 2018
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Alcoholism: Clinical and Experimental Research
Licensed Content Title	Emerging Role of Epigenetic Mechanisms in Alcohol Addiction
Licensed Content Author	Tiffani D.M. Berkel, Subhash C. Pandey
Licensed Content Date	Feb 18, 2017
Licensed Content Volume	41
Licensed Content Issue	4
Licensed Content Pages	15
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	The role of histone and DNA methylation mechanisms in alcohol drinking and anxiety-like behaviors
Expected completion date	Jun 2018
Expected size (number of pages)	160
Requestor Location	Miss. Tiffani Berkel 1623 N Wood St Apt 1FB CHICAGO, IL 60622 United States Attn: Miss. Tiffani Berkel
Publisher Tax ID	EU826007151
Total	0.00 USD
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that

What rights do I retain as an Oxford Journal author?

- The right, after publication by Oxford Journals, to use all or part of the Article and abstract, for their own personal use, including their own classroom teaching purposes;
- The right, after publication by Oxford Journals, to use all or part of the Article and abstract, in the preparation of derivative works, extension of the article into book-length or in other works, provided that a full acknowledgement is made to the original publication in the journal;
- The right to include the article in full or in part in a thesis or dissertation, provided that this not published commercially;

For the uses specified here, please note that there is no need for you to apply for written permission from Oxford University Press in advance. Please go ahead with the use ensuring that a full acknowledgment is made to the original source of the material including the journal name, volume, issue, page numbers, year of publication, title of article and to Oxford University Press and/or the learned society.

The only exception to this is for the re-use of material for commercial purposes. Permission for this kind of re-use is required and can be obtained by using Rightslink:

With Copyright Clearance Center's Rightslink ® service it's faster and easier than ever before to secure permission from OUP titles to be republished in a coursepack, book, CD-ROM/DVD, brochure or pamphlet, journal or magazine, newsletter, newspaper, make a photocopy, or translate.

- Simply visit: academic.oup.com and locate your desired content.
- Click on (Order Permissions) within the table of contents and/ or at the bottom article's abstract to open the following page:

VITA

Tiffani Diane Maria Berkel
University of Illinois at Chicago
Graduate Program in Neuroscience
Department of Psychiatry
Chicago, IL 60622
Email: tberkel2@uic.edu

EDUCATION

University of Illinois at Chicago, Medical Scientist Training Program *2011 – Present*
M.D. and Ph.D. in Neuroscience candidate, anticipated 2020
Advisor: Subhash C. Pandey, Ph.D. Department of Psychiatry

University of Illinois at Urbana-Champaign
B.S. in Molecular and Cellular Biology, *cum laude* *2007 – 2011*
GPA: Cumulative 3.84/4.00, Major: 3.78/4.00
Senior Thesis: *In vivo* Analysis of Myxoma Virus Replication in Response to Rapamycin in B16-SIY Tumors

RESEARCH EXPERIENCE

University of Illinois at Chicago, Chicago, IL *June 2013 – Present*
Graduate Research Assistant
Laboratory of Dr. Subhash C. Pandey

University of Illinois at Chicago, Chicago, IL *June 2011 – August 2012*
Graduate Neuroscience Rotations/MSTP Fellow
Laboratory of Dr. Orly Lazarov
Laboratory of Dr. Toru Nakamura

University of Iowa, Iowa City, IA *May 2010 – July 2010*
Interdisciplinary Summer Undergraduate Research Program Participant
Laboratory of Dr. Pedro Gonzalez-Alegre

University of Illinois at Urbana-Champaign, Urbana, IL *May 2009 – May 2011*
Undergraduate Research Assistant
Laboratory of Dr. Edward J. Roy

PUBLICATIONS

Geraghty JR, Young AN, **Berkel TDM**, Wallbruch E, Mann J, Park YS, Hirshfield LE, & Hyderi A (In Press) *Empowering Medical Students as Agents of Curricular Change: A Value-Added Approach to Student Engagement in Medical Education*. Perspectives in Medical Education.

Berkel TDM, Zhang H, Teppen T, Sakharkar A, Pandey SC (2019) *Essential Role of Histone Methyltransferase G9a in Rapid Tolerance to the Anxiolytic Effects of Ethanol.* International Journal of Neuropsychopharmacology 22(4):292-302.

Berkel TDM & Pandey SC (2017) *Emerging Role of Epigenetic Mechanisms in Alcohol Addiction.* Alcoholism Clinical and Experimental Research 41(4): 666-680.

Krishnan HR, Sakharkar A, Teppen T, **Berkel TDM**, Pandey SC (2014) *The Epigenetic Landscape of Alcoholism*. Epigenetics, Vol 115, IRN, UK: Academic Press, 2014, pp. 75-116.

PUBLISHED ABSTRACTS

Berkel TDM, Zhang H, Teppen T, & Pandey SC. Histone Methyltransferase, G9a, and its Epigenetic Manipulation of Rapid Ethanol Tolerance. Alcoholism: Clinical and Experimental Research. 2018, Jun; 42(S1): 85A.

Berkel TDM, Zhang H, Teppen T, Kyzar EJ, Krishnan HR, Pandey SC (2017) Interactive role of DNA methylation and histone methylation in alcohol drinking behaviors. Alcoholism: Clinical and Experimental Research. Jun; 41(S1): 189A.

Berkel TDM, Zhang H, Kyzar EJ, Krishnan HR, & Pandey SC (2017) Potential role of amygdaloid histone and DNA methylation mechanisms in anxiety and alcohol drinking behaviors. Biological Psychiatry. May; 81(10): S345.

Berkel TDM, Zhang H, & Pandey SC (2016) Histone methylation in the amygdala: A potential role in rapid ethanol tolerance. Alcoholism Clinical & Experimental Research. Jun; 40(S1): 17A.

FUNDED AWARDS

- **Dr. Erminio Costa Scholarship** *2019*
  University of Illinois at Chicago MSTP, Chicago, IL
- **Scaife Medical Student Fellowship in Substance Use Disorders** *2019*
  Institute for Research, Education, and Training in Addictions, Pittsburgh, PA
- **Combining Research and Clinical Careers in Neuroscience Symposium** *2019*
  National Institute of Health, Bethesda, MD
- **Health Professions Student Council Travel Grant** *2018*
  University of Illinois at Chicago HPSC, Chicago, IL
- **Neuroscience Day First Place Poster Presentation** *2017*
  University of Illinois at Chicago Graduate Program in Neuroscience, Chicago, IL
- **Student Merit Award** *2017*
  Research Society on Alcoholism, Austin, TX
- **Pre-Doctoral Scholars Travel Fellowship Award** *2017*
  Society of Biological Psychiatry, Jacksonville, FL
- **Pre-Doctoral Education for Clinical and Translational Scientists Fellowship** *2015-2017*
  Center for Clinical and Translational Science, Chicago, IL
- **Summer Institute for Medical Students (SIMS) Inpatient Program** *2012*
  Hazeldon Betty Ford, Rancho Mirage, CA
- **Clarence E. Brehm Scholarship** *2009 – 2011*
  University of Illinois, Urbana-Champaign, IL
- **Phi Eta Sigma Scholarship** *2009 – 2010*
  University of Illinois, Urbana-Champaign, IL

RESEARCH POSTERS AND PRESENTATIONS

Berkel TDM, Zhang H, Teppen T, Pandey SC. *Histone methyltransferase, G9a, and its epigenetic manipulation of rapid ethanol tolerance.* Research Society on Alcoholism, 41st Annual Scientific Meeting, June 16-20, 2018 AND University of Illinois College of Medicine Research Forum, Nov 9, 2018.

Berkel TDM, Zhang H, Teppen T, Pandey SC. **Berkel TDM**, Zhang H, Teppen T, Sakharkar A, Pandey SC. *G9a-Mediated Regulation of Tolerance to the Anxiolytic Effects of Alcohol*. Society of Biological Psychiatry, May 9-12, 2018.

Berkel TDM, Zhang H, Teppen T, Pandey SC. *Histone methyltransferase, G9a, and its epigenetic manipulation of rapid ethanol tolerance.* University of Illinois Graduate Program in Neuroscience: Neuroscience Day, Oct 13, 2017 and University of Illinois College of Medicine Research Forum, Dec 1, 2017.

Berkel TDM, Zhang H, Teppen T, Kyzar EJ, Krishnan HR, Pandey SC. *Interactive role of DNA methylation and histone methylation in alcohol drinking behaviors.* Research Society on Alcoholism, 40th Annual Scientific Meeting, June 24-28, 2017.

Berkel TDM, Zhang H, Kyzar EJ, Krishnan HR, Pandey SC. *Potential role of amygdaloid histone and DNA methylation mechanisms in anxiety and alcohol drinking behaviors.* Society of Biological Psychiatry, May 18-20, 2017.

Geraghty J, Young A, **Berkel TDM**, Mann J, Wallbruch E, Hyderi A. *Empowering medical students as partners in program evaluation of pre-clerkship courses.* Central Group on Educational Affairs Regional Spring Meeting, Innovations in Medical Education, March 29-31, 2017.

Berkel TDM, Zhang H, Pandey SC. *Histone methylation in the amygdala: A potential role in alcoholism and anxiety.* University of Illinois College of Medicine Research Forum, Nov 18, 2016.

Berkel TDM, Zhang H, Kyzar EJ, Pandey SC. *Emerging role of histone methyltransferase, G9a, in alcohol drinking behaviors.* University of Illinois 7th Annual Department of Psychiatry Research Forum, September 14, 2016.

Berkel TDM, Zhang H, Pandey SC. *Histone methylation in the amygdala: A potential role in rapid ethanol tolerance.* Research Society on Alcoholism, 39th Annual Scientific Meeting, June 25-29, 2016.

Berkel TDM, Zhang H, Moonat S, Pandey SC. *Histone Methylation and Alcoholism.* University of Illinois College of Medicine Research Forum, Nov 21, 2014.

Berkel TDM, Gonzalez-Alegre P. *Investigating transcriptional changes in miRNAs in a mouse model of DYT1 dystonia.* University of Iowa Biomedical Sciences Undergraduate Research Conference, July 27, 2010.

Berkel TDM, Roy EJ**.** *Viruses need love, too! An investigation of the effects of rapamycin on myxoma virus replication in established brain tumors.* University of Illinois at Urbana-Champaign 3rd Annual Undergraduate Research Symposium, April 15, 2010.

TEACHING EXPERIENCE

Lecturer at University of Illinois at Chicago College of Medicine *2015 & 2016*
- Summer pre-matriculation program, **Course:** Neurophysiology

Teaching Assistant at University of Illinois at Urbana-Champaign *2010*
- Department of Physics, **Course:** Mechanics and Heat

Tutor at Southwestern Illinois College *2010*
- Private tutor; **Course:** Statistics

MEMBERSHIPS AND INVOLVEMENT

University of Illinois at Chicago College of Medicine

- **Student Curricular Board** Co-Creator and Lead Student Member *2012 – Present*
- **Curriculum Committee** Appointed Voting Member *2011 – 2017*
- **Chicago Medical School Council** President, Vice President *2012 – 2015*
- **Mentor's Program** Neurology Department Participant *2013 – 2014*
- **Computer Based Testing Committee** Appointed Student Representative *2012 – 2014*

University of Illinois at Urbana-Champaign

- **Carle Hospital** Student Leader Volunteer, Neurological/Surgical and NICU *2008 – 2011*
- **Illini Medical Screening Society**
  President, Volunteer Coordinator, Screening Supervisor *2008 – 2011*
- **Phi Eta Sigma** Member *2008 – 2011*
- **National Society of Collegiate Scholars** Inductee *2008*

HONORS

- Outstanding Student Innovation in Medical Education Poster *2017*
- College of Medicine Research Forum, Honorable Mention *2014, 2017, & 2018*
- Abbas Hyderi Leadership Award Recipient *2013 & 2014*
- Chancellor's Student Service Award *2013 & 2014*
- Mentor's Program Best Overall Performance, Neurology *2013*
- James Scholar *2007 – 2011*