EPIGENETIC EFFECTS OF ETHANOL

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B.S., University of California, Los Angeles, 2008

Submitted to the Graduate Faculty of the

University of Pittsburgh School of Medicine

in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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July 15, 2014

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Alcohol use disorder (AUD) is prevalent and associated with significant mortality and socioeconomic costs globally. Despite the tremendous burden of AUD, mechanisms of alcohol (ethanol) action that underlie the development of AUD are still not known. This dissertation addresses the role of epigenetic modifications in heritability of AUD and mechanisms of ethanol by testing three distinct hypotheses: 1) paternal ethanol exposure regulates ethanol drinking and ethanol-related behavior in offspring; 2) acute ethanol induces conserved changes to histone modifications at model gene promoters in the cerebral cortex; 3) chronic intermittent vapor ethanol and withdrawal induce dynamic changes in gene regulation via discrete histone modifications in accumbal dopamine 1 receptor positive medium spiny neurons. To test these hypotheses, this dissertation developed new models for studying heritability of ethanol drinking using paternal vapor ethanol exposure and epigenetic modifications in a neuronal subtype using fluorescence activated cell sorting. The results presented in this thesis provide evidence for epigenetic effects of ethanol in mediating heritability of ethanol drinking and sensitivity to ethanol selectively in male offspring as well as ethanol-induced gene regulation in the cerebral cortex. Additionally, they set up future studies of ethanol's epigenetic mechanisms in neuronal subtypes, which will increase sensitivity of current assays to detect cell-specific changes in gene regulation. These results are expected to have important implications for the development of drugs that target epigenetic modifying enzymes for treatment of AUD.

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LIST OF FREQUENTLY USED ABBREVIATIONS

Abbreviation	Full terminology
AUD	Alcohol Use Disorder
BAC	Bacterial Artificial Chromosome
BDNF	Brain-Derived Neurotrophic Factor
BEC	Blood Ethanol Concentration
CCx	Cerebral Cortex
D1R	Dopamine D1 Receptor
DMR	Differentially Methylated Region
DNMT	DNA Methyltransferase
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, 5th edition
EPM	Elevated Plus Maze
FACS	Fluorescence Activated Cell Sorting
GWAS	Genomewide Association Study
HAT	Histone Acetyltransferase
HC	Hippocampus
HDAC	Histone Deacetylase
IAP	Intracisternal A Particle
MeCP2	Methyl-CpG Binding Protein 2
mPFC	Medial Prefrontal Cortex
MSN	Medium Spiny Neuron
NAc	Nucleus Accumbens
RIN	RNA Integrity Number
RT	Reverse Transcriptase
RT-qPCR	Reverse Transcription Quantitative PCR
SNP	Single Nucleotide Polymorphism
TSS	Transcriptional Start Site

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PREFACE

My time in the Molecular Pharmacology graduate program at the University of Pittsburgh has been intellectually demanding and, because of its rigor, immensely rewarding. I have grown to appreciate the challenges of designing and implementing research projects that I could have only dreamed of conducting before starting graduate school. Despite these challenges and several failures along the way, I always felt like I was moving forward – toward a better understanding of the question I had asked. Nothing in this dissertation could have been accomplished without the tremendous support of my mentors, graduate program, friends, and family.

I would first like to thank my thesis advisor, Gregg Homanics, for his mentorship and for giving me the opportunity to develop the projects in this dissertation. I could not have asked for an advisor more generous with his time and patient with the growing pains of a graduate student. This dissertation is a direct reflection of our daily interactions and willingness to push into the unknown. I would also like the thank the members of the Homanics lab for their tremendous support, especially Carolyn Ferguson, Matt McKay, Mark Zimmerman, and Greg Rompala, who were always there to lend a hand. I am lucky to have had such a supportive group of co-workers who made coming to work every day enjoyable and productive.

I am indebted to the members of my dissertation committee, J. Richard Chaillet, Don DeFranco, Colleen McClung, and W. Chet de Groat, for their valuable input throughout this process. I am appreciative for their time and thoughtful insights during our meetings and each

member has added their distinct viewpoints to this dissertation. I would also like to thank Patrick Pagano, Guillermo Romero, and Bruce Freeman for leading the Molecular Pharmacology Training Program and Pat Smith for her support. The MPTP has provided a strong and nurturing structure to my training that would not be possible without their dedication to graduate student education. The MD/PhD program has also been immensely supportive and I would like to thank Richard Steinman, Manjit Singh, and all those involved with the MSTP for not letting me lose sight of the big picture during my time in the lab. All of these academic mentors and many others were instrumental to my growth as a scientist and development as a person.

Finally, I am immensely grateful for the support of my friends and family in every aspect of my life. My loving and supportive wife, Alison, has been my best friend through this process and pushes me to be my best in every aspect of my life; I do not know where I'd be without her by my side. My family has always guided me and I am grateful to my parents, grandfather, and sister, who were always there to listen. My friends and especially those in the 2009 Pitt P/MSTP class were there to help me through any problems and provided a much-needed outlet from the stress of graduate school. All of you have added life to my years.

1.0 INTRODUCTION

1.1 ALCOHOL USE DISORDER AND ITS HERITABILITY

The Diagnostic and Statistical Manual of Mental Disorders, 5th edition, (DSM-V) classifies pathological alcohol consumption under a single entity known as alcohol use disorder (AUD) [1]. AUD is diagnosed by a range of criteria related to tolerance to the subjective effects of alcohol, severity of withdrawal symptoms, and impairment of psychosocial functioning. AUD is a chronically relapsing disorder and its etiology is complex, overlapping with other substance use disorders. Initially, alcohol use is impulsive and characterized by positive reinforcement that converts to compulsive use with increasing negative reinforcement, the development of tolerance, and escalation of alcohol drinking. Ultimately, there is loss of control of alcohol drinking and the development of a negative emotional state, which makes it challenging to overcome alcohol use (for review, see [2]).

AUD is a major public health concern and associated with substantial costs to both individuals and society. In the United States, alcohol consumption contributes to $223 billion in societal costs annually [3]. Excluding accidents and homicides by people under the influence of alcohol, alcohol contributes to 1% of deaths annually in the United States [4]. This places alcohol use as the third leading modifiable factor contributing to death after tobacco use and poor diet and physical activity [5]. Despite its tremendous burden and ubiquitous use, there are few

available pharmacological treatments for AUD and relapse rates are ~50% three years after onset of treatment [6,7]. These issues demonstrate the urgency for developing novel agents for treatment of AUD and uncovering potential biomarkers associated with its risk.

The lifetime prevalence of AUD is 30% in the United States [8], highlighting the significant genetic and environmental differences that are likely among such a large cohort. This diversity is further complicated by the wide spectrum of alcohol consumption among humans, so that rate and quantity of alcohol consumed are not considered in DSM-V criteria for AUD [1]. Heterogeneity has made drug development for AUD challenging, since factors promoting pathological alcohol consumption are likely to be complex and varied between subjects. Despite this diversity, twin and adoption studies consistently find that AUD has a heritability of ~50% [9-11], indicating transmission of risk alleles from parents to offspring independent of environment. Heritability of AUD is comparable to other common, complex human diseases with significant gene-environment interactions like diabetes and major depressive disorder [12,13]; though, notably, it is lower than the ~75% heritability reported for nicotine dependence [14]. The discovery of AUD heritability has allowed researchers to search for specific gene polymorphisms associated with alcohol consumption.

Early candidate gene studies for AUD risk identified alcohol metabolism genes as modifiers of alcohol consumption. Nineteen aldehyde dehydrogenase (ALDH) and seven alcohol dehydrogenase (ADH) genes are present in the human genome and involved in metabolizing endogenous and ingested alcohols. Polymorphisms inactivating the ALDH2 gene have been found almost exclusively in Asian populations and are associated with decreased risk for developing AUD [15-17]. ADH1 and ADH7 single nucleotide polymorphisms (SNP) have been associated with alcohol metabolism and consumption in European and African populations

[18,19]. These associations underlie the mechanism of action of disulfiram, one of the three FDA approved drugs for treatment of AUD. Disulfiram inhibits ALDH and leads to an accumulation of acetaldehyde, an ethanol metabolism intermediate associated with “hangover” symptoms. By associating alcohol consumption with an aversive reaction, disulfiram decreases alcohol consumption and reduces rates of relapse [20]. This example illustrates the potential of identifying variants protective for AUD for the development of pharmaceuticals to treat AUD.

Studies of larger populations have been less successful at identifying risk alleles for AUD. One recent genome-wide association study (GWAS) did not identify any SNPs significantly associated with alcoholism risk and estimated that all of the SNPs studied accounted for only 0.1% of the genetic risk for developing alcoholism [21]. Other groups have found SNPs significantly associated with AUD using GWAS but later failed to replicate their results [22,23]. More recent meta-analyses and expanded studies have identified novel SNPs significantly associated with AUD [24,25], though differences in the SNPs discovered between studies suggest they may not be meaningful across the entire population. “Missing heritability” is a recent concept that refers to the failure of GWAS to uncover risk alleles for diseases with high heritability. While technical issues and the contribution of rare genetic variants may be masking these alleles [26], it is also possible that heritable variants outside the DNA sequence, known as “epi-alleles,” may be contributing complex phenotypes like AUD.

1.2 EPIGENETICS

1.2.1 Epigenetic regulation of gene expression

Epigenetics refers to a broad group of modifications to DNA and RNA structure without a change in nucleotide sequence. These mechanisms are the primary drivers for regulating gene expression, so that a single genome can give rise to the hundreds of cell lineages within an organism. The ability of epigenetic mediators to interact at several points along the genome makes them vital to normal cell function and aberrant epigenetic programming has been implicated in the development of cancer [27], addiction [28], and other human diseases. Epigenetic modifications are also mitotically and meitoically heritable [29], contributing to their role in development and heritability of phenotypes. With the sequencing of the human genome, there is now a shift toward mapping of the "epi-genome" to better understand the role of epigenetic modifications in disease [30].

In eukaryotes, DNA is condensed around a core of eight histone proteins into a nucleic acid-protein complex known as chromatin (Fig. 1). The functional unit of chromatin is the nucleosome, which consists of a single histone octamer and the ~147 bp of DNA that wrap around it. Histone octamers consist of homo- and heterodimers of four subunits, H2A, H2B, H3, and H4, with all but H4 encoded by multiple genes to generate histone subunit variants [31]. All histone proteins are rich in basic amino acids that carry a net positive charge within the cell, imparting a strong affinity for the negatively charged DNA phosphodiester backbone.

The affinity between histones and DNA is critical for regulation of gene expression and is altered by covalent modifications to histone N-terminal tails [32]. For example, acetylation of lysine residues neutralizes the positive charge on lysine's ammonium group, reducing its affinity

for DNA; weaker histone-DNA interactions increase accessibility of DNA to transcription factors, which recruit RNA polymerase to initiate transcription [33]. Methylation of lysine's ammonium group can promote or repress gene expression depending on its location on the histone subunit N-terminal tail [34]. It is important to note that histone modifications are rapidly reversible and catalyzed by a diverse group of histone modifying enzymes, so that they are a key mechanism of cellular adaptation to the environment [32]. However, their role in gene regulation is complex, as recent studies have identified over 100 post-translational modifications to histones and the function of most of these is unknown [35]. Combinatorial approaches are only recently emerging to study how modifications interact with one another to alter gene expression [36].

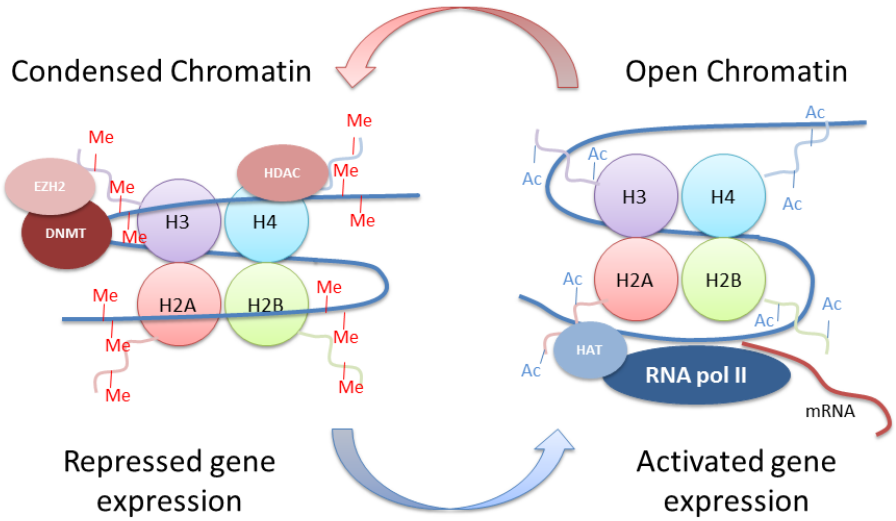

Figure 1. Epigenetic regulation of gene expression

DNA (blue strand) wraps around a histone octamer to form the nucleosome. Chromatin condensation is promoted by histone and DNA methylation (Me) via DNA methyltransferases (DNMT), lysine methyltransferases (e.g., EZH2), and histone deacetylases (HDAC). These modifications are reversible and an open chromatin conformation is induced by histone acetylation (Ac) via histone acetyltransferases (HAT), which weaken histone-DNA interactions to promote transcription factor binding, recruitment of RNA polymerase, and transcription.

The primary covalent modification to DNA in vertebrates is the methylation of cytosine preceding guanine (CpG dinucleotide). CpG dinucleotides occur much less frequently throughout the genome than would be expected by chance, most likely due to deamination of methylcytosine to thymine [37]. However, near transcriptional start sites of most mammalian genes, the density of unmethylated CpG dinucleotides is increased at regions known as "CpG islands" [38,39]. CpG islands show tissue specific patterns of methylation and only ~8% are hypermethylated in most cell types, with methylation associated with gene silencing [40]. DNA methylation is also a key mechanism for repression of retroviral elements in the genome [41]. The role of DNA methylation outside of CpG islands, at intra- and intergenic regions that account for 98% of CpG dinucleotides in the genome, is less clear. One recent study proposed that many of these form lower-density CpG islands with tissue-specific patterns of methylation [40]. Recent evidence is also emerging that intragenic methylcytosine may promote gene expression (for review, see [42]). Therefore, while DNA methylation is primarily associated with gene silencing, it may also play a role in transcription.

DNA methylation at CpG islands represents a more stable mechanism of transcriptional repression than histone modification. Several studies have shown that nucleosome repression, through histone methylation and induction of the polycomb repressive complex, precedes DNA methylation and that DNA methylation may "lock" gene promoters into a repressive state [42-44]. Timing of DNA methylation during development also supports this idea. The *de novo* DNA methyltransferases, DNMT3a and DNMT3b, are highly expressed following genome-wide demethylation of the early embryo [45]. These enzymes are also present in adult somatic tissue; however, the rate of *de novo* DNA methylation is much slower after embryogenesis, on the order of weeks to months [46,47]. Slow induction of *de novo* DNA methylation may represent a

cellular mechanism to maintain transcriptional plasticity. This idea is further supported by the lack of a well-defined mechanism for active demethylation in adult somatic tissue [48]. Ten-eleven translocase (TET) enzymes have recently been proposed to mediate active demethylation by oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) [49], and this process is necessary for early embryo development [50]. However, while 5-hmC is present in the brain and other tissues [51], its function is still unclear. While DNA methylation appears to be mostly fixed in adult tissue, more research is necessary to identify how DNA methylation and demethylation are induced in terminally differentiated cells and whether they represent normal homeostatic mechanisms for altering gene expression or are vestiges of early developmental programming.

1.2.2 The role of epigenetic modifications in drug addiction

This section summarizes general epigenetic mechanisms of drugs of abuse; a more detailed discussion of ethanol-induced epigenetic modifications is included in Section 1.3.2.

Epigenetic modifications are emerging as critical regulators of drug-related behaviors in rodent models. These studies developed from research implicating drug use in widespread changes in gene expression and activation of transcription factors (for review, see [52]). Focus has shifted from manipulating drug-induced transcription factors, like cAMP response element binding protein (CREB) and activator protein 1 (AP-1), to studying how these proteins interact with chromatin modifying enzymes to alter gene expression. Studying these epigenetic modifications is especially attractive because drug-induced priming of gene promoters may underlie long-lasting changes to neurons and glia that promote drug use [53].

While drugs of abuse possess distinct mechanisms, all alter the mesolimbic pathway that supplies dopamine to the nucleus accumbens (NAc) and nearly all activate the AP-1 complex in

NAc [54,55]; moreover, there is converging evidence that histone modifications play a critical role in this modulation. This idea has been especially relevant for acute cocaine exposure, which is associated with increased histone acetylation and decreased histone methylation in NAc [53,56,57]. Interestingly, both chronic cocaine and morphine decrease dimethylation of lysine 9 on histone subunit H3 (H3K9me2) and also decrease expression of the histone lysine methyl-transferase G9a in NAc [53,58]. Additional evidence comes from studies using histone deacetylase (HDAC) inhibitors to modify drug-related behaviors. Systemic administration of the HDAC inhibitor sodium butyrate enhances locomotor sensitization to ethanol, cocaine, and morphine [56,59] and blocks anxiety-related behaviors during ethanol withdrawal [60]. Histone methyltransferases also appear to play a role in drug sensitivity and show cell-type specificity. Modulation of G9a in subpopulations of NAc neurons alters cocaine sensitization and reward as well as gene expression that varies between different dopamine receptor neuronal subtypes [61]. These studies highlight the potential for modulating drug-induced histone modifications for altering drug-related behaviors.

Studies of drug-induced effects on DNA methylation are more limited, potentially due to the slow induction of this chromatin modification. However, several studies have focused on interactions between methyl CpG binding protein 2 (MeCP2) and drug administration. MeCP2 is a transcriptional repressor that binds hypermethylated DNA and has recently been implicated in maintenance of neuronal plasticity [62]. Mice with a truncated form of MeCP2 were found to be more sensitive to ethanol and consume less ethanol in a continuous drinking paradigm [63]. This finding parallels a study showing knockdown of MeCP2 in the dorsal striatum decreases cocaine intake [64]. Other studies have found changes in DNA methylation at specific gene promoters after cocaine, ethanol, and opioid use [65-67], though these results have been more challenging

to associate with a behavior. Therefore, while studies are still limited, modulating enzymes associated with methylated DNA appears to affect drug consumption in rodents.

While ethanol, cocaine, and opioid exposure have all been associated with differential expression of hundreds of genes across multiple brain regions [53,57,68-70], drug-induced epigenetic modifications that regulate changes in gene expression are only recently emerging. It remains to be seen whether a uniform set of chromatin modifying enzymes regulate the structural and cellular changes that underlie addiction.

1.2.3 Intergenerational inheritance

This section summarizes the field of intergenerational inheritance; a more detailed discussion of the potential role of paternal ethanol on offspring behavior is included in Section 1.3.4.

Improved tools to study epigenetic modifications and a search for epi-alleles to explain missing heritability of complex phenotypes has led to the discovery of heritable epigenetic modifications that contribute to offspring phenotype. This idea is especially intriguing because of widespread environmentally-induced epigenetic modifications, some of which may become encoded in the germ line (for review, see [71]). New insights into how these epi-alleles escape epigenetic reprogramming during early embryogenesis and primordial germ cell development are emerging [29,72,73]. In particular, whole genome analysis of DNA methylation has identified large regions of the genome around intracisternal A particles (IAP) and imprinted genes that retain DNA methylation even as neighboring regions are demethylated [29,74,75]. Small RNAs retained in gametes are also emerging as mediators of epigenetic inheritance [71,76,77]. While detailed mechanisms of epigenetic inheritance are still developing, a large group of rodent

studies have now shown that environmental perturbations can produce phenotypic alterations in subsequent generations via inheritance of epigenetic modifications.

Early experiments of environmentally-induced epigenetic inheritance in rodents arose from epidemiological studies in humans, which show that parental and grandparental nutritional status can influence metabolism and longevity of offspring [78-80]. Rodent studies built on these findings and allow for rigorous control of genetic background while manipulating environmental parameters. Additionally, early studies utilized maternal exposures, which may be especially powerful because of effects on *in utero* development and offspring rearing long after exposure has stopped. Maternal obesity appears to have the most pronounced effect of parental nutritional changes, with metabolic changes in offspring present through three generations [81,82]. Interestingly, both small RNAs and DNA methylation have been proposed to mediate these effects [82-84]. Maternal reproductive toxins alter offspring development as well. Administering the antiandrogenic pesticide vinclozolin to gestating female rats during primordial germ cell development *in utero* results in male offspring with impaired fertility for three subsequent generations [85]. Preconception maternal ethanol exposure may also act as a teratogen, as offspring of these dams show an increased rate of skull deformities and changes in DNA methylation at an IAP regulating coat color [86].

Paternal exposures provide a more direct way of studying epigenetic inheritance, since sires are not present during offspring rearing and *in vitro* fertilization can eliminate any contribution of the sire apart from its germ cell. An early study found that paternal low protein diet after weaning was associated with aberrant gene expression and DNA methylation at several metabolically relevant loci in offspring [87]. Paternal fasting and high fat diet have both been associated with altered glucose metabolism in offspring [88,89]. Paternal folate deficiency was

recently found to influence offspring brain development, including levels of methylated cytosine [90]. Interestingly, perturbations to the paternal environment may influence maternal investment in offspring care, a concept termed maternal provisioning. For example, offspring of sires exposed to social enrichment were licked and nursed more frequently than those of sires raised in isolation [91]; along these lines, some paternal effects on offspring phenotypes disappear after *in vitro* fertilization [92].

Studies have now extended beyond preconception nutritional exposures to study how manipulations of parental behavior alter offspring development. An early study noted that maternal licking behavior during the early postnatal period programs offspring behavior across generations and is associated with changes in DNA methylation at the glucocorticoid receptor promoter [93,94]. Paternal exposures have been especially prominent in mediating behavioral effects in offspring. Paternal chronic stress altered sperm miRNA content and led to blunting of the hypothalamic-pituitary-adrenal (HPA) axis in offspring [95]. Paternal olfactory fear conditioning enhanced response to conditioned odors in offspring and decreased methylation of an olfactory receptor [96]. Sires exposed to social defeat stress had offspring that displayed increased anxiety-like behaviors [92]. Studies are now identifying mechanisms of paternal exposures on offspring phenotypes. A recent study injected sperm miRNAs from males exposed to early life stress in the form of maternal separation and found increased depression-like behaviors in offspring [77]. While mechanisms that underlie most effects of paternal exposure on offspring behavior are still unknown, they raise questions about whether transmission of epi-alleles in humans can modify risk for psychiatric disease.

Parental exposure to drugs of abuse has now been found to alter drug-induced behavior and drug consumption in offspring. Adult offspring derived from dams exposed to pre-

conceptional morphine had enhanced behavioral sensitivity to morphine and other behavioral alterations [97]. Male offspring of cocaine-exposed sires surprisingly displayed a cocaine-resistance phenotype and increased expression of BDNF in the prefrontal cortex [98]. Studies have now extended to cross-drug interactions. Parental methamphetamine exposure enhanced cocaine-induced locomotion and reward as well as altered DNA methylation in offspring brains [99]. Parental tetrahydrocannabinol (THC) exposure was associated with increased heroin-seeking and altered heroin withdrawal in offspring [100]. It remains to be seen whether these drugs are acting on common pathways to modulate drug-induced signaling in offspring or if their effects are specific. Considering complex pathways that regulate drug intake, including roles for memory, learning, stress, and reward pathways, it may prove difficult to identify a single mechanism driving altered drug preference in offspring following a parental exposure.

1.2.4 Epigenetic programming during spermatogenesis

This section summarizes epigenetic modifications in sperm; a more detailed discussion of ethanol-induced effects on the male reproductive axis is included in Section 1.3.3.

Epigenetic modifications during spermatogenesis are highly plastic and regulated by coordinated induction of DNA methylation, histone modifications at developmentally important loci, and small RNAs. These systems interact to produce a cell with a compact and transcriptionally silent nucleus, minimal RNA content, and a paternal imprinting pattern. However, while sperm have historically been viewed as passive carriers of genetic information, recent studies have demonstrated the importance of sperm-encoded epigenetic modifications for offspring development.

Condensation of sperm chromatin in mammals involves replacement of nearly all (95-99%) histones with highly basic protamines [101]. In general, protamines facilitate nuclear condensation by tightly binding DNA, which provides more efficient delivery of genetic material and may protect DNA from damage [101]. Retained histones in mature sperm were largely ignored until recently, when it was discovered that histone subunit H3 methylated at either lysine 4 and/or 27 was associated with several loci important for early embryogenesis [102]. A later study took an unbiased approach to characterizing sperm chromatin by measuring DNase sensitivity across the genome; regions with higher DNase sensitivity, indicating less compaction and therefore likely occupancy by a histone rather than a protamine, were enriched in CTCF binding sequences [103]. These observations led to a recent study on the role of histone modifications in regulating gene expression in embryos. Perturbing protamine incorporation into the sperm nucleosome by blocking poly-ADP riboslyation caused aberrant histone retention and altered expression of hundreds of genes in two-cell embryos [104]; this study supports the idea that sperm histones are potential mediators of epigenetic inheritance. While mechanisms for selecting histone vs. protamine occupancy within the sperm genome are still unknown, modifying this process affects offspring gene expression and may lead to altered phenotypes.

The paternal genome undergoes specialized encoding of DNA methylation during spermatogenesis. In primordial germ cells, DNA is actively demethylated and *de novo* re-methylated by DNMT3 enzymes to establish parental imprinting patterns and pluripotency [74]. After primordial germ cell development, DNA methylation patterns are maintained during spermatogenesis by DNMT1 with limited *de novo* methylation occurring postnatally [105]. While not as plastic as primordial germ cell development, DNA methylation during postnatal spermatogenesis is vital to sperm and offspring development. In rodent sires, systemic inhibition

of DNA methylation by 5-azacytidine resulted in decreased sperm count, infertility, and a 30-fold increase in the number of abnormal preimplantation embryos [106]. The role of DNA methylation in sperm extends beyond transcriptional repression. Hypomethylated regions of the sperm genome are enriched in transcription factor binding sites and mark promoters of genes expressed in early embryogenesis [102,107]. Therefore, changes to DNA methylation in sperm can affect offspring development and locus-specific effects have begun emerging [108,109].

Sperm RNAs have gained significant attention for their role in gametogenesis and, more recently, offspring development. Because the mass of RNA in oocytes (~1 ng per cell) dwarfs that of sperm (~10 fg per cell) [110], particular attention has been paid to small noncoding RNA (sncRNA) that have regulatory functions beyond serving as a template for translation. Studies have now identified tens of thousands of distinct populations of sncRNAs in spermatozoa, comprised of miRNAs, piRNAs, tRNAs, and other sequences [111,112]. piRNAs are critical for silencing of retrotransposable elements during primordial germ cell development and spermatogenesis [113,114]. Studies of epigenetic inheritance have specifically focused on the role of miRNAs for modifying offspring development. While an early study found only a minimal contribution of sperm miRNAs to zygotes [115], others have found a role for specific miRNAs in embryo cleavage [116] and offspring phenotypes [76]. Recent studies have found a more general role of altered sperm miRNA populations in heritability of paternally transmitted phenotypes [77,95]. Therefore, while sncRNA populations in sperm are complex and only recently studied, altering their composition can affect offspring phenotypes.

1.3 ETHANOL AND EPIGENETICS

1.3.1 Ethanol-induced gene expression

Despite decades of research, a definitive mechanism of ethanol that underlies its addictive potential has yet to be uncovered. In particular, ethanol is known to modify several neurotransmitter receptors; however, understanding how these receptor systems interact to promote AUD has proven challenging (for review, see [117]). A novel strategy for uncovering potential mechanisms of ethanol is to study its effects unrelated to its ability to bind neurotransmitter receptors. In particular, ethanol alters gene expression in several organ types, including brain [118,119], liver [120], and intestine [121]. Gene expression studies have focused on networks of genes that may be important in mediating adaptive responses to ethanol; however, identifying those genes also permits study of epigenetic modifications that may be conserved across gene promoters after ethanol exposure. Studying transcription factors or chromatin modifying enzymes that mediate these effects could represent a more robust approach to uncovering mechanisms of ethanol that promote AUD.

In humans, AUD is associated with differential expression of genes in the amygdala, cortex, and hippocampus [119,122-124]. These changes drive neuronal and glial adaptations to repeated ethanol exposure that are thought to incentivize further ethanol consumption [125]. Several whole genome expression studies of AUD using the frontal cortex have now shown widespread, though relatively modest (~50%), changes in expression of thousands of genes [118,122,124,126]. They have also identified several networks altered by chronic alcohol consumption, including genes involved in myelination and neurodegeneration that may underlie white matter loss due to AUD [119,124]. While informative, technical issues, like quality of

post-mortem tissue and donor history, have made it difficult to compare datasets; for example, one study used RNA-seq and found only a few dozen differentially expressed genes in the hippocampus of adults with AUD [123]. Studies are now moving past simply identifying genes and utilizing whole genome data to uncover mechanisms of gene regulation, including measuring miRNA expression and GC content of differentially expressed genes [122,127].

Technical challenges in human studies are overcome using rodent models, where animals are tested together and alcohol exposure can be controlled. In rodents, a single, binge-like exposure to ethanol is also associated with up- and down-regulation of genes in the cortex, nucleus accumbens, and ventral tegmental area [68,69,128]. Changes in gene expression are also associated with compensatory changes in miRNA regulation [129], suggesting post-translational mechanisms that may also underlie escalation of ethanol drinking. Gene expression microarrays have also been used to identify genes conferring altered preference for ethanol between different strains [128,130]. These studies have culminated in the identification of a mutation in a metabotropic glutamate receptor that confers increased alcohol preference in rats [131]. While rodent studies have had greater success identifying genes that confer risk for alcohol drinking compared to human gene expression analyses, few have examined mechanisms of ethanol-induced gene regulation.

1.3.2 Ethanol-induced epigenetic modifications in the brain

Studies are emerging on epigenetic mechanisms that regulate ethanol-induced changes to gene expression. Two studies using post-mortem tissue from people with AUD identified altered distribution of histone trimethylation in the hippocampus and cortex, which corresponded to gene expression changes in these regions [122,123]. Additionally, dysregulation of miRNAs,

retrotransposable elements, and DNA methylation have been discovered in similar post-mortem tissue [122,127]. Research has now extended to using pharmaceuticals with epigenetic mechanisms for treatment of AUD. The HDAC inhibitor valproic acid has been studied as a potential therapeutic and was found to reduce relapse rates [132] and alcohol drinking [133] in small cohorts of patients. While still limited, these studies suggest a role for epigenetic modifications in the maintenance of AUD.

Histone Modifications	Brain Region	Role	Reference
H3K9ac and H3K9me2	Primary Neuronal Cell Culture	↑H3K9ac and ↓H3K9me2 underlie ↑NR2B expression after CIE	Qiang et al. [134]
H3K9ac, H3K4me3, andH3K27me3	Amygdala	Chronic ethanol alters histone modifications at prodynorphin and pronociceptin promoters	D'Addorio et al. [135]
pan-H3 acetylation and H3K4me3	Hippocampus	Chronic ethanol alters histone modifications at *Bdnf* exons in hippocampus	Stragier et al. [136]
pan-H3 and H3K9 acetylation	Central Nucleus of Amygdala	Histone acetylation underlies ethanol preference differences between P and NP rats	Moonat et al. [137]
pan-H3 and H4 acetylation	Prefrontal Cortex	Histone acetylation underlies conditioned place aversion extinction in adolescents vs. adults	Pascual et al. [138]
pan-H3 and H4 acetylation	Medial Nucleus of Amygdala	Histone acetylation underlies tolerance to the effects of ethanol	Sakharkar et al. [139]
pan-H3 and H4 acetylation	Central Nucleus of Amygdala	Histone acetylation underlies ethanol-induced anxiolysis and withdrawal-induced anxiety	Pandey et al. [60]
pan-H3 and H4 acetylation	Ventral Tegmental Area	Altered histone modifications at gene promoters following 9 day chronic vapor ethanol and withdrawal	Shibasaki et al. [140]

Table 1. List of histone modifications altered by ethanol in the brain

Rodent studies of ethanol-induced epigenetic modifications have focused on specific brain regions important for regulating ethanol-related behavior (Table 1). Acute ethanol has now

been associated with increased histone acetylation in the central nucleus of the amygdala [60], altered histone modifications at the prodynorphin and pronociceptin promoters in the amygdala [135], and altered expression of histone modifying enzymes in the striatum and prefrontal cortex [141]. Chronic ethanol consumption was recently associated with increased expression of *Bdnf* in the hippocampus and ethanol-induced histone modifications at the *Bdnf* promoter [136]. Modulating histone modifications appears to have a dramatic effect on ethanol-related behaviors. Systemic administration of the HDAC inhibitor sodium butyrate blocked ethanol-induced behavioral sensitization, indicating histone deacetylation plays a critical role in the neuroadaptive response to ethanol administration [142]. Systemic HDAC inhibition also blocked the effects of ethanol withdrawal by reducing anxiety-like behaviors and increasing expression of *Bdnf*, *Arc*, and *Npy* in the amygdala [60,139,143,144]. Specific HDACs are being implicated in regulating ethanol drinking and withdrawal. A recent study pointed to HDAC2 expression in the amygdala as a critical regulator of ethanol preference and anxiolytic response to acute ethanol [137]. All of these studies provide evidence that ethanol-induced histone modifications in the brain are critical regulators of ethanol-related behaviors and consumption in rodents.

Despite emerging evidence for the role of histone modifications in mediating the effects of ethanol, only the HDAC2 studies have attempted to correlate changes in gene expression with ethanol-induced changes to histone modifying enzymes. Additionally, there have been no unbiased, genome-wide studies of histone occupancy after ethanol exposure using a rodent model. These studies will provide mechanistic insight into the well-established effects of ethanol on gene expression; additionally, they could identify networks of transcription factors induced by ethanol based on DNA sequences associated with altered histone modifications.

1.3.3 Ethanol-induced epigenetic modifications in gametes

Ethanol has notable effects on the male reproductive axis, including altering how epigenetic modifications are programmed in germ cells. In humans, research has mainly focused on potential effects of alcohol consumption on fertility. In general, while moderate alcohol consumption has limited effects on fertility parameters [145,146], several studies have noted abnormalities in sperm morphology among men with AUD and/or high alcohol consumption (for review, see [147]). The effects of chronic alcohol on fertility are thought to be mediated in part by central hypogonadism in men with AUD, leading to decreased circulating testosterone levels and altered testicular physiology [148,149]. Additionally, spermatogenesis may be especially susceptible to aberrant one carbon metabolism after chronic alcohol use, in particular alcohol-induced deficits in s-adenosylmethionine (SAM) [150,151], though no studies have examined DNA methylation in spermatocytes of men with AUD. A recent study in men with moderate alcohol consumption found a trend for hypomethylation of paternally imprinted genes in those who consumed alcohol compared to those who did not drink alcohol [152]. No studies have examined whether RNA and histone modifications are altered by AUD in spermatocytes, which will yield further information on potential effects on offspring development.

Rodent studies have focused on mechanistic aspects of ethanol-induced effects on the reproductive axis. In particular, studies are able to use a range of ethanol exposures that are difficult to replicate in humans. Two weeks of ethanol injections were found to decrease sperm counts and plasma testosterone as well as increase oxidative stress in mice [153]. Increased oxidative stress has been associated with global DNA hypomethylation in human sperm [154], suggesting a role for oxidative stress in ethanol's effects on sperm DNA methylation. Ethanol gavage 3 times per week for nine weeks in rats was associated with decreased expression of

DNMT1 in sperm [155]. Studies have focused on the effect of ethanol consumption on DNA methylation at heavily methylated, imprinted regions in sperm that are resistant to genomewide demethylation. One study did not find changes in sperm one week after ethanol exposure but noted significant hypomethylation at paternally imprinted regions in the offspring of ethanol-exposed sires [156]. Another study found hypomethylation at one of these paternally imprinted regions in sperm after four weeks of ethanol treatment [157].

1.3.4 Effects of paternal ethanol exposure on offspring

The effects of ethanol on multiple aspects of the male reproductive axis discussed in Section 1.3.3 suggest that ethanol is altering sperm development. Moreover, as emerging evidence points to a role of the sperm epigenome and microenvironment in offspring development (see Section 1.2.3), these ethanol-induced effects on sperm development are likely to have implications for children of men with AUD. There are several studies that support an association between fathers with AUD and changes across multiple cognitive domains in their offspring. These have shown increased risk for psychiatric disorders [158,159], decreased performance on measures of intelligence [160], personality changes [160,161], and increased incidence of attention deficit hyperactivity disorder (ADHD) [162] in children of men with AUD. Some of these effects are specific to fathers who had active AUD compared to those who were in remission [159]. Physiological deficits in offspring of alcoholic fathers have also been noted, including electroencephalographic changes [163], neuroimaging findings [164], and decreased intracranial volumes [165]. In particular, decreased amplitude of the P300 event related potential (ERP) has been associated with AUD and shows evidence for inheritance [163,166]. While these results are confounded by social and environmental factors associated with being raised by a father with

AUD, they also raise the possibility that acquired changes to male gametes are being transmitted to offspring.

Based on human studies that show a role for paternal ethanol on offspring development, rodent studies have used controlled alcohol exposures to study these effects in depth. Most of these animal studies have been undertaken from a fetal alcohol syndrome (FAS) perspective, which posits that parental ethanol use induces a spectrum of morphological and cognitive deficits in offspring. In rodents, paternal ethanol induces physiological abnormalities in offspring in the absence of maternal ethanol exposure, including low birth weight [155,167], increased number of runts [155], altered organ weights [167,168], thickening of layers of the cerebral cortex [169], and low testosterone levels [170]. Several behavioral abnormalities have also been noted, including decreased spatiotemporal learning [171], decreased novelty seeking behavior [167], increased immobility on the forced swim test [172], and decreased grooming [173]. More recent studies have found decreased open arm entries on the elevated plus maze [157] and increased impulsivity [174] in offspring of ethanol-exposed sires. One of these groups found altered expression of DNMT1 and MeCP2 in brains of paternal ethanol-sired offspring [174], suggesting potentially widespread epigenetic abnormalities in these animals. While these effects are varied, it is important to note that animals show deficits in some but not all behaviors, suggesting paternal ethanol affects discrete neurobiological pathways. Moreover, conserved changes in behavior across multiple animals would be challenging to explain by random mutations in the sperm genome. Unfortunately, gene-specific alterations in mice sired by alcohol-exposed fathers are still lacking, though improved methodology to study epigenetic modifications may lead to their identification in the future.

Despite the range of effects reported for paternal ethanol exposure on offspring, no groups have studied its effects on ethanol-induced behaviors or ethanol drinking. With the emergence of several groups reporting intergenerational inheritance of drug seeking behavior, it is possible epi-alleles account for the heritability of AUD.

1.4 HYPOTHESIS AND SPECIFIC AIMS

Despite its ubiquitous use and access, knowledge of basic neurobiological mechanisms of ethanol remains limited, delaying the development of pharmacological treatments for alcoholism. Moreover, GWAS has accounted for only a fraction of a percent of the heritability of AUD, delaying identification of individuals at high risk for alcohol dependence. To test the hypothesis that epigenetic modifications contribute to neurobiological mechanisms of ethanol and heritability of AUD, I developed three specific aims:

- Specific Aim 1 tests the hypothesis that paternal preconception ethanol exposure alters ethanol consumption and response to ethanol in offspring.
- Specific Aim 2 tests the hypothesis that conserved histone modifications regulate ethanol-induced gene expression in the cerebral cortex.
- Specific Aim 3 develops a method for studying histone modifications in a neuronal subtype *in vivo* and tests the hypothesis that chronic intermittent vapor ethanol and withdrawal induce dynamic changes in gene regulation via discrete histone modifications in accumbal dopamine 1 receptor positive medium spiny neurons.

2.0 PATERNAL ETHANOL ALTERS OFFSPRING BEHAVIOR

Adapted from: Finegersh A, Homanics GE (2014) Paternal Alcohol Exposure Reduces Alcohol Drinking and Increases Behavioral Sensitivity to Alcohol Selectively in Male Offspring. *PLoS ONE* 9(6): e99078. [175]

2.1 INTRODUCTION

Although the heritability of AUD is estimated to be ~50% among men [9], the genetic basis for this disease is poorly understood despite considerable scientific investment. Like many other complex, polygenic diseases, DNA sequence variations are associated with risk of acquiring AUD [24,25]; however, these variants account for a tiny fraction of the total risk [23].

Emerging evidence from several converging fields has reinvigorated the idea that inheritance of acquired characteristics, "epigenetic inheritance," is an adjunct to traditional modes of genetic inheritance. In rodent studies in which genetics and environment can be rigorously controlled, it is now established that environmental perturbations can produce phenotypic alterations in subsequent generations without further exposures. For example, in isogenic rodents, exposure to stress [77,95], endocrine disruptors [85], high fat diet [89], low protein diet [87], and olfactory fear conditioning [96] all result in phenotypic changes in subsequent generations. There is also a growing literature on intergenerational effects of drugs of abuse. Adult offspring derived from dams exposed to morphine prior to conception displayed

enhanced behavioral sensitivity to morphine and other behavioral alterations [97]. Male offspring of cocaine-exposed sires surprisingly displayed a cocaine-resistance phenotype [98]. Finally, prenatal exposure to ethanol was associated with transgenerational effects on POMC expression that was inherited through the male germ line [176].

A number of studies support the idea that parental ethanol exposure prior to mating can alter the phenotype of offspring. In rodents, paternal preconception ethanol exposure induced developmental abnormalities including altered organ weights including brain [168,177], thickening of cortical layers [169], and decreased testosterone levels [170]. Paternal ethanol exposure also induced numerous behavioral abnormalities, including decreased grooming behavior in response to novelty or water immersion [173], altered spatial learning [167], decreased novelty seeking [171], and decreased immobility in a forced swim test [172]. These changes did not appear to be related to stress or undernutrition associated with ethanol exposure.

The studies cited above led us to hypothesize that ethanol drinking behavior and neurobiological sensitivity to ethanol are due in part to paternal ethanol exposure prior to conception. To test this hypothesis in a model system that was free from confounding genetic and environmental influences, adult male mice were chronically exposed to ethanol (or control conditions) and subsequently mated to ethanol naïve females. Adult offspring were tested for ethanol drinking on the two bottle choice test and drinking in the dark assays as well as a range of ethanol-induced and basal behaviors.

2.2 MATERIALS AND METHODS

2.2.1 Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Eight-week-old, ethanol-naïve, specific pathogen free C57BL/6J and Strain 129Sv/ImJ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used to generate the F1 generation of offspring as described below. Mice were group-housed under 12 hour light/dark cycles and had ad libitum access to food and water.

2.2.2 Paternal ethanol exposure

Vapor ethanol inhalation was used because it allows for ad libitum access to food and water, no stress-inducing injections or gavage, and animals remaining in their home cage. Two identical custom-built vapor chambers were used (16” x 16” x 24” constructed from 0.5” plexiglass) to deliver either room air or vaporized ethanol. Flow rate, vaporization temperature, and exposure time were optimized to achieve consistent blood ethanol concentrations (BEC) without the use of pyrazole. Room air was flowed into two heated Erlenmeyer flasks at a rate of 8 L/min; one flask received ethanol at a rate of ~250 μl/min while the other flask received no ethanol. Air from the ethanol and control flasks flowed into separate chambers so that only one chamber received vaporized ethanol.

Male C57BL/6J mice were placed in vapor chambers from 08:00 to 16:00 for 5 consecutive days/week for at least 5 weeks. Five weeks of exposure was chosen because it represents a complete cycle of murine spermatogenesis [178]. Temperature of the chambers was

monitored daily and averaged 78˚ F at the end of 8 h of exposure. Mice were weighed at the beginning of each week and blood was collected from the tail vein at the end of each week. Total ethanol in plasma was measured using an Analox ethanol analyzer (AM1, Analox Instruments, London, UK).

2.2.3 Breeding scheme and offspring rearing

Immediately following the final day of exposure, male mice were removed from group housing and housed with two eight-week-old, ethanol-naïve Strain 129Sv/ImJ female mice. Strain 129Sv/ImJ females were chosen because they do not erase epigenetic marks at intracisternal A particles (IAPs) in offspring in utero while C57BL/6J females do erase these marks [108]. After 48 hours, males were removed from the females' cages. Strain 129xC57 F1 hybrid offspring were reared normally and weighed weekly beginning at 3 weeks of age. All offspring used in behavioral experiments were at least 8 weeks of age.

2.2.4 Isolation of motile sperm DNA

Male mice were group housed and exposed to vapor ethanol or room air for an additional 3 days following mating. This additional exposure was done so that ethanol-induced epigenetic marks were not lost during this time period and that the effects of ethanol withdrawal on gene expression and epigenetic processes would not affect germ cells. Sixteen hours following exposure, the cauda epididymis was dissected from the testes and placed into 4 ml of 1% bovine serum albumin (BSA) in PBS. Sperm and DNA were extracted using a double swim up assay as previously described [179]. Briefly, several longitudinal cuts were made through the cauda

epididymis using a scalpel and the tissue in 1% BSA was collected into a 15 ml conical tube. The tissue was incubated for 30 minutes at 37˚ C and the top 2 ml of liquid was collected into a new 15 ml conical tube, which was incubated for an additional 15 minutes at 37˚ C. The top 1 ml containing motile sperm was collected and used for analysis. Sperm were pelleted at 6000 rpm for 5 min at 4˚ C, resuspended in sperm lysis buffer with proteinase K [179], and incubated at 50˚ C overnight. DNA was extracted using a phenol-chloroform-isoamyl alcohol extraction and an ethanol precipitation and eluted in 100 µl TE buffer.

2.2.5 Isolation of RNA and DNA from the VTA and mPFC

Adult offspring were sacrificed by cervical dislocation and the brain extracted and placed into an ice cold adult mouse brain slicer matrix with 1 mm coronal section slice intervals (Zivic Instruments, Pittsburgh, PA). Razor blades were inserted starting at the rostral end through the midbrain. The ventral tegmental area (VTA) was collected on the first slice where the hippocampus wrapped around the midbrain as the region medial to the substantia nigra. The medial profrontal cortex (mPFC) was collected on the first two consecutive slices where the cortex was first visible. These structures were collected, flash frozen in liquid nitrogen, and stored at -80˚ C. RNA and DNA were extracted using Trizol (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. RNA was purified using the RNeasy mini kit with DNase digestion (Qiagen, Valencia, CA) and eluted in 30 µl water. DNA was eluted in 100 µl TE buffer.

2.2.6 Bisulfite sequencing

Bisulfite treatment was carried out using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Briefly, 20 µl of DNA in TE buffer was treated with sodium bisulfite, desulphonated, and eluted in 10 µl of water. Bisulfite-treated DNA was used as a template for a nested PCR reaction. Primer sequences used are summarized in Table 1. PCR conditions for the outside reaction were: 4 min at 94˚ C, 2 min at 55˚ C, and 2 min at 72˚ C repeated once then 1 min at 94˚ C, 2 min at 55˚ C, and 2 min at 72˚ C repeated 35 times. The outside reaction was used as a template for the inside reaction, whose conditions were: 4 min at 94˚ C, then 1 min at 94˚ C, 2 min at 55˚ C, and 2 min at 72˚ C repeated 35 times, then 7 min at 72˚ C. The inside PCR reaction was run on a 1.2% agarose gel and a ~300 bp product excised and gel purified using the Purelink Quick Gel Extraction kit (Life Technologies) according to the manufacturer's protocol. Gel-purified PCR products were cloned into a TOPO TA vector (Life Technologies) according to the manufacturer's protocol and transformed into TOP10 competent cells (Life Technologies). Cells were plated onto LB agar plates with ampicillin. Individual colonies were selected and grown overnight separately in LB with ampicillin, then plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's protocol.

Plasmids were checked for the presence of an insert using an EcoRI digestion. Those with an insert were sent for sequencing (Genewiz, South Plainfield, NJ). After sequencing, trace files and the original BDNF exon IXa CpG island, *IG* DMR, or *H19* DMR sequence were loaded into CpGviewer, an automated bisulfite sequencing analysis program that detects the methylation status of potentially methylated cytosines [180], and results were used for analysis. At least two

separate bisulfite treatments for each animal's tissue were performed and at least four animals per group were used for analysis.

Amplicon	Forward primer (5'-->3')	Reverse primer (5'-->3')
IG DMR (Outside)	TTAAGGTATTTTTTATTGATAAAATAATGTAGTTT	CCTACTCTATAATACCCTATATAATTATACCATAA
IG DMR (Iniside)	TTAGGAGTTAAGGAAAAGAAAGAAATAGTATAG	TATACACAAAAATATATCTATATAACACCATACAA
H19 DMR (Outside)	GAGTATTTAGGAGGTATAAGAATT	ATCAAAAACTAACATAAACCCCT
H19 DMR (Inside)	GTAAGGAGATTATGTTTATTTTTGG	CCTCATTAATCCCATAACTAT
BDNF exon IXa promoter (Outside)	ATAAAAAAAATAATAACCATCCTTTTCCTTACTA	ATTTAGGTAATTTTTGTATTTTTTTAGTAGAAA
BDNF exon IXa promoter (Inside)	TACCATCCTTTTCCTTACTATTTTTATTTCAT	GAGTAGAGGAGGTTTTAAAGGTATTTG

Table 2. Primer sequences for bisulfite sequencing reactions

2.2.7 Reverse Transcription Quantitative PCR (RT-qPCR)

300 ng of RNA was converted into cDNA using reverse transcriptase (RT) (Bio-Rad, Hercules, CA). Reactions were carried out in duplicate for each gene. SYBR green fluorescent master mix (Bio-Rad) was added to each well and visualized using a Bio-Rad iCycler. All primers were optimized for 90% to 110% efficiency at the following conditions: 10 min at 95˚C followed by 40 cycles of 30 s at 95˚C, 1 min at 60˚C, and 30 s at 72˚C. Primer sequences used are summarized in Table 2. Threshold cycle (Ct) values were calculated for each well and duplicate values averaged. The difference between specific genes and β-actin (ΔCt) was calculated for each animal and normalized to the average of room air sired offspring (ΔΔCt). Fold change over room air sired offspring was calculated for each animal using the following formula: 2-ΔΔCt.

Amplicon	Forward primer (5'-->3')	Reverse primer (5'-->3')
BDNF exon IV	CAGGAGTACATATCGGCCACCA	GTAGGCCAAGTTGCCTTGTCCG
BDNF exon Ixa	AGCCTCCTCTACTCTTTCTGCTG	GTGCCTTTTGTCTATGCCCCTG
Dlk1	GGCCATCGTCTTTCTCAACA	ATCCTCATCACCAGCCTCCT
β-actin	TCATGAAGTGTGACGTTGACATCCGT	CCTAGAAGCATTTGCGGTGCACGATG

Table 3. Primer sequences used for RT-qPCR experiments on offspring

2.2.8 Forced Swim Test

Mice were tested for their performance on the forced swim test, which correlates with anxiety- or depression-like behaviors in rodents [181]. Mice were placed into a standard 1000 mL beaker filled to 900 mL with 26˚ C tap water. The water level was 10 cm above the bottom of the beaker before the mouse was placed into the beaker. Swimming behavior was scored for latency to immobility and total immobility after the first 2 minutes for 4 minutes. Immobility was defined as a cessation in swimming behavior for greater than 5 seconds. After testing, mice were quickly dried with paper towels and placed under a heat lamp for half an hour, then returned to their home cage.

2.2.9 Grooming behavior

One week following testing for the forced swim test, mice were randomly divided into two groups and tested for basal and induced grooming behaviors. For basal grooming, mice were removed from their home cage and singly housed in an empty cage. After acclimating to the new cage for 15 minutes, grooming behavior was measured for 5 minutes and scored for latency to groom, frequency of grooming episodes, and duration of grooming. For induced grooming, mice were also acclimated to single housing in a new cage for 15 minutes. Then, they were placed on top of the new cage and gently sprayed twice with water on the dorsal surface from a distance of ~2 inches. They were placed back in their cage and scored for latency to groom, frequency of grooming episodes, and duration of grooming over 5 minutes.

2.2.10 Two bottle choice

The two bottle choice test was used to assess the effect of paternal ethanol exposure on ethanol preference and consumption. Mice were acclimated to individual housing with food available ad libitum for one week. After one week, water bottles were replaced with two modified 25 ml polystyrene serological pipets (Thermo Fisher, Waltham, MA) fitted with ball bearing sipper tubes and filled with drinking water. Every 4 days, mice were weighed, total volume consumed measured by reading volume markers on the tubes, and tubes were removed, washed, and replaced. After 4 days of drinking water, tubes were replaced with one tube containing a 3% (w/v) ethanol solution and the other containing drinking water. This was immediately followed by 4 days each of a choice between 6%, then 9%, then 12%, and then 15% ethanol solutions and drinking water. The position of the ethanol tube was changed every 4 days, and there were no mice exhibiting a side preference for either tube position. A subset of mice was also tested for their preference for saccharin (0.033% and 0.066% w/v) then quinine hemisulfate (0.03 and 0.06 mM) following 15% ethanol solution. There was a 1 week washout period between ethanol, saccharin, and quinine tastants where both tubes contained water. Quantity of ethanol, saccharin, quinine, and water consumed was calculated for each mouse (g/kg/day or ml/kg/day) and preference was calculated as a ratio of solution consumed over total volume consumed. All solutions were also placed in an empty cage for 4 days to determine evaporation and spillage estimates, which were subtracted from the total volume consumed of each solution for each mouse.

2.2.11 Elevated plus maze, open field, and accelerating rotarod

Offspring were tested for their performance on three consecutive behavioral tests after a single intraperitoneal injection with either 1 g/kg ethanol (0.02 ml/g body weight of 5% ethanol in 0.9% saline) or 0.02 ml/g 0.9% saline. Prior to injection, mice were individually housed with no food or water for 1 hour.

The elevated plus maze was used to assess the effect of paternal ethanol exposure on basal anxiety-like behavior and ethanol-induced anxiolysis. Ten minutes after injection, mice were place in the center of an elevated plus maze facing an open arm. Sessions were video recorded for 5 minutes and manually scored for number of arm entries and time spent in each arm; arm entries were defined as all four limbs within an arm. At the conclusion of the elevated plus maze assay, mice were returned to individual housing.

The open field test was used to assess the effect of paternal ethanol exposure on basal and ethanol-induced locomotor activity. Five minutes after the conclusion of the elevated plus maze (20 minutes post-injection), mice were placed in the corner of a 43.2 x 43.2 x 30.5 cm open field box with a white floor and clear plexiglass walls (Med Associates Inc., St. Albans, VT). The open field box was placed in a sound attenuating cubical (Med Associates Inc.) and illuminated by a 1W bulb and a small fan provided a low level of background noise. Movement was tracked using infrared beam sensors over a 10 minute trial.

The accelerating rotarod test was used to assess the effect of paternal ethanol exposure on basal motor coordination and ethanol-induced ataxia. Five minutes after the conclusion of the open field test (35 minutes post-injection), mice were habituated to the rotarod (Ugo Basile, Italy) for 30 seconds at 5 rpm. Then, the rotarod was accelerated from 5 to 50 rpm over 180

seconds. The amount of time mice remained on the accelerating rotarod was measured for 5 separate trials spaced 60 seconds apart.

2.2.12 Drinking in the dark assay

The drinking in the dark assay is a model of scheduled, limited access ethanol consumption that produces elevated BECs in high drinking mouse strains [182]. In this assay, offspring were singly housed for one week before testing with *ad libitum* food and a standard water bottle. Beginning on Monday each week, the water bottle was replaced with one 25 ml polystyrene serological pipet 3 hours after onset of the dark cycle. For Monday, Tuesday, and Wednesday, mice had access to the solution for 2 hours; on Thursday, mice had access to the solution for 4 hours. At the end of the time period, total volume consumed measured by reading volume markers on the tubes and the standard water bottle was placed back in the cage. This experiment was completed first with water, then 10% ethanol solution in water the following week, then 20% ethanol solution in water the following week. Therefore, there was a 3 day washout period between each solution. All mice were weighed and cages changed at the beginning of the week.

2.2.13 Ethanol metabolism

The rate of ethanol clearance was determined to assess the effect of paternal ethanol on ethanol metabolism. Mice were injected with 3.5 g/kg ethanol i.p. (0.02 ml/g of 17.5% ethanol in 0.9% saline). Blood was collected by tail nick at 60 minutes and 240 minutes post-injection. Total ethanol in plasma and standards was measured using an Analox ethanol analyzer.

2.2.14 Statistical analysis

Behavioral experiments were analyzed using a Student's t-test, $\chi 2$ test, and two-way ANOVA with or without repeated measures where appropriate. For ANOVAs, post-hoc pairwise comparisons were made using the Fisher's LSD test. For analysis of DNA methylation, groups were analyzed using the Mann Whitney U test. For RT-qPCR data, groups were analyzed using a Student's t-test. Data from mice that differed by 2 SDs from the mean were considered outliers and excluded from analysis. All data are presented as mean +/- standard error bars.

2.3 RESULTS

2.3.1 Paternal ethanol exposure

This chronic intermittent vapor ethanol exposure paradigm induces dependence [183] and increases voluntary ethanol drinking [184]. Blood ethanol concentrations (BEC) were measured weekly on the final day of ethanol exposure and averaged 147.1 +/- 7.52 mg/dl (mean +/- SEM) (Fig. 2B). Sires were weighed at the beginning of each week and there was a significant effect of time on sire weight over the course of five weeks ($F_{(4,200)} = 54.50$, $p < 0.0001$) but no significant effect of treatment or interaction, indicating generally normal weight gain among ethanol exposed sires (E-sires) compared to control sires (C-sired) (Fig. 2C).

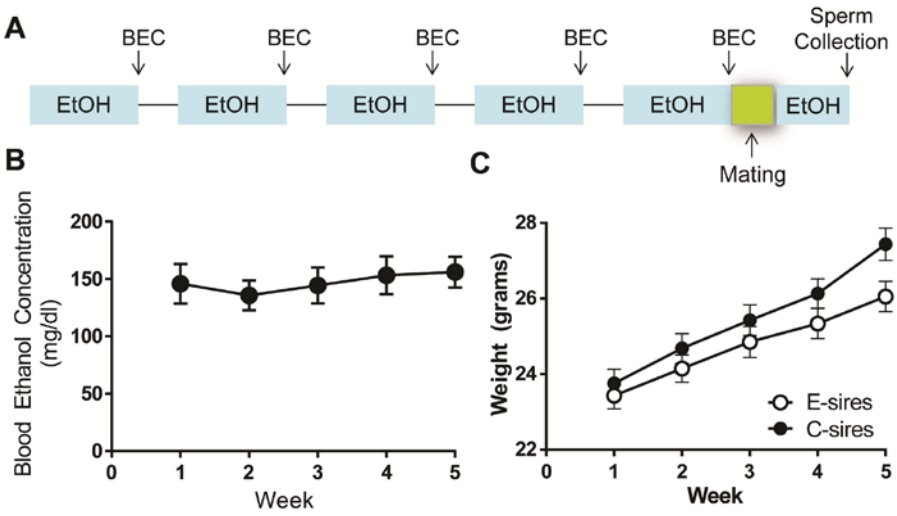

Figure 2. Chronic vapor ethanol exposure

(A) 8-week-old C57BL/6J mice were exposed to ethanol vapor or room air for 8 hours/day, 5 days/week, for 5 weeks and immediately housed with 2 ethanol-naïve Strain 129Sv/ImJ females for 48 hours; after mating, they were re-exposed for 3 days and motile sperm was collected. (B) Blood ethanol concentrations showed limited variability across 5 weeks and averaged 147.1 +/- 7.52 mg/dl (mean ± SEM) (n = 25). (C) There was no difference in weight gain between ethanol (E-sires) (n = 25) and room air (C-sires) (n = 27) exposed sires during the 5 weeks of exposure.

2.3.2 Ethanol exposure hypomethylates the *IG* DMR in motile sperm

Alcohol consumption was found to decrease DNA methylation of paternally imprinted regions in motile sperm [152]; therefore, these regions were studied following the chronic vapor ethanol exposure in this study. There was a ~5% reduction in DNA methylation at the intergenic (*IG*) differentially methylation region (DMR) in sperm ($p < 0.001$), which regulates expression of *Dlk1* from the paternal chromosome [185] (Fig. 3A,C); however, there was no difference in DNA methylation of the *H19* DMR, which regulates expression of *Igf2* from the paternal chromosome [186] (Fig. 3B,D). These results suggest locus-specific changes to DNA methylation in motile sperm following chronic ethanol exposure.

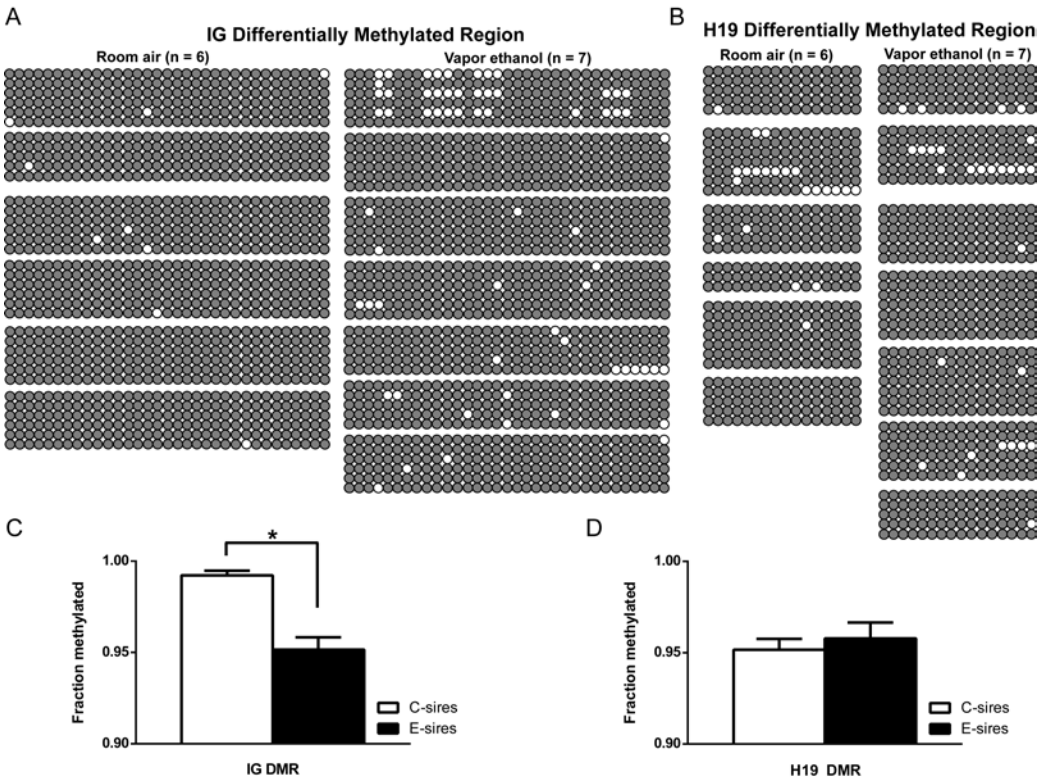

Figure 3. Ethanol decreases DNA methylation of the *IG* DMR in motile sperm

DNA methylation at the intergenic (*IG*) and *H19* differentially methylated regions (DMR) were measured in motile sperm using bisulfite sequencing. (A,C) DNA methylation is significantly reduced at the *IG* DMR in motile sperm of ethanol-exposed sires relative to room air controls. (B,D) There is no change in DNA methylation of the *H19* DMR following ethanol exposure. Each circle represents a potentially methylated cytosines in the DMR; filled circles are methylated and unfilled circles are unmethylated. Each block of rows represents sequenced colonies from a single independent animal. n = 6-7/group. Data presented as mean ± SEM. *$p < 0.0001$.

2.3.3 Paternal ethanol increases male offspring weight in 129xC57 offspring

Immediately following the 5th week of exposure, each sire was mated to 2 ethanol-naïve Strain 129Sv/ImJ females for 48 hours; there was no significant difference in the number of offspring sired or litter size from E-sires compared to C-sires (Fig. 4A-B). For body weight of male offspring, there was a significant effect of time ($F_{(3,297)} = 900.7$, $p < 0.0001$) and sire exposure

($F_{(1,99)}$ = 17.35, p < 0.0001) but no interaction of time and treatment; post-hoc analysis revealed that E-sired male offspring weighed more than C-sired male offspring at four (p < 0.01), five (p < 0.001), and six weeks (p < 0.01) of age (Fig. 4C). For body weight of female offspring, there was a significant effect of time ($F_{(3,258)}$ = 508.3, p < 0.0001), but no effect of sire exposure or interaction between time and treatment (Fig. 4D).

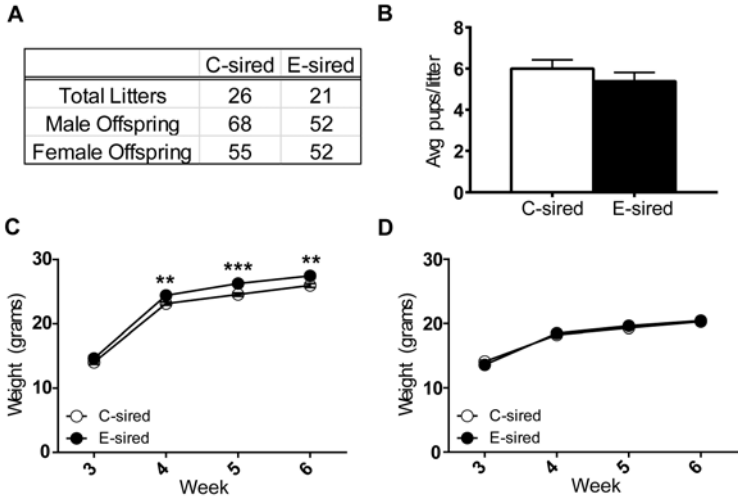

Figure 4. Litter characteristics of ethanol-exposed sires

(A) There were no differences in number of litters, number of male and female offspring, or (B) number of offspring per litter between E- and C-sires. (C) E-sired male offspring (n = 40) gained significantly more weight after weaning at 3 weeks and maintained increased weight through week 6 compared to C-sired male offspring (n = 61) (p < 0.001). (D) There was no significant difference in weight between E- (n = 43) and C-sired (n = 45) female offspring. Note: Error bars in C and D are obscured by the data points), **p < 0.01, ***p < 0.001.

2.3.4 Paternal ethanol on forced swim test behavior

Mice were tested forced swim behavior based on studies showing an effect of paternal ethanol on this assay [172,187]. There was a significant effect of sex on latency to immobility ($F_{(1,31)}$ = 17.04, p < 0.001) as well as a trend for sire exposure ($F_{(1,31)}$ = 3.10, p = 0.08) and a trend for an interaction between sex and sire exposure ($F_{(1,31)}$ = 3.46, p = 0.07) (Fig. 5A); post-hoc analysis

revealed E-sired females had increased latency to immobility compared to C-sired females ($p < 0.05$). There was a near-significant trend for an effect of sex on total immobility ($F_{(1,31)} = 4.03$, $p = 0.053$) but no effect of sire exposure or interaction between sex and sire exposure (Fig. 5B).

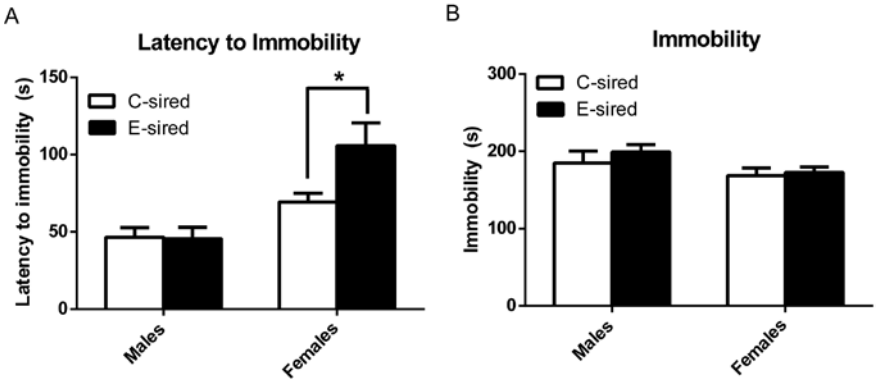

Figure 5. Paternal ethanol on forced swim test behavior

(A) Female offspring of sires exposed to ethanol (E-sired) had significantly increased latency to immobility compared to control-sired female offspring (C-sired); there were no significant effects between E- and C-sired male offspring. (B) There were no significant effects between E- and C-sired offspring on total immobility. n = 9/group; *p < 0.05.

2.3.5 No effect of paternal ethanol on grooming behavior

There were no significant effects of sex or sire on basal grooming or induced grooming following misting with water, suggesting no effect of paternal ethanol on grooming (Fig. 6).

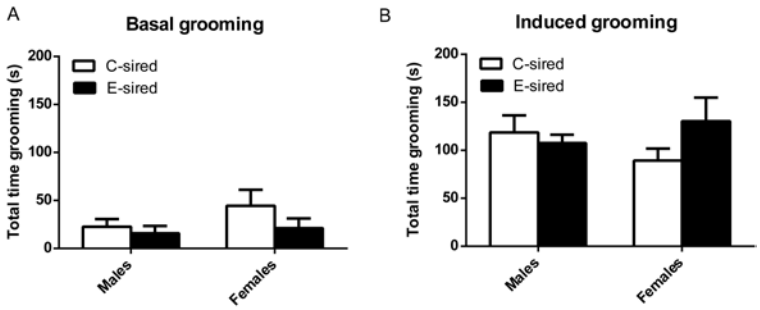

Figure 6. No effect of paternal ethanol on grooming behavior

There was no effect of paternal ethanol on (A) basal or (B) induced grooming.

2.3.6 Paternal ethanol reduces ethanol consumption in male offspring

Offspring were tested for ethanol preference and consumption versus water using a standard two bottle choice drinking paradigm. Singly-housed mice were tested sequentially for consumption of 3%, 6%, 9%, 12%, and 15% ethanol (w/v%) for 4 days each. For ethanol preference in male offspring, there was an effect for sire exposure ($F_{(1,32)}$ = 7.22, $p < 0.05$) but not for ethanol concentration and no interaction between sire exposure and concentration; post-hoc analysis revealed E-sired male offspring had significantly decreased preference for 3% ($p < 0.05$), 6% ($p < 0.05$), and 9% ($p < 0.05$) ethanol solutions compared to C-sired male offspring (Fig. 7A). For ethanol consumption, there was a significant effect of sire exposure ($F_{(1,32)}$ = 6.63, $p < 0.05$) and

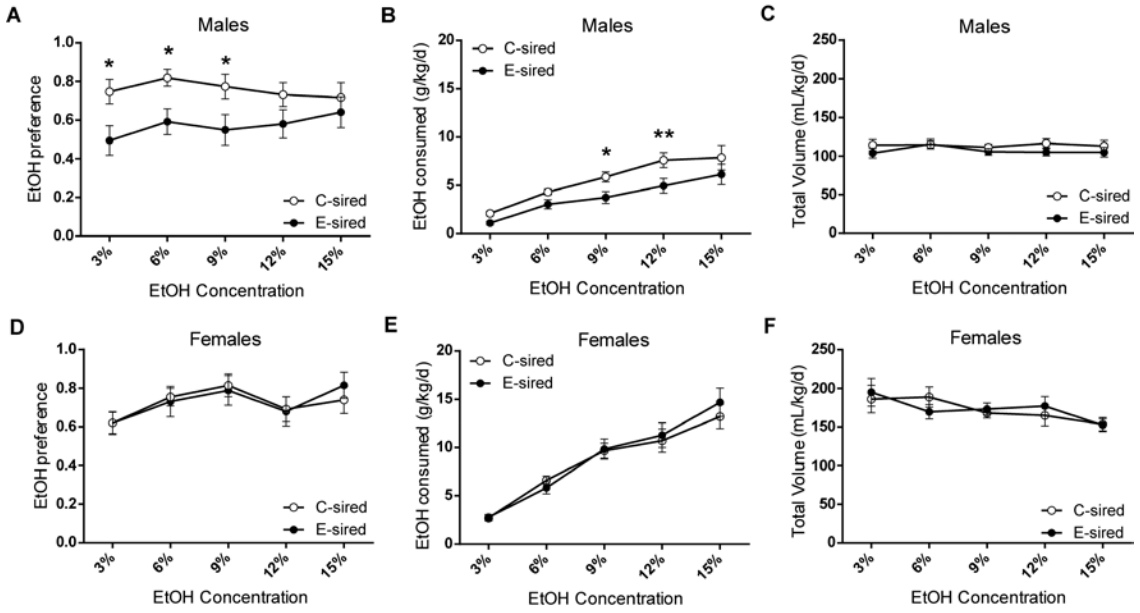

Figure 7. Paternal ethanol on two bottle free choice ethanol drinking

Offspring were tested for ethanol consumption vs. water on a 2 bottle, free choice drinking assay. (A) E-sired male offspring (n = 17) had significantly decreased preference for ethanol compared to C-sired male offspring (n = 17) as well as (B) decreased ethanol consumption and (C) no change in total volume consumption per body weight. There were no significant differences between E-sired female offspring (n = 12) and C-sired female offspring (n = 12) on (D) ethanol preference, (E) ethanol consumption, or (F) total volume consumed. *$p < 0.05$, **$p < 0.01$.

ethanol concentration ($F_{(4,128)}$ = 27.82, $p < 0.0001$) but no interaction; post-hoc analysis revealed E-sired male offspring consumed significantly less of 9% ($p < 0.05$) and 12% ($p < 0.01$) ethanol solutions compared to C-sired male offspring (Fig. 7B). There was no effect of sire exposure, ethanol concentration, or interaction on total volume consumed (Fig 7C).

For females, there was an effect of ethanol concentration on preference ($F(4,88) = 4.84$, $p < 0.01$; Fig. 7D), consumption ($F(4,88) = 67.63$, $p < 0.0001$; Fig. 7E), and total volume consumed ($F(4,88) = 4.01$, $p < 0.01$; Fig. 7F). However, there were no effects of sire exposure and no interaction of sire exposure with concentration on any parameter measured.

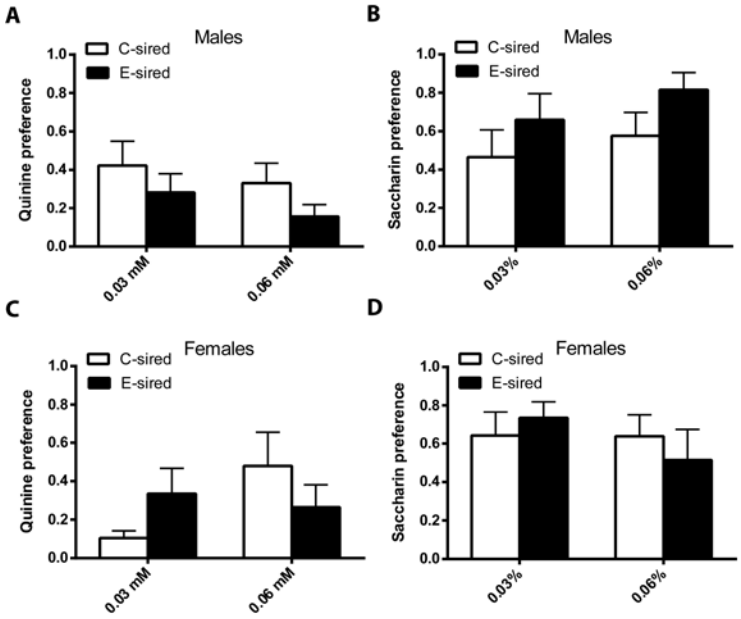

Figure 8. Paternal ethanol on two bottle choice saccharin and quinine drinking

There were no significant differences between E- (n = 11) and C-sired (n = 10) male offspring on (A) quinine drinking or (B) saccharin drinking; there were also no significant differences between E- (n = 6) and C-sired (n = 5) female offspring on (C) quinine drinking or (D) saccharin drinking.

To investigate if any observed changes in ethanol drinking behavior were influenced by alterations in taste perception, mice were tested for preference of quinine or saccharin versus water in a similar two bottle choice assay. No effects of sire exposure, concentration, or

interaction were observed for males consuming quinine (Fig. 8A) or saccharin (Fig. 8B) or for females consuming quinine (Fig. 8C) or saccharin (Fig. 8D).

2.3.7 No effects of paternal ethanol on the drinking in the dark assay

Offspring were tested for their ethanol consumption on a scheduled, limited access assay, where they had access to ethanol for 2 hours (days 1 through 3) or 4 hours (Day 4). There were no significant effects of sire exposure or an interaction between sire exposure and time in either male or female offspring on this assay (Fig. 9).

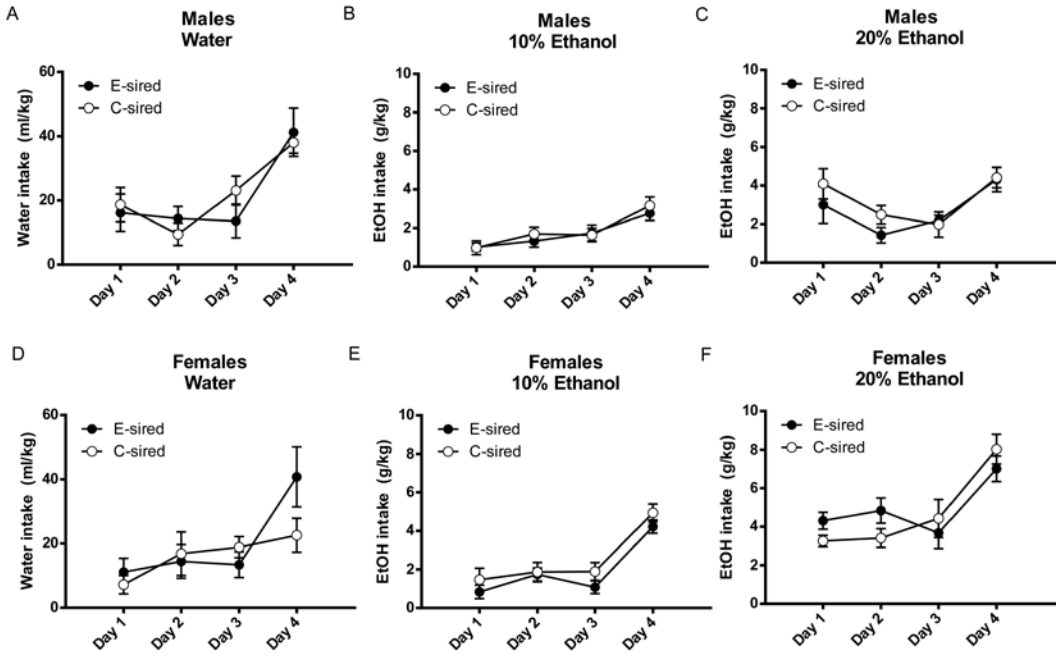

Figure 9. Paternal ethanol on ethanol drinking in the dark

Offspring were tested on a scheduled, limited access drinking model. There were no significant differences in (A,D) water, (B,E) 10% ethanol, or (C,F) 20% ethanol in male or female offspring. n = 6-9/group

2.3.8 Paternal ethanol effects on the elevated plus maze

A separate group of ethanol naïve offspring were tested for performance on the elevated plus maze (EPM) 10 minutes after treatment with 1 g/kg ethanol or saline i.p.. For male offspring, there was a significant effect of treatment ($F_{(1,20)} = 5.50$, $p < 0.05$) and sire exposure ($F_{(1,20)} = 7.95$, $p < 0.05$) as well as a significant interaction between treatment and sire exposure ($F_{(1,20)} = 7.21$, $p < 0.05$) on percent time spent in open arms; post-hoc analysis revealed E-sired male offspring treated with ethanol spent significantly more time in the open arms compared to those treated with saline ($p < 0.01$) as well as C-sired male offspring treated with ethanol ($p < 0.001$) (Fig. 10A). There was also a significant interaction between treatment and sire exposure ($F_{(1,20)} = 4.97$, $p < 0.05$) on percent of open arm entries relative to total arm entries; post-hoc analysis revealed E-sired male offspring treated with ethanol had a significantly greater percent of open arm entries compared to those treated with saline ($p < 0.01$) as well as C-sired male offspring treated with ethanol ($p < 0.01$) (Fig. 10B). These results demonstrate that although basal levels of anxiety-like behavior did not differ between groups, male mice born to ethanol-exposed sires were more sensitive to the anxiolytic effect of ethanol.

There was also a significant effect of treatment ($F_{(1,20)} = 5.91$, $p < 0.05$) but not sire exposure on total arm entries; post-hoc analysis revealed E-sired male offspring treated with ethanol made significantly more arm entries compared to those treated with saline ($p < 0.05$) as well as C-sired male offspring treated with ethanol ($p < 0.01$) (Fig. 10C). These results demonstrate that although basal levels of locomotor activity on the EPM did not differ between groups, male mice born to ethanol-exposed sires were more sensitive to the stimulatory effects of ethanol compared to male mice born to sires that were not exposed to ethanol.

For female offspring, there was no significant effect of treatment or sire exposure and no interactions for percent time spent in open arms (Fig. 10D). There was a significant effect of treatment ($F_{(1,22)} = 4.56$, $p < 0.05$) but no effect of sire exposure or interaction on number of open arm entries (Fig 10E). There was no significant effect of treatment or sire exposure and no interactions for total arm entries (Fig. 10F).

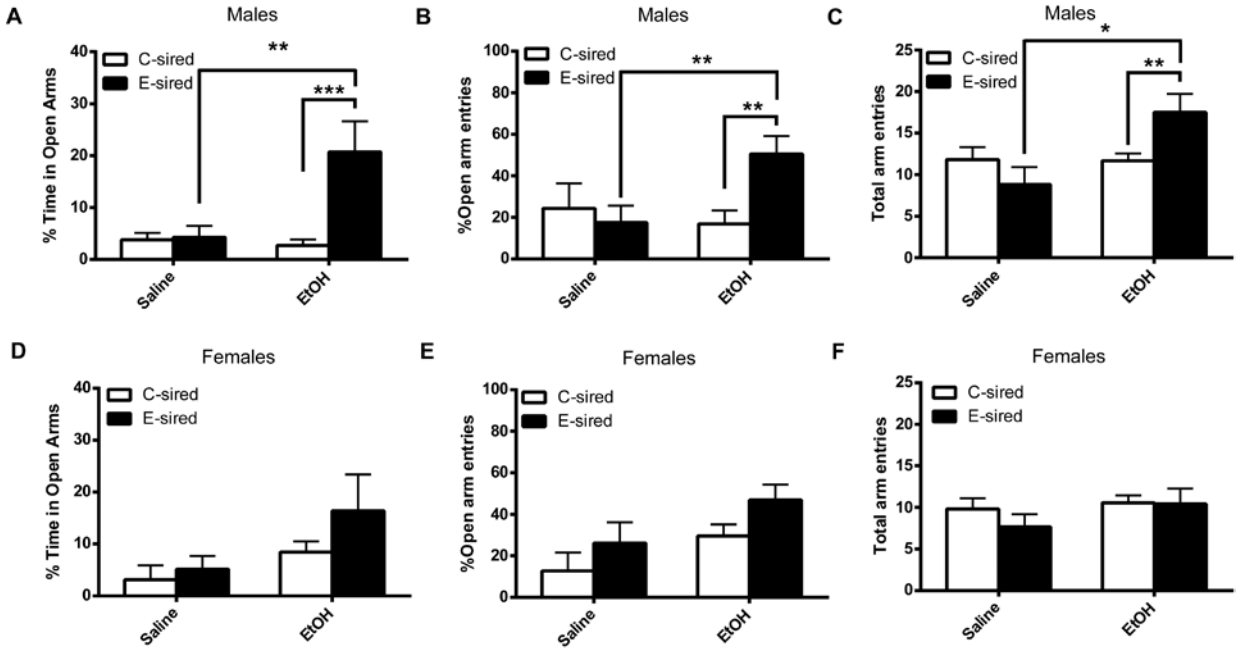

Figure 10. Paternal ethanol on the elevated plus maze assay

Offspring were tested for ethanol-induced anxiolysis by testing performance on an elevated plus maze 10 minutes after i.p. injection of 1 g/kg ethanol or saline. (A) E-sired male offspring spend greater time in open arms after treatment with ethanol compared to C-sired male offspring and E-sired male offspring treated with saline; E-sired male offspring treated with ethanol also have (B) increased percent of open arm entries and (C) total arm entries relative to C-sired male offspring and E-sired male offspring treated with saline. There were no significant differences between E- and C-sired females treated with ethanol or saline on (D) time spent in open arms, (E) percent open arm entries, or (F) total arm entries. n = 6-7/group, *p < 0.05, **p < 0.01, ***p < 0.001.

2.3.9 No effect of paternal ethanol on open field performance

Five minutes after the completion of the EPM (i.e., 20 minutes after treatment with either 1 g/kg ethanol or saline), the same mice were placed in an open field activity monitor and distance traveled was measured for 10 minutes. For male offspring, there was a trend for treatment ($F_{(1,24)}$ = 3.152, p = 0.09) but no effect of sire exposure or interaction on distance traveled (Fig. 11A). For female offspring, there was no significant effect for treatment, sire exposure, or interaction on distance traveled (Fig. 11B).

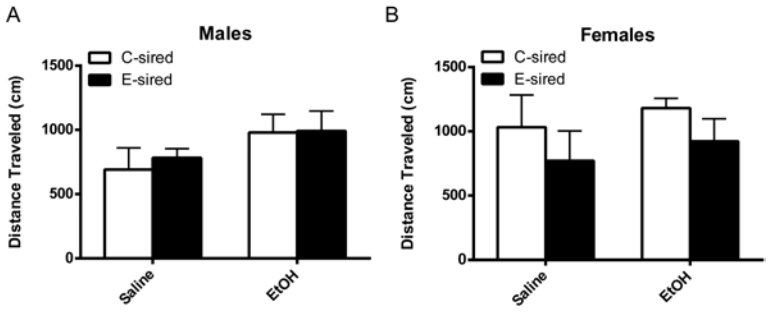

Figure 11. Paternal ethanol on open field performance

Offspring were tested for locomotor activity in an open field 20 minutes after i.p. injection of 1 g/kg ethanol or saline. There were no significant differences among (A) E- and C-sired male offspring or (B) female offspring after treatment with ethanol. n = 7-8/group.

2.3.10 Paternal ethanol effects on accelerating rotarod

Five minutes after completion of the open field test (i.e., 35 minutes after treatment with 1 g/kg ethanol or saline), the same mice were tested on five consecutive trials for their ability to remain on an accelerating rotarod. For E-sired male offspring, there was a significant effect of trial ($F_{(4,56)}$ = 5.93, p < 0.001), a trend of treatment ($F_{(1,14)}$ = 3.21, p = 0.09), and a significant interaction between trial and treatment ($F_{(4,56)}$ = 2.86, p < 0.05); post-hoc analysis revealed E-sired male offspring treated with ethanol performed significantly better on the 5th trial compared

to those treated with saline ($p < 0.01$) (Fig. 12A). For C-sired male offspring, there was a significant effect for trial ($F_{(4,56)} = 3.48$, $p < 0.05$) but not treatment or interaction of trial with treatment (Fig 12B).

For E-sired and C-sired female offspring, there were no significant effects of trial, treatment, or interaction between trial and treatment (Fig. 12C,D).

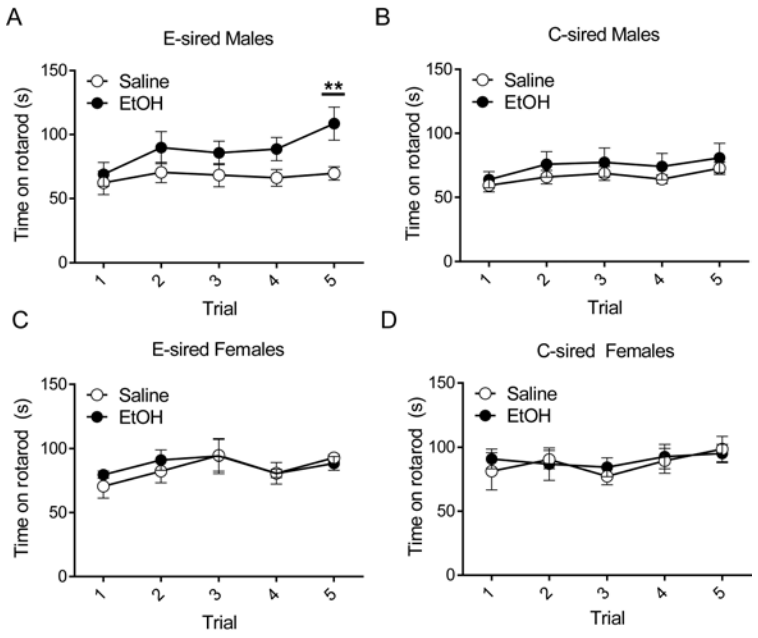

Figure 12. Paternal ethanol on accelerating rotarod performance

Offspring were tested for performance on an accelerating rotarod 35 minutes after i.p. injection of 1 g/kg ethanol or saline. (A) E-sired male offspring treated with ethanol performed significantly better on the 5th trial compared to those treated with saline. There are no significant differences associated with ethanol treatment in (B) C-sired male offspring, (C) E-sired female offspring, or (D) C-sired female offspring. n = 7-8/group, *$p < 0.05$.

2.3.11 Paternal ethanol effects on ethanol clearance rates

To ensure that differences observed on behavioral assays were not confounded by changes in ethanol pharmacokinetics, offspring were tested for the rate at which ethanol was cleared from

the venous circulation. There was no significant effect of sire exposure on ethanol clearance in males or females (Fig. 13).

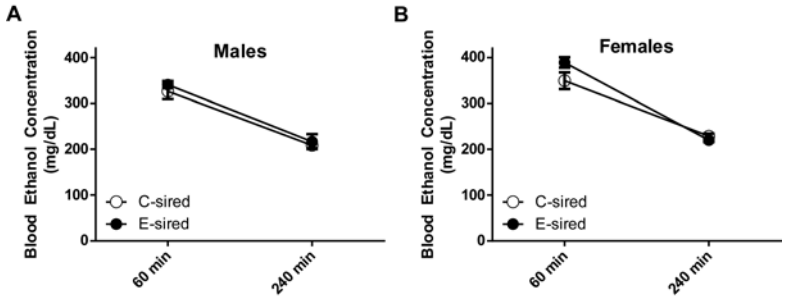

Figure 13. Paternal ethanol on ethanol clearance rates

Ethanol metabolism was measured after i.p. injection of 3.5 g/kg ethanol in saline. There were no significant differences in blood ethanol levels 60 minutes and 240 minutes after ethanol treatment between E- and C-sired (A) male or (B) female offspring. n = 4-5/group.

2.3.12 Increased *Bdnf* expression in VTA of E-sired offspring

To determine if paternal ethanol exposure leads to changes in gene expression in offspring, *Bdnf* and *Dlk1* expression was measure in the VTA and mPFC of offspring. *Bdnf* is a known regulator of ethanol drinking behavior [188-190] whose expression is up-regulated in the mPFC of male offspring of cocaine-exposed sires [98]. *Dlk1* is expressed from the paternal chromosome and regulated by methylation at the *IG* DMR, which was decreased in motile sperm following ethanol exposure in this study; *Dlk1* has important roles in neurogenesis [191] and adipogenesis [192]. The VTA was chosen because *Dlk1* is enriched in this region but has limited expression in other regions of the brain [193,194]; *Bdnf* expression is also enriched at the VTA relative to other brain regions [195]. *Bdnf* exon IXa expression was studied because it is invariably expressed with all *Bdnf* mRNA sequences while *Bdnf* exon IV is an activity-associated splice variant that was increased in male offspring of cocaine-exposed sires [98,196,197]. In the VTA, there was increased expression of *Bdnf* exon IXa but not *Bdnf* exon IV or *Dlk1* in E-sired male

offspring (Fig. 14A); there were no differences in gene expression between E and C-sired female offspring in the VTA (Fig 14B). Expression of *Bdnf* exons IV and IXa was also measured in the mPFC to study whether changes in expression generalized to another brain structure and to test potential similarities between E- and cocaine-sired male offspring. There were no significant differences in *Bdnf* expression between E- and C-sired offspring in the mPFC (Fig. 14C,D).

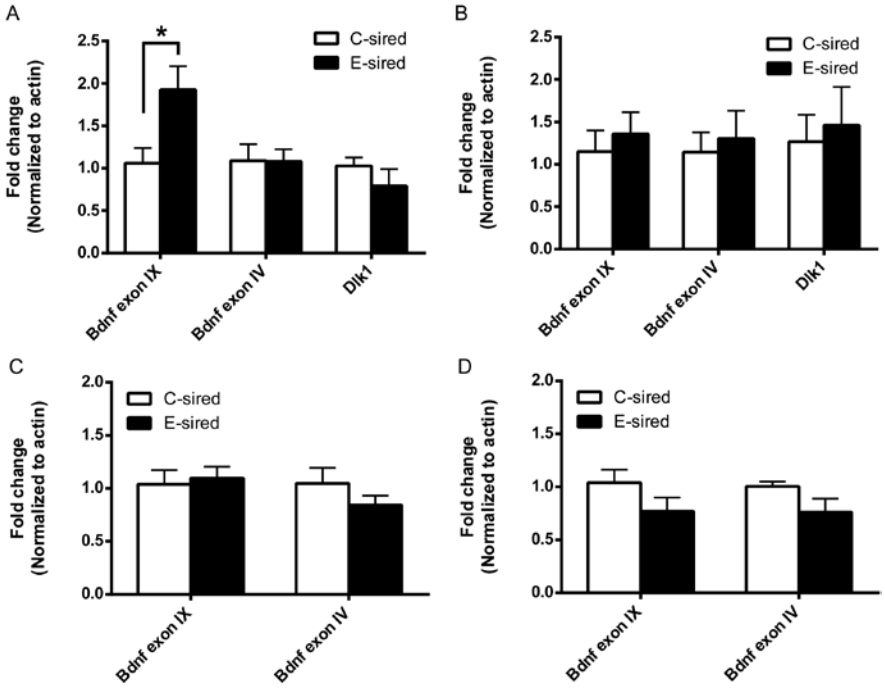

Figure 14. VTA and mPFC gene expression

Expression of *Bdnf* and *Dlk1* were measured in the VTA and mPFC of 129xC57 offspring. (A) E-sired male offspring had significantly increased expression of *Bdnf* exon IXa but not *Dlk1* or *Bdnf* exon IV in the VTA relative to C-sired males. (B) There were no significant differences in *Bdnf* or *Dlk1* expression between E- and C-sired females in the VTA. There were no significant differences between (C) male and (D) female E- and C-sired offspring in expression of *Bdnf* exons IV and IXa. n = 5-6/group.

2.3.13 Paternal ethanol regulates DNA methylation at the *Bdnf* exon IXa promoter

DNA methylation of the *Bdnf* exon IXa promoter regulates its expression [198,199]; therefore, it is possible that ethanol exposure alters DNA methylation at the *Bdnf* exon IXa CpG island in

sperm and that this epigenetic mark is inherited by male offspring and maintained in the VTA. Ethanol exposure significantly decreased DNA methylation of the *Bdnf* exon IXa promoter in motile sperm (Fig. 15A,D) as well as in the VTA of E-sired male offspring (Fig. 15B,E). Surprisingly, E-sired female offspring also had decreased DNA methylation at the *Bdnf* exon IXa promoter (Fig. 15C,F).

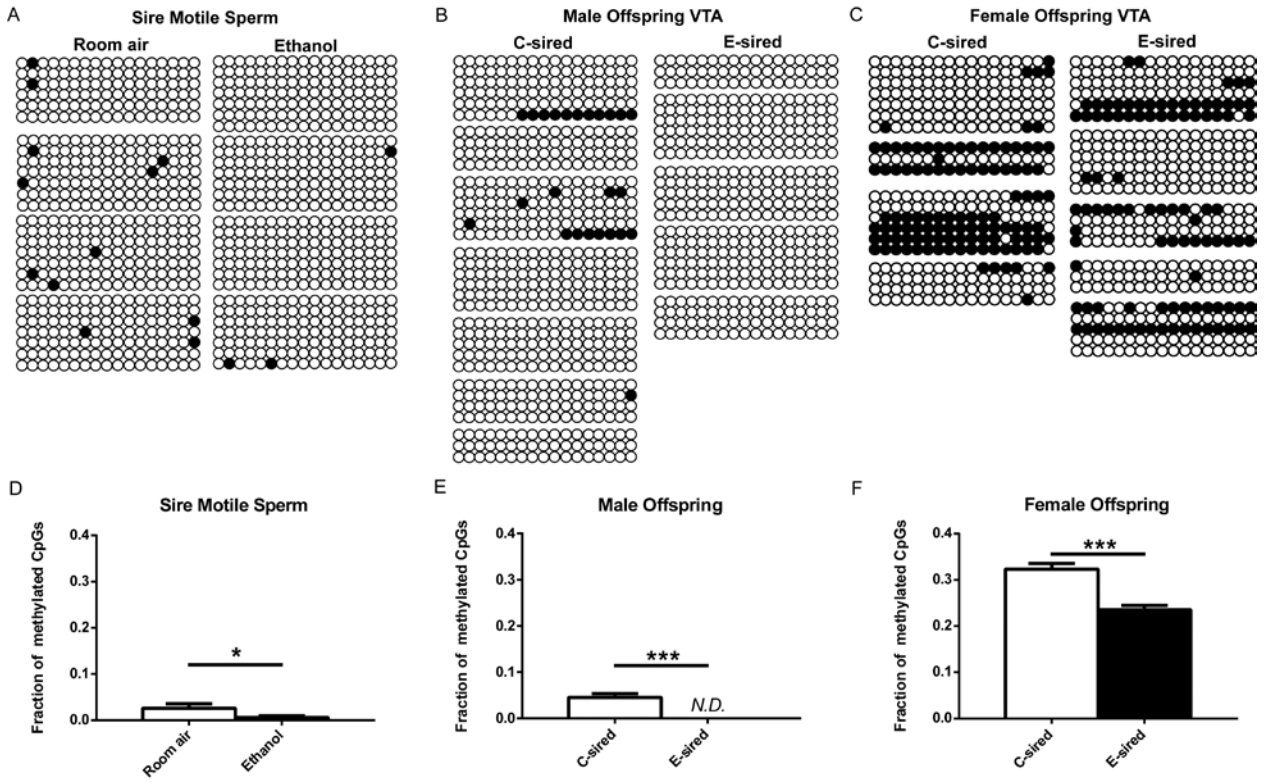

Figure 15. DNA methylation at the *Bdnf* exon IXa promoter

Based on increased expression of *Bdnf* exon IXa in the VTA of E-sired offspring, DNA methylation was measured in sires and offspring VTA. (A,D) Ethanol exposure was associated with decreased methylation at the *Bdnf* exon IXa promoter in motile sperm. Decreased methylation was maintained in the VTA of both (B,E) male and (C,F) female offspring. (A-C) Each circle represents one of the 17 potentially methylated cytosines in the *Bdnf* exon IXa promoter; filled circles are methylated and unfilled circles are unmethylated. Each block of rows represents sequenced colonies from a single independent animal. (D-F) Quantification of bisulfite sequencing. n = 4-7/group, *$p < 0.05$, ***$p < 0.001$

2.4 DISCUSSION

The results in this aim indicate that ethanol drinking behavior and sensitivity to the behavioral effects of ethanol may be epigenetically transmitted through the male lineage. Using isogenic mice, these studies demonstrate that exposure of sires to ethanol prior to mating increases sensitivity to the anxiolytic and motor effects of ethanol and reduces ethanol preference and consumption in male offspring on the two bottle drinking assay. E-sired male offspring also weighed significantly more than C-sired males after weaning, suggesting potential metabolic effects of paternal ethanol exposure. An increase in *Bdnf* expression in the VTA of male but not female offspring was also found, which was associated with ethanol-induced changes to DNA methylation of the *Bdnf* promoter in motile sperm as well as the VTA in offspring. While no behavioral or gene expression changes were observed in female offspring, they retain hypomethylation at the *Bdnf* promoter in the VTA, though methylation at this region was much more variable than compared to males. Notably, no differences were observed in several other behavioral assays, suggesting a specific effect of paternal ethanol on ethanol consumption when measured using a free choice continuous access drinking paradigm as well as on ethanol-induced anxiolysis.

One particularly striking feature of the study presented here is how closely the results parallel those observed following paternal cocaine [98] and maternal preconception morphine [97,200]. Vassoler et al. (2013) demonstrated that consumption of cocaine by sires imparted a cocaine resistant phenotype that was restricted to male offspring and females were phenotypically normal. The study also found changes in *Bdnf* expression in the brain in male offspring of cocaine-exposed sires. These similarities raise important questions about how drugs of abuse with distinct mechanisms of action produce a phenotype of drug resistance in male

offspring. One explanation is that both are acting on a common pathway important for encoding and maintaining epigenetic marks in germ cells that ultimately control gene expression in brains of offspring. The work of Vassoler et al. (2013) implicates cocaine-induced changes in posttranslational histone modifications that ultimately influence brain expression of *Bdnf* in male, but not female offspring, as a causative contributor to the observed phenotype. In this study, there was increased *Bdnf* exon IXa expression in only male offspring but DNA methylation changes at the *Bdnf* exon IXa promoter were found in both male and female offspring. This result suggests that while ethanol-induced changes to DNA methylation may be inherited by both sexes, these changes are not the primary driver of the observed changes in *Bdnf* exon IXa expression. Additional studies are needed to establish a causal role of increased *Bdnf* exon IXa expression in the VTA on ethanol-induced behavioral changes observed in male offspring.

While this study did not establish causality, *Bdnf* expression in the VTA regulates drug sensitivity, preference, and stress responses (for review, see [201]), indicating it could mediate several aspects of our paternal ethanol phenotype. In particular, increased *Bdnf* expression in the striatum, of which a major component is derived from the VTA [202], is associated with decreased ethanol consumption [188-190]. Increased *Bdnf* expression in the VTA also sensitizes rodents to the effects of cocaine [203] and amphetamine [204] and decreases morphine preference [205]. These studies support increased expression *Bdnf* in the VTA as contributing to decreased ethanol preference and increased sensitivity to drugs of abuse. While studies of VTA-derived *Bdnf* on drug-related behaviors support its role in this phenotype, studies of stress signaling are more difficult to reconcile. In particular, social defeat stress increases *Bdnf* expression in the VTA and knocking out *Bdnf* expression in the striatum is associated with stress

resilience [206,207]. Moreover, increasing *Bdnf* expression in the VTA blocks the antidepressant-like effects of electroconvulsive therapy [208]. These studies imply that increased VTA-derived *Bdnf* may increase basal stress or depression-like behavior, though this was not noted in our EPM or forced swim studies. However, the role of VTA-derived *Bdnf* in stress and depression-like behaviors is not clear cut, as one study noted that treatment with the antidepressant fluoxetine increased expression of *Bdnf* in the VTA [209]. Therefore, studies of VTA-derived *Bdnf* are inconclusive for a general effect on anxiety and stress-related behaviors, though effects on specific behaviors have been noted. These studies also highlight the difficulty in studying a nonspecific neurotrophic factor, like *Bdnf*, in behavior and attributing a causal effect of increased VTA-dervied *Bdnf* in our phenotype.

Sexually dimorphic effects in offspring have been noted by several studies of environmental perturbations in parents [87,89,95,96,98,176]. One explanation for sex-specific effects is the influence of the estrous cycle on behavior by daily variation in hormone levels. Previous studies have shown that the estrous cycle influences ethanol consumption in female rodents [210,211], so that endocrine variation may be masking the effects of paternal ethanol in female offspring. Of importance, *Bdnf* expression is altered by circulating estrogens [212-214], so that the estrous cycle may also be masking the effects of hypomethylation at the *Bdnf* promoter on its expression in the VTA. If the estrous cycle was masking the effects of paternal ethanol exposure, ovariectomizing females to attenuate the influence of circulating estrogens may reveal behavioral and molecular effects of paternal ethanol in female offspring. An alternative explanation for lack of an effect in females is that the critical ethanol-induced epigenetic modifications in gametes are being passed through the Y chromosome. While the Y chromosome contains only ~100 genes across mammals, several of these were found to have

critical roles in gene regulation aside from those involved in sex-determination [215]. Therefore, we expect future experiments to study expression and epigenetic regulation of these genes.

Decreased ethanol preference and consumption for E-sired male offspring is in apparent conflict with the familial nature of alcoholism observed in humans. Based on observations in human populations that sons of alcoholics have increased risk for alcoholism [216], the initial hypothesis of this study was that rodent E-sired offspring would consume more ethanol. While the discrepancy between the findings in this study and human studies could simply reflect species differences, experimental design issues may also play a role. Whereas humans voluntarily consume high quantities of ethanol, this study used forced ethanol exposure via vapor inhalation. It is also possible that in genetically heterogenous human populations, genetic influences on drinking behavior mask the epigenetic effects of paternal ethanol exposure. Lastly, the effects in this study were observed in a free choice drinking assay and there was no difference in ethanol drinking on the scheduled, limited access drinking in the dark assay. Considering the range of factors regulating ethanol consumption on these assays, including memory, learning, and stress associated with social isolation, it is possible that paternal ethanol induces behavioral changes that are specific for particular ethanol drinking paradigms. Other behavioral tests of rodent drinking behavior, such as stress-escalated ethanol consumption or reward based drinking are necessary to identify specific mechanisms of paternal ethanol of ethanol drinking.

While the ethanol drinking data are in conflict with human studies, paternal ethanol exposure also increased sensitivity to the anxiolytic and motor enhancing effects of ethanol in male offspring. In humans, increased sensitivity to the subjective effects of ethanol is associated with decreased risk of developing alcoholism [216]. Notably, in this study, increased sensitivity

to ethanol in E-sired male offspring was associated with decreased ethanol consumption. Heritability of ethanol sensitivity on motor tests has also been noted in humans. Of note, static ataxia (body sway) after ethanol consumption shows heritability and is associated with a sexually dimorphic pattern of inheritance [217]. Compared to C-sired male offspring, E-sired males were more sensitive to low dose ethanol enhancement of motor coordination on the accelerating rotarod assay and to locomotor stimulation (and anxiolysis) on the elevated plus maze but not on the open field assay. The discrepancy between the elevated plus maze and open field locomotor results was likely due to the ethanol dose and timing, since several studies have reported 1 g/kg ethanol did not induce changes in locomotion on the open field assay [218-220]. It is also notable that E-sired male offspring demonstrated subtle changes on the accelerating rotarod assay, with only the 5^{th} trial reaching statistical significance compared to C-sired male offspring. This finding is more suggestive of an effect on motor learning than locomotor enhancement. These issues raise the possibility that paternal ethanol exposure affects discrete pathways to alter sensitivity to ethanol. There was also no effect of paternal ethanol exposure on ethanol metabolism in offspring, which is consistent with human studies of children of alcoholics [221]. These findings suggest that behavioral differences in E-sired offspring are being driven by neurobiological changes that alter sensitivity to ethanol to decrease drinking.

Ethanol exposure induced hypomethylation at two loci studied in motile sperm and hypomethylation at one of these, the *Bdnf* exon IXa promoter, was maintained in the VTA of E-sired offspring. This finding is consistent with studies that show both maternal and paternal preconception ethanol exposure alter DNA methylation at imprinted loci in offspring [86,156]. While ethanol's effects on DNA methylation in offspring are striking, ethanol is known to alter several epigenetic marks across tissue types and could act as a broader epimutagen in sperm. For

example, ethanol alters histone modifications in the amygdala, which contribute to its acute anxiolytic effects and withdrawal-induced anxiety [60,137]. miRNA regulation by ethanol has been shown to underlie changes in expression of BK channel splice variants [222]. Therefore, it is conceivable that ethanol induces multiple heritable epigenetic modifications in germ cells and these marks are maintained in the brain of offspring sired by alcohol exposed fathers.

This idea is especially intriguing considering epigenetic reprogramming during spermatogenesis is highly plastic. These changes include chromatin compaction by replacement of most histones with protamines, de novo DNA methylation and maintenance, and silencing of retrotransposable elements through numerous small regulatory RNAs [223]. As discussed previously, ethanol acts as an epimutagen in other tissues and may be affecting multiple epigenetic processes during spermatogenesis. A rodent study demonstrated chronic ethanol exposure decreases cytosine methyltransferase levels in the testes [155], which is consistent with decreased DNA methylation at the IG DMR and *Bdnf* exon IXa promoter in this study. A recent human study also demonstrated that ethanol consumption was correlated with decreased methylation of imprinted genes that are normally hypermethylated in human sperm [152]. Ethanol's effects on DNA methylation in gametes are further supported by the observation that chronic ethanol alters methionine metabolism [224], which is critically involved in the function of cytosine methyltransferases [225]. Additional studies should expand on DNA methylation as well as begin to study ethanol-induced changes to retained histones and noncoding RNAs.

In conclusion, upon paternal ethanol exposure, ethanol likely functions as an epimutagen imparting long lasting effects on germ cells that ultimately impact the next generation. Prior rodent studies demonstrated an impact of paternal ethanol exposure on brain development and numerous basal behaviors. The results presented here demonstrate an effect on behavioral

sensitivity to ethanol, ethanol drinking behavior, and gene expression that is restricted to male offspring. If these rodent studies apply to humans drinking alcohol, the results have far reaching implications considering the large percentage of the human population that consume alcohol prior to procreation.

3.0 EPIGENETIC MECHANISMS OF ETHANOL IN THE CORTEX

Adapted from: Finegersh A, Homanics GE (2014) Acute Ethanol Alters Multiple Histone Modifications at Model Gene Promoters in Cerebral Cortex. *Alcohol Clin Exp Res* 38: 1865-73. [226]

3.1 INTRODUCTION

AUD is associated with widespread changes in gene expression across multiple brain structures [118,119,122,123]. These changes underlie neuronal and glial adaptations to the environmental stress of repeated ethanol exposure and may contribute to the reinforcing effects of ethanol that incentivize further consumption [125]. In rodents, a single, binge-like exposure to ethanol is also associated with up- and down-regulation of genes in the cortex, nucleus accumbens, and ventral tegmental area [68,69,128]. Despite the importance of differential gene expression for ethanol action, mechanisms of gene regulation by ethanol are poorly understood.

Gene expression is regulated by epigenetic mechanisms, which include covalent modifications to histones and DNA [227]. These modifications alter affinity of histones for DNA to inhibit or promote transcription factor binding. In particular, modifications to histone N-terminal tails, including acetylation and methylation of lysine residues, are catalyzed by a large group of histone modifying enzymes and represent a rapid, reversible method of chromatin

alteration [32]. These enzymes can be induced by drugs of abuse, like ethanol, to establish chromatin alterations that promote drug-seeking behavior and addiction [28].

Recent evidence highlights the role of ethanol in inducing epigenetic disruptions and modulating histone modifying enzyme expression in the brain. Studies of patients with AUD have identified altered distribution of histone trimethylation in the hippocampus and cortex, which correspond to gene expression changes in these regions [122,123]. In rodents, acute ethanol is associated with increased histone acetylation in the central nucleus of the amygdala [60], altered histone modifications at the prodynorphin and pronociceptin promoters in the amygdala [135], and altered expression of histone modifying enzymes in the striatum and prefrontal cortex [141]; moreover, pre-treatment with the HDAC inhibitor sodium butyrate blocks ethanol-induced behavioral sensitization, indicating histone deacetylation plays a critical role in the neuroadaptive response to ethanol administration [142]. A recent study pointed to HDAC2 expression in the amygdala as a critical regulator of ethanol preference and anxiolytic response to acute ethanol, identifying a specific histone modifying enzyme in mediating ethanol action [137].

While epigenetic effectors of ethanol are now seen as potential targets for the treatment of AUD, several important questions remain to be resolved. Of note, studies of acute ethanol have focused on epigenetic modifications at up-regulated genes; however, gene expression studies indicate both up- and down-regulation of genes in response to acute ethanol [68,69,128]. Moreover, while acute ethanol-induced histone modifications have been characterized in the amygdala using chromatin immunoprecipitation (ChIP) [135,137], no studies have examined broader effects of acute ethanol in the cerebral cortex (CCx). The CCx is a critical site of ethanol's effect on $GABA_A$ receptor potentiation [228,229], motor control [230], and neuronal

toxicity [231,232]. Due to its sensitivity to ethanol and diversity of cell types, the CCx may be especially important for identifying general epigenetic effects of ethanol in the brain that will be useful for screening potential drug targets. Therefore, this study examines the effects of acute ethanol on multiple histone modifications at promoters of both up- and down-regulated genes in the CCx.

In this section, a single binge-like dose of ethanol (3 g/kg i.p.) known to produce sustained alterations in gene expression in mouse CCx [233] was used to study how acute ethanol alters histone acetylation and methylation at the promoters of three model up- and down-regulated genes, global levels of those histone modifications, and the expression of histone modifying enzymes in CCx.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Treatments

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All experiments were performed using 8-week-old, ethanol-naïve, specific pathogen free male C57BL/6J mice from the Jackson Laboratory (20 – 25 g). Mice were habituated to the University of Pittsburgh animal facility for 1 week prior to initiation of experiments. Mice were housed under 12 hour light/dark cycles and had *ad libitum* access to food and water.

All treatments were administered during the light cycle between 08:00 and 10:00. Mice were given i.p. injections containing 0.02 ml/g of either 15% ethanol solution in saline (3 g/kg ethanol) or saline alone. After injections, mice were individually housed for 6 hours with *ad libitum* access to food and water.

At 6 hours post-injection, mice were rapidly sacrificed by carbon dioxide asphyxiation and decapitated. The brain was immediately removed and placed on a petri dish on ice. The cerebellum was removed and cerebral hemispheres separated at midline. The olfactory bulbs were removed and the telencephalon (CCx) was carefully dissected from the diencephalon and midbrain. The hippocampus (HC) was dissected and removed from the CCx. The remaining left and right CCx and HC were flash frozen separately in liquid nitrogen. All experiments were performed using either the left or right CCx or HC.

3.2.2 Reverse Transcription Quantitative PCR (RT-aPCR)

Total RNA was isolated using TRIzol according to the manufacturer's protocol (Invitrogen), purified with DNase digestion (Qiagen), and 1 μg of RNA was synthesized into cDNA using reverse transcriptase (RT) (Bio-Rad). A no-RT reaction was used as a negative control. Reactions were carried out in duplicate for each gene. SYBR green fluorescent master mix (Bio-Rad) was added to each well and visualized using a Bio-Rad iCycler. All primers were optimized for 90% to 110% efficiency at the following conditions: 10 min at 95˚C (initial denaturation) followed by 40 cycles of 30 s at 95˚C (denaturation), 1 min at 60˚C (annealing), and 30 s at 72˚C (extension). Primer sequences for *β-actin, Gad1*, *Mt1, Mt2, Egr1, Hdac2, Hdac11, Csrp2bp,* and *Kat2b* are shown in Table 4. Threshold cycle (Ct) values were calculated for each well and duplicate values averaged. The difference between specific genes and *β-actin*

(ΔCt) was calculated for each animal and normalized to the average of saline-treated animals (ΔΔCt). Fold change over saline controls was calculated for each animal using the following formula: $2^{-\Delta\Delta Ct}$.

3.2.3 Chromatin Immunoprecipitation (ChIP)

Chromatin was isolated from the CCx using a standard protocol and reagents (Millipore EZ-Magna ChIP). Briefly, the CCx was minced on a petri dish over ice using a razor blade. DNA was cross-linked to histones by incubating minced tissue in 1 ml of 1% formaldehyde in phosphate buffered saline (PBS) at 37˚ C for 10 minutes. The formaldehyde reaction was quenched using glycine and the tissue was washed 3 times in PBS with protease inhibitor cocktail (Roche #04693116001). Cell lysis buffer (Millipore) with protease inhibitor was added and nuclei pelleted. The nuclear pellet was incubated on ice in 500 µL nuclear lysis buffer (Millipore) with protease inhibitor to generate chromatin. Chromatin was sheered in an ice water bath using 4 bursts of 15 s at 35% output and 80% duty cycle on a Branson Sonifier S-250A. An aliquot of sheered, cross-linked DNA was removed and run on a 1.5% agarose gel to ensure the majority of DNA was between 200 and 600 bp. Chromatin was aliquotted and stored in dilution buffer (Millipore) with protease inhibitor at -80˚C until immunoprecipitation experiments.

For immunoprecipitation, chromatin in dilution buffer was thawed on ice and 2% of the volume was removed and saved as the input. The remaining chromatin was incubated at 4˚C overnight with antibody and Protein A/G magnetic beads (Millipore) with end-over-end rotation. The following antibodies were used for immunoprecipitation reactions: histone subunit H3 acetylated at lysines 9 and 14 (H3K9,14ac) (Millipore, #06-559b), histone subunit H3 tri-methylated at lysine 4 (H3K4me3) (Epigentek, #A-4033), and histone subunit H3 tri-methylated

at lysine 27 (H3K27me3) (Millipore, #17-622). IgG (Millipore, #PP64B) was used as a negative control. Antibodies were screened by assessing enrichment of the constitutively active gene, *β-actin*, over the neuronally repressed gene, *ε-globin*; there was no difference in enrichment between ethanol and saline treated animals (Fig. S1) [234]. Antibodies were also validated by assessing binding to peptide arrays containing 46 histone modifications to the H3 N-terminal tail (Millipore #16-667); H3K27me3 and H3K4me3 antibodies bound to their stated histone modifications while the H3K9,14ac antibody bound H3K9ac but not H3K14ac (data not shown). After incubation, magnetic beads containing antibody-chromatin complexes were immobilized on a magnetic rack and washed once with low salt, high salt, and LiCl immune complex wash buffers and TE. Elution buffer (Millipore) with proteinase K was added and the complexes were incubated at 65˚C for 2 hours to elute enriched DNA. Immunoprecipitated and input DNA was purified using a ChIP DNA kit (Zymo Research) and eluted in 100 μL elution buffer.

For qPCR, 5 μL of immunoprecipitated or input DNA was used in each well and carried out in duplicate or triplicate for each primer pair. qPCR conditions were the same as reported in the RT-qPCR section and all ChIP-qPCR primers were optimized to perform at 90% to 110% efficiency. Primer sequences for promoter regions of *β-actin*, *ε-globin*, *Gad1*, *Mt1*, *Mt2*, *Egr-1*, *Hdac2*, and *Hdac11* are listed in Table 4. Ct values were normalized to input DNA and a negative control region not enriched for the histone modification [235-237]. For H3K9,14ac and H3K4me3, *ε-globin* served as the negative control region; for H3K27me3, *β-actin* served as the negative control region. Data are presented as fold enrichment over saline controls.

RT-qPCR			
Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)
β-actin	TCATGAAGTGTGACGTTGACATCCGT	CCTAGAAGCATTTGCGGTGCACGATG	384
Csr2bp	ACATCCCAACCATCAACTCC	TCACATCAGGAACCATGAAGC	124
Egr-1	TGAGCACCTGACCACAGAGTCCTT	ATGGGAGGCAACCGAGTCGT	102
Gad1	TACTACCAACCTGCGCCCTA	GGTGGAGCGATCAAATGTCT	348
Hdac2	GACATATGAGACTGCAGTTGC	ACCTCCTTCACCTTCATCCTC	354
Hdac11	AATGGGGCAAGGTGATCAAC	AGCCACCACCAACATTGATG	299
Kat2b	TACCTCTTCACCTGCGTCCACAAA	TCACACCCTGTTCAATACTGGGCT	168
Mt1	CGACTTCAACGTCCTGAGTACC	ATAGGAAGACGCTGGGTTGGT	291
Mt2	GGGGTCCCCACATCTGTGTAAAT	GTCAACGGCTTTTATTGTCAGT	117
ChIP-qPCR			
Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp) / Relative to TSS* (bp)
β-actin	CGGAGGCTATTCCTGTACATC	GCGAGAGAGAAAGCGAGATT	91 / -290
Egr-1	GCCATGTACGTCACGGCGGA	GGATCTCTCGCGACTCCCCGA	82 / -74
Gad1	AAAACAGAGCGTGCTGAGTG	TGCCTCTGGAGCTTTGTAGG	118 / -264
ε-globin	CTGACCCTCCCATGACCT	TCTGACCCTTTGTTCTGCAT	90 / -134
Hdac2	TAAGACCGAGGGGTGAACCT	CCAGGGCGACAGTAGTGTTT	120 / -239
Hdac11	ACTCCATTGGGTGCTTCTCC	CTACGCAGAACACACGCCTC	112 / -155
Mt1	GGACATGATGTTCCACACGTCACA	CCCTGGAGCGCCAGTGTGC	90 / -94
Mt2	TGTGCTGGCCATATCCCTTGAG	TGGGTCGAGCGCAAAAGC	100 / +23

Table 4. Primer sequences for qPCR experiments

*TSS, Transcriptional Start Site

3.2.4 RT-qPCR array

A RT-qPCR array containing primers for 84 chromatin modifying enzymes was used to screen ethanol-induced gene expression changes according to the manufacturer's protocol (SA Biosciences, #PAMM-085; full gene list shown in Supplementary Table 2). RNA was extracted and purified according to the *RT-qPCR* section, converted to cDNA, and 8.5 ng of cDNA used per well according to the manufacturer's protocol. A total of 6 PCR arrays were used (3 saline-treated and 3 ethanol-treated animals). qPCR conditions were 10 min at 95˚C (initial denaturation) followed by 40 cycles of 15 s at 95˚C (denaturation) and 1 min at 60˚C (annealing and extension). Ct values from each PCR array were normalized to the median Ct value of that array (median normalization). Median normalized Ct values were further normalized to the

average of saline controls (ΔΔCt) and fold change values calculated using the following formula: $2^{-\Delta\Delta Ct}$.

Genes whose expression was changed >100% after ethanol treatment or had a p-value <0.1 and change in expression >25% were chose for validation by RT-qPCR using an additional 6 mice per group.

3.2.5 Western Blot

Histone lysates were extracted using the Qiagen Qproteome kit according to the manufacturer's protocol and quantified using a Bradford assay. Twenty ug of histone lysate was loaded onto 4%-20% Novex Tris-glycine gel (Invitrogen) and transferred to a nitrocellulose membrane. Membranes were blocked in Odyssey buffer (LiCor Biosciences) and incubated overnight with primary antibodies. The following antibodies were used for Western blots according to the manufacturer's protocol: histone subunit H3 (Santa Cruz Biotechnologies, #sc-8654), H3K9ac (Cell Signal Technologies, #9671s), H3K14ac (Millipore, #07-353), H3K4me3 (Epigentek, #A-4033), and H3K27me3 (Millipore, #17-622). After overnight incubation, membranes were incubated with secondary fluorescent antibodies according to the manufacturer's protocol (LiCor Biosciences) and visualized using the Odyssey Infrared Imaging System (Licor Biosciences). The total intensity of each band was divided by the total intensity of histone subunit H3 and presented as a percent change relative to the average of saline controls. For CCx, A total of 6 animals per group were assessed on 2 separate membranes. For HC, a total of 3 animals per grouped were assessed on 2 separate membranes. Membranes were stripped four times using stripping buffer (LiCor Biosciences) between incubations with primary antibody.

3.2.6 Statistical Analysis

For RT-qPCR, ChIP-qPCR, and Western blot quantification, a two-tailed, unpaired t-test was used to compare the ethanol and saline treated groups. Statistical significance was defined by a p-value < 0.05. All data are presented as mean +/- SEM.

3.3 RESULTS

3.3.1 Ethanol-induced histone modifications at down-regulated genes

Genes down-regulated by acute ethanol exposure were identified using previously published microarray data [68,69,128] and the RT-qPCR array used in this study. Six hours after injection of 3 g/kg ethanol or saline there was significantly decreased expression of glutamic acid decarboxylase 1 (*Gad1*) ($p < 0.05$), *Hdac*2 ($p < 0.05$), and *Hdac11* ($p < 0.05$) in the CCx of ethanol-treated compared to saline-treated mice (Fig. 16A).

ChIP assays revealed changes to histone modifications at the *Gad1* and *Hdac2* promoters six hours after injection of 3 g/kg ethanol (Fig. 16B-D). There was a significant decrease in the association of the *Gad1* and *Hdac2* promoters with H3K9,14ac in the CCx of ethanol-treated compared to saline-treated mice ($p < 0.05$). There was also a paradoxical decrease in the association of the *Gad1* promoter with H3K27me3 in the CCx of ethanol-treated compared to saline-treated mice ($p < 0.05$). We did not identify ethanol-induced changes in the association of either promoter with H3K4me3 or the association of the *Hdac11* promoter with the studied histone modifications.

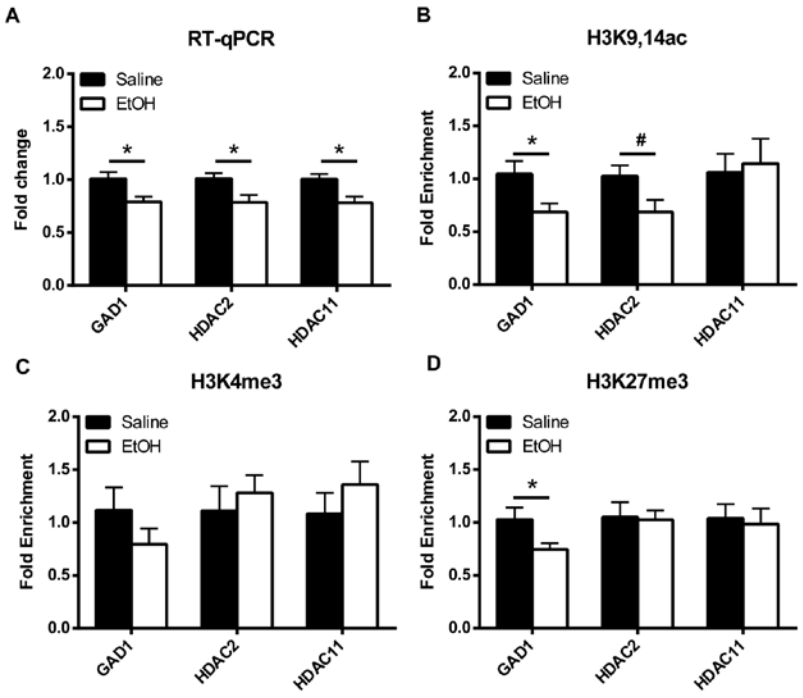

Figure 16. Ethanol-induced histone modifications at down-regulated genes

Ethanol-induced histone modifications were studied at the model down-regulated genes, *Gad1*, *Hdac2*, and *Hdac11*. (A) Acute ethanol significantly decreased expression of *Gad1*, *Hdac2*, and *Hdac11* ChIP-qPCR revealed that (B) the *Gad1* and *Hdac2* promoters had a significantly decreased association with H3K9,14ac, (C) no change in association of either gene with H3K4me3, and (D) that the *Gad1* promoter has a significantly decreased association with H3K27me3. n = 6/group, *$p < 0.05$.

3.3.2 Ethanol-induced histone modifications at up-regulated genes

Metallothioneins are components of the cellular response to oxidative stress whose expression is robustly increased by ethanol exposure [69,128]. Early growth response 1 (*Egr1*) is an immediate early gene whose expression also increases following ethanol exposure [238]. Six hours after injection of 3 g/kg ethanol or saline there was significantly increased expression of metallothioneins 1 (*Mt1*) ($p < 0.05$), 2 (*Mt2*) ($p < 0.01$), and *Egr1* ($p < 0.05$) in the CCx of ethanol-treated compared to saline-treated mice (Fig. 17A).

ChIP assays revealed changes to histone modifications at the *Mt1* and *Mt2* but not *Egr1* promoters six hours after injection of 3 g/kg ethanol (Fig. 17B-D). There was a significant decrease in the association of the *Mt1* promoter with H3K27me3 but not H3K9,14ac or H3K4me3 in the CCx of ethanol-treated compared to saline-treated mice ($p < 0.05$). There was a significant increase in the association of the *Mt2* promoter with H3K4me3 but not H3K9,14ac or H3K27me3 in the CCx of ethanol-treated compared to saline-treated mice ($p < 0.05$). Surprisingly, there were no ethanol-induced changes in the association of model up-regulated gene promoters with H3K9,14ac.

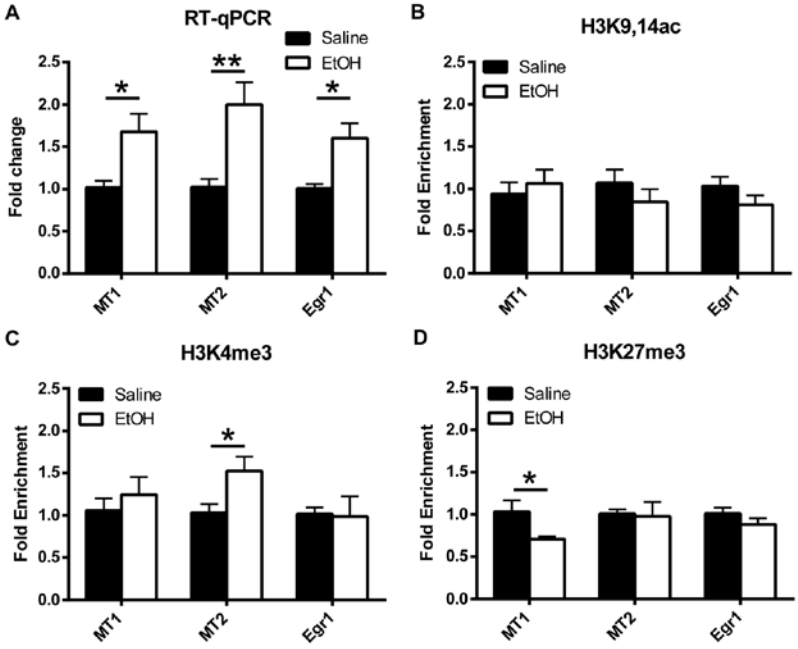

Figure 17. Ethanol-induced histone modifications at up-regulated genes

Ethanol-induced histone modifications were studied at the model up-regulated genes, *MT1*, *MT2*, and *Egr-1* (A) Ethanol exposure significantly increased expression of *Mt1*, *Mt2*, and *Egr1*. ChIP-qPCR studies revealed (B) no changes in association of up-regulated genes with H3K9,14ac, (C) increased association of the *Mt2* promoter with H3K4me3, and (D) decreased association of the *Mt1* promoter with H3K27me3. n = 6/group, *$p < 0.05$, **$p < 0.01$.

3.3.3 Acute ethanol alters histone modifications in CCx

To study whether acute ethanol alters global levels of the histone modifications assessed using ChIP, Western blot was performed on histone lysates generated from the CCx. There was a significant increase in global levels of H3K4me3 but no significant change in H3K9ac, H3K14ac, or H3K27me3 levels in the CCx six hours after injection of 3 g/kg ethanol compared to saline (Fig. 18).

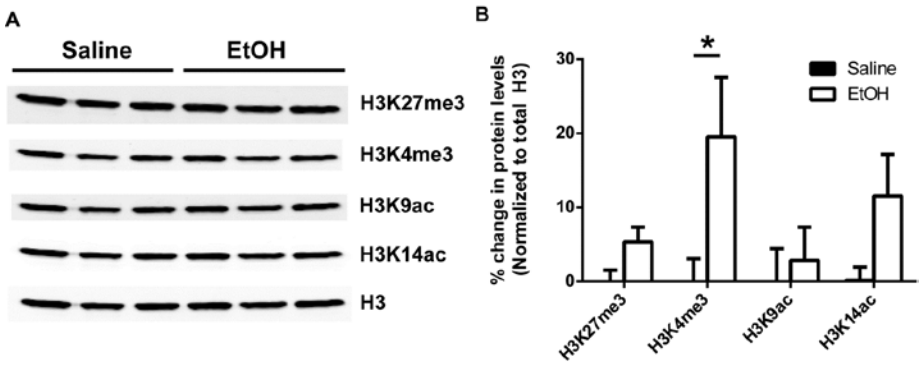

Figure 18. Histone modifications in CCx after acute ethanol

Western blot was used to assess global levels of histone modifications; data were quantified from 6 independent samples run across 2 separate gels. (A) Representative Western blot of ethanol and saline-treated animal histone lysates. (B) Quantification of protein levels normalized to total H3 presented as percent change over saline controls. n = 6/group, *$p < 0.05$.

3.3.4 Acute ethanol alters histone modifying enzyme expression

To identify mechanisms of ethanol-induced changes to histone modifications, we used a RT-qPCR array containing primers for all known HDACs as well as histone acetyltransferases (HAT), methyltransferases, and other enzymes involved in covalent modification of chromatin (full results in Appendix B). The RT-qPCR array demonstrated low variability between ethanol and saline-treated animals with few changes in the expression of chromatin modifying enzymes

associated with ethanol treatment in the CCx (Fig. 19A). This low power, screening assay revealed that *Csrp2bp*, *Hdac2*, *Hdac11*, and *Kat2b* met criteria for validation with an additional 6 animals per group.

PCR validation revealed six hours after injection of 3 g/kg ethanol or saline there was significantly decreased expression of *Hdac2*, *Hdac11*, and *Csrp2bp* and a near-significant trend for increased expression of *Kat2b* in the CCx of ethanol-treated compared to saline-treated mice (Fig. 19B).

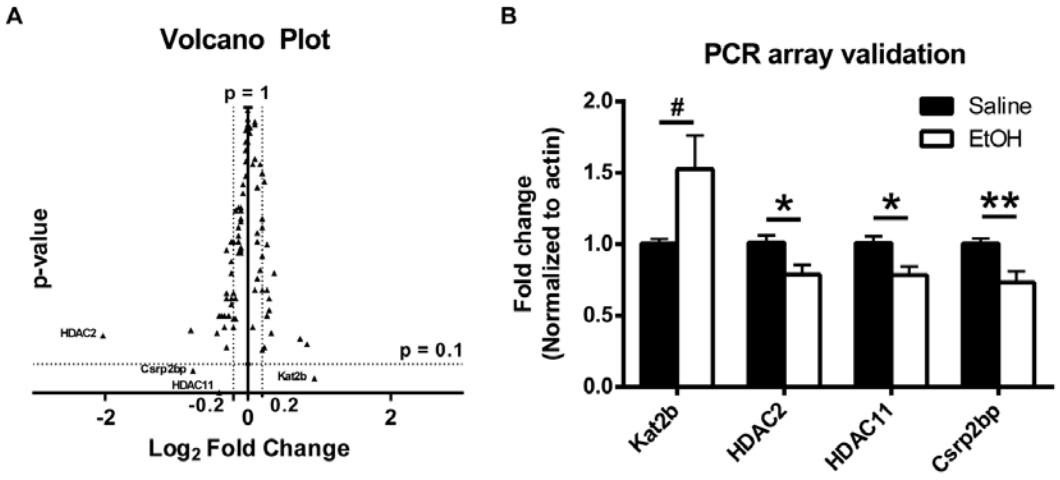

Figure 19. Acute ethanol alters chromatin modifying enzyme expression in CCx

(A) Volcano plot showing the p-value (y-axis) and fold regulation [$\log_2$(fold change)] (x-axis) of all 84 chromatin modifying enzymes with positions of *Csrp2bp*, *Hdac2*, *Hdac11*, and *Kat2b* indicated; vertical dashed lines indicate 25% change in expression and the horizontal dashed line indicates a p-value of 0.1 for ethanol-treated animals compared to saline controls. (B) PCR validation of *Csrp2bp*, *Hdac2*, *Hdac11*, and *Kat2b*. n = 9/group, *p < 0.05, **p < 0.01, #p = 0.056. Note: *Hdac2* and *Hdac11* expression data is identical to that presented in Figure 16.

3.3.5 Ethanol-induced epigenetic modifications in the hippocampus

To study whether epigenetic modifications generalized to another brain structure, global levels of histone modifications and expression of chromatin modifying enzymes that were altered in CCx

were measured in HC from the same mice. Western blot results revealed a significant increase in global levels of H3K9ac and H3K14ac as well as a nonsignificant trend for an increase in H3K4me3 with no change in levels of H3K27me3 six hours after injection of 3 g/kg ethanol compared to saline (Fig. 20A,B). There were no significant differences in expression of *Hdac2*, *Hdac11, Csrp2bp, or Kat2b* in ethanol compared to saline-treated mice in the HC (Fig. 20C).

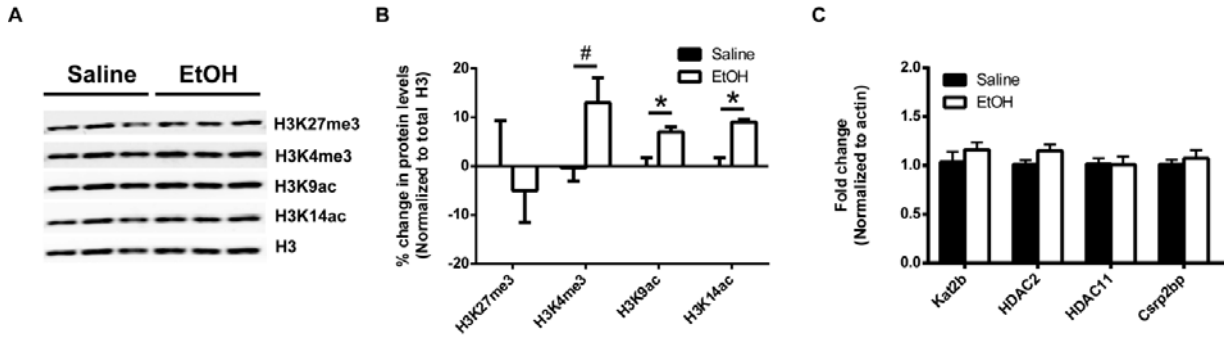

Figure 20. Ethanol-induced histone modifications in the hippocampus

Western blot was used to assess global levels of histone modifications; data were quantified from 3 independent samples per ethanol and saline groups run across 2 separate gels. RT-qPCR was used to study expression of chromatin modifying enzymes (n = 9/group) (A) Representative Western blot of ethanol and saline-treated animal histone lysates. (B) Quantification of protein levels normalized to total H3 presented as percent change over saline controls. (C) *Csrp2bp, Hdac2, Hdac11,* and *Kat2b* expression was not altered in the HC in ethanol-treated compared to saline-treated mice. *$p < 0.05$, #$p = 0.0873$.

3.4 DISCUSSION

These data demonstrate novel epigenetic mechanisms of ethanol-regulated gene promoters, which indicate a diverse pattern of histone modifications induced by acute ethanol. Of note, this is the first study to identify histone deacetylation at gene promoters in the brain following acute ethanol exposure. Altered expression of four histone modifying enzymes, *Hdac2*, *Hdac11*, *Kat2b*, and *Csrp2bp*, was also discovered as well as an increase in H3K4me3 throughout the

CCx and at the *Mt2* gene promoter. Finally, this study revealed increased histone acetylation in the HC with no change in expression of chromatin modifying enzymes that were altered in CCx, suggesting differences in ethanol-induced epigenetic changes between these structures.

Histone deacetylation at our model down-regulated gene promoters indicates that acute ethanol leads to recruitment of HDACs to a subset of gene promoters. This finding is surprising considering several studies show acute ethanol exposure is associated with decreased HDAC activity and expression [60,138,139,141]. However, there is also compelling evidence for histone deacetylation after acute ethanol exposure. In particular, down-regulation of genes by acute ethanol in the brain has been reported by several micro-array studies [68,69,128] and implies a role for HDACs in mediating the acute effects of ethanol. A recent study also revealed decreased acetylation of histone subunit H4 in the nucleus accumbens 4 hours after the onset of binge drinking [239]. These studies highlight the diversity of epigenetic mechanisms induced by acute ethanol and importance of characterizing ethanol-induced histone modifications at specific gene promoters. This is especially important since several studies have proposed a role for HDAC inhibitors for modulating ethanol consumption [60,137,139,239]. Future studies should expand on the current findings by studying both temporal and gene-specific effects of ethanol on histone acetylation.

Ethanol-induced epigenetic changes at two model up-regulated gene promoters were identified by this study. Notably, H3K4me3 was increased at the *Mt2* promoter and H3K27me3 was decreased at the *Mt1* promoter. Ethanol-induced changes to these histone modifications have been reported previously [135]. However, surprisingly, there was no increase in H3K9,14ac at any of the model up-regulated gene promoters in this study. This finding was surprising, since several studies have found that acute ethanol increases histone acetylation in the brain

[60,137,139]. One explanation for no change in histone acetylation at these gene promoters is that they have returned to a baseline state. Importantly, *Mt1*, *Mt2*, and *Egr1* expression has been shown to be elevated four hours following ethanol exposure [128], so that it is possible that the promoters are no longer active at the 6 hour time point studied in this paper. This finding highlights the dynamic nature of epigenetic gene regulation induced by ethanol and future studies should identify gene promoter states at multiple time points.

Increased levels of H3K4me3 have been reported in the cortex of patients with alcoholism [122] and the results in this study indicate that this occurs after a single ethanol exposure. Importantly, H3K4me3 exclusively marks active and poised promoters near transcriptional start sites [34,240], so that a global increase in this histone modification likely reflects ethanol-induced chromatin remodeling to promote gene expression. While the RT-qPCR array did not detect a change in expression of lysine methyltransferases or demethylases, these enzymes are regulated by post-translational modifications that could account for increased histone tri-methylation after ethanol exposure [34]. Further studies are needed to identify how ethanol induces tri-methylation of lysine 4 on histone subunit H3 without altering expression of histone methyltransferases.

The analysis of chromatin modifying enzyme expression following acute ethanol exposure identified two HDACs and two HATs whose expression is altered in the CCx. Notably, decreased expression of *Hdac2* and *Hdac11* after acute ethanol exposure has been reported in the striatum [141], indicating down-regulation of these genes is a fundamental neurobiological mechanism of ethanol. *Hdac2* expression has been implicated in conferring preference for ethanol [137] and down-regulation promotes memory formation [241], so that decreased expression may be important for promoting further ethanol consumption. Differential expression

of *Hdac11* was found to regulate ethanol drinking behavior [242], though specific mechanisms of *Hdac11* in the brain have not been identified. Based on these findings and similar changes in expression in the striatum [141], studying ethanol-induced regulation of *Hdac2* and *Hdac11* will likely help elucidate mechanisms of ethanol-induced gene expression.

Changes in expression of two HATs in the CCx after ethanol exposure likely reflect competition between HAT complexes. *Csrp2bp* is a component of the ATAC complex, which acts as a HAT that regulates cell cycle progression [243]. *Kat2b* is a component of a HAT complex with CREB binding protein, which is critical for memory formation [244]. Interestingly, the ATAC complex was found to be mutually exclusive with a HAT complex containing *Kat2b* [245], so that inverse expression of *Kat2b* and *Csrp2bp* may reflect ethanol-induced mechanisms that activate one HAT complex and repress the other. This idea is supported by studies indicating the importance of CREB for mediating ethanol action [246] and also suggest repression of the ATAC complex as a mechanism of ethanol. Studying how modulating the ATAC and CREB complexes affects ethanol consumption would further support the idea that ethanol induces reciprocal activation of these HAT complexes.

One important limitation of this study is that it analyzes one relatively short (80 - 120 bp) region of the gene promoter near the transcriptional start site (TSS) (Table 4). Importantly, the histone modifications studied are associated with differential states of the TSS, including H3K27me3 at poised and repressed genes, H3K4 methylation at poised and active genes, and H3K9ac at active genes [247]. Altering levels of these modifications at the TSS likely involves moving between repressed, poised, or active states and changes in gene expression. However, several of the genes studied did not have changes to these modifications at the TSS. As discussed above, this may reflect the dynamic nature of these modifications and negative feedback to a

baseline state following an ethanol-induced change in gene expression. Alternatively, it is possible that changes to histone modifications occur far from the TSS at regulatory regions that govern transcription factor binding to the gene promoter. These regions primarily function as enhancers, which recruit transcription factors to potentiate gene expression [248]. For example, metallothioneins have glucocorticoid, metal, and antioxidant response elements in their gene promoters, which can be bound by transcription factors to increase expression [249]. The *Mt2* gene also contains a consensus *Egr1* binding sequence (5'-GCGGGGGCG-3') near the TSS and the primers used for ChIP in this study spanned this region, though only changes in H3K4me3 levels were noted (Fig. 17). Given the potential for transcription factor interactions with histone modifying enzymes at enhancers, tiling primer sequences across larger spans of differentially expressed gene promoters may have identified changes in histone modifications at regulatory elements further from the TSS. Additionally, genome-wide studies of histone interactions using ChIP-chip or ChIP-seq may identify changes to histone modifications at cis-acting regulatory elements that may be up to 1 Mb away from the TSS.

This study raises important points about studying epigenetic mechanisms of ethanol. It identified general mechanisms of acute ethanol on histone modifications and chromatin modifying enzyme expression as well as specific mechanisms at gene promoters using ChIP in the CCx. The findings indicate that changes seen on a global level do not generalize to all or even most gene promoters across the CCx. This is likely due to the dynamic nature of histone modifications and differences among neuronal subpopulations that influence ethanol-induced epigenetic changes, which limit the study's ability to identify specific effects at gene promoters. It is also interesting that chromatin modifying enzymes whose expression was altered in CCx were not altered in HC, suggesting changes in CCx expression may be driven by a specific

region or population of cells. Recent work indicates that ethanol-induced epigenetic changes are different even between different subregions of the same brain structure [60,137,139,141], so that it is not unexpected ethanol-induced gene expression changes differ between CCx and HC. These issues may have precluded identification of a discrete pattern of ethanol-induced histone modifications shared by any of the six studied promoters. Future studies should examine additional time points of ethanol exposure as well as study neuronal subtypes by utilizing laser capture microdissection or fluorescence activated cell sorting. Lastly, since the current study utilized C57BL/6J male mice, it is of interest to determine if the results observed generalize to females and to mice of other genetic backgrounds.

In conclusion, this study introduces new epigenetic mechanisms of ethanol, including histone deacetylation at down-regulated gene promoters, increased global H3K4me3, and altered expression of histone modifying enzymes in the CCx following acute ethanol exposure.

4.0 NEURONAL SUBTYPE SPECIFIC ANALYSIS OF ETHANOL-INDUCED EPIGENETIC MODIFICATIONS

4.1 INTRODUCTION

In Section 3 of this dissertation, epigenetic effects of ethanol were revealed in CCx and HC. Those experiments relied on identifying model genes altered by acute ethanol and studying histone modifications at the promoters of those genes. While this study detected changes in histone modifications at several gene promoters, it may have been limited from detecting more by technical issues inherent to studying histone modifications in a complex tissue like the brain.

These issues relate to the ability to detect changes in RNA transcript levels vs. the ability to detect changes in histone modifications at gene promoters using ChIP. For instance, while a cell may produce hundreds of RNA molecules of the same transcript, it only contains two loci for those RNA sequences in the genome (excluding the potential contribution of copy number variation). Therefore, it may be difficult to detect changes in histone modifications at a specific gene promoter using chromatin made from millions of cells if only a few hundred cells are contributing to a detectable increase in RNA transcript number. This issue may be especially relevant in the brain, which is composed of hundreds of neuronal subtypes, glia, and other cell types with distinct transcriptional profiles [250]. Additionally, substantial loss of chromatin occurs during formaldehyde cross-linking, multiple cell lysis steps, and sonication that are

components of nearly all standard ChIP protocols, so that most protocols recommend using tens of millions of cells as input [251,252]. Some of these limitations are overcome using cell culture systems where histone modifications within a distinct cell type can be studied; however, this lacks complexity relative to whole animal models.

With new evidence emerging for cell-type specific transcriptional regulation by drugs of abuse [55,61,253], studying epigenetic modifications specific to these cell types would greatly improve understanding of drug-induced gene regulation. Additionally, studying a homogenous cell population with similar transcriptional profiles will likely increase the sensitivity of ChIP to detect changes in histone modifications at gene promoters. Several recent advances in methods to isolate neuronal subtypes in the brain have made this possible. In particular, laser capture microdissection allows for visualization and selection of cell types in brain tissue [254]. Fluorescence activated cell sorting (FACS) is now being applied to sorting cell types in the brain based on antibody-mediated or endogenous cell fluorescence. For this study, FACS was chosen to isolate a neuronal subtype because it allows for quantifiable sorting of live cells and collection of thousands of cells in a short time (~1 hour) for downstream analysis.

Several recent studies have utilized FACS to identify transcriptional profiles of neuronal subtypes after drug administration. Many of these studies were made possible by the Gene Expression Nervous System Atlas project, which developed transgenic mice with promoter-specific fluorescent proteins integrated into the genome using bacterial artificial chromosomes (BAC) [255]. Identification of neuronal subtypes using BAC transgenic strains is highly specific and these animals have been used in anatomical, electrophysiological, and optogenetic studies [255-257]. An early paper utilized BAC transgenic mice and FACS to identify transcriptional differences between dopamine D1 receptor (D1R) and dopamine D2 receptor (D2R) medium

spiny neurons (MSN) in the striatum [258]. Eric Nestler's group expanded on this study by showing cocaine has differential effects on gene expression in D1R and D2R MSNs [259]; moreover, epigenetic mechanisms intrinsic to these cells were found to underlie how they respond to cocaine [61]. A recent study utilized BAC transgenic strains along with antibody-mediated tagging of histone modifications to examine how cocaine alters histone modifications in D1R and D2R MSNs [260]. Other groups have used antibody-mediated FACS to study gene regulation induced by cocaine [261], opioids [262,263], and methamphetamine [264] in cellular subtypes. However, while antibody-based approaches allow for selection of a wider array of cell types compared to BAC transgenic strains, they also require fixing cells and have issues with off-target binding of antibodies that may reduce their specificity. Though several groups have now used FACS to show neuronal subtype specific gene regulation by drugs of abuse, I did not identify any studies that utilized this approach for ethanol exposure. Additionally, while one group reported using antibody-mediated FACS to study chromatin in neurons [265], I did not identify reports of a ChIP protocol for use in neuronal subtype-specific BAC transgenic strains.

This study utilized a commercially available BAC transgenic strain where the D1R promoter drives expression of tdTomato, a variant of the red fluorescent protein. This strain has been well-characterized with tdTomato expression restricted to D1R MSNs and no apparent behavioral differences relative to wild type mice [266]. D1R MSNs in the NAc are critical components of reward signaling, integrating dopaminergic inputs from the VTA and glutamatergic inputs from limbic structures to guide goal-directed behaviors [267]. Importantly, ethanol consumption potentiates the NAc in both rodents [268] and humans [269], suggesting this is a critical feature in the development of AUD. In particular, chronic ethanol exposure was found to hyperactivate the VTA and increase PKA activity, a downstream effector of the D1R, in

the NAc [270]. Studies have now specifically implicated the D1R in the NAc in mediating several effects of ethanol. A study utilizing D1R and D2R BAC transgenic strains found increased ΔFosB staining in D1R but not D2R neurons following ethanol exposure [55]. Knocking down or pharmacologically inhibiting D1R in the NAc leads to decreased ethanol consumption [271], blocked behavioral sensitization to ethanol [272], and reversal of ethanol-induced changes to $GABA_A$ receptor-mediated tonic currents [273]. These studies indicate that ethanol preferentially modulates D1R MSNs, but studies are lacking on gene expression and chromatin regulation that may underlie mechanisms of ethanol in this cell type.

Improved rodent models that better replicate some aspects of human drinking have recently been developed. In particular, Howard Becker's group developed a chronic ethanol exposure model in mice that escalates ethanol drinking three days following vapor ethanol exposure [184,274,275]. The procedure relies on four cycles of vapor ethanol and withdrawal, which increases ethanol drinking in a limited access paradigm up to 80 hours following the final ethanol vapor session. Studies have recently implicated changes to glutamate levels and neurophysiology in the NAc induced by this paradigm [184,276]. Additionally, long-lasting effects of ethanol suggest changes in gene expression and chromatin remodeling also underlie drinking escalation. Based on research implicating D1R MSNs in mediating the effects of ethanol as well as changes in the NAc after chronic vapor ethanol exposure, we chose to study the effects of chronic intermittent vapor ethanol on gene expression and chromatin regulation in D1R MSNs of the NAc. This study tests the hypothesis that chronic ethanol exposure and withdrawal induces dynamic changes in gene regulation via discrete histone modifications in accumbal D1R MSNs.

4.2 MATERIALS AND METHODS

A step-by-step protocol for NAc dissociation and RNA and ChIP protocols is provided in Appendix A.

4.2.1 Animals and treatment

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Two heterozygous transgenic males containing a gene for the tdTomato fluorescent protein driven by the D1R promoter were purchased from the Jackson Laboratory (Stock #016204). These males were bred to isogenic C57BL/6J females and offspring were checked for the presence of the transgene with PCR after weaning. Male offspring that possessed the transgene were housed with their wild type littermates and used for all experiments. Mice were housed under 12 hour light/dark cycles and had *ad libitum* access to food and water.

Transgenic mice were used for experiments at 8-10 weeks of age. The experimental design is summarized in Fig. 21. Briefly, mice were weighed and injected with either 1 g/kg of 10% ethanol solution in saline (0.01 ml/g) (ethanol-treated) or 0.01 ml/g saline (room air controls). Mice were immediately placed into one of two identical custom-built vapor chambers described in Section 2.2.2 that were used to deliver either room air or vaporized ethanol. Flow rate, vaporization temperature, and exposure time were optimized to achieve consistent BECs without the use of pyrazole. Room air was flowed into two heated Erlenmeyer flasks at a rate of 7.5 L/min; one flask received ethanol at a rate of ~80 μl/min while the other flask received no

ethanol. Air from the ethanol and control flasks flowed into separate chambers so that only one chamber received vaporized ethanol. Mice were housed for 16 hours in the chambers from 17:00 to 09:00. At the end of the first exposure, tail blood was collected and used to analyze BECs. From 09:00 to 17:00, mice were placed back onto a ventilated cage rack and only exposed to room air.

Injections and exposures were done on 4 consecutive nights and mice were sacrificed either immediately following the 4th exposure (chronic ethanol group) or 72 hours following the 4th exposure (72 hour withdrawal) (Fig. 21); room air controls from both cohorts were included in this study. Any mouse that exhibited an anesthetic effect of ethanol intoxication was excluded from the experiment.

4.2.2 PCR genotyping

Tail snips (< 0.5 cm) were taken at the time of weaning and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instruction. DNA was used for a PCR assay containing GoTaq HotStart Polymerase (Promega, Madison, WI), primers for the transgene and positive control region (20 μM), dNTPs (2.5 mM), 5x reaction buffer, and $MgCl_2$ (2.5 mM). Primer sequences used were Transgene, F: 5'-CTT CTG AGG CGG AAA GAA CC-3' and R: 5'-TTT CTG ATT GAG AGC ATT CG-3', Positive control region, F: 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3' and 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'. PCR conditions were 3 min at 94˚ C, then 30 s at 94 ˚ C, 1 min at 59 ˚ C, 1 min at 72 ˚ C repeated 35 times, then 2 min at 72 ˚ C. PCR products were run on a 1.5% agarose gel. Transgenic animals were identified by the presence of two bands: a 750 bp band for the transgene and 324 bp band for the positive control region.

4.2.3 Dissociation of the nucleus accumbens into a single cell suspension

Tissue dissociation was adapted from a previous report of FACS using BAC transgenic mouse strains [277]. The following reagents were made fresh before the start of each experiment. HABG: 200 µl of 50x B27 supplement (Life Technologies) and 25 µl of 100x Glutamax (Life Technologies) into 9.8 ml of Hibernate A cell culture media (Life Technologies). Papain dissociation buffer: 14 µl of 100x Glutamax (Life Technologies) into 5.5 ml Hibernate E cell culture media (BrainBits LLC, Springfield, IL). Papain: 5 ml of papain dissociation buffer into one vial containing 100 U of papain (Worthington Biochem, Lakewood, NJ). DNase: 500 µl of papain dissociation buffer into one vial containing 1000 U of DNase (Worthington Biochem).

Adult animals were sacrificed by cervical dislocation at the time points indicated (Fig. 21). The brain was extracted and placed into an ice cold adult mouse brain slicer matrix with 1 mm coronal section slice intervals (Zivic Instruments). The NAc was identified under a dissecting microscope as the region surrounding the anterior commissure; it was differentiated from the overlying dorsal striatum by its homogenous appearance relative to the striated appearance of the dorsal striatum. The NAc was placed into a small culture dish containing 1 mL HABG supplemented with 100 U/ml of RiboLock RNase inhibitor (Thermo Scientific, Waltham, MA). Tissue was cut into ~1 mm^3 pieces using a scalpel. Tissue pieces were gentled aspirated using a cut 1 ml pipet tip and allowed to fall to the edge of the tip. The pipet tip was placed into 2 ml of Papain supplemented with 110 µl DNAse that was pre-warmed to 37˚ C and the tissue pieces were allowed to gently fall into the Papain so that there was minimal transfer of HABG into Papain. Tissue pieces in Papain were covered in aluminum foil and incubated at 32˚ C with gentle rotation for 15 minutes.

After incubation in Papain and DNase, tissue pieces were collected with a cut 1 ml pipet tip and allowed to fall to the edge of the tip. The pipet tip was placed into 2 ml of HABG supplemented with 100 U/ml of RiboLock RNase inhibitor and the tissue pieces were allowed to gently fall into the Papain so that there was minimal transfer of Papain into HABG. The tissue pieces in HABG were covered with aluminum foil and incubated for 5 minutes at room temperature. Tissue pieces were triturated using a latex pipette bulb and autoclaved, cotton-plugged Pasteur pipette that was fire-polished to a ~0.5 mm tip. For trituration, tissue pieces in HABG were sucked into the pipette and ejected over ~5 seconds until large tissue pieces were no longer visible, which usually took ~10 cycles of trituration. Care was taken to avoid air bubbles in the cell suspension.

The dissociated cell suspension was passed through a 70 μm cell strainer (Fisher Scientific, Pittsburgh, PA) by aspirating 800 μl of solution at a time through a cut pipet tip and gently placing the tip over the cell strainer. After the cell suspension was strained, 5 ml of HABG supplemented with 100 U/ml of RiboLock RNase inhibitor was passed over the same cell strainer. Therefore, the total volume of the cell suspension was ~7 ml. The cell suspension was covered in aluminum foil and immediately transported for cell sorting. It is important to note that this process takes ~45 minutes from the time the animal is sacrificed to cell sorting.

4.2.4 Fluorescence activated cell sorting of tdTomato+ cells

Neurons were sorted in collaboration with the Rheumatology Flow Cytometry Core Facility at the University of Pittsburgh. Briefly, the dissociated cell suspension was sorted on a FACS ARIA II (Becton Dickinson Inc.) using an 85 μm nozzle with a pressure of 45 p.s.i. debris and doublets in the sample were excluded from sorting by gating for intact cells using forward and

side scatter profiles. tdTomato was excited by an argon-ion laser (488 nm) and detected using an emission spectra between 564 nm and 606 nm. Fluorescence gating thresholds were established by sorting wild-type animals that do not express tdTomato and isolating events that were above the highest detectable limit in wild type animals (Fig. 22). tdTomato+ and tdTomato- cells were separated using fluorescence gating and collected into two separate tubes.

4.2.5 RNA extraction

For RNA studies, cells were sorted directly into 750 µl of Trizol LS (Invitrogen). After sorting, volume of Trizol LS with sorted cells was measured and nuclease free water was added to bring the volume up to 1 ml. RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol without DNase digestion and eluted in 30 µl of nuclease free water.

4.2.6 RT-qPCR

RNA was converted into cDNA using RT (Bio-Rad, Hercules, CA). Reactions were carried out in duplicate for each gene. SYBR green fluorescent master mix (Bio-Rad) was added to each well and visualized using a Bio-Rad iCycler. All primers were optimized for 90% to 110% efficiency at the following conditions: 10 min at 95˚C followed by 40 cycles of 30 s at 95˚C, 1 min at 60˚C, and 30 s at 72˚C. Primer sequences used were β-actin, F: 5'-TCA TGA AGT GTG ACG TTG ACA TCC GT-3' and R: 5'-CCT AGA AGC ATT TGC GGT GCA CGA TG-3', D1R, F: 5'-GAA CCC AGA AGA CAG GTG GA-3' and R: 5'-GCT TAG CCC TCA CGT TCT TG-3', Glial Fibrillary Acidic Protein (GFAP), F: 5'-AGA AAA CCG CAT CAC CAT TC-3' and R: 5'-TCA CAT CAC CAC GTC CTT GT. Threshold cycle (Ct) values were calculated for

each well and duplicate values averaged. The difference between specific genes and β-actin (ΔCt) was calculated for each animal and normalized to the average of room air sired offspring (ΔΔCt). Fold change over room air sired offspring was calculated for each animal using the following formula: 2-ΔΔCt.

4.2.7 Native FACS-ChIP

Reaction buffers were adapted from a previous report of low cell number ChIP [278]. To prevent histone deacetylation and protease activation during tissue dissociation for ChIP studies, all media and buffers were supplemented with 5 mM sodium butyrate and, beginning after papain treatment, all media and buffers were supplemented with cOmplete protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were sorted directly into 250 µl of 2x micrococcal nuclease (MNase) buffer (100 mM Tris, pH 8.0, 2 mM $CaCl_2$, 0.4% Triton X-100) and volume was measured and brought up to 500 µl by adding nuclease free water. MNase (New England Biolabs, Ipswich, MA) was diluted by adding 1 µl of MNase (2,000 gel units) to 500 µl 1x MNase buffer (50 mM Tris, pH 8.0, 1 mM $CaCl_2$, 0.2% Triton X-100). For MNase digestion of chromatin, 15 µl of diluted MNase was added to sorted cells and cells were incubated in a 37˚ C heat block for 5 minutes. To stop the MNase digestion, 50 µl of 10x MNase stop buffer (110 mM Tris, pH 8.0, 55mM EDTA) was added to the cells. From this point, chromatin was kept on ice or in a 4˚ C room through immunoprecipitation. To lyse cells, 550 µl of ice cold 2x RIPA cell lysis buffer (280 mM NaCl, 1.8% Triton X-100, 0.2% SDS, 0.2% Na Deoxycholate, and 5 mM EGTA) was added. The solution was mixed by inverting the tube and the tube was left on ice for 15 minutes. To remove cell debris, the tube was centrifuged at 4˚ C at 13,000 rpm for 10 minutes. Chromatin was decanted into a new 1.5 ml Eppendorf tube and placed on ice.

Chromatin was divided into two 400 µl aliquots for immunoprecipitation and one 200 µl aliquot used as input. For immunopreciptiation reactions, 22 µl of MagnaChIP Protein A/G magnetic beads (Millipore, Billerica, MA) were added to each tube. To one tube, 5 µl of an anti-H3K27me3 antibody (#17-622, Millipore) was added and to the other tube 9 µl of an anti-H3K18ac antibody (#9675, Cell Signaling Technology, Danvers, MA) was added. As a negative control for optimization experiments, nonspecific IgG antibody (#PP64B, Millipore) was also used. The immunoprecipitation tubes were incubated at 4˚ C with gentle end-over-end rotation for 3.5 hours while the input tube was kept at 4˚ C with no rotation. After incubation, tubes were placed onto a magnetic rack and the supernatant was removed using a pipet. Magnetic beads were washed 3 times with 500 µl RIPA buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% Na Deoxycholate) and 1 time with 500 µl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA); between washes, the beads were incubated for 5 minutes at 4˚ C with gentle end-over-end rotation and the supernatant was removed using a pipet.

Following the TE wash step, magnetic beads were eluted in 200 µl elution buffer (50 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1% SDS, 50 µg/ml proteinase K); for the input, proteinase K was added to a concentration of 50 µg/ml. The magnetic beads in elution buffer and input were incubated at 65˚ C with gentle rotation for 45 minutes. Following proteinase K digestion, tubes were briefly spun on a tabletop centrifuge and placed into a magnetic rack. The supernatant (200 µl) was transferred to a tube containing 10 µl 3 M Na Acetate and 1 ml buffer PB (Qiagen). DNA was purified using the Qiagen MinElute Reaction Cleanup Kit according to the manufacturer's protocol and eluted in 14 µl nuclease free water.

ChIP DNA samples were quantified using a Qubit fluorometer (Life Technologies). For optimization of FACS-ChIP experiments using tdTomato+ neurons, samples were diluted to 30

µl and 5 µl were analyzed by ChIP-qPCR in duplicate for each primer set. qPCR conditions were the same as reported in the RT-qPCR section. Primer sequences used were: D1R promoter, F: 5'-GCC TCT GGT TTC CTA CAC CC-3' and R: 5'-AGG GAA AAG CAT GGT CGA GG-3', GFAP promoter, F: 5'-ACA AAA GGC CTG GGT TGA CA-3' and R: 5'-CTC TGG ATC TGG AAC TCG CC-3', LINE1 5' UTR, F: 5'-CCG GGA CTC CAA GGA ACT TA-3' and R: 5'-CCT CCT GGC CGA AGA AGA-3'. Ct values for antibodies were normalized to the input Ct value for each primer set. Data are present as fold enrichment over input DNA.

4.2.8 RNA expression microarray

RNA collected from chronic ethanol and room air controls (n = 7/group) as well as 72 hour withdrawal and room air controls (n = 4-5/group) was sent to Dr. R. Adron Harris' lab at the University of Texas—Austin. RNA concentration and RNA integrity numbers (RIN) were determined on an Agilent Bioanalyzer using the Eukaryotic Total RNA Nano Series II kit (Agilent Technologies, Santa Clara, CA). The EpiCentre TargetAmp Pico 2-round amplification kit (Illumina, San Diego, CA) was used to amplify 180 pg of RNA. cRNA expression will be analyzed using the Illumina mouse WG6 v2 whole genome expression array.

4.2.9 Statistics

For RT-qPCR, a Student's t-test was used to compare groups. For ChIP-qPCR, a two-way ANOVA with Bonferroni post-hoc testing was used to compare antibody enrichment. Statistical significance was defined by a p-value < 0.05. All data are presented as mean +/- SEM.

4.3 RESULTS

4.3.1 Chronic intermittent ethanol exposure

Mice were injected with either 1 g/kg ethanol or saline and exposed to vapor ethanol or room air, respectively, overnight for 4 consecutive days (Fig. 21). BECs were measured after the first exposure and averaged 231.7 +/- 12.7 mg/dl (mean +/- SEM).

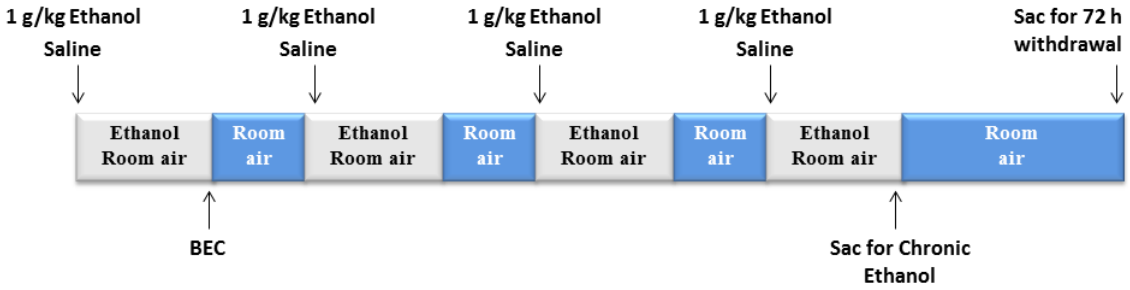

Figure 21. Chronic intermittent vapor ethanol exposure

Chronic intermittent vapor ethanol exposure escalates ethanol drinking in a limited access drinking assay [274]. Mice were injected with either 1 g/kg ethanol or saline and placed into a chamber receiving either vapor ethanol or room air, respectively, for 16 hours. After 16 hours, all mice were placed back into a ventilated cage rack for 8 hours. Mice received four cycles of exposure over consecutive days and were either sacrificed immediately (chronic ethanol or room air) or 72 hours following the final exposure (72 hour withdrawal or room air).

4.3.2 FACS of tdTomato+ neurons

Cells were sorted by their size based on forward and side scatter profiles to exclude debris and doublets; additionally, they were sorted based on tdTomato intensity (Fig. 22). A distinct group of cells with high tdTomato expression could clearly be discriminated using FACS. For RNA collection, there were no significant differences in average number of cells collected between the chronic ethanol (n = 7; 24143 +/- 2703, mean +/- SEM) and room air control (n = 7; 28429 +/-

3032, mean +/- SEM) groups as well as the 72 hour withdrawal (n = 5; 24900 +/- 3508; mean +/- SEM) and 72 hour room air control (n = 5, 35720 +/- 4580, mean +/- SEM) groups.

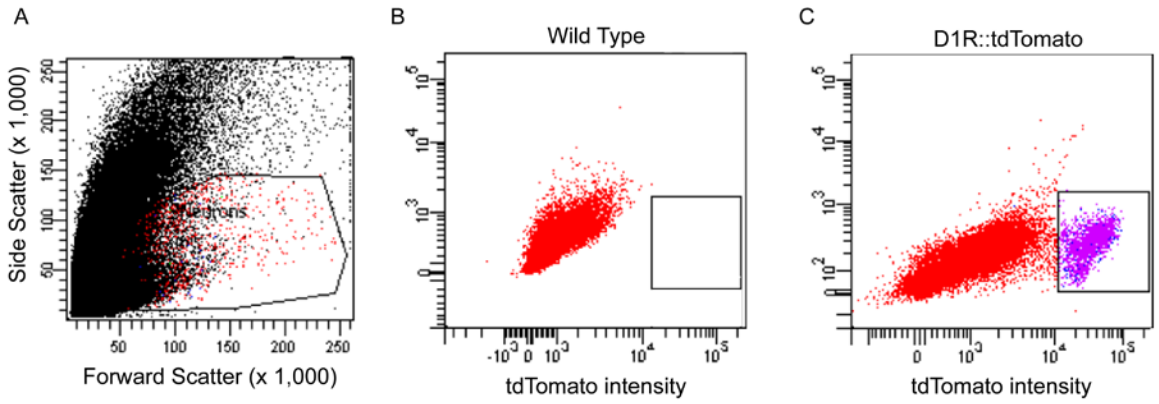

Figure 22. FACS of D1R::tdTomato cells from BAC transgenic mice

A dissociated neuronal suspension derived from the NAc of a BAC D1R::tdTomato transgenic mouse was sorted on a FACS Aria II. (A) Neurons were selected by Forward and Side Scatter profiles. Fluorescence gates were established by comparing tdTomato intensity in (B) wild type and (C) D1R::tdTomato mice. Purple cells were denoted as tdTomato+ and collected for analysis.

4.3.3 RNA analysis

Optimization of cell sorting indicated that the cell population was enriched in D1R with almost undetectable levels of GFAP; therefore, tdTomato+ cells likely represent D1R MSNs (Fig. 23).

RNA was collected from FAC-sorted neurons after ethanol or room air exposure and sent to Dr. R. Adron Harris' lab at UT-Austin for whole genome expression analysis. There, it was quantified and assessed for quality using the Agilent Bioanalyzer. RNA integrity numbers (RIN) were used to determine RNA quality based on height of 28s and 18s ribosomal subunit peaks [279]. RIN values below 7 are associated with poor performance on gene expression microarrays [280]; importantly, all samples had RIN values greater than 7 (Fig. 24A). For the 24 samples collected for whole genome expression analysis, RNA recovery averaged 43.7 +/- 4.0 ng (mean +/- SEM) (range: 13.5 ng – 80.6 ng) with no differences between groups (Fig. 24A). There was a

near-significant trend for a correlation between cells collected and RNA yield (r = 0.38, p = 0.06). In preparation for the Illumina mouse WG6 v2 whole genome expression array, 180 pg of RNA was amplified using the EpiCentre TargetAmp Pico 2-round amplification kit (Fig. 24B). All RNA has been amplified and is currently in queue for the whole genome expression array.

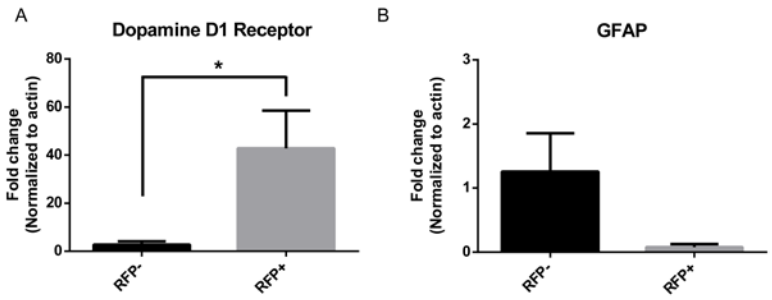

Figure 23. tdTomato+ cells are enriched in D1R and lack GFAP expression

Using RT-qPCR, tdTomato+ were found to have (A) 40-fold enrichment of the Dopamine D1 receptor (n = 8/group) and (B) nearly undetectable expression of GFAP (n = 3/group). *p < 0.05.

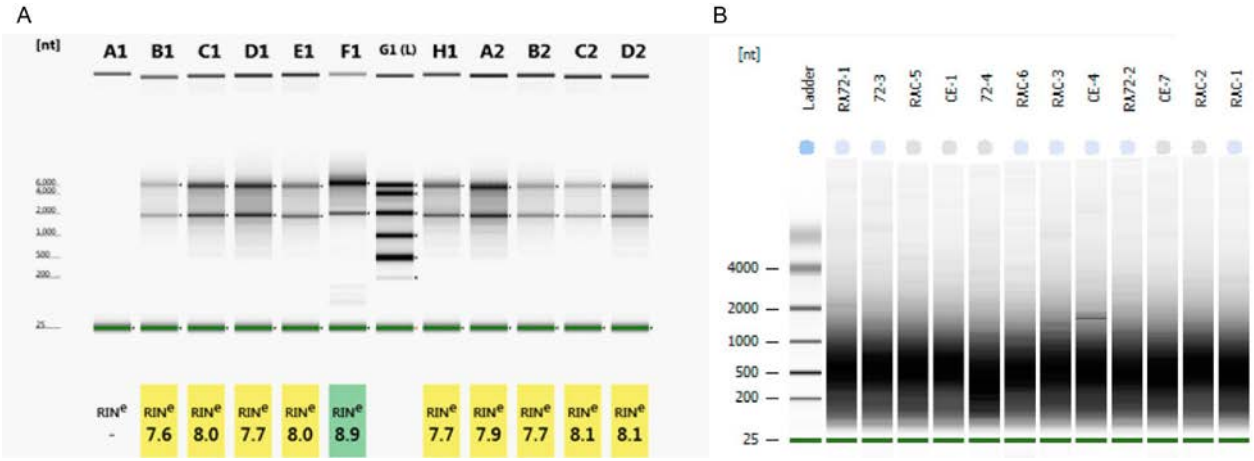

Figure 24. Example of FACS RNA analysis and amplification

RNA was collected form FAC-sorted neurons following ethanol or room air exposure. (A) Agilent Bioanalyzer analysis of RNA indicated strong ribosomal peaks (dark bands) and RIN values above 7. Lanes: A1 is a negative control (water), B1-E1 are D1R-derived RNA, F1 is a positive control (RNA derived from HeLa cells), G1 is a ladder, and H1-D2 are D1R-derived RNAs. (B) RNA (180 pg) from each sample was used for 2-step cRNA amplification in preparation for Illumina mouse WG6 v2 whole genome expression array; peak size of cRNA was ~600 bp across all samples; lanes are indicated above, where numbers correspond to the sample (CE = chronic ethanol; RA = room air; 72 = 72 hour ethanol withdrawal; RA72 = room air 72 hour withdrawal).

4.3.4 Chromatin analysis

Chromatin was collected from sorted tdTomato+ cells and digested using MNase. Chromatin was left in its native state and immunprecipitated immediately, so that there were no freeze-thaw cycles. Optimization using several antibodies revealed that anti-H3K18ac and anti-H3K27me3 antibodies could be used to discriminate active and repressed gene promoters (Fig. 25). Notably, H3K18ac is enriched at actively transcribed gene promoters while H3K27me3 is a component of the polycomb repressive complex and associated with transcriptional repression [247]. To test enrichment of D1R MSNs, H3K18ac and H3K27me3 levels were measured relative to input at the D1R and GFAP promoters, which were enriched and repressed, respectively, based on RT-qPCR analysis of this cell type (Fig. 23). Therefore, the D1R promoter should have high levels of histone acetylation and low levels of histone methylation while the GFAP promoter should have the opposite pattern. The long interspersed nuclear element (LINE1) 5' UTR was used as a control that could be easily quantified, since there are ~500,000 loci for this retrotransposable element in the genome and it is normally associated with histone methylation [247,281].

Using H3K18ac, H3K27me3, and nonspecific IgG antibodies, DNA quantity was assessed relative to input using qPCR. There was a significant interaction between histone modification and promoter regions studied ($F_{(4,13)} = 14.45$, $p < 0.0001$) (Fig. 25). Bonferroni post-hoc testing revealed significantly increased levels of H3K27me3 relative to H3K18ac at the ($n = 4$; $p < 0.001$) and the GFAP promoter ($n = 2$; $p < 0.05$); conversely, there were increased levels of H3K18ac relative to H3K27me3 at the D1R promoter ($n = 3$; $p < 0.05$) (Fig. 25). Additionally, there were nearly undetectable levels of DNA relative to input using a nonspecific IgG antibody, indicating specificity for the histone modifications studied (Fig. 25). DNA was also quantified by the Genomics and Proteomics Core Laboratory at the University of Pittsburgh

using the Qubit fluorometer (Life Technologies) and could be detected in the input as well as anti-H3K18ac, and anti-H3K27me3 immunoprecipitations; no DNA was detected using the IgG antibody. These data indicate that ChIP can be used to discriminate active and repressed genomic regions in FAC-sorted neuronal subtypes with low variability between animals.

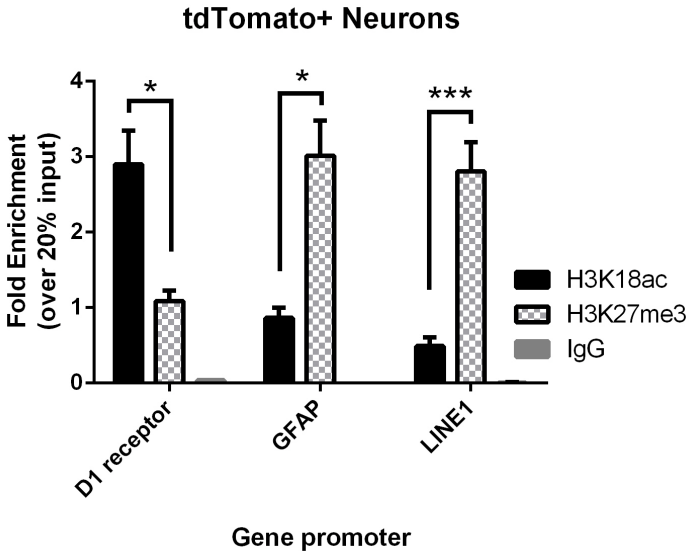

Figure 25. ChIP using FAC-sorted tdTomato+ cells from BAC transgenic animals

tdTomato+ cells were collected from D1R::tdTomato BAC transgenic mice and used to analyze histone occupancy at gene promoters. There was a significant increase in H3K27me3 levels relative to H3K18ac levels at the repressed retrotransposable element LINE1 (n = 4) and GFAP promoter (n = 2). Conversely, there was a significant increase in H3K18ac levels relative to H3K27me3 at the D1R promoter (n = 3). For the IgG antibody, there was nearly undetectable signal of LINE1 (n = 2), D1R (n = 1), or GFAP (n = 1) promoter regions, suggesting specificity of the H3K18ac and H3K27me3 antibodies. *$p < 0.05$, ***$p < 0.001$

4.4 DISCUSSION

This section describes optimization of RNA extraction for whole genome expression and chromatin analysis in a neuronal subtype collected from a BAC transgenic mouse strain. Using FACS, ~25,000 tdTomato+ cells were collected from the NAc with minimal tissue processing

time (~45 min), providing sufficient RNA and chromatin for downstream analysis. Importantly, this may be the first description of a ChIP protocol for neuronal subtypes that were sorted by FACS using BAC transgenic strains. While results of ethanol-induced gene regulation in this cell type are pending, experimental validation shows that tdTomato+ cells have 40-fold enrichment of D1R relative to tdTomato- cells with nearly undetectable expression of GFAP, indicating cell type specificity for D1R MSNs. Despite low input for traditional chromatin studies, the ChIP protocol optimized in this study discriminated transcriptionally active and repressed regions on the basis of H3K18ac and H3K27me3 levels at gene promoters. Importantly, it showed elevated histone acetylation the D1R promoter in tdTomato+ cells, indicating cell type specificity at the level of chromatin. The discussion will be limited to experimental considerations and interpretation of potential effects of chronic intermittent ethanol exposure on this cell type.

FAC-sorting of the NAc from D1R::tdTomato BAC transgenic mice was efficient, with a distinct population of cells with high tdTomato expression (Fig. 22). Gene expression and chromatin analysis of the D1R promoter in these cells confirmed that these are highly enriched D1R MSNs. While collection of ~25,000 tdTomato+ cells per sort is a substantial improvement over previous studies using BAC transgenic stains [258,259,277], this amount is much smaller than the total population of D1R MSNs in the NAc, suggesting considerable loss of neurons during tissue processing. Improving yields will likely require optimization of tissue trituration, which induces considerable mechanical stress on cells and is likely the greatest source of cell loss. Unfortunately, neuronal FACS studies are still limited, so there are no reports of how changing experimental parameters affects yield. Identifying optimal sorting conditions will improve downstream analysis by providing more input for RNA and chromatin studies.

The FACS protocol developed for this study was able to provide sufficient and high quality (RIN > 7) RNA for whole genome expression microarray analysis. This has been demonstrated previously [55,258,259]; however, it is notable that in this study, compared to other FACS whole genome expression studies, RNA yields for the majority of samples collected were sufficient for one-round amplification (> 25 ng input) (Fig. 24) [258,259,261]. While one- and two-round amplification generate gene expression levels that correlate well with one another [282], using one-round amplification increases the number of transcripts detected [282]. Therefore, since this study used a two-round amplification procedure, it may be limited to detecting changes in expression of highly expressed transcripts in D1R MSNs. Using an RNA collection kit optimized for elution in a small volume would have increased the concentration of sample collected, but it may have also reduced yield. Therefore, it is unclear whether changing the RNA collection protocol would allow for one-round amplification. While the current study is sufficiently powered to detect ethanol-induced changes in gene expression, further optimizing RNA collection may improve detection of gene transcripts by allowing for one-round cRNA amplification.

The goal of this study is to examine the dynamics of ethanol-induced histone modifications with high sensitivity by using a specific cell type enriched by FACS. Our optimization experiments show that genomic regions with differential expression in D1R MSNs can be discriminated based on H3K18ac and K3K27me3 levels (Fig. 25). Importantly, this study demonstrates that histone modification levels are detectable with low variability after cell sorting using the current protocol. Therefore, we are poised to examine whether chronic intermittent ethanol exposure induces long-term changes to chromatin that could underlie its effect on ethanol drinking escalation [274]. Pending results from the RNA microarray will be used to

identify genes whose expression is altered by ethanol immediately and 3 days following exposure. Then, ChIP will be used to study the balance of H3K18ac and H3K27me3 at differentially expressed gene promoters. This method will uncover dynamic mechanisms of ethanol-induced gene regulation in D1R MSNs by identifying the time course of ethanol-induced epigenetic modifications. It will be especially interesting to identify long-term, ethanol-induced changes in chromatin despite no detectable changes in expression. Importantly, this is a proof of principle study, so that in the unlikely event we do not identify any ethanol-induced changes to levels of H3K18ac and H3K27me3 at gene promoters, this study appears to be the first to develop a ChIP protocol for highly specific BAC transgenic strains. Therefore, while D1R MSNs are clearly modified by ethanol and critical for goal-oriented behavior like ethanol drinking [267,273] our protocol is scalable to other cell types or histone modifications that may be more relevant for ethanol action.

In conclusion, this section describes a method for isolating D1R MSNs with high specificity and studying whole genome expression using a microarray as well as histone modifications using ChIP. Future studies will identify the role of chronic intermittent ethanol and withdrawal on gene regulation in this cell type *in vivo*.

5.0 FINAL DISCUSSION

5.1.1 Conclusions and significance

Despite the well-studied effects of ethanol on gene expression, little is known about mechanisms by which ethanol alters chromatin to regulate gene promoters. Moreover, it is not known whether ethanol induces long-lasting effects on chromatin that are associated with changes in ethanol drinking and whether ethanol-induced epigenetic modifications are heritable. Therefore, the experiments in this thesis tested three distinct hypotheses related to identifying epigenetic effects of ethanol: 1) paternal ethanol exposure regulates ethanol drinking and ethanol-related behavior in offspring; 2) acute ethanol induces conserved changes to histone modifications at model gene promoters in CCx; 3) chronic intermittent vapor ethanol and withdrawal induce dynamic changes in gene regulation via discrete histone modifications in accumbal D1R MSNs. The results presented in this thesis provide evidence for epigenetic effects of ethanol in mediating both heritability of ethanol drinking and sensitivity to ethanol as well as ethanol-induced gene regulation in CCx. Additionally, they set up future studies of ethanol's epigenetic mechanisms in neuronal subtypes, which will increase sensitivity of current assays to detect cell-specific changes in gene regulation.

Heritability of AUD has been extensively studied and several mutations in alcohol metabolizing enzymes are associated with risk for developing AUD [17-19]; however, GWAS

has failed to uncover genetic variants that explain more than a fraction of a percent of risk for AUD [21]. Therefore, searching for alternative explanations to explain missing heritability is an important step toward uncovering mechanisms of how risk for AUD is inherited. Importantly, dozens of studies have now uncovered metabolic and behavioral effects of parental environmental manipulations on offspring (presented in Section 1.2.3). Additionally, several of these have associated offspring behavioral changes with transmission of epi-alleles, which include altered histone modifications, DNA methylation, and/or sncRNA populations in gametes [77,96,98]. Considering epimutagenic effects of ethanol and reports of altered behavior in offspring of ethanol-exposed animals (presented in Section 1.3.4), it is not completely surprising that paternal ethanol exposure altered ethanol drinking and ethanol-related behaviors in offspring in this study.

To identify mechanisms mediating paternal ethanol's effects on offspring, it is important to consider what is currently known about mechanisms of epigenetic inheritance. While the field is still relatively new, several potential mechanisms underlying epigenetic inheritance have been proposed in mammals. In particular, Wolf Reik's group identified large regions of DNA surrounding IAPs, a type of retrotransposable element in mice, that escape genomewide demethylation during development [74]. Another recent study identified nearly 5,000 paternally imprinted DMRs in sperm that also retain their DNA methylation levels during genomewide demethylation [283]. DNA methylation at these regions can affect offspring phenotype. Two studies have found that DNA methylation patterns at gene promoters regulating coat color [109] and tail kinking [108] are inherited by offspring. Considering DNA methylation levels are maintained between cell divisions [42], these studies provide evidence for mitotic and meiotic heritability of this mark, which is a critical aspect of a modification that must survive rounds of

cell divisions following fertilization to be maintained in adult offspring. These features have led several groups to study how parental exposures alter the DNA methylation landscape in offspring [84,87,89,92,96]. This study also found a change in DNA methylation at the *Bdnf* promoter in sperm that was maintained in the brains of offspring. Therefore, it will be important to characterize DNA methylation at other genes whose expression is altered by paternal ethanol.

Other sources of epi-alleles in gametes are also important to consider. Sperm have a small population of RNAs (~10 fg/sperm) that appear to have an important role in development. Direct mechanisms of RNA-mediated epigenetic inheritance are emerging. One study identified an intronic insertion in the c-kit gene that led to a white tail and feet phenotype in mice, which was inherited even without inheritance of the intron; interestingly, the phenotype could be replicated by injecting RNA derived from the intronic insertion into the pronucleus [76]. A recent study of paternal stress also found that injecting sperm-derived miRNAs from stressed sires into an embryo could replicate its effect on offspring [77]. Semen is also a large source of cell-free RNAs that exist in vesicles and are stable up to 24 hours in solution [284], though their function is still unknown. Additionally, retained histones in sperm have a critical role in early offspring development [102,104]. However, several questions remain regarding heritability of sperm-derived RNAs and histones. Mainly, how is a small population of RNAs or retained histones transferred between rapid cell divisions in early development? The studies above suggest that they are and it will be important to characterize the effect of ethanol on sperm-derived RNAs and retained histones, as they are likely to affect offspring development.

In Section 2 of this dissertation, paternal ethanol exposure was associated with decreased ethanol drinking and increased sensitivity to ethanol in male offspring using a mouse model. Chronic ethanol was also associated with a modest reduction in DNA methylation at the

paternally imprinted *IG* DMR and the *Bdnf* promoter in motile sperm as well as the *Bdnf* promoter in offspring VTA. These studies introduce the idea that parental ethanol consumption can alter ethanol-related behaviors in the next generation. While this finding is surprising, it is important to place its effect in context with its magnitude. The 2 bottle choice drinking experiment required a relatively large number of male offspring (n = 17/group) (Fig. 7) to uncover a statistically significant effect on ethanol preference and consumption. This finding suggests that penetrance or heritability of epi-alleles is much lower than 100%, which is also implied by analyzing individual clones used in bisulfite sequencing results that show some gametes have no changes to DNA methylation after ethanol exposure (Fig. 3 and Fig. 15). While it is possible that ethanol's effects on DNA methylation in sperm are not necessary for its effects on offspring behavior, these results may also be explained by recent findings in humans. Specifically, variable inheritance of epi-alleles is supported by human studies of colorectal cancer, which indicate some but not all offspring inherit altered DNA methylation at a promoter region for a mismatch repair enzyme that confers risk for colorectal cancer [285]. Though mechanisms that underlie susceptibility to epigenetic inheritance are unknown, their impact on human behavior may be large even if the number of susceptible individuals is small. In reference to this study, the lifetime prevalence of AUD is 42% among men [8], so that if the results apply to humans at any level they are likely to have a meaningful impact on drinking behavior.

Another important aspect of the study presented in Section 2 of this dissertation is its potential to uncover biomarkers associated with resistance to ethanol consumption. Despite not identifying an increase in ethanol drinking in ethanol-sired offspring as hypothesized, studying mechanisms that decrease ethanol consumption in this cohort may have greater impact. Notably, ethanol consumption among 129xC57 offspring in this study was variable – with some mice

drinking nearly no ethanol and others drinking only ethanol versus water. More male offspring had low ethanol preference in the ethanol-sired group, which contributed to a group effect for decreased preference. The data hint at a mechanism that may protect some male offspring from high ethanol drinking. Ethanol-sired male offspring had increased *Bdnf* expression and both male and female offspring had a modest reduction in DNA methylation at the *Bdnf* promoter. While the findings between *Bdnf* expression and ethanol consumption are only correlative and DNA methylation changes are present in both sexes, several studies suggest *Bdnf* may be playing a role in this phenotype. Intra-VTA infusion of *Bdnf* enhances sensitivity to cocaine [203] and amphetamine [204] as well as decreases morphine seeking behavior [205]. Moreover, increased *Bdnf* expression in other brain regions is associated with decreased ethanol drinking [188-190]. A genomewide study of epigenetic reprogramming during early development suggests that DNA methylation at the *Bdnf* promoter remains relatively constant throughout this process [74]. These studies support a role for *Bdnf* in our phenotype, though it remains to be seen if epigenetic regulation of *Bdnf* plays a causative role. Establishing a causative role would likely require modulating *Bdnf* signaling, possibly through use of a TrkB receptor antagonist like ANA-12 [98], and testing offspring on ethanol drinking and ethanol-related behavior assays.

Section 3 of this dissertation studied general mechanisms of ethanol that regulated gene expression in CCx and HC. The study was able to identify several epigenetic mechanisms of ethanol, including decreased expression of *Hdac2* and *Hdac11* (Fig. 19) and increased H3K4me3 in CCx (Fig. 18) as well as increased histone acetylation in HC (Fig. 20). Additionally, despite decreased expression of HDACs, the study identified histone deacetylation at down-regulated gene promoters in CCx (Fig. 16), indicating that some HDAC activity is important for ethanol-induced gene regulation. However, inability to identify conserved epigenetic mechanisms at gene

promoters indicates that acute ethanol has complex mechanisms, likely relying on cell-type and gene-specific specific induction of transcription factors across a range of secondary mechanisms. By highlighting the complexity of ethanol action, this study indicates that targeting chromatin modifying enzymes, including HDAC inhibitors, in treatment of AUD may have variable effects on ethanol-related behaviors. This idea is also indicated by published studies to date that show varied roles of HDAC inhibitors on ethanol drinking and behavioral sensitization [139,142-144] (presented in section 1.3.2). Finally, while the study did not identify a conserved ethanol-induced epigenetic program, it is possible that one may be discovered with larger-scale studies of histone modifications or *in vitro* studies of single cell types.

Based on challenges of studying a diverse tissue type at one time point in Section 3, we attempted to focus epigenetic studies of ethanol on a single cell type of the NAc in Section 4. This study required developing a novel ChIP protocol to study chromatin from a relatively small number of cells isolated by FACS. While several groups have reported studying gene expression from FAC-sorted neuronal subtypes, being able to use ChIP to study induction of histone modifications at gene promoters is potentially a major step toward understanding how neuronal subtypes adapt to their environment. Importantly, this type of dynamic, cell-specific data is being endorsed by the recently proposed NIH Brain Initiative [286], so that it may be relevant to several fields in neuroscience. It may be especially relevant for addiction research, since drugs of abuse modulate several neurotransmitter systems to program the brain into a state of drug-seeking behavior [28]. Uncovering which of these is most sensitive to drug-induced gene regulation may allow for more targeted approaches to modify addictive behaviors. Since the ChIP protocol developed in this study is adaptable to potentially any BAC transgenic strain or

virally-transfected fluorescent protein reporter and histone modification, it has potential significance for uncovering a broad range of epigenetic mechanisms in neuronal subtypes.

Specific mechanisms that underlie the development of AUD are still unclear, potentially because ethanol consumption alters several neurotransmitter systems and interactions between these systems are complex [117]. Identifying ethanol-induced changes to neuronal subtypes may uncover which of these subtypes and adaptations are most relevant for ethanol action. As a proof of principle, Section 4 studied D1R MSNs in the NAc, which are potentiated by ethanol and involved in several ethanol-related behaviors (presented in Section 4); additionally, this study used a chronic intermittent vapor ethanol exposure that escalates drinking to identify ethanol-induced gene regulation in D1R MSNs. While microarray and ChIP results are currently pending, optimization studies reveal that the current protocol for studying gene regulation in D1R MSNs is technically sound. Taking an unbiased approach by using a whole genome expression array may also be a hypothesis-generating experiment, since it could identify as yet unknown mechanisms of chronic ethanol important in D1R MSNs. Though this study is new and relatively limited in scope, developing tools for epigenetic analysis of neuronal subtypes *in vivo* has potential for improving understanding of ethanol action.

In conclusion, these three varied experimental sections revealed new roles of epigenetics in ethanol action and ethanol-related behaviors. While the significance of these effects is still not fully known, they introduce the idea that ethanol has diverse roles in regulating gene expression and these are enacted by several mechanisms at the level of chromatin. More focused studies that manipulate chromatin modifying enzymes while studying ethanol-related behaviors are necessary to identify which of these are most critical for ethanol action.

5.1.2 Future Directions

This dissertation develops two distinct models. The first is a paternal alcohol model that studies heritability of AUD. The second is a cell-type specific model for studying ethanol-induced transcriptional regulation. Both of these models have a broad range of potential experiments to identify mechanisms of ethanol that are important for the development of AUD.

For the paternal alcohol model, questions surround both the molecular mechanisms and specific behavioral phenotype that underlies this effect. In particular, decreased ethanol consumption was noted at the beginning of a two bottle choice continuous access experiment when ethanol concentrations were low. However, there was no effect of paternal ethanol exposure on a limited access drinking in the dark experiment using solutions with a high ethanol concentration. Considering the complexity of this behavior and variability of individual mice in the experiment, it will be challenging to uncover specific mechanisms that regulate this effect. Changes to any one of several behavioral pathways could regulate this phenotype in mice – taste perception of alcohol, operant learning, stress associated with social isolation, or sensitivity to the effects of ethanol. Importantly, earlier studies found an effect of paternal ethanol on learning [171] and behaviors that are affected by stress, like grooming [173] and the forced-swim test [187]. While we did not see changes in basal behaviors in mice, other measures of stress, like measurement of corticosterone levels during a stressful paradigm, or learning may uncover effects on these measures in our model. Studying other operant models of ethanol preference or drinking, like the conditioned place preference test [287], may disentangle complex effects of paternal ethanol on neurodevelopment from a specific effect on ethanol preference.

Stress studies in ethanol-sired offspring may be especially important because of the previously identified effects of paternal stress on male offspring, which showed blunting of the

hypothalamic-pituitary axis and changes in several behavioral measures [77,95]. Additionally, considering ethanol exposure potentiates the hypothalamic-pituitary-adrenal axis and is a potent stressor [288], sires exposed to vapor ethanol may also experience chronic stress prior to mating. Stress experiments in offspring can be tied to ethanol-drinking and ethanol-related behaviors. For instance, mice escalate ethanol drinking under certain types of stress [289], so that the two bottle choice experiment may have detected an increase in ethanol drinking in the room air group following social isolation that was absent in ethanol-sired offspring. Therefore, exposing offspring to more potent stressors may reveal a greater difference between ethanol and room air sired groups on measures of drinking escalation. There was also a specific effect of paternal ethanol on ethanol sensitivity on the EPM; however, even this effect may be explained by blunting of a stress response following an ethanol injection rather than a change in sensitivity to ethanol-induced anxiolysis. Characterizing the corticosterone response after injection of 1 g/kg ethanol may reveal this effect, which could account for increased open arm entries in ethanol-sired male offspring. Additional effects of pain sensitivity after ethanol injection may also play into changes in stress response. Importantly, ethanol stimulates nociceptor receptors in gut afferents [290], so that increased ethanol-induced anxiolysis in ethanol-sired male offspring may reflect decreased response to the noxious effects of an i.p. injection with ethanol. Using capsaicin in the control condition or another mode of ethanol exposure may reduce the potentially confounding effects of pain on measures of anxiety. These studies will be important components of fully characterizing ethanol drinking and ethanol-related behaviors in offspring of ethanol-exposed sires.

There are several important studies related to other aspects of our paternal ethanol model. This study found decreased DNA methylation in sperm at the *IG* DMR and *Bdnf* promoter. It

will be important to characterize when these changes occur in sperm and how long they last by studying sperm at different time points of ethanol exposure. If changes to DNA methylation in sperm are found to underlie behavioral effects in offspring, they may be used as a biomarker for risk of inheriting ethanol-induced epigenetic modifications. Importantly, this type of biomarker can be tested non-invasively through sperm collection, so that our findings in rodent studies can also be tested in humans. While dams were only housed with sires for 2 days, it is possible that there is an effect of maternal provisioning, a change in maternal care related to exposure to a sire's fitness [91,94]. Cross-fostering experiments will identify the contribution of this component to the phenotype. Finally, better characterizing the sperm epigenome following ethanol exposure, through studies of sperm RNA populations and histone modifications, may identify other epi-alleles contributing to this phenotype.

Another important step for uncovering mechanisms of paternal ethanol's effects on offspring is studying changes in gene expression that underlie altered behaviors. Additionally, identifying altered genes that drive this phenotype may reveal genes that protect against high ethanol consumption. While this study identified increased *Bdnf* expression in the VTA of male offspring, several other changes are likely to exist considering the complexity of the phenotype presented. However, it is challenging to separate primary genes altered by a heritable epigenetic modification from those that have secondary changes in expression related to altering the primary gene. Whole-genome expression studies of offspring are an important future experiment that would identify networks of genes altered by paternal ethanol and key-in on affected pathways. These studies may also assist in establishing causation, since gene network analysis could identify which changes in expression are most robust and drive altered gene networks. Coupling these studies to DNA methylation analysis and/or miRNA expression analysis would

establish a mechanism by which altered gene expression is encoded in the genome. This type of analysis could also provide regions to screen in the F1 generation for potential transfer of epialleles to the F2 generation and the study of transgenerational effects.

In section 3, significant changes in global levels of histone modifications in CCx and HC following acute ethanol suggest broad epigenetic effects of ethanol. Unfortunately, a conserved program of histone modifications was not uncovered at the model gene promoters studied. Expanding this study to other histone modifications, like histone phosphorylation that was found to be altered by memory induction [291], may identify such an effect. Additionally, studying smaller brain regions affected by ethanol, like the NAc and VTA, or individual cell types may reduce background signal and increase sensitivity to identify histone modifications at gene promoters using ChIP. This type of study may also be done using *in vitro* cell culture experiments, where the effects of ethanol can be studied on a homogenous cell type in a very controlled environment. *In vitro* experiments would also separate primary effects of ethanol on histone modifications from secondary effects of altered neurotransmitter signaling that may be driving some ethanol-induced histone modifications *in vivo*. However, these *in vitro* findings will likely be challenging to replicate *in vivo* considering complexity of ethanol action. Therefore, while a gene-specific epigenetic program was not identified in CCx, one may be identified in the future by expanding histone modifications studied and changing other parameters of the experiment.

The model in Section 4 of this dissertation presents a more focused, cell-type specific method for studying ethanol-induced epigenetic regulation of gene expression. As described, this section is currently a proof of principle experiment to show that ethanol alters gene expression and histone modifications in D1R MSNs. However, if ethanol-induced effects are established in

this model, it can easily be adapted to mechanistic studies of behavior. Notably, variability in drinking escalation following chronic vapor ethanol exposure may be explained by epigenetic or transcriptional differences in key cell types. It would be especially interesting if mice resistant to drinking escalation failed to show expression of a relevant gene or epigenetic priming of a gene promoter activated by ethanol. Aside from epigenetic studies, microarray results on their own can be used to develop hypotheses about cell-type specific effects of ethanol. These can be investigated using cell-type specific knockdown of differentially expressed genes or developing integrated ethanol response networks by studying whole genome expression in multiple cell types. Finally, FACS and BAC technology allows for comparing multiple cell types within a single animal, so that cell types can be compared for their response to ethanol. A D1R vs. D2R study will be an important follow-up to this experiment, since these cell types appear to mediate different properties of ethanol [292,293]. All of these approaches will likely answer important questions regarding which cell types and genes are most critical for ethanol action.

In conclusion, this dissertation introduces several important aspects of epigenetic effects of ethanol by developing new models of ethanol-induced gene regulation; however, several important questions remain and the alcoholism field is only beginning to study specific mechanisms by which ethanol regulates gene expression. The future studies presented here will expand on the effects of ethanol on heritability of ethanol drinking behaviors and changes in histone modifications in the brain, providing more specific mechanisms that underlie the results. These will form the foundation to broader experiments on heritability of AUD and targeted approaches to modulate chromatin modifying enzymes to reduce the burden of AUD.

APPENDIX A

DISSOCIATION AND FLUORESCENCE ACTIVATED CELL SORTING OF THE NUCLEUS ACCUMBENS AND DOWNSTREAM APPLICATIONS: RNA ISOLATION AND CHROMATIN IMMUNOPRECIPITATION

Papain dissociation and cell sorting are adapted from Crook and Housman, 2012, PNAS, 109: 7487. We are using the Drd1a:tdTomato mouse (Jax #016204) but this protocol can be used for any mouse expressing a fluorescent protein.

Reagents and materials:

- **HABG** (prepare fresh day of experiment):
  - 9.8 ml Hibernate A (Gibco # A12475-01)
  - 200 ul B27 (Gibco # 17504-044)
  - 25 ul Glutamax (Gibco #35050-061)
- **Papain dissociation buffer** (prepare fresh day of experiment):
  - 5.5 mL Hibernate E (Brain Bits)
  - 14 ul Glutamax (Gibco #35050-061)
- Papain (Worthington Biochemical #LK003176)
- Trizol LS (Ambion #10296-028)
- DNase (Worthington Biochem #LK003170)
- Ribolock RNase Inhibitor (Fermentas #EO0381)
- Sodium butyrate (Sigma, #B5887)
- Protease Inhibitor Cocktail (Roche cOmplete, # 04693116001)
- Magna ChIP protein A/G beads (Millipore, #16-663)
- Micrococcal nuclease (New England Biolabs, #M0247S)

- Antibodies: anti-H3K18ac (#9675, Cell Signaling Technology), anti-H3K27me3 (#17-622, Millipore), nonspecific IgG as a negative control (#PP64B, Millipore)
- Aluminum foil
- Rodent stainless steel brain matrix for 1 mm coronal slices (Zivic Instruments)
- Pasteur pipets fire polished to a 0.5 mm opening
- Surgical equipment: PBS, razors, scalpels, forceps, etc.
- 15 ml and 50 ml RNase-free tubes
- 70 ul cell strainer
- Nuclease free 15 ml (Invitrogen, # AM12500) and 50 ml (#AM12501) conical tubes
- Petri dishes
- Small Tissue Culture Dish (Falcon #353001)
- Dissecting microscope
- Pipets and cut pipet tips
- Nuclease free water, not DEPC-treated (Ambion, #AM9937)
- RNeasy mini kit
- RNase Zap (Ambion, # AM9780)
- Qiagen MinElute Reaction Cleanup Kit
- Magnetic tube rack (Millipore)

Prepare ChIP reagents (These can be stored at room temperature for weeks):

- **2x MNase buffer**: 100 mM Tris, pH 8.0, 2 mM $CaCl_2$, 0.4% Triton X-100
- **1x MNase buffer**: 50 mM Tris, pH 8.0, 1 mM CaCl2, 0.2% Triton X-100
- **10x MNase stop buffer**: 110 mM Tris, pH 8.0, 55mM EDTA
- **2x RIPA lysis buffer**: 280 mM NaCl, 1.8% Triton X-100, 0.2% SDS, 0.2% Na Deoxycholate, and 5 mM EGTA
- **1x RIPA wash buffer**: 10 mM Tris, pH 8.0, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% Na Deoxycholate
- **TE buffer**: 10 mM Tris, pH 8.0, 1 mM EDTA
- **Elution buffer**: 50 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1% SDS, 50 µg/ml proteinase K
- **3 M Na Acetate**

Prepare DNase, Papain, and HABG fresh on the day of the experiment:

- DNase: add 500 ul Papain dissocation buffer to vial of DNase and invert to mix; store on ice
- Papain: add 5 ml Papain dissociation buffer to vial of Papain and invert to mix; pipet 2 ml into 15 ml nuclease-free tube

    - Incubate papain in 15 ml tube at 37 degrees C for ~20 minutes prior to dissociation
- HABG: Pipet 1 ml HABG into Small Tissue Culture Dish and add 2.5 ul Ribolock (~100 U/ml)

Prepare surgical equipment:

- Clean all equipment thoroughly with 70% EtOH
- Incubate brain matrix on ice for at least 15 minutes
- Place 4 razors in ice cold PBS for brain matrix
- Cut ~1 cm off the tip of four 1 ml pipet tips using a clean razor blade

Dissociation Protocol:

1. Sacrifice mouse by cervical dislocation and decapitate using razor blade
2. Extract whole brain from skull and place into chilled brain matrix
3. Insert razor blades into matrix to generate 1 mm thick coronal slices (about 5 razor blades starting at the rostral end of the brain are sufficient to cover the full length of the NAc)
4. Dissect NAc core from slices carefully; do not collect overlying caudate (caudate tissue appears striated under the microscope while NAc tissue is solid and greyish surrounding the anterior commissure)
5. Place NAc pieces into 1 mL HABG supplemented with Ribolock (100 U/ml) and cut into ~1 mm pieces
6. Remove Papain from incubator and add 110 ul DNase to tube
7. Use cut pipet tip to collect NAc pieces and place tip into incubated Papain – allow the tissue pieces to slowly fall into Papain and gently agitate the pipet tip to encourage them to leave the pipet; DO NOT pipet HABG directly into Papain, as the ions will inhibit dissociation
8. Cover with aluminum foil and incubate in papain at 32 degrees C for 15 minutes with gentle rotation
9. While dissociating, pipet 2 ml HABG into a 15 ml RNase-free tube; add 5 ul Ribolock (~100 U/ml), sodium butyrate to 5 mM (10 ul of 1 M stock), 200 ul of 10x protease inhibitor in PBS
10. **For ChIP only,** prepare 250 ul MNase buffer supplemented with sodium butyrate to 5 mM and protease inhibitor in a 15 ml RNase-free tube
11. **For RNA only,** prepare 750 ul Trizol LS in a 15 ml RNase-free tube
12. After 20 minutes, pipet tissue pieces from papain using a cut pipet tip and place tip into HABG supplemented with Ribolock; allow tissue pieces to slowly fall into HABG and agitate tip as necessary (you may have to repeat this a couple of times as tissue pieces are stickier in Papain)
13. Cover tube with aluminum foil and incubate at room temperature for 5 minutes

14. While incubating, fit a 70 um cell strainer over the top of a 50 ml RNase-free conical tube
15. Triturate the tissue pieces using a fire polished Pasteur pipet with a small opening (~0.5 mm) – pipet pieces up and down over 4 seconds about 10-20 times until there are no longer large visible pieces; the HABG should now appear cloudy
16. Use a cut pipet tip to pipet the dissociated cell suspension in HABG (800 ul at a time) through the 70 um cell strainer and let solution filter
17. Add ~5ml HABG supplemented with 10 ul Ribolock (~100 U/ml), sodium butyrate to 5 mM (25 ul of 1 M stock), 500 ul of 10x protease inhibitor in PBS directly through the cell strainer so that the total volume is ~7 ml
18. Cover tube with aluminum foil to prevent quenching of fluorescence and proceed directly to sorting; keep tube at room temperature

Cell sorting:

These parameters will change depending on the machine and type of fluorescent protein. Work with the flow cytometry core to get the best separation of fluorescent cells with highest yield. Using the FACS Aria II (Becton Dickinson) with an 85 μm nozzle and a pressure of 45 p.s.i, we have had success isolating neurons by setting the gates to side scatter (SSC) less than 150,000 and forward scatter (FSC) greater than 50,000. Set gates for fluorescence by comparing wild type strain to fluorescent strain. Sort cells directly into Trizol LS (for RNA) or 2x MNase (for ChIP) to prevent degradation of RNA and chromatin, respectively.

RNA isolation:

1. Prepare an RNase free surface and clean pipets using an RNase inhibitor (we use RNase Zap)
2. Centrifuge Trizol LS with cells at 200 g for a few seconds to bring down cells into Trizol
3. Transfer Trizol LS with sorted cells to RNase free 1.5 mL eppendorf tube and check volume; add RNase free water to bring volume up to 1 ml
4. Add 200 ul chloroform and shake vigorously for 15 seconds
5. Allow tube to sit at room temperature for 2 minutes
6. Centrifuge at 10,000 g for 8 minutes at 4 degrees C
7. Transfer aqueous layer to a new tube (~550 ul)
8. Add 1 volume of 70% EtOH to aqueous layer and mix by pipetting mixture
9. Add 700 ul of solution to RNeasy mini column and centrifuge at 10,000 g for 1 minute at room temperature with slow acceleration
10. Repeat this step with remaining solution
11. Add 700 ul buffer RW1 and centrifuge at 10,000 g for 30s at room temperature
12. Add 500 ul buffer RPE and centrifuge at 10,000 g for 30s at room temperature
13. Repeat step 10
14. Transfer to a new collection tube and centrifuge at 10,000 g for 1 minute at room temperature to remove residual ethanol

15. Transfer to an RNase free 1.5 mL eppendorf tube, add 30 ul RNase free water directly to the membrane, and incubate at room temperature for 2 minutes
16. Centrifuge at 10,000 g for 1 minute at room temperature
17. Store RNA at -80 degrees C until ready to use
18. For RT-qPCR, this protocol used a 30 ul Bio-Rad RT reaction (23 ul RNA in water, 6 ul 5x RT reaction buffer, 1 ul RT); 5 ul of this this reaction per well. This can be done for assessing expression of 3 genes in duplicate; diluting the final RT reaction can increase the number of detected genes, but will reduce signal.

ChIP procedure:

1. Sort cells directly into 250 µl of 2x MNase buffer supplemented with sodium butyrate to 5 mM and protease inhibitor cocktail
2. Centrifuge briefly at 100 g to bring any residual buffer to the bottom of the tube
3. Collect 2x MNase buffer with sorted cells into a 1.5 ml nuclease free Eppendorf tube
4. Bring volume up to 500 µl by adding nuclease free water (all steps are at room temperature at this point)
5. Dilute MNase by adding 1 µl of MNase (2,000 gel units) to 500 µl 1x MNase buffer
6. For MNase digestion of chromatin, add 15 µl of diluted MNase to sorted cells and cells and incubate in a 37˚ C heat block for 5 minutes
7. To stop the MNase digestion, add 50 µl of 10x MNase stop buffer to the solution.
8. From this point, chromatin is kept on ice or in a 4˚ C room
9. To lyse cells, add 550 µl of ice cold 2x RIPA cell lysis buffer supplemented with sodium butyrate up to 5 mM and protease inhibitor cocktail
10. Mixed by inverting the tube and leave on ice for 15 minutes
11. To remove cell debris, centrifuge chromatin at 4˚ C at 13,000 rpm for 10 minutes
12. Decant the supernatant into a new 1.5 ml Eppendorf tube and place on ice
13. Chromatin volume should be ~1 mL at this point. Divide this volume into two 400 µl aliquots for immunoprecipitation (IP) and one 200 µl aliquot to be used as input
14. For IP reactions add 22 µl of MagnaChIP Protein A/G magnetic beads to each tube
15. Add antibodies to each IP tube (this will vary for each antibody and needs to be optimized). It may be challenging to quantify chromatin if it is divided into more than 2 IP reactions; though this could be possible with a larger number of cells collected. For this experiment, 5 µl of an anti-H3K27me3 antibody was added to one tube and 9 µl of an anti-H3K18ac antibody was added to the other tube. It is also important to perform a negative control initially to ensure the ChIP reaction is specific, which can be done by adding 5 µl of nonspecific IgG antibody in place of one of the antibodies.
16. Incubate the immunoprecipitation tubes at 4˚ C with gentle end-over-end rotation for 3.5 hours; keep the input tube at 4˚ C with no rotation during this time

17. After incubation, place IP tubes onto a magnetic rack and remove the supernatant using a pipet (be careful not to agitate the magnetic beads; no magnetic beads should be removed by the pipet tip with the supernatant at any point).
18. Elute magnetic beads in 500 µl RIPA wash buffer and incubate at 4˚ C with gentle end-over-end rotation for 5 minutes; after incubation, place tubes on the magnetic rack and remove supernatant as before
19. Repeat the RIPA wash buffer step two more times (3 total washes in RIPA wash buffer), then wash the magnetic beads 1 time with 500 µl TE buffer (these may need to be optimized as well depending on the antibody, though these washes are sufficient to remove background using the antibodies in this study)
20. Following the TE wash step, elute magnetic beads in 200 µl elution buffer
21. For the input, add proteinase K to a concentration of 50 µg/ml
22. Incubate the magnetic beads in elution buffer and input at 65˚ C with gentle rotation for 45 minutes
23. Following proteinase K digestion, briefly spin tubes on a tabletop centrifuge and place them into a magnetic rack
24. Transfer the supernatant (200 µl) to a tube containing 10 µl 3 M Na Acetate and 1 ml buffer PB
25. Purify DNA using the Qiagen MinElute Reaction Cleanup Kit according to the manufacturer's protocol and elute in 14 µl nuclease free water
26. For this study, DNA from IP reactions was quantified by the Pitt Genetics and Proteomics Core Lab using a Qubit Fluorometer; the amount of DNA recovered will depend on the number of cells collected but should be ~1 ng/ul for the input and H3K27me3 reactions and ~0.7 ng/ul for the H3K18ac reaction from ~20,000 cells; there should be no DNA detected using the nonspecific IgG antibody.
27. Following IP reactions, levels of enrichment for a particular locus can be quantified using qPCR or other downstream quantitative application
28. For this study, 16 ul of nuclease-free water were added to eluted DNA (14 ul) for a total of 30 ul. 5 ul of diluted DNA were used in each well as a template for a qPCR reaction. Therefore, 3 primer pairs were tested in duplicate, though this can be optimized depending on number of cells collected and antibody enrichment.

APPENDIX B

EXPRESSION OF CHROMATIN MODIFYING ENZYMES IN THE CEREBRAL CORTEX FOLLOWING ACUTE ETHANOL

Gene	Fold Regulation	p-value	Gene Function
Kdm1a	-0.27	0.27	Lysine (K)-specific demethylase 1A
Ash1l	-0.30	0.33	Ash1 (absent, small, or homeotic)-like
Atf2	0.17	0.80	Activating transcription factor 2
Aurka	0.37	0.42	Aurora kinase A
Aurkb	0.20	0.48	Aurora kinase B
Aurkc	-0.10	0.64	Aurora kinase C
Carm1	-0.13	0.58	Coactivator-associated arginine methyltransferase 1
Cdyl	-0.30	0.35	Chromodomain protein, Y chromosome-like
Ciita	0.03	0.93	Class II transactivator
Csrp2bp	**-0.77**	**0.08**	**Cysteine and glycine-rich protein 2 binding protein**
Dnmt1	0.23	0.74	DNA methyltransferase 1
Dnmt3a	0.20	0.36	DNA methyltransferase 3A
Dnmt3b	0.20	0.77	DNA methyltransferase 3B
Dot1l	-0.20	0.26	DOT1-like, histone H3 methyltransferase
Dzip3	0.00	0.96	DAZ interacting protein 3, zinc finger
Ehmt1	0.10	0.94	Euchromatic histone methyltransferase 1
Ehmt2	-0.30	0.33	Euchromatic histone lysine N-methyltransferase 2
Esco1	-0.30	0.50	Establishment of cohesion 1 homolog 1
Esco2	-0.43	0.21	Establishment of cohesion 1 homolog 2
Hat1	-0.10	0.49	Histone aminotransferase 1
Hdac1	0.27	0.52	Histone deacetylase 1
Hdac10	0.13	0.40	Histone deacetylase 10
Hdac11	**-0.40**	**0.00**	**Histone deacetylase 11**

Gene	Fold Regulation	p-value	Gene Function
Hdac2	**-2.03**	**0.20**	**Histone deacetylase 2**
Hdac3	0.33	0.21	Histone deacetylase 3
Hdac4	-0.10	0.51	Histone deacetylase 4
Hdac5	-0.13	0.53	Histone deacetylase 5
Hdac6	-0.30	0.16	Histone deacetylase 6
Hdac7	-0.17	0.33	Histone deacetylase 7
Hdac8	-0.80	0.22	Histone deacetylase 8
Hdac9	-0.33	0.27	Histone deacetylase 9
Kdm5b	-0.23	0.33	Lysine (K)-specific demethylase 5B
Kdm5c	-0.10	0.61	Lysine (K)-specific demethylase 5C
Kdm4a	-0.10	0.50	Lysine (K)-specific demethylase 4A
Kdm4c	-0.03	0.92	Lysine (K)-specific demethylase 4C
Kdm6b	-0.37	0.27	KDM1 lysine (K)-specific demethylase 6B
Kat2a	-0.17	0.64	K(lysine) acetyltransferase 2A
Kat2b	**0.93**	**0.05**	**K(lysine) acetyltransferase 2B**
Kat5	0.83	0.17	K(lysine) acetyltransferase 5
Mll3	-0.03	0.76	Myeloid/lymphoid or mixed-lineage leukemia 3
Mll5	0.73	0.19	Myeloid/lymphoid or mixed-lineage leukemia 5
Mysm1	-0.33	0.27	Myb-like, SWIRM and MPN domains 1
Myst1	0.23	0.16	MYST histone acetyltransferase 1
Myst2	0.03	0.91	MYST histone acetyltransferase 2
Myst3	0.13	0.56	MYST histone acetyltransferase (monocytic leukemia) 3
Myst4	-0.40	0.27	MYST histone acetyltransferase monocytic leukemia 4
Ncoa1	-0.20	0.23	Nuclear receptor coactivator 1
Ncoa3	0.10	0.95	Nuclear receptor coactivator 3
Ncoa6	0.27	0.36	Nuclear receptor coactivator 6
Nek6	-0.13	0.49	NIMA (never in mitosis gene a)-related expressed kinase 6
Nsd1	0.10	0.36	Nuclear receptor-binding SET-domain protein 1
Pak1	-0.10	0.60	P21 protein (Cdc42/Rac)-activated kinase 1
Prmt1	-0.17	0.26	Protein arginine N-methyltransferase 1
Prmt2	-0.23	0.46	Protein arginine N-methyltransferase 2
Prmt3	-0.03	0.81	Protein arginine N-methyltransferase 3
Prmt5	0.07	0.80	Protein arginine N-methyltransferase 5
Prmt6	0.30	0.29	Protein arginine N-methyltransferase 6
Prmt7	-0.10	0.65	Protein arginine N-methyltransferase 7
Prmt8	0.13	0.72	Protein arginine N-methyltransferase 8
Rnf2	-0.13	0.55	Ring finger protein 2
Rnf20	-0.20	0.53	Ring finger protein 20
Rps6ka3	-0.33	0.23	Ribosomal protein S6 kinase polypeptide 3
Rps6ka5	0.17	0.43	Ribosomal protein S6 kinase, polypeptide 5

Gene	Fold Regulation	p-value	Gene Function
Setd1a	0.00	0.94	SET domain containing 1A
Setd1b	-0.03	0.85	SET domain containing 1B
Setd2	-0.07	0.73	SET domain containing 2
Setd3	0.10	0.82	SET domain containing 3
Setd4	0.30	0.33	SET domain containing 4
Setd5	0.20	0.61	SET domain containing 5
Setd6	-0.13	0.65	SET domain containing 6
Setd7	-0.07	0.70	SET domain containing (lysine methyltransferase) 7
Setd8	0.20	0.77	SET domain containing (lysine methyltransferase) 8
Setdb1	-0.03	0.88	SET domain, bifurcated 1
Setdb2	0.27	0.27	SET domain, bifurcated 2
Smyd1	0.20	0.15	SET and MYND domain containing 1
Smyd3	0.00	0.99	SET and MYND domain containing 3
Suv39h1	0.07	0.23	Suppressor of variegation 3-9 homolog 1
Suv420h1	-0.23	0.31	Suppressor of variegation 4-20 homolog 1
Ube2a	0.89	0.51	Ubiquitin-conjugating enzyme E2A, RAD6 homolog
Ube2b	0.91	0.44	Ubiquitin-conjugating enzyme E2B, RAD6 homology
Usp16	1.04	0.92	Ubiquitin specific peptidase 16
Usp21	0.89	0.61	Ubiquitin specific peptidase 21
Usp22	1.20	0.45	Ubiquitin specific peptidase 22
Whsc1	1.14	0.40	Wolf-Hirschhorn syndrome candidate 1

*Bold genes were validated using additional samples (Fig. 19)

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