EPIGENETIC EFFECTS OF FETAL ALCOHOL EXPOSURE ON HYPOTHALAMIC PROOPiomelanocortin GENE

by

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Dipak Sarkar, PhD

and approved by

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Hypothalamic POMC neurons, one of the major regulators of the HPA axis, immune functions, and energy homeostasis, are vulnerable to the adverse effects of fetal alcohol exposure (FAE) exhibiting a significant decrease in POMC gene expression and functions in the arcuate area of the hypothalamus of adult offspring. This permanent deficit in gene expression could be caused by epigenetic mechanisms such as histone modifications and DNA methylation induced by alcohol exposure during critical period of development. We found that FAE decreased significantly the protein and mRNA levels of histone-modifying enzymes that methylate H3K4me2,3 (Set7/9), acetylate H3K9 (CBP) or phosphorylate H3S10. These are activation marks that correlate with gene expression. FAE significantly increased the protein levels and gene expression of G9a and Setdb1 that methylate the repressive mark H3K9me2 in β-endorphin-producing POMC neurons of adult offspring. These changes were associated with increased levels of the DNA-methyltransferase Dnmt1 and the methyl-CpG-binding protein 2 MeCP2 but not Dnmt3a. Microarray analysis confirmed that alcohol exposure modulated the gene expression profile of the epigenetic machinery in LCM-captured POMC neurons. ChIP assay revealed a significant reduction in the activation mark H3K4me3 along Exon 3 of POMC gene in alcohol-exposed rats associated with no change in the repressive mark H3K9me2 in Exon 3 and promoter
region of \textit{POMC} gene. We then examined whether gestational choline supplementation, a major methyl donor, could mitigate alcohol adverse effects on POMC neurons. Gestational choline normalized in alcohol-exposed rats the methylation of H3K4 and H3K9 with no significant effect on other histone marks such as acetylated H3K9 or phosphorylated H3S10. Similarly, gestational choline normalized the protein levels and gene expression of histone-modifying and DNA-methylating enzymes in POMC neurons. This data correlated with normalization of \textit{POMC} gene methylation, \textit{POMC} gene expression and β-EP peptide production. In conclusion, these studies demonstrate that FAE induces long-lasting epigenetic modifications of \textit{POMC} gene in the hypothalamus by altering histone marks and methylation state along \textit{POMC} gene. The hypermethylation state of \textit{POMC} gene might be a cause for induction of lower β-endorphin activity and its inhibitory regulation of stress axis function in the adult offspring.
DEDICATION

I dedicate this work to my husband Ghassan, for his consistent help and support throughout my professional career and to my kids, Lynne and Omar, my greatest accomplishments and the joy of my life.

I also dedicate this work to my beloved family, especially my mother Leila, for their support throughout my journey.
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AD</td>
<td>Ad-libitum</td>
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<tr>
<td>AF</td>
<td>Alcohol-fed</td>
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<tr>
<td>Aza</td>
<td>5'-Aza-2'-deoxycytidine</td>
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<td>ADH</td>
<td>Alcohol Dehydrogenase</td>
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<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
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<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
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<tr>
<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
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<tr>
<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation Assay</td>
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<tr>
<td>CHD</td>
<td>Chromohomeodomain</td>
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<tr>
<td>CRH</td>
<td>Corticotropin-Releasing-Hormone</td>
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<tr>
<td>CBP</td>
<td>CREB-Binding Proteins</td>
</tr>
<tr>
<td>Dnmt</td>
<td>DNA Methyltransferase</td>
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<tr>
<td>DOR</td>
<td>Delta opioid receptor</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EP</td>
<td>Endorphin</td>
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<tr>
<td>ETOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FAE</td>
<td>Fetal Alcohol Exposure</td>
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<tr>
<td>FAS</td>
<td>Fetal Alcohol Syndrome</td>
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<tr>
<td>FASD</td>
<td>Fetal Alcohol Spectrum Disorder</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GD</td>
<td>Gestational day</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HDM</td>
<td>Histone demethylase</td>
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<tr>
<td>5-hmC</td>
<td>5-hydroxymethylcytosine</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
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<tr>
<td>HP1</td>
<td>Heterochromatin protein 1</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalmic-Pituitary-Adrenal</td>
</tr>
<tr>
<td>ING</td>
<td>Inhibitor of growth protein 2</td>
</tr>
<tr>
<td>KAT</td>
<td>Lysine acetyltransferase</td>
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<tr>
<td>KMT</td>
<td>Lysine methyltransferase</td>
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<tr>
<td>KOR</td>
<td>Kappa opioid receptor</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser Capture Microdissection</td>
</tr>
<tr>
<td>mPFC</td>
<td>Medial prefrontal cortex</td>
</tr>
<tr>
<td>MBD</td>
<td>Methyl Binding Protein</td>
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<tr>
<td>5-mC</td>
<td>5-methylcytosine</td>
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<tr>
<td>MeCP2</td>
<td>Methyl CpG binding protein</td>
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<td>MOR</td>
<td>Mu opioid receptor</td>
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<tr>
<td>MCR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid</td>
</tr>
<tr>
<td>MSH</td>
<td>Melanocyte-Stimulating-Hormone</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methyltetrahydrofolate reductase</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neuronal Cell Adhesion Molecule</td>
</tr>
<tr>
<td>ncRNA</td>
<td>noncoding RNA</td>
</tr>
<tr>
<td>NURF</td>
<td>Nucleosome remodeling factor</td>
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ORFs Open Reading Frames
PC Phosphotidylcholine
PC1/2 Pro-hormone Convertase 1 or 2
PEA Phosphatidylethanolamine
PcG Polycomb Group of proteins
PCNA Proliferating cell nuclear antigen
PD Postnatal day
PF Pair-fed
PFC Prefrontal Cortex
PHD Plant homeodomain
PMSF Phenylmethylsulfonyl fluoride
POMC Proopiomelanocortin
PTMs Posttranslational modifications
PVN Paraventricular nucleus
RER Rough Endoplasmic Reticulum
ROS Reactive oxygen species
SAM S-Adenosyl-Methionine
SNP Single nucleotide polymorphism
Sp1 Specific protein 1
TFs Transcription factors
THF Tetrahydrofolate
TRD Transcriptional Repressor Domain
TSA Trichostatin
TSS Transcription Start site
VTA Ventral Tegmental Area
CHAPTER 1

1 Chapter 1: Review of the literature

1.1 Introduction

Alcohol drinking during pregnancy is an intractable health problem worldwide and a leading cause of mental retardation and other neurological disorders in the United States (Sokol et al., 2003). Center for Disease Control and Prevention (CDC) reported that 1 in 8 women in the US drank alcohol during pregnancy which is a major risk factor for embryonic development especially fetal brain development. Fetal alcohol syndrome (FAS) (Jones & Smith, 1973) and fetal alcohol spectrum disorder (FASD) (Sokol et al., 2003) are the most recognized outcomes of fetal alcohol exposure (FAE) on the brain during critical period of development. The overall prevalence of FAS in the United States is estimated to be 0.5 to 2 per 1,000 births. The prevalence of FASD is three times as frequent as FAS and it is estimated to be 1 per 100 births (May & Gossache, 2001 & Sokol et al., 2003). FAE has wide range of effects specifically on the child’s central nervous system with wide range of long-lasting phenotypes such as morphological, behavioral and neurological (Mattson et al., 2001; Goodlett & Horn, 2001 & Guerri et al., 2009).

The long-term effects of prenatal alcohol exposure are wide and more complex than previously thought. Hypothalamic-pituitary-adrenal (HPA) axis is particularly vulnerable to the effects of alcohol exposure where its ramifications could persist throughout life (Weinberg, 1988; Rivier, 1996 & Weinberg et al., 2008,). For example, children who are exposed to alcohol during fetal life often show behavioral and physiological changes in adulthood such as depression, anxiety, hyperactivity, attention deficit and reduced ability to cope with stressful situations (Famy et al., 1998; Riley & McGee 2005; Haley et al., 2006; Hellmans et al., 2008 & Weinberg et al., 2008).
Behavioral deficits, hyperresponses to stress, altered metabolic functions and aberrant immune function are also observed endophenotypes in rodents which are exposed to alcohol during embryonic development (Rivier et al., 1988; Weinberg, 1988; Berman & Hannigan, 2000; Ting & Lautt, 2006 & Boyadjieva et al., 2009). Proopiomelanocortin (POMC) neurons of the arcuate area (ARC) of the hypothalamus are one of the major regulators of the HPA axis, immune functions and energy homeostasis (Sarkar, 1996; Pritchard et al., 2002; Raffin-sanson et al., 2003; De Souza et al., 2005; Arjona et al., 2006 & Sarkar et al., 2007). At the organismal or system level, the dysregulation of the POMC system could have diffuse effects on many physiological processes and could increase the risk of many diseases. At the molecular and cellular levels, prenatal alcohol exposure causes a decrease in POMC gene expression and the death of β-endorphin-producing POMC neurons of the hypothalamus in rats (Chen et al., 2006; Sarkar et al., 2007 & Kuhn & Sarkar, 2008). However, the causes of this deficit in POMC gene expression and its derived peptide, β-endorphin (β-EP), in POMC neurons are not very well delineated.

There is compelling evidence that acute and chronic exposure to drugs or toxicants during embryonic development alter histone marks and/or DNA methylation of specific genes in different areas of the brain thus modulating gene expression and resulting in long-lasting adverse effects on phenotypes (Kumar et al., 2005; Jirtle & Skinner, 2007; Pandey et al., 2008; Hellmans et al., 2008; Novikova et al., 2008; Hunter et al., 2009 & Maze et al., 2010). Previous studies suggest that epigenetic mechanisms are involved in mediating the effects of gene-environment interaction on adult behavioral patterns. Example of these long-lasting effects is the profound impact of the excessive or deficient maternal care on HPA axis functioning of adult offspring and on the development of individual differences in response to stress in adulthood (Liu et al., 1997; Weaver et al., 2004 & Meany & Szyf, 2005). FAE could alter the expression of a network of genes in the brain as well as POMC gene expression in hypothalamic POMC neurons and this
change in gene expression could be mediated by epigenetic mechanisms. The epigenetic mechanisms of FAE in POMC neurons have never been explored before nor has the mechanism of POMC gene expression regulation been elucidated. We hypothesized that FAE causes decrease in the availability of folate and the methyl-donor, S-adenosylmethionine (SAM), resulting in hypomethylation state in POMC neurons. As a compensatory mechanism and to maintain homeostasis in term of methylation requirements during embryonic development, the expression as well as the activity of enzymes that causes histone modifications and DNA methylation is upregulated in the arcuate area (ARC) of the hypothalamus. This upregulation persists in the adult offspring and results in POMC gene promoter hypermethylation. This hypermethylation decreases POMC gene expression and production of one of its peptide, β-EP, causing dysregulation of the stress axis response in the adult offspring.

Our lab demonstrated that FAE causes hypermethylation of four CpG sites at positions -62, -216, -224 and -238 in POMC gene promoter. This hypermethylation state of the upstream CpG island correlates with a decrease in POMC gene expression and functions (Govorko et al., 2011). This thesis work particularly focuses on understanding the role of the components of the epigenetic machinery that regulate histone modification and DNA methylation in β-EP-producing POMC neurons and explore how these components could alter POMC gene expression and β-EP peptide production upon alcohol exposure.

1.2 Fetal Alcohol Exposure
Alcohol exhibits wide range of conspicuous effects on the brain. These effects are more prominent during fetal life and could be long-lasting (Izumi et al., 2005; Goodlett et al., 2005; Harper 2007 & Haycock, 2009). These effects are not uniform and are not manifested the same in all individuals but are diffuse with some areas of the brain are particularly more vulnerable than others (Goodlett & Horn, 2001; Guerri et al., 2009 & Spanagel, 2009). Brain imaging
studies revealed that the effects of prenatal alcohol on the brain are not global but specific. For example, there is consistent overall reduction in brain size in FASD individuals especially in the frontal, temporal and parietal lobes as well as specific morphological changes in different areas of the brain (Clarren et al., 1978 & Lebel et al., 2008). Examples of these regional morphological changes are size reduction in basal ganglia (Mattson et al., 1996a), corpus callosum (Riley et al., 1995 & Sowell et al., 2001a), cerebellum (Sowell et al., 1996 & Archibald et al., 2001), hippocampus (Berman & Hannigan 2000), hypothalamus (Harper, 2009) and significant cortical thickness abnormalities (Yang et al., 2011 & Zhou et al., 2011). Abnormalities in the density of white and gray matter in specific brain regions were also reported in FASD individuals (Archibald et al., 2001 & Sowell et al., 2002b). These observed anomalies adversely affect essential functions such as neuronal connectivity, coordination, mental abilities, behavior, learning and memory.

FAS and FASD both describe the adverse effects of FAE on brain during critical period of embryonic development. The severity of phenotypes between the two depends on the amount, duration and frequency of exposure to alcohol during critical periods of brain development. FAS, first delineated in 1973, results from exposure to large amount of alcohol and shows severe phenotypes such as facial dysmorphism, growth deficiency and severe brain damage (Jones et al., 1973). FASD or Alcohol-Related Neurodevelopmental Disorder (ARND) is an umbrella term that includes FAS. Unlike FAS, FASD results from short exposure to alcohol and exhibits in exposed children wide spectrum of neurological and behavioral changes later in adulthood such as depression, anxiety, hyperactivity, attention deficit, alcohol addiction or reduced ability to cope with different types of stress (Schneider et al., 2002; Del Arbol et al., 2007 & Kelly et al., 2009). FAS and FASD share common phenotypes such cognitive and neurological deficits (Harper & Matsumoto, 2005; Harper, 2007 & Guerri et al., 2009).
The vulnerability to alcohol exposure usually occurs during the first and second trimester of pregnancy which correlate with the timing of essential developmental processes such as neurogenesis, cell migration, cell adhesion and synaptogenesis (Riley & McGee; 2005 & Guerri et al., 2009). Excessive maternal consumption of alcohol during these two periods in humans causes severe phenotypes that we see in FAS children. Exposure to alcohol during the third trimester of pregnancy targets particularly the cerebellum, the hippocampus and the prefrontal cortex and causes other abnormalities such as cognitive and intellectual malfunctions (Riley & McGee, 2005).

1.3 Epigenetic Insights into POMC System Dysregulation

POMC neurons play pivotal role in the regulation of the stress axis besides other functions (Pritchard et al., 2002 & Raffin-sanson et al., 2003). Dysregulation of the POMC system and POMC gene expression could incite physiological abnormalities in terms of production of important biological peptides such as adrenocorticotropin hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH) or β-endorphin (β-EP) which are all derived from POMC gene and have important physiological functions (Raffin-sanson et al., 2003 & Millington, 2007). We focused in this study on understanding the effects of FAE on POMC gene expression and β-EP peptide production and the possible ramifications of its dysfunction on regulation of the stress axis.

Epigenetic alterations emerged lately as major players in a variety of neurological disorders including stress, depression, schizophrenia, bipolar disorder, Rett syndrome, synaptic plasticity and memory formation and alcohol addiction (Jaenisch & Bird, 2003; Eger et al., 2004; Hsieh & Gage, 2005; Fyffe et al., 2008; Sharma et al., 2008; Bokhoven & Kramer, 2010 & Maze & Nestler, 2011). Deciphering the enigma underlying epigenetic mechanisms in these neurological diseases, e.g. stress regulation, could explain the short or long-lasting changes that
are observed in behavior of many individuals and would identify potential therapeutic targets and tangible solutions for treatment of diseases. Dysregulation of the HPA axis or stress axis has many negative effects at all system levels especially at the central nervous system (CNS) level (Lightman, 2008).

The wide and continuum spectrum of behavioral and neuropsychological abnormalities that are seen in FASD individuals and the increase in susceptibility of exposed children to a variety of diseases in adulthood are not only triggered by genetic factors, as was originally thought, but also by epigenetic factors such as histone modifications and DNA methylation (Haycock, 2009 & Ramsay, 2010). Thus, exposure to alcohol during critical periods of development such as prenatal and/or early postnatal periods could alter the chromatin architecture of POMC gene, modulate the whole genome landscape and alter negatively and specifically hypothalamic POMC gene expression and β-EP peptide production.

The role of DNA methylation in neurological disorders was highlighted with the discovery of the methyl-CpG-binding protein 2 (MeCP2) mutation as the causative agent of Rett syndrome (Amir et al., 1999). Since then, many neuronal genes were identified as MeCP2 target and DNA methylation was implicated as a possible confounder of disease (Martinowich et al., 2003 & Chahrour et al., 2008). Similarly, histone marks underlie neuronal plasticity. For example, histone acetylation and methylation were implicated in long-term memory formation (Levenson et al., 2004), behavior (Kumar et al., 2005 & Maze et al., 2010) and alcoholism (Pal-Bhadra et al., 2007 & Pandey et al., 2008). The epigenetic insights into the regulation of POMC gene expression upon alcohol exposure such as possible alterations in histone marks and/or DNA methylation would provide essential information into the molecular as well as physiological underpinnings of FAE and its ramifications on POMC system and on the HPA axis functioning in the adult stage.
1.4 Mechanisms of Alcohol Actions

There are multiple molecular mechanisms or targets for alcohol actions that explain the wide range of phenotypes that we see in children with FAS or FASD. These molecular mechanisms are not very well understood but are most likely involved. Alcohol is conspicuous in actions. It interacts with tissues in a variety of ways inducing at the cellular level alterations of downstream signaling mechanisms resulting in short-term or long-term adverse effects on phenotypes in exposed individuals.

Alcohol exposure during embryonic development negatively alters many physiological processes. For example, it causes reduction of retinoic acid biosynthesis resulting in embryonic malformations (Ribes et al., 2006; Chen et al., 2009 & Leibovich et al., 2009), alteration in the expression and localization of neuronal cell adhesion molecules (NCAMs) (Minana et al., 2000) and modulation in the balance between excitatory or inhibitory neurotransmitters at synapses such as glutamate, serotonin, dopamine, catecholamines, GABA and glycine (Rudeen & Weinberg, 1993; Valenzuela, 1997; Goodlett & Horn, 2001 & Clapp et al., 2008). Alcohol could also cause neuronal death by oxidative stress due to an increase of reactive oxygen species (ROS) (Bredensen 1996 a, b), as well as upregulation of cell-death genes (Ikonomidou et al., 2000; Goodlett et al., 2005; Chen et al., 2006 & Sarkar et al., 2007). In the context of membrane interactions, alcohol affects the bilayer substantially and in complex ways by interacting directly or indirectly with membrane proteins and membrane lipids (Klemm, 1998 & D’Azzo et al., 2006). The secondary effects of these primary interactions could be on other biological molecules such as receptors or channels. The outcome of these interactions could be immense due to modulation of downstream signaling pathways that are interconnecting in complex ways and affecting neuronal functions in different ways.
There are other physiological processes that are adversely affected by fetal alcohol exposure (FAE). For example, alcohol affects negatively glial cells that guide neurons to their appropriate destinations thus affecting neuronal migration (Goodlett & Horn, 2001 & Guerri et al., 2001). The adverse long-lasting effects of prenatal alcohol exposure on the functioning of the HPA axis and on phenotype were demonstrated (Rivier, 1996; Sarkar et al., 2007 & Weinberg et al., 2008). The role of alcohol in inducing epigenetic modifications such as DNA methylation (Garro et al., 1991 & Haycock, 2009), histone modifications (Shukla et al., 2008; Haycock, 2009 & Wang, 2010) and small noncoding RNAs (Haycock, 2009; Wang et al., 2009 & Miranda et al., 2010) is emerging but not very well elucidated.

### 1.5 Alcohol Metabolism

Alcohol has adverse and conspicuous effects on many system levels and affects negatively the functions of many organs mainly the liver, the pancreas and the brain and has no identified specific receptor (Spanagel, 2009). At the central nervous system level (CNS), it induces neurochemical changes resulting in a wide range of phenotypes. These observed changes are due to the primary accumulated effects and incremental damages caused by alcohol exposure itself or by its metabolites or it could be due to the secondary effects of alcohol actions. Alcohol metabolism is mediated by the enzymatic activity of alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), cytochrome P4502E1 (CYP2E1) and catalase (CAT) (Hipolito et al., 2007).

Humans have seven different ADH genes located on chromosome 4 (ADH1A, ADH1B, ADH1C, ADH4, ADH5, ADH6, and ADH7) and two main ALDH genes located respectively on chromosome 9 (ALDH1) and chromosome 12 (ALDH2). ADH1B and ALDH2 are candidate genes related to the increase risk for alcoholism (Kimura & Higuchi, 2011). The bulk of alcohol is metabolized in the liver into acetaldehyde by ADH. Acetaldehyde, a toxic byproduct of
alcohol, is converted in the liver and in the blood brain barrier (BBB) to acetate by ALDH then converted to CO2 and H2O via the citric acid cycle. This could suggest that alcohol’s insult on the brain is unlikely to be caused primarily by acetaldehyde (Queterment et al., 2005; Harper, 2007 & Hipolito et al., 2007). In the brain, alcohol is metabolized by three major enzymes CAT, CYP2E1, ADH1 and ADH4. CAT is the key enzyme of brain alcohol oxidation, contributing to 60% to overall alcohol oxidation. CYP2E1 contributes to 20% of alcohol oxidation and ADH1 and ADH4 contribute to additional 20% (Dietrich et al., 2006 & Hipolito et al., 2007). Although the bulk of alcohol oxidation in the brain is mediated by CAT, CYP2E1 has also gained an important role. Alcohol metabolism by CYP2E1 leads to the formation of ROS that causes, if accumulated in excess amount, severe damage to cellular components such as DNA, lipids and proteins (Seitz et al., 2006).

There is no doubt that genetic factors and epigenetic factors contribute to the long-lasting adverse effects of FAE and to the increase in individual’s susceptibility to diseases. There are individual variabilities in alcohol metabolism and elimination as well as variabilities in individual susceptibilities to alcohol exposure. The presence of single nucleotide polymorphisms (SNPs) in alcohol-metabolizing enzymes is a determinant and contributor to individual’s susceptibility to alcoholism (Quetermont, 2004). These SNPs affect enzymes’s functions by altering their activity or their expression. Genome-Wide Association Studies identified SNPs in a set of genes that are associated with individual’s vulnerability to alcoholism such as ADH, ALDH, dopamine, serotonin, GABA receptors, endogenous opioids and opioid receptors (Kimura & Higuchi, 2011). In a human study, Edenberg et al. (2006) identified 12 SNPs in ADH4 gene located on chromosome 4. These identified SNPs are closely associated with alcohol dependence and increase individual’s risk to alcoholism. Moreover, polymorphism in ALDH2 gene located on chromosome 12 is also linked to alcohol-seeking behaviors in humans (Higuchi et al., 2004). In addition to genetic factors, exposure to environmental factors such as drugs or toxicants lately
emerged as major confounders in inducing epigenetic changes and demarcating individual’s susceptibility to many diseases later in life including stress, altered behavior and alcohol addiction (Jirtle & Skinner, 2007 & Govorko et al., 2011).

1.6 Proopiomelanocortin Neurons

POMC neurons are particularly vulnerable to alcohol exposure. Prenatal alcohol causes POMC neuronal death and malfunctioning of the stress axis response in alcohol-exposed rats (Sarkar et al., 2007 & Boyadjieva et al., 2009). The physiological role and significance of POMC system in feeding circuitry, stress axis regulation and immune modulation are very well documented (Pritchard et al., 2002; Luger et al., 2003; Raffin-sanson et al., 2003; Millington, 2007 & Sarkar et al., 2007). Thus, malfunctioning of this system could have wide and adverse consequences at the organismal level.

POMC cells are localized in the anterior and intermediate lobes of the pituitary, in the ARC of the hypothalamus and to a lesser extent in the nucleus tractus solitaries (NTS) of the brain stem. In the brain, POMC neurons are primarily located in the ARC and they project to various areas in the brain such as the ventral tegmental area (VTA), nucleus accumbens (Nac), amygdala, hippocampus, frontal cortex and periaqueductal gray. They send descending projections to the brain stem via the VTA and the periaqueductal gray and dorsomedial tegmentum (Gianoulakis, 2004 & Marinelli et al., 2004). De Souza et al. (2005) reported that 3,000 POMC-expressing neurons exist in the ARC of the mouse hypothalamus.

1.6.1 POMC gene structure, expression and function

POMC gene structure

Vulnerability of POMC gene expression and functions to alcohol exposure is intriguing and
POMC gene requires a thorough understanding of its structure. POMC gene architecture has been conserved among species implicating its evolutionary functional significance (De Souza et al., 2005). This gene was initially cloned in 1981. It codes for a 35KDa versatile protein precursor, POMC peptide, which is proteolytically processed in a tissue-specific manner to give rise to a wide variety of biological peptides. Specifically, this gene gives rise to two classes of peptides: the melanocortins and the endorphins. The Melanocortin system consists of α, β and γ-melanocyte-stimulating hormone and ACTH. In the hypothalamus, the processing of POMC peptide yields primarily α-MSH and a negligible amount of ACTH. The other class of peptide derived from POMC gene yields primarily β-endorphin (β-EP) (Eberwine & Roberts, 1983). This later exerts an inhibitory effect at the level of the paraventricular nucleus (PVN) to regulate the stress axis (Plotsky et al., 1986; Jessop, 1999 & Sarkar et al., 2007).

POMC gene has unique genomic organization and complex mode of spatial regulation. POMC transcript is 7665 bp in size and is located on chromosome 2 in humans, on chromosome 6 in rats and on chromosome 12 in mice. Structurally, it consists of three exons (Exon 1, 2 & 3), two large intervening intronic regions (Intron 1 & 2), 5’ flanking region and a 3’ untranslated region (3’UTR) (Fig. 1) (Eberwine & Roberts, 1983; Newell-price, 2003 & Raffin-sanson et al., 2003). Exon 1 (87 bp) is untranslated and acts as a leader sequence. Exon 2 (152 bp) codes for the N-terminal region and the signal peptide sequence which is required for POMC transport during its synthesis across the RER and its translocation to the membrane. Exon 3 (833 bp), “the protein-coding sequence”, codes for the majority of the translated mRNA and gives rise to a number of peptides with diverse biological functions such as ACTH, α-MSH and β-EP in the hypothalamus (Newell-Price, 2003). There is high homology and conservation in Exon 2 sequence among species. Exon 3 sequence conservation depends on the type of hormone produced from POMC. For example, ACTH and endorphin regions are the most highly conserved regions of Exon 3 of
POMC gene and have greater than 90% nucleic acid homology among species (Eberwine & Roberts, 1983).

Specific regulatory sequences that play a role in POMC gene expression were identified in the promoter region of POMC gene as well as in the 5’ upstream region. The transcriptional control sequences TATATAA and CAAT are present directly 5’ to POMC Exon 1 and act as binding sites for TFs essential for transcriptional initiation and activation by RNA Polymerase II (Cochet et al., 1982). Interestingly, POMC gene contains two CpG islands, an upstream 5’CpG island located in the promoter region and another 3’CpG island located 5 Kb downstream in Exon 3 (Gardiner-Garden & Frommer, 1994). The 5’ CpG island extends more than 400 bp upstream and 800 bp downstream of Exon 1 in human POMC gene. The methylation of this upstream 5’ CpG island plays a critical role in regulation of POMC gene expression (Newell-Price, 2003). The downstream 3’ CpG island is located in Exon 3 next to a transcription start site (TSS) that could generate short RNA transcripts with a critical role in posttranscriptional regulation of POMC gene (Gardiner-Garden & Frommer, 1994).

Figure 1 Human POMC gene structure

Schematic representation of the human POMC gene. Translated regions in colour: green, the 26 amino acid signal sequence; blue, remainder of POMC; red represents the region corresponding to ACTH (Adopted from Newell-Price, 2003).
De Souza et al. (2005) identified along the *POMC* gene two sequences, termed enhancers nPE1 (600 bp) and nPE2 (150 bp) that are found in all mammals and located 10 to 12 Kb upstream of the *POMC* gene TSS (Fig. 2A). These identified sequences are necessary for *POMC* gene expression in the hypothalamus. They serve as docking sites for essential transcription factors (TFs) (Jenks, 2009). For example, the 3’ end of the nPE1 has six binding sites and nPE2 has three binding sites for TFs (Fig. 2B).

**Figure 2 POMC gene structure**

(A) The regions with a high degree of sequence identity, analyzed by De Souza et al. 2005 and designated as neuronal POMC enhancer 1 (nPE1) and 2 (nPE2), are indicated as are POMC exons 2 and 3 (note lack of sequence identity in exon 1)’ (B) Details of the distal enhancer region of the *POMC* gene responsible for expression of POMC in hypothalamic neurons showing some putative responsive elements, as revealed in the study of De Souza et al. 2005 Abbreviations: COUP; chicken ovoalbumin upstream promoter; ERRα, estrogen-related receptor alpha; STAT3, signal transducer and activator of transcription 3; Brn, brain transcription factor; NERF, new Ets-related factor; ELF-2, E7 4-like factor (Adopted from Jenks, 2009).

Among the sites present in nPE1 are two-conserved cAMP-responsive element binding sites (CREB-like), and signal transducer and activator of transcription 3 response element (STAT3-RE) which is located upstream of the TATAA box. STAT3 is a downstream effector of leptin that regulates *POMC* gene expression in the hypothalamus. The other enhancer nPE2 has binding sites for the estrogen-related receptor alpha (ERRα), the homeobox gene NKx6.1 and POU domain gene Brn2. In the context of chromatin, nPE1 and nPE2 could act as recognition sites for transcriptional complexes that regulate chromatin remodeling and *POMC* gene expression. This raises the question of the impact of the spatial regulation of the epigenetic
machinery on POMC gene expression. Interestingly, the distal enhancer region of POMC gene (nPE1 and nPE2) is not conserved among species unlike Exon 2 and Exon 3 (De Souza et al., 2005).

In the context of alcohol exposure, an association between POMC polymorphisms and alcoholism was reported in human subjects with a history of alcoholism and psychiatric disorders. For example, in a German and Swedish population studies, “two-marker haplotype”, rs934778 and rs3769671, in POMC gene locus were associated with alcohol dependence and drinking behavior in women alcoholics. However, this study did not exclude the possibility that the small group of alcoholic women tested could have caused an overestimation of their results. Moreover, this study did not deny the fact that other human studies conducted in a population of European Americans alcoholics did not find any association between alcohol dependence and the haplotype marker rs934778 (Racz et al., 2008). Thus, more comprehensive human studies of alcoholic subjects from representative groups would provide conclusive results. Moreover, future studies should link any common identified SNPs in POMC gene of alcoholics to common specific phenotypes and specific physiological functions. More importantly, the inheritance or the detection of these SNPs in POMC gene of subsequent generations could confirm the association between these polymorphisms and alcohol addiction. Finally, the detection of these SNPs in the region of POMC gene that codes for β-EP could provide an additional proof of the role of β-EP in modulation of alcohol seeking behavior.

**POMC gene expression and POMC peptide processing**

POMC (35 KDa) is a versatile multifunctional precursor protein that is posttranslationally processed in a tissue-specific manner to a number of peptides with unique and diverse biological functions (Raffin-Sanson et al., 2003 & De Souza et al., 2005). Long-term exposure to external factors such as alcohol during critical period of embryonic development might affect POMC gene
at different levels: at the transcriptional, posttranscriptional or translational level. In either case, the outcome would be negative in terms of the number of functional generated transcripts and the number of peptides, such as β-EP, derived from these transcripts.

*POMC* gene is expressed in corticotrophs of the anterior pituitary, melanotrophs of the intermediate lobe, placenta, testes, ovaries, skin, immune cells, NTS of the brain stem and in the ARC of the hypothalamus (Eberwine and Roberts, 1983; Pritchard et al., 2002 & Newell-price, 2003). After its synthesis, POMC peptide is targeted via a specific signal peptide into secretory granules. It is then postranslationally cleaved within these granules, at dibasic amino acid residues such as lysine-arginine, arginine-lysine or lysine-lysine cleavage sites, in a tissue-specific manner by the subtilisin-like superfamily of enzymes, the prohormone convertases PC1 and PC2, into different peptides (Fig. 3) (Pritchard et al., 2002; Raffin-Sanson et al., 2003 & DeSouza et al., 2005). These peptides include ACTH in the corticotrophs of the anterior pituitary and α-MSH in the intermediate lobe of the pituitary (Whitefeld et al., 1982; Eberwine & Roberts, 1983; Pritchard et al., 2002 & De Souza et al., 2005). In the pituitary, PC1 cleaves POMC and generates ACTH as the major end-product. In melanotrophs of the intermediate lobe and in the hypothalamus, POMC is cleaved by PC1 and PC2 into N-terminal peptide (NT), junctional peptide (JP), ACTH and β-Lipotropin (β-LPH). This later gives rise to γ-LPH and β-EP (Raffin-Sanson et al., 2003).

![Figure 3 POMC processing into active peptides by PC1 and PC2](image-url)
Figure 3 is adopted from Raffin-Sanson et al. (2003).

Tissue-specific expression of *POMC* gene is quite complex and includes the coordinated action of a variety of TFs, the presence of specific regulatory elements and DNA methylation status of its promoter (Newell-price, 2003). Tpit, a TF of the T-box family, and the pituitary homeobox 1 TF (Pitx1), are two TFs that activate *POMC* gene transcription in the pituitary gland. Neurogenic differentiation 1 factor (NeuroD1) that belongs to the family of basic-helix-loop-helix TF binds the E-box and regulate *POMC* gene expression in corticotrophs (Jenks, 2009). The factors that regulate *POMC* gene expression in the hypothalamus are not very well known. The two identified enhancers (nPE1 and nPE2) in the 5’ upstream region are required for hypothalamic *POMC* gene expression (Pritchard et al., 2002; De Souza et al., 2005 & Millington 2007).

Despite the lack of CREB-response element (CRE) in *POMC* gene promoter, it has been suggested that CREB influences *POMC* gene expression indirectly by binding to the AP-1 site in Exon 1 leading to activation of gene expression (Boutillier et al., 1998). It has also been reported that the methylation status of the human *POMC* gene promoter “maybe differentially methylated” in expressing and nonexpressing normal and cancerous tissues (Newell-Price et al., 2001). For example, bisulfite sequencing analysis demonstrated that CpG island of POMC gene promoter was methylated in normal non-POMC expressing tissues such as pancreas, spleen, lung and kidney. On the other hand, this CpG island was unmethylated in normal POMC-expressing tissues such as corticotroph cells of the pituitary and in lung cancerous DMS79 cell line transfected with a vector that expresses *POMC* gene (Newell-Price et al., 2001). These findings could suggest that methylation of CpGs in POMC gene promoter correlates with changes in *POMC* gene expression.
1.6.2 POMC neurons, the HPA axis and Alcohol Exposure

Alcohol exposure negatively impacts hypothalamic POMC neurons and the HPA axis. This axis that regulates the stress response has emerged as a main target of the long-lasting prenatal environmental influences such as alcohol exposure (Rivier et al., 1988; Weinberg, 1988; Berman & Hannigan, 2000 & Boyadjieva et al., 2009). Besides the HPA axis, two other adaptive mechanisms are involved in the regulation of stress response in mammals, the sympathetic adrenomedullary system and the limbic system (Cook et al., 2002; Herman et al., 2003 & Kvetnansky et al., 2009). We focused in this study on the effect of alcohol exposure on POMC gene expression, β-EP peptide production and implication on regulation of the HPA axis functioning.

What is the HPA axis and how is it regulated? The HPA axis is a critical endocrine system and a vital regulator of the adaptation of an organism to stress. It includes the hypothalamus, the pituitary and the adrenal glands (Herman et al., 2003 & Lightman, 2008). The hypothalamus has many nuclei with extensive neuronal connections with other brain regions. The paraventricular nucleus (PVN) of the hypothalamus is particularly important in the regulation of the stress axis. This region receives and sends many neuronal projections from and to different areas of the brain. For example, the PVN projects into the median eminence and into the ARC and receives ascending catecholaminergic (locus ceorulus LC noradrenergic neurons and adrenergic neurons) and serotonergic neuronal projections from the brainstem and from noradrenergic and adrenergic neurons of the nucleus tractus solitarius (NTS), descending projections from the limbic region (mainly the hippocampus and amygdala) and from the medial prefrontal cortex (mPFC) (prelimbic cortex and infralimbic cortex) to influence the HPA axis (Kvetnansky et al., 2009). The hippocampal and amygdaloid modulation of the HPA axis is mediated by the Bed nucleus of the stria terminalis (BNST) where the posterior BNST intergrates
inhibitory inputs from the hippocampus while the anterior BNST integrates excitatory inputs from the amygdala (Herman & Cullinan, 1997). In the PVN region of the hypothalamus, corticotropin-releasing hormone (CRH) – producing neurons are considered major mediators of the organismal response to stress. Some of these CRH neurons are GABAergic and exert inhibitory effect on the HPA axis. However, neuronal excitatory mainly glutamatergic projections to the peri-PVN region from limbic areas such as the amygdala or the hippocampus block GABA inhibition mediated by CRH on this axis (Kvetnansky et al., 2009). Moreover, CRH neurons receive neuronal terminals of β-EP-producing POMC neurons which are localized in the arcuate area (Buckingham, 1986 & Jessop, 1999).

Once activated upon stress, the HPA axis triggers corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) release from the PVN region of the hypothalamus through the hypophysial vasculature system to prompt the corticotroph cells of the anterior pituitary to stimulate POMC gene expression and the release of its derived peptide such as ACTH at the level of the pituitary. Elevation of CRH also stimulates POMC gene expression in the arcuate area and the production of its derived peptide β-EP. At the level of the pituitary, elevation of ACTH then activates the adrenal cortex to secrete glucocorticoids (GCs) and mineralocorticoids (MCs) into the peripheral circulation that will exert a negative feedback mechanism at the level of the PVN to inhibit CRH release. GCs and MCs also exert a negative feedback mechanism at the level of the pituitary and the hippocampus (Cook, 2002 & Ulrich & Herman, 2009). Similarly, elevation of β-EP upon stress by β-EP-producing POMC neurons exerts a negative feedback at the level of the PVN to inhibit CRH release from these neurons (Buckingham, 1986; Jessop, 1999; Boyadjieva et al., 2006 & Sarkar et al., 2007). In addition to the activation of the HPA axis upon stress, the activation of the sympathetic adrenomedullary system results in the release of epinephrine (E) and norepinephrine (NE). Many of the neurons of the sympathetic nervous system (SNS) project into the PVN and impact CRH release and the stress response. Under
abnormal circumstances, where stress cannot be controlled, this negative feedback inhibition exerted by β-EP at the level of the PVN will be dysfunctional resulting in elevation of CRH. Similarly, the negative feedback inhibition by GCs and MCs at the level of the PVN, pituitary and the hippocampus becomes dysfunctional with detrimental effects especially that glucocorticoids receptors (GRs) and mineralocorticoids receptors (MCRs) are widely distributed with considerable variation in expression in different tissues (Ulrich & Herman, 2009).

It has been demonstrated that alcohol exposure decreases POMC gene expression and β-EP peptide production in the arcuate area of the hypothalamus (Sarkar & Minami, 1990 ; Sarkar et al., 2007 & Kuhn & Sarkar, 2008). β-EP peptide belongs to one of the family of endogenous opioid peptides, the proopiomelanocortin (POMC), from which it is derived (Gianoulakis, 2004). It is a 31 amino-acids peptide, has potent analgesic activity and is connected to the mesolimbic reward system that plays an important role in alcohol addiction (Zalweska-Kaszubska & Czarnecka, 2005). β-EP peptide also plays a role in stress regulation. For example, low level of central β-EP has been associated with psychiatric diseases and stress (Darko et al., 1992; Bernstein et al., 2002 & Sarkar et al., 2007). β-EP exerts its function by binding to opioid receptors. There are at least three major opioid receptor types mu (µ) (MOR), delta (δ) (DOR) and kappa (κ) (KOR). These receptors show 60% homology in amino acid sequence (Herz, 1997), are coupled to inhibitory G-proteins and are found in the arcuate nucleus and in the PVN (Pennock & Hentges, 2011). β-EP is particularly selective for MORs and has a lower affinity binding to DORs (Brownstein, 1993). Both MORs and DORs are expressed on POMC neurons and on CRH neurons. Thus, a decrease in β-EP production upon alcohol exposure would impact these two neuronal systems. The inhibitory feedback of β-EP at the level of the PVN on CRH release from CRH neurons has been demonstrated but β-EP feedback on POMC neurons in the arcuate area is not known. For example, Plotsky (1986) demonstrated that intracerebroventricular injection of β-EP resulted in decrease of CRH secretion from CRH-
producing neurons into the circulation. Boyadjieva et al. (2009) demonstrated that β-EP neuronal cell transplant into the hypothalamus reduced CRH hyperresponse to stress in fetal alcohol exposed rats. Interestingly, the regulation of POMC neurons activity by β-EP itself was demonstrated to be mediated presynaptically and postsynaptically. Presynaptic and postsynaptic sites have binding sites for opioid receptors. Pennock & Hentges (2011) demonstrated an autoinhibitory effect of β-EP on POMC neurons activity in mouse brain slices.

What is the effect of alcohol exposure on β-EP peptide production and what are the physiological implications? Alcohol exposure decreases POMC gene expression in the ARC of the hypothalamus with a decrease in production of its derived peptide β-EP (Sarkar & Minami, 1990; Sarkar et al., 2007 & Kuhn & Sarkar, 2008). It has been reported that modulation in β-EP release upon alcohol exposure alters the binding properties of opioid receptors and modifies alcohol seeking behavior in animals and humans. Thus, opioid antagonists such as nalaxone and naltrexone are considered as potential drugs to modify alcohol drinking behavior (Herz, 1997). In vivo and in vitro studies demonstrated that the modulation of β-EP peptide production upon alcohol exposure is dependent on the amount of alcohol, timing and duration of exposure. For example, initiation of alcohol drinking in humans results in a “priming effect” with an increase in β-EP peptides production in the hypothalamus, pituitary and plasma resulting in a short-term state of reward and good feeling. Chronic heavy drinking alters brain circuitries, POMC system is one of them, leading to a state of enhanced neuronal excitability that could lead to dependence (Rivier & Vale, 1988; Gianoulakis, 2004 & Zalweska-Kaszubska & Czarnecka, 2005). In vitro studies also confirmed a differential release of β-EP peptides upon alcohol exposure. For example, acute alcohol exposure stimulates β-EP release from hypothalamic neuronal cultures. Chronic alcohol exposure desensitizes these neurons and leads to a decrease in its release (Sarkar & Minami, 1990 & Boyadjieva & Sarkar, 1994). The physiological implications of β-EP deficit caused by prenatal alcohol exposure are evident on the dysregulation of the stress axis and the
malfunctioning of the immune system in adult exposed offspring (Arjona et al., 2006; Sarkar et al., 2007; Boyadjieva et al., 2009 & Govorko et al., 2011). The malfunctioning of the POMC system and the modulation of β-EP production upon alcohol exposure could be one of the causes that increase individual’s vulnerability to alcohol seeking behaviors or altered behavior later in life (Darko et al., 1992; Bernstein et al., 2002 & Sarkar et al., 2007).

1.7 Epigenetics

The causes of the deficit in POMC gene expression and β-EP production in fetal alcohol-exposed rodents (Sarkar et al., 2007 & Boyadjieva et al., 2009) are not very well known. We propose that FAE causes these changes by inducing long-lasting epigenetic alterations such as histone modifications and/or DNA methylation in POMC neurons.

Prenatal and early postnatal exposure to environmental factors such as drugs, toxicants or stress could cause long-lasting epigenetic mechanisms in the brain with stable adverse effects on phenotype later in life. These observed long-lasting phenotypes demonstrate the impact of the gene-environment interactions in shaping the landscape of the human genome and hence human behavior (Jirtle & Skinner, 2007).

What is epigenetics? Historically, the term “epigenetics” was first defined in 1942 by Conrad Waddington as the influence of gene-environment interaction in shaping behavior. Epigenetics is now more precisely described as a type of molecular and cellular “memory” that results in heritable stable changes in gene expression which are unrelated to changes in DNA sequence in response to environmental factors (Holliday, 2002 & Haig, 2004). These changes are caused by postranslational modification (PTMs) of histones that package the chromatin, DNA methylation or by small noncoding RNAs (Goldberg et al., 2007). These changes affect the translation of genetic information found in the genome language. In our study, we focused on understanding
the effects of FAE on the first two mechanisms in POMC neurons although the role of the third is rapidly emerging and revealing essential regulatory functions.

1.7.1 Histone modifications

Exposure to drugs and alcohol causes selective histone modifications in a tissue-specific manner and results in changes in gene expression and function (Kumar et al., 2005; Pal-Bhadra et al., 2007 & Pandey et al., 2008). Dysregulation of histone modification and/or DNA methylation machineries is linked to physiological changes and diseases (Eger et al., 2004 & Bhaumik et al., 2007). No study was done before to elucidate the effects of FAE on the components of the epigenetic machinery in β-EP-producing POMC neurons of the hypothalamus and to reveal their physiological contribution to regulation of POMC gene expression and functions.

POMC gene expression is not a simple one-way mechanism but rather it is an intricate process that requires the orchestrated effort of a combination of factors such as regulatory factors or TFs as well as a variety of effector proteins and multiprotein complexes at different stages of development (De Souza et al., 2005). Malfunctioning of any of these players or their interactions could place the transcriptional or the translational process in POMC gene out of tune and modulate POMC gene expression with adverse effects on the stress axis function. The identity of these players is not very well known in hypothalamic POMC neurons.

What are histone modifications, what are their physiological functions, how these modifications affect each other and how they contribute to regulation of gene expression? DNA is packaged around an octamer of highly basic proteins called histones to form nucleosomes, the building blocks of chromatin. There are 5 types of histones: H1, H3, H4, H2A and H2B and histone variants. H1 is the linker histone that helps in the packaging of nucleosomes into a higher order
structure and acts as an “exit/entry” point to nucleosomes (Zlatanova et al., 2010). The other histones form the core of a nucleosome which is wrapped by a 147 bp of DNA. There are many types of histone variants. H2AZ and the isoforms of H3 (H3.1/H3.3) particularly play a role in gene activity. For example, H2AZ plays a role in gene activation and gene repression. H3.1 associates with the chromatin during DNA replication while H3.3 associates with the ORFs of genes and plays a role in gene transcription (Kamakaka & Biggins, 2005). Structurally, each histone consists of a globular domain and a charged NH2-terminus tail that protrudes out of the nucleosome. The switch between chromatin compaction and relaxation state is regulated by the ability of this tail to perform malleable posttranslational modifications (PTMs) that affect the access of the transcriptional machinery to genetic information. These modifications occur at specific residues such as lysine (K), arginine (R), serine (S) or threonine (T) and alter the accessibility of TFs to regulatory sites along the DNA (Strahl & Allis, 2000 & Jenuwein & Allis, 2001).

Histone modifications are critical for DNA methylation and are key regulators of gene expression during growth and differentiation in all tissues including the brain (Roberston, 2002; Bhaumik et al., 2007; Cedar & Bergman 2009 & Kondo, 2009). These modifications are dynamic. Once they occur, they can either hide or expose binding sites on the DNA and affect directly the accessibility of TFs to these sites. They can also potentiate the recruitment of chromatin modifying complexes or interact with other proteins such as DNA methyltransferases (Dnmts), repressors or co-repressors such as methyl-binding proteins (MBDs) or histone deacetylase complex (HDAC) to mediate downstream functions essential for cellular functions (Strahl & Allis 2000; Berger, 2007 & Margueron and Reinberg, 2010). The contribution of these PTMs in gene activation or repression depends on the type of histone tail modifications and the machinery that will read and interpret these modifications.
These PTMs are diverse, occur on specific residues, have particular function and could influence each other in the same nucleosome as well as in neighbouring nucleosomes in a synergistic or antagonistic way (Jenuwein & Allis 2001). They occur in dividing cells as well as in adult postmitotic neurons (Feng et al., 2007) and could be altered by alcohol exposure in a time and tissue-dependent manner (Pandey et al., 2008 & Shukla et al., 2008). There is a variety of histone modifications including methylation, acetylation, phosphorylation, sumoylation or ubiquitination. They mostly target histones H3 and H4 which have central role in chromatin structure and function (Strahl & Allis 2000; Bird, 2001 & Jenuwein & Allis 2001). Lysine methylation and acetylation play critical role in transcriptional regulation (Kouzarides, 2007). In this study, we focused on the first three modifications: methylation, acetylation and phosphorylation.

**Histone methylation**

Histone methylation plays a critical role in gene expression and is altered upon alcohol exposure (Pandey et al., 2008; Shukla et al., 2008 & Govorko et al., 2011). It is a biochemically static stable process that involves the addition of the uncharged methyl group (CH3) to lysine or arginine residues of H3 or H4 and recruits other proteins (Bannister & Kouzarides, 2005). Lately, the discovery of the lysine demethylase (LSD1/KDM1) suggested that this modification could be reversible and dynamic (Shi et al., 2004). This reversibility is essential in the context of neuronal plasticity in response to environmental cues. Unlike other histone marks, histone methylation is an inert modification that does not alter the residue charge but act as a “nucleation site” for effector proteins. It is specifically one of the most significant PTMs with essential functions in transcriptional regulation, gene expression, and heterochromatin formation. Histone lysine (K) marks occurs at positions 4, 9, 27, 36 and 79 in H3 and at position 20 in H4 (Lachner & Jenuwein, 2002). Histone methylation occurs in the nucleus and is catalyzed by the activity of histone methyltransferases (HMTs/KMTs) that utilize S-AdenosylMethionine (SAM)
as a methyl donor. The effects of this modification on chromatin structure and transcriptional control depend on which residue of a specific histone is methylated and the number of methyl groups added. Histone H3 at lysine 4 (K4) or lysine 9 (K9) can be mono (me1), di-(me2) or tri-(me3) methylated on their amine (Zhang & Reinberg, 2001 & Jenuwein, 2006).

Several studies suggested that some PTMs such as H3K4 and H3K9 marks could have bidirectional effects on gene transcription depending on the type of effector proteins that will be recruited to the chromatin and on the type of interactions that occurs between effectors and other multiprotein complexes (Vacok et al., 2005; Shi et al., 2006; Ooi et al., 2007 & Wang et al., 2011). H3K4 methylation correlates with activation of gene expression (Santos-Rosa et al., 2002) but also can function in gene repression (Shi et al., 2006). It is mostly found next to a hyperacetylated residue but excluded from a histone carrying a repressive mark such as methylated H3K9 or H3K27. H3K4 methylation is catalyzed by the Set domain containing histone methyltransferase Set7/9 which specifically localizes to the 5’ ends of open reading frames (ORFs) to activate gene expression (Berger, 2000; Lachner & Jenuwein, 2002 & Izzo & Schneider, 2011). Specifically, trimethylated H3K4 mark (H3K4me3) mark occurs at the 5’ region of all active genes, dimethylated H3K4 mark (H3K4me2) is found in the coding region and monomethylated H3K4 (H3K4me1) is most abundant at the 3’ end (Santos-Rosa et al., 2002 & Ng et al., 2003). In the context of H3K4 interactions with other proteins, H3K4me3 mark is recognized by the chromohomeodomain (CHD1) of the ATPase chromatin remodeling complex. This later has the ability to recruit histone acetyltransferases (HATs/KATs) as well as components of the splicing machinery to regulate gene transcription and splicing (Sims et al. 2005).

H3K4me3 also binds to the PHD-finger domain of proteins that initiates transcriptional activity by RNA Polymerase II at promoters such as TFIID, a multiprotein complex that consists of TATA-binding protein (TBP) and other associated factors (Vermeulen et al., 2007 & Gardner et al., 2011). The methyl group of H3K4 is also recognized in Drosophila by the the PHD domain
of the nucleosome remodeling factor (NURF) (Wysocka et al., 2006) and the ING4 containing histone acetyltransferase (HAT) complex (Saksouk et al., 2009). All of these interactions demonstrate that H3K4 mark associates with a transcriptionally active chromatin and correlates with activation of gene expression.

Other studies reported a repressive role for H3K4me3 mark. For example, Shi et al. (2006) demonstrated in vitro that H3K4me3 is linked to transcriptional repression via its binding to the plant homeodomain (PHD) of the inhibitor growth protein 2 (ING2). This binding promoted Sin3a-HDAC1 complex activity resulting in histone deacetylation and gene repression. In vivo, ChIP assay demonstrated the specificity of ING2 binding to H3K4me3 not to H3K4me2 or other histone marks. It was suggested that H3K4me3 recognition by ING2 could be a protective cellular mechanism in response to cellular stress such as DNA damage by directing ING2-HDAC1 complex to the promoter of gene and causing gene repression (Shi et al., 2006).

Similarly, Wang et al. (2011) demonstrated that the H3K4me2,3 methyltransferase Set1 mediated the repression of the PHO5 gene, that codes for an acid phosphatase, in the yeast Saccharomyces cerevisiae. Deletion of Set1 resulted in reduction of nucleosome occupancy at the PHO5 gene promoter. Thus, this bimodal function of H3K4 mark in gene activation or repression is still elusive and not very well understood.

The link between histone modifications, DNA methylation and gene expression has been demonstrated. H3K4me3 mark inhibits binding of de novo methyltransferases, Dnmt3a and Dnmt3L, indicating that the absence of this mark is a necessary step for inducing DNA methylation (Ooi et al., 2007).

**H3K9 methylation** correlates with gene repression. Similarly, it forms complex interactions with other proteins to modulate gene expression. This mark is critical for establishment of DNA
methyltransferases G9a (KMT1C) and the lysine-histone-methyltransferase set domain bifurcated 1 Setdb1 (KMT1E). H3K9me3 mark is mediated by position effect variegation suppressor SUV39H1, H2 (KMT1A &B). It has been shown that G9a has 5 to 10 fold higher activity in fetal brain than SUV39 with dual function in methylating H3 at positions K9 (H3K9) and K27 (H3K27), thereby inducing gene repression (Lachner & Jenuwein, 2002). These HMTs with SET domain contain a potential methyl-CpG-binding domain (MBD) which could suggest interplay between histone methylation and regulation of gene expression (Bird & Wolffe, 1999).

There is a “cross-talk” between H3K9 methylation and DNA methylation (Fuks et al., 2003; Guibert et al., 2009 & Mechedint et al., 2010). For example, methylated H3K9 creates a binding site for the chromodomain of the heterochromatin protein (HP1) which interacts with HDAC and forms complex with Suv39H and G9a to promote histone deacetylation and gene repression (Bannister et al., 2001). H3K9 methylation itself recruits MBDs or MeCP2 and other chromatin-modifying factors to the promoter of a specific gene and leads to gene repression (Zhang and Reinberg, 2001; Sarraf & Stancheva, 2004; Esteller & Almouzni, 2005 & Vaissiere et al., 2008). Although H3K9 histone mark has a repressive function and usually correlates with gene repression, it also has a role in gene activation. Vakoc et al. (2005) demonstrated the presence of this repressive mark in the coding region of actively transcribed genes. This finding demonstrates that the histone mark H3K9 has dual function. It has a repressive function in the promoter region of genes and an activation function in the coding region with an “attenuating effect” function on the elongation of RNA polymerase II.

**Histone acetylation**

Histone acetylation is another modification and a promising mechanism that plays a dynamic role in chromatin remodeling. It is a specific and reversible modification that is regulated by the
activity of histone acetyltransferases HATs (KATs) and histone deacetylases (HDACs). It involves the transfer of an acetyl group from acetyl-coenzyme A to the NH2-terminal tail of lysine residue (Eberharter & Becker, 2002). HATs (KATs) and HDACs work on nucleosomes that are situated next to a TATA box to which RNA polymerase II binds and hence assist in transcriptional activation or repression (Orphanides & Reinberg 2000). The acetylated mark is not only observed in promoter region but also found in enhancer elements and enriched at boundary or insulator elements (Brown et al., 2000).

Unlike lysine methylation, lysine acetylation is not an inert modification. Via its negatively charged acetyl group, it reduces the positive charge on the N-terminal tail of H3 and reduces the electrostatic interaction of this tail with the negative phosphodiester bond of DNA. This results in loosening histone-DNA interactions and creates an open permissive chromatin and an environment around DNA conducive for transcriptional activation (Berger, 2000; Gregory et al., 2001 & Eberharter & Becker, 2002). More specifically, lysine acetylation acts as a signal that will be read by chromatin-associated proteins and will influence other histone marks on neighbouring residues to affect gene expression (Gregory et al., 2001). For example, an interaction between CREB-binding protein (CBP/KAT3A) that possesses HAT activity and histone methyltransferases has been demonstrated (Vandel & Trouche, 2001). The bromodomain of the yeast ATP-dependent chromatin remodeling complex SWItch/Sucrose NonFermentable (SWI/SNF) binds to acetylated histone to activate gene transcription (Narlikar et al., 2002).

**Histone phosphorylation**

Histone phosphorylation plays a role in gene activation or repression. Phosphorylation of histone H3 at serine 10 (pH3S10) is a reversible process catalyzed by kinases and erased by phosphotases. It is positively correlated with transcriptional activation where it inhibits HP1
binding to methylated H3K9 and promotes the recruitment of HATs resulting in acetylation of this mark on lysine 9 (AceH3K9) (Prigent & Dimitrov & Fischle et al., 2005). Phosphorylated H3S10 also promotes chromosome condensation during mitosis by putting a “ready label” on chromosome during metaphase to anaphase transition (Hendzel et al., 1997).

Depending on what type of PTM occurs on a specific residue, the biological output will be different. All these modifications influence each other and influence DNA but they cannot solely modulate the genetic information (Zhang & Reinberg, 2001). It has been demonstrated that methylation of H3 at lysine 4 (H3K4) (Bannister & Kouzarides, 2005), acetylation of H3 at lysine 9 (AceH3K9) (Eberharter & Becker, 2002) and phosphorylation of H3 at serine 10 (pH3S10) could interact synergistically to promote along the DNA an environment conducive for gene activation (Prigent & Dimitrov, 2003 & Graff & Mansuy, 2008). On the other hand, methylation or deacetylation of histone H3 at lysine 9 (H3K9) exclude other activation marks and is often associated with gene repression (Bird, 2001; Feng et al., 2007 & Wu et al., 2007). More importantly, some of these modifications such as histone methylation marks are altered by environmental factors and are inherited in somatic cells (Haycock, 2009).

This simplistic view of regulation of gene expression solely by histone interactions does not reflect the complexity of gene expression regulation. In the context of synaptic strength, neuronal plasticity and the complex circuitry of the brain, it is clear that no single histone modification or interaction is responsible to generate a complex downstream signaling effect at the neuronal network levels. Rather the cumulative effects of many homotypic or heterotypic interactions between HMTs, HDACs, Dnmts, effector proteins, chromatin-remodeling complexes and RNA polymerase II and possibly other yet unidentified factors could explain the complexity of neuronal function and plasticity as well as vulnerability of the later to the effects of external
factors such as alcohol exposure at different stages of development.

These different types of histone modifications could be altered by FAE in POMC neurons. The role of these histone marks and their interactions in regulation of POMC gene expression is unknown and was never studied before. In this thesis work, we focused on investigating the effects of FAE on the activation marks, di-trimethylated H3K4 (H3K4me2,3), acetylated H3K9 (AceH3K9) and phosphorylated H3S10 (pH3S10) and on the repressive mark dimethylated H3K9 (H3K9me2) in POMC neurons of fetal alcohol-exposed offspring.

1.7.1.1 Histone modifying enzymes

Histone modifications such as methylation, acetylation or phosphorylation are catalyzed by the activity of histone-modifying enzymes or “writers” such as histone-methyltransferases (HMTs/KMTs), histone acetyltransferases (HATs/KATs) or kinases. Once established, these histone marks recruit downstream effector proteins with specific “reader” modules such as chromohomeodomain (CHD), bromodomain, plant homeodomain (PHD) or Tudor domains that read these marks which eventually will be interpreted by the epigenetic machinery. Once interpreted, these marks are erased by “erasers” such as HDMs (KDMs), HDACs or phosphotases (Borrelli et al., 2008). The majority of HMTs/KMTs contain a SET domain that catalyses the transfer of methyl group to the lysine residue (Dillon et al., 2005). H3K4 methyltransferases include the yeast Set1, Drosophila trithorax (Trx), mammalian Set7/9 (PKMT) and human SMYD3 (Rutnberg et al., 2007). G9a and Setdb1 are histone methyltransferases that catalyze the dimethylation of H3K9 (H3K9me2) in vivo (Tachibana et al., 2002).

The role of histone deacetylases (HDACs) in many disease states has been reported and the modulation of their activities has aberrant physiological consequences (Saha & Pahan, 2006; Xu
et al., 2007; & Sharma et al., 2008). Inhibitors of HDACs such as trichostatin (TSA), valproic acid or sodium butyrate are potential therapeutic targets for the treatment of cancer and neurological disorders (Yoshida et al., 1990; Dokmanovic & Marks, 2005 & Guy et al., 2007). So, identifying specific epigenetic factors that are responsible for diseases could have potential therapeutic implications and HDACs could be potential candidates. Govorko et al. (2011) demonstrated that injection of TSA, inhibitor of histone deacetylation or 5’-Azacytidine (5’-Aza), inhibitor of DNA methylation, during the neonatal period normalized POMC gene methylation and expression in prenatal alcohol-exposed rats in the adult stage. This correlated with normalization of corticosterone response to lipopolysaccharide (LPS) challenge in these rats. This could suggest that TSA or 5’Aza could be potential drugs to treat behavioral changes observed in FASD individuals such as stress.

There are three classes of HDACs in mammals that are expressed in a spatial and cell-specific manner in the brain. These enzymes are primarily expressed in neurons but some were also found in oligodendrocytes such as HDAC2-5 and HDAC11. Class I HDACs (HDAC1, 2, 3 and 8) are localized in the nucleus and are widely expressed while Class II HDACs (HDAC4, 5, 6, 7, 9 and 10) shuttle between the nucleus and cytoplasm and have high expression in the brain. Class III HDACs include the NAD-dependent Sirtuin family of proteins (SIRT1-7). Class IV HDACs includes only HDAC11 which is specifically expressed in the hippocampus, Purkinje cells of the cerebellum and in oligodendrocytes (Broide et al., 2007). It is also expressed in other organs such as the heart, muscle and kidney (Gao et al., 2002). Recently, hypothalamic Sirt1 gene was demonstrated to play an anorectic role in a FoxO1-dependent manner (Forkhead transcription factor) in POMC neurons. This finding indicates that Sirt1 gene could play a role in regulation of energy homeostasis in hypothalamic POMC neurons and could be a potential target for treatment of obesity or metabolic disorders associated with POMC gene dysfunction (Cakir et al., 2009).
Those enzymes that catalyze the acetylation of histones and create an environment conducive for transcriptional activation are the HATs/KATs. There are two main classes of HATs, type A nuclear HATs and type B cytoplasmic HATs. There are three families of nuclear HATs: Gcn5-related N-acetyltransferase (GNATs), CREB-binding protein (p300/CBP) and MYST proteins. They all possess a highly conserved acetyl-CoA binding site, domains to interact with transcriptional regulators and modules such as chromo, bromo or plant homeodomains to interact with chromatin-remodelers or target regulators of the transcriptional machinery (Roth et al., 2001). A list of selected histone-modifying enzymes that are relevant to our study are listed in Table 1.

Table 1 Selected histone-modifying enzymes

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Name (“Writers”)</th>
<th>Site of modification (Substrates)</th>
<th>“Readers”</th>
<th>Biological significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set7/9 (KMT7)</td>
<td>Set containing domain lysine methyltransferase</td>
<td>H3K4me1, me2, me3</td>
<td>CHD1</td>
<td>Transcriptional activation</td>
</tr>
<tr>
<td>G9a (KMT1C)</td>
<td>Lysine methyltransferase</td>
<td>H3K9me1, me2</td>
<td>HP1</td>
<td>Transcriptional silencing</td>
</tr>
<tr>
<td>Setdb1 (KMT1E)</td>
<td>Set domain bifurcated 1</td>
<td>H3K9me2, me3</td>
<td>HP1</td>
<td>Transcriptional silencing</td>
</tr>
<tr>
<td>Kinases</td>
<td>Kinases</td>
<td>H3S10 phosphorylation</td>
<td>NA</td>
<td>Silencing/Activation</td>
</tr>
<tr>
<td>HDACs</td>
<td>Histone deacetylases</td>
<td>H3K9</td>
<td>NA</td>
<td>Deacetylation/Repression</td>
</tr>
<tr>
<td>CBP/p300 (KAT3A &amp;B)</td>
<td>CREB-binding protein</td>
<td>H3K4, H3K9</td>
<td>NA</td>
<td>Acetylation/Activation</td>
</tr>
</tbody>
</table>

References: Bannister et al. (2001); Izzo & Schneider (2011)

1.7.1.2 Chromatin remodeling complexes

Chromatin-remodeling complexes play an indispensable role in gene expression. They are multiprotein machinery that possesses a catalytic ATPase activity. They interact with histone marks and with other proteins to modulate chromatin structure and allow the interpretation of
genetic information to be placed within a cellular context. They use the energy derived from ATP hydrolysis to alter histone-DNA, histone-histone interactions and nucleosomal positions thus altering chromatin structure, accessibility of TFs to regulatory sites and gene expression (Henikoff et al., 2008).

There are four families of ATPase chromatin remodelers which are evolutionarily conserved from yeast to humans: the yeast *Saccharomyces cerevisiae* mating type Switching/sucrose Nonfermentable (SWI/SNF), nucleosome remodeling factor (NURF) or *Drosophila melanogaster* Imitation Switch (ISWI), yeast Inositol (INO80) and the Chromodomain helicase DNA-binding (CHD1) ATPases (Allis et al., 2007 & Henikoff, 2008). These complexes interact with histone marks. For example, SWI/SNF binds to acetylated histone to activate transcription (Narlikar et al., 2002). CHD1 ATPase binds to H3K4me3 to initiate elongation of RNA polymerase II and modulate gene splicing (Sims et al, 2005 & Sims et al., 2007). It has been demonstrated that elevated GC and GR in corticotroph adenomas, characteristics of Cushing Syndrome, resulted in GR recruitment to the pituitary POMC gene promoter with subsequent recruitment of the ATPase subunit of the SWI/SNF complex (Brg1) and HDAC2 resulting in decrease of *POMC* gene expression. Brg1-HDAC2 complex inhibited initiation of *POMC* gene transcription by RNA Polymerase II and resulted in histone H4 deacetylation (Bilodeau et al., 2006). No chromatin remodeling complex has been yet identified along the hypothalamic POMC gene promoter.

1.7.2 DNA methylation

The regulation of *POMC* gene expression by DNA methylation was recently reported (Newell-Price 2003; Ehrlich et al., 2010 & Muschler et al., 2010) and the decrease in *POMC* gene expression was demonstrated in alcohol-exposed rats (Sarkar et al., 2007). This deficit of *POMC*
gene expression in fetal alcohol-exposed rats was proven recently to correlate with POMC gene promoter hypermethylation (Govorko et al., 2011).

Regulation of gene expression is not only mediated by histone modifications but also by DNA methylation which could be altered by external factors resulting in abnormal cellular functions and altered phenotypes (Bonsch et al., 2005; Jirtle & Skinner, 2007; Novikova et al., 2008; Muschler et al., 2010 & Govorko et al., 2011). DNA methylation is an epigenetic mark that involves covalent modification of the cytosine residue in CpG dinucleotides in the promoter region to “lock in” the silent state of a gene (Holliday & Grigg, 1993). This modification is faithfully preserved during cell division and catalyzed by the activity of DNA methyltransferases (Dnmts) suggesting that DNA methylation is a promising mechanism to endure environmental prints on the genome.

DNA methylation is essential for many physiological processes such as DNA replication, gene repression, parental imprinting, control of cellular differentiation, normal embryonic development and normal brain development in mammals (Robertson & Wolffe, 2000; Ordway & Curran, 2002 Hermann et al., 2004 & Kim et al, 2009). Most importantly, it is a non-random process which is tightly regulated in tissue-specific manner (Chen & Riggs, 2011). Abnormal methylation has been documented in many neurological disorders (Mill et al., 2008). The use of Dnmt inhibitors such as 5’- Aza or Zebularine demonstrated that DNA methylation is not a static process but it is dynamically regulated and could be reversible in the developed brain thus playing a vital role in many essential neuronal functions such as memory formation (Miller & Sweatt, 2007 & Miller et al., 2008), controlling neuronal excitability and connectivity (Nelson et al., 2008) and normalizing the stress axis in alcohol-exposed rats (Govorko et al., 2011).
In the context of chromatin, there is a cross-talk between DNA methylation and histone modifications in regulation of gene expression (Fuks et al., 2000; Fuks et al., 2003; Ooi et al., 2007 & Cheng & Blumenthal, 2010). This basically raises the question about the functional significance of the interplay between these two mechanisms in gene expression regulation.

1.7.2.1 **Biological significance of CpG islands and their role in regulation of gene expression**

In eukaryotes ranging from plants to humans, DNA methylation occurs on cytosine residues in CG dinucleotides. Although there is scant evidence for a causal role of DNA methylation in gene silencing, CpG methylation in the promoter region of a gene correlates with silencing of its promoter activity (Bird & Wolffe, 1999).

CpG islands are stretches of CpG dinucleotides that are mostly found at the 5’ ends around the TSS or promoter region of most genes and are usually unmethylated (Antequera & Bird, 1999). Abnormal methylation of the promoter CpG islands is usually associated with gene silencing (Razin, 1998; Jones & Takai, 2001; Newell-price, 2003; Guibert et al., 2009 & Jones & Liang, 2009). The methylation of C5 of cytosine (5-mC) acts as the most stable heritable chromatin modification which is conducive for inhibition of transcription. A new nucleotide modification, 5-hydroxymethylcytosine (5-hmC), was recently identified in specific areas of the brain such as the cerebral cortex, brain stem and Purkinje neurons of the cerebellum (Kriaucionis & Heintz, 2009). This modification is catalyzed by hydroxylase like ten-eleven translocation proteins (Tet1, Tet2 & Tet3). It is suggested that the hydroxylation of 5-mC to 5-hmC could modulate the binding of proteins to the chromatin and hence alter transcriptional outcome. It facilitates demethylation and promotes gene transcription but its role in DNA methylation is not yet fully understood (Tahiliani et al., 2009 & Zhang et al., 2010). The methylation of CpG in the binding site of certain transcription factors such as Specificity protein 1 (Sp1), c-AMP responsive element
binding protein (CREB) and CCCTC-binding factor (CTCF) can block the accessibility of these factors to regulatory sequences along the DNA and modulate gene expression (Zhang et al., 2005 & Blackledge & Klose, 2010).

The distribution of CpG dinucleotides is uneven and not random across the human genome. They are concentrated in “pockets” called CpG islands (CGIs). These islands are found in the promoter region of most genes (60-70%). On the other hand, intergenic regions (between genes) and intronic regions are usually CpG poor (Miranda & Jones, 2007). It has been demonstrated that CGIs are located not only in the promoter region area but also in the downstream transcribed region of the gene (intragenic) or in intergenic regions (between genes). They occur at high frequency (1/10bp) in the promoter and the 5’ regions of many genes that have greater than 50% GC content and at low frequency (1/100bp) in coding regions (Ordway & Curran, 2002). Intragenic CGIs with unusual location, far from TSS, as well as intergenic CGIs could function as promoter for the formation of short transcripts or noncoding RNAs (ncRNAs) that could have an important transcriptional regulatory function (Deaton & Bird, 2011).

Interestingly, POMC gene has an upstream CGI in the promoter region and a downstream CGI within Exon 3. The downstream CGI is located close to a transcriptional unit and might initiate the formation of truncated short transcripts of unknown function or ncRNAs with regulatory functions (Gardiner-Garden & Frommer, 1994).

### 1.7.2.2 DNA methyltransferases and methyl-CpG-binding proteins

DNA methylation is catalyzed by the enzymatic activity of DNA methyltransferases (Dnmts). These Dnmts include Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3L (Fig. 4) (Robertson, 2002; Hermann et al., 2004; Sharma et al., 2005; Ladd-Acosta et al., 2007 & Chen & Riggs, 2011). These enzymes differ in structure, functions, expression and interactions. Structurally, they
share a conserved C-terminal catalytic domain made of 10 conserved amino acids motifs important for their enzymatic activity except Dnmt3L. They also contain an N-terminal regulatory domain except Dnmt2. This N-terminal domain is essential for protein-protein interactions such as proteins involved in modulation of chromatin structure and function (Chen & Riggs, 2011).

**Figure 4 Structure of Human Dnmts**

Conserved methyltransferase motifs in the catalytic domain are indicated in *Roman numerals*. NLS, nuclear localization signal; RFT, replication foci-targeting domain; BAH, bromo-adjacent homology domain; PWWP, a domain containing a conserved proline-tryptophan-tryptophan-proline motif; PHD, a cysteine-rich region containing an atypical plant homeodomain; aa, amino acids. DNMT3L lacks the critical methyltransferase motifs and is catalytically inactive (Adopted from Chen & Riggs, 2011).

Functionally, Dnmts catalyze the transfer of CH3 from the methyl donor, SAM, to the C5 of cytosine in the 5’-CpG-3’ dinucleotides. This 5-mC, the “fifth base” of mammalian DNA, is an important carrier of epigenetic information and rarely occurs at non-CG sites (Jeltsch, 2002 & Hermann et al., 2004). Methylation of CG sites usually inhibits promoter activity by preventing the binding of TFs to their binding sites and by recruiting methyl- CpG-binding proteins such as methyl-CpG-binding protein 2 (MeCP2) and methyl-binding domain proteins (MBDs) such as MBD1, MBD2, MBD4 or Kaiso to methylated DNA. Methylated CpGs additionally recruit cofactors such as HDACs, HMTs and corepressors to modify gene expression and create an environment conducive for gene repression (Hermann et al., 2004; Lakowski et al., 2006 & Guibert et al., 2009).
**Dnmt1** is a “maintenance methyltransferase” that is active during embryonic development. It preserves and propagates methylation patterns into daughter strand established by the “de novo methyltransferases”, Dnmt3a and Dnmt3b, during replication (Robertson, 2002; Hermann et al., 2004 & Jones & Liang, 2009). **Dnmt2** has low enzymatic activities, unknown biological function and no obvious role in DNA methylation (Hermann et al., 2004). It has recently been found to play a role in RNA methylation (Goll et al., 2006). Compared to **Dnmt3a, Dnmt3b** expression levels are very low in most tissues but the expression of all these three enzymes diminishes upon differentiation (Robertson, 2002; Liu et al., 2003; Feng et al., 2005; Sharma et al., 2005 & Miller and Sweatt, 2007). The role of **Dnmt3L** in methylation is questionable but it interacts with Dnmt3a and Dnmt3b to modulate their activities and it also interacts with HDACs (Hata et al., 1993 & Deplus et al., 2002). It was recently demonstrated that Dnmt3L recognizes unmethylated H3K4 (H3K4me0) and induces the docking of Dnmt3a to the nucleosome carrying this mark to initiate its methylation (Cheng & Blumenthal, 2010). This suggests the link between histone modifications and DNA methylation in regulation of gene expression.

Dnmts in eukaryotes are not free in the nucleus but are interacting in complex ways with each other and with other nuclear components to perform their functions and modulate transcriptional outcome (Fuks et al., 2000; Robertson, 2000; Rountree, 2000 & Kim et al., 2002). For example, Dnmt1 forms a complex with Proliferating Cell Nuclear Antigen (PCNA) at replication fork implicating Dnmt1 role in the control of the cell proliferation (Chuang et al., 1997). In the context of chromatin, it interacts with SUV39H1 and HP1 that catalyze H3K9 methylation (Fuks et al., 2003 & Jones & Liang 2009). It also interacts with HDAC1 and HDAC2 (Robertson et al., 2000; Rountree et al., 2000 & Jones & Liang, 2009), with MBD2, MBD3 and MeCP2 to initiate gene repression (Tatematsu et al., 2000 & Kimura & Shiota, 2003). The other H3K9 methyltransferases such as G9a recruits Dnmt3a and Dnmt3b to the DNA and forms a complex with Dnmt1 and Setdb1 associates with MBD1 (Margueron & Reinberg, 2010). This later
blocks the binding of the transcription factor Sp1 to the CG in the CpG islands to cause gene repression (Ichimura et al., 2005 & Feng et al., 2007). The versatility of Dnmts to form multiple connections with other proteins made them attractive targets for drugs. For example, 5′Aza, the inhibitor of DNA methylation is a potential agent that could attenuate the adverse effects of some neurological disorders (Levenson, 2007).

The role of DNA methylation in modulating gene expression is better understood in the context of methyl-CpG-binding proteins that recognize these methylated cytosines and modulate gene expression. These proteins interact with the methylated CpG of DNA via their methylbinding domain (MBD) and recruit repressors and HDACs via their transcriptional repressor domain (TRD) to repress transcription (Fig. 5) (Bird & Wolffe, 1999 & Fatemi & Wade, 2006).

![Domains of the methyl CpG binding protein family](image)

Figure 5 Domains of the methyl CpG binding protein family

Figure 5 is adopted from Fatemi & Wade (2006).

The role of these proteins in many neurological diseases has been demonstrated. For example, MeCP2, which is abundantly expressed in mature neurons specifically in the hypothalamus, modulates the expression of many genes by acting both as an activator in recruiting the transcriptional activator CREB or as a repressor of gene expression (Chahrour et al., 2008). It plays a critical role in neuronal functions and maturation of neuronal connectivity. So, its
proper expression is essential for normal brain development (Zhou et al., 2006). Recently, MeCP2 has been shown to play a critical role in chromatin remodeling (Tao et al., 2009; Cohen & Greenberg, 2010 & Skene et al., 2010), alternative splicing of pre-mRNA (Young et al., 2005) and stress and behavior (Fyffe et al., 2008). MeCP2 interacts with complexes such as Sin3a/HDAC complex or with SWI/SNF complex to cause deacetylation and transcriptional repression (Bird & Wolffe, 1999).

The incremental understanding of the physical direct or indirect interactions between the components of the epigenetic machinery is essential to our understanding of gene expression regulation and the molecular underpinnings of diseases such as stress regulation. The effects of FAE on these components such HMTs, HDACs, Dnmts and MBDs were never explored before in POMC neurons in the context of possible induction of permanent changes in POMC gene expression and functions in the adult stage.

1.7.3 Small noncoding RNAs

Although DNA methylation and histone modifications are pivotal mechanisms in regulation of gene expression, miRNAs are now emerging as a group of intriguing small RNAs that will add an additional layer of molecular complexity to our understanding of regulation of gene expression. Although they are very small in size, miRNAs are implicated in many diseases such cancer, neurological diseases and alcohol addiction (Marsit et al., 2006; Barbato et al., 2008; Pietrzykowski et al., 2008; Rouhi et al., 2008; Wang et al., 2009; Miranda et al., 2010 & Pietrzykowski, 2010). Their role in alcoholism is clearly fast emerging but their role in fetal alcohol is not very well known or elucidated.
These regulatory elements are described as short 20 to 22 nucleotides RNAs that regulate 30% of genes in the human genome (Bartel, 2004) and have unique spatial and temporal expression profile in the brain (Krichevsky et al., 2003). They are abundantly expressed in developing and mature brain and modulate gene expression at different stages of neuronal development in diverse organisms (Gao, 2007; Kapsimali et al., 2007 & Barbato et al., 2008). Their mode of gene expression regulation is intriguing and unique. They usually target the 3’UTR of a gene in which the complementarity between the seed region (2-7 nt) of miRNA and 3’UTR of the mRNA will cause translational arrest or mRNA degradation hence leading to gene suppression or silencing (Bartel, 2004; Barbato et al., 2008 & Filipowicz et al., 2008). Recently, it was reported that miRNAs could modulate the expression of the components of the epigenetic machinery such as Dnmts, HMTs, HDACs or methyl-CpG-binding proteins. They also could be regulated by epigenetic mechanisms via histone modifications or DNA methylation of their own promoters in response to external factors (Rouhi et al., 2008 & Guil & Esteller, 2009).

POMC gene has an upstream CpG island (CGI) in the promoter region and a downstream CGI within Exon 3. The downstream CGI is located close to a transcriptional unit in Exon 3 and might initiate the formation of truncated short transcripts of unknown function or ncRNAs with regulatory functions (Gardiner-Garden & Frommer, 1994). Multiple transcription initiation sites for small POMC transcripts were identified in human testis (Lacaze-Masmonteil et al., 1987). These sites generated short POMC transcripts (~ 800 bp) that lack Exon 1 and Exon 2. These short transcripts start 41 to 162 bp downstream from the 5’ end of Exon 3 and could not translate a complete POMC peptide. Interestingly, one short transcript (703 bp) could initiate the translation of a 183 amino acids peptide that lacks the NH2 terminal part of γ-MSH. The other transcript initiates the formation of a 45 amino acids peptide encompassing the COOH terminal of γ-LPH and β-EP. Additionally, these two short transcripts do not possess a signal peptide thus could not undergo postranslational processing to generate functional biological peptides (Lacaze-
Moreover, public genome browsers such as UCSC identified expressed sequence tags (ESTs) in the hypothalamus and pituitary of the *Rattus norvegicus* that lack the sequence that codes for part of Exon 3 (EST sequences: CB795446, CB792926, CB741607, CB762586, CB792925, CB736383, CB740788, CB789985, CB740008, CB696267, CB745360 and CB744114). Other ESTs lack the sequence that codes for part of Exon 1 (CB769262) or part of Intron 2 and whole Exon 3 (CB787880). This raises the question whether these ESTs are noncoding RNAs and whether fetal alcohol exposure could generate from *POMC* gene short nonfunctional truncated transcripts or noncoding RNAs with regulatory function on *POMC* gene expression.

In this study, we determined the effects of FAE on DNA methylation and histone modifications on *POMC* gene expression in POMC neurons. The possibility that miRNAs or small noncoding RNAs could play a role in regulating *POMC* gene expression and β-EP peptide production upon alcohol exposure is an interesting area of research that should be explored in the future.

### 1.8 Choline, Alcohol Exposure and the Brain

The activities of Dnmts and HMTs require the availability of the major methyl donor SAM. SAM formation depends on the availability of essential nutrients such as choline, betaine, VitB12 and folate. Choline, a water-soluble nutrient, is critical during embryonic development especially fetal brain development (Zeisel, 2004). It has been reported that neonates have a high capacity to transport choline across the BBB to ensure enough SAM availability for the activity of the neuronal phosphotidylethanolamine-N-methyltransferase (PEMT) that is responsible for the formation of choline (Zeisel & Wurtman, 1981). Choline deficiency causes neural tube defects in mice and in humans (Zeisel, 2000; Fischer et al., 2002 & Shaw et al., 2004) and has negative effects on neuronal migration, survival and differentiation (Zeisel, 1992; Craciunescu et al., 2003; Zeisel, 2006 & Zeisel, 2011).
Alcohol ingestion inhibits folic acid absorption and methionine synthase ability to convert homocysteine to methionine and SAM, which are both critical for methylation processes during embryonic development. Alcohol also diminishes the expression of several enzymes related to folate or methionine cycle such as methyltetrahydrofolate reductase (Mthfr) and S-adenosylhomocysteine hydrolase (SAHH) (Hamid et al., 2009). Choline is linked to folate and methionine metabolism and via its derivative betaine normalizes the level of SAM in the brain (Finkelstein, 1998; Halsted et al., 2002; Olthof & Verhoef, 2005 & Zeisel et al., 2006).

Besides its role in folate and methionine cycle, choline has important cellular functions and its deficiency has adverse effects on normal brain structure and function (Fig. 6) (Finkelstein, 1998; Zeisel, 2000; Zeisel, 2004 & Hollenbeck, 2010). It plays a role in maintaining the integrity of the phospholipid bilayer via its oxidation to phosphotidylcholine (PC), in DNA methylation (Niculescu & Zeisel 2002; Niculescu et al., 2006 & Kovacheva et al., 2007), in altering histone marks (Pogribny et al., 2008; Davison et al., 2009 & Mehedint et al., 2010) and affecting cholinergic neurotransmission via the synthesis of acetylcholine (Montoya et al., 2000).

**Figure 6 Roles of choline on brain functions**

(Abbreviations: PC=phosphotidylcholine, SAM=S-adenosylmethionine, SAH=S-adenosylhomocysteine, THF=tetrahydrofolate, 5MTHF=5-methyltetrahydrofolate, MTHFR=methyltetrahydrofolate reductase).
Nutritional supplementation such as methionine, betaine, choline, folic acid and VitB12 during gestation has beneficial effects on the offspring and could reverse the negative effects of environmental factors (Brunaud et al., 2003; Weaver et al., 2005 & Haycock, 2009). Supplementation of choline perinatally in rats increased choline metabolites in the blood and the brain and reduced stress (Zeisel et al., 2006). Prenatal and perinatal choline has also been shown to have positive effects on development (Zeisel, 2004; Zeisel, 2006 & Thomas et al., 2009), memory (Zeisel, 2000), behavior (Thomas et al., 2007), attention (Mohler et al., 2000) and activity levels and learning performance (Schenk & Brandner, 1995 & Thomas et al., 2000). The positive effects of gestational choline on behavior and learning were observed in fetal alcohol- exposed rats (Thomas et al., 2000; Thomas et al., 2007 & Thomas et al., 2009).

In this study, we investigated whether gestational choline supplementation during the period of alcohol exposure could mitigate alcohol adverse effects on POMC neurons of the hypothalamus.
2 Aims of the Thesis

The overall aim of this thesis work is to study the epigenetic effects of fetal alcohol exposure on hypothalamic Proopiomelanocortin (POMC) gene. More specifically, this study reveals the role of the components of the epigenetic machinery in altering histone modifications and DNA methylation upon fetal alcohol exposure in POMC neurons thus modulating POMC gene expression and β-EP peptide production in adult offspring.

There are four aims:

1. **Aim 1**: Determine the effects of FAE on protein levels and gene expression of histone-modifying enzymes and DNA-methylating enzymes in β-endorphin – producing POMC neurons

2. **Aim 2**: Determine the effects of FAE on gene expression profile of the epigenetic machinery in LCM-captured POMC neurons

3. **Aim 3**: Determine the effects of FAE on histone marks, H3K4me3 and H3K9me2, along POMC gene

4. **Aim 4**: Determine whether the epigenetic effects of FAE in β-endorphin – producing POMC neurons could be reversed by gestational choline supplementation
CHAPTER 2

3 Chapter 2: Fetal alcohol exposure alters the protein and gene levels of histone-modifying and DNA-methylating enzymes in β-endorphin neurons

3.1 Introduction

The role of alcohol exposure in inducing epigenetic modifications such as histone modifications and DNA methylation in the brain is fast emerging but not very well understood. At the neuronal network levels, the modulation of these modifications could change gene expression and shape the neural, behavioral and pathological state of exposed individuals. Consequently, these pathological manifestations can increase individual’s risk to diseases later in life such as cancer, obesity, cardiovascular disease, neurological diseases and alcohol addiction (Eger et al., 2004; Feng et al., 2007; Jirtle & Skinner, 2007; Pandey et al., 2008; Haycock, 2009 & Bokhoven & Kramer, 2010).

Various studies indicated that drugs of abuse and stress alter DNA methylation and histone modifications in different tissues and in distinct areas of the brain, thereby altering gene expression, function and phenotype (Kumar et al., 2005; Chen et al., 2006; Pal-Bhadra et al., 2007; Novikova et al., 2008; Pandey et al., 2008; Hunter et al, 2009 & Maze et al., 2010). Studies also revealed that the outcome of gene expression is affected by the synergistic or mutually exclusive interactions between histone marks and protein complexes on the same or neighboring nucleosomes. For example, there is a competition in influencing gene expression between H3K4 and H3K9 methylation and between H3K9 methylation and H3K9 acetylation (Tachibana et al., 2002 & Maze et al., 2010). Alteration in DNA methylation also plays crucial role in shaping the landscape of the epigenome and modulating gene expression and phenotypes (Razin, 1998; Bird,
Shahbazian et al. (2002) showed that MeCP2 mutant mice have elevated levels of acetylated histone H3. This finding might indicate that the absence of MeCP2, which usually binds to methylated DNA, induces acetylation of histone H3 to potentiate gene expression and influencing regulatory outcomes. This interplay between histone marks and DNA methylation could be altered by environmental factors and could be manifested in the organism by permanent change in gene expression and altered phenotypes later in life (Bhaumik et al., 2007; Jirtle & Skiner, 2007 & Bell & Beck, 2010).

No studies were done before to elucidate the epigenetic effects of FAE such as histone modifications and DNA methylation in β-endorphin - producing POMC neurons of the hypothalamus and to reveal their physiological implications at the organismal level. In this study, we determined the effects of FAE on the protein levels and gene expression of histone-modifying enzymes such as enzymes that di or trimethylate histone H3 at lysine 4 (H3K4me2,3), dimethylate histone H3 at lysine 9 (H3K9me2), acetylate histone H3 at lysine 9 (AceH3K9) or phosphorylate histone H3 at serine 10 (pH3S10). To establish whether there is any positive or negative correlation between histone modifications, DNA methylation and POMC gene expression, we also determined the effects of FAE on changes in protein and mRNA levels of DNA-methylating enzymes such as Dnmt1 and Dnmt3a and the methyl-CpG-binding protein 2 (MeCP2) in POMC neurons of adult exposed offspring.

3.2 Materials and Methods

Animal Model

Sprague-Dawley female rats were purchased from Charles River and maintained in the Bartlett Animal Facility where they were individually housed with 12-h light/12-h dark cycles (lights on at 7:00 h and off at 19:00 h) at a constant temperature (22ºC) throughout the study. On GD 7-21, a period equivalent to the first and second trimesters of pregnancy in humans (Clancy et al.,

pregnant rats were fed rat chow ad libitum fed (AD), a liquid diet containing ethanol (BioServe Inc., Frenchtown, NJ) alcohol-fed (AF), or pair-fed an isocaloric liquid control diet (with the ethanol calories replaced by maltose-dextrin) (PF). The concentration of ethanol varied (1.7-5.0% v/v) in the diet for the first 4 days to habituate the animals with the alcohol diet. After this habituation period, animals were fed the liquid diet containing ethanol at a concentration of 6.7% v/v that maintained an average blood alcohol level between 130 and 150 mg/dl (Chen et al., 2006), which is within the range of blood alcohol concentrations achieved following binge drinking in humans (White et al., 2011). It should be noted that the rat, an altricial species, is an animal model for the midgestational brain differentiation in humans. Additionally, CDC reports that about 1 in 8 pregnant women drinks alcohol in the United States. Therefore, the animal model we used represents alcohol-drinking effect during the midgestational period. AF and PF litters were cross-fostered using untreated lactating rats to prevent any compromised nurturing by the AF lactating mother rats. Litter size was maintained as 8 pups/dam. Only one pup from each litter was used in an experiment in order to prevent gene homogeneity. At postnatal day PD22, pups were weaned, housed by sex, and provided rodent chow meal and water ad libitum.

**Double Immunofluorescence and Confocal microscopy**

Brains of AD, AF, and PF rats were sectioned at 20 µm thickness and placed on the same glass slide. These sections were collected from plate 19 to plate 23 in the stereotaxic atlas and cover the whole ARC area of the hypothalamus (Paxino & Watson, 1989). Every fifth section from each brain was fixed for 10 minutes with 4% PFA then washed in PBS(1X)+ 0.3% TritonX100 for 5 minutes. After wash with PBS(1X) for 5 minutes, brain sections were blocked with 5% horse serum (Vector labs, S2000), then double-immunostained with β-EP (1:200, T-4045, Rabbit Anti-rat-β-endorphin, Bachem, CA) and H3K4me2,3 (1:500, ab6000, Abcam mouse monoclonal Ab to Histone H3 di + trimethyl K4), H3K9me2 (1:500, ab1220, Abcam, mouse monoclonal Ab to Histone H3 dimethyl K9), Acetylated H3K9 (1:500, ab12179, Abcam, mouse monoclonal Ab
to Histone H3 acetyl K9), phosphorylated H3S10 (1:500, ab14955, Abcam mouse monoclonal Ab to Histone H3 phospo S10), Dnmt1 (1:100, sc10222, Santa Cruz Biotechnology), Dnmt3a (1:100, sc10232, Santa Cruz Biotechnology) or MeCP2 (1:500, ab50005, Abcam mAb to MeCP2). Alexafluor 488 donkey anti-mouse (1:1000, Invitrogen), Alexafluor 488 donkey anti-goat (1:1000, Invitrogen), and AlexaFluor594 donkey anti-rabbit (1:500, Invitrogen) were used as secondary antibodies. After staining, slides were mounted in DAPI (Vectashield, Vector Laboratories, CA) and covered with a 1 mm thick coverslip (VWR). Pictures were taken on the same day using confocal microscopy and a 20X objective lens (Nikon EZ-C1 3.60 build 770, Gold version). The total number of β-EP cells from each slide as well as the total number of β-EP cells that stained positive for either histone modifier proteins, Dnmts or MeCP2 in each brain was calculated. From each slide, we counted β-EP cells which are usually localized on the right and left side of the third ventricle.

**Quantitative Real-Time PCR (qRT-PCR)**

Total RNA was extracted from the mediobasal hypothalamus (MBH) using Micro to Midi Kit with Trizol (Invitrogen, Grand Island, NY). The RNA in each sample was quantitated using the NanoDrop -1000 (version 3.7, Thermo Scientific, Rockford, IL). Before RT-PCR, the RNA was treated with DNase (Qiagen, Valencia, CA) and then stored in 25 µl of Ultrapure DNase/RNase-free distilled water (Invitrogen). Afterward, 1000 ng/µl was converted to cDNA using GeneAmp PCR System 9700 (Applied Biosystems, ABi) and cDNA high-capacity RT. The RT-PCR conditions were 25°C for 10 min, 37°C for 60 min, 37°C for 60 min, 85°C for 5 minutes then kept at 4°C. After the reverse transcription reaction, RT-PCR was performed with a total volume of 25 µl of reaction mixture which contains 2.5 µl of cDNA and 22.5 µl of Universal master mix (10 X RT buffer; 25 X dNTP mix; 10 X RT primers; Multiscribe RT; RNase OUT; Nuclease free H2O; Invitrogen). PCR conditions were 50°C for 2 min for 1 cycle; 95°C for 10 min, 1 cycle, 95°C for 15 sec, and 60°C for 1 min, 40 cycles. All runs were performed in
duplicates. The ratio of mean quantity of gene of interest to the mean quantity of the housekeeping gene GAPDH was compared between different groups. All primers were designed by Abi (Table 2). RT-PCR was performed using the Abi prism 7500HT sequence detection system.

**Table 2 Real-Time PCR primers**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Assay ID</th>
<th>Ref Seq</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>Glyceraldehyde phosphate - 3-dehydrogenase</td>
<td>Rn99999916_s1</td>
<td>NM_017008.3</td>
<td>87</td>
</tr>
<tr>
<td><strong>POMC</strong></td>
<td>Proopiomelanocortin</td>
<td>Rn00595020_m1</td>
<td>NM_139326.2</td>
<td>92</td>
</tr>
<tr>
<td><strong>Dnmt1</strong></td>
<td>DNA methyltransferase 1</td>
<td>Rn01486732_g1</td>
<td>NM_053354.2</td>
<td>103</td>
</tr>
<tr>
<td><strong>Dnmt3a</strong></td>
<td>DNA methyltransferase 3a</td>
<td>Rn01469994_g1</td>
<td>NM_001003958.1</td>
<td>105</td>
</tr>
<tr>
<td><strong>G9a</strong></td>
<td>Histone lysine methyltransferase (H3K9)</td>
<td>Rn01525910_g1</td>
<td>NM_212463.1</td>
<td>104</td>
</tr>
<tr>
<td><strong>Setdb1</strong></td>
<td>Set domain bifurcated 1</td>
<td>Rn01533406_g1</td>
<td>XM_001072340.1</td>
<td>110</td>
</tr>
<tr>
<td><strong>Set7/9</strong></td>
<td>Set domain lysine methyltransferase</td>
<td>Rn01494686_m1</td>
<td>NM_001109558.1</td>
<td>101</td>
</tr>
<tr>
<td><strong>MeCP2</strong></td>
<td>Methyl CpG binding protein 2</td>
<td>Rn01529606_g1</td>
<td>NM_022673.2</td>
<td>148</td>
</tr>
<tr>
<td><strong>CBP</strong></td>
<td>CREB-binding protein</td>
<td>Rn01424795_m1</td>
<td>NM_133381.3</td>
<td>105</td>
</tr>
<tr>
<td><strong>HDAC2</strong></td>
<td>Histone deacetylase</td>
<td>Rn01407865_g1</td>
<td>NM_053447.1</td>
<td>97</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

Statistical analysis of data was performed using Graph Pad Prism software version 4.0 (LA Jolla, CA). For immunohistochemistry and qRT-PCR data, the mean values were calculated and analyzed between all groups using one-way analysis of variance (ANOVA) with Newman’s Keuhl post hoc test. All results are presented as standard error of the mean (SEM). P<0.05 was considered as significant.

**3.3 Results**

FAE altered the protein and mRNA levels of histone-modifying enzymes, DNA-methylating and MeCP2 in β-EP-producing POMC neurons. By double immunofluorescence, we found that FAE decreased H3K4 methylation (P<0.05 AF compared to AD and PF) (Figs. 7 A & B), H3K9 acetylation (P<0.01 AF compared to PF and P<0.001 AF compared to AD) (Figs. 9 A & B) and
H3S10 phosphorylation (P<0.01 AF compared to AD and PF) (Figs. 10 A & B). These are activation marks that correlate with an increase in transcriptional initiation and activation. On the other hand, FAE increased significantly the repressive mark H3K9me2 (P<0.05) in β-EP-producing POMC neurons (Figs. 8 A & B). This repressive mark is usually associated with a transcriptionally inactive or condensed chromatin. The protein data correlated with gene expression data. By qRT-PCR, we found that FAE decreased the expression of Set7/9, the enzyme that methylates H3K4 (P<0.05) (Fig. 7C). The expression of G9a and Setdb1 (Figs. 8C & D), the methylases that catalyze the methylation of H3K9me2, significantly increased in AF rats (G9a, P<0.001 AF compared to AD and P<0.05 AF compared to PF; Setdb1, P<0.05 AF compared to AD and PF). The expression of CBP, the acetyltransferase that catalyzes the acetylation of histones, was downregulated in AF rats (P<0.05) (Fig. 9C). The reduction in CBP gene expression was associated with a significant increase in the expression of HDAC2 (P<0.05) (Fig. 9D), the histone deacetylase that catalyzes the deacetylation of histones.

A.
Figure 7 Fetal alcohol exposure decreased H3K4 methylation in β-EP neurons of the arcuate area in the hypothalamus

(A) Arrows show double-stained cells for β-EP & methylated H3K4 using 20X objective lens. Alexafluor 594 Red represents β-EP staining in the arcuate area (ARC) of the hypothalamus of male rats. Alexafluor 488 green represents staining of H3K4me2,3. (B) Percentage of β-EP neurons positive for H3K4me2,3. N=5. *P < 0.05 Alcohol-fed (AF) compared to Ad libitum-fed (AD) and Pair-fed (PF). (C) Set7/9 (P<0.05 AF compared to AD & PF). N=6-9. Values are considered significant using One-way ANOVA and the Posthoc Newman’s Keuhl test for analysis.
Figure 8 Fetal alcohol exposure increased H3K9 methylation in β-EP neurons of the arcuate area in the hypothalamus

(A) Arrows show double-stained cells for β-EP & methylated H3K9 using 20X objective lens. Alexafluor 594 Red represents β-EP staining in the ARC of the hypothalamus. Alexafluor 488 green represents staining of H3K9me2 (B) Percentage of β-EP neurons positive for H3K9me2. N=5, *P < 0.05 AF compared to AD and PF. (C & D) G9a (*p<0.001 AF compared to AD and p<0.05 AF compared to PF); Setdb1 (*p<0.05 AF compared to AD and PF *P<0.01 AF compared to AD & PF). N=6-9. Values are considered significant using One-way ANOVA and the Posthoc Newman’s Keuhl test for analysis.

Figure 9 Fetal alcohol exposure decreased H3K9 acetylation in β-EP neurons of the arcuate area in the hypothalamus

(A) Arrows show double-stained cells for β-EP & acetylated H3K9 using 20X objective lens. Alexafluor 594 Red represents β-EP staining in the ARC of the hypothalamus. Alexafluor 488 green represents staining of AceH3K9 (B) Percentage of β-EP neurons positive for AceH3K9. N=5, *P < 0.01 AF compared to PF and *p<0.001 AF compared to AD. (C & D) CBP & HDAC2 (*P<0.05 AF compared to AD & PF). N=6-9. Values are considered significant using One-way ANOVA and the Posthoc Newman’s Keuhl test for analysis.
Figure 10 Fetal alcohol exposure decreased H3S10 phosphorylation in β-EP neurons of the arcuate area in the hypothalamus


FAE also altered the protein levels and gene expression of Dnmt1, Dnmt3a and MeCP2 in β-EP-producing POMC neurons. The protein level of Dnmt1 (Figs. 11 A & B), Dnmt3a (Figs. 12 A & B) and MeCP2 (Figs. 13 A & B) was significantly elevated in AF rats compared to controls (Dnmt1, P<0.01 AF compared to AD and P<0.001 AF compared to PF; Dnmt3a, P<0.05 AF compared to AD and P<0.01 AF compared to PF; MeCP2, P<0.05). This change in protein levels correlated with a parallel change in gene expression except for Dnmt3a (Dnmt1, P<0.05;
Dnmt3a, P>0.05; MeCP2, P<0.01 (Fig 11C, Fig. 12C & Fig. 13C).

A.

Figure 11 Fetal alcohol exposure increased Dnmt1 protein levels in β-EP neurons in the arcuate area of the hypothalamus

(A) Arrows show double-stained cells for β-EP & Dnmt1 using 20X objective lens. Alexafluor 594 Red represents β-EP staining in the ARC of the hypothalamus. Alexafluor 488 green represents staining of Dnmt1 (B) percentage of β-EP neurons positive for Dnmt1. N=5. *P < 0.01 AF compared to AD and PF. (C) Dnmt1 (*P<0.05 AF compared to AD and PF). N=6-9. Values are considered significant using One-way ANOVA and the Posthoc Newman’s Keuhl test for analysis.
A. Arrows show double-stained cells for β-EP & Dnmt3a using 20X objective lens. Alexafluor 594 Red represents β-EP staining in the ARC of the hypothalamus. Alexafluor 488 green represents staining of Dnmt3a (B) percentage of β-EP neurons positive for Dnmt3a. N=5. *P < 0.01 AF compared to AD and PF; *P<0.05 AF compared to AD (C) Dnmt3a (P>0.05). N=6-9. Values are considered significant using One-way ANOVA and the Posthoc Newman’s Keuhl test for analysis.

Figure 12 Fetal alcohol exposure increased Dnmt3a protein levels in β-EP neurons in the arcuate area of the hypothalamus
Figure 13 Fetal alcohol exposure increased MeCP2 protein levels in β-EP neurons in the arcuate area of the hypothalamus

(A) Arrows show double-stained cells for β-EP & MeCP2 using 20X objective lens. Alexafluor 594 Red represents β-EP staining in the ARC of the hypothalamus. Alexafluor 488 green represents staining of MeCP2. (B) Percentage of β-EP neurons positive for MeCP2. N=5, *P < 0.05 AF compared to AD and PF. (C) MeCP2 (*P<0.01 AF compared to AD and PF). N=6-9. Values are considered significant using One-way ANOVA and the Posthoc Newman’s Keuhl test for analysis.

Quantitation of gene expression data for POMC, G9a, Setdb1, MeCP2, Dnmt1 and Dnmt3a were also performed using β-actin and 18S rRNA as housekeeping genes in addition to GAPDH gene (Supplementary Data, Fig. 27 A-L).

3.4 Discussion

Our data suggest that FAE alters the components of the epigenetic machinery such as histone-modifying enzymes and DNA-methylating enzymes and induces an environment in POMC neurons conducive for gene repression. We showed that FAE causes significant decrease in the activation marks H3K4me2,3, AceH3K9 and pH3S10 and significant increase in the repressive mark H3K9me2 in β-EP-producing POMC neurons. FAE also increases the protein levels of Dnmt1, Dnmt3a and MeCP2. The protein data correlates with the gene expression data for Set7/9, CBP, HDAC2, G9a, Setdb1, Dnmt1 and MeCP2 except for Dnmt3a.
Studies showed that drugs of abuse and stress alter histone modifications and DNA methylation in different tissues and in distinct areas of the brain, thereby altering gene expression and phenotype (Kumar et al., 2005; Zhang et al., 2005; Pal-Bhadra et al., 2007; Novikova et al., 2008; Pandey et al., 2008; Hunter et al, 2009; Jiang et al., 2010 & Maze et al., 2010). For example, Maze et al. (2010) showed that repeated exposure to cocaine decreased protein levels and gene expression of G9a resulting in a decrease in H3K9 methylation in the nucleus accumbens of mice. This was associated with an altered preference of mice to drug intake. Hunter et al. (2009) showed that restraint stress for seven days reduced the levels of H3K4me3 and increased the levels of H3K9me3 in CA1 of the rat hippocampus. Transgenic mice that overexpress Setdb1 gene in the adult forebrain showed a decrease in NMDA receptor subunit NR2B and a change in behavior which suggest Setdb1 as a potential therapeutic target for treatment of altered behavior or depression (Jiang et al., 2010). In this study, FAE increased the expression of the histone methylases G9a and Setdb1 (Fig. 8 C & D). This change correlates with an increase in the repressive mark H3K9me2 (Fig. 8A & B), decrease in the activation mark AceH3K9 (Fig. 9 A & B), an increase in HDAC2 expression (Fig. 9D) and a decrease in POMC gene expression (Fig. 24A) in β-EP-producing POMC neurons. This data could suggest that G9a and Setdb1 that catalyze H3K9 methylation might have an indirect effect on POMC gene expression which could be mediated by MeCP2 binding to hypermethylated POMC gene promoter and subsequent recruitment of HDACs resulting in deacetylation of histones at lysine 9 and methylation of H3K9 hence creating a state conducive for POMC gene repression. It should be noted here that at this stage we couldn’t predict whether the changes in H3K9me2 levels upon alcohol exposure reflect changes on the entire genome or specifically on POMC gene.

Besides changes in histone modifications, FAE increased protein and mRNA levels of Dnmt1 and MeCP2 except for Dnmt3a in β-EP-producing POMC neurons. It has been demonstrated that FAE causes significant hypermethylation of POMC gene promoter at positions -62 and -216
upstream of the TSS (Govorko et al., 2011). Interestingly, the methylation of CpG at position -62 coincides with the CCAAT box which is a binding site for TFs essential for transcriptional activation. POMC gene hypermethylation in AF rats correlates with a decrease in POMC gene expression and an increase in protein levels and gene expression of Dnmt1 (Fig. 11), MeCP2 (Fig. 13) and an increase in HDAC2 expression (Fig. 9D). It has also been demonstrated in our lab by ChIP assay an increase in MeCP2 binding to POMC gene promoter in the ARC area of alcohol-exposed rats (data not published). Interestingly, MeCP2 binding to POMC gene promoter in the PVN where POMC gene is not expressed was much higher (Data not published). The methylation of the promoter region of the negative control gene Dnmt3a, a gene located very close to POMC gene on chromosome 6, was incomparable between groups (Supplementary data, Fig. 28 A &B). This finding supports our gene expression data for Dnmt3a which was unaltered upon alcohol exposure (Fig. 12C). Moreover, the percentage of global 5-mC in the ARC area was also incomparable between groups in both male and female rats (Supplementary data, Fig. 29). Collectively, these data indicate that the effect of FAE on POMC gene methylation is not global but is gene specific.

MeCP2 is abundantly expressed in postmitotic neurons specifically in the hypothalamus and could either repress or activate gene expression (Chahrour et al., 2008). It also regulates chromatin structure of several genes (Martinowich et al., 2003 & Chahrour et al., 2008). It usually binds to methylated CpG with adjacent A/T sequences in the promoter region (Klose et al., 2005). MeCP2 has been implicated in behavioral response of rodents to drug of abuse and in stress regulation. For example, Im et al. (2010) showed that knockdown (KO) of MeCP2 in the dorsal striatum decreased rat’s intake of cocaine and implicated MeCP2 in the compulsive response of rat to this drug. Fyffe et al. (2008) showed that mice with MeCP2 KO in the hypothalamus exhibited specific phenotypes such as aggression, anxiety, abnormal response to stress and hyperphagia which are endophenotypes observed in fetal-alcohol exposed rodents. In
In our study, the increase in Dnmt levels and the repressive mark H3K9me2 correlates with an increase in MeCP2 protein levels, mRNA expression (Fig. 13B & C) as well as MeCP2 binding to POMC gene promoter in the ARC (data not published). This could suggest that MeCP2 might play a specific role in POMC gene expression in the hypothalamus. To establish such possibility, we determined MeCP2 protein levels in CRH neurons of the PVN which are negatively controlled by POMC and showed high CRH immunoreactivity in the PVN of AF rats compared to controls (Supplementary data, Fig. 30). MeCP2 protein levels were incomparable between groups in CRH-producing neurons (Supplementary data, Fig. 30A & B). This indicates that the effect of FAE on MeCP2 in POMC neurons is specific and might have specific role on hypothalamic POMC gene expression in the ARC where POMC gene is highly expressed.

In the context of chromatin, the phosphorylation status of MeCP2 determines its association with or dissociation from chromatin (Zhou et al., 2006 & Tao et al., 2009). Mutation of the phosphorylation site S80 in MeCP2 decreased its binding affinity to POMC gene promoter in mice with no significant changes in gene expression (Tao et al., 2009). Investigating in the future the phosphorylation status of MeCP2 in POMC neurons of AF rats could further confirm its binding to POMC gene promoter and could confirm whether this binding has any role in POMC gene repression upon alcohol exposure. Furthermore, KO of MeCP2 in hypothalamic neuronal culture in vitro or in the hypothalamus in vivo should elucidate its role in POMC gene expression regulation.

This study provides evidence that FAE causes long-lasting changes in POMC neurons and induces an environment conducive for gene repression. FAE alters the protein levels and gene expression of Set7/9, CBP, HDAC2, G9a, Setdb1, Dnmt1 and MeCP2 except Dnmt3a. This data correlates with hypermethylation of POMC gene promoter and decrease in POMC gene expression and functions in POMC neurons of fetal alcohol-exposed rats (Govorko et al., 2011).
4 Chapter 3: Postnatal alcohol exposure alters the gene expression profile of the epigenetic machinery in LCM-captured GFP-POMC neurons

4.1 Introduction

The current study was conducted to elucidate the effect of early exposure to alcohol on the gene expression profile of other components of the epigenetic machinery in POMC neurons of adult exposed offspring. To establish this goal, we used adult GFP-POMC mice (PD70) that were fed postnatally with alcohol from PD2-PD7. This period is equivalent to the third trimester of pregnancy in humans (Clancy et al., 2007). Using this model allowed us to determine changes in gene expression profile at the cell level, POMC neuron.

Ieraci & Herrera (2007) showed that single exposure to alcohol early in life at PD7 in mice decreased neurogenesis in the adult hippocampus by inducing cell death of neural progenitor cells. Wozniak et al. (2004) showed that mice fed with alcohol at PD7 developed neuronal loss in the hippocampus and deficits in learning and memory at PD14 and PD30. Mice that were tested at PD90 or in adult stage performed well in behavioral test but neurogenesis was compromised.

Using the GFP-POMC postnatal alcohol model together with our fetal alcohol model helped us in better understanding the global effect of early alcohol exposure on the expression of the components of the epigenetic machinery in POMC neurons of adult exposed offsprings.
4.2 Materials and Methods

Animal model

The newborn mice were fed with alcohol twice a day at 1000 and 1200 from PD2-PD7 by intubation with 0.1-0.2 ml milk formula containing 11.34% (Vol/vol) alcohol (Alcohol-Fed AF) or fed an isocaloric volume of maltose dextrin (Pair-fed PF) as in Goodlet et al. (1997) with minor modifications by Sarkar et al. (2007). This milk formula supplemented with alcohol yielded a total dose of 2.5g/kg of alcohol daily. At PD21, pups were weaned and housed by sex. At PD70, AF and PF mice were sacrificed and their brains were used in this study.

GFP-POMC mice

GFP-POMC mice are transgenic mice that express the green fluorescent protein (EGFP) under the transcriptional control of mouse POMC genomic sequences (De Souza et al., 2005). The coding region of EGFP was inserted into the mouse POMC Exon 2. The 4 kb region located between -13 and -9 Kb upstream of the murine POMC gene contains neuronal enhancers (nPE1 and nPE2) required for POMC expression in the ARC of the hypothalamus (Fig. 14A, transgene 5). DeSouza et al. (2005) also reported ectopic expression of the EGFP-POMC neurons in the dentate gyrus of the hippocampus.

![Figure 14](image)

Figure 14 GFP-POMC neurons in the arcuate area of the hypothalamus of transgenic mice

(A) Transgenes 1–4 carry the whole transcriptional unit of the mouse POMC gene with the coding region of EGFP that is inserted into exon 2. Transgene 5 carries the sequences of the neuronal enhancers region (-13 to -9 Kb) (B) Shows the location of Exons 1, 2, 3 and the enhancers nPE1 and nPE2 in the mouse POMC gene (Adopted from De Souza et al. (2005)).


**Laser Capture Microdissection (LCM)**

Brains of 3AF and 3PF GFP-POMC mice were sectioned at 18 µm thickness and placed on a pre-chilled glass slide within the cryostat at -15°C. Coronal sections were collected to cover the whole arcuate area. Frozen brain sections were completely dehydrated in graded absolute ethanol solutions (75% ETOH for 1 min, 95% ETOH for 1 min, 100% pure 200 ETOH for 1 min) followed by xylene/ethanol for 1 min and xylene for 5 minutes. Slides were airdried for 10 minutes in the hood then placed in a slide box with a dessicant (VWR). Dehydrated brain sections were immediately used for capture of POMC neurons by LCM.

**RNA extraction**

Around two thousands (2000) cells that represent the whole arcuate nucleus were collected in Capsure LCM caps (LCM0214, Molecular Devices, Mountain View, CA) from 3AF and 3PF brains (Fig. 15). The thermoplastic film-coated caps containing the captured cells were incubated in 10 µl of extraction buffer at 42°C for 1 hr and 20 min then followed by microscopic observation to ensure complete cell lysis (Figs. 15 B & C). RNA was extracted from the LCM-captured cells using PicoPure RNA isolation kit (KIT0202, Arcturus, Molecular Devices, CA).

(A)  
(B)  
(C)

**Figure 15 GFP-POMC neurons in the arcuate area of GFP-POMC mice**

(A) Arrows show GFP-POMC neurons around the third ventricle. (B) LCM-captured POMC neurons before lysis. (C) LCM-captured POMC neurons after lysis.
**Determination of RNA quantity and quality**

Extracted RNAs were assessed by Agilent 2100 Bioanalyzer using Agilent RNA 6000 Pico Kit. The range of RNA quantity was 20-50 ng/µl. RNA extracted from all samples showed a ratio of 18S/28S close to 2.0, 18S and 28S rRNA peaks and a RIN value between 8 and 10 (Fig. 16). This indicates good RNA quality for downstream application such as microarray analysis.

![Figure 16](image)

**Figure 16 Determination of RNA stability from GFP-POMC mice using the Agilent Bioanalyzer**

(A) Lane L is the ladder. Lanes 2, 1 & 3 show each 2 bands of 2,000 and 4,000 nucleotides for 3 PF samples. (B & D) Shows 2 peaks, 18S and 28S, from RNA samples extracted from 2,000 POMC neurons (N=3) (C) Lane L is the ladder. Lanes 1, 2, & 3 show each 2 bands of 2,000 and 1,000 nucleotides for 3 AF samples. (E) Blank as a negative control.

**Microarray Analysis**

Extracted RNAs was amplified using WTA-Ovation Pico RNA Amplification System and following the manufacturer’s instructions (Cat # 3300, Nugen Technologies Inc., CA).
amplification yield is presented in Table 3. A minimum of 5 µg/μl of amplified DNA was used for downstream applications. The amplified single-stranded cDNA from each sample was labeled using NuGen Encore Biotin Module (Cat#4200-12, NuGen Technologies Inc.) and hybridized to Affymetrix Mouse Genome 430 2.0 Array. Microarray analysis was done by Dr. Paul Vau Hummelen (EOHSI, Rutgers University).

**Table 3 Amplification yield of DNA from LCM-captured POMC neurons**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Amplification yield (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF1</td>
<td>5.58</td>
</tr>
<tr>
<td>AF2</td>
<td>6.21</td>
</tr>
<tr>
<td>AF3</td>
<td>5.37</td>
</tr>
<tr>
<td>PF1</td>
<td>5.34</td>
</tr>
<tr>
<td>PF2</td>
<td>6.75</td>
</tr>
<tr>
<td>PF3</td>
<td>5.19</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

The gene expression data was normalized with a standard RMA method. To analyze statistically differentially expressed genes between AF and PF samples, a LIMMA statistics was applied without multiple correction and a P<0.001 (Statistical analysis was done by Dr. Paul Vau Hummelen at EOHSI, Rutgers University).

**4.3 Results**

Microarray analysis revealed differential significant expression of 20 genes with a P<0.001 with 2 fold change and around 2,000 genes with a P<0.001 and a P<0.05 with less than 2 fold change in linear scale. Genes showing expression less than 2 fold change between groups were considered as non-significant in Microarray analysis. We linked particular sets of genes to biological functions including methylation, transcriptional and translational regulation, alcohol metabolism, folate metabolism and immunity (Table 4). Table 5 shows the P-value for each gene.
and the fold change in expression in AF samples as compared to control PF in a linear and log scale.

*POMC* gene expression in AF mice was lower than that of controls (PFs) but this decrease in expression was found by microarray as non significant (P=0.156, fold change in linear scale 0.75 and fold change in log scale -0.41). qRT-PCR demonstrated significant decrease in *POMC* gene expression in two AF samples out of three compared to PF samples. The mean ratio of POMC/GAPDH in 2 AF samples was 1.10 compared to the mean ratio of POMC/GAPDH of 1.65 in 3 PF samples. The low number of tested samples could not provide statistical significance in terms of *POMC* gene expression in AF compared to PF samples.
Table 4 Biological functions of selected genes from Microarray analysis data

<table>
<thead>
<tr>
<th>Genes related to epigenetics</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met5d1</td>
<td>SAM-dependent methyltransferase</td>
</tr>
<tr>
<td>Ezh2</td>
<td>H3K27 methylation for repression</td>
</tr>
<tr>
<td>Rcor2</td>
<td>Repressor, repress REST and binds MeCP2 &amp; HDAC2</td>
</tr>
<tr>
<td>REST</td>
<td>Associates with CoREST &amp; HDAC1</td>
</tr>
<tr>
<td>Myst3</td>
<td>Histone acetyltransferase (HAT)</td>
</tr>
<tr>
<td>MBD1</td>
<td>Methyl-binding protein 1 – binds methylated DNA</td>
</tr>
<tr>
<td>Metl6</td>
<td>Methyltransferase activity</td>
</tr>
<tr>
<td>Jmjd2d</td>
<td>Lysine demethylase, demethylates H3K4me2,3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes related to mRNA</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lsm2</td>
<td>mRNA splicing - role in transcription</td>
</tr>
<tr>
<td>Stat1</td>
<td>TF - activation of transcription</td>
</tr>
<tr>
<td>Dcp1b</td>
<td>Responsible for decapping of mRNA - role in translation</td>
</tr>
<tr>
<td>Tafl</td>
<td>Responsible for initiation of transcription</td>
</tr>
<tr>
<td>Eif4e2</td>
<td>Phosphorylated form binds to Cap important for transcription</td>
</tr>
<tr>
<td>Creb5</td>
<td>CRE-dependent transactivator</td>
</tr>
<tr>
<td>Eif2ak1</td>
<td>Eukaryotic translation initiation factor 2 alpha kinase 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes related to alcoholism</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gstm1</td>
<td>Glutathione transferase</td>
</tr>
<tr>
<td>Ahcy2</td>
<td>Codes for SAHH</td>
</tr>
<tr>
<td>ADH7</td>
<td>Essential for RA synthesis for cellular differentiation</td>
</tr>
<tr>
<td>Aox1</td>
<td>Oxidizes alcohol to acetaldehyde and H2O2</td>
</tr>
<tr>
<td>Cyp2e1</td>
<td>Responsible for alcohol oxidation in brain</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes related to folate &amp; SAM</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cept1</td>
<td>Role in PC &amp; PEA synthesis</td>
</tr>
<tr>
<td>Mthfr</td>
<td>Role in the folate cycle</td>
</tr>
<tr>
<td>Tpmt</td>
<td>Thiopurine methyltransferase-Use SAM to methylate Thiopurine</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes related to Immunity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Socs3</td>
<td>Role in immunity and POMC gene regulation</td>
</tr>
<tr>
<td>Stat1</td>
<td>TF - activator of transcription that binds Stat3</td>
</tr>
<tr>
<td>Mpeg1</td>
<td>Macrophage-expressed gene</td>
</tr>
</tbody>
</table>

References: Sartre et al., (1994); Alonso et al., (1999); Lachance et al., (2002); Zhan et al., (2004); Ichimura et al., (2005); Olthof & Verhoef (2005); Lakowski et al., (2006); Xu et al., (2007); Hamid et al., (2009)
Table 5 Statistical analysis of Microarray data
(n=3, Green=downregulated, Red=upregulated)

<table>
<thead>
<tr>
<th>Genes related to epigenetics</th>
<th>p value</th>
<th>Fold change in Linear scale</th>
<th>Direction in AF</th>
<th>Fold change in Log scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mett5d1</td>
<td>0.002</td>
<td>1.9</td>
<td>Down</td>
<td>-0.89</td>
</tr>
<tr>
<td>Ezh2</td>
<td>0.006</td>
<td>1.6</td>
<td>Down</td>
<td>-0.68</td>
</tr>
<tr>
<td>Rcor2</td>
<td>0.011</td>
<td>1.4</td>
<td>up</td>
<td>0.53</td>
</tr>
<tr>
<td>REST</td>
<td>0.029</td>
<td>1.5</td>
<td>Down</td>
<td>-0.55</td>
</tr>
<tr>
<td>Myst3</td>
<td>0.012</td>
<td>1.5</td>
<td>Down</td>
<td>-0.56</td>
</tr>
<tr>
<td>MBD1</td>
<td>0.028</td>
<td>2.0</td>
<td>up</td>
<td>1.01</td>
</tr>
<tr>
<td>Mettl6</td>
<td>0.028</td>
<td>1.3</td>
<td>up</td>
<td>0.41</td>
</tr>
<tr>
<td>Jmjd2d</td>
<td>0.032</td>
<td>1.6</td>
<td>up</td>
<td>0.67</td>
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<table>
<thead>
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<th>Genes related to mRNA</th>
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<th>Direction in AF</th>
<th>Fold change in Log scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lsm2</td>
<td>0.000</td>
<td>1.9</td>
<td>Down</td>
<td>-0.96</td>
</tr>
<tr>
<td>Stat1</td>
<td>0.001</td>
<td>1.8</td>
<td>Down</td>
<td>-0.84</td>
</tr>
<tr>
<td>Dep1b</td>
<td>0.005</td>
<td>1.8</td>
<td>up</td>
<td>0.81</td>
</tr>
<tr>
<td>Tafl</td>
<td>0.006</td>
<td>1.5</td>
<td>Down</td>
<td>-0.61</td>
</tr>
<tr>
<td>Eif4e2</td>
<td>0.006</td>
<td>1.7</td>
<td>up</td>
<td>0.78</td>
</tr>
<tr>
<td>Creb5</td>
<td>0.035</td>
<td>1.6</td>
<td>Down</td>
<td>-0.65</td>
</tr>
<tr>
<td>Eif2ak1</td>
<td>0.025</td>
<td>1.4</td>
<td>up</td>
<td>0.48</td>
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</table>

<table>
<thead>
<tr>
<th>Genes related to alcoholism</th>
<th>p value</th>
<th>Fold change in Linear scale</th>
<th>Direction in AF</th>
<th>Fold change in Log scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gstm1</td>
<td>0.012</td>
<td>1.5</td>
<td>up</td>
<td>0.57</td>
</tr>
<tr>
<td>Ahcy2</td>
<td>0.014</td>
<td>1.5</td>
<td>up</td>
<td>0.61</td>
</tr>
<tr>
<td>ADH7</td>
<td>0.015</td>
<td>1.8</td>
<td>Down</td>
<td>-0.83</td>
</tr>
<tr>
<td>Aox1</td>
<td>0.001</td>
<td>1.9</td>
<td>Down</td>
<td>-0.92</td>
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<td>Cyp2e1</td>
<td>0.024</td>
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<td>-0.47</td>
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</table>

<table>
<thead>
<tr>
<th>Genes related to folate &amp; SAM</th>
<th>p value</th>
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<th>Direction in AF</th>
<th>Fold change in Log scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cep1l</td>
<td>0.029</td>
<td>1.4</td>
<td>Down</td>
<td>-0.51</td>
</tr>
<tr>
<td>Mthfr</td>
<td>0.047</td>
<td>1.3</td>
<td>up</td>
<td>0.36</td>
</tr>
<tr>
<td>Tpmt</td>
<td>0.014</td>
<td>1.4</td>
<td>Down</td>
<td>-0.48</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes related to immunity</th>
<th>p value</th>
<th>Fold change in Linear scale</th>
<th>Direction in AF</th>
<th>Fold change in Log scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Socs3</td>
<td>0.000</td>
<td>2.2</td>
<td>Down</td>
<td>-1.17</td>
</tr>
<tr>
<td>Stat1</td>
<td>0.001</td>
<td>1.8</td>
<td>Down</td>
<td>-0.84</td>
</tr>
<tr>
<td>Mpeg1</td>
<td>0.012</td>
<td>1.7</td>
<td>Down</td>
<td>-0.79</td>
</tr>
</tbody>
</table>
4.4 Discussion

The current study showed that alcohol exposure alters the gene expression profile of the components of the epigenetic machinery as well as the expression profile of genes related to transcriptional/ translational machinery, alcohol metabolism, folate metabolism and immunity in POMC neurons. In this study, we focused on those genes related to epigenetics.

Change in gene expression of Mett5d1, Rcor2, Myst3, MBD1 and Jmjd2d indicates that alcohol exposure induced an environment conducive for gene repression in POMC neurons. Mett5d1 gene, a SAM-dependent methyltransferase, was downregulated (1.9 fold change) indicating low SAM availability in POMC neurons which is induced by alcohol exposure. The expression of the repressor Rcor2 which binds to HDAC2 and MeCP2 was upregulated (1.4 fold change). This upregulation correlates with an increase in HDAC2 and MeCP2 expression that we found in Aim 1 (Fig. 9D & Fig.13C). MBD1 (2 fold change) was also upregulated in AF mice. This methylbinding protein inhibits binding of the TF Sp-1 to the promoter region resulting in gene repression (Ichimura et al., 2005). It also forms a complex with Setdb1 and associates with REST to mediate gene repression (Sarraf & Stancheva, 2004; Ichimura et al., 2005 & Lakowski et al., 2006). The changes in MBD1 gene expression indicate that alcohol exposure induces DNA hypermethylation in POMC neurons. This upregulation in MBD1 gene expression mirrors the increase in protein and mRNA levels of another methyl-CpG- binding protein, MeCP2, in POMC neurons (Fig. 13). It should be noted here that unlike MBD1, MeCP2 could play a role in POMC gene expression regulation since it is highly expressed in the hypothalamus and has high affinity binding site on POMC gene in mice (Tao et al., 2009). The HAT MYST3 (1.5 fold change) was downregulated similar to CBP (Fig. 9C) which implicates that alcohol exposure induces deacetylation of histones. Finally, Jmjd2d, a demethylase that demethylates H3K4me2,3, was downregulated (1.6 fold change). This confirms our results in Aim1 where
we found a significant decrease in the activation mark H3K4me2,3 (Fig. 7A) and a significant decrease in Set7/9 expression (Fig. 7B) in AF rats.

Microarray data did not show a significant decrease in POMC gene expression upon alcohol exposure in AF mice compared to PFs. This could be explained by the low number of sample tested (N=3) and to the limitations of this method that might provide nonspecific or definitive results. qRT-PCR confirmed a decrease in POMC gene expression in two AF samples compared to 3 PF samples. We did not achieve statistical significance between control and treated groups because of the low number of sample tested.

This study shows that early alcohol exposure alters the gene expression of the components of the epigenetic machinery in POMC neurons.
5  Chapter 4: Fetal alcohol exposure alters the level of histone marks H3K4me3 and H3K9me2 along POMC gene

5.1  Introduction

The changes that we found in protein and mRNA levels of histone-modifying enzymes in β-endorphin-producing POMC neurons of fetal alcohol-exposed adult offspring reflect global changes in the entire genome in POMC neurons not specifically changes along POMC gene. We found that the deficit in POMC gene expression in fetal alcohol-exposed rats correlates with POMC gene promoter hypermethylation (Govorko et al., 2011). This deficit in POMC gene expression could also be mediated by histone modifications. The goal of the current study is to investigate whether FAE causes any changes in histone marks occupancy along POMC gene. We used Chromatin immunoprecipitation assay (ChIP) to profile H3K4me3 and H3K9me2 changes along POMC gene in response to FAE in adult offspring. Methylation of H3K4 is associated with transcriptionally active relaxed chromatin while methylation of H3K9 is associated with transcriptionally inactive condensed chromatin.

Histone marks have specific distribution along the gene which could be modulated by environmental factors or drug exposure leading to changes in transcriptional outcome (Chen et al., 2006; Im et al., 2010 & Maze et al., 2010). The role of histone modifications in gene splicing has been recently demonstrated and defects in this process have been observed in many diseases (Greene et al., 2007; Kashima et al., 2007; Bell et al., 2010 & Kumari & Usdin, 2010). POMC gene has complex structure and complex mode of regulation in different tissues (Eberwine & Roberts, 1983). The regulatory factors, especially epigenetic factors that could regulate POMC gene expression in the brain, are not identified. Structurally, POMC gene
consists of 3 exons, 2 large intronic regions and 2 CpG islands, a 5’upstream and 3’ downstream CpG islands. Interestingly, Exon 3, “the protein-coding sequence” from which β-EP peptide is derived, is larger in size (833 bp) compared to the other two exons and has the location of the downstream 3’ CpG island (Eberwine & Roberts, 1983; Gardiner-Garden & Frommer, 1994 & Raffin-sanson et al., 2003).

Several studies mapped nucleosome positioning along gene body and reported differences in chromatin landscape in TSS and in exonic versus intronic regions. Most protein-coding genes have well-positioned -1 and +1 nucleosome around their first exon, a nucleosome-free region (NFR) just upstream of the TSS and random distribution away from -1 and +1 nucleosomes (Jiang & Pugh, 2009). For example, nucleosomes around the first exon or TSS usually carry the activation marks H3K4me3 or acetylated H3K9 in actively expressed genes but the repressive marks H3K9me2,3 and H3K27me3 in inactive or suppressed genes (Anderson et al., 2009).

Besides TSS, internal exons and introns carry nucleosomes with specific histone modification and specific function in terms of regulation of gene expression. It is suggested that histone marks in internal exons or in intronic regions might act as “speed bumps” to slow down the rate of RNA Polymerase II during transcriptional elongation or act as recognition elements for the components of the splicing machinery to regulate gene splicing (Ringrose 2010 & Schwartz et al., 2010). For example, the activation mark H3K4me3 plays a role in transcriptional activation and initiation in the TSS. Its presence in internal exons plays a role in modulation of gene splicing (Sims et al., 2007). The repressive marks H3K9me2,3 and H3K27me2,3 are usually depleted in exons of expressed genes (Dhami et al., 2010).

Other studies determined alterations in specific histone marks along genes in response to external changes or in some neurological disorders. For example, Fu et al. (2009) demonstrated that the histone code along the rat hepatic gene Insulin growth factor (IGF-I) changed under the stress of
intrauterine growth retardation (IUGR) in a sex-specific manner and this change persisted
postnatally in the offspring. Greene et al. (2007) found an increase in H3K9me2 levels along
intron 1 of Freidrich Ataxia (FXN) gene which possibly impeded RNA polymerase II elongation
and decreased FXN gene expression. The use of TSA enhanced significantly FXN gene
transcription. Moreover, lower levels of the repressive marks H3K9me2 and H3K27me3 were
detected upstream of Fragile X Mental Retardation 1 (FMRI) gene in Fragile X Syndrome
patients while H3K9me3 was more localized in exon 1 of the gene in the CGGCCG repeat
resulting in gene silencing (Kumari & Usdin, 2010).

No study was done before to map changes in histone marks such as H3K4me3 and H3K9me2
along the length of POMC gene of controls and fetal alcohol-exposed rats. To further
investigate the epigenetic effects of FAE on POMC gene, we quantitated the levels of the
activation mark H3K4me3 and the repressive mark H3K9me2 along POMC gene by Chromatin
immunoprecipitation assay (ChIP).

5.2 Materials and Methods

Animal model

Sprague-Dawley female rats were purchased from Charles River and maintained in the Bartlett
Animal Facility where they were individually housed with 12-h light/12-h dark cycles (lights on
at 7:00 h and off at 19:00 h) at a constant temperature (22°C) throughout the study. On
GD7-21, a period equivalent to the first and second trimesters of pregnancy in humans (Clancy et
al., 2007), pregnant rats were fed rat chow ad libitum fed (AD), a liquid diet containing ethanol
(BioServe Inc., Frenchtown, NJ) alcohol-fed (AF), or pair-fed an isocaloric liquid control diet
(with the ethanol calories replaced by maltose-dextrin) (PF). The concentration of ethanol
varied (1.7-5.0% v/v) in the diet for the first 4 days to habituate the animals with the alcohol diet.
After this habituation period, animals were fed the liquid diet containing ethanol at a
concentration of 6.7% v/v that maintained an average blood alcohol level between 130 and 150 mg/dl (Chen et al., 2006), which is within the range of blood alcohol concentrations achieved following binge drinking in humans (White et al., 2011). It should be noted that the rat, an altricial species, is an animal model for the midgestational brain differentiation in humans. Additionally, CDC reports that about 1 in 8 pregnant women drinks alcohol in the United States. Therefore, the animal model we used represents alcohol-drinking effect during the midgestational period. AF and PF litters were cross-fostered using untreated lactating rats to prevent any compromised nurturing by the AF lactating mother rats. Litter size was maintained as 8 pups/dam. At postnatal day PD22, pups were weaned, housed by sex, and provided rodent chow meal and water ad libitum. Male and female rats (PD60-65) were used in this study.

**Chromatin Immunoprecipitation Assay (ChIP)**

ChIP assay was performed following Belden et al. (2007) with minor modifications. Arcuate punches were isolated from six brains of controls (ADs and PFs) or treated groups (AFs) (N=7-8). Punches were washed in cold PBS (1X). DNA-protein crosslinking was done with 37% formaldehyde to a 1% final concentration at 4°C for 15 minutes. Crosslinking was stopped by 2.5M glycine followed by 10 minutes incubation at 4°C. After centrifugation at 2,000 rpm for 5 minutes, pellet was washed twice in cold PBS (1X) and then homogenized in ice cold 1ml of buffer I (0.3M sucrose, 15mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 15 mM Tris PH=7.5) till no white clumps are visible. After centrifugation (10,000 rpm) at 4°C for 14 mins, the pellet was homogenized in buffer I and overlayed with equal volume of buffer II (0.3M sucrose, 15mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 15 mM Tris PH=7.5 and 0.4% of IGEPAL(CA-630, 18896, Sigma). The mixture was then kept on ice for 10 minutes after which buffer III (1.2M sucrose, 15mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 15 mM Tris PH=7.5) was added and then centrifuged at 12,000 rpm at 4°C for 20 mins. All buffers were supplemented with a cocktail of protease inhibitors (0.1M Pepstatin A, 0.1 M Leupeptin, 0.1M PMSF, 1M DTT). Nuclei were
suspended in Micrococcal nuclease (MNase) digestion buffer (0.32 M sucrose, 50 mM Tris-HCl, PH=7.5, 4 mM MgCl2, 1 mM CaCl2 and 0.1 mM PMSF) and kept at -80°C. The conditions of digestion of nuclei by MNase (Takara, 2910A) were optimized and performed at 37°C. The MNase enzymatic reaction was stopped by 0.1M EDTA PH=8. After MNase digestion, the mixture was treated with lysis buffer (0.1M NaHCO3 and 20% SDS), then 5M NaCl and incubated at 65°C for 5 hrs or O/N to reverse DNA-protein crosslinking. After the reverse crosslink, the mixture was incubated in presence of 0.5M EDTA PH=8, 1M Tris PH=6.5, 10 mg/ml of proteinase K (Invitrogen) at 42°C for 1 hour. Phenol-chlorofrom extraction was done followed by precipitation with 1/10 volume of 3M sodium acetate and ethanol. The digestion of chromatin by MNase was assessed on a 1.5% agarose gel stained with ethidium bromide. At this stage, 5 µl of DNA from each sample was kept aside as a total input for qRT-PCR. H3K4me3 (Active motif 39159) and H3K9me2 (abcam1220) were the test antibodies and rabbit IgG (abcam 46450) was used as a negative control antibody. Test and negative control antibodies and beads, Dyna beads protein A (Invitrogen, 100.01D) or Dyna beads protein G (Invitrogen, 100.03D), were added in separate reactions to the chromatin lysate and incubated on a rotator at 4°C O/N. Beads were then washed 5X with IP buffer (1M HEPES PH=7.4, 5M NaCl, 0.5M EDTA PH=8, 20% TritonX100, 20% SDS, and protease inhibitor cocktail) and the precipitates were recovered with elution buffer. DNA-protein reverse crosslink was done as described above. After pooling, DNA was recovered from the eluate using Qiaquick PCR purification kit (Qiagen 28104) according to the manufacturer’s instructions.

**Quantitative Real-Time PCR (qRT-PCR)**

qRT-PCR was performed using the ABI prism 7500 HT sequence detection system. A total mix of 20 µl containing the input or immunoprecipitated DNA, SYBR green mix, DNAse/RNAase free water and 1 µl of reverse and forward primers was prepared. PCR conditions were optimized and were as follows: 1 cycle 94°C, 5 mins; 35-40 cycles 95°C, 30sec, 58°C, 30sec,
72°C, 30 sec; followed by a dissociation stage, 95°C/15 sec, 60°C/1 min, 95°C/15 sec, 60°C/15 sec.

PCR products were resolved on 10% acrylamide gel and bands were visualized by ethidium bromide staining. Input and immunoprecipitated DNA were run in duplicates and non-template controls (NTCs) were used in each run.

**Design of primers along POMC gene**

A set of 17 pair of primers, forward and reverse, were designed 500 or 200 bp apart along Exon 1, Exon 2, Exon 3, Intronic regions 1 & 2, part of 3’ and 5’UTRs of the rat POMC gene using the reference sequence Ensembl (transcript ID ENSRNOT0000016976) and UCSC genome browser. A set of 6 pair of primers were also designed along Exon 3 of POMC gene. Oligos size was around 24 nucleotides and amplicon sizes were 80-120 bp. The Tm ranges of primers were 60-65°C ((Integrated DNA Technologies, USA). The sequence of POMC primers that showed positive results are listed in Table 6: P2 primer cover the area around the TSS and P18 covers Exon3. We also designed primers for histone H4 gene which is a negative control gene for H3K9 methylation, primers for SAT2 gene which is a negative control gene for H3K4 methylation and primers for GAPDH gene which acts as a positive control for H3K4 and H3K9 methylation (Table 6).

**Table 6 Sequence of ChIP primers for qRT-PCR**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Tm</th>
<th>Bases</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>F: 5’ CTG AGT GGA GAT CCA ACA GCA GCC TCC TT 3’</td>
<td>60.3</td>
<td>25</td>
<td>80-120</td>
</tr>
<tr>
<td></td>
<td>R: 5’ AGC AGA TGT GCC TGG AAA GTG CGG A A 3’</td>
<td>64.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P18</td>
<td>F: 5’ TAT CGG GTG GAG CAC TTT CGC T 3’</td>
<td>62.6</td>
<td>22</td>
<td>80-120</td>
</tr>
<tr>
<td></td>
<td>R: 5’ TGG CTC TTC TCG GAG GTC ATG AAG 3’</td>
<td>60.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>F: 5’ CAG CCA CCA TTA GGG CAC TTG AAA 3’</td>
<td>59.8</td>
<td>24</td>
<td>80-120</td>
</tr>
<tr>
<td></td>
<td>R: 5’ CCC AGG ACA ATT GTT GCT TTG CTC 3’</td>
<td>59.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAT2</td>
<td>F: 5’ TGA GCT GTA GGT CCT TTC TGC GGT T 3’</td>
<td>61.9</td>
<td>25</td>
<td>80-120</td>
</tr>
<tr>
<td></td>
<td>R: 5’ TGG AAG CCA TCC TAA GCC TCA CTG CTA 3’</td>
<td>63.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’ ATG AGC CCT TCC ACG ATG CCA AAG TT 3’</td>
<td>62.7</td>
<td>26</td>
<td>80-120</td>
</tr>
<tr>
<td></td>
<td>R: 5’ AAT GCA TCC TGC ACC ACC AAC TAC TT 3’</td>
<td>63.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Statistical Analysis

Data represent the amount of immunoprecipitated DNA as a percentage of total input. The values were normalized as a ratio of the positive control gene GAPDH. The results represent the values of two trials (N=7-8). Differences between groups were assessed using Friedman nonparametric One way analysis of variance (ANOVA) with a Dunn’s Multiple Comparison post-hoc analysis at the level of α=0.05. A P<0.05 is considered significant difference.

5.3 Results

MNase digestion of nuclei prepared from male and female arcuate punches yielded DNA fragments below 500 bp in size as shown in Fig.17. Bands were visualized on 1.5% agarose gel stained with ethidium bromide (Sigma).

Figure 17 MNase digestion of nuclei isolated from arcuate punches of rats
(A-D) MNase digestion of chromatin of AD, AF and PF male and female samples as seen on 1.5% agarose gel. L = 1 Kb DNA ladder.

We determined the changes upon FAE in the activation histone mark H3K4me3 and the repressive mark H3K9me2 along POMC gene. Using P18 primer which was designed +5.5 Kb downstream of POMC gene TSS (Fig. 18), we found that FAE decreased significantly the activation mark H3K4me3 along Exon 3 of POMC gene in male and female rats (P<0.05) (Figs. 19 A & B). This modification was not seen in the promoter region of POMC gene using P2 primer. H3K4me3 level, as expected, was undetectable in intronic regions 1 & 2 and along the negative control gene satellite 2 (SAT2), which is a sequence of heterochromatic region adjacent to the centromere of chromosome 1, in both treated and control groups. Using P18 primer, we found an increase in the repressive mark H3K9me2 in AF rats compared to controls along Exon 3 of POMC gene but incomparable between groups in both male and female rats (P>0.05) (Figs. 20 A & B). Interestingly, the direction of changes or the occupancy of H3K4me3 and H3K9me2 along Exon 3 was opposite as expected since these two modifications are exclusive. Using primer P2 which was designed -300 bp upstream of POMC gene promoter, we found a moderate increase in the repressive mark H3K9me2 in AF rats compared to controls and present at low levels in that region (P>0.05) (Figs. 21 A & B). Finally, H3K9me2 was undetectable in intronic regions 1 & 2. This modification was also undetectable, as expected, along the negative control gene histone H4. The amount of immunoprecipitated DNA from control and treated samples using the negative control antibody IgG was lower compared to the amount of immunoprecipitated DNA using the test antibodies (H3K4me3 or H3K9me2).
Figure 18 Location of ChIP primers along POMC gene

*POMC* gene structure and location of ChIP primers along *POMC* gene (E1-Exon 1, E2=Exon2, E3=Exon 3, TSS=Transcription start site, green= location of the 5’ upstream CpG island and the 3’ downstream CpG island. P2=primer designed 300 bp upstream of the TSS; P18=primer designed 5.5 Kb downstream of the TSS.

Figure 19 Fetal alcohol exposure decreased H3K4me3 along Exon 3 of POMC gene in the arcuate area of the hypothalamus

(A & B) Percentage of total input of H3K4me3 immunoprecipitation along Exon 3 of *POMC* gene of male and female AD, AF or PF offspring using P18 primer. *P<0.05 AF compared to AD and PF. Values are considered significant using Friedman test and the Posthoc Dunn’s multiple comparison test for analysis.

Figure 20 Fetal alcohol exposure increased insignificantly H3K9me2 along Exon 3 of POMC gene in the arcuate area of the hypothalamus
(A & B) Percentage of total input of H3K9me2 immunoprecipitation along Exon 3 of *POMC* gene of male and female AD, AF or PF offspring using P18 primer. P>0.05 AF compared to PF and AD. Values are considered nonsignificant using Friedman test and the Posthoc Dunn’s multiple comparison test for analysis.

![Bar chart](image)

**Figure 21** Fetal alcohol exposure did not alter H3K9me2 in POMC gene promoter in the arcuate area of the hypothalamus

(A & B) Percentage of total input of H3K9me2 immunoprecipitation in POMC gene promoter of male and female AD, AF or PF offspring using P2 primer. P>0.05 AF compared to PF and AD. Values are considered nonsignificant using Friedman test and the Posthoc Dunn’s multiple comparison test for analysis.

### 5.4 Discussion

We showed in this study that FAE causes distinctive spatial changes in histone modifications such as the activation mark H3K4me3 and the repressive mark H3K9me2 along *POMC* gene. We found depletion of H3K4me3 mark around the TSS of *POMC* gene in controls (AD and PF) and treated groups (AFs) and a significant reduction in its occupancy along Exon3 of AF rats compared to controls in both male and female rats (Fig. 18 A & B). Moreover, FAE increased H3K9me2 levels along Exon 3 (Fig. 19 A & B) and around the TSS of *POMC* gene (Fig. 20 A & B) of AF rats compared to controls.

The activation mark H3K4me3 acts as a “docking site” for CHD1 of the ATPase chromatin-remodeling complex and also acts as a “recognition signal” for the recruitment of the components of the splicing machinery (Sims et al., 2007; Ross & Beggs, 2010 & Schwartz et al., 2010). Our
finding related to H3K4me3 reduction along Exon 3 of *POMC* gene in AF rats is novel and could suggest two scenarios: 1) reduction of H3K4me3 mark along Exon 3 of *POMC* gene could modulate the recruitment of splicing factors, affect the rate of RNA polymerase II elongation and alter the efficiency of Exon 3 splicing resulting in the generation of short ncRNAs with regulatory output on *POMC* gene expression; 2) reduction of H3K4me3 along Exon 3 could affect its recognition by the splicing machinery or the recruitment of the later resulting in partial splicing of that exon and generation of truncated transcripts with no physiological function. The depletion of H3K4me3 mark at the 5’ end around the TSS of *POMC* gene in control rats was unexpected. It could imply that *POMC* gene regulatory process in the promoter region requires multifaceted histone modifications other than H3K4me3 such as acetylated H3K9. Interestingly, *POMC* gene has a 5’ CpG island in its promoter and another downstream 3’ CpG island in Exon 3. Moreover, Exon 3 of *POMC* gene is located next to a transcriptional unit TSS that could generate short transcripts (Gardiner-Garden & Frommer, 1994). It is demonstrated that short transcripts can be produced from TSS distinct from the mRNA TSS in the promoter region and ncRNAs could be generated from an inefficient RNA Polymerase II elongation induced by histone marks (Kaikkonen et al., 2011). These facts reinforce our assumption that reduction of H3K4me3 marks in AF rats along Exon 3 could negatively modulate the efficiency of *POMC* gene splicing resulting in a lower number of generated transcripts. Such attractive assumption requires further investigation in the future. In this study, the CT value for H3K4me3 using primers for Intron 1 and Intron 2, was undetectable in control and treated groups. This indicates the absence of this mark in intronic regions of *POMC* gene, as expected (Barski et al., 2007).

We found an increase in the repressive mark H3K9me2 around the TSS and along Exon 3 of *POMC* gene in AF rats compared to controls but this change was statistically nonsignificant between groups. However, the occupancy of this repressive mark in POMC TSS indicates that FAE induces an environment around the TSS conducive for *POMC* gene repression. It also
suggests that this repressive mark in the promoter region does not play a role in regulation of POMC gene expression rather the latter is regulated by DNA methylation of its promoter (Govorko et al., 2011). Interestingly, the occupancy of the mutually exclusive histone marks H3K4me3 and H3K9me2 changed in opposite direction as expected along Exon 3 of POMC gene. H3K9me2 modification was not seen in intronic regions 1 & 2. Studies reported that the location of the repressive mark H3K9 in the gene body might play a role in gene splicing. For example, Allo et al. (2009) found an interesting association between splicing patterns of genes and changes in the repressive marks H3K9me2 and H3K27me3. Luco et al. (2011) demonstrated that H3K9 methylation in intronic region slowed down the elongation rate of RNA Polymerase II and induced the exclusion of the intron and the inclusion of exon 33 (E33) of fibronectin gene. TSA altered the splicing of E33 and modulated H3K9 levels which could implicate H3K9 methylation in splicing. In our study, the occupancy of H3K9me2 in the gene body was minimal indicating that this mark does not play a role in regulation of POMC gene expression. Future studies should investigate whether the other repressive mark H3K27 might have a role along POMC gene promoter upon alcohol exposure.

Overall, this study shows distinctive spatial distribution of H3K4me3 and H3K9me2 along POMC gene which reflects modulation of its landscape in response to alcohol exposure. FAE causes a significant reduction in the occupancy of H3K4me3 mark along Exon 3 with no change increase in the repressive mark H3K9me2 in the promoter region of POMC gene.
CHAPTER 5

6  Chapter 5: Gestational choline normalizes the protein and gene levels of histone-modifying and DNA-methylating enzymes in β-endorphin neurons

6.1  Introduction

Alcohol drinking during pregnancy is a major health problem worldwide and a leading cause of mental retardation in the United States (Sokol et al., 2003). Children who are prenatally exposed to alcohol show behavioral and physiological changes later in life such as depression, anxiety, hyperactivity, and a reduced ability to cope with stressful situations (Famy et al., 1998; Haley et al., 2006; Hellmans et al., 2008; Weinberg et al., 2008 & Guerri et al., 2009). They also show immune and metabolic-related diseases (Arjona et al., 2006 & Ting & Lautt, 2006). POMC neurons of the hypothalamus are one of the major regulators of the HPA axis activity, immune functions and energy homeostasis (Raffin-sanson et al., 2003; Gianoulakis, 2004 & Boyadjieva et al., 2009). A prenatal alcohol- exposed animal model, which mimics fetal alcohol exposure in humans, shows deficit in the number of POMC neurons in the hypothalamus with a decrease in POMC gene expression and production of its derived peptide β-endorphin (β-EP) (Sarkar et al., 2007). Although the effects of fetal alcohol exposure are very well documented, our understanding of how prenatal alcohol exposure causes dysfunction of POMC neurons in the adult stage is not studied before.

Evidence is emerging that prenatal exposure to environmental factors such as alcohol, drugs or toxins could cause long-lasting epigenetic modifications (Jirtle & Skinner, 2007 & Govorko et al., 2011). Epigenetic mechanisms such as histone modifications and DNA methylation are involved in long-term “maternal programming” of the stress axis or HPA axis and in the development of individual differences in response to stress in adulthood (Weaver et al., 2004).
Abnormal changes in histone modifications and/or DNA methylation alter gene expression and result in abnormal cellular functions with long-term adverse effects on phenotypes (Bird, 2001; Esteller & Almouzni, 2005; Graff & Mansu, 2008 & Vaissiere et al., 2008). For example, these changes have been associated with hyperactivity in children and with some psychiatric disorders (Mill et al., 2008 & Mill & Petronis 2008).

Alcohol ingestion inhibits folic acid absorption and decreases the availability of the methyl-donor, S-Adenosyl methionine (SAM), which is critical for methylation processes during embryonic development (Wang et al., 2009). Folic acid supplementation during the gestational period suppresses the toxic effects of alcohol during development (Xu et al., 2006 & Serrano et al., 2010). Choline is a critical nutrient during both embryonic development and in adult stages (Zeisel, 2006). It plays a role in the folate cycle and regulates methionine synthesis via its derivative betaine (Zeisel, 2011). Both betaine and choline are considered “homocysteine-lowering agents” that normalize SAM level and hence impact DNA methylation (Finkelstein, 1998 & Olthof and Verhoef, 2005). Choline could affect methyl group (CH3) levels in fetal brain and modulate DNA methylation and fetal gene expression (Niculescu et al., 2006; Davison et al., 2009 & Mehedint et al., 2010). Besides its role in methylation, choline also plays an important role in the synthesis of phosphatidylcholine (PC) of cellular membranes and in the maintenance of folate pool for nucleotide synthesis during fetal growth (Shaw et al., 2004). Thus, choline availability during pregnancy is extremely important for normal development of fetal tissues and fetal brain (Zeisel, 2004 & Zeisel, 2006).

Choline was recently found to play a key role in altering histone methylation (Davison et al., 2009 & Mehedint et al., 2010) and DNA methylation in response to external factors (Niculescu et al., 2006 & Kovacheva et al., 2007). It has been demonstrated that choline deficiency decreases SAM levels in the liver (Zeisel et al., 1989). On the other hand, choline supplementation in rats
increases choline metabolites in the blood and the brain and reduces stress (Zeisel et al., 2006). Based on these facts, we rationalized that gestational choline supplementation during the period of alcohol exposure could impact fetal alcohol effects on POMC neurons and attenuate its adverse effects on the stress axis functions in the adult offspring.

In this study, we showed that gestational choline supplementation normalized protein levels and gene expression of histone-modifying and DNA-methylating enzymes in β-EP-producing POMC neurons in the hypothalamus of fetal alcohol exposed offspring. We also found that gestational choline normalized methylation status of the POMC gene promoter, *POMC* gene expression and β-EP peptide production in the hypothalamus. These data suggest that gestational choline supplementation would have positive implications on regulation of stress axis function in exposed offspring.

6.2 Materials and Methods

*Animal model*

Adult male and female rats of Sprague-Dawley strain were purchased from Charles River Laboratory and maintained in environmentally controlled animal vivarium on a 12 h light/dark cycle (light on 0700 and light off 1900 h) at a constant temperature (22°C). Female rats were mated with males, and the presence of seminal plug in a particular day indicated mating and designated at GD1. On GD7-GD21, pregnant rats were fed daily chow ad libitum (AD), a liquid diet (BioServe Inc) containing alcohol (AF) or pair-fed an isocaloric liquid control diet (PF; with the alcohol calories replaced by maltose-dextrin). The concentration of alcohol varied (1.7-5.0% v/v) in the diet for the first 4 days to habituate the animals with the alcohol diet. After this habituation period, animals were fed the liquid diet containing alcohol at a concentration of 6.7% v/v, which provided about 35% of the total dietary calories. Some rats were fed with alcohol-containing liquid diet supplemented with 4.6 mmol/Kg/day choline chloride (CAF) or isocaloric
liquid diet (CPF) from gestational day 11 (GD11) until birth. Previous studies have shown that the peak blood ethanol concentration is achieved in the range of 120-150 mg/dl in pregnant dams fed with this liquid diet (Miller, 1992) and produce significant inhibitory effect on β-EP neuronal function (Sarkar et al., 2007). The choline dose is shown to be effective in altering global methylation in brain tissues (Holler et al., 1996 & Cermak et al., 1998). The day of birth was recorded as PD1. AF and PF litters were crossfostered using untreated lactating rats fed chow ad libitum (AD) to prevent any compromised nurturing by the AF lactating mother rats until postnatal day 22 (PD22) and then weaned, housed by sex, and provided rodent chow meal and water ad libitum. Male rats, 60-65 days old, were used in this study. Animal surgery and care were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy.

**Double Immunofluorescence and Confocal microscopy**

Five brains from each treatment groups (AD, AF, CAF, PF & CPF) were cryosectioned at 20 µm in thickness and sections were placed on a prechilled slide (Superfrost plus; VWR). Brain sections were collected from plate 19 to plate 23 of the stereotaxic atlas (Paxino, 1982) to cover the whole arcuate area of the hypothalamus, and every fifth section was used for staining peptide. Each one of these sections represents one plate in the stereotaxic atlas. Brain sections were fixed for 10 minutes with 4% PFA then washed for 5 minutes in PBS(1X)+ 0.3% TritonX100. After wash with PBS(1X) for 5 minutes, brain sections were blocked with 5% horse serum (Vector labs, S2000), then double-immunostained for the following antibodies; di or trimethylated H3K4 (H3K4me2,3) (1:500), dimethylated H3K9 (H3K9me2) (1:500), acetylated H3K9 (AceH3K9) (1:500), phosphorylated H3 at serine 10 (pH3S10) (1:500), methyl-CpG-binding protein (MeCP2) (1:500) and for β-endorphin (β-EP) (1:200). β-EP antibody was raised in rabbit (Bachem, San Carlos, CA). Other primary antibodies were monoclonal and raised in mouse (Abcam, Cambridge, MA). Secondary antibodies used in this study were Alexafluor 488
donkey anti-mouse (1:500; Invitrogen; NY) and AlexaFluor594 donkey anti-rabbit IgG (1:500; Invitrogen). We have also used Dnmt1 (1:100; Santa Cruz Biotechnology; CA) and Dnmt3a (1:100; Santa Cruz) antibodies raised in goat for double staining with β-EP antibody. For fluorescence labeling the goat antibodies, we used AlexaFluor488 donkey anti-goat IgG (H+L) (1:1000). Specificity of each of the primary antibodies was verified by incubating slides with excess peptide matching the primary antibody. After staining, slides were mounted in DAPI (Vector Laboratories, CA) and covered with a 1 mm thick coverslip (VWR). Pictures were taken on the same day using confocal microscopy and 20X objective lens (Nikon EZ-C1 3.60 build 770, Gold version). Total number of β-EP cells as well as total number of β-EP cells, located on the right and left side of the third ventricle, that are positive for H3K4me2,3, H3K9me2, AceH3K9, pH3S10, Dnmt1, Dnmt3a or MeCP2 were presented.

**Quantitative Real-Time PCR (qRT-PCR)**

Total RNA was extracted from the mediobasal hypothalamus (MBH) using Micro to Midi Kit with Trizol (Invitrogen, Grand Island, NY). The RNA in each sample was quantitated using the NanoDrop -1000 (version 3.7, Thermo Scientific, Rockford, IL). Before RT-PCR, the RNA was treated with DNase (Qiagen, Valencia, CA) and then stored in 25 µl of UltraPure DNase/RNase-free distilled water (Invitrogen). Afterward, 1000 ng/µl was converted to cDNA using GeneAmp PCR System 9700 (Applied Biosystems, ABi) and cDNA high-capacity RT. The RT-PCR conditions were 25ºC for 10 min, 37ºC for 60 min, 37ºC for 60 min, 85ºC for 5 minutes then kept at 4ºC. After the reverse transcription reaction, RT-PCR was performed with a total volume of 25 µl of reaction mixture which contains 2.5 µl of cDNA and 22.5 µl of Universal master mix (10 X RT buffer; 25 X dNTP mix; 10 X RT primers; Multiscribe RT; RNase OUT; Nuclease free H₂O; Invitrogen). PCR conditions were 50ºC for 2 min for 1 cycle; 95ºC for 10 min, 1 cycle, 95ºC for 15 sec, and 60ºC for 1 min, 40 cycles. All runs were performed in duplicates. The ratio of mean quantity of gene of interest to the mean quantity of the housekeeping gene GAPDH was
compared between different groups. All primers were designed by ABi (Table 2). RT-PCR was performed using the ABi prism 7500HT sequence detection system.

**SYBR green methylation-specific (MSP) Real-time PCR**

DNA was extracted from the mediobasal hypothalamus (MBH) of experimental rats using the DNeasy Blood & Tissue kit and following the protocol of Qiagen (Valencia, CA). 25 mg of hypothalamic tissues was homogenized then kept in lysis buffer with 20µl proteinase K at 56°C overnight. RNAase A (100 mg/ml, Qiagen) was added. DNA was eluted in DNase/RNAse – free H2O. DNA was quantitated using the NanoDrop then stored at -20°C for later use. 1.5 µg of DNA extracted from each sample was treated with sodium bisulfite and converted using the EZ DNA methylation Kit protocol (Zymo Research, Orange, CA). The PCR primers were designed using the Methyl Primer Express program, version 1.0 (ABi, Foster City, CA) or MethPrimer program (http://www.urogene.org/methprimer/index1.html) and manufactured by Sigma. The sequences of the oligos are:

- **Methylated**
  - forward: 5' CGTTTTTAGCGGCTTTGTGTTAAC 3',
  - reverse: 5' CTACAACGCAACAACGAATCC 3',
  - probe: 5' CGATCGGGAAGTT 3'

- **Unmethylated**
  - forward: 5' GTGTTTTAGTGGGTTTGTGTTAATGTTAG 3',
  - reverse: 5' ACTTCTACAACACAACAAATCCC 3',
  - probe: 5' GTTTTTGTATTTTTAGGTATATTTTG3'

Primers were designed to be “methylation-specific” or “unmethylation-specific” with respect to the particular cytosine nucleotide in the CpG pair under analysis in POMC gene promoter. The ratios of the methylation-specific to unmethylation-specific responses were quantified by ΔCt method. A total mix of 25 µl containing the converted DNA, Syber green mix, DNase/RNAase free water and either the methylated or unmethylated reverse and forward primers were prepared. Rat high methylated and rat low methylated DNA controls (EpigenDx, Worcester, MA) were also bisulfite converted and used for the preparation of the standard curve. The run was conducted as follows: 50°C for 2 mins 1 cycle, 95°C for 10 mins 1 cycle, 95°C for 15 secs, 58°C for 1 min and 72°C for 15 secs for 50 cycles. The dissociation stage is 95°C for 15 sec and 60°C for 1 min 1 cycle. Run for each
sample was done in duplicates and non-template controls (NTCs) were used.

Statistical Analysis

Statistical analysis of data was performed using Graph Pad Prism software version 4.0 (LA Jolla, CA). For immunohistochemistry and qRT-PCR data, the mean values were calculated and analyzed between all groups using one-way analysis of variance (ANOVA) with Newman’s Keuhl post hoc test. All results are presented as standard error of the mean (SEM). P<0.05 was considered as significant.

6.3 Results

Effects of gestational choline on protein levels of histone-modifying enzymes, DNA methylating enzymes and methylbinding protein MeCP2 in β-EP-producing POMC neurons of the hypothalamus

We assessed the effects of maternal nutrient supplementation with choline upon alcohol exposure on protein levels of histone-modifying enzymes and DNA-methylating enzymes in β-EP neurons of the hypothalamus of alcohol-fed (AF) and controls (pair-fed, PF and ad lib-fed, AD) offspring during adult period. We found that fetal alcohol exposure (FAE) reduced the methylation of the activation mark H3K4me2,3 as demonstrated by the reduced number of β-EP neurons positive for this mark, when compared to PF and AD (P<0.05). FAE increased the methylation of the repressive mark H3K9me2 in β-EP neurons in AF as compared to AD (P<0.01) and PF rats (P<0.05). Gestational choline supplementation normalized H3K4me2,3 (P<0.01) (Figs. 22A & B) and H3K9me2 (P<0.05) levels (Figs. 22 C & D) in AF rats. We also determined the effects of FAE on other activation marks such as acetylated H3K9 and phosphorylated H3S10. We found that both marks were reduced in AF rats compared to AD (AceH3K9, P<0.05; pH3S10, P<0.01) and PF rats (AceH3K9, P<0.05; pH3S10, P<0.05). Unlike H3K4me2,3 and H3K9me2, gestational choline supplementation did not reverse alcohol effects on these two histone modifications in CAF compared to AF rats (Figs. 22 E, G, F & H).
Figure 22 Gestational choline normalized fetal alcohol-induced histone modifications in β-EP neurons in the arcuate area of the hypothalamus

Changes in the number of β-EP cell positive for H3K4me2,3 (A, B), H3K9me2 (C,D), AceH3K9 (E, F) and pH3S10 (G,H) in the ARC of the hypothalamus. Representative photographs show the double-labeled cells (A,C,E & G; red and green colocalized), in each treatment group, and histograms (B, D, F & H) show the mean ± SEM values of the percentage of β-EP cells that were double-labeled. N=5. H3K4me2,3 (*P < 0.05 AF compared to AD and PF, **P<0.01 CAF compared to AF; bP<0.01 CPF compared to AF). H3K9me2 (*P<0.05 AF compared to PF,*P<0.01 AF compared to AD; **P<0.05 CAF compared to AF; bP<0.05 CPF compared to AF). AceH3K9 (*P<0.05 AF compared to AD and PF; bP<0.01 CPF compared to AF). pH3S10 (*P<0.05 AF compared to PF; *P<0.01 AF compared to AD; **P<0.01 CAF compared to CPF and AD; aP<0.05 PF compared to CAF; bP<0.001 CPF compared to AF). Alexafluor 594 red represents β-EP staining and Alexafluor 488 green represents staining of H3K4me2,3, H3K9me2, AceH3K9 or pH3S10. Values are considered significant using the ANOVA analysis and the Posthoc Newman’s Keuhl test for analysis.

We also determined the effects of FAE with or without choline supplementation on protein levels of key enzymes regulating DNA methylation such as Dnmt1 and Dnmt3a and on protein levels of MeCP2 in β-EP neurons. FAE increased the protein levels of both Dnmt1 and Dnmt3a, as
demonstrated by the increase percentage of β-EP neurons containing these proteins as compared to AD (Dnmt1; P<0.01 and Dnmt3a; P<0.05) and PF rats (Dnmt1; P<0.001 and Dnmt3a; P<0.01). Choline supplementation completely reversed alcohol effects on proteins levels of Dnmt1 in CAF rats compared to AF (P<0.01) (Figs. 23A-D) but not Dnmt3a. This protein data correlated with an increase in protein levels of MeCP2 (P<0.05) which is known to bind to methylated CpG to silence or activate gene expression (Chahrour et al., 2008). Choline supplementation also normalized its levels in β-EP neurons of CAF rats (P<0.05) (Figs. 23 E & F).

To assess the physiological consequences of supplemental gestational choline, we compared the percentage of β-EP neurons in treated groups compared to AD during the adult period in rats treated with alcohol alone or together with choline during fetal period. Fetal alcohol treatment decreased β-EP immunoreactivity in POMC neurons of AF rats compared to controls (P<0.05). Choline treatment normalized β-EP count to a level comparable to that of controls (P<0.001) (Figs. 23 G & H). These results suggest that FAE altered the protein level of enzymes critically involved in histone modification and DNA methylation. This change correlated with a decrease in the level of the opioid peptide β-EP in POMC neurons. Gestational choline supplementation prevented alcohol action on several key histone-modifying and DNA-methylating enzymes and normalized their levels as well as β-EP peptide levels during adult period.
Figure 23 Gestational choline normalized fetal alcohol-induced changes in the protein levels of Dnmts and MeCP2 in β-EP neurons as well as the number of β-EP neuron count in the hypothalamus

Changes in the number of β-EP cell positive for Dnmt1 (A, B), Dnmt3a (C, D), MeCP2 (E, F) and the number of β-EP neurons (G, H) in the ARC of the hypothalamus. Representative photographs show the double-labeled cells (A,C,E,G) in each treatment group and histograms (B,D,F) show the mean ± SEM values of the percentage of β-EP cells that were double-labeled. N=5. Dnmt1 (*P<0.001 AF compared to PF; **P<0.01 AF compared to AD; ***P<0.01 CAF compared to AF; bP<0.001 CPF compared to AF). Dnmt3a (*P<0.05 AF compared to AD; **P<0.01 AF compared to PF). MeCP2 (*P<0.05 AF compared to AD, PF; **P<0.05 CAF compared to AF). β-EP count (*P<0.05 AF compared to AD and PF; **P<0.001 CAF compared to AF, bP<0.05 CPF compared to AD; bP<0.001 CPF compared to AF). Alexafluor 594 red represents β-EP staining and Alexafluor 488 green represents staining of Dnmt1, Dnmt3a or MeCP2.

Effects of gestational choline on gene expression of histone-modifying enzymes, DNA methylating enzymes and methylbinding protein MeCP2 in the mediobasal hypothalamus

We next determined the effects of fetal alcohol exposure with or without gestational choline supplementation on mRNA levels of Set7/9 that catalyzes the methylation of H3K4, G9a and
Setdb1 that catalyze H3K9 methylation as well as mRNA levels of Dnmt1, Dnmt3a, and MeCP2 in adult male rat offspring. We also determined the effects of gestational choline on CBP and HDAC2 expression. FAE decreased the mRNA levels of POMC (P<0.05), Set7/9 (P<0.05) and CBP (P<0.05) and increased the mRNA level of G9a (P<0.01), Setdb1 (P<0.05) and HDAC2 (P<0.05). Choline supplementation reversed the alcohol effect on all these genes in CAF rats compared to AF (Set7/9, P<0.05; G9a & Setdb1, P<0.001) (Figs. 24A-D, H-I). Choline treatment also suppressed G9a mRNA levels in PF rats. In addition to its effect on histone methylating genes, fetal alcohol exposure also increased mRNA levels of Dnmt1 (P<0.05) but not Dnmt3a (P>0.05). It also increased mRNA levels of MeCP2 (P<0.01) (Fig. 11C). Choline supplementation reduced Dnmt1 and MeCP2 levels in AF (Dnmt1; P<0.05, MeCP2; P<0.001 compared to controls) and CAF rats (Dnmt1; P<0.001, compared to controls, MeCP2; P<0.01 compared to AF), but it increased Dnmt3a level in both CAF (bP<0.01 compared to AD and AF) and CPF rats (Dnmt3a, bP<0.001 compared to AD and PF) (Figs. 24 E-G).
Changes in mRNA levels of POMC (A), Set7/9 (B), G9a (C), Setdb1(D), Dnmt1(E), Dnmt3a(F), MeCP2(G), CBP(H) and HDAC2 (I). Data presented are mean ± SEM. N=6-9. POMC (*P<0.01 AF compared to AD and PF; **P<0.001 CAF compared to AF; bP<0.01 CPF compared to AF). Set7/9 (*P<0.05 AF compared to AD; **P<0.05 CAF compared to AF; aP<0.01 PF compared to AF; bP<0.001 CPF compared to AF). G9a (*P<0.05 AF compared to PF; *P<0.001 AF compared to AD; **P<0.05 CAF compared to AD; aP<0.01 PF compared to CAF; bP<0.001 CPF compared to AF and PF; bP<0.01 CPF compared to AD). Setdb1(*P<0.05 AF compared to AD and PF; **P<0.001 CAF compared to AD, PF and AF; aP<0.05 PF compared to AF; bP<0.01 CPF compared to AF and CAF). Dnmt1(*p<0.05 AF compared to AD and PF; **P<0.001 CAF compared to AD, AF and PF; bP<0.01 CPF compared to AF and CAF). Dnmt3a (**P<0.01 CAF compared to AD and AF; **P<0.05 CAF compared to PF; bP<0.001 CPF compared to AD, AF and PF; MeCP2 (**P<0.01 AF compared to AD and PF; **P<0.001 CAF compared to CPF, **P<0.01 CAF compared to AF; bP<0.001 CPF compared to AD, AF and PF). CBP (**P<0.05 AF compared to AD and PF; **P<0.001 CAF compared to PF, AD and **P<0.05 compared to AF; bP<0.001 CPF compared to AD, AF and PF). HDAC2 (P<0.05 AF compared to AD and PF; **P<0.001 CAF compared to AD, AF, PF; bP<0.001 CPF compared to AF, PF, bP<0.01 CPF compared to AD and bP<0.05 CPF compared to CAF).

Our gene expression data correlated well with the protein data of all the histone methylating and DNA methylating enzymes except for Dnmt3a. Increase in MeCP2 expression also correlated with an increase in its protein levels. These data support the notion that gestational choline compensated for the lack of the methyl group CH3 induced by FAE and normalized POMC gene promoter methylation, POMC gene expression and the production of its derived peptide β-EP. To establish such possibility, we determined the effects of gestational choline supplementation during the period of alcohol exposure on the changes in methylation status of POMC gene promoter and POMC gene expression.
Effects of gestational choline on POMC gene methylation and mRNA expression in the hypothalamus

It has been shown previously that the methylation of the CpG island in the human POMC gene promoter can lead to gene silencing in nonexpressing tissues (Newell-Price et al., 2001). In order to characterize the extent of cytosine methylation of CpG dinucleotides in the 5’ CpG island of POMC gene promoter, we designed one set of primers specific to either the methylated or the unmethylated state of the CpG sites adjacent to the POMC gene transcription start site. TaqMan methylation-specific real-time PCR, with the probes derived from the sequence in the region -81 to -154, identified significant increase in cytosine methylation between AF and control animals. Choline supplementation normalized fetal alcohol effect on methylation status of these two sites in POMC gene promoter in AF rats (P<0.05) (Fig. 25). Interestingly, these CpG sites reside in the binding site of transcription factors essential for transcriptional activation. Normalization of POMC gene promoter methylation with choline supplementation correlated with normalization of POMC mRNA levels (Fig. 24A). Overall, our data indicate that FAE resulted in POMC gene hypermethylation in adulthood and gestational choline supplementation normalized POMC gene promoter methylation and POMC gene expression in adult male rats.

Figure 25 Effects of gestational choline on methylation of POMC gene promoter

(A) POMC gene promoter methylation in male AD, AF, CAF, PF and CPF rats. Data presented are mean ± SEM. N=7-8. *P<0.05 compared to AD, PF and CAF.
6.4 Discussion

In this study, we determined whether gestational choline supplementation could counteract fetal alcohol effects on histone marks, DNA methylation in β-EP-producing POMC neurons and normalize POMC gene methylation and expression. We showed here that gestational choline supplementation altered the expression patterns of various histone- modifying genes and DNA-methylating genes and normalized fetal alcohol-altered POMC gene expression and β-EP peptide production in the hypothalamus in the adult stage. Choline prevented alcohol effects on the activation mark H3K4me2,3 and the repressive mark H3K9me2. Choline also reversed alcohol repressional effect on Set7/9 expression that controls H3K4 methylation and alcohol activational effect on G9a and Setdb1 that regulate H3K9 methylation. Choline normalized mRNA and protein levels of Dnmt1 and MeCP2 and increased Dnmt3a expression in CAF and CPF rats. It also normalized CBP and HDAC2 expression. Additionally, it normalized fetal alcohol-induced abnormality in POMC gene methylation, POMC mRNA expression and β-EP peptide production. These results could suggest that choline supplementation was able to prevent alcohol-induced epigenetic modifications of POMC gene of adult offspring leading to normalization of POMC gene expression and its control of the stress axis function.

Alcohol ingestion is known to inhibit folic acid absorption and methionine synthase ability to convert homocysteine to methionine and SAM, which are both critical for methylation processes during development (Wang et al., 2009). FAE is also known to cause hypomethylation (Garro et al., 1991). We reasoned that this hypomethylation is the result of low SAM availability caused by a deficiency of one of its methyl donors, choline, and could be compensated by supplementation of choline. Behavioral deficits and hyperresponses to stress have been reported in rodents which were exposed to alcohol during embryonic development (Rivier et al., 1988; Weinberg, 1988; Berman & Hannigan, 2000 & Boyadjieva et al., 2009). Consequently, we
determined whether gestational choline supplementation could mitigate alcohol effects on the stress axis by altering the gene expression of one of its regulators POMC by modulating histone marks and DNA methylation in POMC neurons. We showed here that gestational choline supplementation modified the levels of the methylation marks H3K4me2,3 and H3K9me2, altered the expression of histone-modifying enzymes such as Set7/9, G9a, Setdb1, CBP and HDAC2 and the expression of Dnmt1 and MeCP2 in the mediobasal hypothalamus of adult rats.

Choline deficiency has previously been shown to alter the activity of G9a in neural progenitor cells. It decreased H3K9me1,2 and increased the expression of genes involved in neurogenesis in mice fetal hippocampus (Mehedint et al., 2010). In our study, we found that gestational choline increased H3K4me2,3 methylation, decreased H3K9me2 methylation in β-EP-producing POMC neurons of AF rats as compared to AD and PF rats. Gestational choline did not significantly alter alcohol effects on other histone marks such as H3K9 acetylation or H3S10 phosphorylation. This could be explained by the fact that histone-modifying enzymes that acetylate H3K9 or phosphorylate H3S10 do not require methyl group for their activity and it could also be due to the complex interactions between different histone marks and DNA methylation (Roberston, 2002 & Berger, 2007).

There is a cross-talk between histone modifications and DNA methylation in regulation of gene expression. For example, H3K9 methylation affects DNA methylation by recruiting methyl-binding proteins such as MBDs or MeCP2 and other chromatin-modifying factors to the promoter of a specific gene resulting in gene repression (Vaissiere et al., 2008 & Guibert et al., 2009). MeCP2 is abundantly expressed in the hypothalamus and acts both as a repressor or an activator of gene expression (Chahrour et al., 2008). It has also been associated with regulation of stress and behavior (Fyffe et al., 2008). In our study, we found that gestational choline normalized the protein levels and gene expression of Dnmts and MeCP2 in the mediobasal hypothalamus.
except for Dnmt3a. Dnmt3a gene is located very close to POMC gene on chromosome 6. Govorko et al. (2011) (submitted) demonstrated that alcohol exposure did not alter the methylation status of Dnmt3a promoter compared to POMC gene promoter. This data supports our Dnmt3a gene expression data where the later was unaffected by alcohol exposure (Fig. 24F). The upregulation of Dnmt3a expression in CAF and CPF rats is interesting (Fig. 24F). This upregulation in Dnmt3a gene expression in CAF rats could suggest that under saturating conditions of SAM due to choline supplementation, DNA of fetuses from alcohol-fed rats were a better substrate compared to controls because of fetal hypomethylation condition induced by alcohol exposure. The exaggerated response in terms of Dnmt3a expression in CPF rats could suggest that these saturating conditions of SAM induced by supplemental gestational choline could have adverse effects in control rats compared to exposed rats. SAM excess might increase disproportionately in control rats the activity of DNA-methylating enzymes such as Dnmt3a and result in drastic changes in gene expression. Moreover, this upregulation of Dnmt3a gene expression could also be due to the necessity of its activity to contribute to de novo methylation in the adult stage in response to stimuli.

The quantitative real-time-PCR analysis confirmed a decrease in the expression of Dnmt1 in the mediobasal hypothalamus and a decrease in G9a and Setdb1 upon choline supplementation thus creating an environment conducive for gene activation upon choline supplementation. In KO mouse model for MeCP2, choline supplementation reduced some of the behavioral and anatomical changes observed in mutant mice (Zeisel, 2011). In our study, MeCP2 gene expression was decreased upon choline supplementation and this decrease correlated with a normalization of POMC gene expression and β-EP peptide production. Further studies are needed to determine whether MeCP2 binds to POMC gene promoter and whether choline supplementation could decrease its binding to this promoter and alter POMC gene expression.
Histone modifications and DNA methylation are both essential for regulation of gene expression (Razin, 1998; Zhang & Reinberg, 2001 & Berger, 2007). The changes in histone methylation that we found in β-EP-producing POMC neurons positively correlated with changes in Dnmt1 gene expression but negatively correlated with changes in POMC gene expression in the mediobasal hypothalamus. Choline-supplemented embryos showed attenuation in the expression of Dnmt1 with hypermethylation of the CpG islands of Dnmt1 gene in the brain (Kovacheva et al., 2007). In our study, FAE modulated histone and DNA methylation, and choline reversed alcohol effects. Hence, fetal hypomethylation caused by alcohol exposure possibly upregulated the expression and activity of histone-modifying enzymes and DNA-methylating enzymes in POMC neurons. This upregulation possibly resulted in a state of hypermethylation in the adult offspring as a compensatory mechanism resulting in POMC gene promoter hypermethylation and decrease in expression. Feeding choline from GD11-21 normalized the methylation status in the fetus and prevented abnormal hypomethylation in the fetus and hypermethylation in the adult stage. This is an important finding with clinical implications in FASD etiology.

The normalization of POMC gene expression upon choline supplementation paralleled the normalization of POMC gene promoter methylation and β-EP production in male rats. The ratio of methylated and unmethylated DNA in choline supplemented alcohol-exposed rats was comparable to that of controls. This data suggests that choline is a major methyl group that leads to formation of SAM via betaine and that methylation is possibly regulating POMC gene expression and regulating stress axis function. Alterations in several genes within the mediobasal hypothalamus have been documented in rodents after alcohol exposure (Chen et al., 2006; Sarkar et al., 2007 & Kuhn & Sarkar, 2008). Gestational choline has long-lasting beneficial effects on behavior, memory, attention and learning in the adult offspring (Meck and Williams, 1997; Thomas et al., 2000; Mohler et al., 2001; Thomas et al., 2007 & Thomas et al.,
Our study is the first to report that gestational choline altered alcohol-induced changes in histone marks in β-EP-producing POMC neurons and hypothalamic POMC gene promoter methylation and POMC gene expression in adult exposed offsprings.

In conclusion, these data demonstrate that gestational choline altered alcohol-induced epigenetic changes such as histone marks in β-EP-producing POMC neurons and DNA methylation in hypothalamic POMC gene promoter of the adult offspring. It would be important to investigate in the future the behavioral aspect of choline supplementation in AF rats. Further studies are needed to confirm that choline is an effective and safe choice to mitigate the effects of FAE on the regulation of stress axis in the adult stage.
7 Conclusions and Future Directions

Four main conclusions can be deduced from this dissertation work: 1) FAE decreases the levels of the activation histone marks H3K4, aceH3K9 and pH3S10 in β-EP-producing POMC neurons and causes a significant increase in the repressive histone mark H3K9; 2) FAE alters the protein levels and gene expression of POMC, Set7/9, CBP, HDAC2, G9a, Setdb1, MeCP2 and Dnmt1 except for Dnmt3a; 3) FAE reduces the activation mark H3K4me3 along Exon 3 of POMC gene; and 4) gestational choline supplementation reverses alcohol-induced alteration in histone-modifying and DNA-methylating enzymes and normalizes POMC gene promoter methylation, POMC gene expression and β-EP peptide production in POMC neurons. Overall, these research findings collectively demonstrate that FAE causes long-lasting epigenetic modifications in hypothalamic POMC neurons thereby impairing POMC gene expression and functions in the adult exposed offspring.

Reduction in POMC gene expression and β-EP peptide production contribute to the hyperresponse to stress observed in alcohol-exposed rats (Rivier et al., 1988; Weinberg, 1988; Sarkar et al., 2007 & Boyadjieva et al., 2009). Based on my experimental data as well as other previous data (Govorko et al., 2011), I propose that POMC gene hypermethylation and possibly histone modifications, such as H3K4me3 reduction along Exon 3, are responsible for the deficit in POMC gene expression and β-EP peptide production in fetal-alcohol exposed rats.

Fig. 26 depicts a model that suggests that FAE causes POMC gene hypermethylation by Dnmt1 resulting in the binding of MeCP2 to methylated CpG in the promoter region hence recruiting HDACs and resulting in histone deacetylation and POMC gene repression.
Figure 26 Proposed model of the effect of fetal alcohol exposure on hypothalamic POMC gene expression

Fetal alcohol exposure (FAE) causes upregulation of Dnmt1 expression resulting in hypermethylation of CpG in POMC gene promoter. Methylated CpGs induce MeCP2 binding which in turn is bound to HDACs. HDACs promote deacetylation of histones creating an environment conducive for POMC gene repression. In normal conditions, Dnmt1 expression is normal resulting in normal methylation of POMC gene promoter, decrease in MeCP2 binding, and increase in acetylation of histones creating an environment conducive for POMC gene activation.

Histone marks are distributed along the gene body and have specific function in terms of regulation of gene transcription (Jiang & Pugh, 2009). In the promoter region, these marks could affect accessibility of essential TFs to regulatory sites thus modulating transcriptional activation and initiation. In the gene body, they could modulate the rate of RNA Polymearse II elongation or modulate the accessibility of the splicing machinery thus altering the efficiency of transcriptional elongation or splicing (Luco et al., 2010; Ramakrishna et al., 2010; Ross & Beggs, 2010; Schor et al., 2010 & Tilgner & Guigo, 2010). FAE decreases H3K4me3 mark along Exon 3 of POMC gene and this correlates with a nonsignificant increase in the repressive mark H3K9me2 in Exon 3 and in the promoter region of POMC gene. Interestingly, H3K4me3 and H3K9me2 marks changed in opposite direction along Exon 3 as anticipated. It has been demonstrated that H3K4me3 mark recruits the CHD1 ATPase chromatin-remodeling complex and the components of the splicing machinery to regulate gene splicing (Sims et al., 2007).
Here I propose that H3K4me3 mark could play a crucial role in Exon 3 splicing and affect the number of generated transcripts from POMC gene. H3K9me2 repressive mark does not play a critical role in regulating POMC gene expression rather the later is regulated by DNA methylation of its promoter (Govorko et al., 2011). Future studies should investigate whether the other repressive mark H3K27 could play a role in POMC gene repression along POMC gene promoter.

Fig. 27 depicts a model of the role of H3K4me3 mark in regulating POMC gene expression upon alcohol exposure. This model suggests two possible scenarios: 1) Reduction of the activation mark H3K4me3 along Exon 3 in AF rats could modulate recruitment of splicing factors resulting in an inefficient recognition of Exon 3 by the splicing machinery resulting in inefficient splicing and generation of truncated transcripts with no physiological function; 2) Reduction of H3K4me3 along Exon 3 in AF rats could result in the generation from Exon 3 of short ncRNAs with negative regulatory outcome on POMC gene expression. The validity of these two scenarios should be elucidated in the future.

**Figure 27** Role of H3K4me3 mark in splicing of Exon 3 of POMC gene

Reduction of H3K4me3 along Exon 3 of POMC gene could affect the recruitment of the splicing machinery and the efficiency of Exon 3 splicing resulting in generation of ncRNAs that could
impact negatively *POMC* gene expression or could generate truncated transcripts with no physiological functions (E1=Exon 1, E2=Exon 2, E3= Exon 3, POLII=RNA Polymerase II, CHD=chromohomeodomain of ATPase chromatin-remodeler).

Maternal diet including VitB12, folic acid, choline and betaine has profound impact on the epigenome and on the offspring phenotypes later in life. The availability of choline and betaine, precursors of SAM, during critical period of gestation is needed for normal brain development (Zeisel, 2000; Niculescu & Zeisel, 2002; Zeisel, 2004; Niculescu et al., 2006; Zeisel, 2006 & Zeisel, 2011).

Fig. 28 depicts a model for the role of gestational choline in attenuating alcohol’s adverse effects on POMC neurons. Here I propose that FAE causes low folate and low SAM resulting in hypomethylation. Since normal methylation is essential for normal embryonic development, hypomethylation results in an increase in Dnmts and HMTs expression and an increase in their activity in the ARC as the system’s response to the altered methylation state. This upregulation in expression and activity induces a hypermethylation state that persists into adulthood resulting specifically in *POMC* gene hypermethylation and decrease in its expression and functions.

Gestational choline supplementation during the period of alcohol exposure normalizes the levels of the activation mark H3K4 and the repressive mark H3K9 with normalization of *POMC* gene methylation and expression to a level comparable to that of controls (Bekdash et al., 2011; In the process of submission).
Figure 28 Proposed model of the effects of gestational choline supplementation on POMC gene

Fetal alcohol exposure causes low folate and SAM resulting in hypomethylation. As a compensatory mechanism, the expression and the activity of HMTs and Dnmts were upregulated resulting in hypermethylation state in the ARC that persisted into adulthood. This hypermethylation caused a decrease in POMC gene expression, β-EP production with a reduction in β-EP inhibitory effect on CRH. Gestational choline supplementation normalized folate and SAM pool resulting in normal POMC gene methylation and expression with normalization of the stress axis (PVN=paraventricular nucleus, CRH=corticotrophin-releasing hormone, ACTH=adrenocorticotropic hormone, GC=glucocorticoid, β-EP=β-endorphin, SAM=S-adenosylmethionine).

Some critical questions would need to be addressed in the future:

1. Does FAE cause alteration in the methylation status of the downstream 3’ CpG island in Exon 3 of POMC gene? Is it a transient or a permanent change? Does this change play a role in regulation of POMC gene expression?

2. Could MeCP2 regulate POMC gene expression? In vivo and in vitro KO of MeCP2 as well as the determination of the phosphorylation state of MeCP2 at S421 or S80 upon alcohol exposure could provide an answer.

3. Could histone mark H3K4me3 play a role in Exon 3 splicing and POMC gene expression regulation? Could alteration in H3K4 mark along Exon 3 upon alcohol exposure result in the generation of ncRNAs? If so, what possibly could be the role of these ncRNAs? How are they regulated? Are they conserved? Are they only expressed upon alcohol exposure?
4. What is the behavioral aspect of gestational choline supplementation in alcohol-fed rats? Could gestational choline play a role in regulation of the stress axis? What is the effect of gestational choline supplementation on CRH protein and mRNA levels in the PVN region of AF rats? Our research findings have pathophysiological relevance and implications in FASD etiology. One one hand, our data show that the components of the epigenetic machinery are altered by FAE and cause POMC gene hypermethylation, downregulation of POMC gene expression and a decrease in β-EP peptide production. This suggests that these components could be potential targets for drugs to treat psychiatric or neurological disorders associated with FASD such as stress or altered behavior. On the other hand, our choline data suggest a possible avenue or choice to use choline as a safe supplement to counteract the adverse effects of FAE on normal functioning of POMC system and the stress axis in the adult stage. Future detailed studies are needed to identify specific epigenetic factors that are involved in regulation of POMC gene expression and to target specifically these factors to treat phenotypes associated with FASD such as hyperresponse to stress.
8 Supplementary Data

mRNA expression using β-actin and 18S rRNA as housekeeping genes

A.

B.

C.

D.

E.

F.
Figure 27 Gene expression using β-actin or 18S rRNA

Changes in mRNA levels of POMC (A & B), G9a (C & D), Setdb1(E & F), MeCP2 (G & H), Dnmt1 (I & J), and Dnmt3a (K & L). Data presented are mean ± SEM. N=6-9. *P<0.05 AF compared to AD and PF.
**TaqMan MSP real-time PCR for Dnmt3a**

The PCR primers for *Dnmt3a* gene were designed using MethPrimer program (http://www.urogene.org/methprimer/index1.html) and manufactured by Sigma. Forward and reverse methylated-specific (M) and unmethylated-specific primers (U) were designed in the position -206 upstream of the Dnmt3a gene transcription start site. The sequences of the oligos are:

5’ AGTTTTAATAGTGGGATACGTTATATTTTC 3’ Forward M

5’ AACGCTAAATAACTCTACCTACGAA 3’ Reverse M

5’ AGTTTTAATAGTGGGATATGTTATATTTTT 3’ Forward U

5’ CTGAACACTAAATAACTCTACCTACAAA 3’ Reverse U

qRT-PCR was performed with the help of ABI Prism 7500 Sequence Detection System using Power SYBR Green Master Mix (ABi). Rat high methylated and rat low methylated DNA controls (EpigenDx, Worcester, MA) were also bisulfite converted and used for the preparation of the standard curve. 2.5 µl of bisulfite converted DNA was utilized in each run. The thermal cycling conditions included 50°C for 2 min, then 94°C for 5 min, followed by 40 cycles of amplification at 94°C for 15 sec and at 60°C for 1 min followed by a dissociation stage. The ratios of the mean quantity of methylation-specific to unmethylation-specific responses in each sample were quantified and compared in all groups. All runs were performed in duplicates.

A.  B.
Figure 28 Dnmt3a methylation in the arcuate area of male rats

(A) Shows the location of the *Rattus norvegicus* Dnmt3a gene relative to POMC gene as illustrated in Ensembl Genome Browser. (B) Shows the effects of fetal alcohol exposure on *Dnmt3a* gene methylation in the ARC of the hypothalamus in rats prenatally exposed to alcohol (AF) or control diets (PF and AD) (B). Methylation-to-unmethylation ratio in the -206 position upstream of the *Dnmt3a* gene transcription start site as measured using TaqMan Methylation-Specific Real-Time PCR in ARC of F1 males AF, PF and AD rats. Data are mean ± SEM (N=8, P > 0.05) using One-way ANOVA and Newman's keuhl posthoc test for analysis.

Global DNA methylation status measurement using MethylFlash Methylated DNA Colorimetric Assay

DNA was extracted from the homogenized ARC punches of AD, PF and AF F1 male and female adult rats following Qiagen DNA Blood and Tissue Kit protocol (Qiagen, Valencia, CA).

Quantification of DNA in all samples was performed and DNA quality was assessed using the NanoDrop -1000 version 3.7 (Thermo Scientific, Rockford, IL). 50 ng of input DNA from each sample was used to quantitate and compare the percentage of 5-methylcytosine in all groups following the protocol of MethylFlash Methylated Quantification kit (Epigentek, NY).

Figure 29 Percentage of global 5-methylcytosine in the arcuate area of male and female rats

Effects of fetal alcohol exposure on global DNA methylation in the ARC of the hypothalamus in rats prenatally exposed to alcohol (AF) or control diets (PF and AD). DNA was isolated from the ARC of 60 days old rats of the F1 generation. 5-mC levels in the ARC extracts were measured using the protocol of MethylFlash Methylated Quantification kit. Data are mean ± SEM values. N = 8; P >0.05

Double Immunofluorescence & Confocal microscopy – MeCP2

Brains from 5AD, 5AF and 5PFs were sectioned at 20 µm in thickness. Brain sections were collected from plates 17 and 18 to cover the PVN region. Every tenth section from each brain
was double-immunostained for CRH (1:500, T-4037, Bachem, CA) and MeCP2 (1:500, Abcam 50005 mouse monoclonal Ab to MeCP2). AlexaFluor594 donkey anti-rabbit IgG (1:500, Invitrogen) and AlexaFluor488 donkey anti-mouse IgG (1:500, Invitrogen) were used as secondary antibodies. After staining, slides were mounted in DAPI (H-1200 Vectashield, Vector Laboratories) and covered with a 1mm thick coverslip. Pictures were taken on the same day using confocal microscopy (Nikon EZ-C1 3.60 build 770, Gold version). Total number of CRH cells as well as total number of CRH cells that are positive for MeCP2 was calculated.

A.  

![Image](image1)

B.  

![Image](image2)

C.  

![Image](image3)

**Figure 30 MeCP2 staining in CRH neurons of the PVN**

(A) Changes in the number of MeCP2-positive CRH neurons in the PVN region. Representative photographs show the double-labeled cells (A: red and green colocalized), in each treatment group, and histogram; (B) shows the mean ± SEM values of the percentage of CRH cells that were double-labeled. N=4. P > 0.05 compared with the rest. (C) CRH immunoreactivity in the PVN. Alexafluor 594 red represents CRH staining and Alexafluor 488 green represents staining of MeCP2. Values are considered nonsignificant using the ANOVA analysis and the Posthoc Newman’s Keuhl test for analysis.
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