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LOYOLA UNIVERSITY CHICAGO

MECHANISMS AND CONSEQUENCES OF EPIGENETIC INHERITANCE

FOLLOWING PARENTAL PRECONCEPTION ALCOHOL EXPOSURE

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY

ANNADOROTHEA ASIMES

CHICAGO, IL

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS.....	x
ABSTRACT.....	xii
CHAPTER I: STATEMENT OF THE PROBLEM.....	1
CHAPTER II: INTRODUCTION	3
Literature Review	3
Epigenetics: Environment Meets Genetics	3
DNA Methylation – Writers, Readers, and Erasers.	4
Functional Meaning of DNA in Different Parts of the Genome.	7
Age-Driven Changes in Somatic Methylation.....	9
Epigenetic Reprogramming During Embryonic Development.	10
Multigenerational Epigenetic Inheritance.	12
Adolescence: Development and Drinking.....	15
Inheritance of Parental Alcohol Exposure.....	17
Molecular Actions of Alcohol in Somatic Cells- Non-Genomic.....	18
Molecular Actions of Alcohol in Somatic Cells– Genomic and Epigenomic.	21
Alcohol and the Hypothalamus.....	25
Summary.....	28
CHAPTER III: ADOLESCENT BINGE-PATTERN ALCOHOL EXPOSURE ALTERS GENOME WIDE DNA METHYLATION PATTERNS IN THE HYPOTHALAMUS OF ALCOHOL NAÏVE MALE OFFSPRING	32
Introduction.....	32
Results	35
Parental Ethanol Exposure Induces Differentially Methylated Cytosine Residues in the Hypothalamus Of Male Ethanol-Naïve Offspring.....	35
Differentially Methylated Cytosines Were Distributed Across All Chromosomes and the Extent of Hypo- Versus Hypermethylation was Dependent on Parental Ethanol Exposure. 41	41
DMCs were Primarily Observed Outside Of CpG Islands and in Intergenic Regions.....	46
mRNA Expression of Differentially Methylated Genes is Altered in the Hypothalamus.....	49
Discussion	52
CHAPTER IV: PARENTAL PRECONCEPTION ALCOHOL EXPOSURE IMPACTS OFFSPRING DEVELOPMENT THROUGH PUBERTY.....	59
Introduction.....	59

Results	61
Experiment 1: Baseline Characteristics of Male and Female Offspring from Parents Administered Preconception Binge-Pattern EtOH During Adolescence (Fig 9-13)	61
Experiment 2: Effects of Adolescent EtOH Exposure in Offspring with Parental History of Preconception EtOH Consumption (Fig 14-18)	69
Experiment 3: Tracking Transcriptional Regulation of Candidate Genes in the Offspring Hypothalamus Through Development Following Parental EtOH Exposure (Tables 7, 8).....	77
Discussion	80
CHAPTER V: "FATTY BRAIN" FOLLOWING PUBERTAL BINGE ETHANOL EXPOSURE	88
Introduction.....	88
Results	91
ApoE was Increased in Adult Males Following Pubertal EtOH Exposure.	91
Lipid-Associated Genes are Unaltered in Juvenile and Adolescent Offspring Hypothalamus.	92
Primary Astrocytes Cultured from Adult Males Showed Increased Cholesterol Accumulation.	93
Fatty Astrocytes are Less Neuroprotective Against Toxic Insult.	96
Discussion	97
CHAPTER VI: FINAL DISCUSSION.....	101
Summary.....	101
Key Findings.....	103
Final Thoughts	106
Maternal AND Paternal Preconception Behaviors Influence Offspring.	106
Epigenomics is Invaluable, but Cannot Give the Whole Picture Yet.	107
Epigenetic Inheritance is not Necessarily Adaptive for Offspring.	109
Binge Consumption Patterns Signify a Unique Stressor to the Body.	111
Future Directions	113
CHAPTER VII: GENERAL METHODS.....	120
Ethics Statement.....	120
Animal Paradigms.	120
Chapter III.	121
Methylation Sequencing and Statistics.....	122
Reverse Transcription Quantitative PCR (RT-qPCR)	124
Chapter IV, Experiment 1.....	126
Chapter IV, Experiment 2.....	126
Tissue Collection.	127
Enzyme-Linked Immunosorbent Assay (ELISA).	127
Reverse Transcription Quantitative PCR (RT-qPCR)	128

Testes Histology and Analysis.....	129
Play Behavior.....	129
Statistics.....	130
Chapter V.....	130
Primary Astrocyte Culture.....	130
Neuronal Protection Assay.....	131
Cholesterol Quantification.....	131
Reverse Transcription Quantitative PCR (RT-qPCR).....	132
Statistics.....	132
APPENDIX A: CHAPTER III SUPPLEMENTARY DATA	133
APPENDIX B: CHAPTER IV SUPPLEMENTARY DATA.....	156
APPENDIX C: CHAPTER V SUPPLEMENTARY DATA	163
APPENDIX D: ALCOHOL METABOLISM TIMECOURSE	167
APPENDIX E: HYPOTHALAMIC GENE EXPRESSION CHANGES IN ADULTHOOD.....	169
APPENDIX F: THE EFFECTS OF ALCOHOL ON EPIGENETIC ENZYMES <i>IN VIVO</i> AND <i>IN VITRO</i>	171
LIST OF REFERENCES.....	179
VITA	190

LIST OF TABLES

Table 1. Preconception binge alcohol exposure did not alter litter size, sex ratio or offspring growth.....	37
Table 2. Alignment efficiencies and conversion rates for ERRBS of all samples.	38
Table 3. Top 5 Hyper and 5 Hypomethylated DMCs as ranked by greatest percentage difference for each treatment group.	45
Table 4. Genes associated with differential promoter methylation for each treatment group...	48
Table 5. Summary data for mRNA expression of DMC-associated genes in male offspring.	51
Table 6. Summary data for mRNA expression of DMC-associated genes in female offspring.	52
Table 7. Gene expression changes in male and female offspring at PND 44.	79
Table 8. Gene expression changes in second litter male and female offspring at PND 7.	80
Table 9. RT-qPCR Primer Sequences for Chapter III.	125
Table 10. RT-qPCR primer sequences for Chapter V.	132

LIST OF FIGURES

Figure 1. Cytosine methylation and demethylation.....	5
Figure 2. Schematic representation of HPA axis function and feedback.	26
Figure 3. Adolescent binge alcohol exposure paradigm.	36
Figure 4. Annotated genes associated with hypermethylated residues vary between treatment groups.....	40
Figure 5. Annotated genes associated with hypomethylated residues vary between treatment groups.....	41
Figure 6. Distribution of differentially methylated cytosines across chromosomes.....	43
Figure 7. Location of differentially methylated cytosines within CpG and functional regions of the genome.....	47
Figure 8. Animal treatment paradigm for Experiment 1 and 2.. ..	62
Figure 9. Offspring of EtOH-treated parents were smaller after pubertal onset and displayed fewer play behaviors.. ..	63
Figure 10. Offspring of EtOH-treated parents had changes in baseline HPG axis parameters.. ..	65
Figure 11. Gene markers of mature sperm and testicular morphology were not altered by parental EtOH exposure.. ..	66
Figure 12. Baseline CORT in male, but not female, offspring was decreased following parental EtOH exposure.....	68
Figure 13. mRNA expression of glucocorticoid receptors and neuropeptides that mediate HPA axis were not affected by parental EtOH consumption.....	69
Figure 14. Body weight decreased in female EtOH-treated offspring of EtOH-treated parents..	71
Figure 15. EtOH-treated offspring of EtOH-treated parents had decreased HPG axis parameters.	73
Figure 16. Gene markers of mature sperm and testicular morphology were not altered by offspring EtOH exposure.....	74
Figure 17. Circulating corticosterone (CORT) response to EtOH exposure was not dependent on parental history of EtOH treatment.....	76

Figure 18. Parental history of EtOH exposure did not affect offspring mRNA expression of glucocorticoid receptors and neuropeptides in response to EtOH..	77
Figure 19. Ages of investigation for dysregulation of lipid homeostasis.....	91
Figure 20. ApoE expression was dysregulated in a sex-specific manner in the adult hypothalamus.....	93
Figure 21. Primary astrocytes from EtOH-treated males are “fatty” and overexpress ApoE.....	95
Figure 22. Fatty astrocytes confer less protection to neurons against toxic insult.....	97
Figure 23. Lipid-associated genes with differential methylation in alcohol-naïve offspring following parental preconception EtOH exposure (Experiment 1)	100
Figure 24. Visual representation of summary of findings.	102

LIST OF ABBREVIATIONS

5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine
5fC	5-formylcytosine
5caC	5-carboxylcytosine
ApoE	Apolipoprotein E
Arrdc1	Arrestin domain containing protein 1
ADHD	Attention-Deficient Hyperactivity Disorder
BER	Base excision repair
TET1	Ten eleven translocation methylation dioxygenase 1
BAC	Blood alcohol concentration
bp	Base pair
C	Cytosine
CNS	Central Nervous System
CpG	Cytosine-phosphodiester-guanine dinucleotide pair
CDS	Coding DNA sequence
CGI	CpG island
DMC	Differentially methylated cytosine
DNMT	DNA methyltransferase

Ephb3	Ephrin type B receptor 3
ERRBS	Enhanced Reduced Representation Bisulfite Sequencing
EtOH	Ethanol
Esam	Endothelial cell adhesion molecule
FASD	Fetal alcohol spectrum disorder
GABA	γ -aminobutyric acid
GR	Glucocorticoid receptor
HMGB	High-mobility group box
ICR	Imprinting control region
miR	microRNA
PND	Post-natal day
SAM	S-adenosyl methionine
SAH	S-adenosyl homocysteine
TDG	Thymine DNA glycosylase
THC	Tetrahydrocannabinol
TLR	Toll-like receptor
TSS	Transcription start site
UTR	Untranslated region

ABSTRACT

Recent advances in genomics research have revealed that preconception behaviors and experiences of mothers and fathers, including diet, environmental toxicants, and drug abuse, can impact future offspring through epigenetic mechanisms. This means that the risky behaviors of young people, such as the extremely popular practice of binge drinking, have potentially far-reaching consequences for generations to come. While there has been considerable research into fetal alcohol exposure and parental alcoholism, there has yet to be sufficient investigation into the mechanism of epigenetic inheritance or the functional consequences of parental preconception binge pattern alcohol abuse. The hypothesis tested herein is that parental preconception alcohol exposure can impact offspring through epigenetic inheritance of DNA methylation patterns in the hypothalamus, leading to impaired hypothalamic function during development and a predisposition to neurodegeneration. This dissertation reveals that 1) male offspring of both maternal and paternal preconception alcohol exposure have genome-wide changes in methylation patterns in the hypothalamus, 2) offspring have altered hypothalamic function resulting in modest phenotypic and behavioral changes lasting through pubertal development, 3) parental preconception alcohol exposure does not confer advantages to offspring for improved alcohol metabolism, and 4) lipid homeostasis may be disrupted in the brain of alcohol-naïve offspring following parental alcohol exposure, as well in the brain of the parents themselves. These results suggest that parental preconception alcohol exposure confers maladaptive epigenetic traits to first generation offspring.

CHAPTER I

STATEMENT OF THE PROBLEM

Inheritance extends beyond mere genetics, or DNA sequence, and can impact offspring traits through epigenetic mechanisms. Recent work has shown that preconception behaviors of both mothers and fathers, such as drug abuse, can impact offspring outcomes. The most widely abused drug in the United States is alcohol with more than 5.3 million Americans under the age of 21 engaging in binge pattern drinking. This type of rapid consumption during pubertal development has been linked to increased cognitive health risks late into adulthood, and the mechanism of this transmission is thought to be epigenetic. Our lab has previously shown functional dysregulation of the stress response in the adult brain following repeated alcohol exposure during pubertal development. This dysregulation occurs at both the molecular and behavioral level, stemming from altered gene expression in the hypothalamus, a region of the brain involved in regulation of pubertal development, stress regulation, and behavior.

Additionally, we have shown that alcohol-naïve offspring of animals exposed to alcohol during adolescence show altered gene expression profiles in the hypothalamus as neonates. Although alterations in the epigenetic landscape have been shown to be central to the inheritance of effects of other drugs of abuse, there has yet to be sufficient work into the effects of preconception binge-pattern alcohol exposure on future offspring. Therefore, my current work aimed to investigate the epigenetic landscape in offspring after parental alcohol exposure and

characterize functional consequences in these offspring. My hypothesis is that parental preconception alcohol exposure can impact offspring through epigenetic inheritance of DNA methylation patterns in the hypothalamus, leading to dysfunctional hypothalamic function during development. Once we can determine the mechanisms by which ethanol causes long-term and inherited changes in the brain, we can more easily study these consequences and investigate potential therapeutics. Furthermore, this will provide a better understanding of the mechanisms involved in developmental epigenetic regulation during adolescence and its vulnerability to environmental changes.

CHAPTER II

INTRODUCTION

Literature Review

Epigenetics: Environment Meets Genetics.

Mounting evidence shows that the nucleotide sequence of DNA is not the only important carrier of information in genetic material. Epigenetics is the term ascribed to functionally relevant changes to the expression of genes that do not arise from changes, or mutations, in DNA itself. These can include methylation of the DNA, modifications to the histone proteins which package the DNA, and actions of non-coding RNA species which influence messenger RNA availability. Epigenetic modifications are important for normal organism development, cellular differentiation, sex-specific brain development, and dosage compensation/allele silencing. However, dysregulated epigenetic control has also been found to be involved in the development of many diseases, particularly neurodegenerative and cognitive disorders. Epigenetic modifications can arise or change throughout a lifetime, and many of the changes are attributed to environmental influence, extending from the immediate environment of a cell through the environmental exposures of an individual. The delicate balance of these epigenetic modifications underscores their importance as mediators of environment and genetics. The following literature review and dissertation will focus on DNA methylation, in particular, as it is a relatively stable and directly heritable epigenetic modification. I will

summarize the current understanding of the mechanisms and significance of DNA methylation and outline the field of inheritance of epimutations arising from environmental exposure, particularly those caused by alcohol abuse.

DNA Methylation – Writers, Readers, and Erasers.

Mammalian DNA consists of four nucleotide bases: adenine, thymine, guanine, and cytosine. Epigenetic regulation via methylation in mammals occurs mainly at cytosine residues in the context of CpG dinucleotides, which provides palindromic complementarity on the opposing strand of DNA (Bird, 2002; Jones, 2012). In this way, cytosine methylation can be copied from one DNA strand to the next during replication and meiosis. CpG islands (CGIs), defined as regions of the genome of about 1000 bp in length which are enriched in C and G nucleotides, tend to have uniform methylation patterns such that they are entirely unmethylated or fully methylated along their length. This regulation of CGIs is mostly likely due to spreading of methylation events along the chromosome from neighboring methylated cytosines, where the methylation enzymes can process along the region (Schübeler, 2015).

Cytosine residues are methylated by DNA methyltransferase (DNMT) enzymes. DNMT1 is known as the maintenance methyltransferase, reading hemimethylated DNA after cell division and adding methylation to the newly synthesized strand (Chen and Riggs, 2011). DNMT3a and 3b are *de novo* methyltransferases and can add methylation to previously unmethylated residues (Smith and Meissner, 2013). S-adenosyl methionine (SAM) acts as a universal methyl donor for these reactions and is converted to S-adenosyl homocysteine (SAH).

The cytosine residues are methylated on the 5' carbon and are, therefore, referred to as 5-methylcytosines (5mC, Fig 1). DNMTs have been shown to interact with other epigenetic regulators, such as histone modifiers and RNA polymerase enzymes, and show a concerted effort to regulate gene activity (Chen and Riggs, 2011; Holoch and Moazed, 2015; Matzke and Mosher, 2014).

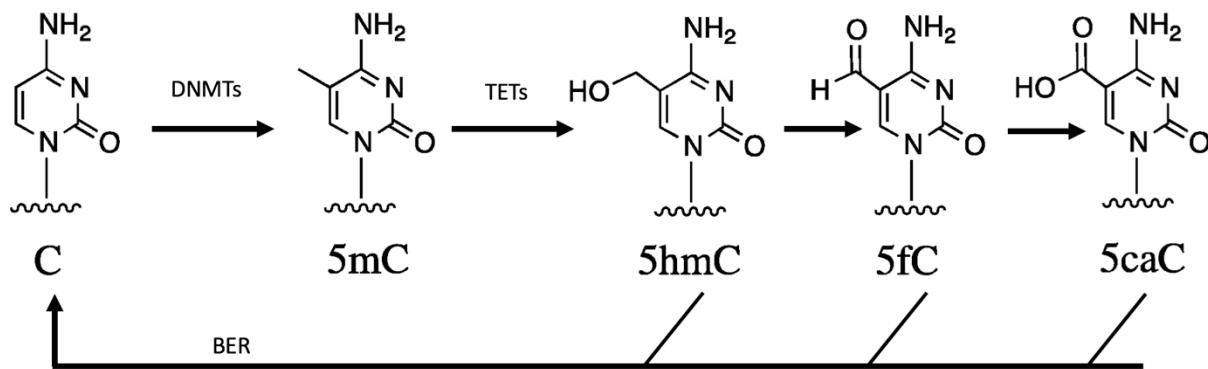


Figure 1. Cytosine methylation and demethylation. The structures of each modified base identified by their abbreviation. TET enzymes are responsible for each subsequent modification from 5mC, which is modified by DNMT enzymes. All modified bases, with the exception of 5mC, are thought to be returned to cytosine, or actively removed, through base excision repair mechanisms. All modified bases may also be removed through passive demethylation during replication.

Demethylation of cytosine is only partially understood, but can occur through both active and passive mechanisms. Passive demethylation is a result of inactive or underactive maintenance DNMTs during replication of the DNA where the new strand fails to receive a methylation mark on the corresponding CpG/GpC (Kohli and Zhang, 2013). After two replication cycles, methylation at that position is lost entirely. Active demethylation, on the other hand, is thought to be a result of several base modifications followed by base excision repair (BER) as opposed to the result of one specific enzymatic reaction (Weber et al., 2015). Methylated cytosines can undergo conversion by the TET family of methylcytosine dioxygenase enzymes

(TET1, 2, 3) to make 5-hydroxymethylcytosine (5hmC, Fig 1). 5hmC can be further oxidized by TETs to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) which can be recognized by the DNA repair protein thymine DNA glycosylase (TDG). TDG, which has been found to interact physically with TET1, excises the modified cytosine base and leads to recognition of the residue as a target for BER enzymes (Weber et al., 2015). However, hydroxymethylation and other intermediaries may also have their own epigenetic meaning (Schübeler, 2015). Although the mechanisms of cytosine methylation have been more or less described, our knowledge on the meaning of methylation marks and the regulatory cues signaling methylation changes are still in their infancy.

Methylated DNA is a true epigenetic mark as there are “readers” which preferentially recognize 5mC and can interpret the meaning of the methylation mark (Zhu et al., 2016). The classical family of these readers is known as methyl binding domain (MBD) proteins. These proteins act as intermediaries between methylated DNA and other proteins such as transcription factors. It has recently been suggested that some transcription factors, such as CEBP and KLF4, can interact with methylated DNA without an MBD (Zhu et al., 2016). There are other transcription factors which are inhibited from binding the DNA when there is methylation in the recognition site, which serves as the opposite signal of the epigenetic mark (Zhu et al., 2016). Methylation of a cytosine residue is a binary state – either methylation or no methylation. However, when a particular tissue is discussed, methylation is given in a percentage or relative level compared to “normal” control states, such that we can classify

something as highly or lowly methylated, also called hypermethylation and hypomethylation, respectively.

Functional Meaning of DNA in Different Parts of the Genome.

It was originally thought, and still widely accepted, that DNA methylation causes gene silencing and/or transcriptional repression. DNA in centromeres and transposable elements, which are non-transcribed regions of the genome, are highly methylated (Schübeler, 2015). Methylation is also the key to gene silencing and X-chromosome inactivation in females (mechanisms of which are discussed in further detail in the following sections). Methylation also often occurs in the genome along with specific histone methylation marks, such as histone 3 lysine 9 and lysine 27 trimethylation (H3K9me3, H3K27me3), which together promote heterochromatin coiling and physically hinder binding of transcriptional activators (Jones, 2012). MBD proteins that recognize methylated DNA have also been found to mostly associate with repressive proteins and complexes, therefore leading the assumption that DNA methylation is a repressive mark (Jones, 2012). While all of these conclusions may be correct, the meaning of DNA methylation is more nuanced and context-specific than previously thought. It is now believed that gene silencing may be the intended effect only in the case of gene promoters and does not hold true in other genetic regions, such as within coding regions or at enhancers (Bird, 2002; Smith and Meissner, 2013).

Methylation of gene bodies has, in fact, been correlated with increased transcript expression (Jones, 2012). Interestingly, MBD proteins such as methyl-CpG-binding protein 2

(MeCP2) are bound to these active genes and do not repress transcriptional elongation (Maunakea et al., 2013). Therefore, methylation readers can interpret, not only the methylation marks, but also the context in which they are situated, allowing for context-specific functions of the readers. Indeed, MeCP2 is known to associate with spliceosome components, and gene body methylation, particularly at exon-intron boundaries, has also been correlated with increased splicing of nascent mRNAs produced from the region. This may be due to the presence of MeCP2 and/or slowing of elongation by methylation “speed bumps”, ultimately, leaving more time for increased splicing. Another explanation of within-gene methylation is silencing of internal, alternative promoters which necessitate silencing for various reasons (Maunakea et al., 2013). Methylation at these sites, which are likely to already be classified as weak promoters, would weaken their ability to initiate transcription and leave the traditional promoter as the prominent start site.

The difficulty in deciphering the meaning of a methylation mark is in the cause-effect relationship. For example, it may be possible that methylation is not present at active gene promoters because of the nature of an active promoter – the region is devoid of nucleosomes, so the DNA is crowded with multiple active protein complexes. The DNA methylation machinery may be simply inhibited from reaching promoters, and therefore, only methylation at silent promoters is detected. However, the causal explanation would interpret the same observation as active gene repression by methylation and, therefore, demethylation allowing for transcriptional activity to begin at targeted promoters. Additionally, some genes are silenced by other epigenetic interventions, and there is considerable redundancy in the epigenetic

programs; therefore, some genes may not be primarily regulated by methylation while others may be solely regulated by methylation.

Age-Driven Changes in Somatic Methylation.

Development, and neuronal development in particular, is reliant on proper methylation pattern establishment and maintenance over a lifetime. Sex-specific maturation has been shown to be dependent on methylation patterning, as seen in the hypothalamic region called the Preoptic area (POA), which is responsible for copulatory behavior in adult males (Nugent et al., 2015). Active DNA methylation is needed to both suppress male-behavior development as well as to activate feminization of the region (Nugent et al., 2015). DNA methylation is not only important for development of the brain, but is largely responsible for the phenomenon of synaptic plasticity. Synaptic plasticity of a single neuron, following repeated stimulation, requires epigenetic rearrangement in order to alter gene expression cascades which is necessary for the process of learning at the organism level (LaPlant et al., 2010; Meadows et al., 2015). These epigenetic changes are reliant on both methylation and demethylation processes, as inhibiting either pathway prevents proper learning and memory (LaPlant et al., 2010).

Theories of an “epigenetic clock” have emerged, which suggest that in the course of normal aging, there is accumulation of some methylation marks and loss of others which are predictable and consistent between individuals - regardless of their sex, tissue examined, or disease-state (Horvath, 2013). In addition, diseases associated with premature aging such as progeria, can be faithfully predicted using the same methylation patterns, suggesting biological

age, as opposed to chronological age, is dependent on DNA methylation (Weidner et al., 2014).

While many of these attempts have a high correlation rate between individuals, it is still unclear if the age-related changes in methylation are functionally relevant or if they merely present a diagnostic tool for use in research or clinic settings. In the developing brain, there is evidence to suggest that there is an overall gain of methylation marks through age, although this is region specific, with some areas such as the hippocampus showing more robust changes in methylation than others (Simmons et al., 2013). What remains to be seen is why some methylation marks are changed through aging, and if they are intentionally altered, how the methylation event is targeted to that particular cytosine residue.

Epigenetic Reprogramming During Embryonic Development.

The coordinated and extensive remodeling of the epigenome that occur during fertilization and embryonic development are illustrative of the potential mechanisms occurring in somatic cells during aging. The transition from primordial germ cells into oocytes and spermatagonia requires almost complete erasure of methylation marks from the DNA, and reestablishment of the germ- and sex-specific patterning are essential for viable embryogenesis (Zaitseva et al., 2007). Failure to properly regulate DNA methylation in germ cells can lead to infertility and/or embryonic lethality. It is thought that maternal methylation marks inherited by the embryo are responsible for allele selection, and paternally-inherited marks are responsible for silencing of repetitive elements and centromeres (Szyf, 2015). DNMTs expressed from the oocyte are responsible for maintenance of imprinting methylation marks during the preimplantation stage, and the choice of allelic silencing is based on gamete methylation

patterns, although how these are initially established is not well understood (Schaefer et al., 2007). However, the imprinting control regions (ICRs) require a certain amount of targeting to maintain their methylation during global demethylation events in development. Some ICRs are targeted for methylation simply by the sequence of the DNA at that point, but mutating the sequence does not eliminate all imprinting (Matsuzaki et al., 2015). Other known imprinting regions, such as *KCNQ1OT1*, are methylated through a self-reinforcing mechanism where a non-coding RNA produced from the paternal locus acts *in cis* to promote methylation and silencing in nearby genes (Korostowski et al., 2012).

The best-understood example of programmed methylation during embryogenesis is X-inactivation through the non-coding RNA *Xist*. *Xist* is expressed from what will become the inactive X chromosome in female embryos, and promotes silencing of the chromosome through recruitment of repressive complex proteins and DNA methylation to the entire length of the chromosome. The *Xist* promoter itself is silenced on the active X chromosome so that its own expression is repressed and the chromosome can be fully active, which is accomplished through an antisense ncRNA to *Xist* termed *Tsix*. This complex regulation occurs in every cell in female mammals and allows for combinatorial expression of X-linked phenotypes, such as with the coloring of calico cats, but also can regulate the development of X-linked diseases. For example, mutated X-linked genes are sometimes present in the silenced X chromosome and, therefore, the disease is not expressed, whereas other individuals may carry the same mutations but it is on the active X chromosome, and the individual will develop the associated disease.

These global changes in epigenetic patterning are highly regulated and still only vaguely understood, but they represent a challenge to understanding transgenerational epigenetic inheritance, as the remodeling process is thought to eliminate almost all non-essential “epimutations”, or epigenetic modifications. However, the complexity of the protein-RNA-DNA interactions needed for faithful recapitulation of methylation in offspring also provide potential mechanisms for inheritance of the non-imprinting methylation. Additionally, passing on of just a few methylation marks may serve as sufficient instructions for multigenerational inheritance.

Multigenerational Epigenetic Inheritance.

Selective pressure of adaptive, spontaneous mutations within a species has been the prevailing theory of continuous directed evolution and species growth within the scientific community since the early 1900s. This Darwinian theory is normally discussed in opposition to Lamarckian inheritance theories which state that physical traits acquired by one generation over their lifetime (i.e. strength, height) can be passed on to offspring. With the dawn of epigenetic research, the followers of Darwin and Lamarck have come to appreciate that adaptation is most likely a combination of both theories. While genetic mutations can produce changes in offspring phenotype, this process is slow and does not allow for quick adaptation to changing environments. On the other hand, Lamarckian theories would allow environmental information to be passed on from parents to offspring between, or even within, generations.

There are several modes of epigenetic inheritance, and it is important to define the terms to use when describing heritable epigenetic information. *Intergenerational* and *cross-*

generational both imply that the parents, and therefore the germ cells that they already possess, were exposed to the environmental factor in question. *Transgenerational* refers to ancestral exposure such that the individuals being examined were never directly exposed to the environmental factor (either as individuals or future germ cells). This can apply to a third generation following *in utero* exposure or a second generation following preconception exposure (Szyf, 2015). Fetal, or *in utero*, exposure to an environmental factor is not considered to be *inheritance* as the offspring are exposed directly; however, the mechanisms of adverse development following *in utero* exposure to drugs, alcohol, diet and other factors often affect the offspring through epigenetic mechanisms.

Preconception exposures of mothers and fathers to environmental toxicants, drugs of abuse, and dietary choices have recently been investigated for their potential influence on epigenetic inheritance (Skinner, 2015). Due to the nature of male and female reproductive systems, most studies on preconception epigenetic inheritance through the germline have focused on paternal effects since sperm production occurs throughout the lifetime. Interestingly, even advanced paternal age, outside of any other particular environmental stressors, can impact offspring health through dysregulation of sperm DNA methylation (Milekic et al., 2015). While offspring of aged fathers are thought to have accumulated more genetic mutations, it is also true that phenotypic differences may arise from epimutations acquired by the sperm progenitors throughout a lifetime. In addition to normal aging experiences, other environmental conditions, such as heat and stress, have been found to impact offspring methylation and/or gene expression following paternal exposure. Offspring of the same male

guinea pigs, before and after chronic high ambient temperature exposure, showed differential methylation patterns and gene expression in the thermoregulatory genes in the liver (Weyrich et al., 2016). Parental preconception high-fat and sucrose-rich diets have been shown in mice and *Drosophila*, respectively, to increase the rate of offspring obesity and diabetes (Huypens et al., 2016; Öst et al., 2014). These experiments demonstrate the rapid and genome-independent effects of epigenetic inheritance.

Parental exposure to drugs of abuse and stress have been of particular interest for offspring brain development as they have been shown to have long-lasting effects in the brain of the individual exposed, and it is well known that early life stress can have detrimental effects on adult brain function. Many research groups, including those of Tracey Bale, Andrea Gore, David Crews and others, have shown that preconception chronic stress induces widespread epigenetic changes in the offspring brain, in particular the hypothalamus and downstream relay centers in the stress response (Dietz et al., 2011; Rodgers et al., 2013; Skinner et al., 2008). Recently, with the legalization of recreational marijuana in many states, researchers have begun to investigate the cross-generational effects of adolescent marijuana exposure (Watson et al., 2015). Tetrahydrocannabinol (THC), which is the psychoactive component in marijuana, has been shown to cause genome-wide changes in methylation patterns of the nucleus accumbens, a brain region involved in positive reinforcement/addiction. In the cases of stress and drug exposure, these heritable epimutations were also associated with altered behavioral phenotypes and wide-spread gene expression alterations, indicating that the epigenetic inheritance does carry some informational content to offspring development.

It is important to note that many studies, including the ones mentioned above, concerning stress, environmental toxicants, and various drugs all involved adolescent exposure. The timing of preconception environmental exposure is important to consider in interpreting their potential effects on offspring (Bale, 2015). There are particular vulnerable periods in development where environmental factors may have more impact on epigenetic inheritance, and adolescence seems to be critical for transmission of epigenetic signals to future offspring.

Adolescence: Development and Drinking.

Adolescence is an important period of development for the brain, where proper neural circuitry is still being formed. It is also characterized by the onset of pubertal development and ends after sexual maturation is complete. These two major development programs are of critical importance to long-term cognitive health and epigenetic inheritance. Disruption of both neurological and sexual maturation can be achieved easily by drug or stress exposure during this time. Unfortunately, a large portion of teenage culture involves risky behaviors such as binge alcohol drinking which can disrupt these maturation processes. Over 90% of all alcohol consumed by underage Americans is done so in a binge pattern, which is defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as raising the blood alcohol concentration (BAC) above 0.08% within 2 hours. Binge alcohol consumption among adolescents is a major health concern, with 21% of teenagers in the United States reporting binge-pattern drinking behavior in the last 30 days. This behavior is not only dangerous at the time but can also lead to various health problems in adulthood such as increased risk for

developing depression, mood disorders, alcohol dependence, and neurodegenerative diseases (Miller et al., 2007; Smyth et al., 2015; Song et al., 2013; White and Hingson, 2013).

Adolescent alcohol exposure has been shown to impact the brain, globally, into adulthood. There is a decrease in myelination of the prefrontal cortex in animals exposed to alcohol during pubertal development, and this lack of myelin persists into adulthood (Vargas et al., 2014). Others have shown that there is a lower threshold of activation for neurons in the CA1 region of the hippocampus following adolescent exposure to alcohol, and this leads to less mature dendritic arbor formation (Risher et al., 2015). Some long-term deficits in the brain following alcohol exposure have also been suggested to be latent, and therefore undetectable, until an “unmasking” of these symptoms through subsequent stress exposure. For example, in a mouse model of early life alcohol exposure, there was no difference in working memory between treated and untreated animals until task complexity was sharply increased or there was further exposure to alcohol (Coleman et al., 2012). The same is thought to be true of alcohol exposure during puberty (Trezza et al., 2015). These symptoms of adolescent alcohol exposure last long into adulthood, suggesting a stable change in neuronal function, however many of these studies use chronic alcohol exposure models. Our lab has developed a rat model of repeated binge alcohol exposure during puberty which closely mimics both the timing of intoxication and blood alcohol levels reached by teens who engage in binge drinking, making it a well-fit rodent model for this human behavior. We have used this model to study the long-term effects of adolescent binge alcohol consumption on hormone expression in the hypothalamus and found persistent changes in adults, even in the absence of further alcohol

exposure (Przybycien-Szymanksa et al., 2010; Przybycien-Szymanska, 2011). More recent work in our lab has shown that adult male rats who were exposed to alcohol in adolescence show a hypervigilant phenotype after further stress, suggesting that the disruptions in stress responsiveness may be mild under normal conditions but exhibit more pronounced deficits when individuals are under further stress (Torcaso et al., 2017). The molecular and behavioral changes lasting into adulthood, in our models as well as others', provide experimental evidence for observations made in humans with a history of alcohol exposure.

Inheritance of Parental Alcohol Exposure.

Clinical studies have long suggested that children of alcoholics have an increased risk of mood disorders and a greater propensity for abusing alcohol throughout life, but these behaviors were often thought to be the result of child-rearing in homes where alcohol abuse was easily visible. Multiple genetic mutations have been investigated for their role in transmission of alcoholism through families, but this is normally in cases where alcohol use is categorized as an addictive disease and not in sub-clinical usages (Anstee et al., 2013). However, our lab and others have shown experimental evidence of intergenerational transmission of adolescent alcohol exposure in animal models where offspring are never exposed to alcohol, suggesting a molecular mechanism of transmission (Przybycien-Szymanska et al., 2014). These changes in offspring include altered gene expression in the brain as well as altered preference for alcohol drinking and dysfunctional stress responsiveness (Burne et al., 2014). Offspring of chronic alcohol-treated male mice, who had been exposed to alcohol daily for seven weeks, showed a phenotype similar to Attention-Deficient Hyperactivity Disorder (ADHD) along with

decreases in dopamine transporter expression and promoter methylation (Kim et al., 2014).

Transgenerational epigenetic inheritance following fetal alcohol exposure has also been examined, and it is thought that only the male germline transmits the effects of fetal alcohol spectrum disorder (FASD), changing the expression of Proopiomelanocortin (POMC) in the hypothalamus (Govorko et al., 2012). POMC is central in the regulation of stress response, metabolism, and energy homeostasis.

It has been hypothesized, yet inconclusively tested, that epigenetic inheritance is the mechanism by which preconception adolescent alcohol exposure in binge patterns can affect naïve offspring (Finegersh et al., 2015; Shukla et al., 2008b). By studying the molecular actions of alcohol in other paradigms, such as chronic alcoholism, fetal alcohol syndrome, and acute molecular mechanisms, we will form a better understanding of the potential mechanisms which alcohol is acting through to cause epimutations in future generations.

Molecular Actions of Alcohol in Somatic Cells- Non-Genomic.

Alcohol, unlike other drugs of abuse, does not have an agreed-upon receptor pathway through which it exerts its actions. Several mechanisms have been proposed, and many in the alcohol research field believe in different primary mechanisms of action. It is, however, widely accepted that ethanol may work through different mechanisms in different tissues. Some of the proposed mechanisms include lipid bilayer disruption, production of reactive oxygen species (ROS), and ligand-gated ion channel modulation. Ethanol (EtOH) is a small molecule, which is able to diffuse easily out of the digestive tract and circulate through the blood. It is thought to

cross the blood brain barrier and effect central nervous system (CNS) function directly, as well as have some function through its metabolite acetaldehyde or further downstream products. Cell-surface receptors for EtOH have been proposed, as have intracellular mechanisms which assume EtOH can diffuse through the plasma and/or nuclear membranes. I will focus on the mechanisms of action within the nervous system, assuming that the CNS effects of EtOH exposure are due to EtOH directly and not its metabolites.

It is important to differentiate between the acute actions of a single EtOH exposure and chronic treatment with EtOH, and between high and low doses. During acute EtOH exposure, EtOH acts as a depressant through upregulating inhibitory pathways and downregulating excitatory pathways. EtOH molecules can bind to and modulate most glutamate receptors, including NMDA and AMPA, causing inhibition of their ionotropic action. EtOH can also act as an agonist of the inhibitory GABA receptors. These effects together are thought to underlie the initial sedation and sluggish effects of EtOH, at least in adults. Long-term EtOH use can lead to downregulation of surface expression of GABA receptors and a switch in EtOH action at glutamate receptors, causing an excitatory and potentially addictive effect (McClintick et al., 2016). Binge EtOH exposures, which are characterized by intermittent exposure to EtOH disrupted by periods of abstinence, sometimes with withdrawal symptoms, are thought to act through a combination of these acute and chronic mechanisms. However, there is less known about the molecular changes that occur due to intoxication compared to the changes that occur because of withdrawal. This is because of the nature of binge intoxication— it is difficult to design a system where an experimenter can cause the repeated intoxication without causing a

chronic alcohol exposure paradigm, while at the same time removing the withdrawal component. Likewise, it is impossible to cause withdrawal without first causing intoxication. Doses of EtOH exposure used *in vivo* and *in vitro* can also vary. BAC of animals and humans is often reported in terms of percent, or units of mg/dl, and in *in vitro* experiments is often addressed in units of mM. For example, the legal driving limit of 0.08% is equivalent to 80 mg/dL and ~17 mM. This is considered to be a low dose of alcohol. Some of the highest doses used in *in vitro* experimental models are around 350 mM, equivalent to 1600mg/dL or 1.6%, which would not be tolerable in animal models or humans.

The lipid bilayer-disrupting properties of alcohol and increased generation of ROS may also cause the psychoactive and molecular consequences of intoxication, and may also change between acute, binge, and chronic EtOH exposure. It is well-known that EtOH molecules cause increased fluidity of membranes, and even at low doses (i.e. 20 mM) are able to disrupt properties of the synaptic membranes, mitochondrial membranes, and myelin composition (Ingolfsson and Andersen, 2011). It was also found to be dose-related, such that higher EtOH concentrations (150 to 350 mM) caused more membrane disruption than lower doses. EtOH is known to cause both an increase in generation of ROS as well as a decrease in the reductive capacity of the cell (Hoek et al., 2002). These combinatorial effects cause dangerous levels of ROS, which have been linked to long-term changes in cognitive health and an increased risk for neurodegeneration. It has also been hypothesized that lipid disruptions could be causal to some long-term effects of EtOH because of the “leakiness” between different compartments. For example, mitochondrial membrane fluidity may allow ROS to leave the mitochondria and

damage cytoplasmic cellular components. Similarly, plasma membrane fluidity can limit the electrical ability of neurons to generate action potentials and continue neurotransmission, as well as allow extracellular debris to enter the cell. The mechanistic summaries discussed here are only a fraction of what has been reported in the literature, and there is still much disagreement about the critical mechanism(s) of EtOH action.

It is highly likely that all of these mechanisms are working together in the brain to cause the psychoactive effects of alcohol as well as the long-term consequences of intoxication. Molecular mechanisms seem to change between exposure conditions, doses, frequency of intoxication, type of alcohol, age of intoxication, and even between individuals. “Intoxication reversal” drugs, which are designed to act as competitive agonists at EtOH sites of action, have been relatively unsuccessful in inhibiting the actions of alcohol. This is further evidence for the fact that multiple mechanisms of action are most likely occurring simultaneously.

Molecular Actions of Alcohol in Somatic Cells—Genomic and Epigenomic.

Many of the longer timescale effects of EtOH are thought to be accomplished through genomic, and epigenomic, changes following exposure. Rapid and widespread changes in gene expression, measured through RNA sequencing and targeted measurements, have shown many genes to be differentially regulated by EtOH exposure. The functional categorization of these genes is also varied, with chaperone proteins, transcription factors, hormones, and neurotransmitters as some of the classes represented among the EtOH-sensitive genes. Some attempts have been made to determine how a transcriptional signal is initiated following EtOH

exposure, but there has yet to be consistent evidence to support a single direct transcriptional mechanism of EtOH. Multiple transcription factors such as NF κ B, GR, and Oct1 have been shown to have differential activity following EtOH treatment. GR and Oct1 have both been found to have less binding to their promoter recognition sequences during and after EtOH exposure (Lin et al., 2013; Przybycien-Szymanska, 2011). NF κ B has been shown to increase and subsequently upregulate its target genes. Work from our lab has shown that EtOH uniquely represses the ability of glucocorticoid receptor (GR) to bind to its transcription factor recognition site within the promoter of its target genes. This effect is not seen with other agonists of GR, such as the synthetic agonist dexamethasone, and thus must be a specific effect of EtOH on the transcriptional regulation of GR (Przybycien-Szymanska, 2011).

The neuroinflammatory pathway has been proposed as a transcriptional mechanism through which EtOH can act to cause its widespread effects in the peripheral organs as well as in the CNS (Crews et al., 2013; Goral et al., 2004). In particular, the activation of High-mobility group box 1 (HMGB1) and the Toll-like receptor (TLR) family may lead to an increase in NF κ B transcription which causes a host of downstream inflammatory signals, including upregulation of TLRs (Crews et al., 2013). While the neuroinflammatory consequences of alcohol exposure are intriguing, they have only been reliably studied in animal and human models of extreme alcohol dependence and chronic abuse. Therefore, this does not provide many insightful clues as to the mechanism of short bouts of binge EtOH exposure.

Epigenetic changes following a variety of EtOH exposure paradigms have been reported, including microRNA expression, histone modifications, and global changes in methylation.

However, these are rarely in agreement (Chastain and Sarkar, 2017). Some groups, mostly studying chronic alcohol administration, have reported global decreases in genome methylation as well as decreased expression and activity of the methylation machinery in the brain following EtOH exposure (Krishnan et al., 2014; Kyzar et al., 2016; Pandey et al., 2015; Sakharkar et al., 2014). Other groups have focused on histone modification, finding enrichment of lysine methylation (activation mark) and reduced acetylation (repressive mark) throughout the genome (Kyzar et al., 2016). Our lab has measured long term changes in microRNA (miR) expression following adolescent EtOH exposure and found discrete changes, with some miRs increasing expression and others decreasing in a brain region specific manner (Prins et al., 2014). Some of the notable downstream targets of these differentially regulated miRs were brain-derived neurotrophic factor (BDNF) and sirtuin 1 (SIRT1). Others, using different doses and administration paradigms have reported little to no change in epigenetic marks, or sometimes opposing effects in different tissues (Chastain and Sarkar, 2017).

The widespread effects of EtOH on genomic control have also been partially attributed to changes in several key epigenetic modifiers. MeCP2 represents an interesting inflection point for EtOH's epigenetic regulation. MeCP2 expression itself has been shown to be sensitive to EtOH treatment, but MeCP2 also seems to be an important mediator for other epigenetic changes. For example, a single dose of EtOH in neural stem cell culture caused hypermethylation of the MeCP2 regulatory elements and reduced gene expression. However, repeated EtOH exposures caused the opposite effect to occur, which was attributed to the withdrawal between EtOH doses (Rasangi et al., 2015).

Some cases and symptoms of FASD, especially disruptions of the stress axis, are thought to persist through epigenetics causing long-lasting changes in methylation, histone modifications, and gene expression. In particular, these disrupted patterns are thought to be set up during gestation as a product of the reduction of SAM within the developing fetus. EtOH is known to reduce the absorption of folic acid and production of SAM, and the work of Dipak Sarkar has shown that choline supplementation during gestational EtOH exposure can ameliorate the epigenetic effects of EtOH as well as many of the symptoms of FASD (Bekdash, Zhang and Sarkar 2013).

Looking at the field as a whole, it is clear that postmortem alcoholic tissue can be extremely variable, and does not provide a satisfactory model to study epigenetic changes caused by EtOH. Animal models, where dose, timing, and environmental exposures can be controlled, have more easily repeatable phenotypes. However, the amount of alcohol and the developmental stage seem to play a large role in the effects of EtOH. Lower or intermittent doses during adulthood do not seem to produce any appreciable effects on genomic or epigenomic patterns. Chronic EtOH administration seems to cause overall decreases in methylation in the brain as well as particular sensitivity of pro-growth/anti-apoptotic genes. *In utero* exposure causes widespread epigenetic and transcriptional alterations, but is extremely dependent on the amount and developmental stage of the offspring.

Epigenetics and alcohol have been shown to have interacting effects in the development of many neurological diseases including mood disorders and neurodegeneration. The hypothalamus is a particular region of interest as it is a master regulator of physiologic function

and a relay center for many of the altered behavioral phenotypes resulting from binge alcohol exposure. The hypothalamus has also recently been appreciated for its potential role in development of neurodegenerative diseases such as Alzheimer's disease (AD), as we begin to learn more about the effects of alcohol and peripheral signals on CNS health.

Alcohol and the Hypothalamus.

Alcohol acts as a physiologic stressor in the body, and has been found to trigger a response of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Allen et al., 2011; Rivier, 2014). This axis is responsible for properly managing stress and acts through positive and negative feedback loops. Stimulation of the hypothalamus by a stressor results in release of the hormones Corticotrophin Releasing Factor (CRF) and Arginine Vasopressin (AVP) to the anterior pituitary gland where they trigger production of Adrenocorticotropic Hormone (ACTH). ACTH then acts to increase release of glucocorticoids from the adrenal gland, which travel throughout the body. Glucocorticoids are pleiotropic hormones, and act to allow the animal to physically address the stressful situation at hand by increasing glucose mobilization and vasodilation. Glucocorticoids act through binding to their receptor, GR, which is a member of the nuclear receptor family. GR serves as a transcription factor, binding to the Glucocorticoid Response Element (GRE) upstream of target genes. In the HPA axis, the GREs of interest are negative GREs, and therefore inhibit transcription of ACTH, CRF and AVP mRNA. The release of these hormones is then halted, which serves as negative feedback in order to shut down the stress response once the stressor is no longer present (Fig 2).

This HPA feedback system is known to be dysregulated in patients with anxiety, depression, and other mood disorders. This can manifest as perpetually high levels of circulating glucocorticoids, and a desensitization of the hypothalamus to negative feedback. Previous work in our lab has shown many steps of the pathway to be altered in the adult male brain following adolescent exposure to alcohol, including increased expression of CRF and decreased AVP expression as well as elevated circulating ACTH and glucocorticoids (Przybycien-Szymanksa et al., 2010). These alcohol-induced changes in the HPA axis have not been observed in female rodents, as they seem to be resistant to the EtOH exposures during the same periods of development.

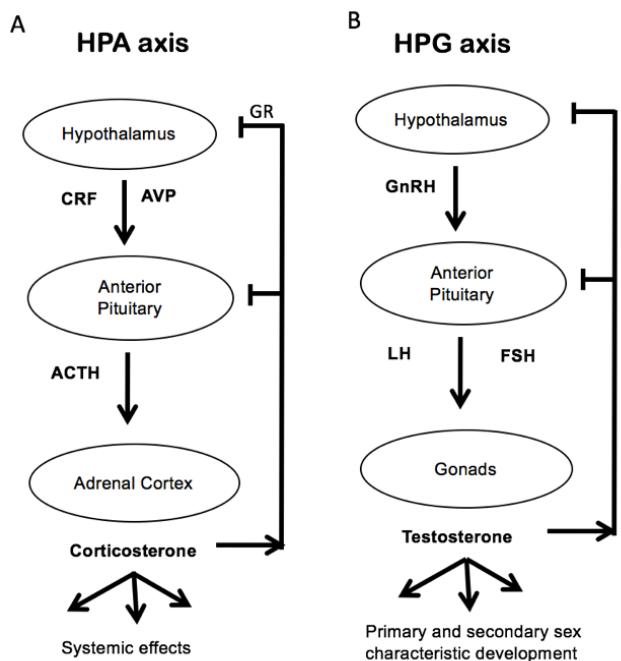


Figure 2. Schematic representation of HPA axis and HPG axis function and feedback. The hypothalamus acts as a regulatory region for both the HPA and the HPG axis. A) Upon encountering a stressor, the hypothalamus releases CRF and AVP hormones, which travel to the pituitary gland and cause the synergistic release of ACTH. ACTH stimulates release of glucocorticoids (corticosterone in rats) which can in turn regulate the axis activity through

negative feedback via GR and transcriptional repression of CRF, AVP, and ACTH. B) The hypothalamus works in a similar fashion to allow for stimulation of pubertal development by releasing the hormone GnRH to the pituitary, stimulating the release of LH and FSH to the gonads, where, in males, testosterone is released into circulation. Testosterone has pleiotropic effects throughout the body, and can also regulate the activity of the HPG axis through negative feedback.

Alcohol is also known to impact the functioning of the hypothalamus in the Hypothalamic-Pituitary-Gonadal (HPG) axis. Human studies have shown for decades that alcohol use during puberty can lower estrogen levels in females and cause reductions in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in males, all of which seem to persist into adulthood (Diamond Jr et al., 1986). Additionally, disruptions in the release of other growth-inducing hormones have been shown in animal models of chronic alcohol administration during puberty. Chronic alcohol exposure of pubertal males was found to lower circulating testosterone, without causing changes in other hormones like LH and FSH (Ren et al., 2005). Gonadal development appears to be permanently damaged by alcohol exposure during this important period of maturation, although many of these studies were carried out in chronic alcohol abuse models. In adults, alcohol is also known to disrupt the HPG axis; however, these changes seem to be more transient. They include alterations in menstruation and/or decreases in fertility in females, and low testosterone coupled with higher estrogen production in men, which can lead to low or abnormal sperm production. Overall, alcohol has been shown to play a disruptive role in many of the endocrine functions of the hypothalamus, and these disruptions may lead to long-term changes in the hypothalamic regulation of normal physiology.

Summary

Recent technological advances have allowed breakthroughs in our understanding of the importance of the epigenome for growth and development, learning, disease progression, and generation-to-generation communication. The debate between Darwinian and Lamarckian inheritance theories seems to be less of a question of “either, or” and more of a blending of the two ideas. Along with this understanding has come a drive to uncover which parental choices can impact offspring, the degree of their importance, and the mechanisms of this molecular memory. These epimutations inherited by offspring have been shown to be adaptively advantageous in some contexts and deleterious in others. The goal of this dissertation is to elucidate the effects of parental preconception binge alcohol exposure on the offspring epigenome and the potential functional consequences for these offspring. This objective is significant as adolescent binge drinking has been on the rise in the United States in the last several decades, with underage drinkers consuming over 90% of alcohol in binge-like patterns. Binge drinking has been linked to increased cognitive health risks late into adulthood as well as similar health risks in offspring. Children of alcoholics display an increased risk of suicide and a greater propensity for abusing alcohol throughout life. Previous work in our lab has shown several changes in the adult brain following adolescent binge-pattern exposure to alcohol including dysfunction of the stress response and altered gene expression in alcohol-naïve offspring of these animals.

The work presented herein is novel in that it is the first study of its kind to investigate a mechanism by which *either* maternal or paternal preconception exposure to binge alcohol

conditions can impact DNA methylation in offspring. My overarching hypothesis is that parental preconception alcohol exposure can impact offspring through epigenetic inheritance, from sperm and eggs, of DNA methylation patterns in the hypothalamus, leading to dysfunctional hypothalamic function during development and a predisposition to neurodegeneration. The contribution of this research to the field is significant as it delineates a pathway through which preconception binge alcohol treatment acts to cause epigenetic dysregulation in alcohol-naïve future generations.

I tested this hypothesis by pursuing two specific aims:

Aim 1 was to interrogate changes in DNA methylation in the hypothalamus of offspring following parental alcohol exposure. This aim tested the working hypothesis that binge alcohol exposure during puberty will cause alterations to the DNA methylation pattern in the hypothalamus of their alcohol-naïve offspring in a sex-specific manner.

We employed Enhanced Reduced Representation Bisulfite Sequencing as an unbiased approach to test the hypothesis that parental exposure to binge-pattern alcohol during adolescence alters DNA methylation profiles in their alcohol-naïve offspring. Analysis of male PND7 offspring revealed that both maternal and paternal preconception alcohol exposure caused differential methylation patterns in the offspring hypothalamus. The differentially methylated cytosines (DMCs) were distinct between offspring depending on which parent was exposed to ethanol. Moreover, novel DMCs were observed when both parents were exposed to ethanol, and many DMCs from single parent ethanol exposure were not recapitulated with dual

parent exposure. We also measured mRNA expression of several differentially methylated genes and some, but not all, showed correlative changes in expression. Importantly, methylation was not a direct predictor of expression levels, underscoring the complexity of transcriptional regulation. Overall, we demonstrated that adolescent binge ethanol exposure causes altered genome-wide DNA methylation patterns in the hypothalamus of alcohol-naïve offspring.

Aim 2 was to determine the functional consequences of parental preconception ethanol exposure on offspring development. We hypothesized that the altered methylation marks might be setting the offspring up for altered development and/or leaving them more susceptible to alcohol or other stress exposure later in their life. The epimutations may leave the offspring poised for further environmental influence, which could manifest as more pronounced phenotypic differences in adulthood. We tested several parallel hypotheses concerning offspring development: 1) Parental preconception alcohol exposure will alter offspring juvenile behavior and hypothalamic control of developmental cascades, 2) parental preconception alcohol will confer adaptation to offspring such that they have less severe physiologic response to alcohol exposure themselves, and 3) adolescent alcohol exposure leads to a “fatty brain” phenotype in offspring as well as the aging exposed animal themselves, through dysregulation of lipid homeostasis.

Using our repeated binge alcohol exposure paradigm, we produced a first generation of offspring whose parents had been exposed to alcohol and characterized their development, which included decreased juvenile play behavior, diminished body weights, and lower

expression of pubertal drivers. Additionally, there were no adaptive advantages in offspring exposure to alcohol with parental preconception drinking. Interestingly, when the parents of these offspring were mated again one month later, the same differences in hypothalamic gene expression were not observed in infant offspring. This represents a novel finding in that there is a temporal component to binge alcohol epigenetic inheritance.

Another functional component of methylation dysregulation is an increased risk for neurodegeneration. In order to study this potential effect, we utilized young offspring as well as aged animals exposed to alcohol themselves. Apolipoprotein E (ApoE) is a carrier protein necessary for the delivery of cholesterol from astrocytes to neurons and an important risk factor for the development of Alzheimer's disease. We found a decrease in *ApoE* mRNA expression in the hypothalamus of adult males exposed to EtOH, but no difference was measured in females. Correspondingly, there was an increase in cholesterol accumulation in cortical astrocytes of binge-EtOH exposed males compared to vehicle treated counterparts, but there was no change in females. In the EtOH-naïve offspring of EtOH-exposed parents, we also found downregulation of *ApoE* in the hypothalamus. These results suggest that EtOH-induced downregulation of *ApoE* results in a decrease in astrocytic secretion of lipids, leading to "fatty astrocytes" and cholesterol-starved neurons.

CHAPTER III

ADOLESCENT BINGE-PATTERN ALCOHOL EXPOSURE ALTERS GENOME-WIDE DNA METHYLATION PATTERNS IN THE HYPOTHALAMUS OF ALCOHOL-NAÏVE MALE OFFSPRING

Modified from Asimes et. al, *Alcohol*, 2017

Introduction

Binge alcohol consumption among adolescents is a major health concern in the United States, with 21% of teenagers reporting binge-pattern drinking behavior in the last 30 days (White and Hingson, 2013). Americans under the age of 21 consume over 90% of alcohol in binge-like patterns, which is defined by the Centers for Disease Control as raising the blood alcohol concentration (BAC) above 0.08% within 2 hours (CDC 2014) (Miller et al., 2007). This behavior is not only dangerous at the time, but can also lead to various health problems in adulthood such as increased risk for developing depression, mood disorders, alcohol dependence and neurodegenerative diseases (Allen et al., 2011; Coleman Jr et al., 2011; Vargas et al., 2014).

Clinical studies have shown that children of alcoholics are at an increased risk for attention-deficit/hyperactivity disorder and have a greater propensity for abusing alcohol throughout life, but the root of these behaviors is confounded by child-rearing practices in homes of alcoholics (Hairston et al., 2016; Hill et al., 2011; Sundquist et al., 2014). However, experimental evidence from animal models suggests that molecular inheritance mechanisms

could underlie these clinical findings (Finegersh et al., 2015; Przybycien-Szymanska et al., 2014).

Data from our lab and others have demonstrated ethanol-induced long-term changes in gene expression in the hypothalamus as well as behavioral changes, such as increased preference for alcohol drinking and dysfunctional stress responsiveness in alcohol-naïve offspring (Burne et al., 2014; Govorko et al., 2012; Przybycien-Szymanska et al., 2014; Rompala et al., 2016). The hypothalamus has been investigated for its vulnerability to binge alcohol exposure as it has central importance in regulating the stress response (Przybycien-Szymanksa et al., 2010). Together, these studies raise the possibility that epigenetic inheritance is one mechanism by which adolescent alcohol exposure can affect naïve offspring (Finegersh et al., 2015; Shukla et al., 2008a).

DNA methylation is a heritable epigenetic mark that is relatively stable but varies throughout development and can be influenced by environmental factors (Carone et al., 2010; Jones, 2012). Aberrant DNA methylation is implicated in many cognitive disorders such as schizophrenia, depression, and addiction (Gavin et al., 2013; Grayson and Guidotti, 2012; Manzardo and Butler, 2013). In the brain, DNA methylation is intimately involved in cellular differentiation as well as synaptic plasticity (Tognini et al., 2015). Therefore, proper patterning of the epigenetic landscape is necessary for neuronal function. Environmental factors are known to cause differential methylation of the brain during early development, which is a potential mechanism for lifetime adaptation. For example, early life stress through maternal deprivation can alter methylation of genes involved in mediating the physiological stress response and these methylation marks are persistent throughout adulthood (Chen et al., 2012).

Exposure to adverse environmental factors and drugs of abuse during adolescence has also been demonstrated to have transgenerational consequences (Carone et al., 2010; Minnes et al., 2014; Öst et al., 2014; Weyrich et al., 2016). For example, paternal cocaine exposure during puberty has been shown to alter DNA methylation and behavior in offspring (Killinger et al., 2012). Paternal preconception exposure to alcohol has also been associated with increased anxiety and depression in offspring (Liang et al., 2014). However, there have been very few studies to examine the effects of maternal preconception exposure to drugs of abuse on offspring (Vassoler et al., 2014).

Methylation occurs primarily at cytosine residues in the context of CpG dinucleotides, although other modified bases have recently been reported (Schübeler et al., 2011). CpG islands (CGIs) are GC-rich regions of the genome, an average of 1,000 bp in length, that are often close to transcription start sites (TSS) and tend to be unmethylated, which allows for active gene transcription (Jones, 2012; Schübeler, 2015). Methylated DNA found in promoter regions is thought to inhibit gene transcription by encouraging heterochromatin formation, therefore preventing binding of transcriptional activators, as well as recruiting repressive proteins to inhibit transcription of downstream genes (Jones, 2012; Schübeler, 2015; Smith and Meissner, 2013). Less is known about the role of methylation in other genic and intergenic regions, although it has been suggested that methylated DNA in coding regions of a gene can promote gene expression and/or alternative splicing (Maunakea et al., 2013).

We employed Enhanced Reduced Representation Bisulfite Sequencing (ERRBS) as an unbiased approach to test the hypothesis that preconception parental exposure to binge-

pattern alcohol consumption during adolescence alters DNA methylation in the hypothalamus of alcohol-naïve male offspring. ERRBS uses a genome-wide sequencing platform to allow for any relevant changes in methylation to be sequenced, as opposed to targeted sequencing approaches which investigate only those methylation sites deemed relevant by the experimenters. Our experimental design also allowed us to compare between the discrete maternal vs. paternal contributions to altered DNA methylation in offspring. This is the first study of this scope to analyze genome-wide changes in DNA methylation of offspring as a result of adolescent binge alcohol exposure of both parents.

Results

Parental Ethanol Exposure Induces Differentially Methylated Cytosine Residues in the Hypothalamus of Male Ethanol-Naïve Offspring.

We used a rat model of pubertal binge alcohol treatment previously established in our laboratory to determine intergenerational changes in DNA methylation patterns associated with parental adolescent exposure to binge alcohol (Fig. 1) (Przybycien-Szymanska et al., 2014). Genome-wide DNA methylation in the hypothalamus was compared between male offspring of control x control mating pairs and all other combinations (maternal ethanol only, paternal ethanol only, both parents ethanol). Corresponding gene expression was measured in the male offspring as well as female offspring of the same litters at PND7. There were no differences in offspring weight, litter size, or sex ratio at birth between any of our mating pairs (Table 1). Genomic DNA of three pups per treatment group, but from different litters, was analyzed with

an average of 113,445,354 reads generated for each sample. Both pre-trimming and post-trimming QC reports indicated that the read data passed basic quality control, with exceptions following the expected patterns in ERRBS experiments. Alignment efficiencies and conversion rates were typical of ERRBS experiments and consistent across samples (Table 2). Raw methylation files will be deposited for public access upon publication.

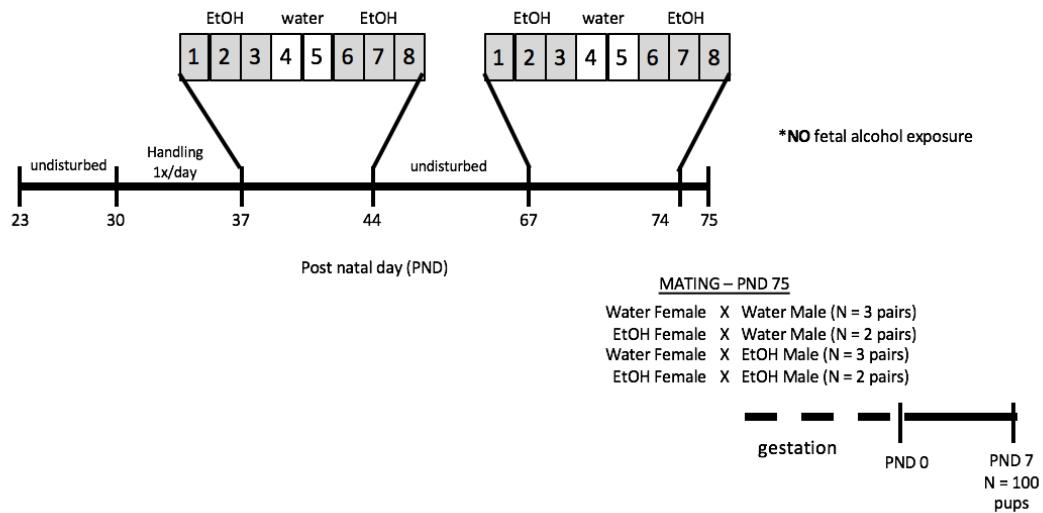


Figure 3. Adolescent binge alcohol exposure paradigm. An 8-day treatment paradigm was administered to male and female Wistar rats where animals received 3 g/kg body weight of ethanol (20% v/v in water) via oral gavage once per day. Starting at PND 37 (peri-puberty), ethanol-treated animals received 3 days ethanol, 2 days tap water, and 3 days ethanol, whereas control animals received tap water only for all 8 days. Animals were then left undisturbed until PND 67, when they underwent the same 8-day treatment. 24 hours after last ethanol dose, animals were mated (n=2-3 pairs/treatment) and offspring were born approximately 23 days later. Litters were culled to 10 pups per dam at PND 0 and animals were euthanized at PND 7.

Table 1. Preconception binge alcohol exposure did not alter litter size, sex ratio or offspring growth. Weights are displayed as mean \pm SEM

Litter	Total Pups	Sex Ratio	Average PND0 Weight (g)	Average PND7 Weight (g)
Control 1	15	8M:7F	6.50 \pm 0.07	17.81 \pm 0.20
Control 2	14	6M:8F	6.11 \pm 0.07	14.54 \pm 0.17
Control 3	13	5M:8F	5.80 \pm 0.09	14.44 \pm 0.22
Maternal Exposure 1	15	9M:6F	6.09 \pm 0.15	14.86 \pm 0.32
Maternal Exposure 2	11	4M:7F	6.86 \pm 0.14	14.28 \pm 0.15
Paternal Exposure 1	15	5M:10F	6.84 \pm 0.08	17.37 \pm 0.23
Paternal Exposure 2	8	5M:3F	6.93 \pm 0.12	15.09 \pm 0.31
Paternal Exposure 3	15	5M:10F	6.09 \pm 0.06	17.59 \pm 0.39
Both Exposed 1	13	5M:8F	6.47 \pm 0.06	14.47 \pm 0.19
Both Exposed 2	13	6M:7F	5.73 \pm 0.07	13.13 \pm 0.25

Table 2. Alignment efficiencies and conversion rates for ERRBS of all samples.

Sample	Total Reads	Trimmed Reads	Unique Alignment	Alignment Efficiency	Conversion Rate
Control 1	101,048,410	100,855,039	62,220,409	61.70%	99.40%
Control 2	127,821,721	127,215,674	78,361,090	61.60%	99.00%
Control 3	102,862,686	102,170,841	58,944,438	57.70%	99.10%
Maternal Ethanol 1	109,878,778	108,966,846	52,870,280	48.50%	98.90%
Maternal Ethanol 2	124,275,848	123,559,339	74,581,912	60.40%	99.20%
Maternal Ethanol 3	107,454,830	106,658,607	64,249,678	60.20%	99.30%
Paternal Ethanol 1	114,893,371	113,982,695	69,455,351	60.90%	99.30%
Paternal Ethanol 2	106,580,485	105,705,397	69,203,843	65.50%	99.20%
Paternal Ethanol 3	99,166,858	98,773,966	61,786,238	62.60%	99.20%
Maternal + Paternal 1	139,906,554	138,640,440	81,779,772	59.00%	99.10%
Maternal + Paternal 2	121,250,474	120,463,927	69,505,491	57.70%	99.10%
Maternal + Paternal 3	106,204,238	105,806,869	66,903,713	63.20%	99.40%

In general, we found more instances of hypermethylation in all groups compared to control and differentially methylated cytosines (DMCs) were distinct for all treatment groups with very little overlap; only 4 hypermethylated DMCs were common to all three groups (Fig. 4). The largest number of hypermethylated residues was found when both parents were exposed to ethanol (Fig. 4). Specifically, dual parent preconception ethanol exposure resulted in 168 hypermethylated DMCs. We also examined the differences in offspring DNA methylation when only one parent was exposed to binge ethanol preconception (the other parent received

water), since both maternal and paternal gametes can affect offspring methylation patterns.

We observed 95 hypermethylated DMCs in offspring where only the mother was exposed to ethanol and 54 hypermethylated DMCs when there was only paternal ethanol exposure (Fig. 4). Several genes were associated with more than one hypermethylated DMC, such as Rn5-8s, Bmp3 and Atg5 (Fig. 4; boldface type).

There were also a large number of hypomethylated DMCs among all groups with only 5 hypomethylated DMCs shared between all treatments (Fig. 5). In offspring where both parents were exposed to ethanol there were 105 hypomethylated DMCs compared to offspring from water-treated parents (Fig. 5). In maternal ethanol treated offspring, there were 79 hypomethylated residues compared to 47 hypomethylated residues when only the father was exposed to ethanol (Fig. 5). There were also multiple genes associated with more than one DMC, for example Exo5 was hypomethylated on multiple residues in both maternal ethanol and paternal ethanol offspring (Fig. 5; boldface type).

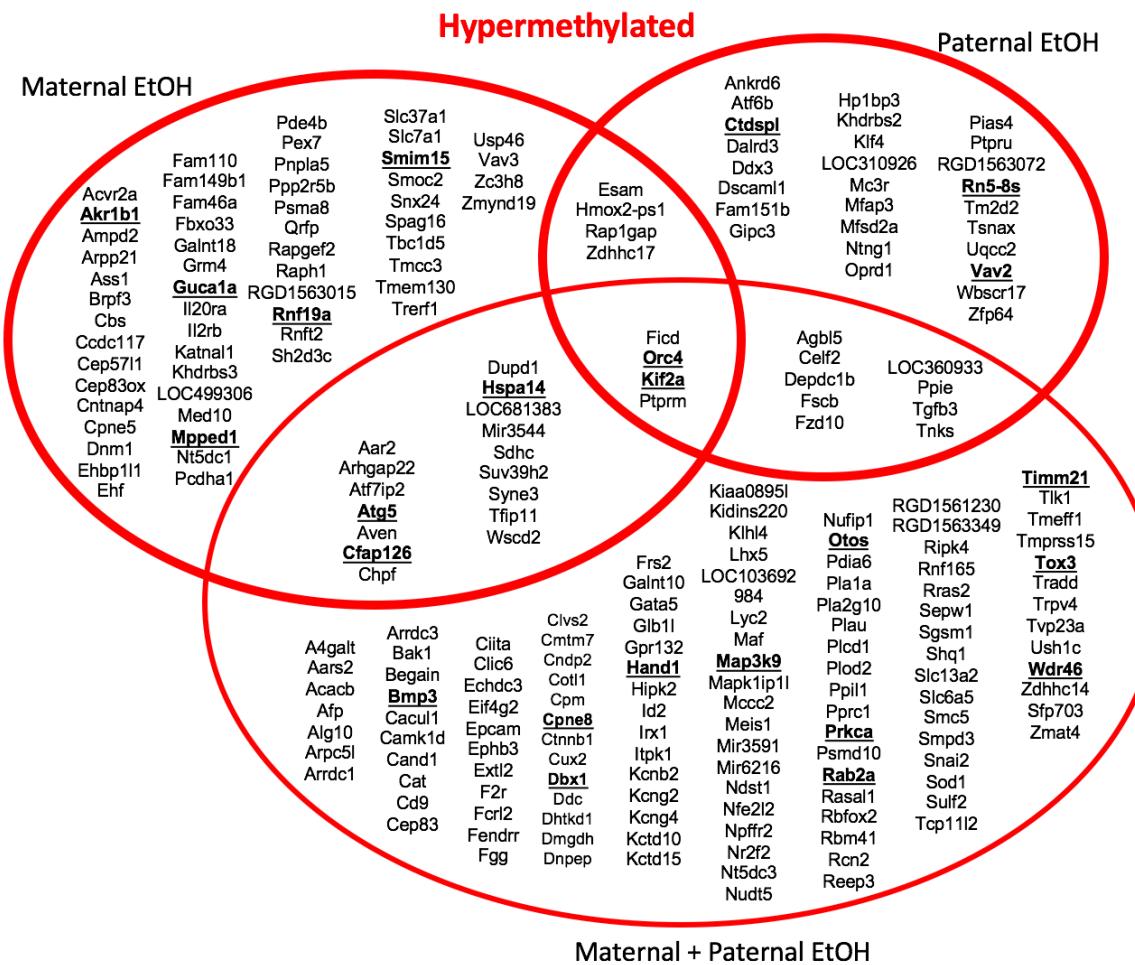


Figure 4. Annotated genes associated with hypermethylated residues vary between treatment groups. Venn diagram describing bioinformatics results using UCSC Genome Browser for analysis. Statistically significant differentially methylated cytosine (DMC) residues between treatment groups were associated with the nearest downstream gene for hypermethylated cytosines. Genes are listed in alphabetical order for each treatment group. Gene names underlined in bold face type indicate that more than one cytosine was differentially methylated near that gene.

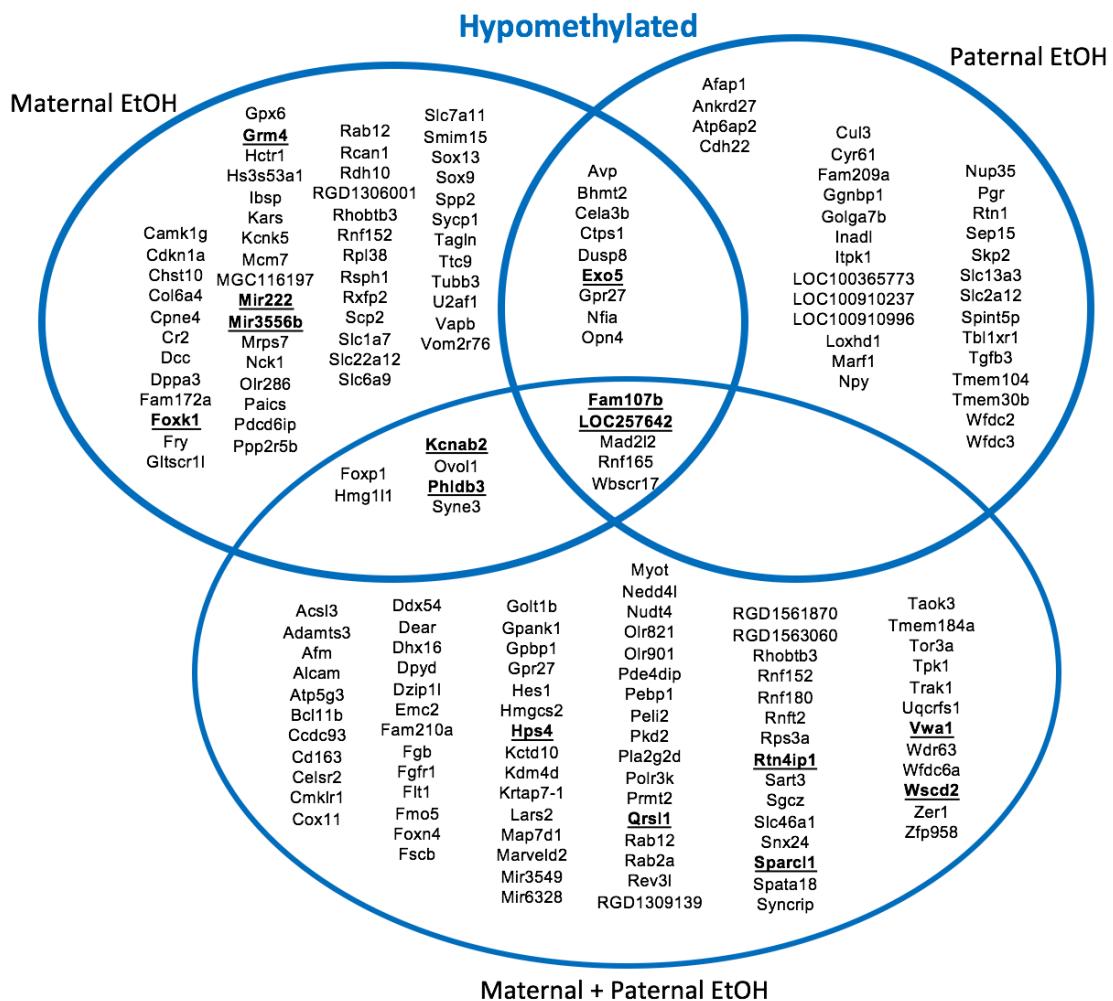


Figure 5. Annotated genes associated with hypomethylated residues vary between treatment groups. Venn diagram describing bioinformatics results using UCSC Genome Browser for analysis. Statistically significant differentially methylated cytosine (DMC) residues between treatment groups were associated with the nearest downstream gene for hypomethylated cytosines. Genes are in alphabetical order for each treatment group. Gene names underlined in bold face type indicate that more than one cytosine was differentially methylated near that gene.

Differentially Methylated Cytosines were Distributed Across All Chromosomes and the Extent of Hypo- Versus Hypermethylation was Dependent on Parental Ethanol Exposure.

Overall, there were discrete yet robust changes in DNA methylation across the genome and we did not observe clustering of DMCs on a particular chromosome or region of the

genome (Fig. 6). In addition, there were no ethanol-induced global changes in DNA methylation and ethanol exposure to both parents did not have an additive effect on DMCs for individual chromosomes (Fig. 6). For example, maternal ethanol exposure caused hypomethylation of cytosine residues on chromosome 11, whereas paternal ethanol exposure had no effect (Fig. 6A, B). By contrast, ethanol induced a combination of both hypo- and hypermethylated sites on chromosome 11 when both parents were exposed (Fig. 6C). As another example, the X chromosome was hypomethylated in the offspring of both maternal-only ethanol and paternal-only ethanol exposed animals (Fig. 6A, B), but the X chromosome was hypermethylated when both parents were treated (Fig. 6C). Similarly, the Y chromosome had hypermethylated DMCs only when the father was ethanol exposed (Fig. 6B). These examples underscore the lack of an additive effect from dual parental exposure and the complexity of offspring DNA methylation.

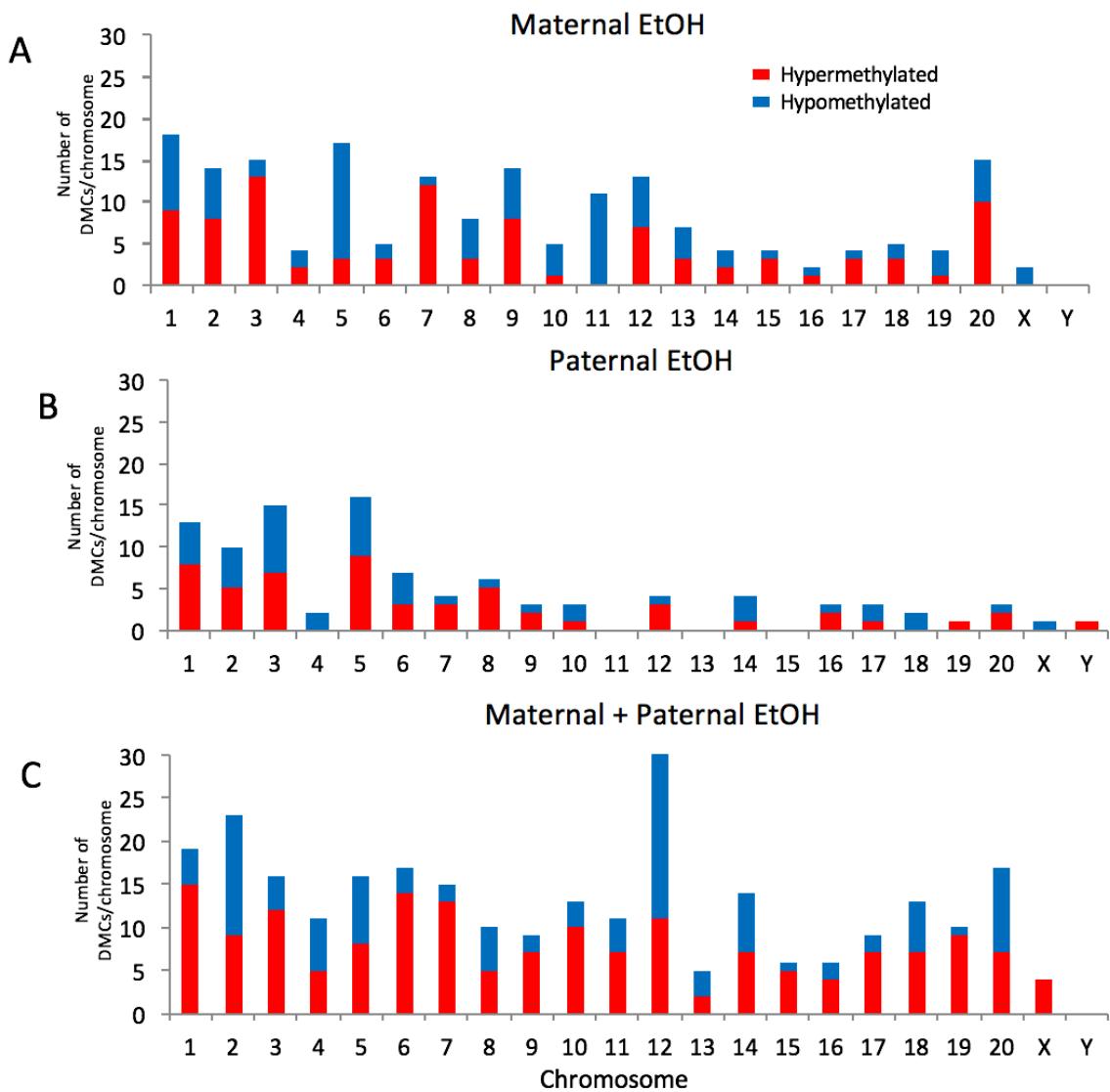


Figure 6. Distribution of differentially methylated cytosines across chromosomes. Histogram analysis of differentially methylated cytosines (DMCs) on each chromosome in (A) offspring from maternal ethanol-exposed, (B) offspring from paternal ethanol-exposed, and (C) offspring from both maternal and paternal ethanol-exposed. Blue region indicates number of hypomethylated DMCs and red region indicates number of hypermethylated DMCs on each chromosome.

In addition to genome-wide changes in methylation, we examined the percentage differences in methylation at each residue. Residues with the greatest percent difference in

methylation were ranked for hyper- and hypomethylation and the top 5 hypermethylated and 5 hypomethylated DMCs for each group are listed in Table 3. The same cytosine on chromosome 2, upstream of Hmox-ps1, was the most hypermethylated residue in maternal ethanol and paternal ethanol offspring, but was not changed in offspring of maternal and paternal ethanol exposure (Table 3).

Table 3. Top 5 Hyper and 5 Hypomethylated DMCs as ranked by greatest percentage difference for each treatment group. Residues with significant hypermethylation compared to control are in white rows, with significant hypomethylated residues in gray rows.

Treatment	Location	Position	P value	Q value	Methylation Difference (%)	Nearest Gene	Distance to gene body (bp)	Region
Maternal Ethanol	chr2	215352989	1.38E-07	3.89E-03	92.44	Hmox2-ps1	17205	Inter CGI
	chr2	177162515	1.59E-07	4.12E-03	90.63	Rapgef2	673686	Inter CGI
	chr12	8190387	5.32E-06	4.17E-02	86.25	Slc7a1	81414	Inter CGI
	chr3	33440426	5.97E-06	4.40E-02	85.81	Orc4	364739	Inter CGI
	chr15	2798203	7.63E-11	3.02E-05	85.10	Dupd1	0	Coding
	chr9	45676004	2.21E-07	5.29E-03	-84.74	Chst10	116862	Coding
	chr16	11212749	6.04E-08	2.39E-03	-85.30	Opn4	260198	Coding
	chr20	6421481	1.18E-07	3.66E-03	-87.00	Cdkn1a	62616	Exon
	chr15	4643007	9.53E-11	3.02E-05	-89.17	Kcnk5	46006	Inter CGI
Paternal Ethanol	chr1	215031715	7.29E-08	2.64E-03	-95.36	Dusp8	0	Exon
	chr2	215352989	6.01E-10	2.68E-04	95.30	Hmox2-ps1	17205	Inter CGI
	chr5	71464665	6.25E-07	1.74E-02	89.32	Klf4	818645	Inter CGI
	chr9	39855580	2.37E-06	3.27E-02	80.00	Khdrbs2	0	Coding
	chr14	42190320	1.63E-06	2.59E-02	78.80	LOC360933	756401	Inter CGI
	chr6	26853900	3.29E-07	1.16E-02	77.01	Agbl5	0	Exon
	chr10	103823050	2.81E-06	3.55E-02	-67.20	Tmem104	29835	Coding
	chr1	114898684	1.12E-07	6.84E-03	-68.63	LOC100365773	0	Coding
	chr16	11212749	2.23E-06	3.21E-02	-78.36	Opn4	260198	Coding
Maternal + Paternal Ethanol	chr9	86252196	3.07E-06	3.76E-02	-80.77	Cul3	149036	Inter CGI
	chr1	215031715	6.91E-07	1.89E-02	-91.00	Dusp8	0	Exon
	chr2	186682537	6.68E-08	2.78E-03	93.39	Fcrl2	77443	Inter CGI
	chr3	2137673	1.06E-05	4.32E-02	92.26	Arrdc1	736	Promoter
	chr12	48646837	4.00E-08	2.13E-03	91.05	Ficd	12829	Coding
	chr2	39211484	1.31E-09	2.18E-04	90.32	Kif2a	1002717	Inter CGI
	chr7	131964758	2.75E-06	2.34E-02	89.12	Alg10	628574	Inter CGI
	chr17	78631069	7.02E-07	1.12E-02	-81.86	Fam107b	69454	Inter CGI
	chr18	73900477	3.46E-06	2.63E-02	-82.27	Rnf165	27232	Inter CGI
	chr2	42671444	1.20E-07	3.68E-03	-82.33	Gpbp1	330599	Inter CGI
	chr12	45206285	9.70E-07	1.31E-02	-85.42	Pebp1	175136	Inter CGI
	chr2	185450313	4.10E-11	2.73E-05	-86.61	Rps3a	5465	Inter CGI

DMCs were Primarily Observed Outside of CpG Islands and in Intergenic Regions.

The distribution of DMCs throughout the genome can have important functional implications for their role in gene expression. Therefore, we identified the relationship between DMC location and the defined functional genomic region for each treatment group. Fig. 7 (A-C) shows the percentage of differentially methylated residues that fall within the defined classes of CpG rich regions. Interrogation was similar between CpG islands and InterCGI regions in all samples (48% and 40%, respectively), but the majority of DMCs were found outside of CpG islands. The priority analysis for functional overlapping elements was gene promoter, coding DNA sequence (CDS), noncoding region, 5'UTR, and then 3'UTR. Fig. 7 (D-F) shows the distribution of differentially methylated residues that fall within defined genic elements according to RefGene. The majority of DMCs were found at nucleotides outside genic regions (intergenic), as well as in the introns of coding genes, regardless of parental ethanol exposure (percent DMCs in intergenic + introns = maternal 86%; paternal 88%; dual parent 89%; Fig. 7D-F). The functional role of methylation in intergenic regions is not completely understood, but these might mediate the activity of distant enhancer elements and non-coding RNAs, or modulate overall chromatin structure (Schübeler, 2015). The other chromosomal regions had very low incidence of ethanol-induced DMCs and were similar between treatment groups (Fig. 7).

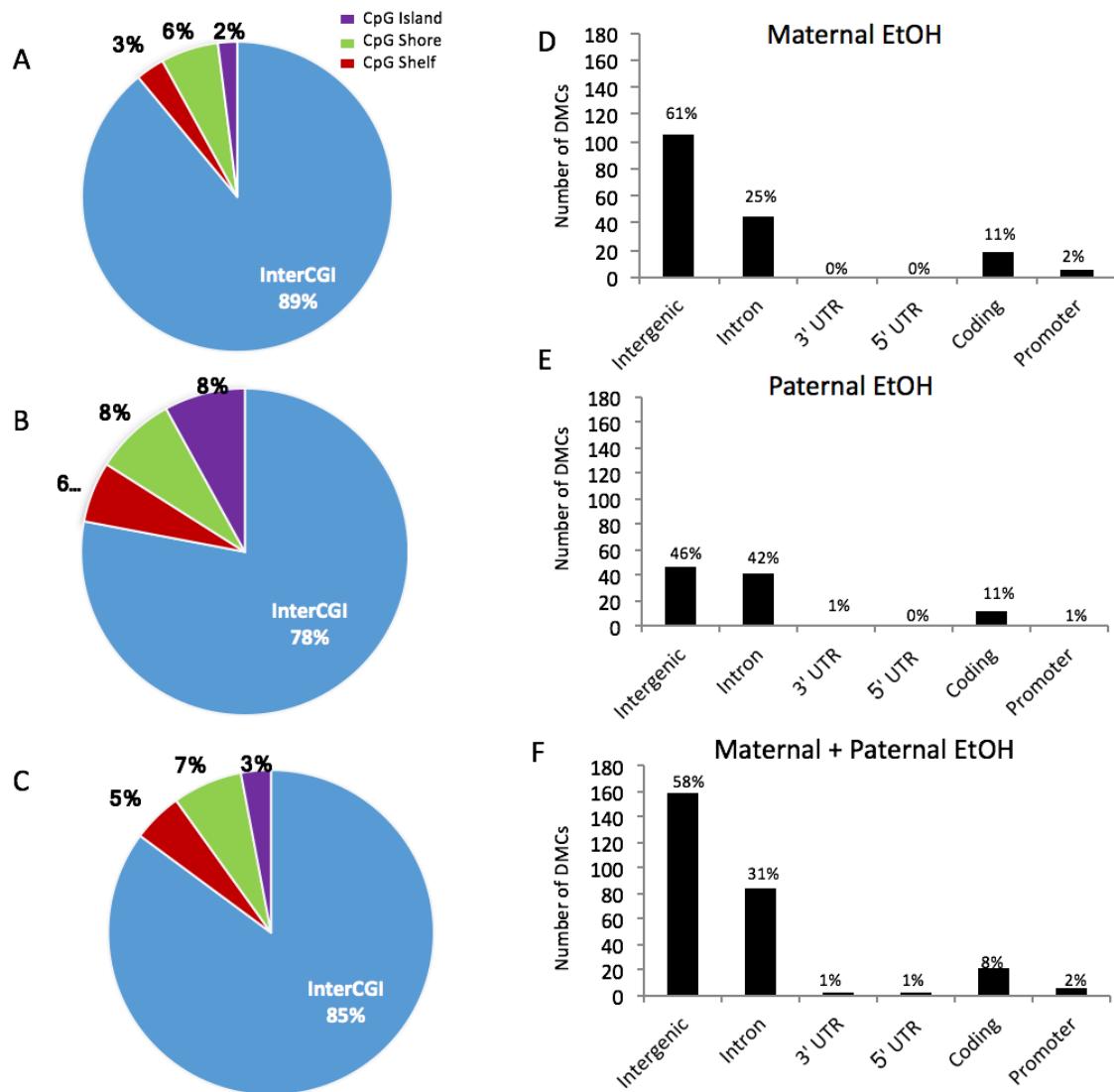


Figure 7. Location of differentially methylated cytosines within CpG and functional regions of the genome. Pie charts represent the percentage of DMCs found in each region as defined by CpG status in pups from (A) maternal ethanol, (B) paternal ethanol, and (C) dual parent exposure. Bar graphs representing the number of DMCs detected in each defined genic regions in (D) offspring from maternal ethanol-exposed, (E) offspring from paternal ethanol-exposed, and (F) offspring from both maternal and paternal ethanol-exposed. UTR = untranslated region; CDS = coding DNA sequence

Methylation of gene promoter regions is considered to have the greatest potential impact on transcriptional gene activity (Jones, 2012). Our data revealed that 35-45% of all annotated promoters were interrogated with sufficient depth in each sample to detect reliable differences. Of those interrogated, only 10 genes had DMCs in their promoter regions (Fig. 7, Table 4). Residues found in the promoters of Fam110a and Esam were hypermethylated and Olr286 was hypomethylated in offspring of mating pairs with only maternal exposure to ethanol. The same hypermethylated cytosine was found in the Esam promoter with only paternal exposure, and this was the only instance of differential promoter methylation in these offspring (Table 4). Unexpectedly, the Esam promoter was not differentially methylated in offspring where both parents were exposed. Instead, adolescent ethanol exposure of both parents induced hypermethylation of cytosine residues in the offspring gene promoters of Arrdc1, Ephb3, and miR6216. Conversely, this same treatment induced hypomethylation in the gene promoters for Golt1b, Gpank1, and Sparcl1 (Table 4).

Table 4. Genes associated with differential promoter methylation for each treatment group. Those with significant hypermethylation compared to control are in red rows, with significant hypomethylation in blue rows. Average percent methylation for groups are given in parentheses \pm SEM (control; treatment).

Maternal Ethanol	Paternal Ethanol	Maternal + Paternal Ethanol
Esam (3.8 ± 4.7 ; 32.66 ± 12.3)	Esam (3.8 ± 4.7 ; 28.5 ± 3.11)	Arrdc1 (0 ± 0 ; 93.3 ± 9.4)
Fam110a (28.2 ± 3.7 ; 56.2 ± 5.0)		Ephb3 (8.7 ± 12.3 ; 89.3 ± 14.0)
Olr286 (63.95 ± 14.7 ; 2.9 ± 2.6)		Mir6216 (71.3 ± 10.2 ; 91.15 ± 4.2)
		Gpank1 (92.3 ± 13.3 ; 62.4 ± 9.6)
		Golt1b (47.6 ± 12.3 ; 1.1 ± 1.9)
		Sparcl1 (95.1 ± 4.3 ; 41.5 ± 4.5)

mRNA Expression of Differentially Methylated Genes is Altered in the Hypothalamus.

Next, we measured the hypothalamic mRNA expression of several genes associated with DMCs that are known to have functions in the nervous system in order to determine if the gene methylation status correlated with gene expression. Genes harboring differential promoter methylation were expected to show a reduction in gene expression, however four genes with differential promoter methylation were excluded because they have not been well characterized (Fam110a, Olr286, Mir6216, Golt1b). Several genes associated with coding or intergenic differential methylation were also measured as they are related to neuro-development or function, with uncertainty as to the effect of methylation at these positions on their mRNA expression.

Hypermethylation of arrestin domain containing protein 1 (Arrdc1), a gene involved in vesicle formation, correlated with a 50% reduction in mRNA expression in offspring where both parents were exposed to alcohol (Table 5). The modified methylated cytosine residue was located within the promoter region (736 bp upstream of the TSS), suggesting that promoter hypermethylation of Arrdc1 reduced downstream gene transcription. This methylation site is within the canonical binding site of the transcription factor family E2F, particularly the sequence which E2F1 and E2F2, both transcriptional activators, bind to (Saadeh and Schulz, 2014). It has recently been shown that methylation within the E2F1 recognition site can prevent binding of E2F1 and subsequent downstream gene expression (Campanero et al., 2000). In these experiments, and others, it has been shown that differential methylation of a single residue is sufficient to change binding of MBD proteins and/or transcription factors to the DNA.

This change in protein:DNA interaction is, therefore, likely to elicit changes in transcription. In this way, a single 5mC change can impact transcription (Campanero et al., 2000). However, there was little correlation between gene expression and DMCs for the other genes we tested, suggesting that the relationship between DNA methylation and gene expression may be more complex (Table 5). For example, Ephrin-type B receptor (Ephb3), a receptor tyrosine kinase thought to inhibit synaptic stability, had a hypermethylated residue in the promoter region, 863 bp upstream of the TSS, in offspring in which both parents were exposed to alcohol, yet there were no changes in Ephb3 gene expression (Table 5).

Table 5. Summary data for mRNA expression of DMC-associated genes in male offspring.

Gene	Expression Fold Change ± SEM			P value	DMC state	Location of DMC
	Maternal Ethanol	Paternal Ethanol	Maternal + Paternal Ethanol			
Sparcl1	0.61 ± 0.15	0.71 ± 0.12	0.38 ± 0.10	0.047*	Both Exposed (2): -53%, -54%	Promoter, CpG shore
Arrdc1	0.90 ± 0.16	1.15 ± 0.04	0.53 ± 0.13	0.005*	Both Exposed :+92%	Promoter
Esam	1.04 ± 0.14	1.25 ± 0.15	0.51 ± 0.10	0.005*	Maternal: +28%, Paternal: +26%	Promoter
Gpank1	0.99 ± 0.06	1.25 ± 0.08	0.89 ± 0.10	0.009*	Both Exposed : -35%	Promoter, exon
Ephb3	1.35 ± 0.10	1.30 ± 0.11	1.19 ± 0.12	0.223	Both Exposed: +85%	Promoter
Grm4	1.10 ± 0.12	1.25 ± 0.10	1.28 ± 0.16	0.454	Maternal (3): +48%, +60%, -82%	Promoter (3)
AVP	3.52 ± 1.30	3.00 ± 0.52	2.22 ± 0.61	0.122	Maternal: -36%, Paternal: -37%	Coding
Acvr2a	0.91 ± 0.07	0.93 ± 0.07	0.98 ± 0.11	0.943	Maternal: +67%	Intergenic
Begain	1.55 ± 0.24	1.20 ± 0.14	1.41 ± 0.27	0.351	Both Exposed: +75%	Intergenic
FGFR1	1.19 ± 0.13	1.06 ± 0.17	0.998 ± 0.10	0.756	Both Exposed : -62%	Intergenic
Fzd10	1.21 ± 0.32	1.65 ± 0.36	1.58 ± 0.28	0.462	Paternal +37%, Both Exposed +26%	Intergenic
MC3r	1.29 ± 0.19	1.05 ± 0.13	1.26 ± 0.24	0.668	Paternal: +53%	Intergenic
NPY	1.23 ± 0.11	0.93 ± 0.17	0.84 ± 0.21	0.335	Paternal: -43%	Intergenic
Rtn4ip1	1.16 ± 0.09	1.17 ± 0.9	1.08 ± 0.08	0.650	Both Exposed : -34%	Intergenic

One-way ANOVA (n=7-10 per group) with p <0.05 considered significant. When there was a significant difference, pairwise comparisons were performed with Tukey's post hoc analysis. Values in boldface type represent statistically significant differences in those offspring as compared to offspring of control mating pairs.

Corresponding gene expression was measured in hypothalamic samples of female offspring of the same litters. There was only one consistent expression profile between male and female offspring, which was for the gene Esam (Table 6). Esam functions in cell-to-cell adhesion, particularly at tight junctions of the blood brain barrier in the brain. Sparcl1 and Arrdc1 were both more highly expressed in female offspring of dual parent EtOH exposure than

was the case for the same group in male offspring. Gpank was more lowly expressed in female offspring whose parents were given EtOH than the male offspring of these mating pairs (i.e. no significant difference in Gpank for female offspring whereas male offspring had significantly higher expression). AVP was significantly changed in female offspring whereas there was no difference in expression in male offspring.

Table 6. Summary data for mRNA expression of DMC-associated genes in female offspring.

Gene	Expression Fold Change ± SEM			P value	Relationship to Male Offspring Expression
	Maternal Ethanol	Paternal Ethanol	Maternal + Paternal Ethanol		
Sparcl1	0.93 ± 0.11	0.78 ± 0.15	0.74 ± 0.17	0.748	Higher in M + P
Arrdc1	0.63 ± 0.17	0.34 ± 0.05	3.16 ± 0.94	0.005*	Higher in M + P
Esam	0.55 ± 0.19	1.27 ± 0.21	0.46 ± 0.06	0.014*	Similar
Gpank1	0.91 ± 0.17	0.93 ± 0.08	0.92 ± 0.02	0.880	Lower in Paternal
AVP	0.61 ± 0.21	0.40 ± 0.18	1.78 ± 0.50	0.032*	Lower in all groups

One-way ANOVA (n=5 per group) with p <0.05 considered significant. When there was a significant difference, pairwise comparisons were performed with Tukey's post hoc analysis. Values in boldface type represent statistically significant differences in those offspring as compared to offspring of control mating pairs.

Discussion

The results from this study revealed three novel findings and highlight the potential for both maternal and paternal preconception binge-like alcohol abuse during adolescence to alter the epigenetic landscape of first-generation offspring. First, there was a lack of global DNA methylation changes in offspring as a result of parental preconception exposure to binge ethanol treatment, suggesting that ethanol mobilizes distinct molecular machinery that confers specificity to DNA methylation sites within the genome. This observation would also support

the conclusion that intergenerational ethanol effects are not due to broad ethanol-induced dysfunction in the gametes. Second, the modes of epigenetic inheritance are more complex than that of classical genetic inheritance, and do not necessarily reflect equal contributions of both parents. Unexpectedly, genes that were differentially methylated with either maternal or paternal ethanol exposure (i.e. Esam, See Table 5), were not differentially methylated when both parents were exposed. These results suggest that recombination events during early conception may mask or redefine individual parental epigenetic marks. Third, there was a high prevalence of intergenic, non-promoter methylation in the genome and, methylation of gene promoter regions did not always correspond to changes in gene expression. Additionally, gene expression between male and female offspring was discrepant. The stringent analysis parameters used along with our mild animal paradigm underscore the remarkable nature of our results, showing preconception exposure of either parent to just a few episodes of binge-pattern alcohol consumption can cause differential methylation in the hypothalamus of offspring.

To our knowledge, this is the first report of a genome-wide approach examining DNA methylation patterns in ethanol-naïve offspring of parents exposed to binge ethanol treatment during puberty. Previous work examining adult brain tissue reported that repeated adolescent ethanol treatment reduces the function of enzymes involved in epigenetic patterning, including DNA methyltransferases and histone deacetylases (Sakharkar et al., 2014). Therefore, it could be predicted that epigenetic marks as a whole would be reduced in all tissues following ethanol exposure. Instead, our results showed that adolescent binge ethanol exposure had specific

consequences at particular residues within the genome for first generation offspring. The lack of global changes in DNA methylation of the hypothalamus in these animals suggests that alcohol does not cause a deficiency in the epigenetic machinery as a whole, especially in the gametes of these exposed animals. Rather, our results suggest that there might be a wide range of nucleotide residues in gametes that are susceptible to alcohol-induced modifications, but the underlying molecular basis for vulnerability at these cytosine residues requires further research. Additionally, further research into the epigenetic changes in specific nuclei of the hypothalamus, such as the paraventricular nucleus, may reveal more noticeable changes in methylation patterns as each nucleus has distinct gene expression patterns and functional outputs.

Our experimental design allowed us to differentiate between the maternal and paternal contributions to offspring methylation patterns, providing some insight into sex-specific mechanism(s) by which epigenetic marks are transmitted to offspring. DNA methylation was altered in the hypothalamus of alcohol-naïve offspring regardless of which parent was exposed. However, very few of the detected DMCs were common to all treatment groups and there were very few DMCs that could be attributed to maternal and paternal exposure, separately, that were then combined in offspring when both parents were treated. Additionally, the differentially methylated residues fall outside regions of known parental imprinting. These results highlight the complexity of epigenetic inheritance and also allow us to speculate about the epigenetic vulnerabilities of gametes to binge alcohol exposure. One possibility is that offspring methylation is reflective of changes in parental gamete methylation that are simply

passed on to offspring. Alternatively, it is possible that the gametes of both parents transmit dysfunctional epigenetic machinery to the offspring, preventing proper epigenetic patterning. For example, hypermethylated residues that we observed in alcohol naïve offspring might have escaped demethylation during normal embryogenesis, while hypomethylated residues were skipped during remethylation processes. Another possibility is that post-natal treatment of offspring by the mother is changed when either parent is exposed to alcohol, as has been a suggested mechanism in other preconception treatment experiments (Mashhood et al., 2012). Our experimental design precludes determining which of these possibilities represents the mechanistic basis for sex-specific contributions of offspring methylation patterns following preconception alcohol use. Future studies that include maternal cross-fostering and quantification of gamete methylation prior to conception will further refine our understanding.

Based on published literature, we can speculate that site specific changes to methylation patterns, such as the ones observed in this study, would require protein or nucleic acid "guides" that would direct these epigenetic events to specific cytosine residues in the genome. Putative molecular candidates for this process include non-coding RNAs, which can be transmitted via gametes to the embryo and are known to be critical for embryonic development (Rodgers et al., 2013; Rodgers et al., 2015). Recently, mechanisms demonstrating that non-coding RNAs can mediate DNA methylation were described and this process has been hypothesized to effect transgenerational epigenetic inheritance (Holoch and Moazed, 2015; Matzke and Mosher, 2014; Peschansky and Wahlestedt, 2013; Yan, 2014). Similarly, previous work in our lab demonstrated that adolescent binge ethanol treatment can alter the long-term

expression of microRNAs in the hypothalamus, and these small non-coding RNAs could also dictate changes in offspring gene expression (Prins et al., 2014). Taken together, the emerging evidence supports the hypothesis that adolescent exposure to binge alcohol alters the expression of non-coding RNA in both the sperm and egg and those RNAs can direct a different epigenetic landscape in multiple organ systems in the offspring.

This study revealed that a large percentage of discrete changes in DNA methylation were located in different functional regions of the genome, with the highest prevalence in intergenic regions as well as in introns and coding regions. One possible conclusion is that the intergenic methylation sites correspond to enhancer regions, which may influence gene expression of proximal or more distal genes. The current analysis only examined the relationship of DMCs to the nearest downstream gene and further work needs to be done to test the possibility of their interaction with distant elements. Differential methylation within the coding region of a gene has been previously shown to have case-dependent impacts on gene expression. Some reports have shown that gene body methylation can increase transcription, while others have shown that it might inhibit transcription (Jones, 2012; Watson et al., 2015). Still others have shown that intron methylation may cause alternative splicing of the transcript (Maunakea et al., 2013). In this study we did not measure a direct relationship between the methylation status and expression pattern for all of the select genes we investigated, however, it is important to carefully interpret the causal relationship between methylation and gene expression (Birney et al., 2016). We also observed sex differences in the gene expression patterns of differentially methylated genes in male offspring. This was not

unexpected as our previous study of F1 gene expression using a microarray determined many genes were differentially expressed in alcohol-naïve male and female offspring, but many of them were not shared between the sexes (Przybycien-Szymanska et al., 2014). Future research will examine the consistency of methylation marks between male and female offspring, as well as the stability of methylation in the offspring as they develop and age.

Many biological systems have shown that differential methylation at an individual residue can impact gene transcription, mainly through altering interactions of transcription factors with the genome (Wyatt et al., 2013). However, recent studies have demonstrated that the relationship between hypermethylation of promoters and gene expression is both gene- and tissue-specific (Birney et al., 2016; Jones, 2012). Therefore, the reported methylation marks in the young offspring could lead to altered hypothalamic development and/or predispose them for adverse responses to alcohol or other stressors later in life. The DNA may be “poised” for further environmental influence, which could manifest as more pronounced phenotypic differences in adulthood. Alternatively, these methylation marks could represent evolutionary adaptation to environmental toxins and will confer resilience in the offspring. For example, a recent study in Wild guinea pigs found that exposure of fathers to high heat causes adaptive responses in offspring via DNA methylation and differential expression of a key thermoregulation gene, *Stat3* (Weyrich et al., 2016).

Preconception use of other common drugs such as nicotine, opioids and marijuana has been a focus of several previous studies, but this study is the first of our knowledge to examine the effects of maternal preconception exposure to alcohol on offspring (Vassoler et al., 2014).

Our results are consistent with a recent report on the use of marijuana and the intergenerational effects of its active ingredient tetrahydrocannabinol (THC) (Watson et al., 2015). In that study, DNA methylation profiles from the nucleus accumbens brain region of drug-naïve offspring revealed discrete, yet genome-wide, changes with some correlating with altered gene expression when both parents were treated with THC throughout pubertal development (Watson et al., 2015). The parents were given THC every third day during a 21-day period in pre-puberty (starting at PND 28) and were mated after drug abstinence for two weeks. Taken together these results provide evidence that there is epigenetic vulnerability to drugs of abuse that extend beyond the exposed individual, and that these changes may affect global epigenetic regulation of the genome.

In conclusion, this study provides the first genome-wide interrogation of the intergenerational effects of adolescent binge-pattern alcohol consumption in rats. Remarkably, we demonstrated that there were altered DNA methylation patterns in alcohol-naïve male offspring, regardless of which parent was exposed to alcohol. These changes were at discrete residues throughout the genome and differed between maternal and paternal ethanol exposure, underscoring the complexity of epigenetic inheritance. Additionally, DMCs were mostly found in intergenic, intronic and coding functional regions and did not directly correlate with gene mRNA expression. These results provide insight into the mechanism of intergenerational epigenetics and the potential vulnerability of offspring to both maternal and paternal preconception binge-pattern alcohol consumption.

CHAPTER IV

PARENTAL PRECONCEPTION ALCOHOL EXPOSURE IMPACTS OFFSPRING DEVELOPMENT

THROUGH PUBERTY

Under review, Journal of Endocrine Society

Introduction

Epigenetic modifications, or epimutations, are defined as functionally relevant changes to the genome that do not alter DNA sequence. They can include methylation of cytosine residues within DNA, histone protein modifications, and non-coding RNA mediated changes in gene expression. These epigenetic mechanisms work together to modify gene expression throughout the body and are heritable from cell to daughter cell and from parent to offspring. In this way, parental epigenetic patterns can impact offspring traits independent of Mendelian genetics (Carone et al., 2010; Minnes et al., 2014; Öst et al., 2014; Weyrich et al., 2016).

DNA methylation is a relatively stable epigenetic modification by which environmental information can be transmitted to first generation offspring faster than evolutionary adaptation similar to Lamarckian inheritance theories (Carone et al., 2010; Jones, 2012). DNA methylation patterns can be induced through preconception behaviors and/or experiences of parents including diet (Öst et al., 2014), exercise (Denham, 2017), and drug exposure (Minnes et al., 2014). Using adolescent alcohol exposure as a model for both physiological and psychological stress, our lab previously showed that preconception parental binge-pattern alcohol exposure

altered DNA methylation patterns in the hypothalamus of their alcohol naïve offspring, which is a region of the brain involved in regulation of pubertal development, stress regulation, and social behaviors (Asimes et al., 2017). In that study, differentially methylated cytosine residues were observed genome wide and occurred in offspring regardless of which parent (maternal, paternal, or both) was exposed to alcohol during adolescence. These observations have potentially serious implications for human health, as alcohol is the most widely abused drug in the United States, with more than 4.6 million Americans under the age of 21 engaging in binge-pattern alcohol abuse (SAMHSA, 2017). Binge-pattern alcohol drinking is distinguished from casual consumption by the large volumes of alcohol that are consumed in a short amount of time, reaching a minimum blood alcohol content (BAC) of 0.08% within 2 hours. It is known that this type of rapid consumption during adolescent development can have long-lasting effects in the brain (Crews et al., 2016; Govorko et al., 2012; Przybycien-Szymanksa et al., 2010; Przybycien-Szymanksa, 2011; Spear, 2016; Trantham-Davidson et al., 2017; Vetro et al., 2017), and our recent evidence suggests these effects may extend beyond the individual to directly impact first-generation offspring (Asimes et al., 2017; Govorko et al., 2012; Hill et al., 2017; Przybycien-Szymanksa et al., 2014). However, these epimutations were measured during the early infant stage, and the long-term functional consequences of these changes for the offspring are unclear.

It has been hypothesized that epigenetic marks passed to offspring can serve as adaptive aids, such that encountering similar environmental stressors or compounds would be more easily tolerated (Skinner, 2015; Weyrich et al., 2016). If this were the case for parental

binge alcohol consumption, we would expect that offspring of parents who were exposed to binge pattern alcohol consumption would have an increased ability to metabolize alcohol, resulting in lower BAC when exposed to alcohol, and have an attenuation to the normal physiological stress response observed following alcohol consumption. Therefore, in this study we tested two parallel hypotheses: 1) offspring of binge EtOH-exposed parents will have altered normal development through puberty; and 2) parental preconception EtOH exposure will provide adaptive protection against the effects of EtOH exposure in offspring. Our goal was to first determine functional consequences of parental EtOH exposure in offspring as they develop through puberty and, secondarily, to assess how parental EtOH exposure would impact offspring when they were exposed to alcohol themselves.

Results

Experiment 1: Baseline Characteristics of Male and Female Offspring from Parents

Administered Preconception Binge-Pattern EtOH During Adolescence (Fig 9-13).

Offspring of alcohol-exposed parents had decreased body weight after puberty, but not at birth. In order to assess the impacts of preconception parental EtOH exposure on offspring outcomes, we mated males and females in adulthood after adolescent exposure to EtOH (Fig 8). Impregnated females were left undisturbed through normal gestation and birth. There were no statistically significant differences in the time from pair-housing to birth (indicative of gestation length), number of pups, or sex ratio of offspring between EtOH- and water-treated mating pairs. Within 1 hour of birth all animals were weighed and litters were

culled to 10 rats per dam (5 male, 5 female). Offspring (male or female) weights were not different at birth (PND 0) or at PND 7 between treatment groups (Fig 9A). However, as the offspring grew through weaning and entered puberty (PND 36), the male offspring of parents who received EtOH were significantly smaller than the offspring of water-treated parents, with average weights of 176 g and 188 g, respectively (Fig 9A). This was also true for female offspring at PND 36 with average weights of 144.05 g for offspring from EtOH-treated parents and 152.75 g for offspring from water-treated parents. The offspring weight remained consistently lower for both male and female offspring, although the rate of growth, as analyzed by calculated differences in the slope of the growth curves as 4 g/day for females and 8 g/day for males, was not different than control counterparts (Fig 9A).

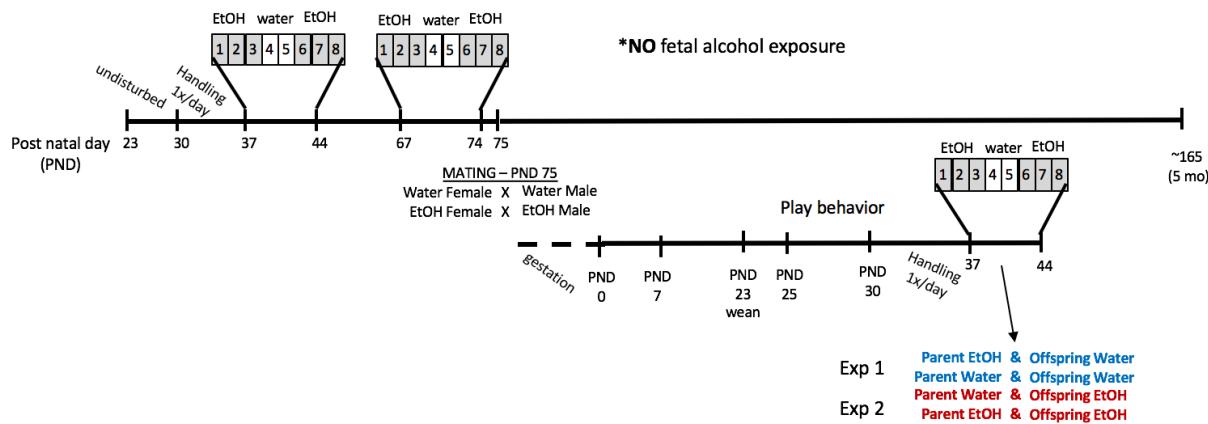


Figure 8. Animal treatment paradigm for Experiment 1 and 2. Wistar rats received EtOH exposure during early and late puberty (control animals received water only), and were paired housed for mating 24 hours following the last dose in pairs of Water-Water or EtOH-EtOH. After normal gestation and birth, litters were culled to 10 pups per dam (5 male, 5 female) and left undisturbed until weaning at PND 23. Offspring were then moved into same-sex group housing of 5 animals, all from the same parental treatment group, and home-cage play behavior was recorded daily from PND 25 to PND 30. Animals were then moved to paired housing and

randomly assigned to Experiment 1, where all offspring received water, or Experiment 2, where offspring underwent the same EtOH treatment paradigm as their parents.

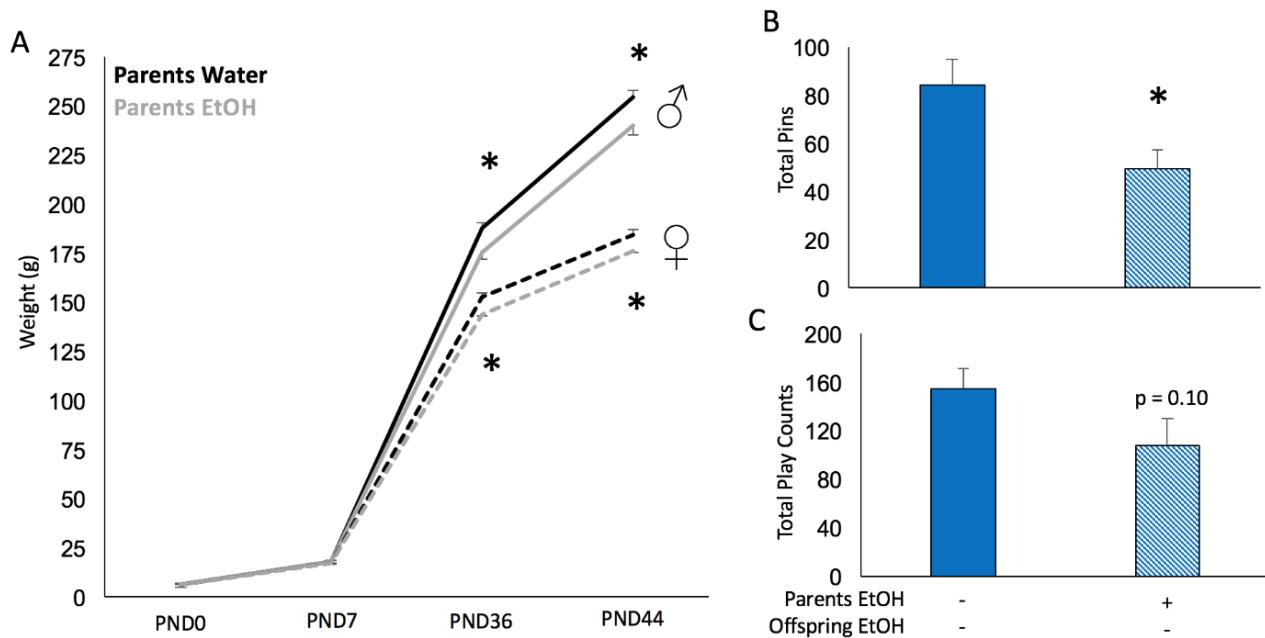


Figure 9. Offspring of EtOH-treated parents were smaller after pubertal onset and displayed fewer play behaviors. A) Male and female offspring of EtOH-treated parents were smaller at PND36 and PND44 compared to offspring of water-treated parents, although there was no difference at birth or PND7 and growth rate between PND 7 and PND 36 was the same. (B) Offspring of EtOH-treated parents displayed fewer pinning behaviors compared to offspring of water-treated parents. (C) There was a modest decrease in overall play behaviors exhibited by offspring of EtOH-treated parents compared to water-treated parents. Two-sample t-test, * $p < 0.05$, mean \pm SEM, $n=10$ /group.

Juvenile play behavior was altered in offspring of alcohol-exposed parents. Offspring were scored on their home-cage play behaviors for 5 consecutive days during the pre-pubertal juvenile period from PND 25 to PND 30. During this time, animals were housed in groups of five, which allowed for quantification of the group play behaviors of biting, chasing, pouncing, pinning and boxing along with normal grooming behaviors. There were no differences in the scored behaviors between the sexes; therefore, males and females were combined for

statistical analysis. Overall play counts were slightly lower in offspring of EtOH-treated parents (Fig 9C, $p=0.10$), but there were no statistically significant differences in other play behaviors or grooming behaviors; however, our results demonstrated a significant reduction of 42% in pinning behaviors, a core component of juvenile play, in offspring whose parents were exposed to EtOH preconception (Fig 9B). Pinning behavior in juvenile rats is considered the most reliable and high frequency component of juvenile play, indicating disrupted juvenile social interactions in offspring of EtOH-treated parents (Panskepp et al., 1984).

Pubertal markers were dysregulated by parental preconception exposure to alcohol.

The hypothalamus not only controls the physiological stress response, but is also central to pubertal regulation as part of the hypothalamo-pituitary-gonad (HPG) axis. Considering that both male and female offspring of parents who were given EtOH displayed stunted growth following the onset of puberty, we hypothesized that pubertal hormones would be dysregulated. First, *GnRH* mRNA expression was measured in the hypothalamus to determine if there was pubertal perturbation at the level of the brain. *GnRH* mRNA was not statistically different in male or female offspring from parents given EtOH treatment, although there was a consistent decreased trend in both sexes (Fig 10A, B). Next, we measured circulating luteinizing hormone (LH; pituitary function) and testosterone (T; gonadal function) from trunk blood collected at the time of euthanasia (PND 44). LH was significantly decreased in male (Fig 10C), but not female (Fig 10D), offspring of parents exposed preconception to EtOH compared to offspring of water-treated parents. This had potentially functionally relevant consequences, as T

levels were also modestly reduced ($p = 0.08$ for males, Fig 10E, and $p = 0.10$ for females, Fig 10F).

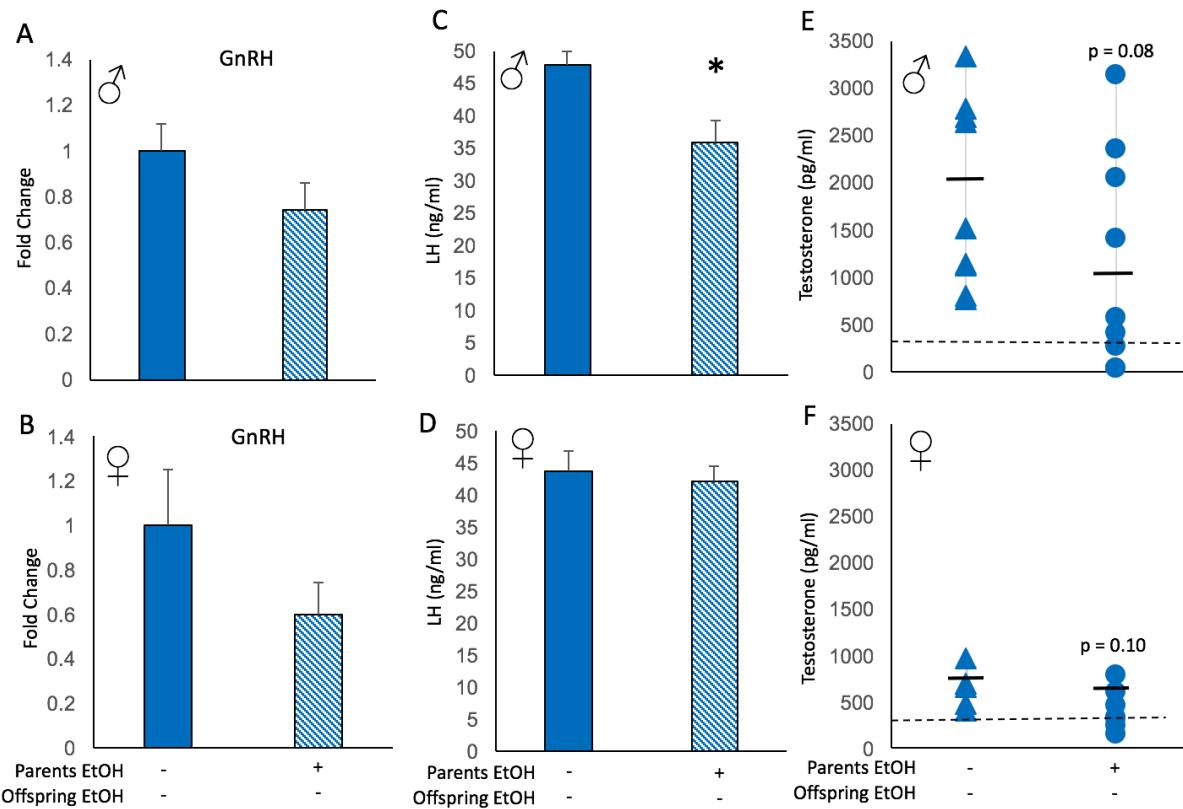


Figure 10. Offspring of EtOH-treated parents had changes in baseline HPG axis parameters. Hypothalamic gonadotropin-releasing hormone (GnRH) mRNA expression was decreased in male (A) and female (B) offspring of parents who were exposed to EtOH-treatment before conception. Circulating luteinizing hormone (LH) was decreased in male (C), but not female (D) offspring of EtOH-treated parents. Circulating testosterone (T) was reduced in male (E) and female (F) offspring of EtOH-treated compared to offspring of water-treated parents. Assay limit of detection indicated by dashed horizontal line in E and F. Two-sample t-test, * $p < 0.05$, mean \pm SEM, $n=10$ /group.

Testicular development was not overtly impacted by parental EtOH exposure.

Spermatogenesis is dependent on high intratesticular T levels, which greatly exceed circulating concentrations and are maintained independently through local production by Leydig cells (Coviello et al., 2004). Our results demonstrated that preconception parental EtOH exposure

reduced male offspring LH and to a lesser extent T, suggesting that spermatogenesis could be impacted. Therefore, we measured genetic markers of mature sperm (Acrosomal Vesicle Protein 1 (*Acrv1*), Lactate Dehydrogenase C (*LHDC*) and androgen binding protein (*ABP*) in the testes. Our results showed no significant differences in mature sperm (Fig 11A, B) or ABP (Fig 11C) between male offspring of EtOH- and water-treated parents. Additionally, testes were cryosectioned and stained with H & E and scored for presence of mature (i.e. elongated) spermatids and opening of a central lumen in the seminiferous tubules. Consistent with the lack of changes in genetic markers, we also observed no gross anatomical differences in testes size, spermatid count, and luminal opening (Fig 11D).

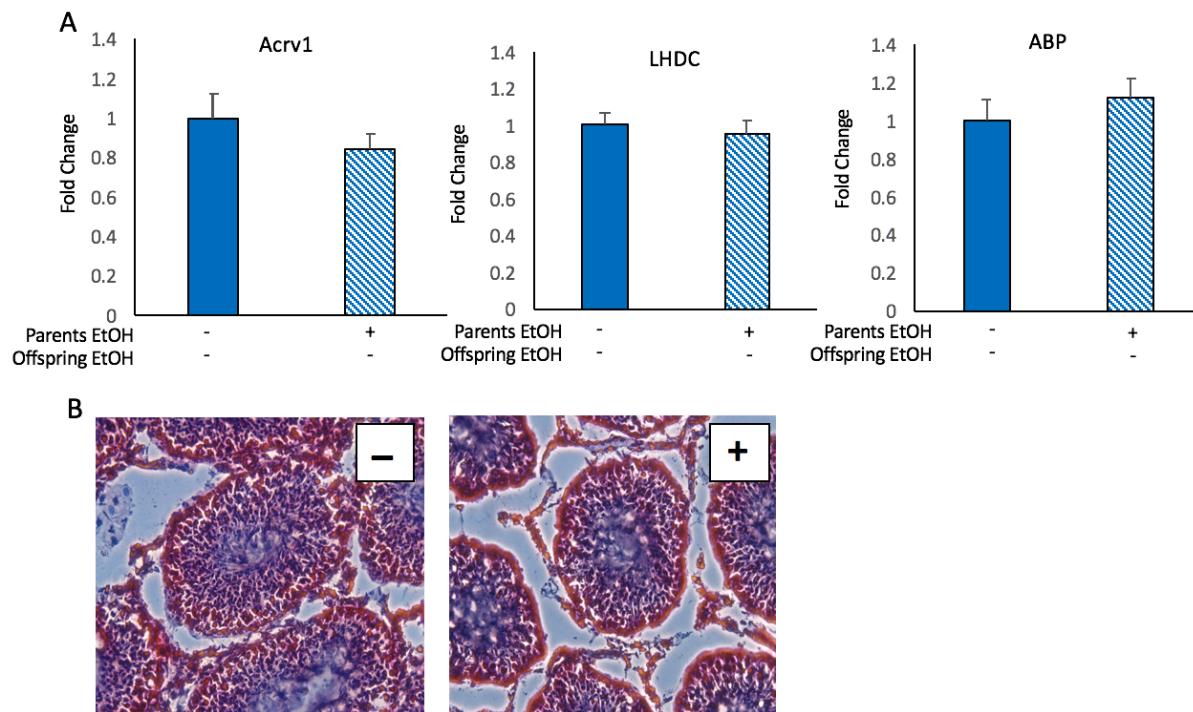


Figure 11. Gene markers of mature sperm and testicular morphology were not altered by parental EtOH exposure. A) mRNA expression of mature sperm markers *Acrv1*, *LHDC*, and *ABP* was not different in testes of male offspring of EtOH- or water-treated parents. (B) Hematoxylin and eosin staining of the testes showed no apparent difference in gross morphology or number

of elongated spermatids between offspring whose parents received vehicle (left -) or EtOH (right +). Two-sample t-test, * p<0.05, mean ± SEM, n=10/group.

Male offspring of EtOH-treated parents had lower baseline CORT levels than offspring of control animals but expression of feedback genes was unchanged. Next, we assessed baseline function of the HPA axis in offspring whose parents were treated with EtOH, as alcohol is a known potent activator of the hypothalamo-pituitary-adrenal (HPA) axis. We found that male offspring had a statistically significant lower circulating CORT level when parents were given EtOH preconception (Fig 12A). By contrast, circulating CORT levels of female offspring were not dependent on parental preconception EtOH exposure (Fig 12B). Hypothalamic mRNA expression of *GR*, *CRF* and *AVP*, genes which are controlled by CORT negative feedback and mediate the HPA axis response, were not different between offspring of EtOH-treated or control-treated parents, for either male or female offspring (Fig 13A-F).

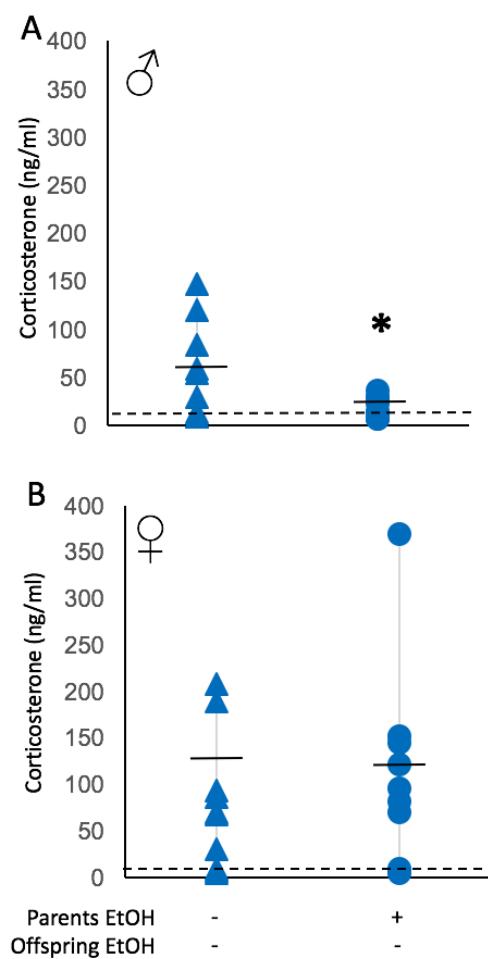


Figure 12. Baseline CORT in male, but not female, offspring was decreased following parental EtOH exposure. Male (A), but not female (B) offspring of EtOH-treated parents had significantly lower levels of circulating CORT compared to offspring of water-treated parents. Assay limit of detection indicated by dashed horizontal line. Two-sample t-test, * p<0.05, n=10/group.

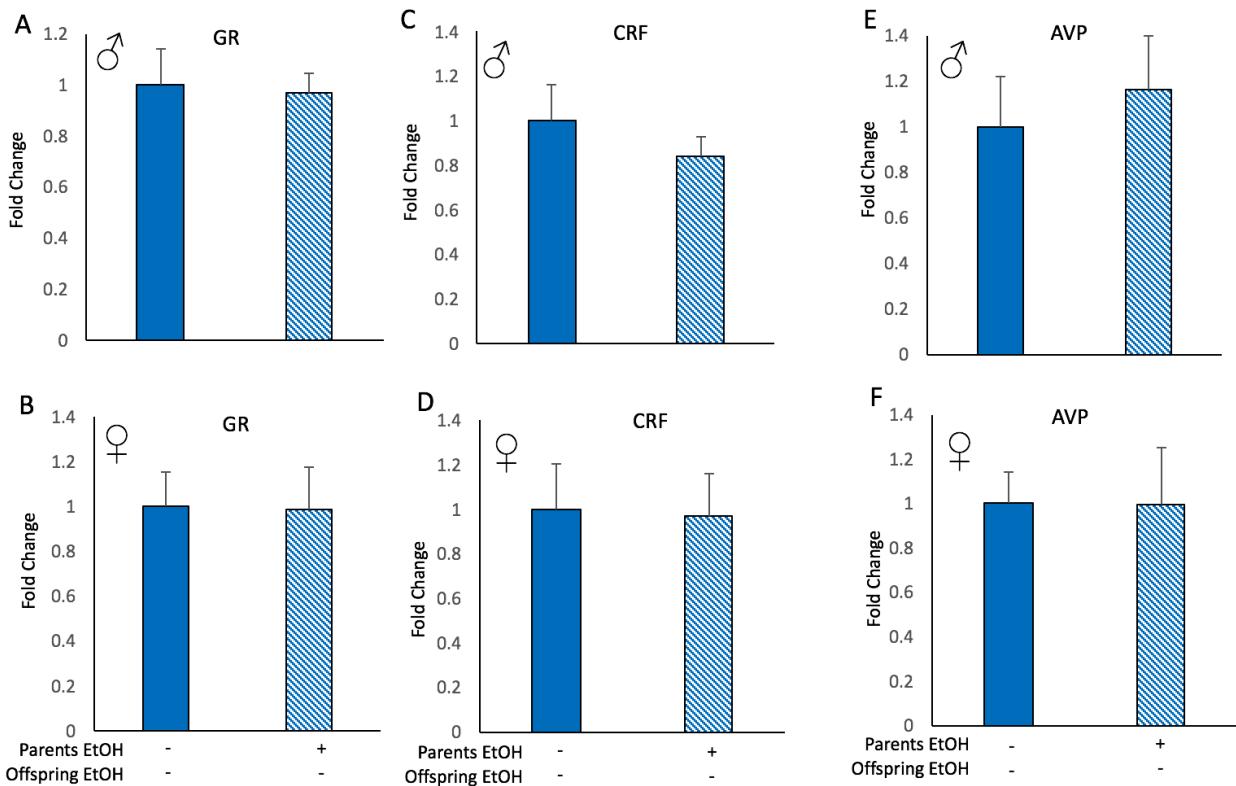


Figure 13. mRNA expression of glucocorticoid receptors and neuropeptides that mediate HPA axis were not affected by parental EtOH consumption. Hypothalamic mRNA expression of glucocorticoid receptor (*GR*), corticotrophin-releasing factor (*CRF*), and arginine vasopressin (*AVP*) was not affected by parental EtOH exposure in either male (A, C, E) or female (B, D, F) offspring. Two-sample t-test, * p<0.05, mean ± SEM, n=10/group.

Experiment 2: Effects of Adolescent EtOH Exposure in Offspring with Parental History of

Preconception EtOH Consumption (Fig 14-18).

In these studies, all offspring were administered the binge-pattern EtOH paradigm (see Fig 8) during early puberty (PND 37 – PND 44) and euthanized one-hour following the last dose of EtOH at PND 44. The variable of interest was whether the offspring responded differently to the EtOH treatment depending on parental history of preconception binge EtOH exposure, thereby testing our hypothesis that parental EtOH exposure predisposed offspring towards an adaptive response to adolescent binge-pattern EtOH (i.e. first-generation consequence).

Adolescent binge-pattern EtOH exposure significantly decreased body weight of female offspring whose parents had history of binge-drinking. Similar to what we observed with offspring body weight during normal development, adolescent binge-pattern EtOH exposure significantly decreased body weight in females, but not males (Fig 13A, B). Further, pursuant to our hypothesis that heritable epimutations in offspring may be adaptive aids in preparation for exposure to alcohol, we measured blood alcohol concentrations (BAC) in offspring. Blood alcohol levels were not impacted by parental exposure to alcohol, with male offspring of EtOH-treated parents averaging 126.5 mg/dl compared to control counterparts averaging 107.0 mg/dl. Female offspring also showed no significant effect of parent treatment on BAC, with 130 mg/dl average for control parents and 104.4 mg/dl average for parental-EtOH group (Fig 13C, D), suggesting that the offspring were not able to metabolize EtOH differently based on parental EtOH-exposure history.

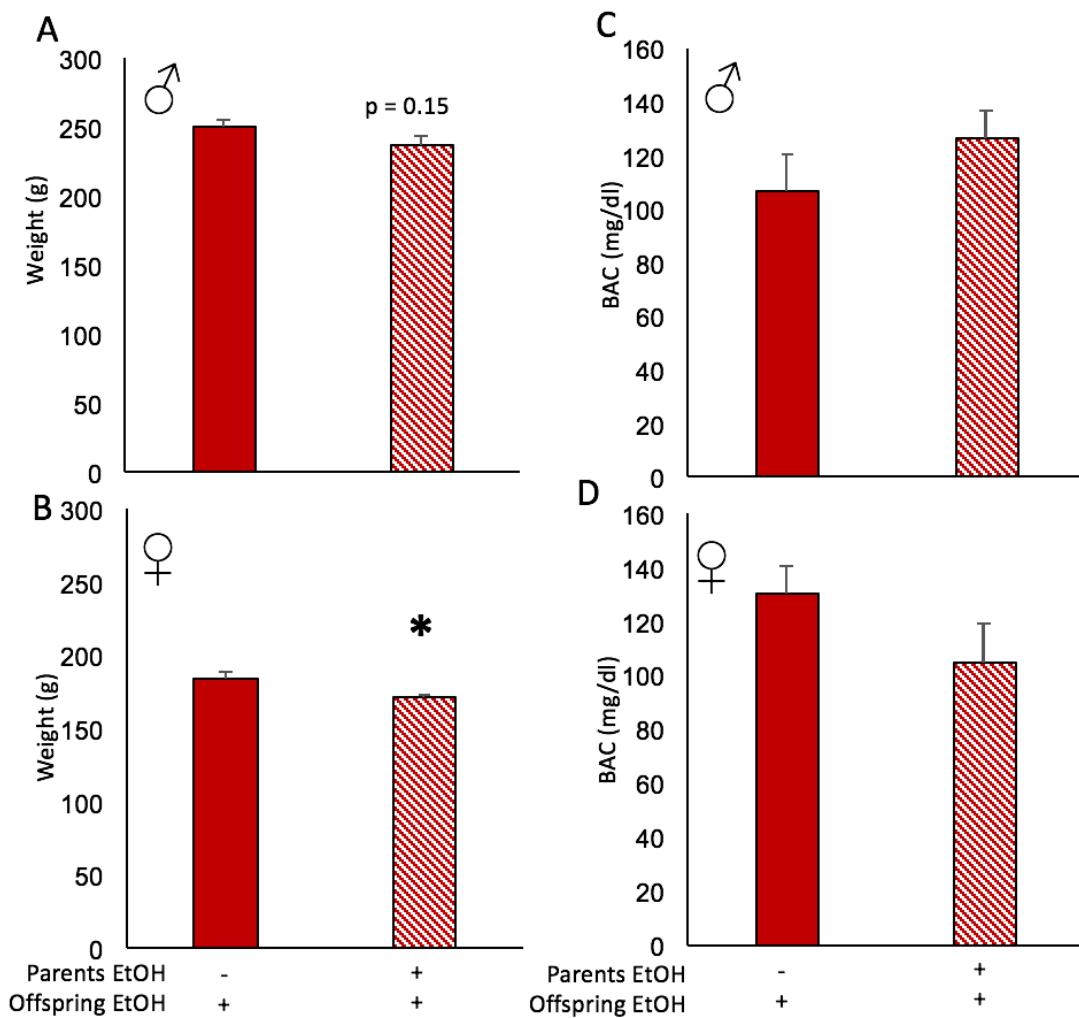


Figure 14. Body weight decreased in female EtOH-treated offspring of EtOH-treated parents.

A) Male EtOH-treated offspring of EtOH-exposed parents did not have significantly lower body weights compared to EtOH-treated offspring from water-treated parents. (B) Female offspring treated with EtOH had significantly lower body weights if parents had also been treated with EtOH. There was no difference in the blood alcohol concentration (BAC) between male (C) or female (D) offspring of EtOH-treated or vehicle-treated parents. Two-sample t-test, * p<0.05, mean ± SEM, n=10/group.

Effects of offspring EtOH exposure on pubertal development were exacerbated by parental preconception exposure to alcohol. Previous studies have shown that EtOH treatment during pubertal development can impair pubertal progression, dampening T and LH rises, and

slowing gonad maturation (Rachdaoui and Sarkar, 2013). In our alcohol-naïve offspring, we found a similarly attenuated HPG profile dependent on parental preconception EtOH treatment (see Fig 10). In this experiment where the offspring were treated with EtOH, we observed a more severe decrease in some of the HPG parameters, which depended on paternal history of EtOH exposure. First, there was a marked decrease in hypothalamic *GnRH* mRNA expression in EtOH-treated offspring if parents had been exposed to EtOH, but not water, before conception. This reduction was around 50% for both male and female offspring (Fig 15A, B). However, the reduction in *GnRH* mRNA was not reflected in correspondingly reduced LH levels, as LH levels were unaffected by EtOH treatment in both male and female offspring (Fig 15C, D). Circulating T levels were also significantly reduced in male offspring whose parents had been exposed to EtOH preconception (Fig 15E). By contrast, EtOH exposure did not significantly affect T levels in female offspring, although there was a strong trend for decreased levels in most of the animals tested (Fig 15F).

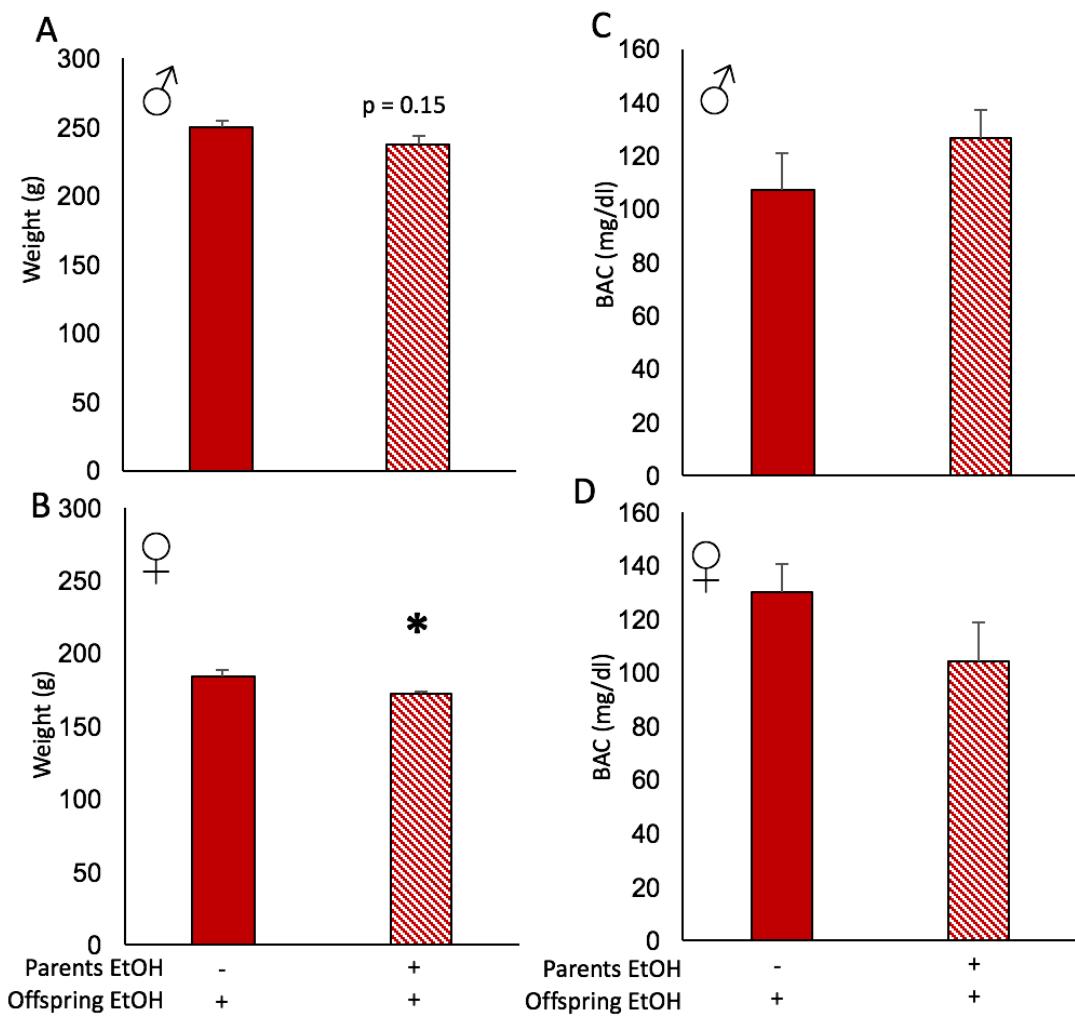


Figure 15. EtOH-treated offspring of EtOH-treated parents had decreased HPG axis parameters. Hypothalamic mRNA expression of gonadotropin-releasing hormone (*GnRH*) was significantly decreased in both male (A) and female (B) offspring who were treated with EtOH, if their parents were exposed to EtOH before conception. Circulating luteinizing hormone (LH) was not different between EtOH-treated male (C) or female (D) offspring whose parents received EtOH or water treatment. Testosterone (T) was significantly decreased in EtOH-treated male (E), but not female (F) offspring of EtOH-treated parents compared to EtOH-treated offspring of water-treated parents. Assay limit of detection indicated by dashed horizontal line in E and F. Two-sample t-test, * p<0.05, mean ± SEM, n=10/group.

EtOH treatment in offspring did not affect testicular development irrespective of

parental EtOH exposure. The observed reductions in HPG parameters suggested that testicular development might be further impacted if offspring of EtOH-treated parents were exposed to EtOH themselves during puberty. Therefore, we measured the same mature sperm markers (*Acrv1*, *LHDC*) and *ABP* in the testes and scored H&E stained cross sections of EtOH-treated offspring testes. Our results showed that there were no differences in mRNA expression for any of the genes measured (Fig 16A). In addition, there was no effect of offspring EtOH exposure on seminiferous tubule lumen opening or number of elongated spermatids irrespective of parental EtOH exposure (Fig 16B).

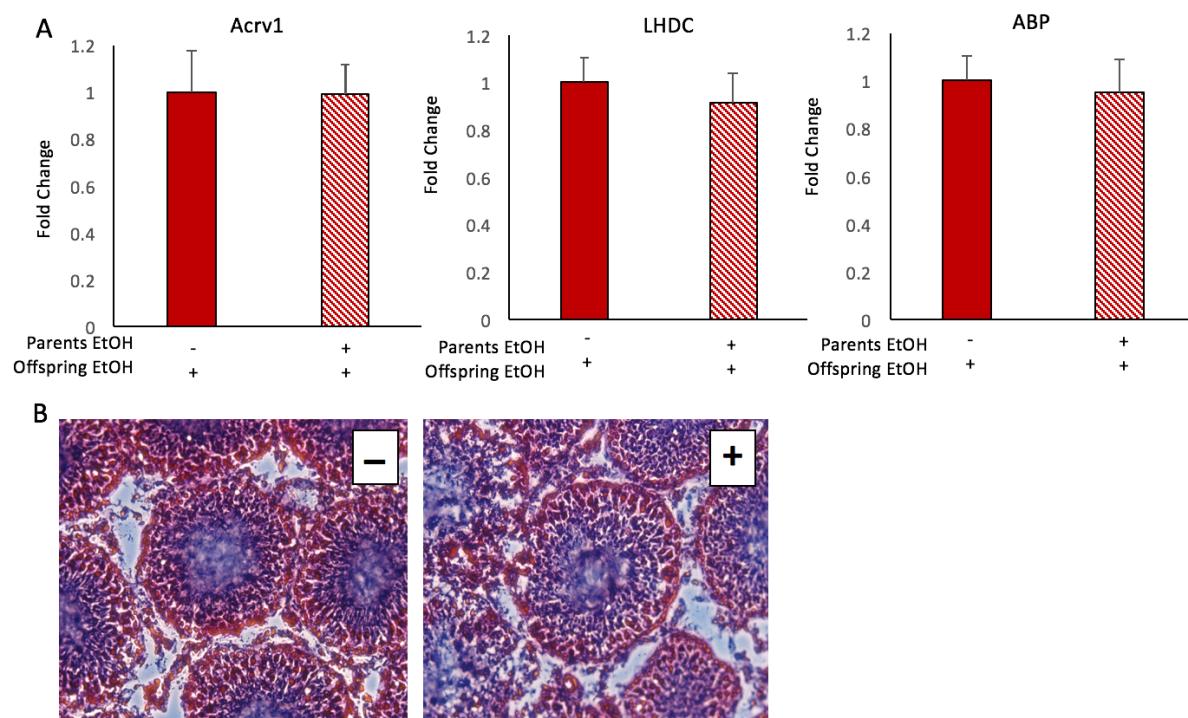


Figure 16. Gene markers of mature sperm and testicular morphology were not altered by offspring EtOH exposure. A) mRNA levels of *Acrv1*, *LHDC*, and *ABP* were measured in testes following offspring EtOH treatment and were not differentially expressed in male offspring of EtOH-treated or water-treated parents. B) Hematoxylin and Eosin staining of the testes revealed no apparent morphological differences or numbers of elongated spermatids between

EtOH-treated offspring whose parents received water (left -) or EtOH (right +). Two-sample t-test, * p<0.05, mean \pm SEM, n=10/group.

Offspring of EtOH-exposed parents had no adaptation in the stress response to EtOH. EtOH is a physiological a stressor, as evidenced by a sharp spike in circulating CORT shortly after exposure. Our previous studies demonstrated that adolescent binge-pattern alcohol exposure resulted in dysfunction of the HPA axis; specifically, negative feedback in the adult brain was impaired, and CORT remained significantly elevated after subsequent alcohol exposure (Przybycien-Szymanksa et al., 2010). Therefore, our hypothesis was that offspring would display a resistance to this potentially harmful spike in CORT. Our results showed that EtOH induced an increase in circulating CORT in all our EtOH-treated offspring, but these levels were not different between offspring whose parents received EtOH or water (Fig 17A, B). Similarly, the hypothalamic mRNA expression of GR, CRF and AVP following EtOH treatment was not impacted by preconception parental EtOH exposure for either male or female offspring (Fig 18A-F).

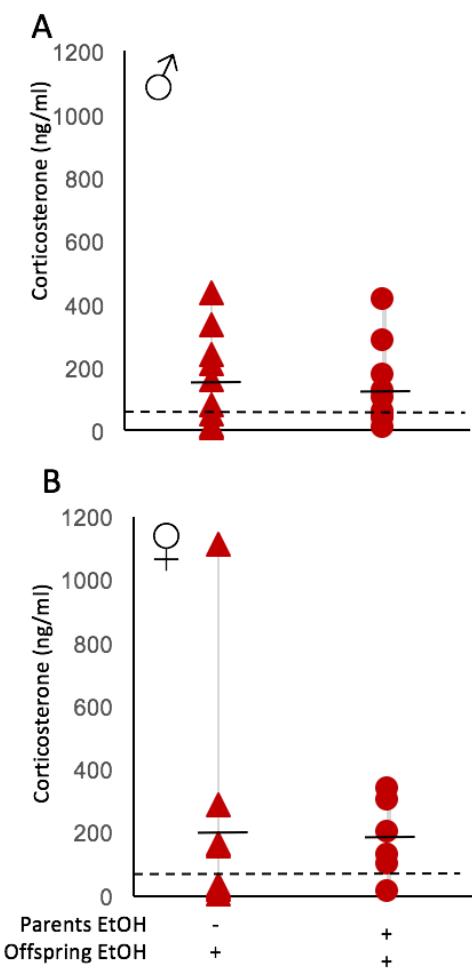


Figure 17. Circulating corticosterone (CORT) response to EtOH exposure was not dependent on parental history of EtOH treatment. Parental EtOH exposure did not change the levels of circulating CORT in male (A) or female (B) offspring after EtOH treatment. Assay limit of detection indicated by dashed horizontal line. Two-sample t-test, * p<0.05, n=10/group.

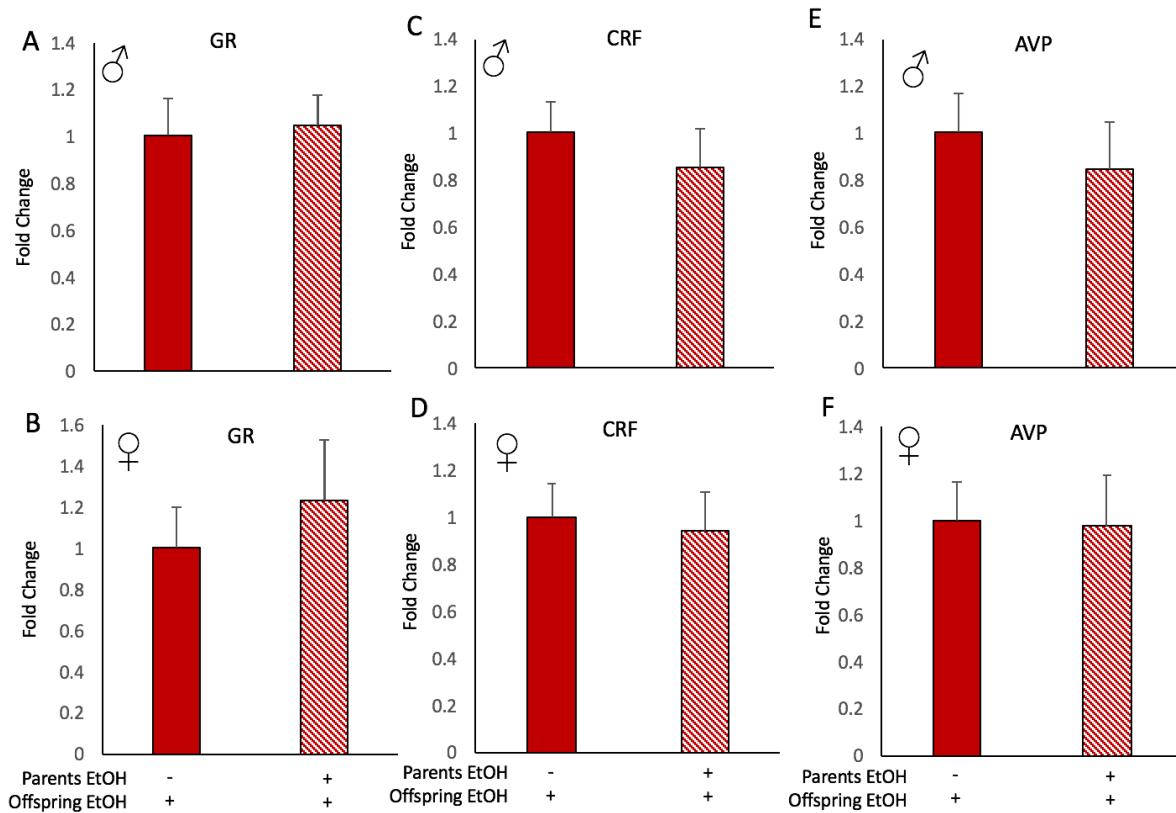


Figure 18. Parental history of EtOH exposure did not affect offspring mRNA expression of glucocorticoid receptors and neuropeptides in response to EtOH. mRNA expression of glucocorticoid receptor (GR), corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) following EtOH treatment in offspring were unaffected by parental EtOH exposure in either male (A, C, E) or female (B, D, F) offspring. Two-sample t-test, * p<0.05, mean ± SEM, n=10/group.

Experiment 3: Tracking Transcriptional Regulation of Candidate Genes in the Offspring

Hypothalamus Through Development Following Parental EtOH Exposure (Tables 7, 8).

In light of our previous findings that juvenile offspring showed altered genome-wide methylation patterns and that, as the offspring develop, there were modest changes to their physiology, the constancy of transcriptional regulation of several candidate genes which were shown to be differentially methylated and expressed at PND 7 were examined (Asimes et al.,

2017). I tested the hypothesis that changes in mRNA expression of differentially methylated genes measured in offspring at PND 7 would remain altered compared to age-matched counterparts at PND 44 and in a second litter. mRNA expression of candidate genes was measured in PND 44 offspring, as described in Experiments 1 and 2, as well as in PND 7 offspring of a second litter of the same mating pairs, which were conceived approximately 45 days following the last EtOH exposure of parents (see Fig 8). The candidate genes examined were chosen from Table 5 (Chapter III) and are meant to serve as a sampling of the potential regulation of many genes.

Adolescent offspring show no difference in gene expression of candidate genes except for Sparcl1, with or without exposure to EtOH themselves. The discrete changes in gene expression measured at PND 7 did not consistently correlate with methylation patterns, which is not unexpected. However, this leaves open the possibility that transcriptional regulation of these genes will fluctuate throughout the offspring life. Comparing the expression pattern of a few candidate genes between our earlier study in PND 7 male offspring and PND 44 male offspring (who did not receive any alcohol themselves) shows an opposite pattern of expression for Sparcl1 (Table 7). In PND 7 male offspring, Sparcl1 was significantly decreased in expression, but at PND 44 it was higher in offspring whose parents received EtOH compared to control offspring. Arrdc1 and Esam showed no difference in expression at PND 44, compared to a decrease in expression of both genes at PND 7. Gpank1 also showed a different expression pattern compared to offspring at PND 7 (where there was no difference in expression in alcohol-naïve offspring whose parents received EtOH). Female offspring showed no significantly

different patterns of gene expression at PND 44 between EtOH-treated and control parentage (Table 7). There were no differences in expression of these candidate genes in offspring who received alcohol themselves, for either male or female offspring (Table 7).

Table 7. Gene expression changes in male and female offspring at PND 44.

Gene	Experiment 1 (No Offspring EtOH) Expression Fold Change ± SEM			
	Male Offspring	P value	Female Offspring	P value
Sparcl1	1.43 ± 0.15	0.02*	1.21 ± 0.20	0.34
Arrdc1	0.87 ± 0.02	0.39	0.97 ± 0.09	0.79
Esam	0.92 ± 0.10	0.58	0.94 ± 0.10	0.63
Gpank1	1.21 ± 0.10	0.06	1.04 ± 0.07	0.69
Gene	Experiment 2 (All Offspring EtOH) Expression Fold Change ± SEM			
	Male Offspring	P value	Female Offspring	P value
Sparcl1	0.92 ± 0.06	0.90	1.08 ± 0.06	0.34
Arrdc1	1.20 ± 0.12	0.12	1.27 ± 0.14	0.08
Esam	0.78 ± 0.06	0.29	1.50 ± 0.26	0.09
Gpank1	0.87 ± 0.06	0.32	1.10 ± 0.05	0.20

Two-sample t test (n=5 per group) with p < 0.05 considered significant.

Second litter offspring show no difference in gene expression, except for Grm4

expression in males. It is a possibility that the heritable changes in gene expression we measure in offspring following parental EtOH exposure are due to the short amount of time between the EtOH exposures and conception (2 to 10 days). Here I wanted to examine a second litter of the same mating pair in order to determine if, at the same offspring age, there were differences in a first and second litter (i.e. a longer abstinence period before conception). Parents were pair housed for mating again around PND 105, 30 days after the first pair housing began. The candidate genes examined here were the same as listed above with the addition of

AVP, CRF, GnRH and GR. There were no differences in mRNA expression of any genes in female offspring and only one gene, Grm4, was altered by parental EtOH exposure in male offspring (Table 8). Grm4 was decreased to 61% of the expression level in offspring of control treated parents. Interestingly, Grm4 was not differentially expressed in any treatment group measured in the first litter at PND 7, although there were three DMCs within the gene promoter when only the mother received EtOH (see Table 5).

Table 8. Gene expression changes in second litter male and female offspring at PND 7.

Gene	Male Offspring (Parental EtOH) Fold Change ± SEM	P value	Female Offspring (Parental EtOH) Fold Change ± SEM	P value
Sparcl1	0.64 ± 0.10	0.248	1.66 ± 0.37	0.224
Arrdc1	0.96 ± 0.04	0.645	0.94 ± 0.11	0.756
Esam	0.74 ± 0.10	0.118	2.37 ± 0.99	0.210
Gpank1	1.13 ± 0.04	0.096	1.18 ± 0.19	0.412
Grm4	0.61 ± 0.13	0.05*	0.69 ± 0.12	0.169
MC3r	1.12 ± 0.21	0.661	1.42 ± 0.37	0.321
AVP	1.94 ± 0.68	0.283	1.13 ± 0.34	0.827
CRF	1.51 ± 0.46	0.342	1.01 ± 0.32	0.989
GnRH	1.57 ± 0.73	0.528	2.08 ± 1.81	0.239
GR	1.37 ± 0.12	0.423	0.79 ± 0.35	0.124

Two-sample t test (n=5 per group) with p < 0.05 considered significant.

Discussion

Taken together, our results demonstrated that parental preconception EtOH exposure did not confer any apparent adaptive phenotypic traits for the offspring, but rather, had potentially maladaptive consequences on offspring growth, social interactions, and pubertal development. In these studies, we used adolescent binge-pattern EtOH consumption as a model

for preconception epigenetic modifications induced by a mild physiological/toxicological stressor. Adolescent binge-pattern alcohol consumption is a major public health concern and many studies have documented the long-term negative consequences of this risky behavior for the individual (Crews et al., 2016; Przybycien-Szymanksa et al., 2010; Przybycien-Szymanska, 2011; Spear, 2016; Torcaso et al., 2017; Trantham-Davidson et al., 2017; Vetroeno et al., 2017).

These altered traits in offspring demonstrate the potential of parental preconception behaviors to impact future offspring, most likely through transmission of altered epigenetic patterning, or epimutations. A significant number of studies have shown that these epimutations exist, but often the physiological consequences for offspring are less evident. We have previously showed that the F1 generation offspring of animals administered binge-pattern EtOH during the adolescent period had numerous differentially methylated cytosine residues throughout the genome as well as wide-spread alterations in gene expression (assessed at PND 7), despite never having been exposed to EtOH directly, but the functional consequence of these epimutations conferred by the parents were unclear (Asimes et al., 2017; Przybycien-Szymanska et al., 2014). Since these changes occurred genome wide, and primarily outside of promoter regions, we chose to pursue a systems-level characterization of offspring hypothalamic function as opposed to a focus on any particular gene associated with differential methylation. Intragenic DNA methylation may have the ability to influence the expression of distal genes and/or non-coding RNA, which have a broader physiologic impact than simply assigning the methylation mark to the nearest gene (Schübeler, 2015).

Somatic growth from birth through puberty was assessed based on prior evidence that offspring exposed to EtOH *in utero* tend to have lower birthweights (Carter et al., 2013; Sampson et al., 1994). Our results revealed that parental preconception EtOH did not affect offspring weight at birth or at PND 7, and body weight differences in both male and female offspring arose only after weaning from the mother. These data suggest that there was no overt malnutrition of these offspring, but a latent difference in control of body weight that was only evident later in life during the peripubertal growth spurt. Along with the decreased body weight, the offspring showed a perturbation in post-weaning juvenile play behavior with less pinning behaviors in both male and female offspring of EtOH-treated parents. Decreased pinning and engagement in other bouts of play has been linked to social withdrawal and social anxiety phenotypes, and represents a less-masculine/dominant display of play (Meaney and McEwen, 1986). Therefore, these data indicate that offspring of EtOH-treated parents have disrupted juvenile social interactions that may suggest increased social anhedonia or social withdrawal, which are markers of atypical juvenile social development and social pathology.

Testosterone is an anabolic steroid hormone that is critical for somatic growth in males and plays a role in both males and females during early puberty. Further, T is associated with increased play behaviors in juveniles (Meaney and McEwen, 1986). Therefore, we measured circulating T along with genetic markers of HPG axis function in the alcohol naïve offspring. Parental preconception adolescent binge EtOH-exposure decreased circulating T levels in both male and female offspring, although the high variability of this outbred rat strain resulted in no statistically significant differences ($P=0.08$ male; $P=0.10$ female). These, along with the slight

changes in LH concentration in circulation for male offspring and the differential GnRH expression in the hypothalamus, may indicate a disturbance in the synchronization of puberty, where changes in hormone balance can impact the timing of body growth and development. While EtOH has been shown to disrupt HPG function in the individual exposed, this is the first study, of our knowledge, to find HPG disruption in offspring of parents exposed to only preconception EtOH doses (Rachdaoui and Sarkar, 2013). In conjunction with circulating hormones, we examined several markers of sperm development in the males and did not observe any changes in testes or sperm maturation, which argues that the hormone imbalances are not impacting sexual development. Future experiments testing the fertility of these offspring would provide more concrete evidence of the impacts of parental EtOH exposure on offspring sexual maturation.

We also measured a similar if not exacerbated effect of parental EtOH exposure on HPG function in offspring when they were given EtOH. GnRH expression in the hypothalamus was reduced by almost 50% in both males and females after EtOH exposure. Additionally, testosterone was significantly reduced in male offspring and trended towards a decrease in female offspring. These measures, as above, indicate a dysfunction in the hypothalamic control of hormone balance. However, we did not see a difference in gonad development in male offspring who were exposed to EtOH themselves, indicating that sexual maturation was progressing, but the viability of the mature sperm and functional capacity to fertilize ova was not assessed.

Our original hypothesis was that the epimutations we had previously observed in offspring following parental EtOH exposure would confer to them an adaptation to EtOH exposure themselves. Indeed, in EtOH-naïve offspring we measured a lower baseline CORT level in male offspring whose parents had been treated with alcohol, which was consistent with the phenotype we, and others, have previously reported in adult animals after exposure to adolescent EtOH exposure who exhibited a lower baseline CORT level in adulthood (Crews et al., 2016; Fernandez et al., 2016; Przybycien-Szymanksa et al., 2010; Vetreno et al., 2017). However, an alternative hypothesis is that preconception parental binge alcohol consumption leaves lasting epigenetic marks that may result in phenotypic disadvantages for the offspring, such as a heightened stress response when exposed to a similar dose of alcohol. Previous studies from our lab and others have demonstrated adolescent alcohol-induced long-term dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, the primary mediator of the physiological stress response that persisted even after prolonged periods of alcohol abstinence (Przybycien-Szymanksa et al., 2010). These data suggest that alcohol-induced epimutations could result in a maladaptive phenotypic response to EtOH. We examined blood alcohol levels, circulating corticosterone, and stress-responsive gene expression patterns in the hypothalamus and found none of these measures to be different. Our results, instead, seem to indicate that there were no differences in offspring stress response based on parent treatment, neither adaptive nor maladaptive. However, in the EtOH-naïve male offspring we observed a reduction in baseline circulating CORT and in the EtOH-treated offspring we saw no difference between offspring whose parents received EtOH or water. This would indicate that the increase in CORT caused by

EtOH exposure is almost twice the magnitude in male offspring whose parents received EtOH compared to offspring whose parents received water. Therefore, the spike in stress response may actually be greater in offspring of EtOH treated parents, indicating a maladaptive response to EtOH. Additionally, the lack of adaptive response to EtOH does not preclude a lack of adaptation from parental drinking as a whole. These offspring may be adept at handling heterotypic stressors, such as psychological stress, or may have more efficient glucose mobilization with the same corticosterone surge.

Previous work in our lab demonstrated genome-wide changes in DNA methylation as well as widespread changes in gene expression patterns in the hypothalamus at PND 7, a period in rat neurodevelopment similar to humans at birth (Dobbing and Sands, 1979). These changes, resulting from preconception EtOH exposure of the parents only, may underlie the moderate changes in hypothalamic function we observed in the experiments presented here. However, there were more changes in both gene expression and methylation pattern that do not seem to be attributable to the phenotypic profiles we have presented here. Additionally, there were some changes in gene expression, such as a decrease in AVP and CRF expression at PND 7 that do not directly match in the PND 44 hypothalamus, where there was no change in expression of either gene (Przybycien-Szymanska et al., 2014). Additionally, of the genes examined in Experiment 3, there were no major differences in either the PND 44 hypothalamus or in a second litter at PND 7, with only two significantly differentially expressed genes (Sparcl1 and Grm4, see Tables 7 and 8). This is further evidence that the heritable effects of EtOH are not measurable later into development, and with proper rearing can be mitigated. Additionally, the

lack of differences in a second litter suggests that a longer period of abstinence before conception would prevent transmission of methylation patterns across generations. It is well known that development is accompanied by changes in gene expression and DNA methylation patterns, but future studies are needed to investigate how and when these changes may occur. The inheritance of epigenetic marks may be important in the establishment of offspring gene expression profiles, but developmental environments may provide the stimulation to ameliorate some of the inherited marks. This is important to keep in mind when interpreting data describing heritable epigenetics and their potential implications.

A potential confounding factor in our experimental design was the rearing of the litter by the biological (i.e. EtOH-exposed) mother, which may have conferred compensatory factors for phenotypic changes in the juvenile offspring. For example, increased/decreased maternal grooming behaviors have been linked with epigenetic changes in DNA methylation and histone acetylation marks in the offspring brain (Weaver et al., 2005). Our design in this study was intended to better mimic the human societal situation where the majority of offspring would be reared by the biological parents. In addition, cross-fostering could induce its own set of stressors and epigenetic changes that would be difficult to assess and beyond the scope of these studies. Therefore, future studies investigating the growth and development of offspring after cross-fostering to EtOH naïve mothers, and likewise of control animals raised by EtOH-exposed mothers, would shed light on the interaction of maternal behavior with preconception EtOH exposure on these offspring.

The belief that parental alcohol exposure can cause phenotypic disturbances of offspring is hardly a new idea (Warner and Rosett, 1975). One of the first records of this concept is from the 1720s, where children of parents who were frequently intoxicated, but not necessarily alcoholics, were described as “weak, feeble, and distempered” by the English College of Physicians, who warned against the consequences of intoxication of either parent. Although we would not use the same language now, our data presented here seems to be in agreement with the observations at that time. We have shown evidence of smaller body size, disrupted juvenile social play, and decreased circulating testosterone, in conjunction with no apparent beneficial phenotypic changes. However, with advances in medicine and research technology, we are now able to understand on a biological level what these phenotypic changes truly are, how they may be caused, and, ultimately, what we can do to prevent their perpetuation.

CHAPTER V

“FATTY BRAIN” FOLLOWING PUBERTAL BINGE ETHANOL EXPOSURE

Introduction

Recent advances in research on neurodegenerative diseases, such as Alzheimer’s disease (AD), points to the importance of a cholesterol carrier molecule called Apolipoprotein E (ApoE). ApoE is a carrier protein necessary for brain function as it is responsible for trafficking lipids, mainly cholesterol, from the astrocytes where it is made and deliver it to neurons (Mahley et al., 2009; Rebeck, 2017). Neurons then use the cholesterol for maintenance of their expansive plasma membranes, for the formation of synaptic vesicles, and for metabolism through its byproducts (Zhang and Liu, 2015). During juvenile and adolescent development, there is a faster rate of cholesterol synthesis in the brain, which corresponds to the synaptic growth and extensive myelination that is required during this period (Quan et al., 2003). In the juvenile brain, neurons retain the ability to synthesize cholesterol themselves, but this is lost in adulthood, leaving the aging brain particularly dependent on proper ApoE function (Xu, 2006). In fact, human allele variants of ApoE have been found to correlate with risk for developing AD, with patients carrying the ApoE ϵ 4 variant having increased risk (Deary et al., 2002; Tai et al., 2014).

ApoE works by packaging lipids, and other molecules such as amyloid β , in discoidal shapes for delivery to neurons. Lipids and ApoE are transported out of astrocytes by the ATP-binding cassette transporter ABCA1, which is also known as the cholesterol efflux regulatory

protein (Vitali et al., 2014). This extracellular ApoE-cholesterol disc is recognized by the low-density lipoprotein receptor (LDLR) on neurons, and taken up through endocytosis (Yancey, 2003; Zhang and Liu, 2015). Free cholesterol is then made by lysosomal breakup of the ApoE-cholesterol complex. Interestingly, membrane fluidity has been shown to relate to the rate of cholesterol flux between cells in the brain, but it remains to be seen how membrane fluidity is sensed and subsequently reacted to (Los and Murata, 2004). Human ApoE ε 4, in comparison to ApoE ε 2 and ApoE ε 3, has diminished capacity for lipid packaging and formation of the proper discoidal shape (Tai et al., 2014). This depreciation in proper lipid delivery as well as movement of amyloid β , over the course of aging, can lead to cognitive decline and neurodegenerative diseases (Huang and Mahley, 2015). Rats only harbor one isoform of ApoE, and it is most similar to that of human ApoE ε 4 (LaDu et al., 1997).

Cholesterol synthesis and efflux from the brain are also important factors in lipid homeostasis throughout life (Zhang and Liu, 2015). Many of the genes involved in cholesterol synthesis are transcribed by the lipid-sensing transcription factor sterol-regulatory binding protein (SREBP1). SREBP1 is normally present as a transmembrane protein in the endoplasmic reticulum, but is cleaved and enters the nucleus upon sensing low intracellular sterol levels. The target genes of SREBP1 include many members of the cholesterol synthesis pathway. On the other hand, Cyp46a1, a member of the cytochrome p450 family, is responsible for conversion of cholesterol to 24-hydroxylersterol, which can exit the brain through the blood-brain barrier and travel to the liver for degradation (Kölsch et al., 2009; Zhang and Liu, 2015). The expression and

activity of all these components determines the level of cholesterol in the brain on both short and long timescales.

It is well known that chronic alcohol consumption can cause fatty liver development. This is thought to be through several parallel mechanisms involving upregulation of SREBP1 and lipogenic cellular components in liver cells (Lieber, 2004). Cholesterol is unable to cross the blood brain barrier, so the pools of peripheral and brain cholesterol are not continuous; therefore, fatty liver is not thought to impact neuronal lipid homeostasis (Zhang and Liu, 2015). However, it is possible that similar mechanisms taking place in the liver are also occurring in the brain. Alcohol consumption has been associated with higher risks of developing several neurodegenerative diseases later in adulthood, in particular AD (Corella et al., 2001). Additionally, our lab has shown that there is a severe reduction in ApoE expression in offspring of parents who received binge EtOH doses before conception (Przybycien-Szymanska et al., 2014) . The knowledge of a relationship between EtOH and SREBP1 along with this reduction in offspring ApoE expression led me to further explore the impact of EtOH consumption on lipid homeostasis in the brain. I tested the hypothesis that adolescent binge alcohol exposure of parents decreases the expression of ApoE in the offspring brain, leading to altered cholesterol stasis. As aging is an important component of lipid dysregulation, I also examined the effect of adolescent binge drinking on the brain cholesterol homeostasis in the same animals as adults, following a prolonged period of EtOH abstinence.

Results

These experiments were carried out using similar paradigms as already described by our laboratory. Wistar rats were administered our repeated binge-EtOH exposure paradigm at early and late puberty (PND 37, 67). In order to minimize the animals needed, we utilized tissues from multiple experiments already described in this document. Tissue for the experiments herein were taken from adult animals at approximately 5 months of age, and offspring hypothalamus was isolated from animals at PND 7 as well as at PND 44 (Fig. 19). Primary astrocyte cultures were prepared from cortical tissue of both pups (PND 7) and parents (5 months).

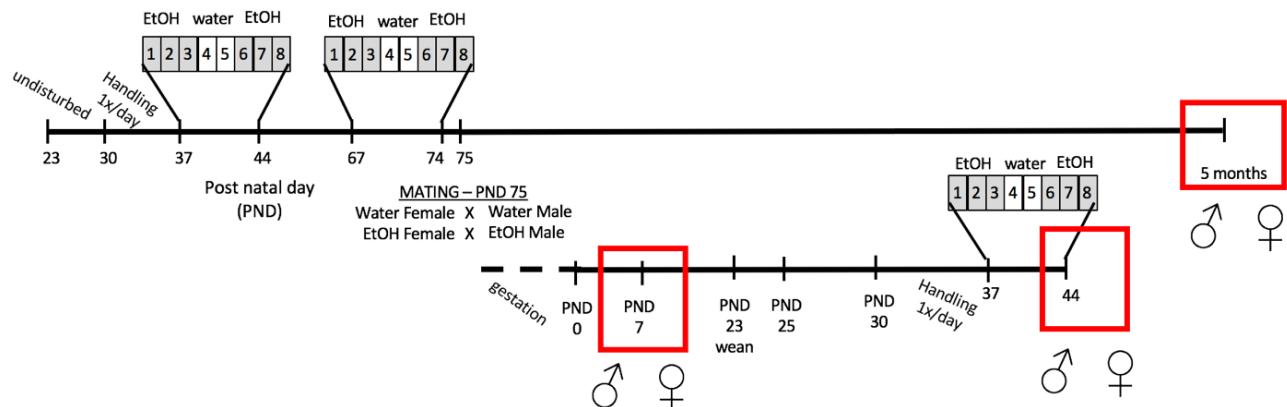


Figure 19. Ages of investigation for dysregulation of lipid homeostasis. Following repeated binge EtOH exposure during puberty, animals were mated and left undisturbed through normal gestation and birth. Hypothalamic tissue of PND 7 offspring as well as hypothalamic tissue from PND 44 offspring were used for these experiments. Cortex and hypothalamus were isolated from parents, who were exposed to EtOH themselves, after they reached adulthood and were abstinent from EtOH for approximately 2 months.

ApoE was Increased in Adult Males Following Pubertal EtOH Exposure.

Previous work in our lab showed that ApoE was lowly expressed in the PND 7 hypothalamus after parental exposure to EtOH. I wanted to measure the expression of ApoE in

the aged brain in order to determine if lipid trafficking might be disrupted at a more physiologically relevant age. Interestingly, there was a significant increase in ApoE expression in 5 month old males who had been exposed to EtOH during adolescence (Fig 20a). This was the opposite of the trends seen in the neonates. There was no difference in ApoE expression in the female hypothalamus at this age (Fig 20b). We also examined the expression of other genes whose proteins play roles in lipid homeostasis, including ABCA1, LDLR, SREBP1 in the adult male hypothalamus and found no differences in gene expression (see Appendix C). Female hypothalamus was not examined for expression of other lipid-associated genes as there was no other appreciable phenotypes in adult females.

Lipid-Associated Genes are Unaltered in Adolescent Offspring Hypothalamus.

We also examined the expression of lipid-associated genes in the PND 44 offspring hypothalamus of both alcohol-treated and alcohol-naïve offspring. None of these showed any significant differences in mRNA expression at this age, in either male or female offspring (see Appendix C). I also measured mRNA expression of these lipid-related genes in the offspring from second litters, whose parents had a longer period of abstinence following EtOH exposure (as described in Chapter IV). The only alteration in expression was of SREBP1 mRNA, which was significantly downregulated (74% of control) in male offspring whose parents had been treated with EtOH (Appendix C, Table 3). None of the other lipid-associated genes, including ApoE were significantly altered, therefore, these age groups were not explored any further.

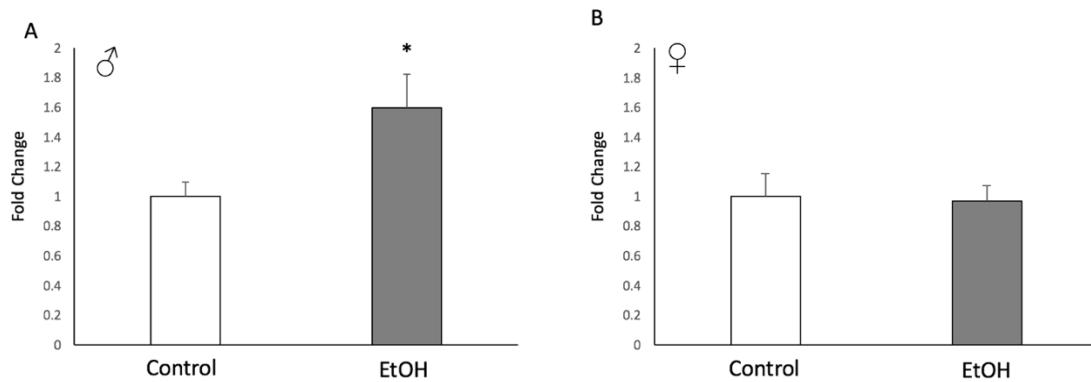


Figure 20. ApoE expression was dysregulated in a sex-specific manner in the adult hypothalamus. mRNA expression of ApoE was examined in male and female hypothalamus of adult animals who were exposed to EtOH during puberty. A) Adult males showed an increase in mRNA expression of *ApoE*. B) There was no difference in expression of *ApoE* in adult females.
* $p<0.05$, Two sample t test, $n = 4-6$ per group, error bars = SEM

Primary Astrocytes Cultured from Adult Males Showed Increased Cholesterol Accumulation.

In order to determine if this change in ApoE expression impacted astrocyte function, and therefore the supply of cholesterol to neurons, we cultured primary astrocytes from the adult and offspring cortex. Primary astrocytes cultured from PND 7 offspring showed no apparent phenotype while *ex vivo*, nor any differences in function, and are therefore not shown in remaining figures. The primary astrocyte cultures from adult males showed increased accumulation of lipids within intracellular droplets (Fig 21a). I quantified the amount of cholesterol present in each culture, and found a significant increase in cholesterol content of astrocyte cultures from adult males who had been treated with EtOH (Fig 21b). There was no change in cholesterol content or lipid droplet appearance in astrocyte cultures from adult female rats (Fig 21c). Primary astrocytes from adult males also showed a significant increase in ApoE expression (Fig 21d), suggesting that the whole tissue increases in expression may be

driven primarily by astrocyte expression profiles. We have termed this cellular phenotype "fatty astrocytes."

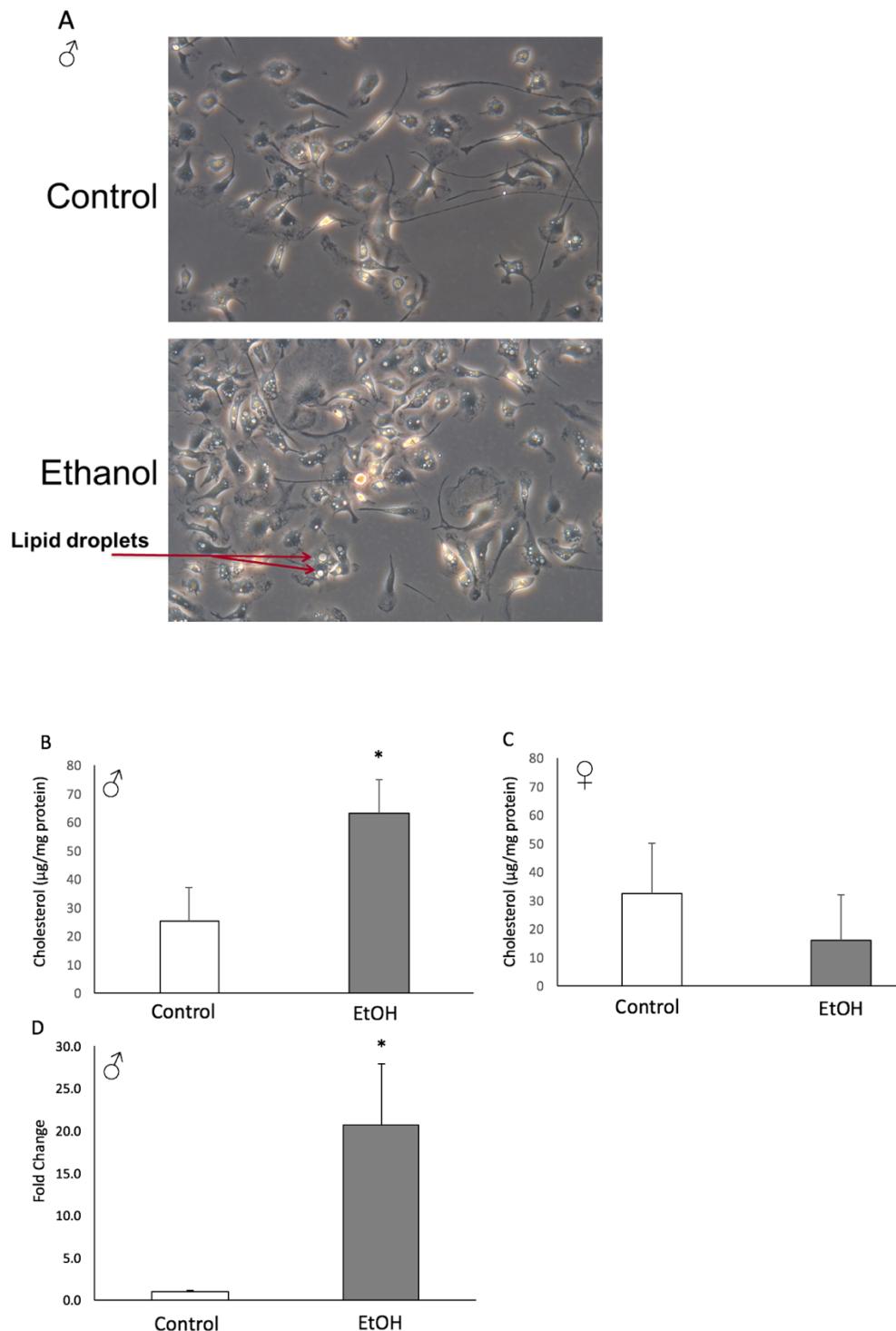


Figure 21. Primary astrocytes from EtOH-treated males are “fatty” and overexpress ApoE. A) Primary astrocytes cultures from cortical tissue of adults following pubertal EtOH exposure showed increased accumulation of lipids within intracellular droplets. This was true mainly for

male astrocyte cultures, and not for female astrocytes (not pictured). B) Overall cholesterol content of astrocytes from adult males was higher when they had been treated with EtOH compared to control animals. C) There was no difference in cholesterol content of astrocyte cultures from females treated with EtOH or control counterparts. D) mRNA expression of ApoE was also elevated in primary astrocyte cultures from males treated with EtOH. * $p<0.05$, Two sample t test, n= 3 per group, error bars = SEM

Fatty Astrocytes are Less Neuroprotective Against Toxic Insult.

Since the adult male astrocytes showed increased lipid retention, I wanted to test the ability of these astrocytes to serve their protective function against neuronal injury. I used a differentiation paradigm for neurons in culture to produce dopaminergic neurons from the neuronal precursor N27 cell line. I then pretreated the neurons with astrocyte-conditioned media from adults who either received EtOH or water during adolescence and then used the potent toxin MPP⁺ to induce cell death (Fig 22a). Fatty astrocytes from males treated with EtOH conferred less protection to the neurons than did astrocytes from males treated with water during adolescence (Fig 22b). Female astrocytes, which did not have a fatty phenotype, showed no difference in protection against MPP⁺-mediated cell death (Fig 22c). Interestingly, astrocytes from control females did not confer any protection to the neurons, except for a minor increase in cell viability at the highest dose of MPP⁺.

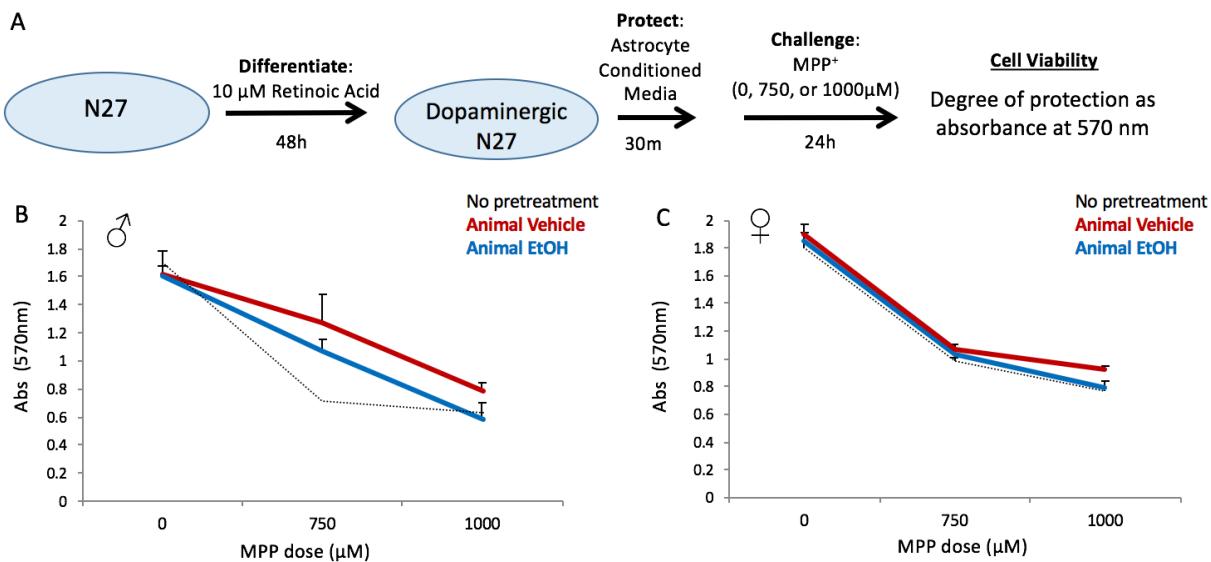


Figure 22. Fatty astrocytes confer less protection to neurons against toxic insult. A) Schematic representation of neuronal differentiation and cell death assay. Increased absorbance at 570 nm correlates with increased cell survival of the preconditioned neurons, suggesting more protection of the astrocytes used for pretreatment. B) Astrocytes from adult male control animals conferred the most protection to injured neurons, whereas astrocytes from EtOH-treated males were somewhat protective but less-so than control counterparts. Dotted line indicates no pretreatment, representing a normal dose-dependent cell death following MPP⁺ treatment. Two-sample t-test at each dose, n= 3/group, p = 0.99, 0.41, 0.18, respectively. C) Astrocytes from adult females, either EtOH- or control-treated, did not confer any protection to neurons.

Discussion

The results presented here demonstrate that repeated binge EtOH exposures during pubertal development cause increased cholesterol accumulation in adult male astrocytes, which results in decreased astrocytic protection of neurons from toxic injury. These data suggest that EtOH-induced disruption of ApoE may lead to development of “fatty astrocytes” and cholesterol-starved neurons in adulthood. A decrease in ApoE expression in young animals, followed by overexpression in the adult brain, may complicate trafficking of lipids from astrocytes to neurons, which could lead to a decline in brain resiliency later in adulthood. We

have termed this phenotype “Fatty Brain,” but the effects of this phenomenon require further characterization.

The association between EtOH and membrane fluidity may play a key role in regulating lipid homeostasis in the brain (Los and Murata, 2004). It seems reasonable that repeated cycles of increasing and decreasing membrane fluidity could cause disruptions in the normal lipid signaling pathways. In fact, adolescence may be a particularly vulnerable time for this cycling to occur due to the high rate of cholesterol synthesis at this time for the purposes of myelination (Pfrieger, 2003). Most of the brain’s cholesterol is found within myelin sheaths, and it has been shown that EtOH exposure during puberty can cause long lasting reductions in the amount of white matter (i.e. myelin) in the adult brain (Vargas et al., 2014). By causing too much fluidity in the plasma membrane, cholesterol may be diverted to filling in these gaps in the neuronal plasma membrane as opposed to building myelin sheaths. As the individual ages, the astrocytes may still be producing high amounts of cholesterol, and with no other options for deposit, accumulate lipid droplets intracellularly, while myelination is still diminished.

A more comprehensive study is needed to determine how developmental timing of Fatty Brain is impacted by EtOH exposure, as well as EtOH withdrawal. The reductions seen in ApoE expression in young offspring may set these offspring up for lower myelination of the brain during growth. This may be due, in part, to an overall reduction in the freely available cholesterol levels in the brain. Additionally, our genome-wide methylation study of preconception EtOH offspring showed differential methylation of several genes which may be involved with lipid homeostasis (see Figs 4 and 5, recapitulated in Fig 23). Some of these genes

include Gpr27, which is a G-protein coupled receptor, expressed mainly in the nervous system and a proposed lipid receptor molecule. Wbscr17, also known as Galnt17, was hypomethylated in all offspring groups, suggesting it has a particular sensitivity to parental EtOH exposure. Galnt17 is a known membrane trafficking protein, and may play a role in the formation of the lipid droplets I observed. miR6216, which was hypermethylated if both parents were exposed to EtOH, is in the miR-466 family of microRNAs, which are predicted to target ApoE in the rat (TargetScan). This represents another potential level of transcriptional control of lipid homeostasis. Although I did not measure expression of these genes in the offspring or adults, their regulation may be an important indication of a more global change in brain metabolism.

Alcohol abuse is frequently associated with fatty liver disease, but this study is novel in that it is the first description of a “fatty brain” phenotype following alcohol exposure. The previous findings in our lab of ApoE depletion in offspring following parental preconception alcohol exposure led me to test the effects of EtOH on the mediators of lipid homeostasis. Many of the known lipogenic genes, such as SREBP1, were not altered by a history of EtOH exposure in the offspring or adults that I tested. However, I was able to begin to characterize the fatty brain phenotype in the adult brain following pubertal EtOH exposure, and found there are sex-specific differences in development of this phenomenon. Lipid droplet accumulation in male astrocytes, accompanied by a diminished capacity to provide neuroprotection *in vitro* implied a dysfunctional lipid trafficking system. Lipid-poor neurons and overly fatty astrocytes caused by

teen drinking may underlie many of the long term deficits in neuronal function and in the increased vulnerability to development in neurodegenerative diseases.

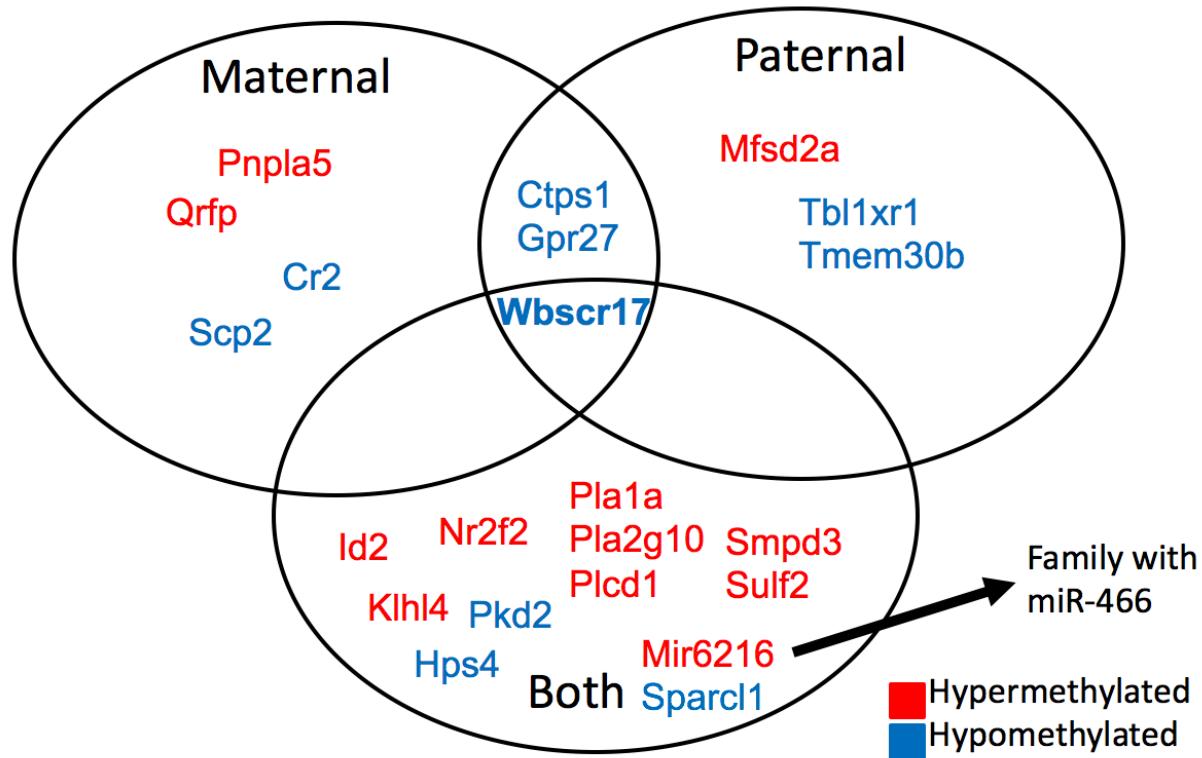


Figure 23. Lipid-associated genes with differential methylation in alcohol-naïve offspring following parental preconception EtOH exposure (Experiment 1). Genes associated with hypermethylated residues are in red; genes associated with hypomethylated residues are in blue.

CHAPTER VI

FINAL DISCUSSION

Summary

Parental behavior can impact offspring development. This is certainly true during child rearing and parenting, and recent advances in our understanding of genome regulation suggest that this is also true of *preconception* parental behaviors. The epigenome, or transcriptional control of genetic material, is now known to be central for proper growth of animals, disease initiation and progression, *and* heritable communication of environmental signals. Epimutations inherited by offspring can serve as adaptive aids or impediments to offspring survival. The goal of this dissertation was to elucidate the effects of parental preconception binge alcohol exposure on the offspring epigenome and characterize the potential functional consequences for these offspring. Binge drinking has been linked to increased cognitive health risks late into adulthood as well as similar health risks in offspring. The novel work presented herein is the first study of its kind to investigate a mechanism by which *either* maternal or paternal preconception exposure to binge alcohol conditions can impact DNA methylation in offspring and to track the impact of this inheritance through offspring development into adulthood. The results show that parental preconception alcohol exposure can impact offspring through epigenetic inheritance of DNA methylation patterns in the hypothalamus, which leads to hypothalamic dysfunction during development and a predisposition to neurodegeneration. The contribution of this research to the field is significant as it delineates a pathway through which

preconception binge alcohol treatment acts to cause epigenetic and functional dysregulation in alcohol-naïve future generations.

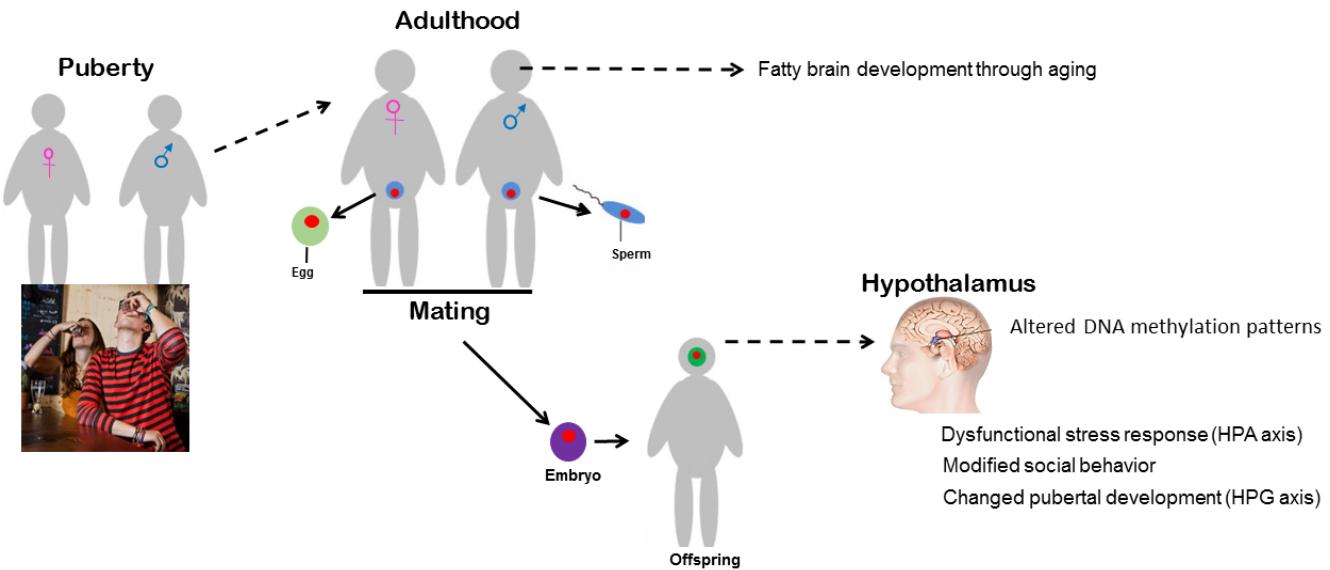


Figure 24. Visual representation of summary of findings.

Key Findings

Chapter III: Male offspring of parents exposed to alcohol during puberty have differentially methylated DNA in the hypothalamus.

- Parental preconception binge alcohol exposure during puberty alters DNA methylation in the hypothalamus of future offspring.
- Both maternal and paternal preconception exposure contribute to offspring epimutations, but to different marks throughout the genome.
- There are only a few methylation marks which were common to offspring of maternally, paternally and dual parent exposure.
- Affected genes span a wide array of cell and neurological functions.
- Male offspring show altered gene expression profiles in the hypothalamus, but methylation didn't always predict expression.
- Female offspring show gene expression profiles which are different than male offspring, suggesting sex-differences in the epimutations inherited.

Chapter IV: Parental preconception alcohol exposure alters normal offspring hypothalamic development and does not confer adaptation to offspring when encountering alcohol themselves.

- Offspring of EtOH-treated parents were smaller after pubertal onset and displayed fewer play behaviors.
- Offspring of EtOH-treated parents had changes in baseline HPG axis parameters, displaying lower GnRH expression in the hypothalamus and decreased circulating testosterone. However, testicular maturation did not seem to be effected by parental drinking.
- Baseline CORT in male, but not female, offspring was decreased following parental EtOH exposure. However, mRNA expression of GR and neuropeptides that mediate HPA axis were not affected by parental EtOH consumption.
- There was no difference in offspring blood alcohol level of EtOH- or control-treated mating pairs when exposed to the same paradigm as their parents had received.
- EtOH-treated offspring of EtOH-treated parents also had decreased HPG axis parameters, with decreased hypothalamic GnRH expression and circulating testosterone in males. Testicular development was, however, not altered by offspring EtOH exposure.
- Parental history of EtOH exposure did not affect offspring CORT response or HPA axis reaction to EtOH exposure themselves.

Chapter V: Adolescent alcohol exposure leads to fatty astrocytes in adult males.

- ApoE expression was dysregulated in a sex-specific manner in the adult hypothalamus, with increased expression only in the male brain after adolescent alcohol exposure.
- Primary astrocytes cultured from adult males showed increased cholesterol accumulation, giving a “fatty” appearance and causing a diminished capacity to be neuroprotective against toxic insult in culture.

Final Thoughts

Maternal AND Paternal Preconception Behaviors Influence Offspring.

In Chapter III, I demonstrate that parental preconception binge alcohol exposure can cause genome-wide changes in DNA methylation in the hypothalamus of male offspring. The offspring tested were unrelated individuals from an outbred strain of rats, and the analysis criteria used were quite stringent. Results in this context give confidence to the idea that this epigenetic inheritance is not simply an artifact of genetic background or of “omics” over-generalization. These differentially methylated sites throughout the genome represent a potentially conserved mechanism of heritable marks from mothers and fathers, and my study design allows us to infer which marks may be altered by maternal and paternal alcohol consumption independently. Interestingly, I found that inherited epigenetic marks were not additive between parental exposures, such that there were mostly unique genes impacted by single or dual parent exposure. This was intriguing as it implies that, while there may be changes within the sperm and eggs individually that are passed on to offspring, there must also be some impact of preconception alcohol on the epigenetic rearrangement happening after fertilization occurs, where the information from each parent’s epigenome is becoming integrated. The interplay between these signals is still being determined for normal patterns of imprinting and epigenetic regulation in embryos, and perhaps these endeavors would be aided by including data on the impact of drugs on this process.

Maternal behavior has long been the focus of setting up offspring for a healthy life, especially when it comes to alcohol consumption. For most of society, a majority of the burden is placed on mothers to make the right health choices in preparing for healthy offspring development. However, we now know, through our research and others', that paternal preconception behaviors are also central to development of offspring. Alcohol, along with other drugs of abuse, can have impacts for many generations through the male germline. This is extremely important to keep in mind in public-health endeavors to combat risky teen drinking behaviors. A shared focus on future offspring health should be included in both girls' and boys' educational materials on dangers of adolescent binge drinking. Additionally, research to determine how the combined parental history of an animal mating pair may impact the epigenome of offspring may shed light on heritable genetic and epigenetic disease prediction.

Epigenomics is Invaluable, but Cannot Give the Whole Picture Yet.

While the field of epigenetics, and in fact the mere definition of the word, focuses on the functional transcriptional control mediated by epigenetic marks, it is important to study the epigenome with some reservations as to the physiological relevance of each mark. In theory, each methylation mark along the DNA has the potential to be read by molecular readers and interpreted as a certain signal. What that signal is, however, remains to be appreciated by researchers, including our lab. Our genome-wide methylation changes in offspring did not necessarily correlate with microarray expression data we collected from PND 7 offspring in a previous publication, nor did they seem to predict the phenotypic differences we observed in Chapter IV as the offspring grew into puberty. The standard description of upstream

methylation silencing nearby gene expression does not seem to hold true for our methylation data. However, this may be due in part to the nature of “omics” research, where parameters of analysis must be set, but may not be the only method for examining relationships. For example, the DMCs we found were attributed to the nearest downstream gene in order to determine which genes may be differentially regulated by methylation with each parent’s EtOH treatment. The consistency of discrete DNA methylation marks between animals in each treatment group suggests that, while these methylcytosines may be single point epimutations, they likely are purposeful modifications to the genome. However, these marks may in fact alter expression of something else not directly near the methylation site and/or work through indirect mechanisms to alter the final expression profile of the cell. Reanalysis of the data in another way (i.e. methylation in enhancers, insulators, transcription factor binding sites) would still be bioinformatically correct, and may give us a different list of “regulated genes” with which to pursue functional characterization.

Indeed, the functional changes I measured in offspring indicate that there is inheritance of phenotypic information by offspring following parental alcohol exposure. The behavioral changes we observed in juvenile play bouts, with decreased play and pinning in particular, are similar to the phenotype we have previously measured in adults who were exposed to EtOH during puberty (Torcaso et al., 2017). These adults displayed a heightened risk assessment behavior, or hypervigilance, during anxiety test procedures. Alcohol’s known effects on the hypothalamus and the function of the HPA axis predict dysfunction of the stress response, and this seems to manifest in the exposed animals as adults as well as in young offspring. There was

a significant reduction in GnRH expression in the hypothalamus, decreased testosterone in some groups, and decreased baseline CORT level in males. Therefore, even though the epigenetic modifications do not seem to be obviously tied to the phenotypic changes, there is likely a yet-undiscovered underlying mechanism which connects them.

Epigenetic Inheritance is Not Necessarily Adaptive for Offspring.

The other functional changes we observed in the hypothalamus of offspring, in total, do not seem to be adaptive, nor particularly maladaptive to the offspring development. The Lamarckian inheritance theory would assume that inheritance of a trait, or the instructions for creating that trait, serve an adaptive purpose for the next generation. Otherwise, there would be no advantage to passing on of the trait. However, it is also true that if the mechanism for passing on information about parental health and positive experiences (i.e. regular exercise) is already in place, then potentially harmful experiences (i.e. drug abuse) can also be passed on in the same way. Indeed, there was no apparent increased ability to cope with EtOH treatment in offspring whose parents had received EtOH before them; but, at the same time, these offspring were not exceedingly injured by EtOH exposure, meaning they were not particularly sensitized to it either. Overall, it seems as though the mild stressor of preconception binge alcohol exposure *can* impact offspring development and behavior, but perhaps is not as detrimental as we may think.

Another way to examine the gravity of parental preconception alcohol exposure is to look at multiple litters and the offspring as they age, which I demonstrate briefly in Chapters III

and IV. By examining the expression of a few candidate genes, I was able to show that there is no consistency in enrichment between male and female offspring, the first and second litters, or juvenile and adolescent hypothalamic samples. This shows that changes to the transcriptome in juvenile offspring may not be permanent, and serves as a reminder that gene expression is very temporally dependent. Previous work in our lab has shown that sex-difference in EtOH responses are prevalent, with females normally showing a resistance to the effects of EtOH. Therefore, the sex differences in gene expression were not unexpected, but future work examining the methylation patterns in females may shed light on those relevant mechanisms. It is possible that the methylation patterns are different between age-matched males and females, and therefore expression is not similar, or it is also possible that some methylation marks are inherited by both sexes and interpretation of the transcriptional signals vary.

One of the characteristics that we did not fully explore in Chapter IV is the fertility of offspring whose parents received EtOH preconception. There were no physical changes in testicular development of the male offspring, but the reduction in GnRH expression in both males and females suggests that sexual maturation may be impacted in these animals. This could manifest as a late onset of puberty, complete infertility, or no deficiency in fertility. However, decreased reproductive capacity in offspring who have inherited epimutations would present an interesting mechanism of trait extinction in a Lamarckian theory of inheritance. In this case, subsequent generations would produce fewer offspring and therefore, the disadvantageous behavior would lead to extinction of that germ line. More research into the

second and third generations resulting from parental preconception drug exposure would be an interesting investigation of this theoretical implication.

Binge Consumption Patterns Signify a Unique Stressor to the Body.

Many researchers claim to study adolescent binge alcohol consumption with their experimental models, however the interpretation of the clinical definition by basic science researchers is not consistent. The most-clinically relevant models should reflect the most prevalent forms of the teenage behavior: not chronic, but repeated throughout pubertal development, alcohol which is administered via intragastric modes, in doses which reflect a BAC of between 0.08% and 0.3%, and occur at the transition between dark and light hours ("evening" for the rodent), and use animals which have already initiated puberty but have not yet become reproductively competent. Chronic exposure, vapor or injection, pre-puberty, and high dose or increasing dose models too often cause disruptions to the normal development pattern far beyond the effects of the alcohol itself, including unresponsiveness (i.e. no eye blink response), lack of feeding or drinking, addiction to alcohol (i.e. purposefully bred alcohol-preferring strains or alcohol/sucrose solutions), and non-specific stress with handling. The variability in model systems causes a confusing array of results interpretations, particularly in finite molecular studies. These discrepancies may be mitigated by a more stringent or conservative analysis of data, so that the human condition which is being mimicked is more accurately reflected in the model. Overall, I have found that preconception binge alcohol consumption is truly a unique stressor on the body and deserves a more strongly defined clinical definition.

Comparisons of model systems are particularly important for genome-wide analyses, such as the DNA methylation patterns I examined here because the data are very sensitive to the experimental conditions. The data sets are such that comparisons are relatively easy to accomplish technically, but the interpretation is less straightforward. Useful comparisons between different genome-wide and transcriptomic analyses of preconception drug studies, fetal alcohol models, and others can be done in order to determine similarities in methylation patterns. Within the last few years, there have been more and more published raw data, allowing us to compare the actual data points between various experiments. One study of interest is a recent publication on the effects of preconception THC, the active ingredient in marijuana (Watson et al., 2015). Genome-wide methylation analysis in offspring (also a rat model) showed that all differentially methylated cytosines (DMCs) were different than the ones identified in our parental EtOH offspring. However, when comparing the genes nearby these DMCs, there were several genes which were indeed shared between parental preconception THC and EtOH exposure. These include Begain, Dmgdh, Galnt10, LOC681383, Map7d1, and Marveld2 between dual parent alcohol and dual parent THC. Their offspring also showed common differential methylation with our single-parent groups, including Bhmt2, Cpne4, Ehf, and LOC681383 with maternal EtOH exposed offspring and Afap1, Bhmt2, Cdh22, Mfsd2a, Pias4 with paternal EtOH exposed offspring. Interestingly, LOC681383 and Bhmt2 were two of the genes which were present in more than one of our offspring groups. This suggests that some genes may have multiple sites of epigenetic transcriptional regulation, and these genes may be particularly important because they are regulated by both drugs in different chromosomal

locations. These could potentially be termed “preconception recreation-drug sensitive”, and represent a more narrowed class of genes to study for potential functional implications.

Also important is the lack of overlap between genes we found to have DMCs and known regions of parental imprinting. When comparing both the genes and the DMCs themselves, there is no overlap between these data (Morison and Reeve, 1998). This is further evidence that parental preconception EtOH exposure does not just cause perturbations in the normal imprinting regulation that occurs during embryogenesis. Interestingly, experiments examining genome-wide transcriptome patterns of chicken embryos exposed to fetal alcohol-like conditions shared three alcohol-related genes with methylation patterns from offspring in our experiments which were Ctnnb1, Eif4g2, and Id2 (Garic et al., 2014). This was an interesting finding because, although both preconception and fetal alcohol exposure can cause widespread methylation changes in offspring, these do not appear to overlap very extensively, and the three genes which were modified in both experiments may represent particularly sensitive genes within the embryogenesis period. The methylation control of genes which seem to be modified in preconception exposure to drugs of abuse (i.e. EtOH and THC) along with the shared regulatory consequences of fetal alcohol exposure on the methylation patterns should be investigated in the future to determine the exact mechanism of regulation at these sites.

Future Directions

The work presented herein contributes novel information to the field of multigenerational epigenetic transmission and advances our understanding of heritable

phenotypes following parental drug exposure. However, many questions still remain about the molecular mechanisms by which parental alcohol can impact the offspring epigenome, and subsequently, physical characteristics. Therefore, future work should evaluate the sex-specific contributions of maternal and paternal preconception binge-like alcohol exposure to the epigenetic landscape of offspring and identify a targeting mechanism for this disrupted epigenetic function.

In Experiment 1, I found discrete residues throughout the alcohol-naïve offspring genome that were both hyper and hypomethylated and very few of these residues were shared between maternal alcohol (EtOH) exposure and paternal EtOH exposure. The results from this study revealed 1) that there was a lack of global DNA methylation changes in offspring, suggesting a molecular targeting mechanism and not epigenetic dysfunction overall, and 2) that modes of epigenetic inheritance were more complex than that of classical genetics, as they did not reflect equal contributions of both parents. Non-coding RNAs (ncRNAs) make a strong putative molecular candidate for these mechanisms as they can be transmitted from parents to offspring via gametes and, recently, non-coding RNA-mediated DNA methylation has been described in mammals (Mercer and Mattick, 2013; Mercer et al., 2010; Mohammad et al., 2010; Peschansky and Wahlestedt, 2013; Quan et al., 2015; Rinn and Chang, 2012). Additionally, ncRNAs are known to be under sex-specific regulation and may, therefore, provide a way in which each parent contributes differently to offspring patterns of DNA methylation (Bhan et al., 2013; Ottaviani et al., 2014). Taken together, the data I have presented along with emerging evidence from the field supports the putative mechanism that adolescent exposure to binge

alcohol alters the expression of non-coding RNA in both the sperm and egg and those RNAs can direct a different epigenetic landscape in gametes and, subsequently, in offspring (Fig 24).

While the work of our lab and others have shown changes in gene expression and/or behavior in offspring of alcohol-exposed parents, there has yet to be a study into the mechanism of how such discrete changes occur in the offspring (Bekdash et al., 2014; Burne et al., 2014; Finegersh et al., 2015; Govorko et al., 2012; Rompala et al., 2016). Investigating a central role of non-coding RNA as the mediator of epigenetic changes in the parental gametes would uncover a mechanism through which parental preconception alcohol exposure alters offspring gene expression. This contribution will be significant as it will delineate a pathway through which preconception binge alcohol treatment acts to cause epigenetic dysregulation in alcohol-naïve future generations. Once we can determine the mechanism by which alcohol causes transgenerational changes in the brain, we can more easily study these consequences and investigate potential therapeutics.

Results of Experiment 1 revealed that across the genome, each treatment group had distinct epigenetic marks (Figs 4 and 5). The observed differential methylation patterns suggest that the parental gametes (sperm and egg) may also harbor these differential methylation marks following binge EtOH exposure and pass them on to offspring. DNA methylation is known to be influenced by environmental factors and is also heritable, making it the most studied epigenetic mark in systems of transgenerational inheritance (Bird, 2011; Chen and Riggs, 2011; Jones, 2012; Schübeler, 2015). Global DNA methylation patterns are known to change during embryogenesis, but many methylation marks escape the reprogramming process and can retain

epigenetic memory from parents to offspring (Hackett et al., 2013; Tobi et al., 2014; Zhou, 2012). A select few of the DMCs that I identified in PND7 offspring could be traced to maternal and paternal EtOH exposure separately, and combined when both parents were exposed to alcohol (Figs 4 and 5). For example, GPR27 was hypomethylated as compared to control in all three treatment groups. By contrast, AVP was hypomethylated in both maternal-EtOH and paternal-EtOH offspring and then not differentially methylated in offspring where both parents were treated. Similarly, Esam was hypermethylated in maternal-EtOH and paternal-EtOH offspring, but not different than control if both parents were exposed to EtOH. These examples, among others, highlight the complexity of inheritance of DMCs and the inheritance patterns of these marks should be pursued in future research. This could be accomplished by investigating the methylation at certain candidate residues in sperm and eggs at different time points after pubertal exposure, during embryogenesis and during offspring development. I would expect to find some differential methylation in eggs and sperm that are attributable to the observed DMCs in each offspring treatment group. However, I do not think that all of the offspring DMCs would be detectable in parental gametes. Specifically, I predict that DMCs seen in offspring where both parents were treated with EtOH that do not appear in offspring with single parent exposure (i.e Begain, Sparcl) would not be detected in sperm or eggs, as methylation at these sites was not consistent across all treatment groups. There are likely both directly-inherited and post-fertilization changes in the epigenome following preconception exposure to alcohol.

The data presented here show that DNA methylation, and subsequently developmental potential, is altered in offspring following preconception alcohol exposure of parents; however,

it is not clear if the methylation itself is transmitted across generations or if the methylation machinery is disrupted and subsequently inherited. Preliminary data collected from our lab (see Appendix F) suggest that repeated alcohol exposure may have an impact on methylation machinery, however, there were no *global* changes in offspring methylation (i.e. complete hypomethylation or complete hypermethylation). This suggests there is a targeting mechanism by which alcohol is being directed to certain loci in the genome, whether in the gametes or in the offspring (Fig 6). Global changes in methylation potential may, in fact, be affected, but there must also be a targeting mechanisms affected by repeated EtOH exposure, which could result in the alterations observed in both expression and methylation profiles (Asimes et al., 2017; Przybycien-Szymanska et al., 2014).

There is strong evidence in the literature to support the hypothesis that long non-coding RNA (lncRNA) are the missing link directing differential DNA methylation in gametes. First, an RNA-based mechanism provides sequence specificity to differential methylation (i.e. complementarity between RNA and DNA sequences allow recognition of only certain sites). Additionally, several lncRNAs are known to associate with DNMTs and are involved in sex-specific parental imprinting (Asimes et al., 2017; Larriba and del Mazo, 2016; Lee, 2012). For example, *Xist* is a functional lncRNA that is necessary for X chromosome imprinting in female offspring (Peschansky and Wahlestedt, 2013). *Air* is another example lncRNA which is paternally expressed only, with imprinting of the maternal allele through placental expression (Nagano et al., 2008). In this manner, sex-specific expression of lncRNA may account for the parental-specific changes we see in offspring DNA methylation and for the fact that there is not an

additive effect of maternal and paternal EtOH exposure. Several of the DMCs in my methylation analysis were also in known non-coding RNA regions; for example, we found hypermethylation upstream of the *Fendrr* sequence when both parents were exposed to alcohol. *Fendrr* is a non-coding RNA that is known to repress transcription at certain neighboring loci, in this case *Fox1* (Grote and Herrmann, 2013; Xu et al., 2014). Therefore, regulation of *Fendrr* by alcohol could lead to further changes in the epigenome.

It is also important to consider the impact of alcohol on the enzymes mediating methylation processes. Cytosine residues are methylated by DNA methyltransferases (DNMT1, DNMT3a, DNMT3b, Fig. 6) (Schübeler, 2015). S-adenosyl methionine (SAM) acts as a universal methyl donor for these reactions, and is converted to S-adenosyl homocysteine (SAH). Alcohol is known to reduce the SAM/SAH ratio in the liver of chronic alcohol patients (Caudill et al., 2001). Preliminary studies from our lab showed that adolescent binge alcohol exposure reduced the SAM/SAH ratio in the testes one hour following the last alcohol treatment (data not shown). This could indicate that DNMT activity overall is reduced, as SAM is a limiting factor, and this competition for methyl donor could push methylation events towards lncRNA-facilitated pathways. Transcription of many non-coding RNAs is known to be under the control of environmental cues, and it has also been shown that alcohol differentially regulates ncRNA in other tissues, such as the liver and brain (Alsina et al., 2013; Krishnan et al., 2014; Sartor et al., 2012). Future work investigating the expression of non-coding RNAs, their association with methylation machinery, and the activity of such machinery would shed light on the short-term molecular mechanisms taking place during EtOH exposure which lead to long-term changes in

the epigenome. The field of ncRNA-directed DNA methylation has greatly expanded in the last few years, and with more knowledge of these concepts it becomes a more plausible mechanism of the transcriptional regulation that I have presented here.

CHAPTER VII

GENERAL METHODS

Ethics Statement.

Animal procedures were approved by the Loyola University Medical Center Institutional Animal Care and Use Committee (permit #2012021). All measures were taken to minimize pain and suffering.

Animal Paradigms.

Male and female Wistar rats were purchased from Charles River Laboratory (Wilmington, MA) at post-natal day (PND) 23 and were allowed to acclimate for 7 days. Then, animals were handled by experimenters for 5 minutes once daily for 7 days to control for non-specific handling stress. Animals were pair-housed within the same treatment group. Food and water were available *ad libitum* and animals were kept on a 12:12 light/dark cycle, with lights on at 7:00 AM and handling/treatment began at 10:00 AM.

Beginning at PND37, which is defined as peri-puberty in the rat (Ketelslegers et al., 1978), animals were exposed to a repeated binge-pattern alcohol paradigm (Fig. 1). This 8-day paradigm has been used previously by our lab and others and is designed to mimic the reported drinking patterns of adolescents (Lauing et al., 2008; Przybycien-Szymanksa et al., 2010; Przybycien-Szymanksa et al., 2014). This pattern of alcohol consumption raises the blood alcohol concentration (BAC) to 150-180 mg/dl in males and 210-240 mg/dl in females without

altering body weight or normal growth patterns (Przybycien-Szymanksa et al., 2010; Przybycien-Szymanksa et al., 2014). Animals were given food grade alcohol (Everclear, Luxco) diluted in tap water at a dose of 3g/kg body weight (20% v/v solution), or an equal volume of vehicle (water) via oral gavage. Treatment was given once per day for 3 days, followed by 2 days tap water and another 3 days alcohol (Fig. 1). Control groups received tap water for all 8 days. Animals were then left undisturbed until PND67, which is considered late puberty, when they underwent the same 8-day treatment.

Chapter III.

Genome-wide DNA methylation patterns in the hypothalamus of alcohol-naïve male offspring. In order to assess the inheritance of epimutations from maternal and/or paternal preconception alcohol consumption, animals were pair housed for mating 24 hours following the last gavage treatment. Pairs consisted of all combinations (maternal vehicle x paternal vehicle, maternal ethanol x paternal vehicle, maternal vehicle x paternal ethanol, maternal ethanol x paternal ethanol), with 2-3 pairs of each treatment group. After 7 days, females were single-housed in order to properly nest and males were returned to pair-housing with previous cage-mate. There was no difference in litter size, pup weight, or sex ratio between treatment groups (Table 1). Maternal care was assessed based on gathering pups into the nest, crouching over pups to facilitate suckling, active licking/grooming, and growth rates of pups (indicative of nutritional status). Twice daily observations showed no apparent differences in maternal care between groups, although these observations were not scored for quantitative analysis. Within 1 hour of birth, litters were culled to 10 pups per dam (5 pups of each sex) and pups were

raised by their biological mother until PND7. Pups were anesthetized on ice and euthanized by rapid decapitation. Brains were immediately removed and whole hypothalamus was microdissected on ice before flash freezing. Tissue was stored at -80°C until use.

Methylation Sequencing and Statistics.

Enhanced Reduced Representation Bisulfite Sequencing (ERRBS) was performed at the University of Michigan Epigenomics Core, as described by Grimes et al. (2012). For ERRBS, genomic DNA from 3 male pups of each treatment group, with at least one from each mating pair, were used (12 total pups). Previous work using this paradigm has shown larger changes in gene expression of male offspring, therefore we chose to perform ERRBS analysis on only males (Przybycien-Szymanska et al., 2014). Tissue from remaining male pups was used for mRNA expression analysis and female offspring were used for subsequent experiments.

FASTQC was employed (version 0.11.3) to assess the overall quality of each sequenced sample and identify specific reads and regions that may benefit from trimming (Rosenbloom et al., 2015). TrimGalore (version 0.4.0) was used to trim low-quality bases (quality score lower than 20), adapter sequences (stringency 6) and end-repair bases from the 3' end of reads (Carone et al., 2010). For alignment and methylation calling Bismark (version 0.14.3), an integrated alignment and methylation call program that performs unbiased alignment (by converting residual cytosines to thymines prior to alignment in both reads and reference) was employed (Krueger and Andrews, 2011). Briefly, reads were aligned to the reference genome (UCSC rn6 from iGenomes, (Manzardo and Butler, 2013) using Bowtie2 (Langmead and

Salzberg, 2012) (version 2.2.1) with default parameters settings, except for maximum number of mismatches in seed alignment (N) set to 1, and length of seed substrings (L) set to 20.

Methylation calls were reported for all nucleotides with a read depth of at least 10.

Initial analysis was done with the methylSig R package (0.3.2) to assess the overall quality of methylation calls and coverage (Park et al., 2014). It was then employed to identify differentially methylated positions by tiling the methylation data across windows of 25 bases. In addition, we used information from nearby CpG sites to improve variance estimates (local window size of 200 bases). For each pairwise comparison methylSig uses a beta-binomial approach to calculate differential methylation statistics, accounting for variation among replicates within each group. The p-values were adjusted for multiple testing using the FDR approach, and considered sites to be differentially methylated when they had a percent change in methylation of at least 20% and a q value < 0.05.

Finally, sites and regions were annotated using UCSC Genome Browser's annotations for CpG islands, promoters and other genic regions (Rosenbloom et al., 2015). CpG shores were defined as the regions outside CpG islands but within 2,000 bp of a CpG island. CpG shelves were defined as the regions within 2,000 bp of a CpG shore. When regions overlapped, the priority was CpG island, followed by CpG shore. Gene promoters were defined as 1,000 bp upstream of reported transcription start sites.

All reported data passed QC analysis. In any given cell, any given cytosine is either methylated or not. Thus looking at a population of cells should yield a pattern where many C

positions have high methylation and many C positions have low methylation. Percent methylation histograms should therefore have two peaks at either end, which we observed for all samples (Appendix A). Histogram analysis of CpG coverage demonstrated the absence of a right-shift secondary peak, indicating that there was no PCR duplication bias in our samples (Appendix A). Differences in methylation at individual cytosine residues were analyzed, as this is a strength of the ERRBS technique. The base-pair resolution of ERRBS allows us to identify the exact residues which are susceptible to modification by parental binge alcohol exposure.

Reverse Transcription Quantitative PCR (RT-qPCR).

mRNA expression of differentially methylated target genes in offspring hypothalamus was measured using reverse transcription quantitative PCR ($n=7-10/\text{group}$). Total RNA (1.0 μg) was reverse transcribed using High Capacity Reverse Transcription Kit (Applied Biosystems) according to manufacturer instructions. FastStart SYBR Green Master Mix (Roche) was used for all RT-qPCR reactions, adding 2 μl of cDNA and final primer concentrations of 0.25 μM for each gene (primer sequences listed in Table 2). RT-qPCR data were analyzed using the $\Delta\Delta\text{Ct}$ method comparing to 18S RNA expression of each sample. Data were compared using one-way ANOVA to determine differences in expression between all treatment groups with Tukey's Post-hoc analysis. P-value < 0.05 was considered significant.

Table 9. RT-qPCR Primer Sequences for Chapter III.

Gene Name		Sequence (5' to 3')
Begain	F	GATGGGCAGCGATCAGTCTTC
	R	AGCTTCTCCAACTTGTGCGT
Rtn4ip1	F	GGACTCCCTGCTTGTGCTT
	R	ATTGCTACTGTGCTCCCCAC
NPY	F	TACTCCGCTCTGCGACACTA
	R	TGGGGGCATTTCTGTGCTT
Grm4	F	ATGCGCGGTTGGTAGGAGTG
	R	CAGCGCAGGTTCAGTAATGC
FGFR1	F	TCACAGCCACTCTCTGCACT
	R	GTGATGCGTGTACGGTTGCT
Acvr2a	F	CGGGGATTGTCATTGTGCG
	R	TCCAGGGTCCTGAGTAGGAA
MC3r	F	TGCAACTCTGTCATCGACCC
	R	CCATTGCAACCAGCAGAGAAAT
Fzd10	F	CTCCATGGACTTAGAGCGCC
	R	TGGTGTGTTAGCCGATGTCC
Esam	F	CCAGCTTACTGCAGGGTTTG
	R	GATGAAGACTCCTCCCGTGC
Arrdc1	F	ACTACCCTCCGAGCTATCCG
	R	TGAAGTAGCTCTCCACCA
Gpank1	F	TGAGGGACTTAGGTGGGTGT
	R	GGACATGGCTCAGGTTAGCG
Ephb3	F	AAGTTGGGGGAGAACCTA
	R	TGAAGAGGTTGGGCACAC
AVP	F	CGCAGTGCCCACCTATGCTC
	R	AGGAAGCAGCCCAGCTCGTC
18S	F	CATTCGAACGTCTGCCCTAT
	R	GTTTCTCAGGCTCCCTCTCC

Chapter IV, Experiment 1.

Baseline characteristics of male and female offspring from parents administered preconception binge-pattern EtOH during adolescence. In order to study the functional implications of parental preconception EtOH exposure, animals were paired (EtOH-EtOH, vehicle-vehicle) for mating 24 h after the last EtOH dose at PND 75, with 5 mating pairs of each treatment. After 7 days, females were moved to single-housing in order to properly gestate and nest, and males were returned to pair-housing with their previous cage-mate. Litters were culled to 10 pups per dam in equal sex ratios within 1 hour of natural birth. Pups were left with their biological mother until weaning at PND 23. After offspring weaning, parents remained undisturbed and housed for future studies. Offspring were housed from PND 23 to PND 30 in same-sex groups of 5 from litters of the same treatment (i.e. parental EtOH housed together, parental vehicle housed together). Behavioral observations were made from recordings of home cage activity daily from PND 25-30. Offspring were separated into paired-housing following play behavior recordings and were handled daily from PND 30-36. Offspring, both male and female, that were used for baseline offspring characterization were administered water during the early puberty gavage days of PND37-44 in order to control for the experimenter handling and gavage stress.

Chapter IV, Experiment 2.

Effects of adolescent EtOH exposure in offspring with parental history of preconception EtOH consumption. Pups of the mating pairs described in Experiment 2 were also used to test

the effects of parental EtOH history on offspring response to EtOH during puberty. Following pair housing and handling, these offspring were administered the same adolescent binge-EtOH paradigm described above during early puberty (PND 37-44). All offspring were anesthetized with isoflurane and euthanized by decapitation 1 h following the last EtOH dose at PND 44.

Tissue Collection.

Trunk blood was collected at the time of decapitation on ice into heparinized tubes, centrifuged at 4500 rpm for 8 min. at 4°C, and plasma was aliquoted and stored at -20°C. Brains and testes were immediately removed and flash frozen in isopentane on dry ice. Tissue was stored at -80°C until microdissection and processed for total RNA isolation with TRIzol reagent (Life Technologies, cat#15596018), according to manufacturer instructions. Briefly, brains were sectioned rostral to caudal at 200 µm using a Leica CM3050 S cryostat, and whole hypothalamus (-0.8 to -3.8 mm relative to bregma) was microdissected using a Palkovit's brain punch tool (Stoelting Co.), according to The Rat Brain in Stereotaxic Coordinates, Fourth Edition Atlas (Paxinos and Watson, 2013).

Enzyme-Linked Immunosorbent Assay (ELISA).

Circulating concentrations of corticosterone (CORT), testosterone (T) and luteinizing hormone (LH) were measured in all animals using ELISA assay kits, according to manufacturer instructions (Corticosterone Enzyme Immunoassay Kit, Arbor Assays, cat #K014-H1, lot #16CS080d; Testosterone Enzyme Immunoassay Kit, Arbor Assays, cat # K032-H1, lot #17T051b; LH ELISA Kit, Enzo Life Sciences, cat #ENZ-KIT107-0001, lot #02061708A). For all assays, samples

which fell below the limit of detection were included in analysis with a value equal to that of the detection limit as indicated by the dashed line in each graph.

Reverse Transcription Quantitative PCR (RT-qPCR).

Complimentary DNA (cDNA) was reverse transcribed from 1.0 µg of total RNA isolated from male offspring hypothalamus and testes using SuperScript IV First-Strand Synthesis System (Invitrogen, cat # 18-091-200) according to manufacturer instructions. Quantitative PCR (qPCR) of hypothalamic genes was performed to measure mRNA expression of gonadotropin-releasing hormone (*GnRH*), corticotrophin releasing factor (*CRF*), arginine vasopressin (*AVP*), and glucocorticoid receptor (*GR*). In addition, qPCR was performed on cDNA prepared from testes to measure mRNA expression of mature sperm marker genes acrosomal vesicle protein 1 (*Acrv1*), lactate dehydrogenase C (*LHDC*), and androgen binding protein (*ABP*). FastStart SYBR Green Master Mix (Roche, cat#04913914001) was used for all RT-qPCR reactions, adding 2µl of cDNA and final primer concentrations of 0.25 µM for each gene. RT-qPCR data were analyzed using the $\Delta\Delta Ct$ method comparing to 18S RNA expression of each sample. Primer sequences used were as follows:

GnRH (F: 5'- CTGCTGACTGTGTGTTGGAAGG, R: 5'- CCTGGCTTCCTCTCAATCA),

CRF (F: 5'-GAGAAAGGGAAAGGCAAAG, R: 5'- ATCAGAATCGGCTGAGGTTG),

AVP (F: 5'-GGGCAGGTAGTTCTCCCT, R: 5'- CACCTCTGCCCTGCTACTTCC),

GR (F: 5'-CACCATGATCCTGTCAGTG, R: 5'-AAAGCCTCCCTGCTAACCC),

Acrv1 (F: 5' - CATGCTTCTACCGAGCACAC, R: 5' -CACCTGAAACTTGTTGCCTG),

LHDC (F: 5' – TTTCTTAAGTGTGCGGGGT, R: 5' – TGTTGAGCCTTCACAGGTGG)

ABP (F: 5' – TCTGAGCCACTGGGTGACAG, R: 5' – CAACAACAGAAGCAGTCGGC)

18S (F: 5'- CATTGAACGTCTGCCCTAT, R: 5'- GTTTCTCAGGCTCCCTCTCC).

Testes Histology and Analysis.

Testes were post-fixed and cryoprotected in sucrose before sectioning at 16 µm thickness on a Leica CM3050 S cryostat. Frozen sections were thaw mounted on Superfrost Plus (Fisher Scientific) slides and dried at 37°C for 30 min. Tissue was stained using hematoxylin and eosin (H&E) and imaged under a light microscope, as previously described (Pak et al., 2002). Images were analyzed by two blinded reviewers, and qualitatively scored for the presence of elongated spermatids and lumen opening.

Play Behavior.

Juvenile social play behavior was assessed using paradigms adapted from those previously reported (Meaney and McEwen, 1986; Olesen et al., 2005; Verdejo García et al., 2013). Recordings of home-cage behavior were made for 5 consecutive days, at 3 hours after lights off (10 PM). For each recording day, every animal was marked with permanent marker on their backs and tails within 2 hours before lights off. Recordings were made by replacing the cage lid with plexiglass and moving the cage to a table under a night vision camera. Cages were left beneath the camera for 5 min trials, for a total observation time of 25 minutes per cage.

Play behavior recordings were scored by a blinded experimenter measuring the following criteria: (1) biting: one rat bites another; (2) chasing: one rat chases another; (3) pouncing: one rat pounces or lunges at another; (4) pinning: one rat stands over another, with its forepaws on the ventral surface of the opposing rat; and (5) boxing: both rats stand on hind legs and engage each other with forepaws. Both auto- and allo-grooming behaviors were also scored. Counts across all observation windows were totaled per cage.

Statistics.

Statistical analysis was performed using a two-sample T-test for each endpoint, using SYSTAT software (Version 13) and a statistically significant difference indicated by $p<0.05$.

Chapter V.

Effect of repeated binge EtOH exposure during puberty on lipid homeostasis in the adult and offspring brain. To test the hypothesis that parental EtOH exposure can impact lipid homeostasis in the brain of offspring, as well as the adolescent exposed later in adulthood, offspring and parents from Experiment 1 were also used for Experiment 4. Additionally, another cohort of males ($n=10/\text{group}$) was treated with water or EtOH during the early puberty 8-day binge and euthanized one hour after the last alcohol dose on PND 44.

Primary Astrocyte Culture.

Cortical tissue was fresh dissected from parents and PND 7 pups and washed in Hanks Balanced Salt Solution (HBSS). Tissue was dissociated with trypsin before manual disruption by

forceful pipetting. Tissue lysate was then passed through a 70 um strainer and cells were left to grow in culture in DMEM/F-12 50:50 with 10% FBS growth medium.

Neuronal Protection Assay.

N27 neuronal cell line was first differentiated by treatment with 10 µM Retinoic Acid (Sigma, #R2625) for 48 hours. Plates were washed and cells were then pre-treated with astrocyte-conditioned media (serum-free DMEM/F-12, conditioned for 24 hours by astrocyte cultures) for 30 minutes. 1-methyl-4-phenylpyridinium (MPP^+) iodide (Sigma, #DO48) was added to each culture at final concentrations of 0, 750, or 1000 µM and incubated for 24h. Standard viability curves were made by pretreating and incubating cells with regular media (DMEM/F-12 with 10% FBS). Cell death was determined using Molecular Probes Vybrant MTT Cell Proliferation Assay Kit (Fisher, #V13154) according to manufacturer instructions. Absorbance at 570nm corresponds to cell viability, with greater absorbance indicating more neuroprotection.

Cholesterol Quantification.

Cholesterol in astrocyte cultures was measured using Amplex Red Cholesterol Assay (Life Technologies, #A12216) according to manufacturer instructions. Incubation with fluorometric probes allows absolute quantification of cholesterol content using a standard curve. For each sample values are normalized to total protein content. Protein was measured using the Pierce BCA Protein Assay and following manufacturer instructions.

Reverse Transcription Quantitative PCR (RT-qPCR).

mRNA expression of differentially methylated target genes in offspring hypothalamus was measured using reverse transcription quantitative PCR (n=7-10/group). Total RNA (1.0 µg) was reverse transcribed using High Capacity Reverse Transcription Kit (Applied Biosystems) according to manufacturer instructions. FastStart SYBR Green Master Mix (Roche) was used for all RT-qPCR reactions, adding 2µl of cDNA and final primer concentrations of 0.25 µM for each gene (primer sequences listed in Table 10). RT-qPCR data were analyzed using the $\Delta\Delta Ct$ method comparing to 18S RNA expression of each sample.

Statistics.

RT-qPCR data were analyzed using the $\Delta\Delta Ct$ method. Offspring data were compared using Two-way ANOVA to determine interactions and main effects of each parent's treatment on pup outcome. Adult comparisons were made using Two-sample t-tests. P-value < 0.05 was considered significant. Error bars = SEM

Table 10. RT-qPCR primer sequences for Chapter V.

Target Gene		Sequence (5' -> 3')
SREBP1	F	CAGGTCTTGAGCTCCACAATC
	R	GCCCACAATGCCATTGAGA
LDLR	F	CTGTATTCACGGTAGCCGCC
	R	TGGGTCACATTGATGCAGCC
CYP46a1	F	CTGCATGGCCAACAGTTT
	R	CAATGGCTTGAGCGTAGCC
ABCA1	F	ACGAGATTGATGACCGCC
	R	AGCATCCACCCCCACTCTCTTC

APPENDIX A

CHAPTER III SUPPLEMENTARY DATA

Genome-Wide Methylation Sequencing Output

Report prepared by Epigenomics Core, University of Michigan

November 23, 2015

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Location on Bfx server /ccmb/BioinfCore/ActiveProjects/EpiCore/Asimes_1385_ERRBS01_AA-9/

Samples

Sample.Ids	Treatment
CCM1	Control
ECM2	Group B
CCM3	Control
CEM1	Group A
EEM2	Group C
CEM3	Group A
ECM1	Group B
CCM2	Control
ECM3	Group B
EEM1	Group C
CEM2	Group A
EEM3	Group C

Differential Methylation Comparisons

Comparison	Numerator	Denominator
Group A vs Control	Group A	Control
Group B vs Control	Group B	Control
Group C vs Control	Group C	Control

Methods

At the UM Epigenomics Core, Enhanced Reduced Representation Bisulphite Sequencing (ERRBS) was performed as described in Akalin et al., 2012. We employed FASTQC [2] (version 0.11.3) to assess the overall quality of each sequenced sample and identify specific reads and regions that may benefit from trimming. We employed TrimGalore [3] (version 0.4.0) to trim low-quality bases (quality score lower than 20), adapter sequences (stringency 6) and end-repair bases from the 3' end of reads. For alignment and methylation calling we employed Bismark [4] (version 0.14.3), an integrated alignment and methylation call program that performs unbiased alignment (by converting residual cytosines to thymines prior to alignment in both reads and reference). Briefly, we aligned reads to the reference genome (UCSC rn6 from iGenomes [5]) using Bowtie2 [6] (version 2.2.1) with default parameters settings, except for maximum number of mismatches in seed alignment (N) set to 1, and length of seed substrings (L) set to 20. Methylation calls were reported for all nucleotides with a read depth of at least 10.

We used the methylSig [7] R package (0.3.2) to assess the overall quality of methylation calls and coverage. We then employed it to identify differentially methylated positions, as well as regions, by tiling the methylation data across windows of 25 bases. In addition, we used information from nearby CpG sites to improve variance estimates (local window size of 200 bases). For each pairwise comparison methylSig uses a beta-binomial approach to calculate differential methylation statistics, accounting for variation among replicates within each group. We adjusted the p-values for multiple testing using the FDR approach, and considered sites to

be differentially methylated when they had a percent change in methylation of at least 20% and a qvalue smaller than 0.05. Finally, we annotated sites and regions using UCSC Genome Browser's annotations for CpG islands, promoters and other genic regions [8].

Results

1. QC

Both pre-trimming and post-trimming QC reports indicated that the read data passed the basic QC, with exceptions following the expected patterns in ERRBS experiments. Alignment efficiencies and conversion rates are typical of ERRBS experiments and consistent across samples (Table 1).

Sample	Total Reads	Trimmed Reads	Unique Alignment	Alignment Efficiency	Conversion Rate
CCM1	101,048,410	100,855,039	62,220,409	61.70%	99.40%
ECM2	106,580,485	105,705,397	69,203,843	65.50%	99.20%
CCM3	102,862,686	102,170,841	58,944,438	57.70%	99.10%
CEM1	109,878,778	108,966,846	52,870,280	48.50%	98.90%
EEM2	121,250,474	120,463,927	69,505,491	57.70%	99.10%
CEM3	107,454,830	106,658,607	64,249,678	60.20%	99.30%
ECM1	114,893,371	113,982,695	69,455,351	60.90%	99.30%
CCM2	127,821,721	127,215,674	78,361,090	61.60%	99.00%
ECM3	99,166,858	98,773,966	61,786,238	62.60%	99.20%
EEM1	139,906,554	138,640,440	81,779,772	59.00%	99.10%
CEM2	124,275,848	123,559,339	74,581,912	60.40%	99.20%
EEM3	106,204,238	105,806,869	66,903,713	63.20%	99.40%

Table 1. Sequencing, alignment, call and conversion rate statistics.

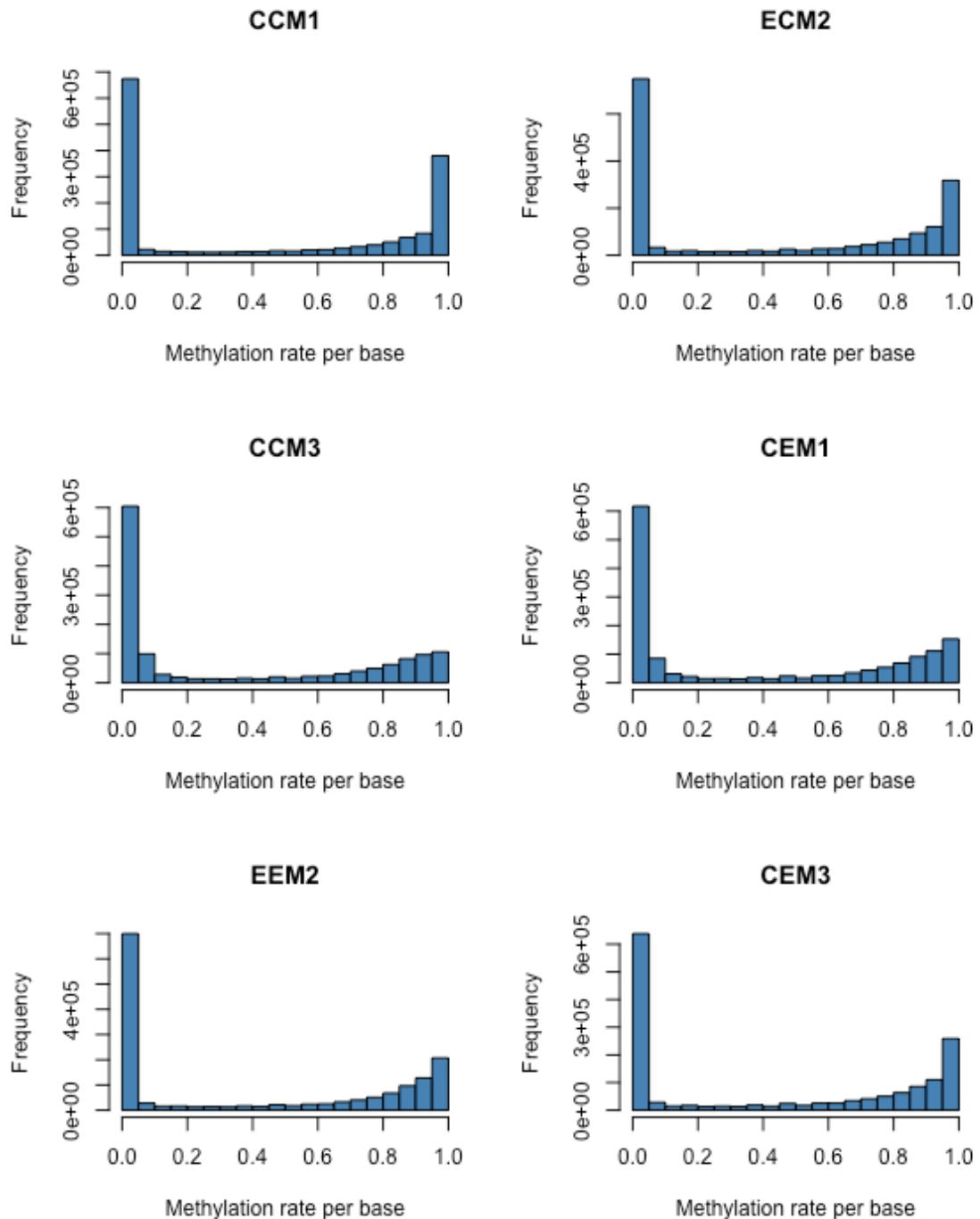
2. Methylation Calls QC (CpG Context only)

Table 2 shows the number of positions with methylation calls in at least one sample, after filtering for coverage (minimum 10, maximum 500).

Sample	No. Positions	No. Calls
CCM1	1,548,430	148,645,065
ECM2	1,746,989	117,515,495
CCM3	1,365,932	105,297,119
CEM1	1,476,996	65,949,787
EEM2	1,545,922	150,105,716
CEM3	1,510,418	81,660,190
ECM1	1,701,483	96,687,947
CCM2	2,052,495	101,198,362
ECM3	1,469,586	161,880,946
EEM1	2,047,820	83,929,723
CEM2	1,648,141	88,324,610
EEM3	1,430,106	186,423,827

Table 2. Number of positions with a methylation call and total number of methylation calls.

In any given cell, any given C is either methylated or not. Thus looking at a population of cells should yield a pattern where lots of C positions have high methylation and lots of C positions have low methylation. Percent methylation histograms should therefore have two peaks at either end. This is what we observe for all samples (Fig 1).



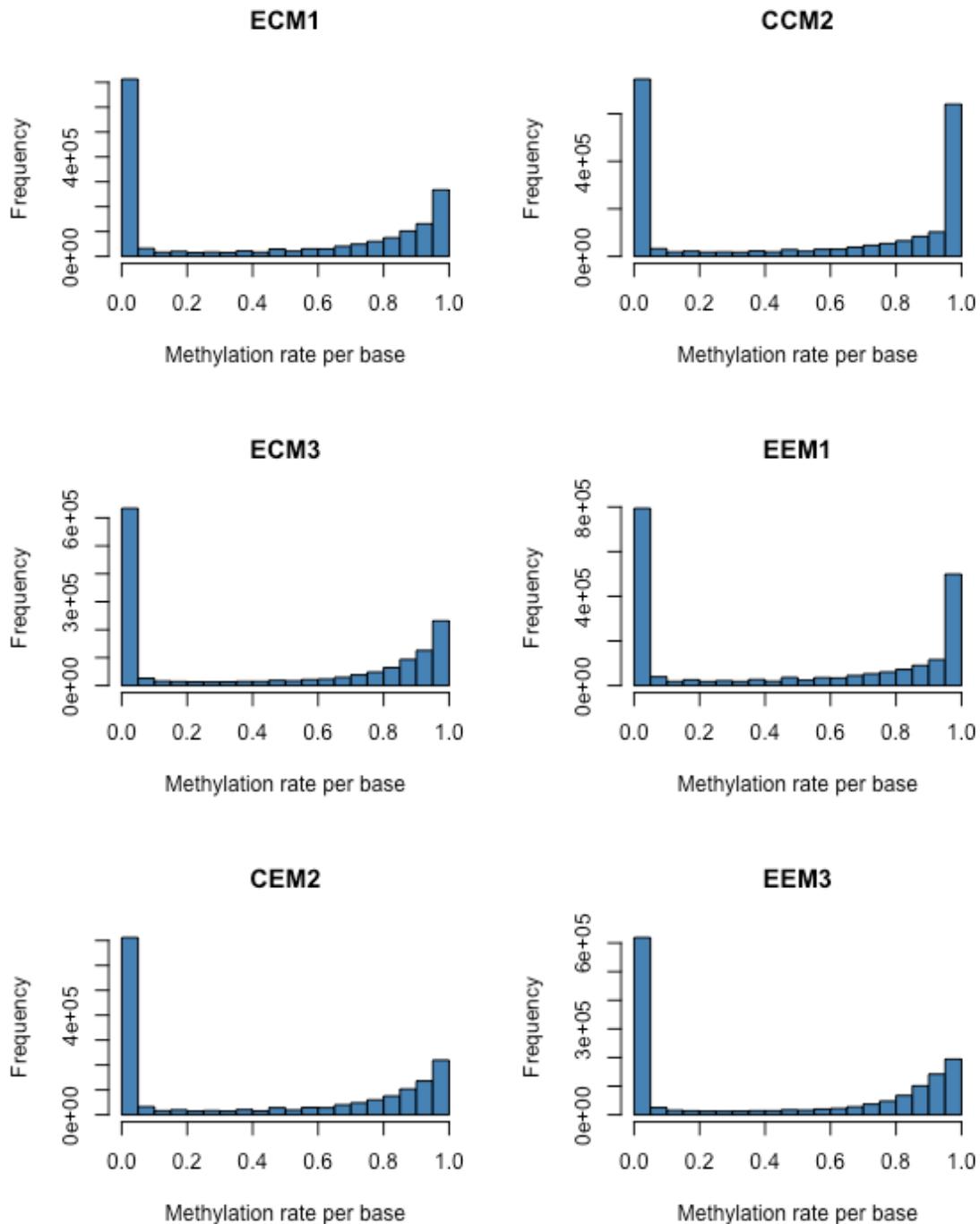
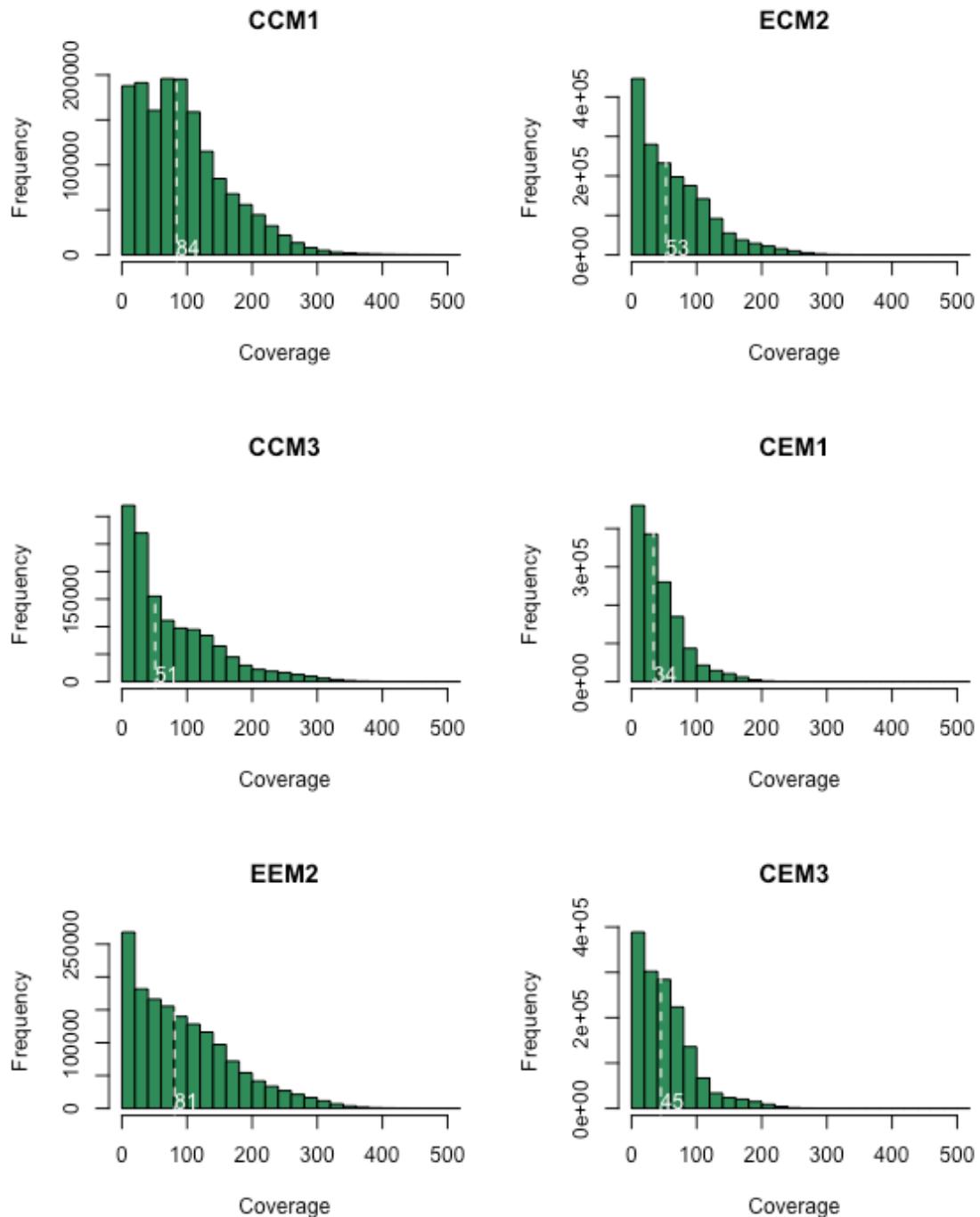


Figure 1. Histograms of percent CpG methylation.

In experiments that suffer from PCR duplication bias, histograms of CpG coverage have a secondary peak towards the right hand side. No samples exhibited such a peak (Fig 2).



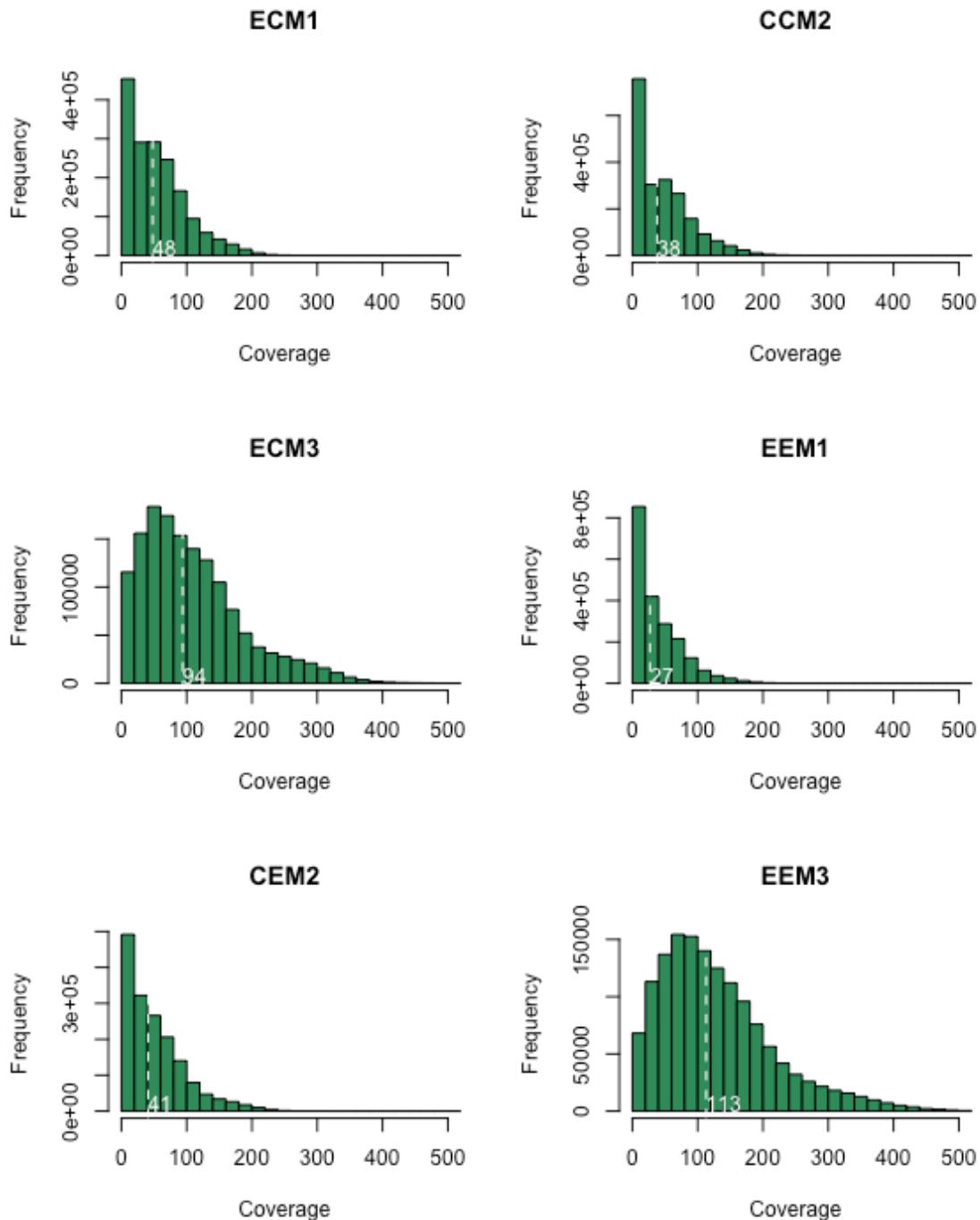


Figure 2. Histograms of CpG coverage. Median coverage is highlighted in white.

Figure 3 shows depth of coverage of CpG islands, CpG shores, CpG shelves and promoter regions. Depth is defined as the sum of all observed reads in the region. CpG shores are defined

as the regions outside CpG islands but within 2,000bp of a CpG island. CpG shelves are defined as the regions within 2,000bp of a CpG shore. When regions overlap, the priority is CpG island then CpG shore. Promoters are defined as the 1,000bp upstream of transcription start sites.

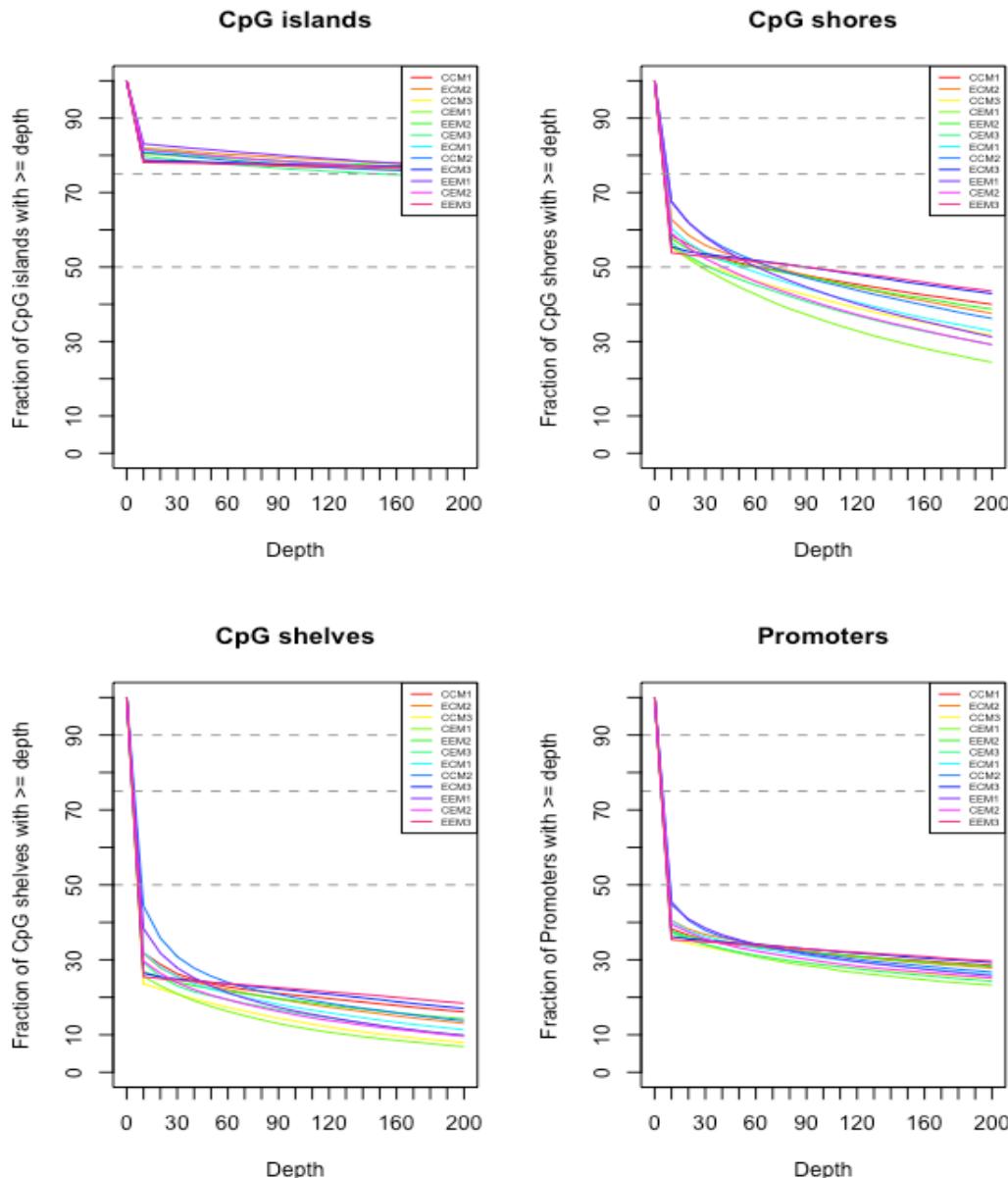


Figure 3. Depth of coverage for CpG islands, shores, shelves and promoters regions.

3. Methylation Correlation Across Conditions

Figure 4 shows the correlation of methylation rates at all CpG sites between samples. All samples are very highly correlated, but there is little grouping of samples by treatment and/or batch.

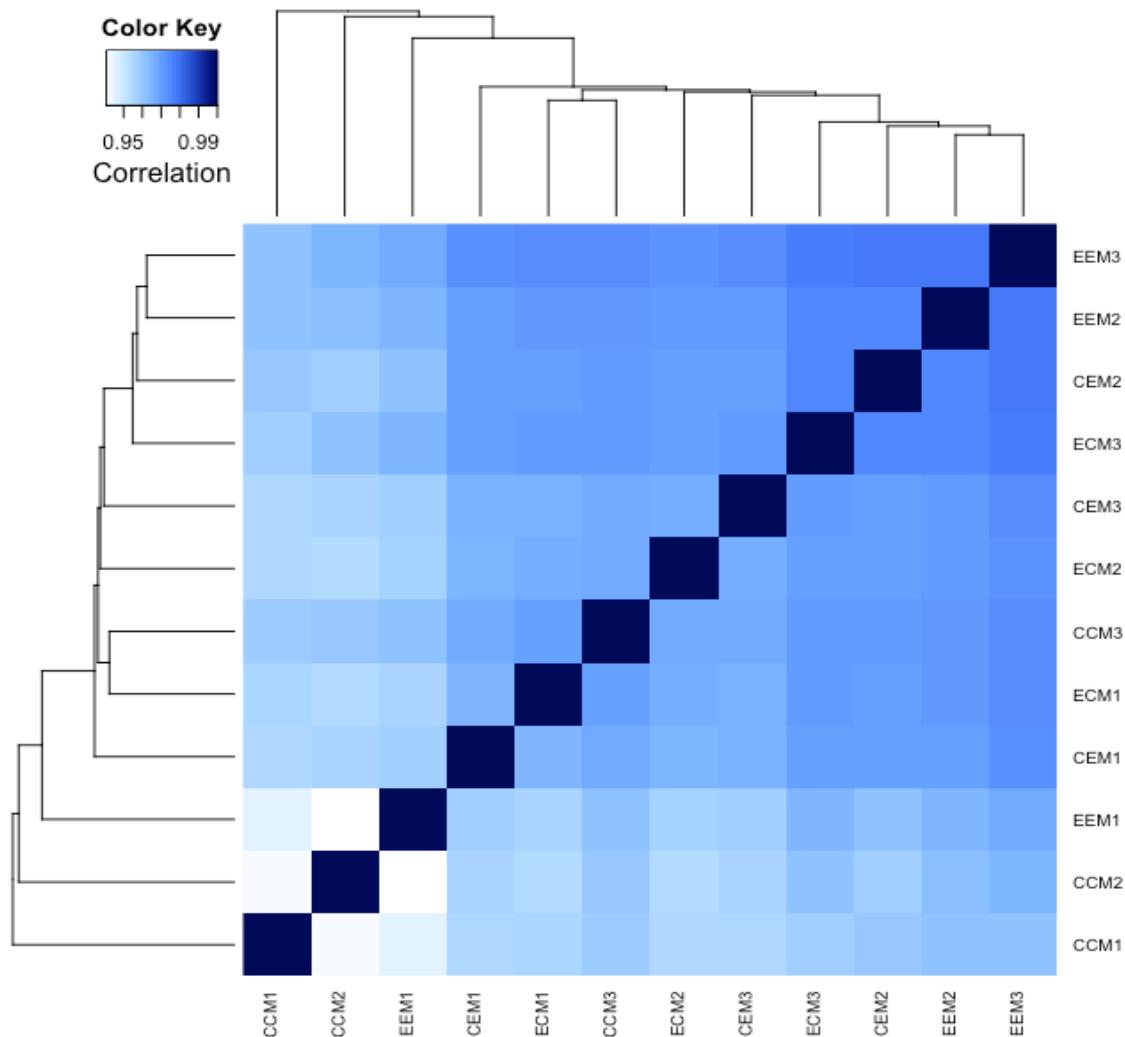


Figure 4. Heatmap based on methylation rate at all CpG sites.

4. Differential Methylation (DM)

The number of differentially methylated positions and regions using the parameters described in the methods section is summarized in Table 3. Volcano plots for sites and regions are shown in figures 5 and 6.

Comparison	Tested Cs	DMCs	HyperMCs	Tested Rs	DMRs	HyperMRs
Group A vs Control	1267728	174	54.6	748871	52	61.5
Group B vs Control	1338950	101	52.5	807509	33	54.5
Group C vs Control	1332919	273	61.5	802273	86	62.8

Table 3. Number of DM positions and regions for each pairwise comparison

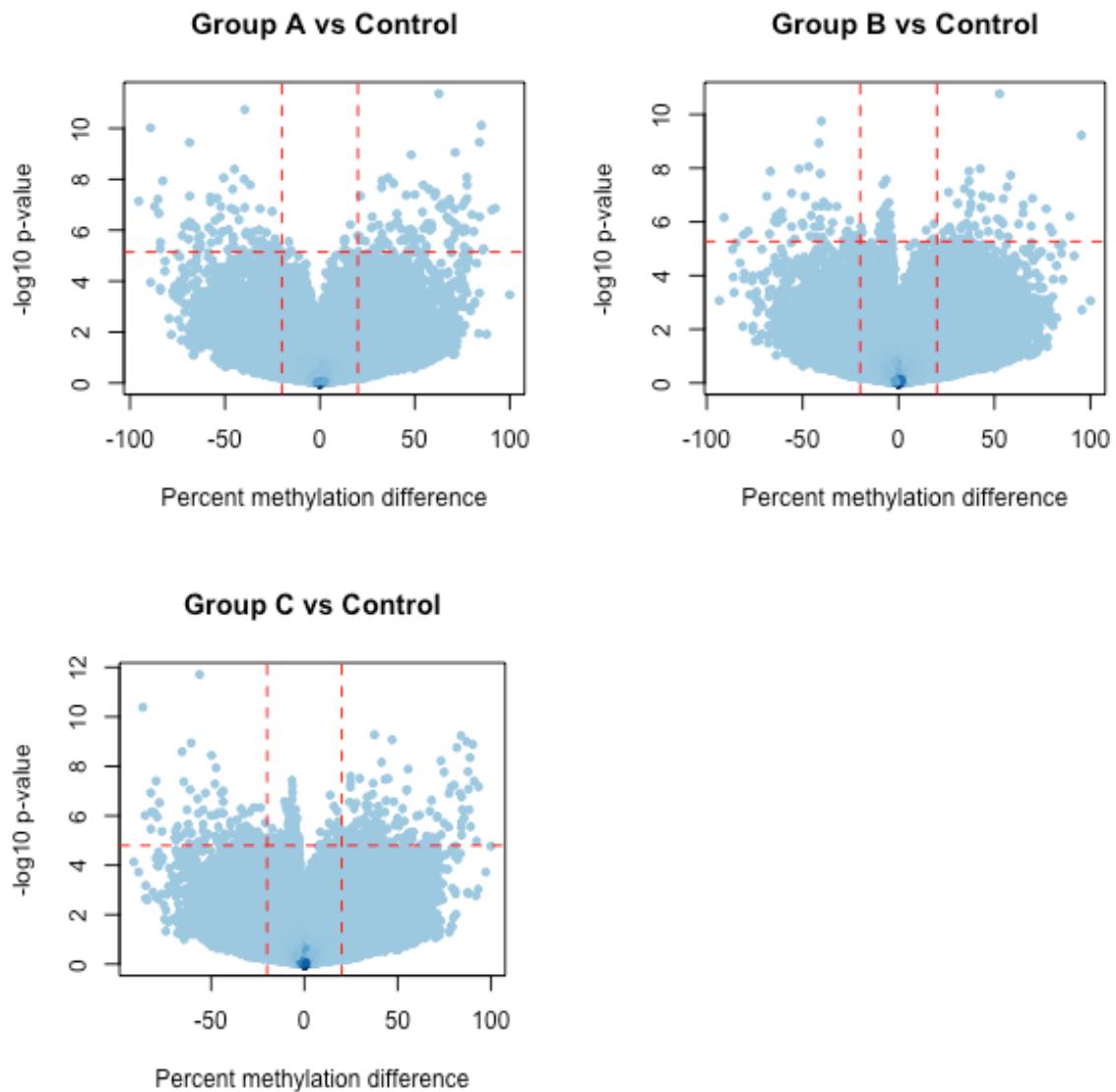


Figure 5. Volcano plots of percent methylation difference against statistical significance for DMCs. Red dashed lines mark the cutoffs used to determine differential methylation (as described in the methods).

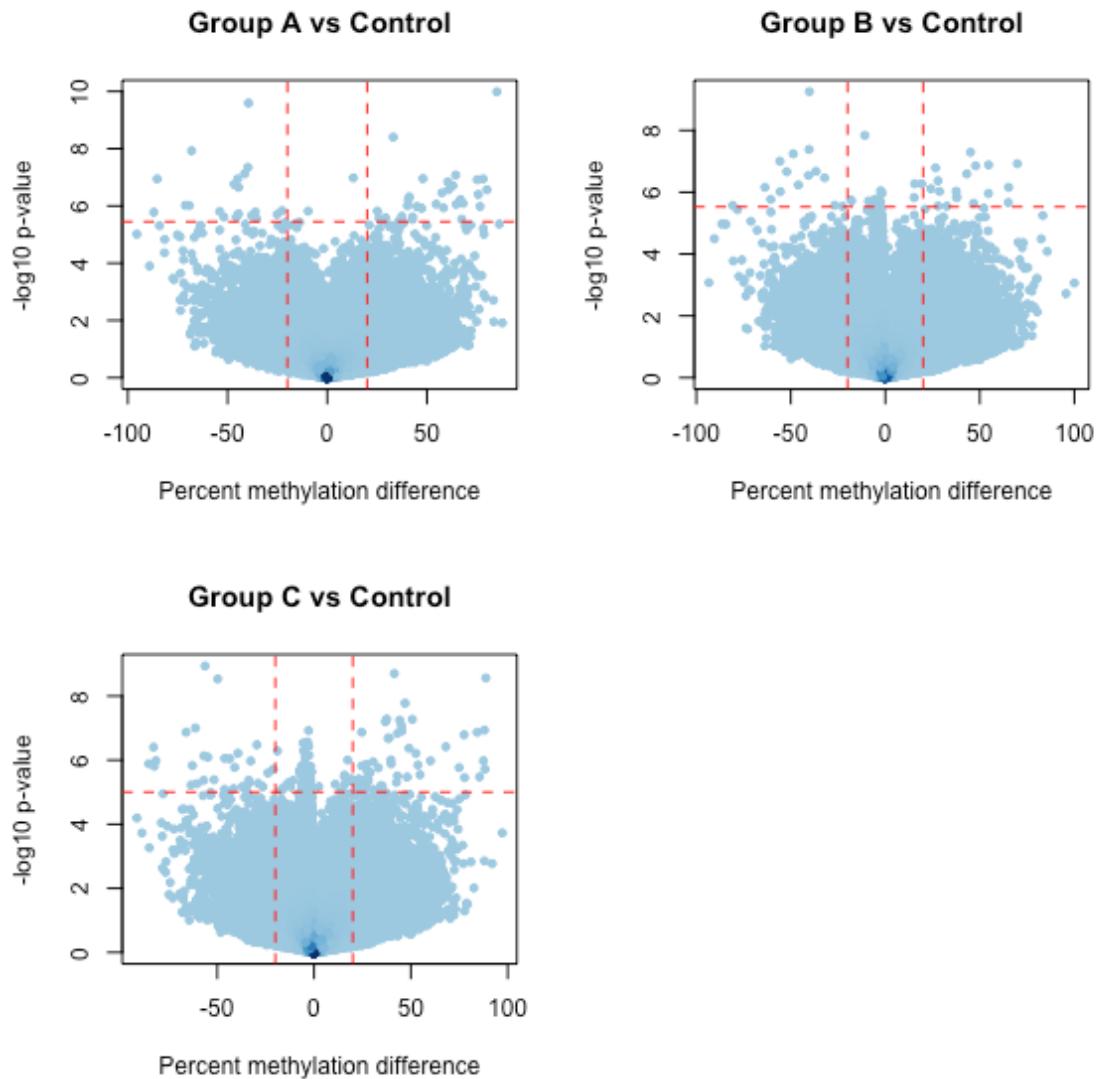


Figure 6. Volcano plots of percent methylation difference against statistical significance for DMRs. Red dashed lines mark the cutoffs used to determine differential methylation (as described in the methods).

Figures 7 and 8 show the distribution of identified sites and regions across chromosomes, and the proportion of sites/regions that were hyper- vs. hypo-methylated.

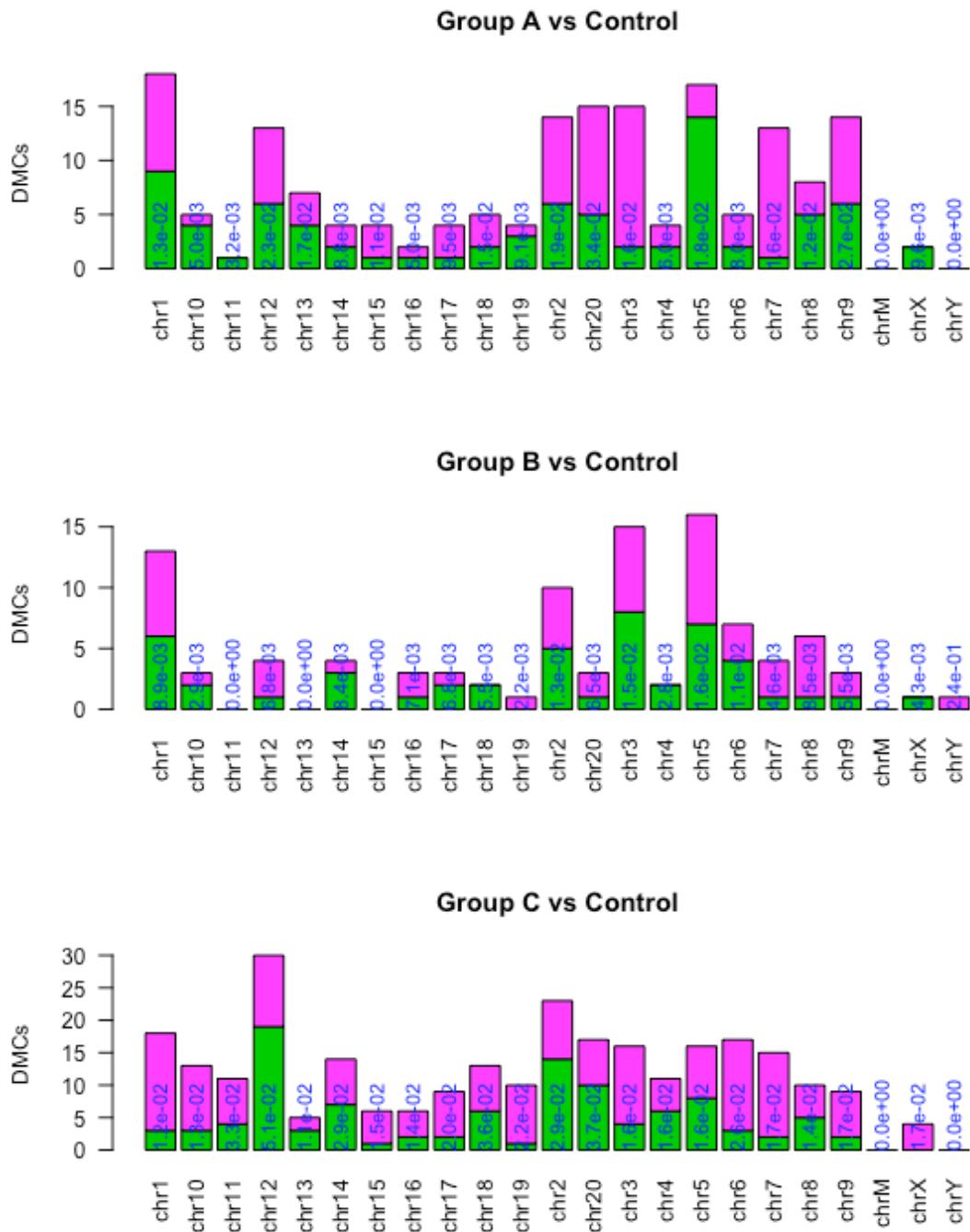


Figure 7. Distribution of DMCs per chromosome, Magenta bars represent hyper-methylated regions, green bars represent hypo-methylated regions. Blue numbers on bars are DMC percentage relative to tested regions per chromosome.

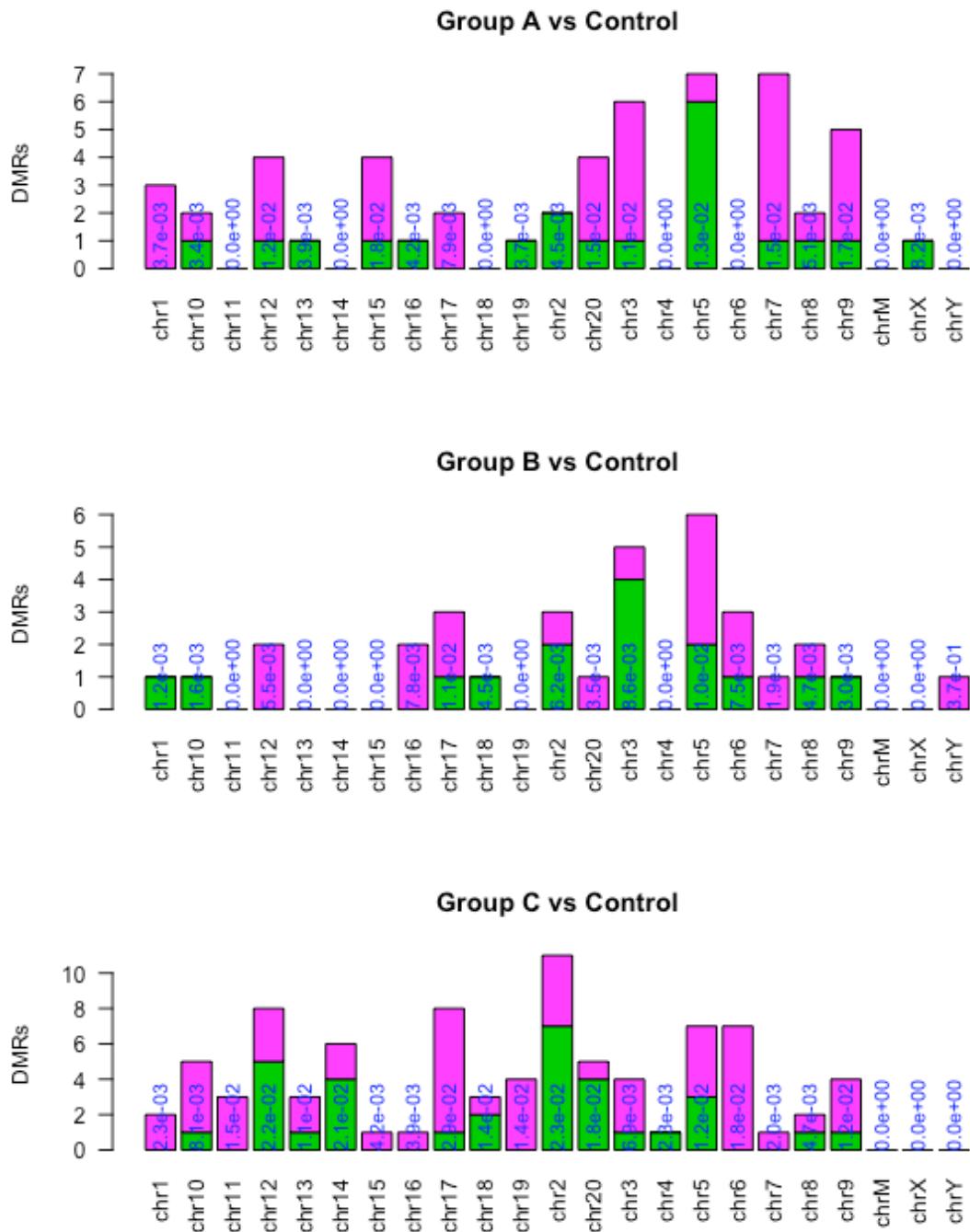
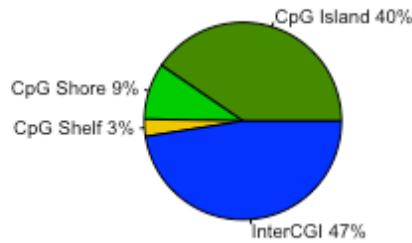
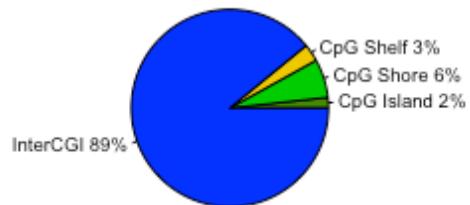
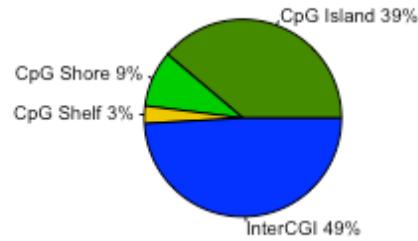
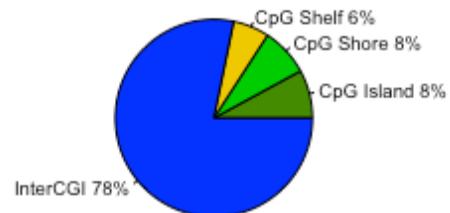
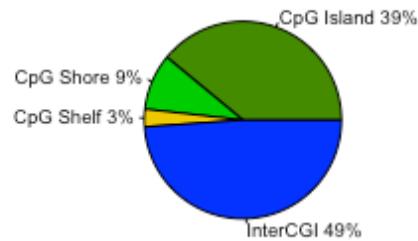
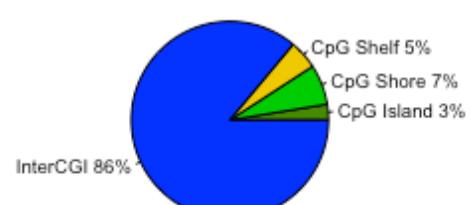


Figure 8. Distribution of DMRs per chromosome, Magenta bars represent hyper-methylated regions, green bars represent hypo-methylated regions. Blue numbers on bars are DMR percentage relative to tested regions per chromosome.

5. Annotation

Figures 9 and 10 show the proportion of tested versus DM positions (and regions) that fall in CpG islands, shores and shelves. CpG shores are defined as the regions outside CpG islands but within 2,000bp of a CpG island. CpG shelves are defined as the regions within 2,000bp of a CpG shore. When regions overlap, the priority is CpG island then CpG shore. Regions more than 4,000bp from a CpG island are annotated as interCGI.

1267728 tested sites in Group A vs Control**174 DM sites in Group A vs Control****1338950 tested sites in Group B vs Control****101 DM sites in Group B vs Control****1332919 tested sites in Group C vs Control****273 DM sites in Group C vs Control****Figure 9. CpG island annotation for tested sites (left) and DM sites (right).**

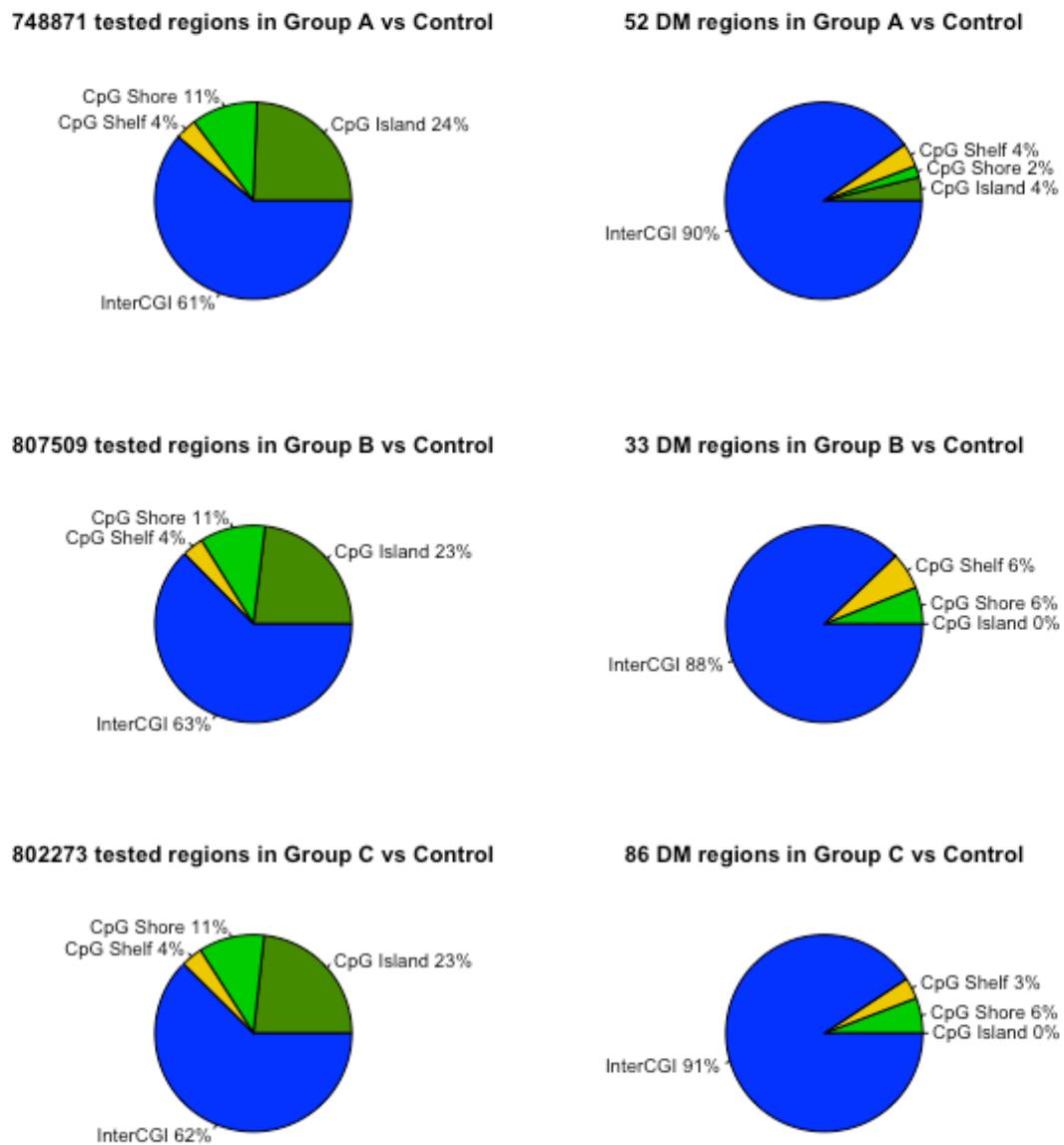


Figure 10. CpG island annotation for tested regions (left) and DM regions (right).

Figures 11 and 12 show the distribution of tested versus DM positions (and regions) that fall within various RefGene defined genic elements. The promoter is defined as the 1,000bp upstream of transcription start sites (TSS). When elements overlap, the priority is promoter, CDS, noncoding, 5'UTR then 3'UTR.

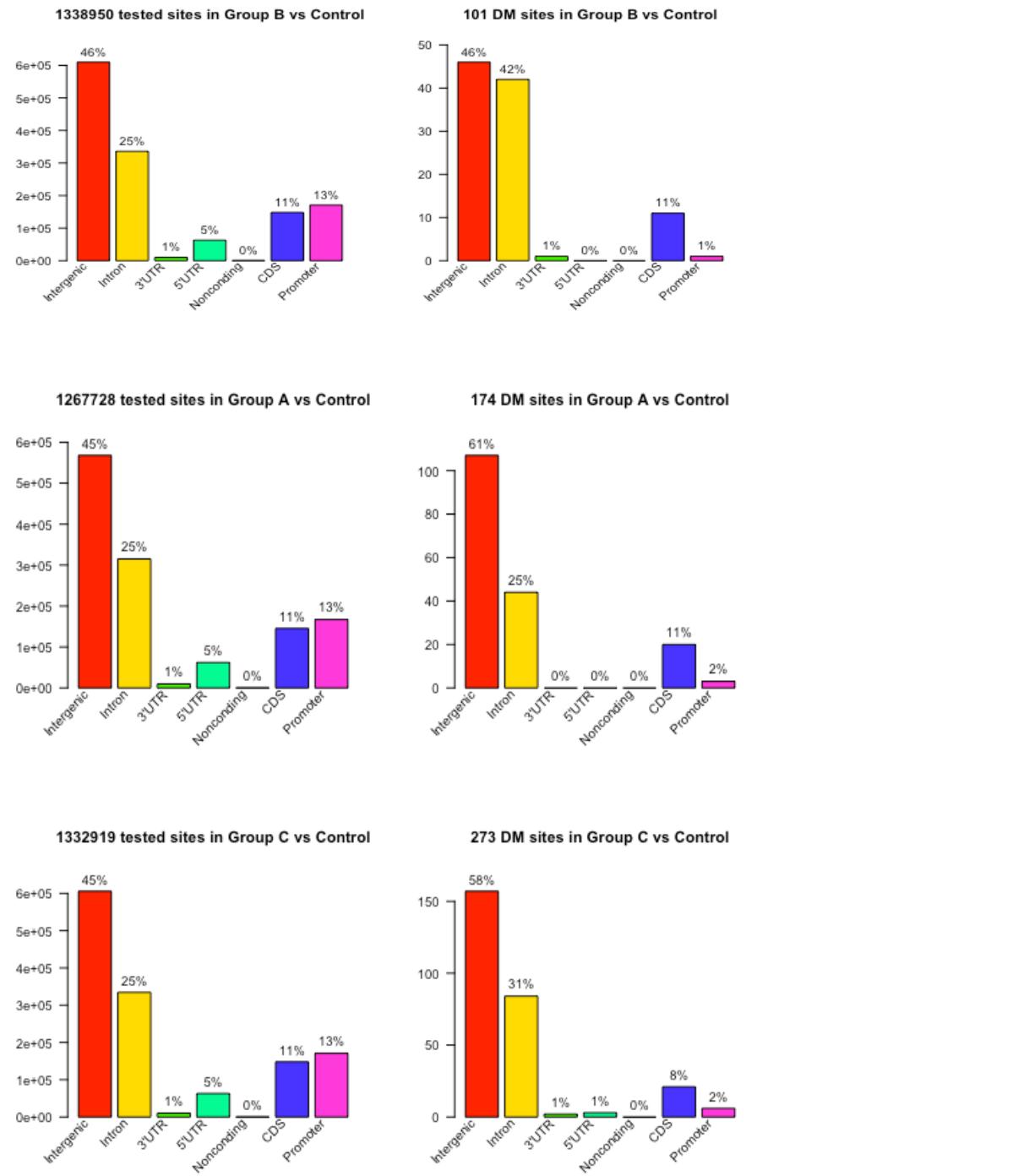


Figure 11. Genic element annotation for tested sites (left) and DM sites (right) according to RefGene.

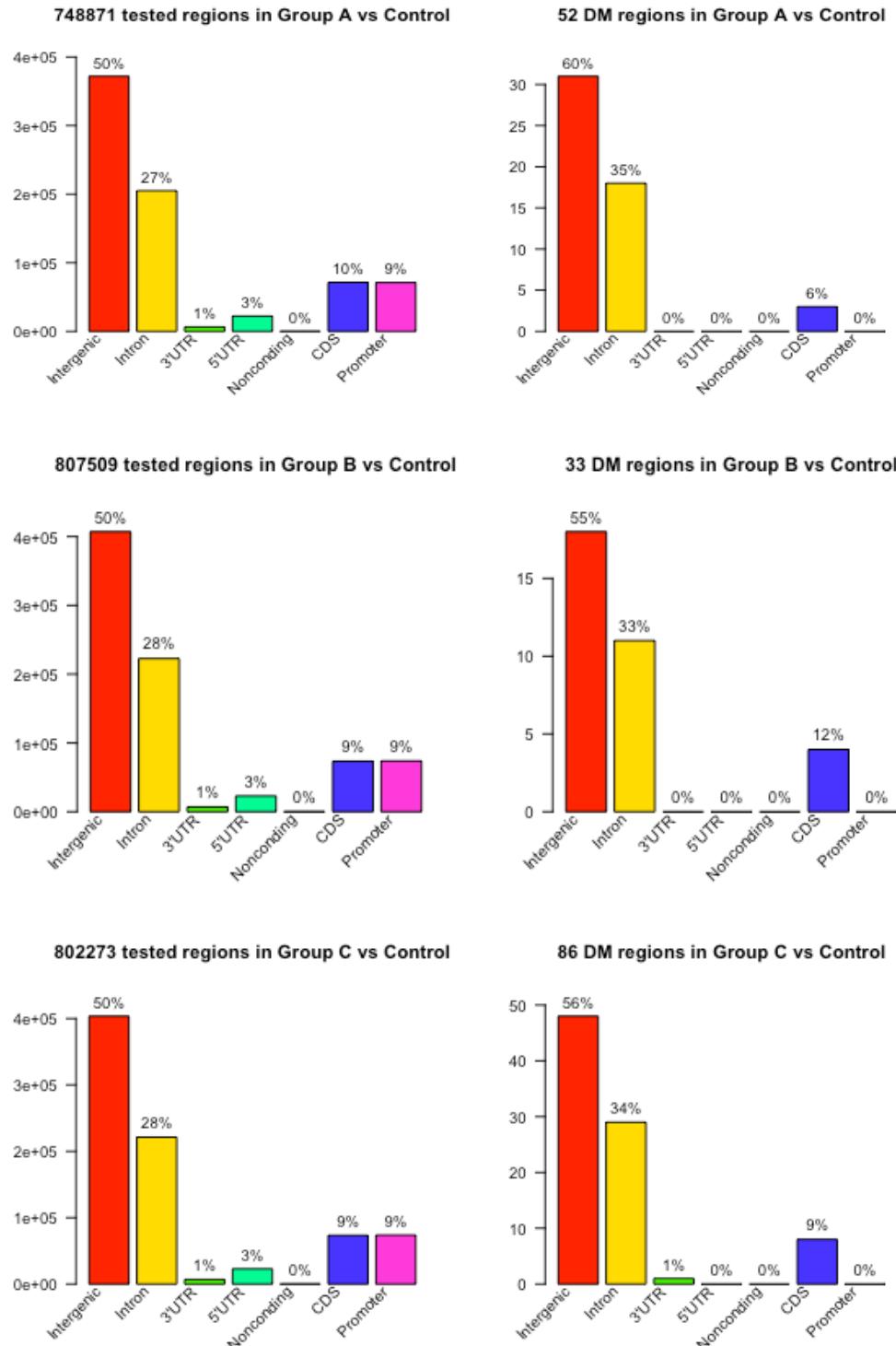


Figure 12. Genic element annotation for tested regions (left) and DM regions (right) according to RefGene.

Attachments

We will share the following data via Globus:

- **01-raw_fastq**: Raw sequence files in FASTQ format (1/sample)
- **02-fastqc**: FASTQC output folders (1/sample)
- **03-methylation_call**: CpG context methylation call files in text format (1/sample)
- **04-methylation_bed**: CpG context methylation call files in BedGraph format (1/sample)
- **05-alignment_bam**: Alignment files in BAM format (1/sample)
- **06-DM_bed**: DMC and DMR files in BED format (1/comparison, differentially methylated sites and regions only, respectively).
- **Stats.xlsx**: Excel spreadsheet with summary statistics
- **DMCs.xlsx**: Excel workbook with position-based differentially methylation output (1 sheet/comparison, with differentially methylated sites, as well as sites with p<=0.001).
- **DMRs.xlsx**: Excel workbook with tiling-based differential methylation output (1 sheet/comparison with differentially methylated regions, as well as sites with p<=0.001).
- **ERRBS Pipeline Statistics Glossary.pdf**
- **DM Output Glossary.pdf**

Follow-on

We will be happy to answer any questions you may have and/or set-up a meeting to go over the results of your experiment.

Citation

Please acknowledge us in your presentations, posters and publications as Epigenomics Core, University of Michigan.

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APPENDIX B
CHAPTER IV SUPPLEMENTARY DATA

Litter and Offspring Characteristics

Table 1. Offspring demographics of litters at PND 0 for Experiments 1 and 2.

	Litter Size	Days to Birth	Male Weight (g)	Female Weight (g)
Control	13 ± 1	23.2 ± 0.37	6.62 ± 0.07	6.19 ± 0.10
EtOH	14.5 ± 0.5	25.0 ± 0.58	6.36 ± 0.11	6.01 ± 0.11

Two-sample t-test, data presented as mean ± SEM.

There were no differences in litter size, days to birth from pair housing, or offspring weights at PND 0. There were 5 mating pairs for control-treated parents and 4 mating pairs for EtOH-treated parents. Days to birth from pair-housing was used as a proxy measurement for gestational length. We did not examine females for evidence of impregnation, although observations showed no evidence of mating behavior defects in EtOH-treated animals. Offspring sex was determined by anogenital distance (longer in males than females) within one hour of birth, and confirmed with measurement of sex-determining region of the Y chromosome (SRY) gene expression after tissue collection. SRY is only expressed from Y chromosomes, and therefore, is used as a method of sex determination in tissue samples. We confirmed correct sex identification of pups after euthanizing and collecting hypothalamic tissue. SRY primer sequences were F: 5' –GTCCCGTGGAGAGAGGCGCAA and R: 5' –CTGCGCCTCCTGGAAAAAGGGC.

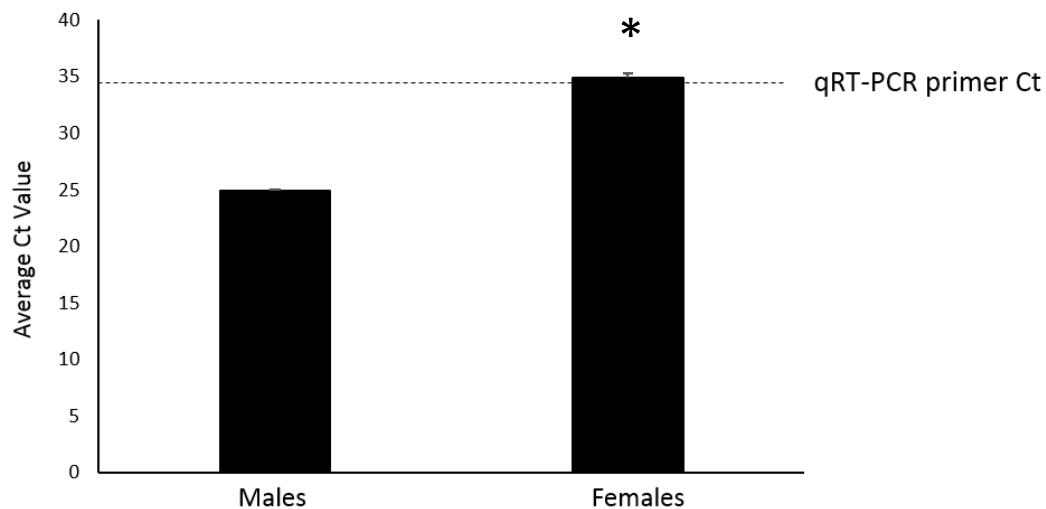


Figure 1. Confirmation of sex assignment in offspring. SRY mRNA was measured in hypothalamic tissue of offspring using RT-qPCR. Data are displayed as mean Ct \pm SEM. The dashed line represents the Ct value of no-template (primer only) conditions, therefore is the limit of detection for SRY in this assay. Two sample t-test, n=10/group, * p<0.00

Table 2. Offspring weight at PND 7 for Experiments 1 and 2.

	Male Weight (g)	Female Weight (g)
Control	18.00 \pm 0.59	17.66 \pm 0.43
EtOH	18.01 \pm 0.41	17.39 \pm 0.36

There was no difference in offspring weight at PND 7 in male or female offspring.

Table 3. Offspring demographics of second mating litters at PND 0 and PND 7 for Experiment 3.

	Litter Size	PND 0		PND 7	
		Male Weight (g)	Female Weight (g)	Male Weight (g)	Female Weight (g)
Control	12.8 \pm 1.66	6.32 \pm 0.07	5.95 \pm 0.09	18.78 \pm 0.24	17.28 \pm 0.33
EtOH	16 \pm 0.82	6.31 \pm 0.08	6.08 \pm 0.07	18.00 \pm 0.47	16.97 \pm 0.40

There was a slight increase in the litter size of EtOH-treated mating pairs during the second mating, however this increase was not statistically significant (Two-sample t-test). There were no other differences in offspring weight, at either PND 0 or PND 7 in male and female offspring.

Juvenile Play Behavior Output

Play behaviors were recorded from the home cages during juvenile development, as described in Chapter VII. The only significantly different behavior was found to be that of pinning, which is shown in Chapter IV. However, there were other scored behaviors which were found to be unchanged by parental EtOH exposure. All behaviors and summary counts are listed below in Table 4.

Table 4. Play behavior summary tables.

A

B

	Bite	Box	Pin	Pounce	Chase	Aggressive Bite	Groom	Any Behavior	Bite	Box	Pin	Pounce	Chase	Aggressive Bite	Groom	Any Behavior	Bite	Box	Pin	Pounce	Chase	Aggressive Bite	Groom	Any Behavior	Bite	Box	Pin	Pounce	Chase	Aggressive Bite	Groom	Any Behavior	Sum of Total Play
Day 1	0	8	20	24	1	0	-	53	0	0	10	4	2	0	-	16	0	6	16	5	0	0	-	27	0	2	25	15	1	0	-	43	139
Day 2	0	4	19	13	3	0	-	39	0	4	15	10	4	0	-	33	0	4	8	5	3	0	-	20	0	0	14	6	1	0	-	21	113
Day 3	0	8	33	22	6	0	-	69	0	2	14	5	0	0	-	21	0	4	6	2	0	0	-	12	0	2	30	12	0	0	-	44	146
Day 4	0	2	9	10	0	0	-	21	0	0	6	2	3	0	-	11	0	4	17	13	1	1	-	36	0	0	6	6	0	0	-	12	80
Day 5	0	0	17	9	2	0	-	29	0	0	11	6	3	0	-	20	0	8	10	16	9	0	-	43	0	2	16	14	3	0	-	35	127
Sum	0	22	98	78	12	0	0	211	0	6	56	27	12	0	0	101	0	26	57	41	13	1	0	138	0	6	91	53	5	0	0	155	605

C

D

Home cage play behaviors were scored for 5 minutes once daily for 5 days. Each outlined square within the panel represents a single cage which was scored in total, with 5 animals in each cage. A) Male offspring of Control-treated parents, B) Female offspring of Control-treated

parents, C) Male offspring of EtOH-treated parents, D) Female offspring of EtOH-treated parents.

Male Offspring Testes Scoring

Testis tissue was cryosectioned and stained with hematoxylin and eosin, as described in Chapter VII. Images were scored by two independent and blinded experimenters on degree of lumen opening using a 5-point scale (1 = no visible lumen, 5 = lumen open, mature sperm tails present). Discrepancies in score were reevaluated by a third experimenter. Average lumen score was taken for each animal and listed below in Table 5. Average group lumen opening score was calculated and compared between each treatment group, with no statistically significant difference in opening.

Table 5. Lumen opening score of male offspring testes at PND 44.

Animal	Lumen Opening Score	Parent Treatment	Offspring Treatment	Group Average
D1M1	2.59	Control	Control	2.64 ± 0.15
D3M1	2.43	Control	Control	
D3M2	3.08	Control	Control	
D4M1	2.44	Control	Control	
D10M3	2.69	Ethanol	Control	2.48 ± 0.05
D10M4	2.39	Ethanol	Control	
D8M1	2.42	Ethanol	Control	
D9M1	2.46	Ethanol	Control	
D9M2	2.45	Ethanol	Control	2.40 ± 0.20
D1M3	2.39	Control	Ethanol	
D1M5	2.06	Control	Ethanol	
D3M3	2.76	Control	Ethanol	
D10M1	3.32	Ethanol	Ethanol	2.48 ± 0.18
D10M2	2.36	Ethanol	Ethanol	
D7M3	2.31	Ethanol	Ethanol	
D7M4	2.59	Ethanol	Ethanol	
D8M4	2.08	Ethanol	Ethanol	2.48 ± 0.18
D9M3	2.25	Ethanol	Ethanol	

There was no difference in lumen opening on a 5-point scale between animals whose parents received EtOH or water treatment, in either alcohol-naïve or alcohol-treated offspring.

APPENDIX C
CHAPTER V SUPPLEMENTARY DATA

Lipid-Associated Gene Regulation

Table 1. mRNA expression of lipid-associated genes in adult male (5 months) hypothalamus.

Gene	EtOH-treated Male Fold Change ± SEM	P value
SREBP1	1.00 ± 0.08	0.96
ABCA1	0.94 ± 0.06	0.64
LDLR	1.27 ± 0.18	0.30
Cyp46a1	0.92 ± 0.08	0.66

Two-sample t test (n=4-6 per group) with p < 0.05 considered significant.

Table 2. mRNA expression of lipid-associated genes in the adolescent (PND 44) offspring hypothalamus.

Gene	Experiment 1 (No Offspring EtOH) Expression Fold Change ± SEM			
	Male Offspring	P value	Female Offspring	P value
ApoE	1.22 ± 0.09	0.23	0.84 ± 0.10	0.41
ABCA1	1.18 ± 0.08	0.33	1.02 ± 0.06	0.91
LDLR	1.12 ± 0.05	0.17	0.83 ± 0.03	0.26
SREBP1	1.17 ± 0.11	0.23	0.97 ± 0.24	0.86
Cyp46a1	1.09 ± 0.15	0.57	1.05 ± 0.18	0.78
Gene	Experiment 2 (All Offspring EtOH) Expression Fold Change ± SEM			
	Male Offspring	P value	Female Offspring	P value
ApoE	1.09 ± 0.19	0.43	1.05 ± 0.07	0.59
ABCA1	1.35 ± 0.49	0.27	0.67 ± 0.09	0.27
LDLR	0.91 ± 0.14	0.22	1.08 ± 0.07	0.33
SREBP1	1.16 ± 0.23	0.27	1.17 ± 0.12	0.21
Cyp46a1	0.77 ± 0.19	0.21	1.02 ± 0.07	0.84

Two-sample t test (n=5 per group) with p < 0.05 considered significant.

Table 3. mRNA expression of lipid-associated genes in second litter offspring hypothalamus at PND 7.

Gene	Male Offspring (Parental EtOH) Fold Change ± SEM	P value	Female Offspring (Parental EtOH) Fold Change ± SEM	P value
ApoE	1.14 ± 0.06	0.12	0.98 ± 0.05	0.89
SREBP1	0.74 ± 0.06	*0.01	1.21 ± 0.33	0.57
ABCA1	0.66 ± 0.08	0.23	4.89 ± 2.75	0.20

Two-sample t test (n=5 per group) with p < 0.05 considered significant.

Astrocyte Image Scoring

Bright field images of primary astrocytes were qualitatively assessed for the presence of lipid accumulation, and semi-quantitatively scored for number of lipid droplets per cell as well as average droplet size (perimeter and area dimensions). Analyses were carried out using ImageJ (NIH). Available images only covered a small sample size, with 3 control and 1 EtOH-treated female samples, and 1 control and 3 EtOH-treated male samples. Due to this small sample size, all of the scores are listed in the table below, but numbers were not statistically analyzed.

Table 4. "Fatty" astrocyte lipid droplet assessment.

Sample Name	Treatment	Image #	Lipids	No Lipids	Total Cells Counted	Average Ratio of cells with droplets	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Average Lipid Droplets Per Cell	Average Droplet Perimeter	Average Droplet Area
D43	Control Female	1	33	29	62	0.44	9	18	19	13	10	12.95	389.90	232.90
		2	14	14	28		15	21	9	21	20			
		3	17	39	56		13	5	10	14	15			
		4	22	29	51		6	11	9	9	12			
D46	Control Female	1	24	19	43	0.52	14	12	8	10	12	10.35	363.34	185.74
		2	22	15	37		15	5	12	12	8			
		3	40	40	80		16	7	8	20	15			
		4	14	18	32		3	6	8	8	8			
D47	Control Female	1	14	32	46	0.44	13	16	8	11	10	13.35	475.29	233.71
		2	26	19	45		7	13	9	10	6			
		3	40	31	71		27	9	16	12	24			
		4	13	27	40		13	27	6	8	22			
D54	EtOH Female	1	12	22	34	0.40	12	10	13	5	10	12.85	452.67	209.81
		2	35	48	83		14	15	11	8	15			
		3	12	26	38		30	6	28	12	9			
		4	31	31	62		13	11	12	8	15			
S36	Control Male	1	20	46	66	0.52	12	16	10	7	6	14.50	413.63	197.75
		2	49	16	65		23	5	8	18	23			
		3	43	42	85		11	18	20	8	18			
		4	45	41	86		22	11	14	11	29			
S40	EtOH Male	1	43	47	90	0.33	7	12	6	13	12	6.73	244.23	119.45
		2	30	65	95		10	8	3	6	4			
		4	8	36	44		6	5	2	4	3			
S41	EtOH Male	1	76	30	106	0.63	6	8	10	13	10	10.33	327.75	153.98
		2	57	47	104		13	6	19	10	9			
		3	46	29	75		7	6	12	17	9			
		4	24	15	39		6	15	10	9	9			
S42	EtOH Male	2	23	28	51	0.60	4	11	13	16	3	11.70	387.07	184.95
		3	30	9	39		3	26	10	22	10			
		4	56	42	98		15	12	19	12	9			

For each animal, 4 independent bright field images were obtained. First, the number of cells with and without apparent droplets were tallied and an average ratio of lipid-positive cells of total cells was calculated for each animal. Second, within each image, 5 cells were randomly selected and the number of lipid droplets within them was counted. These numbers were averaged across all 20 cells for each animal. Third, each of the droplets was measured for its perimeter size and area (given in pixels) and averaged for all images.

APPENDIX D
ALCOHOL METABOLISM TIMECOURSE

In order to ensure we were measuring blood alcohol concentration (BAC) at peak intoxication in our animal model, we performed a time course experiment. We used male Wistar rats ($n=3$ /group), administering the same dose (3 g/kg) and schedule as previously described (Fig 1a). Animals were euthanized at varying times following the last dose of EtOH on PND 44, trunk blood was collected and BAC was measured using the Blood Alcohol Concentration kit (Pointe Scientific). We found that BAC remained high between 30 minutes and 2 hours, then began to fall and was almost undetectable at 6 hours post-EtOH administration (Fig 1b). Therefore, the BAC data we have presented previously is, in fact, near peak BAC at 1 hour post-EtOH exposure.

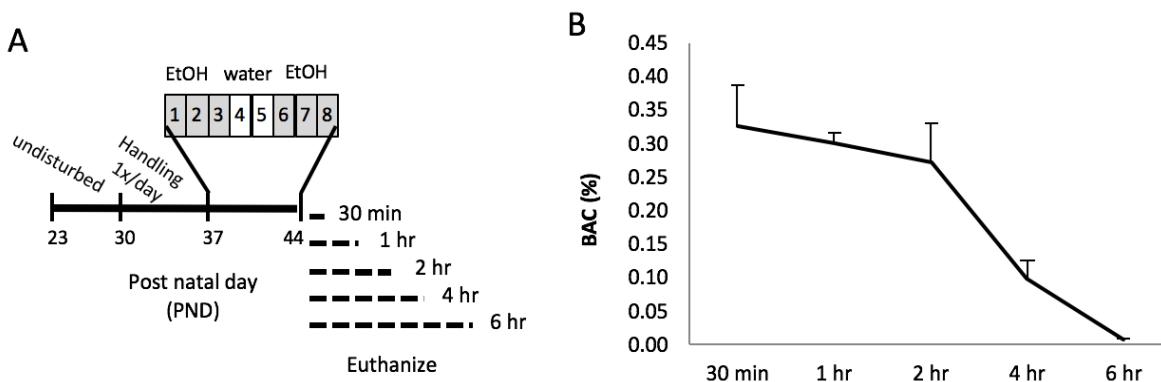


Figure 1. Blood alcohol level peaks for 2 hours following alcohol dose. A) The experimental paradigm was the same as previously described, and animals were euthanized at varying times following the last dose of EtOH. B) BAC level was measured following last EtOH dose, and remained high for the first 2 hours then fell to almost non-detectable levels by 6 hours. Data presented are mean \pm SEM ($n=3$ /group).

APPENDIX E

HYPOTHALAMIC GENE EXPRESSION CHANGES IN ADULTHOOD

Previous work in our lab has shown long-lasting changes in the hypothalamus following adolescent binge alcohol exposure. These studies, however, examined this tissue in early adulthood. I, therefore, wanted to measure the expression of HPA axis mediators in the hypothalamus of the aging parents used for breeding in the experiments described here. These parents, who had been exposed to alcohol during adolescence, were allowed to age normally following mating, and were euthanized around 5 months, or 160 days, old. I measured mRNA expression of AVP, CRF, GR, and POMC using RT-qPCR in the hypothalamus of male and female adult rats. I also measured mRNA expression of MeCP2 for its role in epigenetic regulation. Primer sequences are listed in Chapter VII for AVP, CRF and GR. Primers used for POMC were F: 5' - CTCCTGCTTCAGACCTCCATAGAC; R: 5'- ATTTCAAGGGCTGTTCATC, and MeCP2 F: 5' - GGGCTCAGGGAGGAAAAGTC; R: 5'-CACGAATGATGGAACGTCGC.

Table 1. RT-qPCR measurements from adult hypothalamus.

Gene	Adult Male Fold Change ± SEM	P value	Adult Female Fold Change ± SEM	P value
AVP	0.95 ± 0.21	0.90	1.86 ± 0.45	0.16
CRF	0.96 ± 0.14	0.88	1.16 ± 0.50	0.75
GR	1.63 ± 0.29	0.28	0.72 ± 0.09	0.49
POMC	7.27 ± 4.24	0.27	1.01 ± 0.24	0.99
MeCP2	0.95 ± 0.11	0.75	1.05 ± 0.12	0.82

All data are presented as mean ± SEM for each gene. Fold change was calculated using the $\Delta\Delta Ct$ method, with 18S as a control gene and compared to control animals for each sex. Two sample t-test, n=4-6/group.

APPENDIX F

THE EFFECTS OF ALCOHOL ON EPIGENETIC ENZYMES *IN VIVO* AND *IN VITRO*

We tested the hypothesis that binge alcohol exposure during adolescence alters the activity and expression of the enzymes in the methylation/demethylation cycle. We measured availability of the methyl donor, S-adenosyl methionine (SAM), and the methyl-depleted S-adenosyl homocysteine (SAH) in the male testes following adolescent alcohol exposure at PND 74. Hypothalamic tissue was also extracted from these animals and used to assay enzymatic activity of TETs and quantify protein levels of TET1 and MeCP2. In parallel, we used an *in vitro* model of repeated EtOH exposure, termed “Binge in a Dish,” with a hypothalamic-derived cell line (IVB) and measured DNMT and TET activity in these cells.

Methods.

Animals were treated with our paradigm already described, and euthanized one hour following last EtOH dose on PND 74. Blood alcohol concentration was measured as previously described. For *in vitro* assays, we used a Binge in a Dish model with both repeated and acute EtOH treatments. IVB cells, which are derived from rat paraventricular nucleus of the hypothalamus (PVN), were treated with media containing 50 mM EtOH for 2h for 3 consecutive days. For acute treatment, cells were only treated with EtOH on the last day of experimenting, and control cells received normal media during all changes. Plates were washed with PBS between media changes and before harvest. Total RNA was isolated from cells using TRIzol reagent and RT-qPCR was performed as described in detail in Chapter VII.

Enzymatic activity was assayed from nuclear fractions of both tissue and cell lysate, tissue according to manufacturer’s instructions for EpiQuik Nuclear Extraction Kit I (Epigentek #OP-0002). TET1 and DNMT activity were then measured using Epigenase 5mC Hydroxylase TET

Activity/Inhibition Assay Kit and EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kits, respectively (Epigentek #P-3086, #P-3001).

Protein quantification was accomplished through extraction from frozen hypothalamic tissue punches using TPER (Thermo Scientific #78510) and protein concentration measured using BCA Protein Assay Kit (Pierce). Proteins were separated by SDS-PAGE and transferred to PVDF membrane, then probed with antibodies for TET1 (Epigentek #A-1020), MeCP2 (ECM Biosciences #MP4591) and β -actin (Cell Signaling Technology #4970S).

SAM/SAH quantification was accomplished using HPLC column chromatography, where absolute levels of SAM and SAH were measured in testes of PND 74 males then used to calculate a ratio of SAM/SAH as a measure of methyl availability.

Results.

Our results showed that binge EtOH-exposed adult males showed a slight increase in MeCP2 protein expression at PND 74. We also found that EtOH decreased TET activity slightly in the hypothalamus. There were no changes in total TET1 protein.

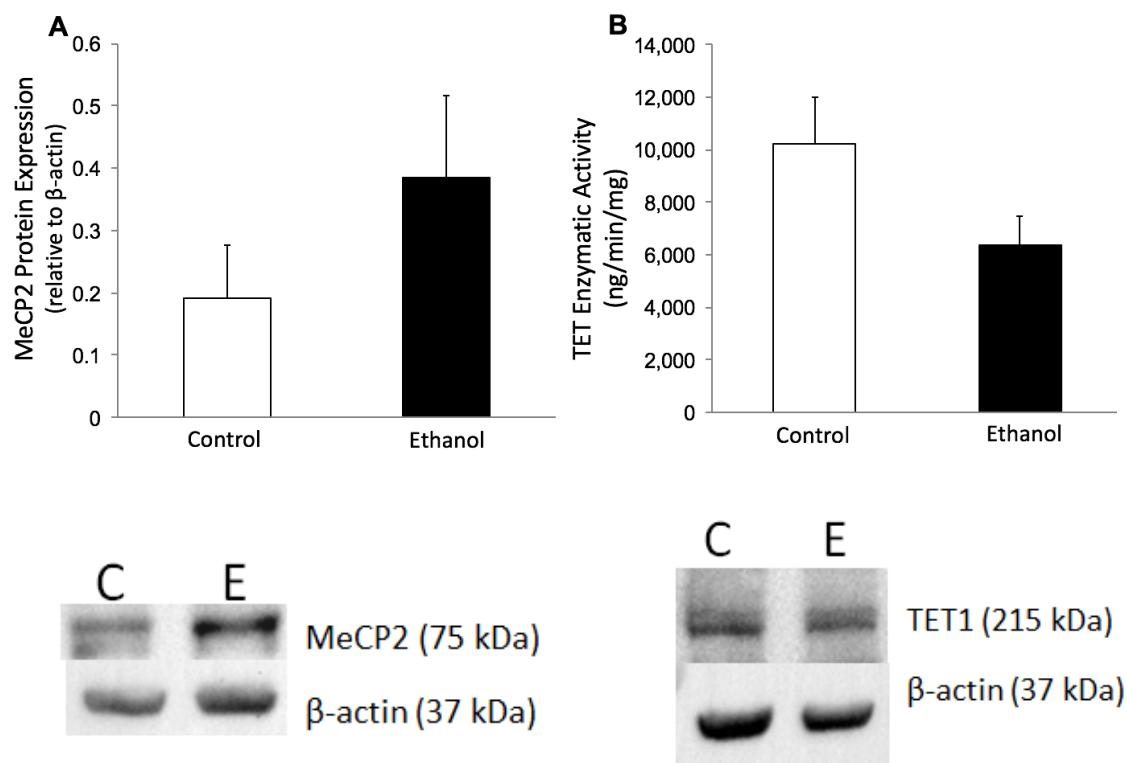


Figure 1. Protein expression and activity in the PND 74 male hypothalamus of epigenetic modifiers. A) MeCP2 protein expression is slightly higher in EtOH-exposed males compared to controls. Two-sample t-test, n=5/group, p=0.255. B) Activity of TET enzymes is slightly decreased in EtOH-treated males. Two-sample t-test, n= 5/group, p=0.117. Bottom panels - Representative Western Blots showing MeCP2 protein slightly increased and TET1 protein level unchanged in the hypothalamus of EtOH-treated males.

In order to determine the relationship between EtOH and TET activity, we correlated the BAC of animals 1 hour after EtOH exposure with the TET activity in the hypothalamus at the same time. We found that TET activity was negatively correlated with BAC, such that higher concentrations of EtOH in the blood were associated with lower TET activity (Fig 2). We also measured the availability of SAM in the testes of these males and found a reduction in SAM/SAH ratio in EtOH-treated animals compared to control counterparts (Fig 3). This reduction

in SAM availability suggests a decrease in methylation in the sperm of these males, which could result in altered epigenetic patterning in future offspring.

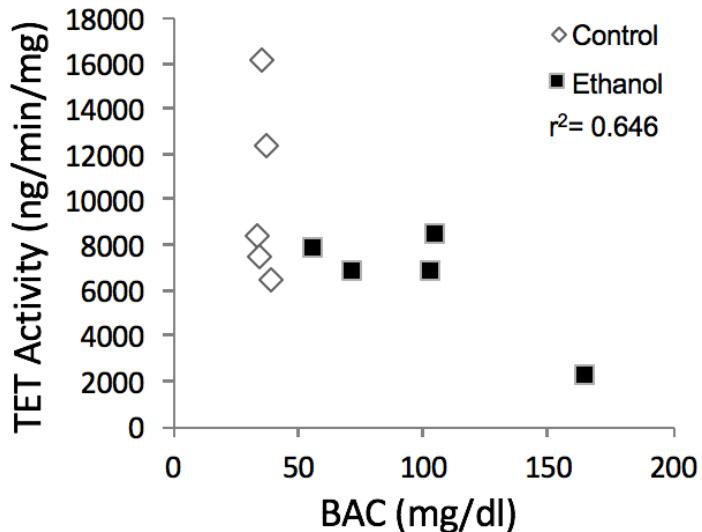


Figure 2. TET activity in the hypothalamus decreases with increasing BAC. Adolescent males showed a strong relationship between hypothalamic TET activity and BAC 1 hour following last EtOH dose. Pearson correlation, n=5 per group, $r^2= 0.646$

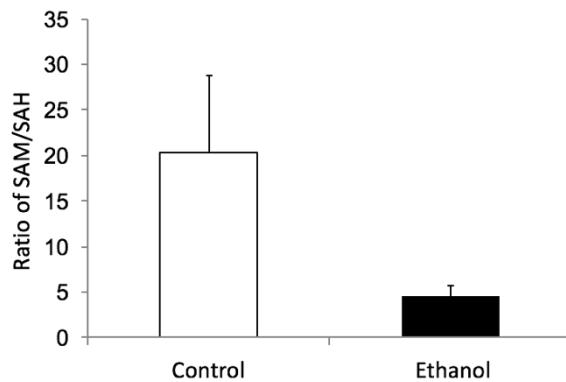


Figure 3. Alcohol exposure decreases the availability of the methyl group donor, SAM, in the adult male testes following adolescent binge. The ratio of SAM/SAH was slightly lower in binge EtOH-exposed male testes than in vehicle treated counterparts 1h following last EtOH dose. Two-sample t-test, n=7-10/group, p=0.097.

We used an *in vitro* model to investigate the direct effect of EtOH on methylation/demethylation machinery. We found that repeated EtOH exposure caused a

decrease in TET and DNMT enzymatic activity following Binge in a Dish. Interestingly, a single EtOH exposure was insufficient to cause changes in DNMT activity. These results are preliminary as they represent averages of just two experiments, however, they suggest that repeated EtOH exposures are required for epigenetic changes to occur.

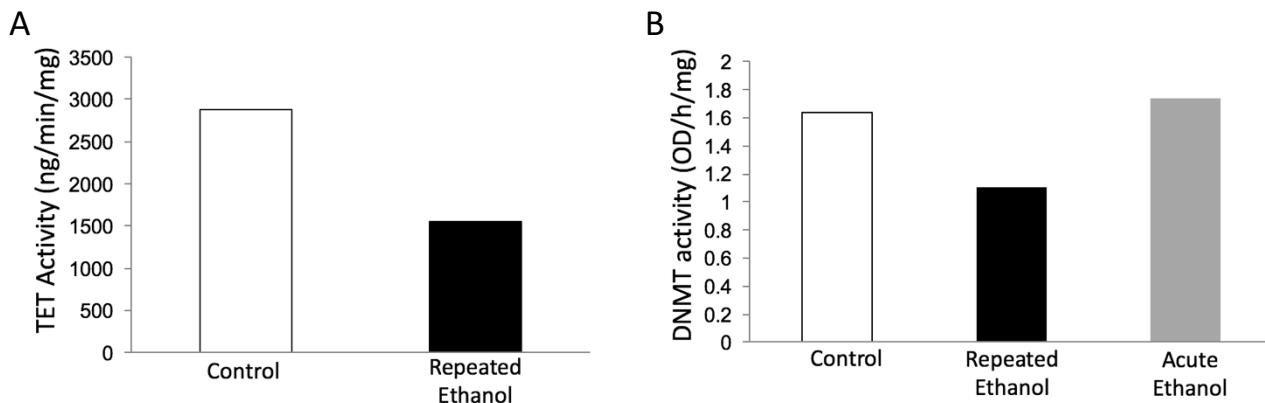


Figure 4. Enzymatic activity of TET and DNMT in Binge in a Dish. A) TET activity was decreased in vitro following 3 days of EtOH treatment. B) DNMT activity was also reduced by repeated EtOH treatments. A single EtOH dose before harvesting cells, however, did not alter DNMT activity. n= 2 per group

In addition, we measured expression of AVP and ApoE mRNA in our *in vitro* model to characterize the effect of our Binge in a Dish system on genes which we have previously found to be differentially expressed following EtOH exposure *in vivo*. We found an increase in AVP expression after 3 days of EtOH treatment, which was in agreement with our animal exposure paradigm (Fig 5a). ApoE expression was not different between our control- and EtOH-treated cells, suggesting that Binge in a Dish may not be suitable for studying the impacts of EtOH on lipid homeostasis (Fig 5b). This is not unexpected, as our data on EtOH and lipids seems to more heavily implicate astrocytes, and this *in vitro* system uses a neuronal cell line.

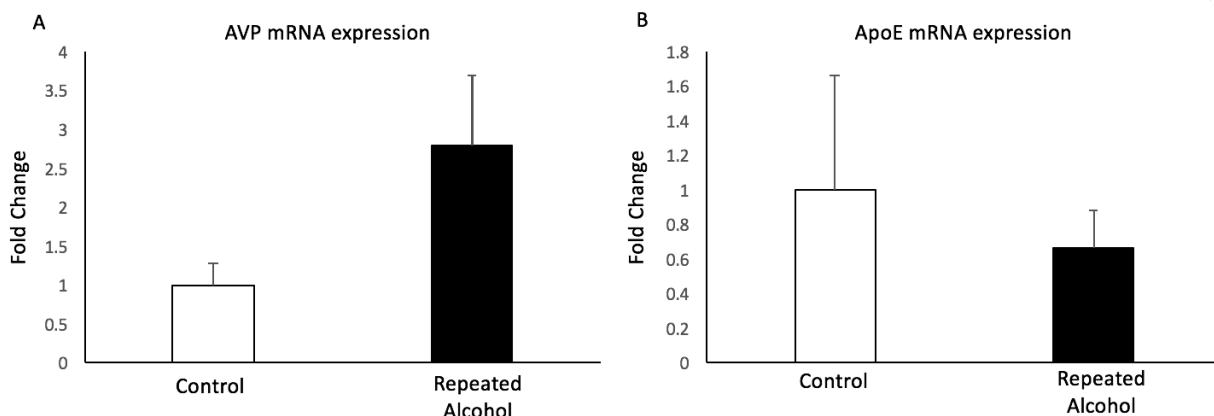


Figure 5. Binge in a Dish reproduces *in vivo* phenotype. A) AVP mRNA expression was slightly increased in cells treated with EtOH. Two-sample t-test, n= 3 per group, p=0.12 B) ApoE mRNA expression was not altered in EtOH-treated cells. Two-sample t-test, n=3 per group, p=0.66.

Taken together, these results suggest that epigenetic mechanisms may be altered in the adolescent hypothalamus following repeated EtOH treatments. We found a slight increase in MeCP2 protein expression, along with a strong reduction in TET activity with increasing BAC levels. Additionally, our *in vitro* model suggested that alterations in TET activity were reliant on repeated EtOH doses, as opposed to a single acute dose or the presence of EtOH at the time of activity measurement. This represents a potential mechanism for long-term changes in gene expression of the adolescent brain after alcohol exposure. Repeated binge intoxication may lead to decreased TET activity, which in turn increases DNA methylation, providing more binding sites throughout the genome for MeCP2. This pathway may underlie the long-term consequences of binge EtOH exposure that we have previously measured. In addition, the reduction in SAM availability in the testes of these adolescent males may alter the epigenetic patterning in sperm, serving as a potential mechanism for the altered DNA methylation patterns we observed in offspring whose father had been exposed to EtOH.

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VITA

The author, AnnaDorothea (Andie) Asimes, was born in Cleveland, Ohio on May 4, 1991 to Dean and Alexandra Asimes. She attended Kenyon College in Gambier, Ohio where she earned a Bachelor's of Arts in Neuroscience in May 2013. After graduation, Andie matriculated into the Loyola University Chicago Integrated Program in Biomedical Sciences, and began her graduate education in the Neuroscience Program under the mentorship of Dr. Toni Pak.

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After completion of her graduate studies, Andie will continue to do research under the mentorship of Dr. Toni Pak as a postdoctoral fellow, and begin teaching in undergraduate neuroscience programs. She will continue living in the Chicago area with her husband, Arry Lazaridis, and their adorable dog, Aero.

