INVESTIGATING THE EFFECT OF CONTINUOUS ETHANOL EXPOSURE ON DIFFERENTIATED NEURAL STEM CELLS; A STUDY FOR POTENTIAL FASD BIOMARKERS

By

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ABSTRACT

Prenatal alcohol exposure (PAE) is considered as a risk factor for the development of fetal alcohol spectrum disorders (FASD). Mounting evidence suggests that PAE affects epigenetic mechanisms and alters the normal differentiation and development of neural stem cells (NSC) in fetal brain. DNA methylation, as an important epigenetic mechanism, contributes to several biological processes during brain development and undergoes significant changes following PAE. In addition, recent studies suggest that DNA methylation is a considerable biomarker for the early diagnosis of FASD. However, effects of PAE are dependent to a variety of factors such as sex and strain of studied subjects. In this regard, we aimed to investigate whether sex and strain play a determinant role in the effects of chronic ethanol exposure on DNA methylation machinery. In addition, based on our previously studied RNA-Sequencing data, we tested the validity of three potential biomarkers (*Dcc*, *Scn3a*, and *Sptbn2*), which were suggested by IPA (Ingenuity Pathway Analysis) software. To do this, we applied a standard and controlled *in vitro* model system, in which we treated male and female differentiating NSC (obtained from the forebrain of CD1 and BL6 embryos at the embryonic day 14.5) with chronic ethanol exposure (70 mM) for 8 days. Our results showed that chronic ethanol treatment induced global DNA hypomethylation and altered the components of DNA methylation machinery in a sex and strain-specific manner. These alterations in DNA methylation status were associated with altered expression of glial markers CNPASE, GFAP, and OLIG2 in CD1 (but not BL6) cells. We also found that *Dcc*, *Scn3a*, and *Sptbn2* and their corresponding proteins were altered by ethanol exposure, but may not be true candidate biomarkers for FASD biomarkers because their alterations were not consistent in all experimental groups. In conclusion, the effects of ethanol on DNA methylation of differentiated NSC depended on sex and strain of cells, and DNA

methylation alone may not be a suitable biomarker for FASD without consideration of sex and strain. In addition, the effects of ethanol on NCS fate commitment were only observed in CD1 cells and not BL6 cells. Taken together, the results of this work provided evidence that chronic ethanol exposure affects DNA methylation and cell fate commitment in differentiated NSC, but in a sex and strain-specific manner. My results also revealed that either DNA methylation and/or candidate biomarkers (*Dcc*, *Scn3a*, and *Sptbn2*) alone may not be suitable biomarkers for FASD.

DEDICATION

To my beloved older brother, ***Shahed****,*

who was like a candle in my darkest hours,

but the wind never let him to see my bright days.

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ABBREVIATION

5-hmC	5- Hydroxymethylcytosine
5-mC	5- Methylcytosine
ACSF	Artificial Cerebral Spinal Fluid
α-MSH	Alpha Melanocyte-Stimulating Hormone
ACTH	Adrenocorticotropic Hormone
A.D	Anno Domini
ADHD	Attention Deficit Hyperactivity Disorder
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ARBD	Alcohol-Related Birth Defects
ASH2L	ASH2 Like, Histone Lysine Methyltransferase Complex Subunit
ARND	Alcohol-Related Neurodevelopmental Disorder
ATP	Adenosine Triphosphate
BAX	Bcl-2-Associated X protein
BBB	Blood Brain Barrier
B.C	before Christ
BDNF	Brain-Derived Neurotrophic Factor

bFGF	basic Fibroblast Growth Factor
BNST	Bed Nucleus of the Stria Terminalis
°C	Degrees Celsius
cAMP	cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CDT	Carbohydrate-Deficient Transferrin
cGMP	Guanosine Monophosphate
CL	Cold Light
CNPASE	2',3'-Cyclic-Nucleotide 3'-Phosphodiesterase
CNR1	Cannabinoid Receptor 1
CNS	Central Nervous System
CREB	Cyclic AMP Response Element-Binding Protein
CREBBP	CREB-Binding Protein
DAMP	Damaged or Danger Associated Molecular Patterns
DAPI	4′,6-Diamidino-2-Phenylindole
DCC	Deleted in Colorectal Cancer
DLX2	Distal-Less Homeobox 2

DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
E	Embryonic Day
EAAT4	Excitatory Amino-Acid Transporter 4
EDTA	Ethylenediaminetetraacetic Acid
EHMT1	Euchromatic Histone Lysine Methyltransferase
ERα	Estrogen Receptor α
ERK	Extracellular signal Regulated Kinases
ESC	Embryonic Stem Cells
EtG	Ethyl Glucuronide
EtS	Ethyl Sulfate
FAEE	Fatty Acid Ethyl Ester
FAE	Fetal Alcohol Effects
FASD	Fetal Alcohol Spectrum Disorders
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor

GABA	Gamma-Aminobutyric Acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GD	Gestation Day
GDNF	Glial-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GGT	γ-Glutamyl Transferase
GSH	Glutathione
h	Hours
HATs	Histone Acetyltransferases
HDACs	Histone Deacetylases
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
IF	Immunofluorescence
IGF-1	Insulin-like Growth Factor-1
IL-1	Interleukin 1
Il3	Interleukin 3
iNOS	Inducible Nitric Oxide Synthase
IQ	Intelligence Quotient

IPA	Ingenuity Pathway Analysis
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
kDa	Kilodalton
KDM1B	Lysine (K)-specific Demethylase 1B
LncRNA	Long ncRNA
LPS	Lipopolysaccharide
LTP	Long-Term Potentiation
MAPK	Mitogen-Activated Protein Kinases
MB	Methylene Blue
MBD2	Methyl Binding Domain 2
MCV	Mean Corpuscular Volume
MeCP2	Methyl CpG Binding Protein 2
µg	Microgram (weight)
mg	Miligram
mGluR1	Metabotropic Glutamate Receptor 1
min	Minutes
miRNA	MicroRNA

µl	Microliter (volume)
mL	Mililiter (volume)
µM	Micromolar (concentration)
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NaOH	Sodium Hydroxide
Na3VO4	Sodium Vanadate
$Na_v1.3$	Voltage-gated sodium channel type III, alpha subunit
NCAM	Neuronal Cell Adhesion Molecules
ncRNA	Non-coding RNA
NE	Neuroepithelial Cells
NDS	Normal Donkey Serum
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
nM	Nanomolar
NMDA	N-methyl-D-aspartate
NPC	Neural Progenitor Cells
NSC	Neural Stem Cells

NT-3	Neurotrophin-3
OLIG2	Oligodendrocyte transcription factor 2
P	Postnatal day
PAE	Prenatal Alcohol Exposure
PAX6	Paired Box 6
PEth	Phosphatidylethanol
pFAS	Partial Fetal Alcohol Syndrome
PI3K/Akt	Phosphatidylinositol 3-kinase and protein kinase B
POMC	Polypeptide proopiomelanocortin
PSA	Polysialylated
PTMs	Post-Translational Modifications
PVDF	Poly-Vinylidene Difuoride
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
rhEGF	recombinant Human Epidermal Growth Factor
RhOA	Ras homolog gene family, member A
ROS	Reactive Oxygen Species
RNA	Ribo-Nucleic Acid

RNA-seq	RNA-sequencing
RPM	Revolutions Per Minute
RT	Room Temperature
SAM	S-Adenosyl Methionine
SCA5	Spinocerebellar Ataxia Type 5
SDS	Sodium Dodecyl Sulfate
SETD7	SET domain containing (lysine methyltransferase) 7
SFK	Src Family Kinases
SRY	Sex-Determining Region Y
SSC	Saline–Sodium Citrate
TBS	Tris EDTA Buffer
TET	Ten-Eleven Translocation
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor alpha
TrkB	Tropomyosin receptor kinase B
TSS	Transcription Start Site
UV	Ultra Violet

UNC-5	Uncoordinated-5
Xist	X-Inactive Specific Transcript
XCI	X Chromosome Inactivation
YAP	Yes-Associated Protein

CHAPTER ONE: INTRODUCTION

1.1. Prenatal alcohol exposure and developmental timing of the brain

Prenatal alcohol exposure (PAE) is associated with a wide range of neurobehavioral, neurodevelopmental, physical and cognitive difficulties in *in utero*-exposed individuals. A large body of evidence indicates that the developing brain is highly vulnerable to pathological genetic variation and environmental insults ranging from prenatal inflammation to toxic effects of alcohol (1-3). During mammalian brain development, there are particular time windows, during which central nervous system (CNS) undergoes significant changes related to distinguished developmental stages, called “critical periods”. During critical periods, significant physiological and cellular changes occur and brain development is accompanied by neuronal migration, circuit formation and synaptic development. Brain development during sensitive periods is controlled *via* several interconnected mechanisms such as epigenetic mechanisms and gene expression, molecular guidance cues, and external environmental factors such as mother-embryo crosstalk during gestation. Indeed, ample evidence indicates that critical periods are sensitive time windows, during which experiencing any environmental insult may cause impairment of CNS development and manifestation of psychiatric and neurodevelopmental disorders (4-6). Although brain development is initiated by early gestation during the *in utero* embryonic patterning, the major brain development milestones take place during the first decades of lifespan when gliogenesis, synaptogenesis, myelination, and finally synaptic pruning occurs in late adolescence (2, 7). On the other hand, the developing brain is an extremely energy-consuming organ due to its highly energy-demanding processes such as neurogenesis, neuronal migration, and the activity of sodium/potassium pumps that generate electrical potentials. This high level of energy is supplied by mitochondria, which are not fully mature and functional during brain

development. In addition, the developing brain is very susceptible to environmental insults such as PAE because it contains high levels of unsaturated fatty acids, immature and proliferative cells, low antioxidant capacity, and high level of oxygen demand (3, 8).

Focusing on the PAE and critical developmental periods, extensive research has shown that both alcohol levels and the duration, through which the developing brain is exposed to alcohol, significantly determine the extent of brain damage. However, timing of alcohol exposure during brain development may determine what brain structures are affected and the degree of the damage (9) (**Fig. 1.1**). The first trimester of pregnancy has been reported as a period, in which PAE is highly associated with the development of fetal alcohol spectrum disorders (FASD). However, several lines of research have shown that PAE during all trimesters is considered to be a risk factor for FASD (10, 11). Both clinical and experimental studies have shown that PAE during gastrulation (first 3 weeks of gestation in humans, first week of gestation in rodents) significantly increases the risk of facial dysmorphology, which is a main feature of fetal alcohol syndrome (FAS). This period comprises of pre-implantation, implantation, and gastrulation when the majority of mothers are unaware of their pregnancy (12, 13). For instance, preclinical studies have shown that PAE on gestation day (GD) 7 specifically affects medial forebrain regions, while PAE on GD 8 particularly induces morphological alterations in the olfactory bulb, hippocampus, and cerebellum. Alcohol exposure on GD 9 reduces the cerebellar volume, increases the volume of ventricles, and induces malformation of the cerebral cortex, hippocampus, and right striatum structures (14-16). Another critical period during fetal brain development is neuroepithelial cell proliferation and migration (week 7-20 in humans and gestation day 11-20 in rodents), which are also affected by PAE (17, 18). The third trimester of human gestation (equivalent to gestation day (GD) 20 to postnatal day (PD) 19) is accompanied

by significant brain growth, outgrowth of glial lineage cells, and dendrite arborization. It has been shown that PAE during this period may induce severe cognitive disabilities, microcephaly and reduced neurogenesis in several brain regions such as hippocampus (9, 19). Collectively, the timing of PAE is a strong factor in determining the brain-specific changes and relevant behavioral phenotypes that are responsible for clinical variability in FASD patients.

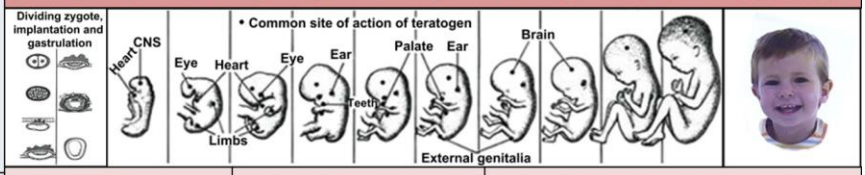
Developmental Stages in Human/Rodents (Mice & Rats)	**Ovum Stage**	**Embryonic Stage**	**Fetal Stage**	**Fetal Stage (human) Postnatal/Fetal Stage (Rodents)**
Developmental Time in Mice & Rats	**E1- E7**	**E8-14**	**E14-21**	**E21-P10**
Developmental Stages of Human	1 2	3 4	5 6 7 8	9 16 20-36 108 Weeks
				
Normal Brain Development & Growth	✓ **Epigenetic Programming** ✓ **Neural tube formation** ✓ **Stem cells proliferation**		✓ **CNS formation and differentiation** ✓ **Proliferation & differentiation of NSCs** ✓ **Neuronal migration,** ✓ **Corpus callosum formation**	✓ **Brain growing at its fastest rate** ✓ **Astrogliogenesis and myelin development** ✓ **Functional neural connections (synaptogenesis)** ✓ **Differentiation of cerebellum**
Brain Development & Growth following Prenatal Alcohol Exposure	X **FAS dismorphia** X **Severe neural tube defects** X **Increased neural crest cell death** X **Reduced neural proliferation**		X **Abnormal cell migration** X **Abnormal radial glia: neuronal and astroglia deficits** X **Neural cell loss** X **Corpus callosum malformations**	X **Prominent microcephaly** X **Abnormal glial development** X **Increase in natural cell death and cell necrosis** X **Alterations in synaptogenesis and plasticity** X **Alterations in the cerebellum**

Figure 1. 1 The effects of PAE on developing CNS in humans and rodents

F; fetal; NSC, neural stem cells; P, postnatal. Adapted from Guerri and Pascual, 2017, (Doi: 10.1016/B978-0-12-804239-7.00024-X).

1.2. An Overview to FASD

1.2.1. History of FASD

Although there are similar reports throughout the history about the negative effects of alcohol consumtion during pregnancy, Lemoine *et al*., reported a disorder called alcoholic embryopathy (20-22). Indeed, authors of this report found the etiology of FAS for the first time and reported that maternal alcohol consumption induced symptoms such as low intelligence quotient (IQ), psychomotor abnormalities, and facial morphology (21-23). Further research by Jones and Smith (1975) led to the introduction of FAS in medicine terminology. These researchers found that PAE is associated with a specific pattern of brain malformations and growth impairment in children (24, 25). Since 1968, researchers have conducted clinical and experimental studies on the consequences of PAE on biological systems using a variety of animal models and human resources, and concluded that adverse effects of PAE are not restricted to FAS and include a way wider spectrum of disorders, called FASD. Indeed, FASD is an umbrella term that identifies the spectrum of PAE-induced consequences, which are classified as partial FAS (pFAS), fetal alcohol effects (FAE), alcohol-related birth defects (ARBD), and alcohol-related neurodevelopmental disorder (ARND) (26).

1.2.2. Diagnosis of FASD

The effects of PAE on fetal development depend on many factors such as pattern and timing of PAE, alcohol dose, genetic background, maternal nutrition, socio-economic conditions, and maternal health. As mentioned above, PAE is able to induce a range of neurodevelopmental disorders under the umbrella term, FASD. Fetal alcohol syndrome is the most severe form of disorders induced by PAE and is known as one of the major causes of intellectual disabilities and behavioral dysfunction in Western countries (27). A diagnosis of FAS relies on three main

features: 1) specific form of facial dysmorphologies (smooth philtrum, thin vermillion border, and short palpebral fissures, please see **Fig.1.2**), 2) evidence for prenatal or postnatal growth defects, and 3) CNS abnormalities in patients such as neurological, behavioral, anatomical, or the combination of them. Similarly, partial FAS describes individuals who have confirmed evidence of PAE and have signs and symptoms associated with severe PAE, but not diagnosed as FAS. In 1978, Clarren and Smith introduced a new term fetal alcohol effects (FAE) that was similar to FAS, but failed to fall into FAS definition (25). Since FAE term was a broad definition and was incorrectly used by clinicians and researchers to address the PAE effects in the literature, usage of this term was discouraged by scientists in both the literature and clinics. Another group of disorders classified as disorders related to PAE is alcohol-related neurodevelopmental disorder that defines people with confirmed PAE who have neurodevelopmental/cognitive/behavioral abnormalities irrelevant to any other genetic or environmental reason (28, 29). This term differs from alcohol-related birth defects, which specifically describe the physical, functional, and anatomical defects induced by PAE in children (30, 31). Overall, FASD diagnosis is done based on detailed diagnostic criteria, which are suggested by valid and standard guidlines (**Table 1.1**).

1.2.3. Prevalence, socio-economic impact and comorbidities

Although the global prevalence of FASD is estimated at ~1% of the population, it should be noted that there is available prevalence rates data for only 10 countries in the world. Globally, a recent meta-analysis research has shown that South Africa has the highest prevalence rate for FASD (~11.3%), FAS (~5.5%), and ARND (~2%) while the highest prevalence rate for pFAS is observed in Croatia (~4.3%) (27). In Canada, PAE-related disorders are underdiagnosed and despite extensive efforts to notify people about the adverse effects of alcohol consumption during pregnancy, it is estimated that about 14% of pregnancies among normal population are

exposed to alcohol. The prevalence rates for PAE during pregnancy differs region by region and it is 50% and 60% among aborginals in north Alberta and Inuits of Quebec, respectively (32-34). It is quite clear that FASD is a socio-economic burden. It has been estimated that only cost of diagnostic servises is more than $5 million per year and overall costs of FASD is around $2 billion Canadian Dollars (34). It is important to note that cognitive and behavioral dificulties of FASD are considered as primary disabilities which may be exacerbated by secondary disabilities such as addictions, crimes and involvement with the law, and poor school performance. These factors increase the socio-economic burden of FASD (35). In addition, increasing lines of reseach have shown that FASD patients suffer from a wide range of comorbid medical conditions. For example, it has been reported that FASD is associated with behavioral comorbidities (impulssive behavior, depression, anxiety), increased risk of cancer, and metabolic disorders (such as obesity) (36-39). FASD is not a diagnostic term and consists of different forms of complex neurodevelopmental disorders without any currently available treatment. Since 50% - 90% of behavioral and cognitive disabilities of FASD are similar to attention deficit hyperactivity disorder (ADHD), treating FASD patients with stimulants was suggested. However, treating FASD patients with stimulants led to mixed results and in many cases stimulant therapy led to poor results in FASD patients (40, 41). To treat the memory and learning deficits in FASD, activation of cyclic AMP response element-binding protein (CREB) was considered as a therapeutic target in order to enhance the poor ability of neuronal plasticity and synaptic formation in the brain (42, 43). Recently, nutriton and diet therapy has gained attention for the treatment of FASD. Clinical and preclinical studies suggest that nutrition interventions are approprite tools to attenuate the negative effects of PAE on the developing fetus (44). Since PAE is able to induce maternal micronutrient deficiency, nutritional supplementation has been

shown to have protective effects against negative impact of PAE. Indeed, nutritional supplementation exerts its effects through a variety of mechanisms such as reducing the alcohol induced-oxidative stress and redox imbalance as well as normalizing the altered epigenetic factors following PAE (45, 46). For example, it is known that PAE is associated with altered methylation patterns of genes during pregnancy. Choline supplementation, as a methyl donor, has been shown to reverse the effects of PAE on DNA methylation machinery in the hippocampus and prefrontal cortex of rats (47, 48). Interestingly, advantagous effects of nutritional interventions are not limited to prenatal stage. Research has shown that omega-3 fatty acids, vitamin D, and choline are micronutrients that attenuate the effects of alcohol and cognition and behavior (49, 50). Collectively, early diagnosis, appropriate environment, adequate support, appropriate socio-economic conditions, nutritional interventions, physical activity, and exercise are some of the many factors that are capable in mitigating the burden of PAE on economy, society, families, and most importantly FASD patients.

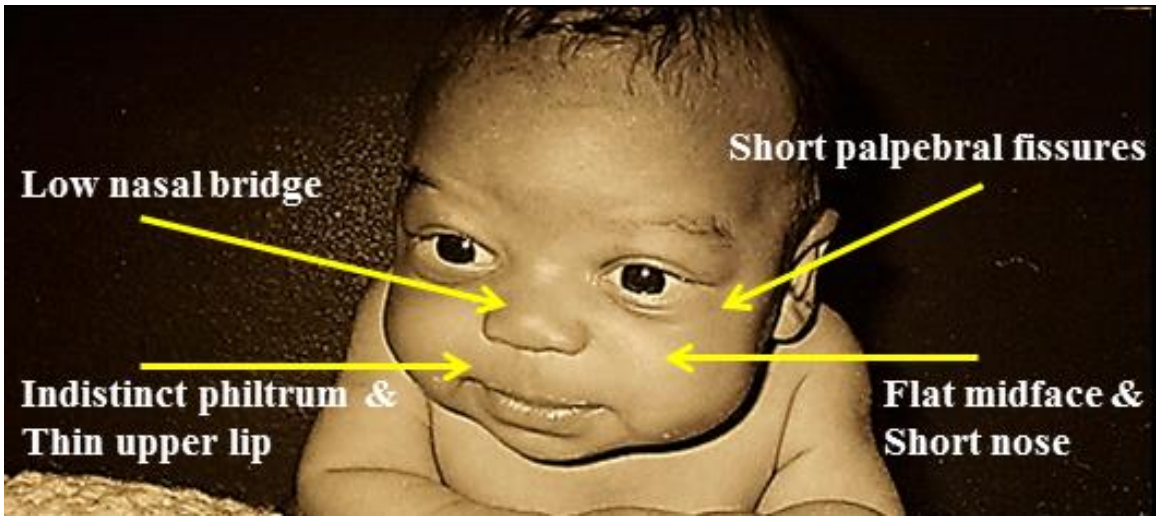


Figure 1. 2 Facial characteristics in FAS

Adopted from http://littleearthlingblog.com/cdc-fetal-alcohol-spectrum/

Table 1. 1 Canadian diagnostic criteria for PAE-related disorders

Adapted from Fitzpatric et al. 2012 (doi: 10.1136/bmjopen-2012-000968)

Diagnostic category	**Fetal alcohol syndrome**	**Partial fetal alcohol syndrome**	**Neurodevelopmental disorder- alcohol exposed**
Diagnostic criteria	Requires all four criteria below to be met	Requires confirmed prenatal alcohol exposure, the presence of two of the three characteristic facial anomalies at any age and CNS criteria to be met	Requires confirmed prenatal alcohol exposure and CNS criteria to be met
Prenatal alcohol exposure	Confirmed or unknown	Confirmed	Confirmed
Facial anomalies	Presence of all three of the following facial anomalies at any age: - Short palpebral fissure length (≤2 SDs below the mean using the Hall charts) - Smooth philtrum (rank 4 or 5 on UW Lip-Philtrum Guide) - Thin upper lip (rank 4 or 5 on the UW Lip-Philtrum Guide)	Presence of any two of the following facial anomalies at any age: - Short palpebral fissure length (≤2 SDs below the mean) - Smooth philtrum (rank 4 or 5 on the UW Lip-Philtrum Guide) - Thin upper lip (rank 4 or 5 on the UW Lip-Philtrum Guide)	No anomalies required
Growth deficit	Birth length or weight ≤ 10^{th} percentile adjusted for gestational age or postnatal height or weight ≤10th percentile	No deficit required	No deficit required
CNS abnormality	Significant CNS dysfunction (evidence of impairment in three or more of the following CNS domains): - Hard and soft neurological signs; seizure disorder; gross and fine motor functioning; articulation, phonology and motor speech - Cognition (IQ or uneven cognitive profile) - Memory - Executive functioning and abstract reasoning - Communication (expressive and receptive language) - Attention deficit/hyperactivity +/- other behavioral problems; abnormal sensory processing - Visual motor integration - Adaptive behavior/social skills/social communication - Academic achievement - CNS structure (including head circumference # 3rd percentile or other structural CNS abnormality)		

1.3. Potential mechanisms of PAE-induced brain abnormalities

Although extensive attempts have been made by many researchers to understand the mechanisms of PAE-induced abnormalities in the developing brain, the exact mechanism(s) through which alcohol exerts its wide-range effects on the CNS are not clear. Indeed, pathophysiology of PAE-related disorders is complex because alcohol interferes with various biological systems. Also, alcohol dosage, duration of exposure, timing of exposure, genetic background are determinant factors for the PAE-induced effects on particular biological system.

1.3.1. Dysregulation of trophic factors

During brain development, trophic factors control a wide range of biological processes such as neuronal survival and growth, neuronal maintenance and maturation, and regulation of apoptosis (51, 52). Ample evidence indicates that PAE alters the signaling and production of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF-1), neurotrophin 3 (NT-3), and fibroblast growth factor (FGF) (53-58). For example, exposure to low levels of alcohol has been shown to reduce the BDNF-dependent plasticity of GABAergic neurons in the developing hippocampus (53). Also, moderate alcohol exposure during brain development (130 mg/dL, maternal blood level) disrupts the BDNF signaling *via* altering the expression of tropomyosin receptor kinase B (TrkB) and its downstream pathway mitogen-activated protein kinases/extracellular signal regulated kinases (MAPK/ERK) and phosphatidylinositol-3-kinase)/AKT or PI3K (54). Similarly, PAE is able to reduce the expression of GDNP, which plays a critical role in neuronal protection against cell death (55, 56). Alcohol exposure has been shown to inhibit IGF-1 function and auto-phosphorylation as well as IGF-1 signaling (57, 58). In addition, there are several reports indicating the negative effects of PAE on neurotrophic factors

and growth factors relevant to cell-cycle, cell division and differentiation (59, 60). Collectively, alteration of trophic factors by PAE during CNS development is associated with neurotoxicity and impairment in neuronal proliferation, survival and maturation, which results in short-term and long-term developmental abnormalities observed in FASD patients.

1.3.2. Alterations in cell adhesion molecules

Cell adhesion molecules play a pivotal role in mediating cell-cell interactions, which determines brain organization during the development. These molecules are members of the immunoglobulin superfamily and are involved in many biological processes such as synapse formation, synaptic plasticity, neuronal migration, and development. Recent studies have demonstrated that PAE alters the function and regulatory role of cell adhesion molecules. For example, it has been illustrated that continuous exposure to moderate levels of alcohol changes the expression of neuronal cell adhesion molecules (NCAM) through increasing the polysialylated NCAM (PSA-NCAM) in the cortical areas during synaptic formation period (61, 62). Since NCAM plays a crucial role in the brain synaptic formation and plasticity, it is clear that disruption of these cell adhesion molecules plays a part in the abnormal morphology and plasticity of brain following PAE. Also, NCAM can be considered as a therapeutic target against the negative impact of PAE. For example, treating mouse embryos with Octanol reversed the L1-mediated cell adhesion and alcohol-induced cell death (63, 64).

1.3.3. Disturbances in glial development and neuronal migration

Both studies on humans and animals have demonstrated that PAE has long-lasting effects on glial function during brain development (65, 66). It is well-known that glial cells play a crucial role during brain development through mediating important processes such as neuronal migration, formation of the corpus callosum and anterior commissure, as well as synaptic

formation. Radial glia are not only multipotent neural precursor cells, but also contribute to neuronal guidance during neuronal migration. Also, glial cells produce several chemicals that regulate and enhance synaptic formation and function. Regarding the multiple roles of glial cells, it is clear that during critical periods of CNS development PAE has profound adverse effects on the brain such as malformation of cortical areas and impaired function of astrocytes and oligodendrocytes. Several lines of research have shown that PAE affects glial differentiation to astrocytes and neurons, reduces the number of radial glia cells, interferes with the glial product and growth factors, and disrupts astroglial survival and function (65, 66). PAE induces a wide range of defects and abnormalities in the developing brain such as the loss of white matter, impaired energy metabolism, and dysregulation of neurotransmission and neuronal plasticity (65, 67, 68).

As mentioned above, evidence indicates that PAE is able to affect normal neuronal migration during brain development. Indeed, alcohol-induced abnormal neuronal migration is one of the most important adverse effects of PAE contributing to the pathophysiology of disease. Microcephaly is observed among individuals diagnosed with PAE-related disorders namely FAS and pFAS. In the case of FAS, PAE-induced abnormal neuronal migration leads to the formation of leptomeningeal neuroglial heterotopia. This structure is an aberrant sheet (made of neuronal and glial cells), which covers the surface of some regions of the brain such as cerebrum and cerebellum. Also, some other anomalies such as corpus callosum agenesis and septo-optic dysplasia were reported in FASD patients (69-71). Alcohol exerts its negative effects on neuronal migration through a variety of mechanisms. Affecting radial glia function is one of the mechanisms by which PAE affects neuronal migration. Most studies have used cerebellar granule cells as a valid model system to investigate the effects of alcohol on neuronal migration.

These studies revealed that alcohol decreases the number of spontaneous calcium spikes in the migrating cells and slows down calcium signaling. In addition, alcohol disrupts cyclic nucleotide metabolism and signaling such as cyclic AMP (cAMP) and cyclic GMP (cGMP) (72, 73). Research has shown that enhancing calcium signaling and cGMP production mitigates negative effects of alcohol on neuronal migration. Researchers have suggested several other molecular pathways that are affected by PAE, but calcium signaling and cGMP seem to be the main molecules affected by PAE. Early diagnosis, namely *in utero*, may provide opportunity to correct the aberrant neuronal migration through enhancing calcium signaling in developing fetus (71).

1.3.4. Oxidative damage and neuro-immune system

As mentioned earlier, the developing brain demands a huge amount of energy to govern crucial processes such as neurogenesis, neuronal migration and the activity of sodium/potassium pumps that generates electrical potentials. These large energy levels are supplied by mitochondria, which are not completely mature and functional during brain development (74). As mentioned above, the developing brain is highly susceptible to environmental insults such as PAE because it contains high levels of unsaturated fatty acids, immature and proliferative cells, low antioxidant capacity, and high level of oxygen demand (3). Numerous clinical and preclinical studies have shown that alcohol and its metabolites are able to induce oxidative stress and toxicity (46). Alcohol is converted into acetaldehyde by 3 different groups of enzymes, which are alcohol dehydrogenase, CYP2E1, and catalase, mostly in the liver. Then, aldehyde dehydrogenase converts acetaldehyde to acetate, and the later compound converts to water and CO2 (**Fig.1.3**). It is important to note that acetaldehyde is a well-known teratogen and toxic compound. It is important to note that alcohol levels are similar between the mother's blood and fetal tissues, but acetaldehyde levels are higher in the mother's blood when compared to fetus.

Interestingly, the activity of enzymes involved in alcohol metabolism is negligible in the fetal liver (46, 75). Alcohol metabolism is the first source of reactive oxygen species (ROS) formation as ROS are side products of alcohol metabolism. However, PAE induces dysfunction in the mitochondrial bioenergetics, which is the main source of ROS by immature mitochondria in the developing brain (46). It should be noted that the function and structure of mitochondria during the prenatal stage differs from adulthood. During fetal development, brain mitochondria have lower respiratory capacity, lower cristae and matrix protein contents, and lower calcium uptake capacity in comparison with mitochondria in the adult brain (74). These differences highlight the fact that mitochondria are vulnerable to toxic effects of alcohol and its metabolites. Evidence indicates that PAE prevents mitochondrial proliferation and differentiation, decreases ATP production and GSH levels, and consequently induces oxidative stress. Further, alcohol reduces both enzymatic and non-enzymatic antioxidant capacity by decreasing the synthesis and activity of glutathione peroxidase and GSH synthase. Studies on the effects of PAE in prenatal stages have shown that mitochondrial dysfunction, imbalanced redox system, and increased nitrosative stress are the consequences of PAE observed in several brain regions (46).

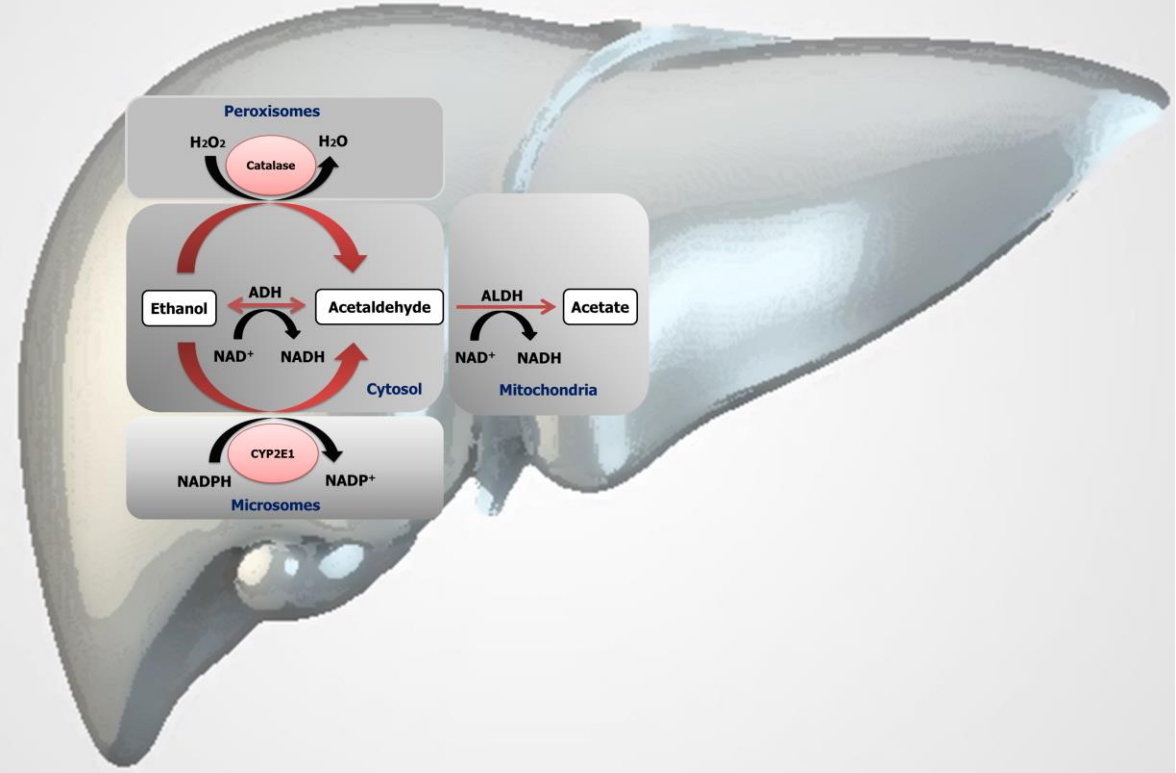


Figure 1. 3 Hepatic metabolism of ethanol. Ethanol is mainly metabolized by three enzymatic mechanism 1) catalase in the peroxisome, 2) ADH and ALDH in the cytosol, and 3) CYP2E1 in the microsomes (76)

As stated earlier, PAE has long-lasting effects on the glial cells and neuro-immune system during the brain development. It is traditionally believed that brain is an immune-privileged organ, namely because of the blood brain barrier (BBB) and lack of lymphatic drainage in the brain. However, we currently know that microglia and astrocytes are serving as brain immune cells and are involved in a wide range of biological events in the brain (65). Recent evidence indicates that oxidative stress and impaired antioxidant system is associated with the activation of sterile inflammation through the formation of damaged or danger associated molecular patterns (DAMPs) (77, 78). These biomolecules are released from the injured or dying cells and are mistakenly recognized as endogenous ligands for innate immunity. It has been shown that innate immunity in uterine and placenta protects the fetus during

gestation, and PAE is able to activate the innate immunity in both placenta and fetus by triggering the immune-inflammatory responses through toll-like receptors (TLRs). Evidence has shown that PAE is able to activate immune-inflammatory responses by increasing pro-inflammatory cytokines. It has been reported that alcohol is able to activate TLRs and their downstream such nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), inducible nitric oxide synthase (iNOS), pro-inflammatory cytokines (e.g. TNF-α, and IL-1). Activation of these pathways in glial and neuronal cells worsens the production of ROS and oxidative damage, and consequently glial and neuronal cell death. Research has shown that anti-inflammatory agents such as minocycline and pioglitazone could alleviate the inflammatory responses in glial cells following alcohol exposure. Thus, PAE is able to alter several developmental processes *via* inducing oxidative stress, neuro-inflammation and deregulation of glial function (79-81).

1.4. Genetics and epigenetics basis of FASD

1.4.1. Genes involved in the susceptibility to PAE

Before introducing the genes that are directly involved in FASD, it is important to note that there are several maternal and paternal factors that affect gene expression in the developing embryo and predispose it to the negative effects of PAE. For example, maternal hormones have significant effects on the pattern of gene expression in the developing fetus (82). The importance of genetic background on the effects of PAE comes from the studies showing that while the effects of PAE on monozygotic twins are 100% the same, dizygotic twins only show 63% concordance (83). Regarding the role of genetic factors in the development of FASD, it has been shown that variant alleles of genes involved in alcohol metabolism, cellular development, and regulation of hypothalamic-pituitary-adrenal (HPA) axis contribute to vulnerability to adverse

effects of PAE (84). Variants of the gene that encode ADH are linked to maternal drinking pattern, addiction and risk for FASD. For example, maternal variant alleles of ADH1B-B3 are associated with fast metabolism of alcohol that decreases the FASD risk, and maternal alcohol intake because of massive production of aldehyde. Similarly, previous studies have shown that variants of *Aldh2* in mice and *Fancd2* in zebrafish that are involved in alcohol metabolism are considered as genes that determine alcohol susceptibility (85-87). Another gene in humans, which is associated with FASD and sudden infant death syndrome is *HTTLPR* that encodes for serotonin transporter and is susceptible to PAE (88, 89). Ample evidence demonstrated the deleterious impact of PAE on HPA axis activity. One of the most important genes in HPA axis is *POMC* that encodes precursor polypeptide proopiomelanocortin or (POMC). Cleavage of POMC results in the release of adrenocorticotropic hormone (ACTH), β- endorphin, and α-MSH. Clinical and experimental studies reported that PAE affects the expression of the *POMC* gene as well as neurons that produce POMC in the arcuate nucleus of the hypothalamus (84, 90, 91). In addition, there are many reports about the genes involved in neuronal migration, nitric oxide pathway, insulin, and retinoic acid signaling, which are affected by PAE (9).

1.4.2. Epigenetics and PAE

Epigenetics refers to a variety of mechanisms, which modulate gene expression without affecting the encoding DNA sequences (92). Epigenetic alterations emerge in different forms of histone modifications, DNA methylation, and non-coding RNAs, among other mechanisms (93, 94). These epigenetic changes play a key role in exerting the effects of environmental stimuli that further shape the brain and behavior during development. Mounting evidence indicates the impact of PAE on epigenetic machinery and the potential role of PAE-induced epigenetic changes in the pathophysiology of FASD. It is because epigenetic mechanisms tightly control the

fetus development through altering the gene expression during different critical stages of development.

1.4.2.1. DNA methylation

DNA methylation is a phenomenon found in both physiologic and pathophysiologic conditions (94). DNA methylation plays a critical role in early stages of the development (including differentiation in the developing brain), and contributes to cortical maturation during childhood and adolescence. The most studied form of DNA methylation refers to the addition of a methyl group to CpG site of the cytosine or C5 that leads to formation of 5-methylated cytosine or 5-mC. This form of DNA methylation has been reported to regulate X chromosome inactivation, imprinting, malignancy, differentiation, and some other cellular functions. DNA methyltransferases (DNMTs) are a family of enzymes, which are responsible for the methylation of cytosine by covalent addition of a methyl group mostly using S-adenosyl methionine (SAM), as a methyl donor. While 5-mC is considered as the fifth base in the literature, oxidizing of 5-mc by TET (Ten Eleven Translocation) enzymes leads to formation of 5- hydroxy-methylcytosine (5-hmC) or sixth base (94, 95). Roughly half of CpG islands in human and mouse are linked to transcription start sites (TSS) and are responsible for gene inactivation and control of gene expression (96, 97). Evidence has shown that PAE is able to affect DNA methylation machinery *via* different mechanisms. Alcohol and its metabolite, acetaldehyde; are known to affect DNMTs by reducing the activity and expression of these enzymes. In *in vitro* studies, acute exposure to ethanol reduced both activity and protein level of DNMTs, and DNA methylation in astrocytes and fibroblasts (98, 99). However, other studies have shown that acute ethanol exposure increases DNMTs activity without altering 5-mC or 5-hmC levels (100, 101). The impact of DNA methylation alterations on the gene expression relies on the binding of methyl-CpG

binding proteins such as MeCP2 (methyl CpG binding protein 2) (102). Results of the previous studies on the effects of alcohol on MeCP2 expression are not consistent. While some studies showed that alcohol reduces MeCP2 expression in fibroblasts, others have reported that alcohol increases MeCP2 levels in NSC (99, 101, 103). In addition, animal studies have shown that PAE decreases DNMTs expression, and DNA methylation levels during neonatal or embryonic stage, but increases same measures in the adolescence and adulthood of exposed subjects (104). Alcohol is also able to induce DNA hypomethylation by interacting with molecules that: 1) serve as methyl-donors (such as SAM), 2) are involved in methyl metabolism (such as folate), and 3) absorption of methyl sources (such as choline and folate) (105-107). Recent research has provided evidence that choline supplementation protects against PAE-induced DNA methylation in a variety of genes and PAE-related symptoms (47). The methylation status of many genes is crucial for the normal development of the fetus during the prenatal stage. For example, 5-mC levels increase in neuroepithelial cells (NE) during early stages of development, and decrease during differentiation of NE to more mature cells. On the other hand, 5-hmC levels are higher in mature cells compared to neural progenitor cells (NPC) (108, 109). In addition, differentiation, and proliferation of embryonic stem cells (ESC) to NPCs and more mature neuronal and non-neuronal cells require a series of well-tunes patterns of methylation and de-methylation of specific genes that govern the differentiation processes during critical periods of development. For example, *Oct4*, *Nanog*, and *Sox2* genes undergo DNA methylation reprogramming during early stages of development in NPCs (110). It has been reported that PAE affects the genes that controls neural stem cell differentiation, proliferation, and cell cycle. In this regard, PAE induced hypermethylation of genes that control cell cycle such as *CcnB1*, *Cdc20*, *Bub1*, and *Plk1*, while reduces the methylation level of other genes that contribute to differentiation such as *Adra1a*,

Tnf, and *Pik3r1* (100, 111). Further, it has been shown that increased DNA methylation of the *Bdnf* gene is associated with deregulation in BDNF pathway and behavioral abnormalities following PAE in rodents (112, 113). Again, it is important to note that the results of previous studies on the effects of alcohol on DNA methylation machinery are not consistent, and this inconsistency depends on various factors such as the dose, timing, and maternal conditions (Please see **Table 1.2 and 1.3**).

Table 1. 2 Summary of studies on the impacts of PAE on 5-mC and 5-hmC

Embryonic day (E), Postnatal day (P), Day (D), Male (M), Female (F), Unclear (U), Both sexes (B). Adapted from Gavin et al. doi:10.3390/genes8050137

5-methylcytosine (5mC)							
Global /Gene	**Effect**	**Exposure time**	**Time of study**	**Tissue**	**Species**	**Sex**	**References**
Global (continued next page)	↓		48 h	Embryonic fibroblasts	Mice	M	(99)
	↓	E8.25	E10	Neural Tube	Mice	U	(111)
	↓	E9-11	E11	Whole Embryo	Mice	B	(105)
	–	E5-16	E17	Ammonic Neuroepithelium	Mice	B	(112)
	↑	E5-16	E17	Intermediate Zone	Mice	B	(112)
	↑	E5-16	E17	Hippocampus	Mice	B	(112)
	↓	P7	P8	Hippocampus	Mice	B	(114)
	↓	P7	P8	Neocortex	Mice	B	(114)
	↑	P2-10	P21	Prefrontal Cortex	Rats	B	(48)
	↑	P2-10	P21	Hippocampus	Rats	B	(48)
	–	E7-21	P60-80	Hypothalamus	Rats	B	(115)

Genome-wide	Mostly↓		48 h	NSC		B	(116)
	↑↓	E8-10	E10	Whole Embryo	Mice	B	(107)
	Mostly ↑	P4 & P7	P70	Hippocampus	Mice	M	(117)
	↑↓		5-18 years old	Buccal Epithelial Cells	Human	B	(118)
***Mecp2* Promoter**	↑	D0-2	D2	NSC	Mice	B	(101)
	↓	D0-8	D8	NSC	Mice	B	(101)
Cell Cycle Genes	↑		48 h	NSC		F	(100)
***Gfap* Promoter**	↑	E1-21	E21	Whole Brain	Rats	B	(119)
***Plat* Promoter**	↓		24 h	Primary cortical Astrocytes	Rat	B	(98)
Imprinted Genes	↓	Correlated with Alcohol Drinking		Sperm	Human	M	(120)
KCNQ1OT1	↓	1 to 26 years old		Blood	Human	B	(121)
***PEG3* Promoter**	↓	1 to 26 years old		Blood	Human	B	(121)
***Slc6a4* Promoter**	↑	E1-21	P55	Hypothalamus	Rats	F	(122)
***Pomc* Promoter**	↑	E7-21	P60-80	Hypothalamus	Rats	B	(115)

Gm9268 **Promoter**	↑	E0.5-8.5	P28	Hippocampus	Mice	M	(123)
Vpreb2 **Promoter**	↑	E0.5-8.5	P28	Hippocampus	Mice	M	(123)
Olfr601 **Promoter**	↓	E0.5-8.5	P28	Hippocampus	Mice	M	(123)
Slc17a6 **Promoter**	↓	E0.5-8.5	P120	Hippocampus	Mice	M	(124)
5-hydroxymethylcytosine (5-hmC)							
Global	↓	E5-16	E17	Ammonic Neuroepithelium	Mice	B	(112)
	↓	E5-16	E17	Intermediate Zone	Mice	B	(112)

1.4.2.2. Epigenetics and PAE; histone modifications

Nucleosomes are biological structures that form the repetitive units of the chromatin structure and consist of histone octamers, including 2 copies of histones H2A-H2B dimers, and two molecules of histone H3 and H4, which are wrapped by the DNA molecule. Upon post-translational modifications (PTMs), histones experience several types of modifications, which lead to their interaction with other molecules such as DNA or other histones (5, 6). Up to now, there are different forms of histone modifications that have been recognized such as acetylation, methylation, phosphorylation, and ubiquitination. There are a large number of enzymes responsible for making and omitting these unstable epigenetic marks at histone levels (125). Focusing on FASD, histone acetylation and methylation have been reported to play a crucial role in the pathophysiology of these disorders (**Table 1.4**) (7). Histone acetyltransferases (HATs) and

histone deacetylases (HDACs) are the main families of enzymes, which account for histone acetylation. Lysine residue in the N-terminal of histones is the target of acetylation (8). On the other hand, histone methylation is also engaged in the mechanisms of alcohol toxicity. Methylation of histones can occur in mono-, di-, or tri-methylation modes and each type of modification may lead to specific and distinct effects on gene transcription (11). For instance, while H3K4 is tightly associated with gene activation, H3K9 and H3K27 have been shown to have repressive effects (12).

Effects of PAE on histone modifications depend on many factors such as time of alcohol exposure, exposed species, dose of alcohol, and the exposed region in the brain. Cell culture studies have shown that low dose ethanol increases the histone acetylation while high dose ethanol treatment reduces the level of histone acetylation in NSC (104). Also, treating NSC with high dose ethanol has been reported to enhance the expression of euchromatic histone lysine methyltransferase 1 (*Ehmt1*), a gene responsible for encoding H3K9 methyltransferase, and reduce the expression of absent, small, or homeotic-like (*Ash2l*) transcripts, which controls the expression of H3K4 methyltranferase complex. These results suggest that high dose ethanol is associated with increased condensed chromatin, which is associated with decreased expression of some genes such as *Pomc* gene (104, 126, 127). Animal studies have shown that PAE reduces histone acetylation levels in the cerebellum and hypothalamus of animals through decreasing the expression of CREB-binding protein (*Crebbp*) (115, 128). Although the effect of PAE on histone acetylation varies in a study-by-study basis, PAE increases the histone methyl marks such as H3K9me2, H3K27me2 in different brain regions. Similar reports indicate the effect of PAE on genes that control histone methylation such as increased in *Ehmt2,* and decreased expression of SET Domain Containing Lysine Methyltransferase 7 (*Setd7)* that encodes H3K4

methyltranferase. Overall, it seems that PAE has significant effects on histone modifications by increasing repressing histone marks and decreasing active histone marks which results in more condensed chromatin and reduced global transcription in FASD (47, 104, 129).

Table 1. 3 Summary of studies on the impacts of PAE on DNMTs and Methyl-CpG-binding domain (MBD) proteins

Embryonic day (E), Postnatal day (P), Day (D), Male (M), Female (F), Unclear (U), Both sexes (B). Adopted from Gavin et al. doi:10.3390/genes8050137

DNMTs							
DNMTs	**Effect**	**Exposure Time**	**Time of Study**	**Tissue**	**Species**	**Sex**	**References**
***Dnmt1* mRNA**	↓		48 h	Embryonic fibroblasts	Mice	M	(99)
	↑		5 Days	Neurospheres	Mice	B	(127)
	↑	D1-3	D7	Neurospheres	Mice	B	(126)
	–		24 h	Primary cortical Astrocytes	Rats	B	(98)
	↓	P90-155	P155	Sperm	Rats	M	(130)
	↓	P7	P8	Hippocampus	Mice	B	(114)
	↑	P7	P8	Neocortex	Mice	B	(114)
	↑	E7-21	P60-65	Hypothalamus	Rats	M	(47)
DNMT1 Protein	↓		48 h	Fibroblasts	Mice	M	(99)
	↑		48 h	NSC		F	(100)
	–		24 h	Primary cortical Astrocytes	Rats	B	(98)
	–	E6-15	P35	Striatum	Mice	U	(110)

	↓	P7	P8	Hippocampus	Mice	B	(114)
	–	E6-15	P35	Cortex	Mice	U	(110)
	↓	P7	P8	Neocortex	Mice	B	(114)
	↑	E7-21	P60-65	Hypothalamus	Rats	M	(47)
DNMT Activity	↑		48 h	NSC		F	(100)
	↓		24 or 48 h	Primary cortical Astrocytes	Rats	B	(98)
	↓	E9-11	E11	Whole Embryo	Mice	B	(105)
	↑	E1-P10	P21	Hippocampus	Rats	B	(131)
***Dnmt3a* mRNA**	↑		48 h	Embryonic fibroblasts	Mice	M	(99)
	–	P7 (high dose)	24 h	Primary cortical Astrocytes	Rats	B	(98)
	↓	P7 (Low dose)	P8	Hippocampus	Mice	B	(114)
	↑	P7 (High dose)	P8	Hippocampus	Mice	B	(129)
	↓	P7 (Low dose)	P8	Neocortex	Mice	B	(114)
	↑		P8	Neocortex	Mice	B	(129)
DNMT3A Protein	↓		48 h	Embryonic fibroblasts	Mice	M	(99)
	↓		24 h	Primary cortical Astrocytes	Rats	B	(98)
	↓	P7 (High dose)	P8	Hippocampus	Mice	B	(114)
	↑	P7(Low dose)	P8	Hippocampus	Mice	B	(129)

	↓	P7 (High dose)	P8	Neocortex	Mice	B	(114)
	↑	P7 (Low dose)	P8	Neocortex	Mice	B	(129)
	↑	E7-21	P60-65	Hypothalamus	Rats	M	(47)
***Dnmt3b* mRNA**	↑		48 h	Embryonic fibroblasts	Mice	M	(99)
DNMT3B Protein	↓		48 h	Embryonic fibroblasts	Mice	M	(99)
Methyl-CpG-binding domain (MBD) proteins							
MBD mRNA/Protein	**Effect**	**Exposure Time**	**Time of Study**	**Tissue**	**Species**	**Sex**	**References**
***Mecp2* mRNA**	↑		48 h	Embryonic fibroblasts	Mice	M	(99)
	↑	D0-2	D2	NSC	Mice	B	(101)
	↑	D0-8	D8	NSC	Mice	B	(101)
	↓	D3-13	D13	Primary cortical Neurons	Mice	B	(103)
	↓	D3-8	D13	Primary cortical Neurons	Mice	B	(103)
	↑	E7-21	P60-65	Hypothalamus	Rats	M	(47)
	–	P7 (Low dose)	P8	Hippocampus	Mice	B	(129)
	–	P7 (Low dose)	P8	Neocortex	Mice	B	(129)
	↓		48 h	Embryonic fibroblasts	Mice	M	(99)
	↑	D0-2	D2	NSC	Mice	B	(101)
	↑	D0-8	D8	NSC	Mice	B	(101)

MeCP2 Protein	↓	D3-13	D13	Primary cortical Neurons	Mice	B	(103)
	↓	D3-8	D13	Primary cortical Neurons	Mice	B	(103)
	↓	E6-15	P35	Striatum	Mice	U	(110)
	↓	E5-16	E17	Hippocampus	Mice	B	(112)
	↑	P7 (Low dose)	P8	Hippocampus	Mice	B	(129)
	↑	E8-21	Adult	Hippocampus	Rats	M	(132)
	↓	E6-15	P35	Neocortex	Mice	U	(110)
	↑	P7 (Low dose)	P8	Cortex	Mice	B	(129)
	↑	E7-21	P60-65	Hypothalamus	Rats	M	(47)
***Mbd2* mRNA**	↓		48 h	Embryonic fibroblasts	Mice	M	(99)
MBD2 Protein	↓		48 h	Embryonic fibroblasts	Mice	M	(99)
***Mbd3* mRNA**	↓		48 h	Embryonic fibroblasts	Mice	M	(99)
MBD3 Protein	↓		48 h	Embryonic fibroblasts	Mice	M	(99)

1.4.2.3. Epigenetics and PAE; Non-coding RNAs

Protein-coding sequences in the human genome, which encode all the functional and regulatory proteins only build up to 2% of the whole genome, and 98% of our genome is known to encode vital biological elements involved in the regulation of our development and physiology. Non-coding RNAs (ncRNAs) is a multifarious class of RNAs that contribute to

various biological processes under normal and pathological conditions, namely through the regulation of gene expression. These molecules are subcategorized into small, medium and long ncRNAs and each class has its own sub-classifications. One of the mostly studied ncRNAs are microRNAs (miRNAs), which are small molecules (17-24 nucleotides) and modulate the post-transcriptional regulation of gene expression through miRNA-binding elements in specific target genes (133). MicroRNAs are capable of suppressing gene expression by a variety of mechanisms such as interfering with the initiation of translation, altering the splicing process, deadenylation of 3′ and decapping the 5′, and finally degradation of mRNAs by nuclease enzymes (134, 135). It has been reported that miRNAs are "master regulators" of gene expression and play a crucial role in neuronal differentiation and synaptogenesis during critical times of CNS development (136, 137). Emerging lines of research have demonstrated the significant effect of alcohol on ncRNAs expression, mostly miRNAs (133, 136). RNA-sequencing (RNA-seq) studies on post-mortem prefrontal cortex samples of alcoholics have revealed that alcohol affects the expression pattern of various forms of ncRNAs, including 35 upregulated miRNAs. Also, studies of the post-mortem human brain samples and animal studies have shown that alcohol can induce epigenetic reprogramming in the brain, which is region-specific and includes different forms of epigenetic alterations such as DNA methylation and histone modifications (133). Human studies revealed that continuous alcohol consumption upregulated the expression of miRNAs such as *miR-377*, *miR-493*, and *miR-293p*, which contribute to cellular proliferation and differentiation as well as neuronal migration (138).

Focusing on FASD, it has been shown that PAE alters the expression of miRNAs and their target genes in the developing fetus (139, 140). For example, one study on the effects of PAE on mice revealed that alcohol-induced teratogenesis was accompanied by upregulation of

miR-10a and *miR-10b* and altered expression of *Hoxa1* gene as their target (141). Another study on the embryonic cortical-derived NSC has shown that PAE down-regulates the expression of *miR-21*, *miR-9*, *miR-355*, and *miR-153*. Down-regulation of *miRNA-21* is mediated through GABA-A receptor and this microRNA has an anti-apoptotic role that its suppression is associated with increased cell death (142). In addition, further research revealed that *miR-355* and *miR-153* are involved in NSC proliferation and locomotor activity, respectively (140). Collectively, miRNAs play a significant role during brain development and PAE may exert some of its effects on brain through alteration in the expression of miRNAs.

Table 1. 4 List of studies on the impact of PAE on histone modifications

Embryonic day (E), Postnatal day (P), Day (D), Male (M), Female (F), Unclear (U), Both sexes (B). Adopted from Gavin et al. doi:10.3390/genes8050137 (- no change)

Histone Acetylation							
Global/Gene	**Effect**	**Exposure Time**	**Time of Study**	**Tissue**	**Species**	**Sex**	**References**
Global	↓	E7-21	P60-80	Hypothalamus	Rats	M	(47)
	↓	P2-10	P2-10	Cerebellum	Rats	B	(128)
	–	P2-12	P12	Cerebellum	Rats	B	(128)
***Ehmt2* Promoter**	↑	P7 (Low dose)	P8	Neocortex	Mice	B	(126)
Growth Factor Genes (22	↑	D1-3 (Low dose)	D3	NSC	Mice	B	(126)
	↓	D1-3 (High dose)	D3	NSC	Mice	B	(126)
	↑	D1-3 (Low dose)	D7	NSC	Mice	B	(126)

genes)	↓	D1-3 (High dose)	D7	NSC	Mice	B	(129)
Cnr1 **Promoter**	↑	P7	P8	Neocortex, Hippocampus	Mice	B	(107)
Dlx2 **Promoter**	↑	E7	E17	Neocortex	Mice	B	(126)
HAT/HDAC Expression							
Crebbp **mRNA**	↓	E7-21	P60-80	Hypothalamus	Rats	B	(115)
CREBBP Protein	↓	P2-10	P2-10	Cerebellum	Rats	B	(128)
	–	P2-12	P12	Cerebellum	Rats	B	(128)
Hdac1 **mRNA**	↓	P7	P7	Whole Brain	Mice	M	(143)
Hdac2 **mRNA**	↑	E7-21	P60-80	Hypothalamus	Rats	B	(115)
H3K4 Methylation							
Growth Factor Genes (22 genes)	↑	D1-3 (Low dose)	D3	NSC	Mice	B	(126)
	↓	D1-3 (High dose)	D3	NSC	Mice	B	(126)
	↑↓	D1-3 (Low dose)	D7	NSC	Mice	B	(126)
	↑↓	D1-3 (High dose)	D7	NSC	Mice	B	(126)
Genome-wide	↑↓	P4 & P7	P70	Hippocampus	Mice	M	(117)
Slc17a6 **Promoter**	↑	E0.5-8.5	P120	Hippocampus	Mice	M	(124)
Sox2 **Promoter**	↓	D1-5	D5	Neurospheres	Mice	B	(127)
Dlx2 **Promoter**	↓	D1-5	D5	Neurospheres	Mice	B	(127)

Pax6 **Promoter**	↓	D1-5	D5	Neurospheres	Mice	B	(127)
H3K4 Methyltransferase/Demethylase Expression							
Ash2l1 **mRNA**	↓	D1-5	D5	Neurospheres	Mice	B	(127)
Setd7 **mRNA**	↓	E7-21	P60-80	Hypothalamus	Rats	M	(47)
Kdm1b **mRNA**	↓	D1-5	D5	Neurospheres	Mice	B	(127)
H3K9 Methylation							
Cnr1 **Promoter**	↓	P7	P8	Neocortex, Hippocampus	Mice	B	(129)
Dlx2 **Promoter**	↑	E7	E17	Neocortex	Mice	B	(127)
Dlx3 **Promoter**	↑	E7	E17	Neocortex	Mice	B	(127)
Growth Factor Genes (22 genes)	↑	P7 (High dose)	P8	Hippocampus	Mice	B	(144)
	↑	P7 (High dose)	P8	Neocortex	Mice	B	(144)
	↓	D1-3 (Low dose)	D3	NSC	Mice	B	(127)
	↓	D1-3 (High dose)	D3	NSC	Mice	B	(127)
	↑	D1-3 (Low dose)	D7	NSC	Mice	B	(127)
	↑	D1-3 (High dose)	D7	NSC	Mice	B	(127)

1.5. Sex differences in FASD

1.5.1. Role of epigenetics in sex differences

Extensive research during recent decades has revealed that there are significant sexually dimorphic differences in the structure, function, and development of mammalian CNS. Sexual differences have been reported in a variety of biological, molecular, behavioral and developmental events in mammals under normal and pathologic conditions (145). Significant efforts have been made to find the underpinning mechanisms, which regulate and govern sex-specific features of mammals during critical timing of the brain development. It has been demonstrated that sex chromosomes and sex hormones are responsible for the majority of sex differences in the brain structure and function, but it is not clear that how they would shape the sex-specific characteristics of the brain during lifespan. Recent studies have shown that epigenetic factors are the main molecular mechanisms that mediate the effects of sex hormones and sex chromosomes during lifespan (146). Involvement of epigenetics in sex differences begins in the very early stages of development during the X chromosome inactivation (XCI) process. It is important to note that epigenetic modifications do not silence all the genes on the X-chromosome during XCI and some of them continue to express and are responsible for sex differences (147, 148). Studies on mice at E10.5 (when there is no effect of gonadal hormones) have demonstrated that there are around 50 sexually dimorphic genes in the developing brain (149). For example, one of these genes is *Gli3*, which has a higher expression in females than males. It has been shown that *Gli3* $^{-/-}$ mice have relatively bigger brains than their wild-type counterparts suggesting that higher expression of *Gli3* in females may be responsible for the smaller size of the brain in females in comparison with males (146, 150). As mentioned above, sex chromosomes are partly responsible for sex differences. In this context, *Sry* (Sex determining

Region Y) gene is on the Y chromosome and encodes SRY protein, which is a transcription factor responsible for the differentiation of the bipotential gonadal primordium into the testes during fetal development at E11.5 in mice (151). Interestingly, SRY is known to control the locomotion through the regulation of tyrosine hydroxylase expression and dopamine synthesis, and several brain regions (such as substantia nigra, midbrain, and ventral tegmental area) express this protein (152-154). Testosterone production in fetal testes and further conversion of testosterone to estradiol in the CNS affect many biological processes at mid- to late-gestation period and build the majority of sex differences through permanent organizational alterations in developing fetus (155, 156). As mentioned earlier, DNA methylation is an important and dynamic epigenetic modification that plays a crucial role in the regulation of gene expression in both development and disease (94). Emerging lines of research have shown that the DNA methylation machinery is involved in sexually dimorphic expression of several genes in the CNS (157). DNA methylation contributes to XCI and controls the expression of the *Sry* gene. One important gene that its expression is controlled by DNA methylation is Estrogen Receptor α (*ERα*). In mice, *Erα* expression is relatively high at birth in both male and female pups, but its expression declines throughout the lifespan in the preoptic area and hypothalamus of male mice *via* hypermethylation of *Erα* promoter (156-158). Similar pattern of gene expression and DNA methylation was reported for progesterone receptor in the brain. In addition, methyl-CpG binding domain (MBD) proteins (such as MeCP2) are involved in the sex differences. A study on P1 mice revealed that male pups express significantly less amount of MeCP2 in the amygdala and ventromedial hypothalamus than females. In addition, silencing *Mecp2* in the amygdala significantly reduces the play behavior in male mice while has no impact on female mice behavior suggesting that *Mecp2* is responsible for sex differences in social behavior (159-161).

Further, a recent study has shown that female mice at P1 have higher expression of amygdalar DNMT3A than male counterparts suggesting that DNMT3A may play a role in sex differences relevant to amygdala-related behaviors (162).

Both testosterone and estradiol have reported to affect histone modifications and contribute to sex differences in the early stages of life (163). Evidence indicates that more acetylation in the different regions of the brain is associated with more masculinization. For example, the bed nucleus of the stria terminalis (BNST), a part of limbic system, has a bigger volume and higher cell number in males than females of many mammals (164, 165). Previous research has shown that applying valproic acid (as an HDAC inhibitor) to neonatal mice during the critical time of sexual differentiation results in the reduced volume and cell number of BNST of male (and not female) mice (166). Applying valproic acid inhibited the masculinization of BNST (bigger size and cell number) in males suggesting that histone acetylation contributes to sex differences. Complementary studies have found that sex differences in BNST size and cell number dependent on the activity of *Bax* gene that encodes a pro-apoptotic protein, and testosterone-mediated histone acetylation controls the expression of this gene (167, 168). Similarly, male rodents have greater expression of vasopressin in the lateral septum, and valproic acid treatment decreases the vasopressin expression to a level similar to the female mice (169). In addition, pioneering research on the role of histone modifications in sex differences has shown that sex differences in histone acetylation are observed at E18 to P1, while histone methylation differences are observed in P6. A later study also revealed that male mice have higher levels of H3K9/14Ac and H3K9Me3 in the cortical and hippocampal formations than female counterparts (163). Evidence indicates that both short and long non-coding RNAs contribute to the sex differences. It has been reported that miRNAs exhibit a sexually dimorphic pattern of expression

in different parts of the mice brain such as hippocampus and cortex (170). In this regard, female mice express higher levels of *miR-181b*, *miR-34c*, and *miR-488* in their hippocampus when compared to male counterparts. In contrast, male mice showed higher expression of *miR-206*, *miR-214*, *miR-329*, and *miR-124a* in the same region than females (171). It is interesting that sex differences in hippocampal dendrite morphology and plasticity have been reported in the literature, and *miR-329* is tightly involved in dendritic spine outgrowth and formation in the hippocampal formation (172). In addition, there are some sex-specific genes (such as *SRY*) that their expression is regulated by specific miRNAs such as *let-7a* (173). Ample evidence indicates that long ncRNAs (lncRNAs) contribute to the epigenetic mechanisms involved in sex differences. X inactive specific transcript (*XIST*) is a well-known gene that is transcribed in the inactive X chromosome and recruits histone methylases and histone deacetylases enzymes in order to alter chromatin structure and X chromosome inactivation (174). It has been reported that not all genes on the X chromosome are inactivated and 15% of them are still active and expressed (175). Collectively, several mechanisms are involved in a wide range of sexual differences between the male and female subjects, and epigenetic mechanisms play a critical role in mediating the effects of sex hormones and sex-specific genes.

1.5.2. Sex differences in FASD

As mentioned earlier, the pathophysiology of PAE is complex and depends on various factors. Although there are numerous clinical and experimental studies about the impact of PAE, sex-specific effects of PAE have not been understood well. Generally, it should be noted that prenatal adversities have more negative effects on males than females during gestation and early postnatal stages (176). Evidence indicates that there is no sex difference among the children in the case of FASD diagnosis rate, but boys (12.9/1000) are diagnosed more frequently than girls

(10.4/1000) with FAS (177). Further, studies on humans revealed that there is no sex difference in cognitive deficits among individuals diagnosed with FASD (178, 179). However, there are number of studies that have reported striking sex-dependent effects of PAE in humans. Evidence indicates that PAE-induced HPA-axis hyper responsiveness in young boys are more than young girls (180). In addition, recent research demonstrated that female human embryo is more vulnerable to very low amount of alcohol during early gestation than male embryos (181). Sex differences in FASD are also observed in the results of neuroimaging studies. These studies have shown that there are sex differences in the activation of cortical areas following auditory stimulation. Also, these studies have found that there is a greater reduction in the brain volume of male patients than female ones (182, 183). While human studies indicates that there are some neuroanatomical, physiological, and endocrinological differences among the male and female patients, animal studies show more detailed data about the sex-dependent impacts of PAE. Previous studies have shown that PAE differentially affects memory and executive function in the male and female rodents. Male rats prenatally exposed to continuous alcohol exhibit more impairment in their spatial memory in the Morris water maze test when compared to female rats. However, acute PAE at E8.5 impaired spatial memory in females more than males (184, 185). Unlike female rats, adolescent male rats show impaired social behavior in the form of play behavior following PAE (179). In addition, male rats that experience PAE show more aggressive behavior in comparison with female counterparts (179). Apart from the behavioral differences among the male and female rodents, there are physiological differences in male and female rodents following PAE. Similar to humans, HPA-axis hyper responsiveness is more observed in male animals than female ones (90). In comparison with female rats, male rats show greater immune-inflammatory responses to lipopolysaccharide (LPS) suggesting that PAE effects on

neuro-immune system of male animals are more devastating than females (186). As mentioned above, epigenetic mechanisms regulate the majority of molecular machinery required for sex hormones- and sex chromosomes- induced sex differences during development. However, it is not clear whether the impacts of PAE on epigenetic mechanisms are sex-specific.

1.6. Biomarkers of PAE-relevant disorders

1.6.1. Overview

Accurate diagnosis of PAE-related disorders requires a series of clinical and behavioral analysis by a multidisciplinary team in both individuals and their mothers. Appropriate diagnosis of these disorders is highly important because it provides patients early treatment, management, and special care of disease. Advances in the understanding of FASD pathophysiology have resulted in a better diagnosis of these disorders. For example, diagnosis of FAS has increased significantly during the past 10 years suggesting more accurate diagnosis of the disease (187). It is important to note that FASD may be misdiagnosed with several mental disorders such as ADHD. A recent research on the role of gender in FASD patients has revealed that half of the subjects, mainly males, in their study were misdiagnosed with ADHD (178). One important point that should be considered is that not all FASD patients exhibit specific facial dysmorphology required for FAS diagnosis. In these cases, further experiments should be done to ascertain the prenatal exposure to alcohol. In this regard, many researchers attempted to find and introduce a valid and reliable biomarker for the diagnosis of FASD. Although there are several definitions for the term “biomarker” in the literature, NIH defined the biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (188). To date, there is no biomarker that is indicative of FASD in the babies suspected to have PAE. Since confirming

maternal alcohol consumption is the key to diagnose children without facial features of FAS, researchers have tried to identify genetic, biochemical and molecular biomarkers that are indicative of PAE (44). Different classes of biomarkers have been reported in the literature, but each of them has their advantages and disadvantages. An ideal biomarker should: 1) be able to detect low levels of alcohol consumption during all gestation period, 2) have high sensitivity to alcohol, 3) have high specificity, 4) require small amount of sample, 5) not be an invasive procedure, and 6) not be an expensive analytical test (189). It is important to note that there is a temporal window for the detectability of FASD biomarkers. This temporal window suggests that each biomarker is able to determine the timing and amount of alcohol exposure during pregnancy, includes those biomarkers that can predict PAE within hours, days, weeks, and months before birth (190). Measurement of biomarkers along with reliable self-reports and questionnaires provides solid helpful data that facilitate the appropriate diagnosis of FASD.

1.6.2. FASD Biomarkers

Many *in vitro* and *in vivo* biomarkers for PAE do not have adequate sensitivity and validity. For example, measurement of blood/breath ethanol, serum γ-glutamyl transferase (GGT), red blood cells mean corpuscular volume (MCV), and carbohydrate-deficient transferrin (CDT) are non-specific indicatives of PAE in the last 3 weeks of pregnancy. Also, many imaging studies on humans and animals have shown that neuroimaging results improve our understanding about the pathophysiology of FASD but are not good indicatives of PAE and cannot be used as biomarkers for FASD (189).

1.6.2.1. Biomarkers associated with alcohol metabolism

Although the detection of ethanol or acetaldehyde is the most promising indicative of PAE, but these compounds have a short half-life in biological samples and other ethanol

metabolites with more stability were considered as metabolic biomarkers of FASD. Fatty acid ethyl esters (FAEEs) are compounds formed following ethanol reaction with free fatty acids and fatty acyl-CoA that can be detected in newborn's meconium and hair. These compounds are direct biomarkers of FASD and have a sensitivity around 27% to 100% to moderate and severe PAE (including binge-drinking) during the second and third trimesters of pregnancy. Presence of FAEEs in the meconium is considered as a poor prognosis for the comorbid mental and psychomotor difficulties in childhood. However, FAEEs have their own drawbacks such as lack of sensitivity to PAE during first trimester, limited time for sample collection (48 hour (h) after birth), lack of meconium in 10% of newborns (190, 191). Other metabolic biomarkers of FASD are Ethyl Glucuronide (EtG) and Ethyl Sulfate (EtS). Reaction of ethanol with glucuronic acid in fetal hepatocytes forms EtG. Both EtG and EtS are detectable in maternal urine (five days and 30 h following maternal drinking, respectively) and are not considered as a reliable biomarker due to high reports of false positive results, and lack of sensitivity to PAE during the most of gestation period (189). Phosphatidylethanol (PEth) is another potential metabolic FASD biomarker that is stable for 21 days in the maternal blood and is sensitive to moderate amount of alcohol consumption during pregnancy. Hair analysis for FAEEs and EtG has shown that hair samples can provide evidence for the presence of these metabolic biomarkers, but very low concentration of these compounds in hair samples, expensive methods of chemical extraction, and high false negative results were reported as disadvantages of hair biomarkers (190).

1.6.2.2. Epigenetic biomarkers

Emerging lines of research have demonstrated that specific types of epigenetic modifications, namely DNA methylation, can be used as a potential diagnostic tool for PAE. Recently, researchers have suggested that investigating the specific changes to methylome

following PAE may lead to finding a specific subset of genes that are unique to the effects of PAE. One of the advantages of DNA methylation biomarkers is that they can detect the early exposure to ethanol during embryogenesis. Also, DNA methylation changes are stable and can be detected in youth and adults with FASD (192-194). As mentioned above, it has been accepted that PAE is associated with general reduction in DNA methylation or hypomethylation in the genome (105). Clinical and experimental studies have shown a subset of genes involved in neurodevelopment and behavioral pathways experience specific patterns of DNA methylation following PAE. For example, human studies have revealed that there is a significant hypomethylation of the promoter of dopamine transporter (*SLC6A3*) and serotonin transporter (*SLC6A4*) in children with the history of moderate and heavy PAE (192). On the other hand, a recent study on children with a history of moderate PAE has revealed that ethanol increased DNA methylation in the regulatory elements of the *MECP2* gene (121). In addition, evidence indicates that miRNAs can be used as potential biomarkers for FASD. Recent studies have shown that *miR-9*, *miR-15b*, *miR-19b*, and *miR-20a* could be considered as potential biomarkers for PAE (195, 196). Using a variety of *in vivo* and *in vitro* models of FASD along with several human studies, researchers are working on the PAE-induced alteration in DNA methylation status of genes and it is believed that these studies may lead to identify solid and reliable biomarkers for PAE. However, there is a significant inconsistency among the results of genome-wide DNA methylation studies on FASD. These inconsistencies are explained by the differences between animal models of FASD, alcohol dose, strain and sex, time of alcohol exposure, and many other factors in these studies. In case of human studies, socioeconomic conditions, age, sex, genetic background, early-life insults, nutrition, and many other variables contribute to the results of such studies that may result in the poor outcome of DNA methylation studies for

biomarker identification (192, 197). Ultimately, there is no approved and reliable biomarker for the diagnosis of PAE-related disorders and current biomarkers may improve the diagnosis and confirmation of PAE in some cases.

Recently, our lab conducted a RNA-seq study on differentiated NSC obtained from the cortical areas of developing forebrain in the fetus (E14.5) exposed to different modes of alcohol exposure *in vitro*. We found that different modes of alcohol exposure have a distinct and unique effect on the transcript levels of genes during development ([a]Xu et al., *Scientific Reports, 2018, accepted*). Using Ingenuity Pathway Analysis (IPA), our results identified two genes that are significantly affected following exposure to both acute (2 days) and chronic (8 days) ethanol treatment (70 mM). *Sptbn2* and *Dcc* were identified as potential biomarkers for alcohol exposure to NSC by IPA biomarker analysis. In addition, we have found another gene (*Scn3a*) of interest that was identified by IPA analysis as a gene that was affected by both modes of alcohol exposure and associates with PAE (Xu et al., *Scientific Reports, 2018, accepted*). These three genes will be introduced in the next section and their relevance to brain development, neurodevelopmental and psychiatric disorders and PAE will be discussed.

1.7. Potential biomarkers for alcohol exposure to differentiated NSC

1.7.1. Deleted in colorectal cancer (Dcc)

Deleted in Colorectal Cancer or DCC is a transmembrane protein that recognizes netrin-1 as its specific ligand and mediates the bi-functional effects of netrin-1 through its long and unique cytoplasmic C-terminal residue. This receptor was initially reported as a tumor marker in

[a] Xu W, Liyanage VBR, McAuley A, Levy RD, Curtis K, Olson CO, Zachariah RM, Amiri S, Buist M Hicks GG, Davie JR, Rastegar M. Genome-wide transcriptome landscape of embryonic brain-derived neural stem cells exposed to alcohol with strain-specific cross-examination in BL6 and CD1 mice. ***Scientific Reports*** 2018, *DOI: 10.1038/s41598-018-36059-y, accepted*

colonic cancer and was then identified as netrin-1 receptor in the CNS. This receptor is encoded by *Dcc* gene on the chromosome 18 in mice, and is considered as a dependent receptor. Dependent receptors are able to initiate/inhibit cell death process and depend on the absence/presence of their specific ligands. In the case of DCC and in the presence of netrin-1, it mediates cell survival and axon guidance; however, DCC is also able to recruit caspase-3, initiating apoptotic cell death (198, 199). Interestingly, netrin-1 is able to induce both attractive and repulsive responses in neurons. Chemo-attraction occurs when netrin-1-activation of DCC leads to homo-dimerization of P3 motif of the cytoplasmic tail of DCC, and consequent activation of non-receptor tyrosine kinases (such as Src family kinases). However, chemo-repulsion occurs when the transmembrane receptor Uncoordinated-5 (Unc5) co-expressed beside DCC (198, 200, 201). Evidence indicates that Rho GTPases are a family of intracellular proteins that play critical roles in chemo-repulsive effects of netrin-1 through activation of Ras homolog gene family, member A (RhoA). However, RhoA inhibition is associated with chemo-attractive responses following DCC activation (202). Netrin-1 and DCC are highly expressed during embryonic development in the brain, namely in the cerebellum and developing cortical plate (203). Although DCC is known for its role in neuronal migration and brain wiring during development, it also plays important roles for brain development and function after birth. Evidence indicates that DCC mediates the migration and axonal arborization of dopaminergic neurons into prefrontal cortex during adolescence (204, 205). In addition, DCC is known to regulate synaptic plasticity and function in the adult brain (206). For example, DCC mediates the process of long-term potentiation (LTP) in hippocampal neurons by Src-related activation of NMDA receptors. Decreased or lack of DCC expression is associated with impaired spatial and recognition memory and short-sized dendritic spines (206, 207). Ultimately, DCC is an

important single-pass transmembrane receptor expressed mostly in the CNS and involved in a variety of biological processes throughout the lifespan from neuronal migration in fetal period to synaptic plasticity in the adulthood.

1.7.2. Voltage-gated sodium channel type III, alpha subunit (Nav 1.3)

Study and research on voltage-gated sodium channels are necessary for understanding the neuronal excitation and neurological disorders associated with neuronal excitation such as seizures, neuropathic pain and migraine. Each voltage-gated sodium channel is made of an α-subunit (in the center) and two β-subunits on each side of the α-subunit. While β-subunits (β1-β4) mediate the effects of α-subunit through cytoplasmic targets, the α-subunit is the gate of channel and conducts Na ions inside the cell (208). The α-subunit has four domains (D1-D4), which each of them has six α-helical transmembrane segments (S1-S6). Voltage-gated sodium channels are closed in quiescent cells, but thousands of them open instantly following depolarization and inward Na ions into cells. Interestingly, these channels become inactivated in milliseconds resulting to the formation of transient influx of huge amount of Na ions that consequently complete the depolarization phase of the action potential in neurons (209). There are nine different subtypes of α-subunits ($Na_v1.1$-$Na_v1.9$), which are encoded by *SCN1A–SCN5A, SCN8A–SCN11A (208, 209).* Expression of these subtypes differs during development is tissue and cell-type dependent. Among α-subunits, $Na_v1.3$ subtype is encoded by *SCN3A* and is expressed highly during fetal life in both humans and rodents. However, while *SCN3A* expression is limited to fetal period in rodents, it is expressed postnatally in humans to some extent and is highly associated with epileptic disorders (210, 211). It is important to note that gradual decrease in the expression of *Scn3a* is regulated by the DNA methylation of its regulatory element (CpG-39) during fetal development (212). Interestingly, altered expression

pattern of *SCN3A* has been reported in people with mental retardation, neurobehavioral, and psychiatric abnormalities (213, 214). Finally, mutations in *SCN3A* are associated with increased seizure susceptibility and abnormal neuronal excitation patterns (215).

1.7.3. Sptbn2

Spectrin β-III is a member of spectrin family, and is encoded by *SPTBN2,* expressed during the brain development. spectrins are structural proteins that are essential for the cellular skeleton and maintenance of cell membrane structure and function. In 1968, Marchesi and colleagues found Spectrins in red blood cells for the first time. Now, we know there are two different α-subunits (encoded by *SPTA1* and *SPTAN1*) and five β-subunits (encoded by *SPTB*, *SPTBN1*, *SPTBN2*, *SPTBN4*, and *SPTBN5*). Transcription of these genes is accompanies by several splicing steps, which leads to the formation of different isoforms of Spectrin. Indeed, the expression of Spectrins is time- and tissue-dependent, and they are expressed in all cell types in the mammals. It is well known that spectrins are involved in shaping the cell skeleton in different ways. For example, spectrins are involved in the transport of organelles, endocytosis, exocytosis, and stabilization of proteins and other structures in the cell membrane (216, 217). Mutations in *SPTBN2* gene cause autosomal dominant Spinocerebellar Ataxia type 5 (SCA5) (218). Spectrin β-III is responsible to stabilize the glutamate transporter EAAT4 in the Purkinje cells, where it is highly expressed during cerebellar development. It has been shown that abnormal signaling of glutamate following EAAT4 mislocalization and malfunction leads to loss of Purkinje cells and development of SCA5 (219-221). In addition, spectrin β-III contributes to vesicle transportation in the cell. It has been shown that this protein is highly expressed in Golgi apparatus and transporting vesicles (222). Spectrin β-III is expressed along with spectrin β-II is detected in dendrites and distal axons, respectively. Since these two proteins are responsible for stabilizing

receptors and channels in the cell membrane and neuronal transport, their abnormal function affect ankyrin G and voltage-gated sodium channels that consequently lead to altered neuronal action potential threshold and cellular excitation (216, 223). Further, it has been shown that mutations in the spectrin β-III are associated with mis-localization and lack of function of metabotropic glutamate recptor 1 (mGluR1) (224). More importantly, spectrin β-III plays a critical role in dendrite arborization, dendritic spine, and synaptic activity (225, 226). Recent studies revealed that a conserved repeat of spectrin-actin regulates the neural stem cell lineage and is present in a variety of neurons (227, 228). Thus, *SPTBN2* is an important gene, which contributes to several important procedures during development and is involved in the pathophysiology of neurodevelopmental disorders.

1.8. Rationale, objectives, hypothesis, and aims of the study

1.8.1. Rationale and objectives

Recently, our lab reported that ethanol alters gene expression and DNA methylation in differentiating NSC obtained from the forebrain of CD1 mice embryos, without sex-separation (101). Subsequently, we used a similar model system and applied RNA-seq study on isolated self-renewing and differentiated NSC exposed to short-term and continuous mode of alcohol exposure (Xu et al., *in revision*). In this study, we identified *Dcc, Scn3a, and Sptbn2* as potential biomarkers for PAE. The highest number of altered genes that were identified in our study appeared to be under continuous ethanol exposure. Therefore, in my thesis, I am extending our previous studies in differentiating NSC exposed to continuous ethanol exposure to further investigate the deregulated biomarkers in a sex- and strain-specific manner. The main objectives of my thesis are:

1) To determine specific biomarkers, which are significantly, altered following continuous ethanol exposure in differentiating embryonic NSC.

2) To investigate the epigenetic mechanisms implicated in FASD pathobiology by studying the sex- and strain-specific effects of ethanol in differentiating embryonic NSC.

To study the effects of alcohol on DNA methylation machinery and potential biomarkers in differentiating NSC, we determined the sex of embryos and used C57BL/6 and CD1 strains of mice that have been extensively used for ethanol-associated studies in the context of FASD. We conducted all experiments in a sex- and strain-specific manner. My project is novel in its approach, as to date, there is no report or study on the sex-specific effects of ethanol on the DNA methylation machinery of NSC, and the results of this study will show the different effects of ethanol on the DNA methylation, and cell fate commitment of NSC during differentiation. In addition, there is no previous report on the effects of ethanol on *Dcc*, *Scn3a*, and *Sptbn2*. The results of this study will not only determine the potential of these genes as FASD potential biomarkers, but they may also show new genes affected by ethanol during development. Identification of these genes may help researchers to better understanding of PAE-induced neurodevelopmental disorders.

1.8.2. Hypothesis and aims

Based on our own preliminary data, we hypothesize that the effects of ethanol on DNA methylation status of differentiating NSC are sex- and strain-specific, and these epigenetic changes are accompanied by deregulation at specific genes (*Dcc*, *Scn3a*, and *Sptbn2)* relevant to FASD. This project has two aims to test the hypothesis.

AIM 1. Investigating the DNA methylation machinery in differentiating NSC following continuous ethanol exposure. This aim is important for two reasons: First, according to our previous unpublished work we have found that continuous alcohol exposure induced significant changes in the transcriptome of differentiated NSC (Xu et al., *in revision*), thus, we have aimed to investigate DNA methylation machinery in a sex- and strain-specific manner. Since evidence indicates that DNA methylation is considered as potential FASD biomarkers, we wanted to test the hypothesis that alcohol-induced changes in DNA methylation are sex- and strain-specific. In line with our previous published work (Liyanage *et al*., 2015), we also investigated the effects of continuous ethanol on cell lineage markers as well.

AIM 2. Investigating potential gene biomarkers in differentiating NSC following continuous ethanol exposure. This aim is based on unpublished previous work from our lab, through which we found three genes (*Dcc*, *Scn3a*, and *Sptbn2*) associated with FASD. In AIM2, we will validate the alteration of these genes in our *in vitro* NSC model system in two mice strains (C57BL/6 and CD1) in both male and female NSC.

1.8.3. Expected outcomes

Regarding AIM1, we expect that chronic alcohol exposure will alter DNA methylation machinery in both strains and sexes. Also, we expect to see sex differences and strain-specific alterations in DNA methylation machinery following ethanol exposure. Regarding AIM2, we expect that chronic alcohol exposure alters gene and protein expression of potential biomarkers, namely *Dcc*, *Scn3a*, and *Sptbn2*. We expect to see some differences in the expression of these genes between sexes and strains.

1.8.4. Significance of the proposed research

In Canada, PAE-related disorders are underdiagnosed and despite extensive efforts done to notify people about the adverse effects of alcohol consumption during pregnancy, it is estimated that about 14% of pregnancies among normal population are exposed to alcohol. It is quite clear that FASD is a socio-economic burden. It has been estimated that only cost of diagnostic servises is more than $5 million Canadian Dollars per year and overall costs of FASD is around $2 billion Canadian Dollars. FASD has no cure, and the mechanism of the disease is not fully understood. The results of this study address the first steps to identify potential biomarkers for early detection of FASD and earlier intervention therapeutic strategies.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Embryonic harvest and sex determination of the embryos

All experimental procedures were conducted in accordance to the standards of the Canadian Council on Animal Care with the approval of the Office of Research Ethics of University of Manitoba and under the approved animal protocol number 16031 (AC11190) for Dr. Mojgan Rastegar. Time-pregnant C57BL/6 and CD1 mice at embryonic day E14.5 were received from the animal facility of the University of Manitoba. In order to investigate the strain-specific effects of alcohol on differentiating NSC, a total number of 14 time-pregnant C57BL/6 and 13 time-pregnant CD1 mice were used in this study. All experiments were conducted using at least 3 independent pregnant mice of each strain and were repeated for reproducibility. Sex determination of embryos was performed based on a previously published study by Wesley *et al*. (229) and molecular confirmation of male-female by Sex-determining region Y (*Sry*) and the Interleukin 3 (*Il3*), as well as RT-PCR for detection of X-inactive specific transcript (*Xist*) transcripts was done as we previously reported (230). To do this, pregnant mice were sacrificed by CO2 on the morning and embryos were immediately harvested from the membranes, transferred into sterile plates, and emerged into sterile phosphate buffered saline (PBS) on ice. Under a Cold-light source CL 6000 LED stereomicroscope (Carl Zeiss, Germany), the anterior half of the each embryo was cut, and after removing the liver and intestine the sex of embryos was determined by visually observing the phenotype of the gonads on the dorsal side of the embryo. Ovaries have no thickened capsule and there is a grain reticular pattern on the body of the structure while testes structure has thickened capsule, vessels and cords (as shown in **Fig.2.1**). The embryo heads were cut following sex determination for the dissection of forebrain from embryos of each sex and strain.

2.2. Neural stem cell differentiation and ethanol treatment

The isolation of NSC was done from the forebrain of CD1 and C57BL/6 embryos (E14.5) of both sexes, and NSC culture was performed based on our previously published studies (101, 231, 232). (Please see **Fig.2.1 and Fig.2.2**). In brief, isolated forebrain tissues were homogenized in the NSC media (DMEM/F12 containing HEPES, antibiotic/antimycotic, and glucose). Cell suspension was filtered through mesh membrane (40 μm), and then neurosphere-forming cells were cultured and plated at the density of 10^5 cells/cm^2 in full NSC media (NSC media containing recombinant human epidermal growth factor (rhEGF; Sigma, 20 ng/ml), basic fibroblast growth factor (bFGF; Upstate, 20 ng/ml), heparin (Sigma, 2 μg/ml) and hormone mix [DMEM:F12, glucose (0.6%), insulin (0.25 mg/ml), transferrin (1 mg/ml), progesterone (0.2 μM), putrescine (0.097 mg/ml), sodium selenite (0.3 μM)]. Following 7 days of NSC culture, primary neurospheres were formed, which were collected and dissociated for seeding. Neural stem cells were cultured and differentiated on matrigel coated-plates (BD Bio-sciences) at a density of 10^5 cells/cm^2 in DMEM media (GIBCO) containing 10% fetal bovine serum (FBS, Invitrogen) for 8 consecutive days with or without continuous ethanol (Sigma) treatment (320 mg/ dl or 70 mM). This concentration of ethanol is illustrative of the blood levels of ethanol reported in the individuals with episodic binge drinking and chronic alcohol users and was chosen according to previously published studies (127, 233). Media and ethanol were refreshed every 48 h for both control and continuous ethanol treatment groups. After 8 days of NSC differentiation, cells were collected, washed with cold PBS, and stored in -80 ºC for further experiments.

2.3. Molecular confirmation of embryos sex determination

In order to confirm the sex of embryos, DNA and RNA were extracted from the NCSs derived from both male and female CD1 and C57BL/6 embryos according to manufacturer's instructions using DNeasy Blood and Tissue kit (Qiagen) and RNeasy Mini Kit (Qiagen), respectively. In order to extract DNA from differentiated NSC, cell pellets were resuspended in 200 µl of PBS (containing 20 µl of proteinase K), and then 200 µl of buffer AL was added to each tube and mixed. Tubes were incubated for 10 min at 56 ºC, and 200 µl of ethanol (96-100%) was added to tubes and mixed. In the next step, contents of each tube was transferred into DNeasy Mini spin columns (placed in collection tubes), and centrifuged (8000 rpm, 1 min). DNeasy Mini spin columns were placed in new collection tubes and 500 µl of the buffer AW1 was added to all columns, followed by centrifugation (8000 rpm, 1 min). Flow through was discarded and 500 µl of AW2 was added to all columns and centrifuged (14000 rpm, 3min). Columns were placed in new collection tubes and centrifuged once again (4000 rpm, 1min) to eliminate any ethanol residue in the columns. Finally, DNeasy Mini spin columns were placed in 1.5 Eppendorf tubes and 200 µl of water was added onto the column membrane, incubated for 1 min and centrifuged (8000 rpm, 1min). Eluted DNA was collected, quantified and stored in -20 ºC. To extract RNA from differentiated NSC, cell pellets were resuspended in 350 µl of RTL buffer (containing β-mercaptoethanol) and mixed by vortex. Then, 350 µl of 70% ethanol was added to all cell lysates and mixed. Then, cell lysate in each tube (~ 700 µl) was transferred into RNeasy spin columns (placed in collection tubes) and centrifuged (13000 rpm, 30s). After discarding the flow through, 700 µl of RW1 buffer was added to all columns (placed in collection tubes) and centrifuged (13000 rpm, 30s). Flow through was discarded and 500 µl of RPE buffer was added to each column (placed in collection tube) and centrifuged (13000 rpm,

30s). The latter step was repeated and columns were centrifuged (13000 rpm, 2min). RNeasy spin columns were placed in new collection tubes and centrifuged (13000 rpm, 1min) to eliminate the RPE buffer residue. RNeasy spin columns were placed in 1.5 ml Eppendorf tubes and 30-50 µl of ultrapure water was added onto the column membrane and tubes were centrifuged (13000 rpm, 1min). Eluted RNA was collected, quantified, and stored in -80 ºC. Synthesis of cDNA and qRT-PCR were performed as previously described in our studies (101, 234). While semi-quantitative PCR was used to amplify *Il3* (as the control gene for both sexes) and *Sry* (specific gene for males), quantitative RT-PCR was used to amplify *Xist*, which is a long non-coding RNA expressed only by X chromosome and is considered as a female-specific sex marker (101, 235, 236). Primers used in this study are listed in **Table 2.1.** Using Applied Biosystems® *7500 Real-Time* PCR machine, the PCR program used for *Xist* was: 95 ºC for 5 min followed by 40 cycles of 95 ºC for 30 s, 53 ºC for 30 s, 72 ºC for 30 s, and 78 ºC for 30 s, followed by a melt curve. In order to do PCR for the amplification of *Sry* and *Il3*, Platinum *Pfx* DNA polymerase kit was used (Invitrogen) and reaction was prepared according to the manufacturer's instructions and as we reported (230). Using a thermal cycler (Eppendorf), the following PCR program was used for the amplification of *Sry* and *Il3*: 95 ºC for 4.5 min, 33 cycles of 95 ºC for 35 s, 50 ºC for 1 min, 72 ºC for 1 min, and final extension at 72 ºC for 5 min. Applying agarose gel electrophoresis, PCR products for *Sry*, *Il3* and *Xist* were run on agarose gel (2%), stained by ethidium bromide and was imaged using Gel Doc (Please see **Fig. 2.1**).

2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from differentiated NSC using RNeasy Mini Kit (Qiagen) as described above. Superscript III Reverse Transcriptase (Invitrogen) was used for cDNA synthesis according to our previous published studies (101, 234). Using SYBR Green-based RT^2

qPCR Master Mix, qRT-PCR was carried out in a Fast 7500 Real-Time PCR machine (Applied Biosystems) as previously reported (101, 234). Specific primer sequences for studied genes in this study are listed in the **Table 2.1.** Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was chosen as housekeeping gene and CT values of all genes were normalized to *Gapdh* in order to calculate ΔCt values of samples. Analysis of relative gene expression was done by comparing the $2^{-\Delta Ct}$ of ethanol-treated NSC with relative control values and results were reported as fold change according to our previous studies (101, 234). The following PCR programs were used; 1) 95 ºC for 3 min followed by 40 cycles of 95 ºC for 1 min, 60 ºC for 30 s, 72 ºC for 45 s followed by a melt curve (for *Gfap, Mecp2e1, Mecp2e2,* and *Tuj1*), 2) 50 ºC for 20 s, 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s, 60 ºC for 1 min followed by a melt curve (for *Olig2, Scn3a, Sptbn2, Cnp,* and *Dcc*), 3) 95 ºC for 10 min followed by 40 cycles of 95 ºC for 30 s, 60 ºC for 30 s, 72 ºC for 30 s with holding stage of 95 ºC for 1 min followed by melt curve (for *Dnmt1, Dnmt3a* and *Dnmt3b*), and 4) 95 ºC for 3 min followed by 40 cycles of 95 ºC for 1 min, 58 ºC for 30 s, 72 ºC for 45 s with holding stage of 72 ºC for 10 min followed by melt curve (for *Tet1, Tet2* and *Tet3*).

2.5. Western Blot (WB)

Protein was extracted from differentiated NSC using lysis buffer containing Tris (Trizma Base, pH 8.0) (50 mM), NaCl (150 mM), EDTA (pH 8.0, 5 mM), sodium deoxycholate (0.2%), NP-40 (1%), NaF (50 mM), Na_3VO_4 (1 mM), and 50X protease inhibitor. Fifteen micrograms of protein was loaded on 10% and 8% SDS-polyacrylamide gels and electrophoresis was performed and protein transferred to PVDF membrane (after activation in methanol). Membranes were blocked in TBST (Tris buffered saline with Tween 0.2%) containing 5% skim milk for 12 h at 4 °C. Membranes were incubated in TBST containing 3% skim milk with primary antibodies

(Please see **Table 2.2**) overnight at 4 °C. Membranes were washed 3 times with TBST and were incubated in TBST containing 3% skim milk and specific horseradish peroxidase-conjugated secondary antibodies (Please see **Table 2.3**) for 1h at room temperature. Protein band were visualized using developing X-ray films and a chemiluminescence kit (Amersham Life Sciences, UK). Adobe Photoshop CS5 software was used for the quantification of bands, and GAPDH was chosen as the loading control protein. The expression of proteins in ethanol-treated samples was compared to control counterparts of the same biological sample and results were reported as fold change according to our previous studies (101, 234).

2.6. Immunofluorescence (IF)

Immunofluorescence for differentiated NSC was carried out as described previously (101, 234). In brief, differentiated NSC on coverslip were washed with phosphate buffered saline (PBS, GIBCO) and fixed in 4% paraformaldehyde. Differentiated NSC were permeabilized with 2% NP40 in PBS for 10 min, and blocked with 10% normal donkey serum (NDS, Jackson ImmunoResearch) in PBS for 1 h. Then, cells were incubated with appropriate concentration of primary antibodies (Please see **Table 2.2**) dissolved in 10% NDS in PBS for overnight at 4 °C. Coverslips were washed 3 times with PBS and then cells were incubated with appropriate secondary antibodies (Please see **Table 2.3**) dissolved in 10% NDS in PBS for 1h at room temperature. Coverslips were washed 3 times with PBS, followed by 15 min incubation with DAPI (1:15000 in PBS). Finally, coverslips were washed 3 times more and mounted on slides using ProLong® Gold Antifade Mountant (Invitrogen). Axio Observer Z1 inverted microscope was used to detect the IF signals and, Zen 2011 software was employed to image the slides. Image J program was used to measure signal intensity of approximately 500 DAPI positive cells

from both ethanol-treated and control groups from 3 biological replicates according to our previously published works (101, 230).

2.7. DNA Dot blot assay

DNA dot blot assay for 5-mC and 5-hmC was carried out according to protocol previously described in our studies (101, 230). In brief, DNA was extracted from differentiated NSC samples using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. Using 1.5-ml Eppendorf microtubes, a total volume of 500 µl of 0.4 M NaOH containing 500 ng DNA and 10 mM EDTA was heat for 10 min at 100 °C for denaturing DNA in each sample. Then, equal volume of ice-cold 2 M ammonium acetate (pH 7.0) was added to the contents of each tube. Applying a dot blot apparatus (Bio Rad), denatured DNA of samples were loaded on wells and transferred to Zeta Probe GT blotting membrane (Bio Rad), followed by rinsing with 0.4 M NaOH. Then, membrane was washed with 2X saline–sodium citrate (SSC) buffer, air-dried and UV cross-linked. Membrane (loaded as duplicate) was cut in 2 parts and blocked in PBST (PBS+ 0.1% Tween) containing 3% and 5% of skim milk for 5-mC and 5-hmC for 3h at room temperature, respectively. Membranes were incubated with primary antibodies against 5-mC and 5-hmC (Please see **Table 2.2**) dissolved in PBST containing 3% skim milk for overnight at 4 °C. Membranes were washed 3 times with PBST and were incubated in PBST containing 3% skim milk and specific horseradish peroxidase-conjugated secondary antibodies (Please see **Table 2.3**) for 1h at room temperature. DNA signals were visualized using developing X-ray films and a chemiluminescence kit (Amersham Life Sciences, UK). Total DNA levels on membranes was stained with 0.02% methylene blue (MB) in 0.3 M sodium acetate (pH 5.2), and dot blot signals were quantified using Adobe Photoshop. The values of DNA dot blot were normalized to the values of total DNA staining, and normalized data for

ethanol-treated samples was compared to control counterparts of the same biological sample and results were reported as fold change.

2.8. DNMT and TET activity

Enzymatic activity of TET and DNMT activity was measured according to previously published studies (237, 238). Nuclear proteins of differentiated NSC were extracted using Nuclear Extraction Kit from Abcam (ab113474) and protein levels were measured using Bio-Rad Protein Assay Kit. In order to extract nuclear extraction from differentiated NSC, cells were collected by scrapers, centrifuged (1000 rpm, 5min), and suspended in 100 µl of ice-cold 1X Pre-Extraction Buffer (including protease inhibitor cocktail (PIC) and Dithiothreitol (DTT)) per 10^7 cells. After incubation on ice (10 s), tubes were vortexed (10 s), and centrifuged (12000 rpm, 1 min). After collecting the supernatant (cytoplasmic fraction), two volumes of extraction buffer (containing DTT and PIC) were added to nuclear pellet, and incubated on ice (15 min), mixing by vortexing every 3 min for 5 s. Then, tubes were centrifuged (14000 rpm, 10 min, 4 °C), and supernatant (nuclear fraction) was collected and stored in -80°C. In order to determine the enzymatic activity of DNMT and TET proteins in our samples, DNMT Activity Quantification Kit (Abcam) and TET Hydroxylase Activity Quantification Kit (ab113467) were used as per the manufacturer instructions. To measure DNMT activity, blank wells (50 µl Adomet working buffer), positive control wells (50 µl of adomet working buffer + 0.5/1 µl of DNMT enzyme control), and sample wells (45 µl - 49 µl of adomet working buffer + 1 µl - 5 µl of nuclear extracts (5 µg) were prepared, and covered with adhesive film and incubated for 120 min at 37 ºC. Then, reaction solution was removed, and wells were washed three times with 150 µl of 1X wash buffer. Diluted capture antibody (50 µl) was added to all wells, and Parafilm-covered wells were incubated for 60 min in room temperature. After removing diluted capture antibody

solution and washing all wells (three times with 150 µl of 1X wash buffer), 50 µl of diluted detection antibody was added to all wells, and Parafilm-covered wells were incubated for 30 min in the room temperature. Then, diluted detection antibody solution was removed and wells were washed (four times with 150 µl of 1X wash buffer). Diluted enhancer solution (50 µl) was added to all wells, and Parafilm-covered wells were incubated for 30 min in the room temperature. For the final washing step, diluted detection antibody solution was removed and wells were washed five times with 150 µl of 1X wash buffer). Then, 100 µl of developer solution was added to all wells and incubation was done in darkness for 1-10 min (until medium blue color appears in positive controls), and then 100 µl of stop solution was added to all wells (at this stage the color of samples is yellow). Using a hybrid multi-mode microplate reader (Synergy H4, BioTek, USA) absorbance of the samples was read at 450 nm (within 2-10 min) with an optional reference wavelength of 655 nm. Average duplicate reading was calculated for sample wells and blank wells. DNMT activity is per OD/h/µg and was calculated using following formula;

$$DNMT\ activity\ = \frac{(\text{Sample OD} - \text{Blank OD})}{(\text{Protein Amount } (\mu g) * \text{Hour})} \text{ X } 1000$$

To measure TET activity in samples, standard samples should be prepared. To do this, TET assay standard was diluted with final TET assay buffer to the final concentration of 2 ng/µl by adding 1 µl of TET assay standard to 9 µl of final TET assay buffer. Then, five concentrations of standard samples (0.05, 0.2, 0.5, 1.0, and 2 ng/µl) were prepared by combining the 2ng/µl diluted TET assay standard with final TET assay buffer. Then, 80 µl of binding solution was added to all wells (Blanks, standards, and samples). Two microliters of 0.5X TET Substrate was added to all blank wells and sample wells (but not standard wells). Instead, 1 µl of diluted TET Assay Standards (0.05-2ng/ µl) was added into standard curve wells. After mixing solution, wells

were covered with Parafilm and incubated for 90 min at 37 °C. Then, reaction solution was removed, and wells were washed three times with 150 µl of 1X wash buffer. Fifty microliters of final TET assay buffer was added to all blank and standard wells. In the case of sample wells, 46-49 µl of final TET assay buffer plus 1-4 µl of nuclear extract (5 µg) were added to each sample wells. Wells were covered with adhesive film and incubated for another 90 min in 37 °C. Reaction solution was removed, and wells were washed three times with 150 µl of 1X wash buffer. Then, 50 µl of the diluted capture antibody was added to all wells, and Parafilm-covered wells were incubated for 60 min at room temperature. Diluted capture antibody solution was removed and wells were washed three times with 150 µl of the 1X wash buffer. On the next step, 50 µl of diluted detection antibody was added to all wells, and Parafilm-covered wells were incubated for 30 min at room temperature. After removing diluted detection antibody solution, wells were washed four times with 150 µl of 1X wash buffer. Then 50 µl of the diluted enhancer buffer was added to all wells and Parafilm-covered wells were incubated for 30 min in room temperature. Diluted detection antibody solution was removed and wells were washed five times with 150 µl of 1X wash buffer. At the final step, 50 µl of fluorescence development solution was added to all wells and after 2-4 min of incubation (until pink color appeared); fluorescence of samples was read within 2-10 min using a hybrid multi-mode microplate reader (Synergy H4, BioTek, USA) at 530-excitation/590 emission nm. To accurate calculation of TET activity, a standard curve was generated based on RFU values of each standard sample versus the amount of TET assay standard at each concentration, and then slope was determined as delta RFU/ng. The amount of Hydroxmethylated product was calculated based on following formula;

$$\text{Hydroxymethylated product (ng)} = \frac{\text{Sample RFU} - \text{Blank RFU}}{\text{Slope}}$$

Then, TET activity was calculated based on following formula;

$$\text{TET Activity (ng/min/mg)} = \frac{\text{Hydroxymethylated product (ng)}}{\text{Protein Amount (µg) x Incubation Time}} \text{ X } 1000$$

Experiments were repeated in triplicates, and the values of ethanol-treated samples were compared to control counterparts.

2.9. Statistical analysis

Student's t-test was used to determine statistical significance between the basal expression of genes and proteins in untreated NSC. Two-way ANOVA test was used to determine statistical significance in all experiments, which the treatment factor (control or ethanol) and sex factor (male or female) were involved. Our results are presented as the mean ± standard deviation (SD) of 3-4 biological samples unless otherwise stated. P values less than 0.05 were considered statistically significant and GraphPad Prism software version 6 was used to generate graphs.

Table 2. 1 List of primers used in qRT-PCR and PCR

Gene	Direction	Sequence (5′ to 3′)	Reference
Sry	Forward	TGGGACTGGTGACAATTGTC	(230)
	Reverse	GAGTACAGGTGTGCAGCTCT	
Il3	Forward	GGGACTCCAAGCTTCAATCA	(230)
	Reverse	GGAGGAGGAAGAAAAGCAA	
Xist	Forward	TTGTGGCTTGCTAATAAT	(230)
	Reverse	AAACCCCATCCTTTATG	
Tuj1	Forward	TCAGCGATGAGCACGGCATA	(101)

	Reverse	CACTCTTTCCGCACGACATC	
Gfap	Forward	GCTCACAATACAAGTTGTCC	(101)
	Reverse	ACCTAATTACACAGAGCCAGG	
Olig2	Forward	GTGGCTTCAAGTCATCTTCC	(101)
	Reverse	GTAGATCTCGCTCACCAGTC	
Cnp	Forward	CATCCTCAGGAGCAAAGGAG	(230)
	Reverse	TGAATAGCGTCTTGCACTCG	
Dnmt1	Forward	AGGGAAAAGGGAAGGGCAAG	(230)
	Reverse	AGAAAACACATCCAGGGTCCG	
Dnmt3a	Forward	CAGCGTCACACAGAAGCATATCC	(230)
	Reverse	GGTCCTCACTTTGCTGAACTTGG	
Dnmt3b	Forward	CCTGCTGAATTACTCACGCCCC	(230)
	Reverse	GTCTGTGTAGTGCACAGGAAAA	
Mecp2e1	Forward	AGGAGAGACTGGAGGAAAAGT	(234)
	Reverse	CTTAAACTTCAGTGGCTTGTCTCTG	
Mecp2e2	Forward	CTCACCAGTTCCTGCTTTGATGT	(234)
	Reverse	CTTAAACTTCAGTGGCTTGTCTCTG	
Tet1	Forward	CCATTCTCACAAGGACATTCACA	(239)
	Reverse	GCAGGACGTGGAGTTGTTCA	
Tet2	Forward	GCCATTCTCAGGAGTCACTGC	(239)
	Reverse	ACTTCTCGATTGTCTTCTCTATTGAGG	
Tet3	Forward	GGTCACAGCCTGCATGGACT	(239)
	Reverse	AGCGATTGTCTTCCTTGGTCAG	

Dcc	Forward	TCTCATTATGTAATCTCCTTAAAAGC	(240)
	Reverse	CTGTTATGGAACGAGTGGTGGC	
Sptbn2	Forward	GTGGCAGAAACACCAGGCATTC	Designed in our lab.
	Reverse	CTCCAGCTTCTCTGACACTACG	
Scn3a	Forward	TCCGAGCCTTATCCCGCTTTGA	Designed in our lab.
	Reverse	GAAGATGAGGCACACCAGTAGC	
Gapdh	Forward	AACGACCCCTTCATTGAC	(101)
	Reverse	TCCACGACATACTCAGCAC	

Table 2. 2 List of primary antibodies used in WB, IF and DNA Dot blot

Primary Antibody	**Application and Dilution**	**Source**
Beta TUBULIN III	IF 1:200, WB 1:500	Chemicon, MAB1637
CNPASE	IF 1:250, WB 1:1:400	Covance, SMI-91R
GFAP	IF 1:1000, WB 1:500	Santa Cruz, sc-6171
OLIG2	IF 1:500, WB 1:1000	Invitrogen, P21954
DNMT1	WB 1:500	Santa Cruz, sc-271729
DNMT3A	WB 1:250	Invitrogen, MA5-16171
DNMT3B	WB 1:500	Invitrogen, PA1-884
MeCP2	WB 1:1000	Abcam, ab50005
TET1	WB 1:500	Millipore, 09-872
TET2	WB 1:1000	Invitrogen, PA5-72804

DCC	IF 1:250, WB 1:500	Santa Cruz, sc-6535
Nav 1.3	IF 1:100, WB 1:250	Santa Cruz, sc-22202
Beta SPECTRIN III	IF 1:150, WB 1:250	Santa Cruz, sc-28273
5-mC	Dot blot 1:1000	Abcam, ab10805
5-hmC	Dot blot 1:10000	Active Motif, 39769
GAPDH	WB 1:5000	Santa Cruz, sc-47724

Table 2. 3 List of secondary antibodies used in WB, IF and DNA Dot blot

Secondary Antibody	Application and Dilution	Source
Peroxidase-AffiniPure donkey anti-rabbit IgG	WB 1:7500 Dot blot 1:7500	Jackson ImmunoResearch 711-035-152
Peroxidase-AffiniPure Goat anti-mouse IgG	WB 1:7500 Dot blot 1:7500	Jackson ImmunoResearch 115-035-174
Peroxidase-AffiniPure donkey anti-goat IgG	WB 1:7500	Jackson ImmunoResearch 705-035-003
Alexa Fluor 594 conjugated donkey anti-rabbit IgG	IF 1:1000	Invitrogen, A21207
Alexa Fluor 594 conjugated donkey anti-mouse IgG	IF 1:1000	Invitrogen, A21203
Alexa Fluor 594 conjugated donkey anti-goat IgG	IF 1:1000	Invitrogen, A11058

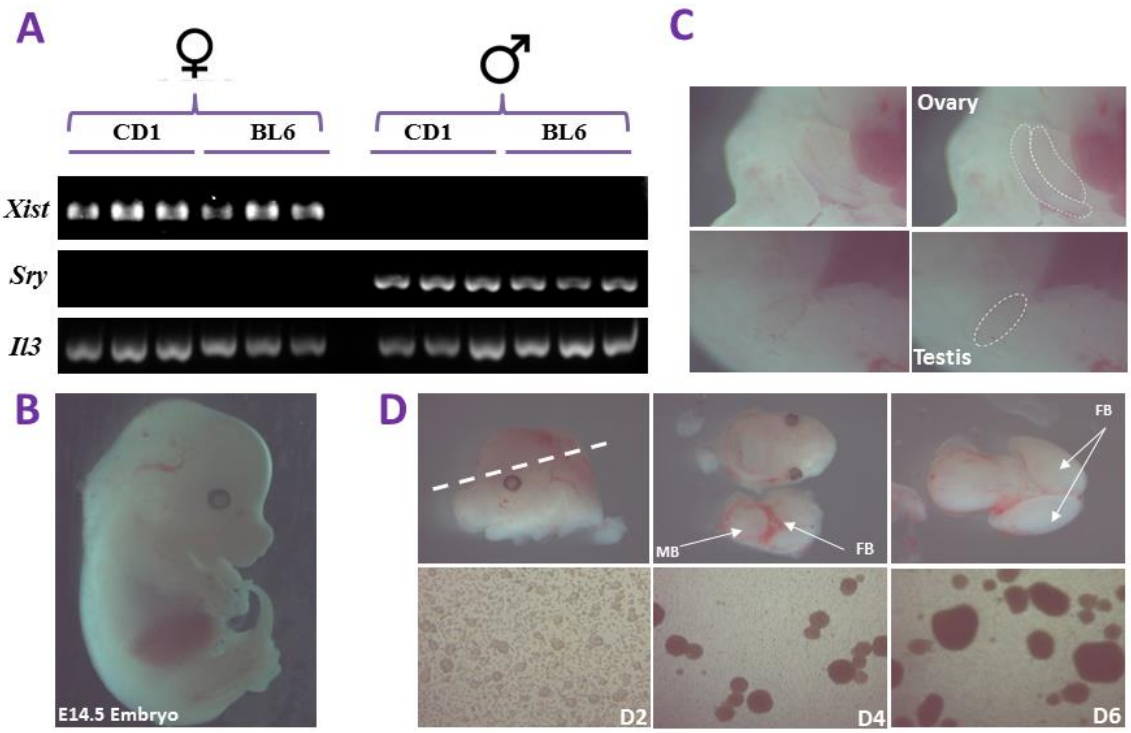


Figure 2. 1 Sex determination in neural stem cells (NSC) and the process of embryo dissection and neurosphere formation.

A) RT-PCR analysis of *Xist* (female-specific marker), PCR amplification of *Sry* (male-specific marker, 402 bp), and *Il3* (control gene for both male and female, 544 bp) in differentiated NSC obtained from male and female CD1 and BL6 embryos (n=3 for each group). B) Right sagittal view of mouse embryo at embryonic day (E) 14.5. C) Macroscopic views of ovaries (upper panel) and testes (lower panel) in E14.5 embryos. D) Dissecting the embryo brain by separating forebrain (FB) from midbrain (MB), and isolation of NSC from FB (upper panel), and harvesting of the NSC and neurosphere formation (lower panel).

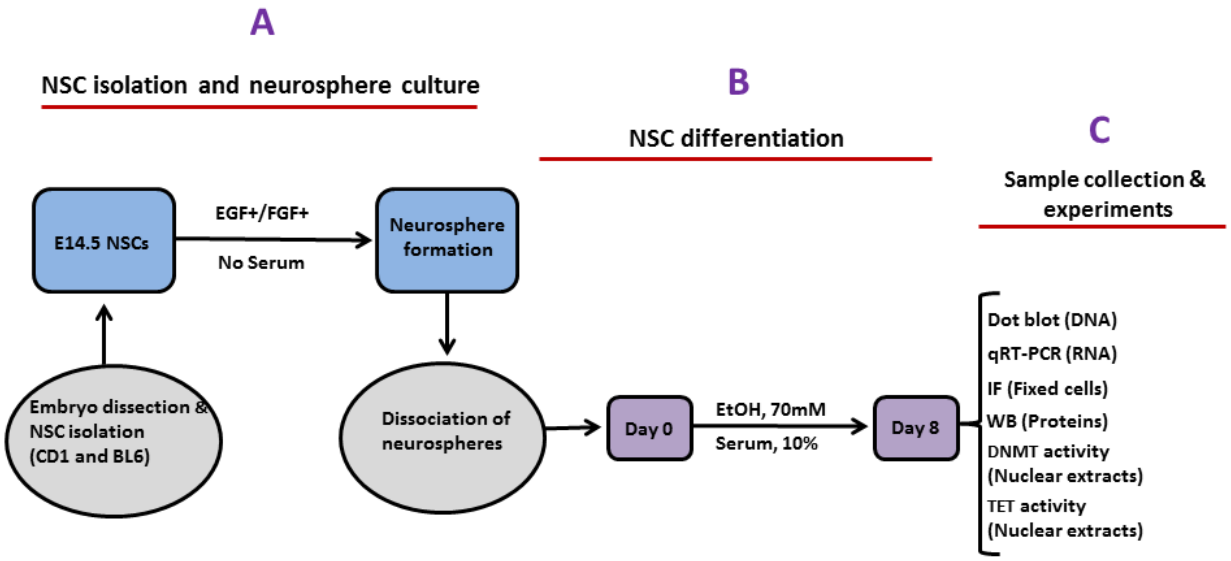


Figure 2. 2 Differentiation of NSC, ethanol treatment and list of experiments

A) Schematic representation of neural stem cell (NSC) isolation and culture in the presence of growth factors (EGF and FGF) to generate neurospheres. B) Schematic representation of NSC differentiation with 10% serum along with chronic ethanol treatment (70mM) for 8 days, and C) collection of differentiated NSC and sample preparation for the experiments in this study.

CHAPTER THREE: RESULTS

3.1. AIM 1. Investigating the DNA methylation-related factors in differentiating NSC following continuous ethanol exposure

3.1.1. Chronic ethanol treatment altered global DNA methylation of differentiated NSC in a sex- and strain-specific manner.

Our lab previously showed that different modes of ethanol exposure changes global DNA methylation in a mixed population of male and female differentiated NSC from CD1 embryos (101). To further investigate the sex- and strain-specific effects of chronic ethanol treatment on differentiating NSC, we dissected the male and female embryos of the two strains and studied them separately. We treated differentiating NSC of male and female CD1 and BL6 embryos with ethanol for 8 days and conducted genomic DNA dot blot experiments to quantify DNA methylation changes in our experimental groups. Our results showed that there were no significant differences in the basal levels of 5-hmC between groups ($p>0.05$). In the case of basal 5-mC levels, our results showed that untreated (control) female CD1 cells had higher basal levels of 5-mC than untreated (control) male CD1 cells (Fig.3.1B, $p<0.001$***). In contrast, untreated (control) female BL6 cells had lower basal levels of 5-mC than untreated (control) male BL6 cells (Fig.3.1B, $p<0.001$***). In addition, untreated (control) female CD1 cells had higher basal levels of 5-mC than untreated (control) female BL6 cells (Fig.3.1B, $p<0.001$***). Regarding the impacts of ethanol treatment on global 5-hmC levels of differentiated NSC, results showed that chronic ethanol treatment had no significant effect on 5-hmC levels in both male and female CD1 cells when compared to untreated (control) groups. In contrast, chronic ethanol treatment significantly decreased 5-hmC levels in female BL6 cells (but not males) when compared to untreated (control) groups (Fig.3.1A, $p<0.001$###). Chronic ethanol treatment significantly

decreased 5-mC levels in female CD1, male BL6, and female BL6 cells when compared to their untreated (control) counterparts (Fig.3.1B, p<0.001###, p<0.05#, and p<0. 01##, respectively). Also, chronic ethanol treatment decreased 5-mC levels in male CD1 cells, but this effect was not significant (Fig.3.1B, p>0.05). These results show that basal levels of 5-mC (but not 5-hmC) differ in male and female differentiating NSC, and this difference is considerable in CD1 strain rather than BL6 strain. Also, there is a difference in the basal levels of 5-mC (and not 5-hmC) between CD1 and BL6 strains. These findings indicate that effects of chronic ethanol treatment on the differentiating NSC are sex-, and strain-specific.

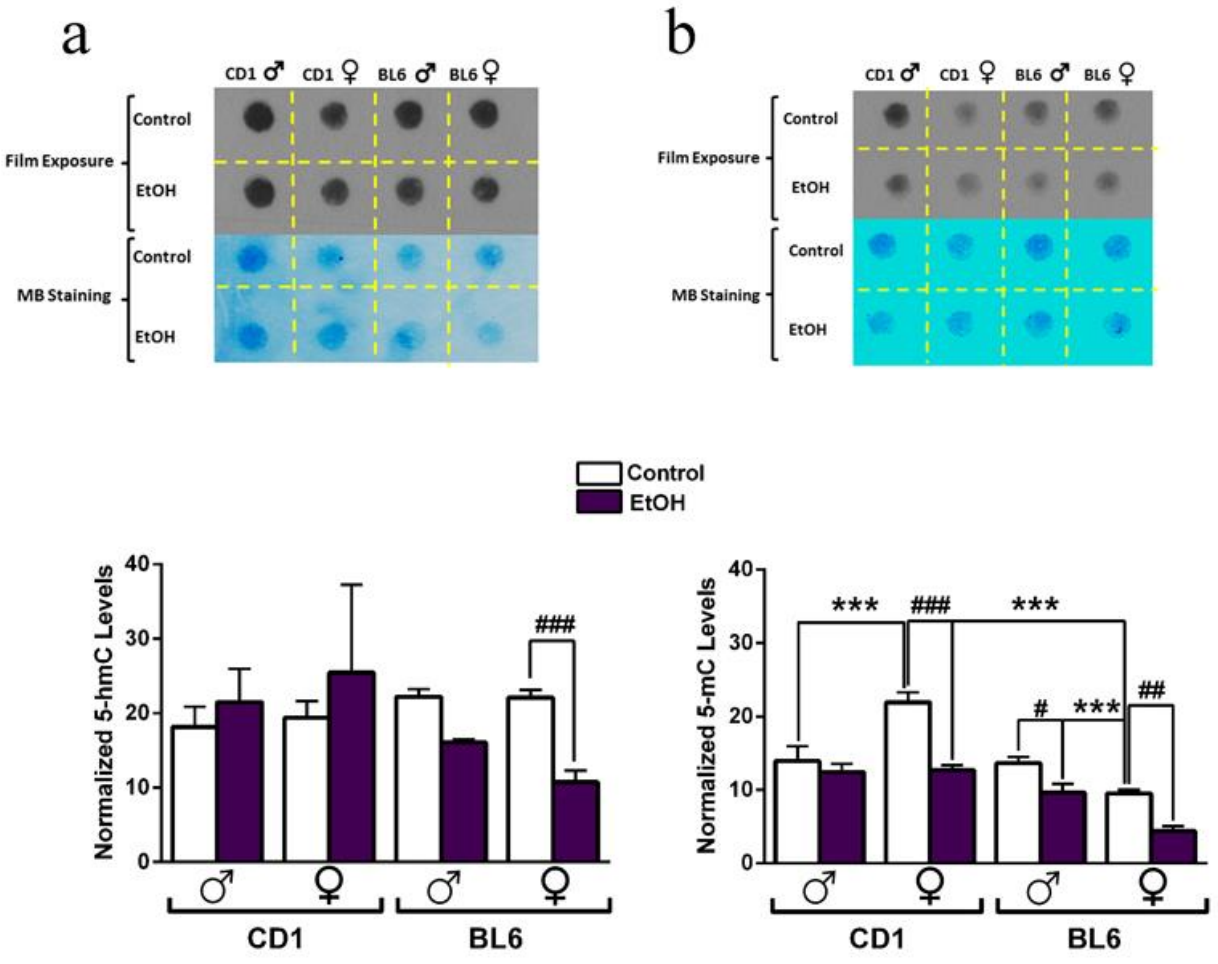


Figure 3. 1 Basal levels of 5-mC and 5-hmC in male and female differentiated neural stem cells (NSC) of CD1 and BL6 embryos and the effects of chronic ethanol treatment on NSC. a) 5-hmC levels in differentiated NSC of male and female cells of CD1 and BL6 embryos with and without ethanol treatment. b) 5-mC levels in differentiated NSC of male and female CD1 and BL6 embryos with and without ethanol treatment. MB refers to methylene blue staining for visualizing the total DNA. Global 5mC and 5hmc levels were normalized to the total DNA levels (N = 3 ± SEM. $p<0.001$*** represents significant differences between basal levels of 5mC among untreated (control) groups. $p< 0.001$###, $p<0.01$##, and $p<0.05$# represent significant differences in 5-mC/5-hmC levels between ethanol-treated (EtOH) and untreated (control) groups.

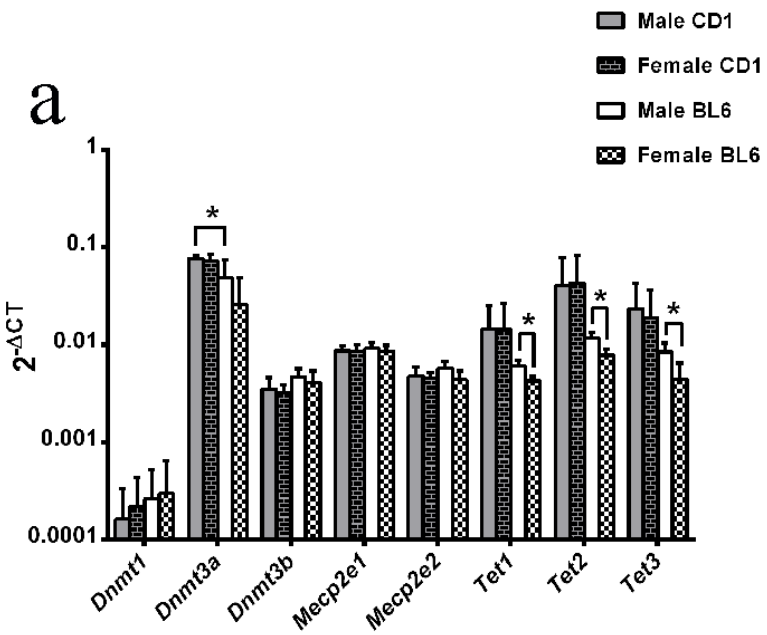
3.1.2. Chronic ethanol treatment altered the transcript expression of DNA methylation-related genes in a sex- and strain-specific manner.

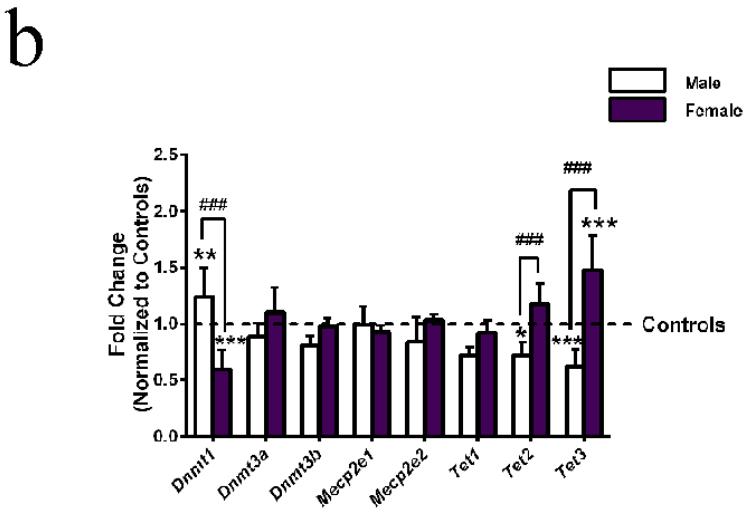
To investigate the effects of chronic ethanol treatment on the expression of genes involved in DNA methylation, we conducted a series of qRT-PCR experiments on ethanol-treated and untreated differentiated NSC of all experimental groups. We first analyzed the basal expression of DNA methylation-related genes in our experimental groups. Our results showed that untreated (control) female CD1cells express significantly higher levels of *Dnmt3a* transcript than untreated (control) female BL6 cells (Fig.3.2A, $p<0.05$*). In addition, untreated (control) male BL6 cells express significantly higher basal levels of *Tet1*, *Tet2*, and *Tet3* transcripts in comparison with untreated (control) female BL6 cells (Fig.3.2A, $p<0.05$*). In comparison with untreated (control) groups, chronic ethanol treatment decreased the transcript expression of *Dnmt1* in female BL6 cells (Fig.3.2B, $p<0.001$***), while the same treatment increased the

transcript expression of *Dnmt1* in male BL6 cells (Fig.3.2B, $p<0.01$**). Chronic ethanol treatment induced no alteration in the transcription of *Dnmt3a* (both male and female) when compared to untreated (control) groups (Fig.3.2B, $p>0.05$). Same treatment had no significant effect on the transcript expression of *Dnmt3b* in both male and female BL6 cells in comparison with untreated (control) groups (Fig.3.2B, $p>0.05$ for both). There were no significant effects of chronic ethanol treatment of the transcription of *Mecp2e1* and *Mecp2e2* in both male and female BL6 cells when compared to controls (Fig.3.2B, $p>0.05$ for both). However, the same treatment reduced the transcription of *Tet2* (Fig.3.2B, $p<0.05$*) and *Tet3* (Fig.3.2B, $p<0.001$***) in male BL6 cells when compared with control counterparts. Chronic ethanol treatment increased transcription of *Tet3* in female BL6 cells in comparison with untreated control groups (Fig.3.2B, $p<0.001$***) and had no effect on the transcription of *Tet1* and *Tet2* in female BL6 cells (Fig.3.2B, $p>0.05$ for both). Regarding the different effects of chronic ethanol treatment on the gene expression in male and female BL6 cells, there were significant differences in the transcription of *Dnmt1* (Fig.3.2B, $p<0.001$###), *Tet2* (Fig.3.2B, $p<0.001$###), and *Tet3* (Fig.3.2B, $p<0.001$###).

Chronic ethanol treatment had significant effects on the expression of genes involved in the DNA methylation of differentiated NSC from the CD1 strain. In comparison with untreated (control) cells, chronic ethanol treatment increased the transcript levels of *Dnmt1* in male CD1 cells (Fig.3.2C, $p<0.001$***). The same treatment decreased the transcript levels of *Dnmt1* in female CD1 cells (Fig.3.2C, $p<0.05$*), and also decreased the transcript levels of *Tet3* in male CD1 cells (Fig.3.2C, $p<0.05$*) (but not females, Fig.3.2C, $p>0.05$) when compared to untreated (control) groups. Chronic ethanol exposure had no significant effects on the expression of *Dnmt3a*, *Dnmtb*, *Mecp2e1*, *Mecp2e2*, *Tet1*, and *Tet2* in both male and female CD1 cells when

compared to control counterparts (Fig.3.2C, $p>0.05$). Regarding the different effects of chronic ethanol treatment on the gene expression in male and female CD1 cells, there were significant differences in the transcript levels of *Dnmt1* (Fig.3.2C, $p<0.001$###) and *Tet3* (Fif.3.2C, $p<0.05$#). Collectively, these findings indicate that effects of chronic ethanol treatment on the expression of genes of DNA methylation-related genes are sex-, and strain-specific in differentiating NSC. Also, the basal expression of some genes in untreated (control) cells differ between the two tested strains (*Dnmt3a*) as well as the two sexes (*Tet1*, *Tet2*, and *Tet3*) in BL6 strain.


a
Male CD1
Female CD1
Male BL6
Female BL6
2-ΔCT
1
0.1
0.01
0.001
0.0001
*
*
*
*
Dnmt1
Dnmt3a
Dnmt3b
Mecp2e1
Mecp2e2
Tet1
Tet2
Tet3


b
Male
Female
Fold Change
(Normalized to Controls)
2.5
2.0
1.5
1.0
0.5
0.0
Controls
**
###
***
###
*
###
***
Dnmt1
Dnmt3a
Dnmt3b
Mecp2e1
Mecp2e2
Tet1
Tet2
Tet3

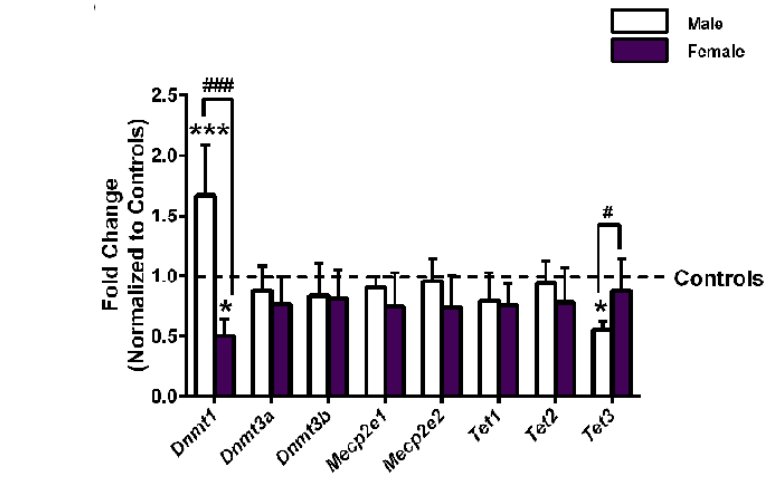

c
Male
Female
Fold Change
(Normalized to Controls)
2.5
2.0
1.5
1.0
0.5
0.0
Controls
###
***
*
#
*
Dnmt1
Dnmt3a
Dnmt3b
Mecp2e1
Mecp2e2
Tet1
Tet2
Tet3

Figure 3. 2 Effect of chronic ethanol treatment on the expression of genes associated with DNA methylation-related genes in male and female NSC of CD1 and BL6 embryos.

a) Comparison of the basal expression of *Dnmt1*, *Dnmt3a, Dnmt3b, Mecp2e1*, *Mecp2e2*, *Tet1*, *Tet2*, and *Tet3* transcript levels in differentiated NSC of male and female cells of CD1 and BL6 embryos (N=4±SEM, for each group). Significant differences between untreated (control) groups are shown with $p<0.05$*. b) Effect of chronic ethanol treatment on the expression of *Dnmt1, Dnmt3a, Dnmt3b, Mecp2e1*, *Mecp2e2*, *Tet1*, *Tet2*, and *Tet3* transcript levels in differentiated NSC of male and female cells of BL6 and c) CD1 embryos (N=4±SEM, for each group). Fold changes are reported relative to untreated control (male CD1, female CD1, male BL6, and female BL6) groups (shown as dashed line). Significant differences from controls are shown with $p<0.001$***, $p< 0.01$** and, $p<0.05$*. Significant differences between male and female groups are indicated with $p<0.001$### and $p<0.05$#. The transcript expression values were normalized to *Gapdh* expression.

3.1.3. Chronic ethanol treatment altered the expression of DNA methylation machinery proteins in a sex- and strain-specific manner.

To investigate the effects of chronic ethanol treatment on the expression of DNA methylation factors, we conducted a series of WB experiments on ethanol-treated and untreated differentiated NSC of all experimental groups. We first analyzed the basal expression levels of DNA methylation-related proteins in our experimental groups. Our results showed that untreated (control) female CD1cells express significantly lower levels of DNMT1 in comparison with untreated (control) male CD1 (Fig.3.3A, $p<0.05$*). In addition, untreated (control) male CD1 cells expressed significantly higher levels of DNMT1 in comparison with untreated (control) male BL6 cells (Fig.3.3A, $p<0.05$#). In the case of DNMT3A, untreated (control) male CD1 cells

expressed significantly higher levels of this protein when compared to untreated (control) female CD1 cells (Fig.3.3A, $p<0.05$*). Also, in comparison with untreated (control) female CD1 cells, significantly higher levels of DNMT3A were observed in untreated (control) female BL6 cells (Fig.3.3A, $p<0.001$###). There were no significant differences in the expression of DNMT3B between male and female untreated (control) CD1 and BL6 cells (Fig.3.3A, $p>0.05$), but there were significant differences between untreated (control) male CD1 and BL6 cells (Fig.3.3A, $p<0.01$##) as well as untreated (control) female CD1 and BL6 cells (Fig.3.3A, $p<0.05$#). Similarly, while there were no significant differences in the expression of MeCP2 between male and female untreated (control) CD1 and BL6 cells (Fig.3.3A, $p>0.05$), but there were significant differences between untreated (control) male CD1 and BL6 cells (Fig.3.3A, $p<0.05$#) as well as untreated (control) female CD1 and BL6 cells (Fig.3.3A, $p<0.05$#). In comparison with untreated (control) male CD1 and female BL6 cells, significantly lower levels of basal TET1 were detected in female CD1 group (Fig.3.3A, $p<0.05$* and $p<0.01$##). Also, untreated (control) male CD1 expressed lower levels of TET1 in comparison with their male BL6 counterparts (Fig.3.3A, $p<0.05$#). There were no significant differences in the expression of TET2 between male and female untreated (control) CD1 and BL6 cells (Fig.3.3A, $p>0.05$), but there were significant differences between untreated (control) male CD1 and BL6 cells (Fig.3.3A, $p<0.001$###) as well as untreated (control) female CD1 and BL6 cells (Fig.3.3A, $p<0.05$#).

Figure 3.3B shows that chronic ethanol treatment has significant effects on the expression of DNA methylation-related proteins of differentiated NSC of CD1 and BL6 strain. In comparison with untreated (control) male CD1 cells, chronic ethanol treatment only decreased protein expression of MeCP2 ($p<0.05$*) in differentiated male NSC, while it has no effects on the expression of other proteins in CD1 male cells (Fig.3.3B, $p>0.05$). On the other hand, chronic

ethanol treatment significantly increased protein expression of DNMT3B ($p<0.05$*) and MeCP2 ($p<0.01$**) in female CD1 differentiated NSC when compared to their controls. Also, same treatments had no significant effect on the expression of DNMT1, DNMT3A, TET1, and TET2 proteins in female CD1 differentiated NSC when compared to control groups (Fig3.3B, $p>0.05$). Regarding the BL6 strain, chronic ethanol treatment significantly increased protein expression of DNMT1 ($p<0.001$***) in male BL6 differentiated NSC in comparison with male controls, and had no significant effects on other proteins (Fig3.3B, $p>0.05$). In comparison with female BL6 controls, chronic ethanol treatment had no significant effect on the expression of all proteins (Fig3.3B, $p>0.05$). Regarding the different effects of chronic ethanol treatment on the gene expression in male and female CD1 cells, there were significant differences in the expression of DNMT3A (Fig.3.3B, $p<0.05$#), DNMT3B (Fig.3.3B, $p<0.001$###), MeCP2 (Fig3.3B, $p<0.001$###), and TET2 (Fig.3.3B, $p<0.05$#). Collectively, these findings indicate that effects of chronic ethanol treatment on the expression of DNA methylation-related proteins are in a sex-, and strain-specific in differentiating NSC. Also, the basal expression levels of these proteins in untreated (control) cells differ between the two tested strains as well as sexes.

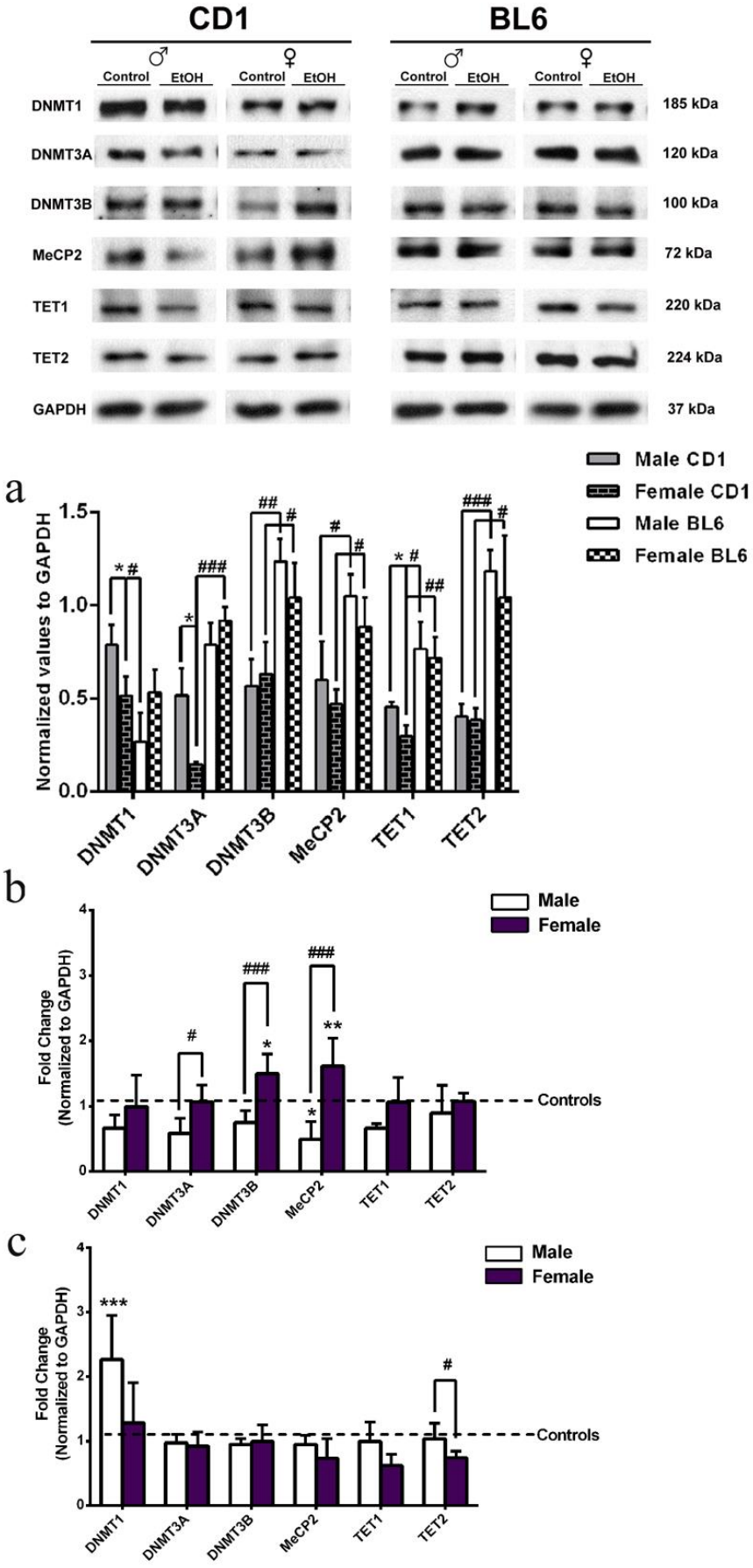

CD1
BL6
♂
♀
Control
EtOH
DNMT1
DNMT3A
DNMT3B
MeCP2
TET1
TET2
GAPDH
185 kDa
120 kDa
100 kDa
72 kDa
220 kDa
224 kDa
37 kDa
a
Male CD1
Female CD1
Male BL6
Female BL6
Normalized values to GAPDH
b
Male
Female
Fold Change (Normalized to GAPDH)
Controls
c
Male
Female
Fold Change (Normalized to GAPDH)
Controls

Figure 3. 3 Effect of chronic ethanol treatment on the expression level of DNA methylation-related proteins in male and female differentiated NSC of CD1 and BL6 embryos.

a) Comparison of the basal protein expression of DNMT1, DNMT3A, DNMT3B, MeCP2, TET1, and TET2 in differentiated NSC of male and female cells of CD1 and BL6 embryos (N=3±SEM, for each group). Significant differences between male and female untreated (control) groups are shown with p<0.01** and p<0.05*. Significant differences between strains (CD1 and BL6) are shown with p<0.001###, p<0.01##, and p<0.05#) b) Effect of chronic ethanol treatment on the expression of DNMT1, DNMT3A, DNMT3B, MeCP2, TET1, and TET2 in differentiated NSC of male and female cells of CD1 and c) BL6 embryos (N=3±SEM, for each group). Fold changes are reported relative to untreated control (male CD1, female CD1, male BL6, and female BL6) groups (shown as dashed line). Significant differences from controls are shown with p<0.001***, p<0.01**, and p<0.05*. Significant differences of ethanol effects on male and female groups are shown with p<0.001### and p<0.05#. GAPDH was used as a loading control for Western blots.

3.1.4. Chronic ethanol treatment altered DNA methyltransferase activity in differentiated NSC.

To investigate the effects of chronic ethanol treatment on the DNMT and TET enzymatic activities, we used DNMT and TET assay activity kits on ethanol-treated and untreated differentiated NSC of all experimental groups. We first analyzed the basal activity of DNMT and TET enzymatic activities in the nuclear extract of our experimental groups. Our results showed that untreated female CD1 cells have higher DNMT activity compared to untreated male CD1 cells (Fig.3.4A, p<0.001###). Also, untreated female CD1 cells had significantly higher DNMT activity when compared to untreated female BL6 cells (Fig.3.4A, p<0.001$$$). There were no

significant differences in the DNMT activity levels between other untreated groups (Fig.3.4A, $p>0.05$). In the case of TET activity, there were no significant differences in the basal levels of TET activity between all groups (Fig.3.4B, $p>0.05$).

In comparison with control groups, chronic ethanol treatment significantly reduced DNMT activity in female CD1 cells (Fig.3.4A, $p<0.05$*), while had no significant effect on female BL6 cells (Fig.3.4A, $p>0.05$). Chronic ethanol treatment had no significant effect on TET activity levels in differentiated NSC when compared with their control counterparts (Fig.3.4B, $p>0.05$). These findings indicate that basal DNMT (but not TET) activity in untreated differentiated NSC differs between the two tested strains as well as the two sexes. Also, the effects of chronic ethanol treatment on DNMT activity are sex-, and strain-specific in differentiating NSC, without any significant effect on TET activity in our experimental groups.

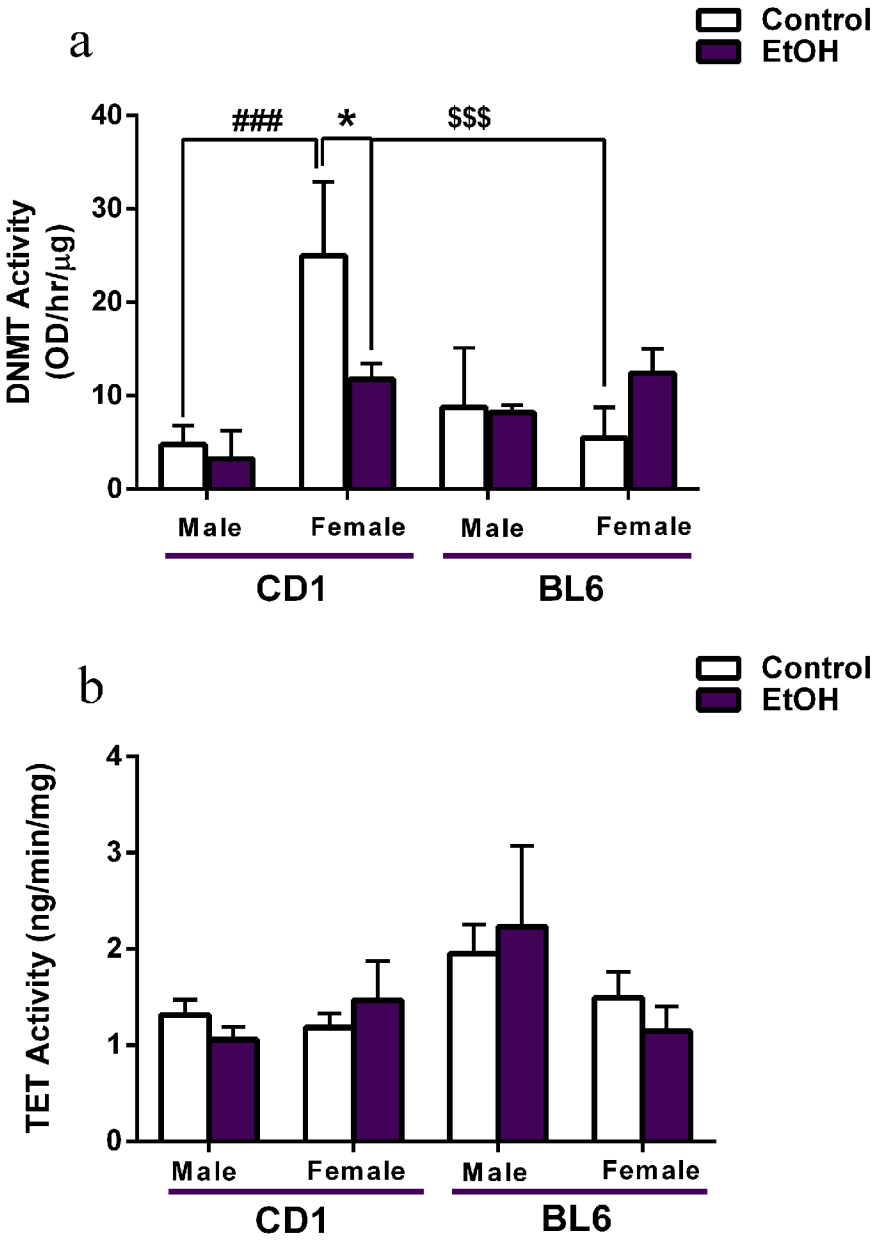


Figure 3. 4 Detection of in vitro DNMT and TET enzymatic activities in differentiated NSC.

a) *In vitro* DNMT enzymatic activity assay in the nuclear extracts of NSC of male and female cells of CD1 and BL6 embryos (N=3±SEM, for each group), $p<0.05$* represents significant differences between ethanol and control groups, $p<0.001$### represents significant differences

between male and female CD1 control groups, p<0.001$$$ represents significant differences between female CD1 and female BL6 control groups. b) *In vitro* TET enzymatic activity assay in the nuclear extracts of NSC of male and female cells of CD1 and BL6 embryos (N=3±SEM, for each group).

3.1.5. Chronic ethanol treatment altered the transcript expression of cell lineage marker genes in a sex-, and strain-specific manner.

To investigate the effects of chronic ethanol treatment on the expression of cell lineage markers, we conducted a series of qRT-PCR experiment on ethanol-treated and untreated differentiated NSC of all experimental groups. We first analyzed the basal expression of cell type-specific markers in our experimental groups. Our results showed that untreated (control) male CD1cells express significantly lower levels of *Tuj1* transcript than untreated (control) male BL6 cells (Fig.3.5A, p<0.05*). In addition, there were no significant differences in the basal expression of *Cnp, Gfap,* and *Olig2* transcripts between untreated differentiated NSC of all groups (Fig.3.5A, p>0.05). In comparison with untreated (control) groups, chronic ethanol treatment decreased the transcript expression of *Olig2* (Fig.3.5B, p<0.001***) and *Tuj1* (Fig3.5B, p<0.01**) in male BL6 cells. Similar treatments induced no significant effects in the expression level of *Cnp* and *Gfap* genes in differentiated NSC when compared with male BL6 controls (Fig.3.5B, p>0.05). Also, chronic ethanol treatment significantly decreased the transcript expression of *Tuj1* (Fig3.5B, p<0.01**), while significantly increased *Olig2* transcript (Fig3.5B, p<0.01**) in female BL6 cells when compared to their control counterparts. Similar treatments induced no significant effect on the transcript expression of *Gfap* and *Cnp* in female BL6 cells in comparison with control groups (Fig.3.5B, p>0.05). Regarding the different effects of ethanol

treatment on male and female differentiated NSC, we observed a significant difference in the transcript expression of *Olig2* between male and female BL6 cells (Fig.3.5B, $p<0.001$###).

In comparison with untreated CD1 controls, chronic ethanol treatment had no significant effects on the transcript expression of all genes in experimental groups (Fig.3.5C, $p>0.05$). Also, there was a significant effect of ethanol on male and female CD1 NSC in the case of *Olig2* transcript expression (Fig.3.5C, $p<0.05$#). These results indicate that the effects of chronic ethanol treatment on the expression of cell lineage marker genes are sex-, and strain-specific in differentiating NSC. Also, the basal expression of these genes in untreated (control) cells did not differ significantly and the only detected difference was observed between male BL6 and CD1 strains in the *Tuj1* expression.

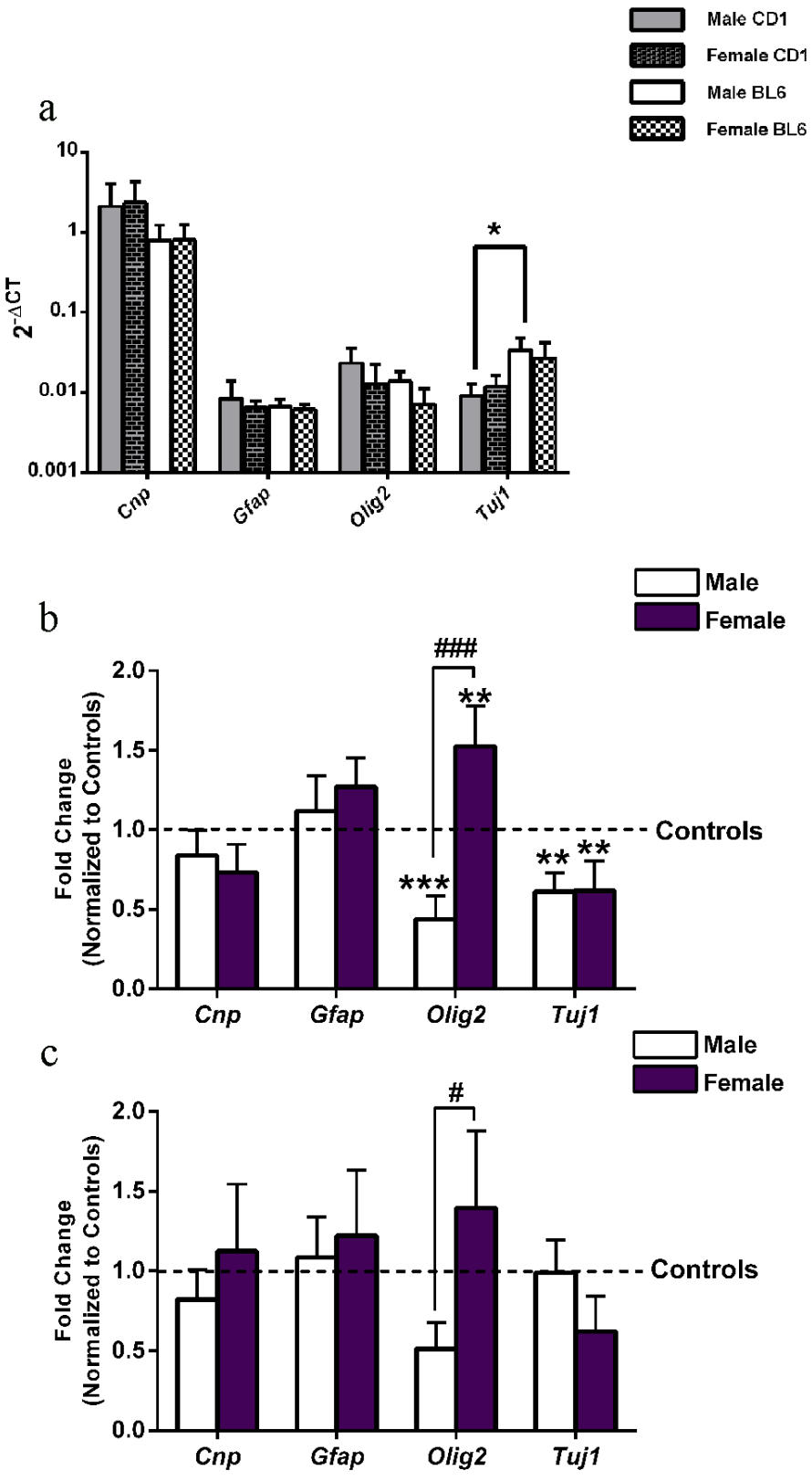

a
Male CD1
Female CD1
Male BL6
Female BL6
10
1
0.1
0.01
0.001
2-ΔCT
*
Cnp
Gfap
Olig2
Tuj1
b
Male
Female
2.0
1.5
1.0
0.5
0.0
Fold Change
(Normalized to Controls)
###
**
Controls
***
**
**
Cnp
Gfap
Olig2
Tuj1
c
Male
Female
2.0
1.5
1.0
0.5
0.0
Fold Change
(Normalized to Controls)
#
Controls
Cnp
Gfap
Olig2
Tuj1

Figure 3. 5 Effect of chronic ethanol treatment on the expression of genes associated with cell lineage markers in male and female differentiated NSC of CD1 and BL6 embryos.

a) Comparison of the basal expression of *Cnp, Gfap, Olig2,* and *Tuj1* transcript levels in differentiated NSC of male and female cells of CD1 and BL6 embryos (N=4±SEM, for each group). Significant differences between untreated (control) groups are shown with $p<0.05$*. b) Effect of chronic ethanol treatment on the expression of *Cnp, Gfap, Olig2,* and *Tuj1* transcript levels in differentiated NSC of male and female cells of BL6 and c) CD1 embryos (N=4±SEM, for each group). Fold changes are reported relative to untreated control (male CD1, female CD1, male BL6, and female BL6) groups (shown as dashed line). Significant differences from controls are shown with $p<0.001$*** and $p<0.01$**. Significant differences between male and female groups are indicated with $p<0.05$# and $p< 0.001$###. The transcript expression values were normalized to *Gapdh* expression.

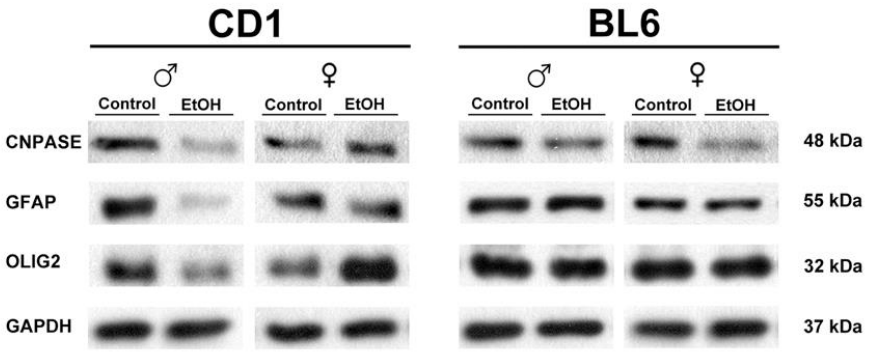
3.1.6. Chronic ethanol treatment altered the expression of glial marker proteins in a sex-, and strain-specific manner.

To investigate the effects of chronic ethanol treatment on the protein expression of glial lineage markers, we conducted a series of WB experiment on ethanol-treated and untreated differentiated NSC from all experimental groups. We first analyzed the basal expression levels of glial marker proteins in our experimental groups. In the case of basal CNPASE expression, our results showed that untreated (control) female BL6 cells expressed significantly lower levels of CNPASE in comparison with untreated (control) male BL6 (Fig.3.6A, $p<0.01$**), and there was no significant difference in basal levels of CNPASE between male and female CD1 cells (Fig.3.6A, $p>0.05$). Also, both male BL6 ($p<0.001$###) and female BL6 ($p<0.05$#) differentiated NSC had higher basal levels of CNPASE in comparison with their CD1 counterparts (Fig.3.6A).

In the case of basal GFAP expression, our results showed that untreated (control) female BL6 cells expressed significantly lower basal levels of GFAP protein in comparison with untreated (control) male BL6 cells (Fig3.6A, $p<0.01$**). Also, untreated (control) female BL6 cells expressed significantly lower levels of GFAP when compared to their CD1 counterparts (Fig.3.6A, $p<0.01$##), and there was no significant difference in the basal GFAP expression between other groups (Fig.3.6A, $p>0.05$). In the case of basal OLIG2 expression, there were no significant differences in the basal OLIG2 expression between male and female CD1 and BL6 cells (Fig.3.6A, $p>0.05$). However, both male BL6 ($p<0.01$##) and female BL6 ($p<0.01$##) differentiated NSC had higher basal levels of OLIG2 in comparison with their CD1 counterparts (Fig.3.6A).

Figure 3.6B shows that chronic ethanol treatment has significant effects on the expression of glial marker proteins of differentiated NSC of CD1 and BL6 strains. In comparison with untreated (control) male CD1 cells, chronic ethanol treatment significantly decreased protein expression of CNPASE ($p<0.01$**), GFAP ($p<0.05$*), and OLIG2 ($p<0.05$*) in differentiated male CD1 NSC (Fig.3.3B). Similar treatment significantly increased protein expression of OLIG2 in female CD1 cells when compared to untreated female CD1 cells (Fig.3.6B, $p<0.01$**). Also, chronic ethanol treatment had no significant effect on the protein expression of CNPASE and GFAP in female CD1 cells (Fig.3.6B, $p>0.05$). In addition, there were significant differences in the protein expression of CNPASE ($p<0.01$##), GFAP ($p<0.05$#) and OLIG2 ($p<0.001$###) between male and female CD1 differentiated NSC following chronic ethanol treatment (Fig.3.6B). In the case of BL6 strain, chronic ethanol treatment significantly decreased CNPASE expression in the female BL6 cells when compared to the controls (Fig.3.6B, $p<0.05$*). In addition, there were no significant effects of ethanol treatment on the protein expression of glial

markers in other groups in BL6 strain (Fig.3.6A, $p>0.05$). Overall, these findings indicate that the effects of chronic ethanol treatment on the expression of some glial lineage proteins are sex-, and strain-specific in differentiating NSC. Also, the basal expression of the aforementioned proteins in untreated (control) cells differs between the two tested strains as well as the two sexes.


CD1
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♀
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CNPASE
GFAP
OLIG2
GAPDH
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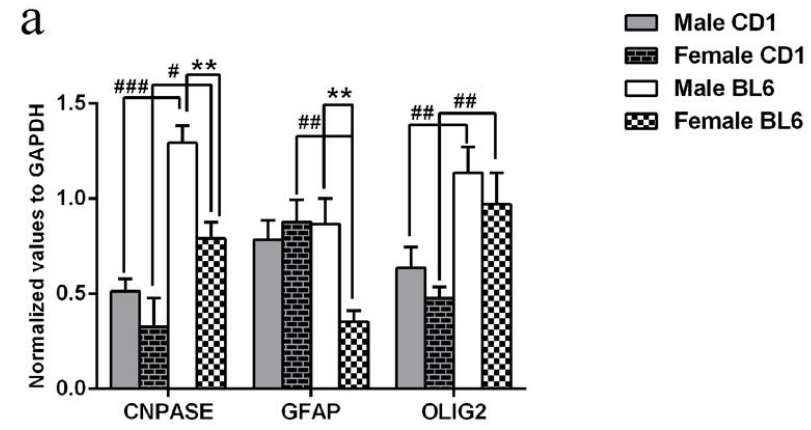
55 kDa
32 kDa
37 kDa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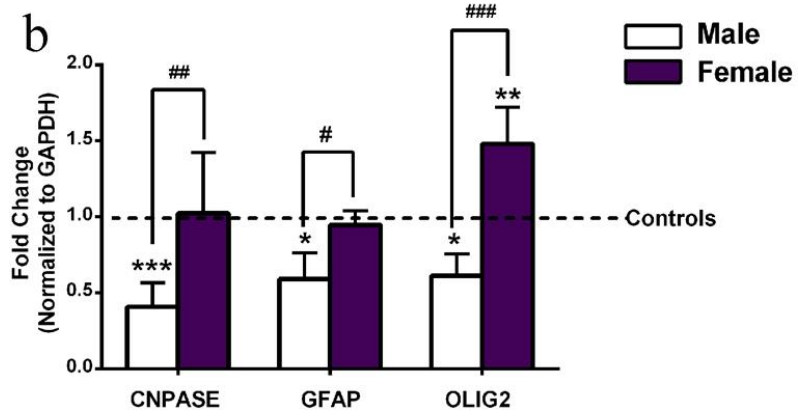
Male CD1
Female CD1
Male BL6
Female BL6
Normalized values to GAPDH
CNPASE
GFAP
OLIG2


b
Male
Female
Fold Change (Normalized to GAPDH)
Controls
CNPASE
GFAP
OLIG2

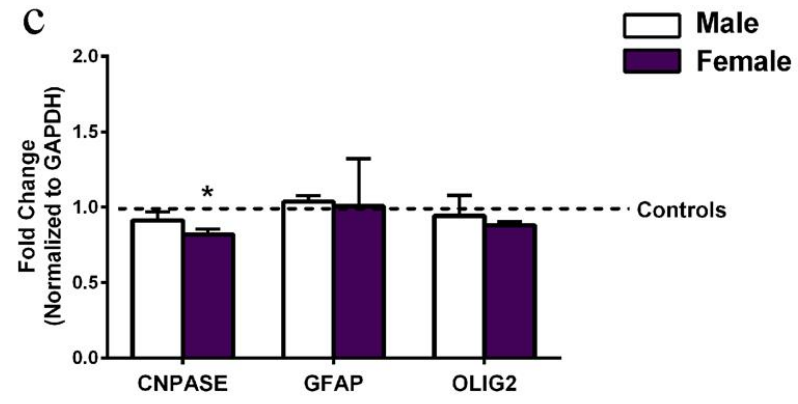

c
Male
Female
Fold Change (Normalized to GAPDH)
Controls
CNPASE
GFAP
OLIG2

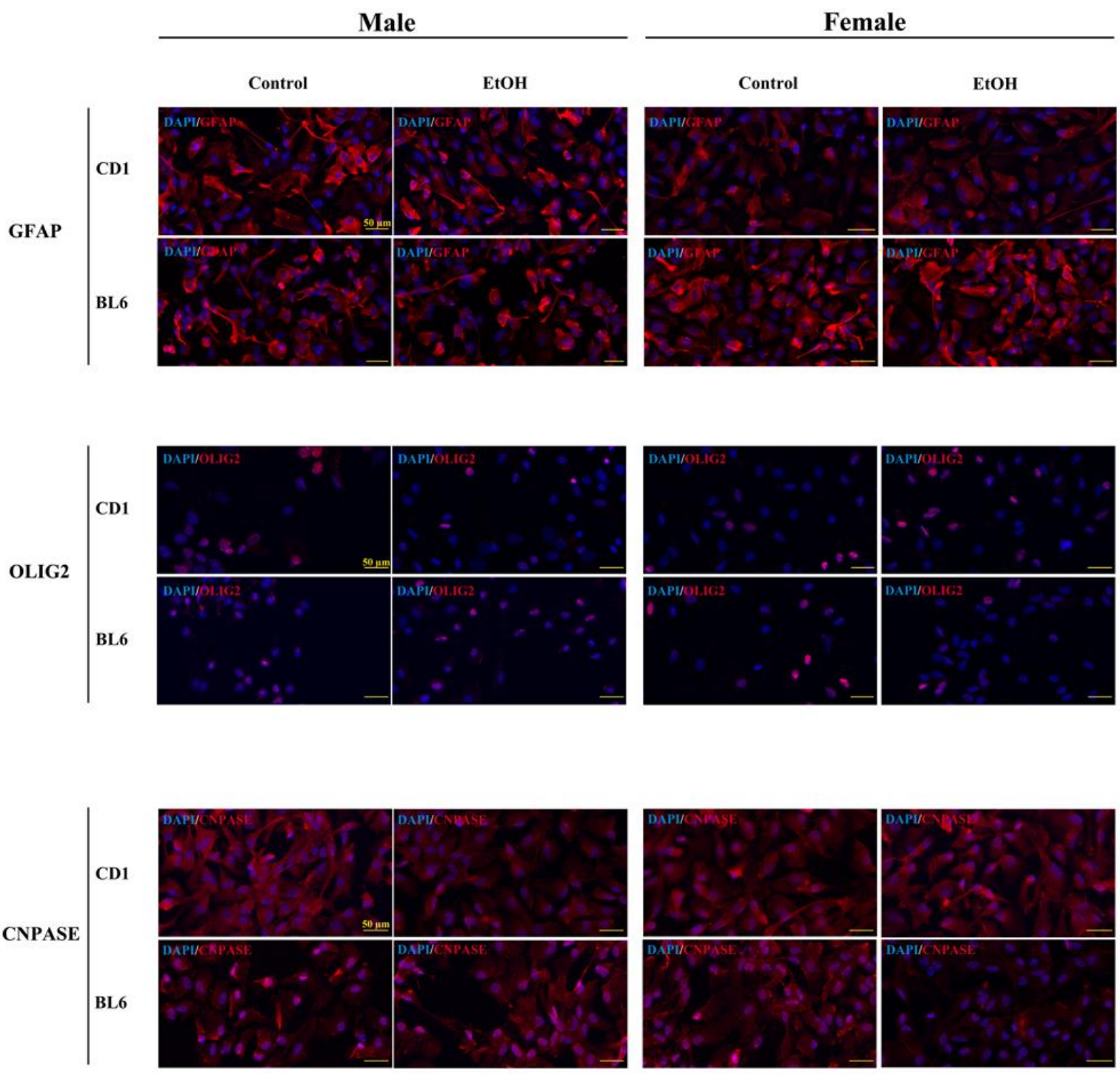
Figure 3. 6 Effect of chronic ethanol treatment on the expression level of glial-lineage marker proteins in the male and female differentiated NSC of CD1 and BL6 embryos.

a) Comparison of the basal protein expression of CNPASE, GFAP, and OLIG2 in the differentiated NSC of male and female cells of CD1 and BL6 embryos (N=3±SEM, for each group). Significant differences between male and female untreated (control) groups are shown with $p<0.01$**. Significant differences between strains (CD1 and BL6) are shown with $p<0.001$###, $p<0.01$##, and $p<0.05$#). b) Effect of chronic ethanol treatment on the expression of CNPASE, GFAP, and OLIG2 in the differentiated NSC of male and female cells of CD1 and c) BL6 embryos (N=3±SEM, for each group). Fold changes are reported relative to untreated control (male CD1, female CD1, male BL6, and female BL6) groups (shown as dashed line). Significant differences from controls are shown with $p<0.001$***, $p<0.01$**, $p<0.05$*. Significant differences of ethanol effects on male and female groups are shown with $p<0.001$###, $p<0.01$## and $p<0.05$#. GAPDH was used as a loading control for Western blots.

3.1.7. Immunofluorescence staining showed a sex-, and strain-specific effect of chronic ethanol treatment on the protein expression of glial markers.

To investigate the effects of chronic ethanol treatment on the expression of CNPASE, GFAP, and OLIG2 in differentiating NSC, we conducted a series of IF experiment on ethanol-treated and untreated differentiated NSC of all experimental groups. We analyzed the basal expression of glial marker proteins by WB in our experimental groups (Fig.3.6A), and we observed significant differences in the basal expression of CNPASE, GFAP, and OLIG2 between untreated NSC of all groups. Chronic ethanol treatment induced significant differences in the expression of glial marker proteins in both CD1 and BL6 cells. In comparison with untreated (control) BL6 cells, results showed that chronic ethanol treatment significantly decreased the

protein expression of CNPASE only in female BL6 differentiated NSC (Fig.3.7, $p<0.001$***) and induced no significant effect on the expression of GFAP and OLIG2 proteins in all other groups (Fig.3.7, $p>0.05$). Also, there were significant differences in the protein expression of CNPASE ($p<0.001$###) and GFAP ($p<0.05$#) between male and female BL6 cells (Fig.3.7). In comparison with untreated (control) male CD1 cells, chronic ethanol treatment significantly decreased protein expression of CNPASE ($p<0.05$*), GFAP ($p<0.05$*), and OLIG2 ($p<0.001$***) in differentiated male CD1 NSC (Fig.3.7). Similar treatment significantly increased protein expression of OLIG2 in female CD1 cells when compared to untreated female CD1 cells (Fig.3.7, $p<0.001$***). Also, chronic ethanol treatment had no significant effect on the protein expression of CNPASE and GFAP in female CD1 cells (Fig.3.6B, $p>0.05$). In addition, there were significant differences in the protein expression of CNPASE ($p<0.05$#), GFAP ($p<0.01$##), and OLIG2 ($p<0.001$###) between male and female CD1 differentiated NSC following chronic ethanol treatment (Fig.3.7).


Male
Female
Control
EtOH
Control
EtOH
GFAP
CD1
BL6
OLIG2
CD1
BL6
CNPASE
CD1
BL6
DAPI/GFAP
DAPI/OLIG2
DAPI/CNPASE
50 μm

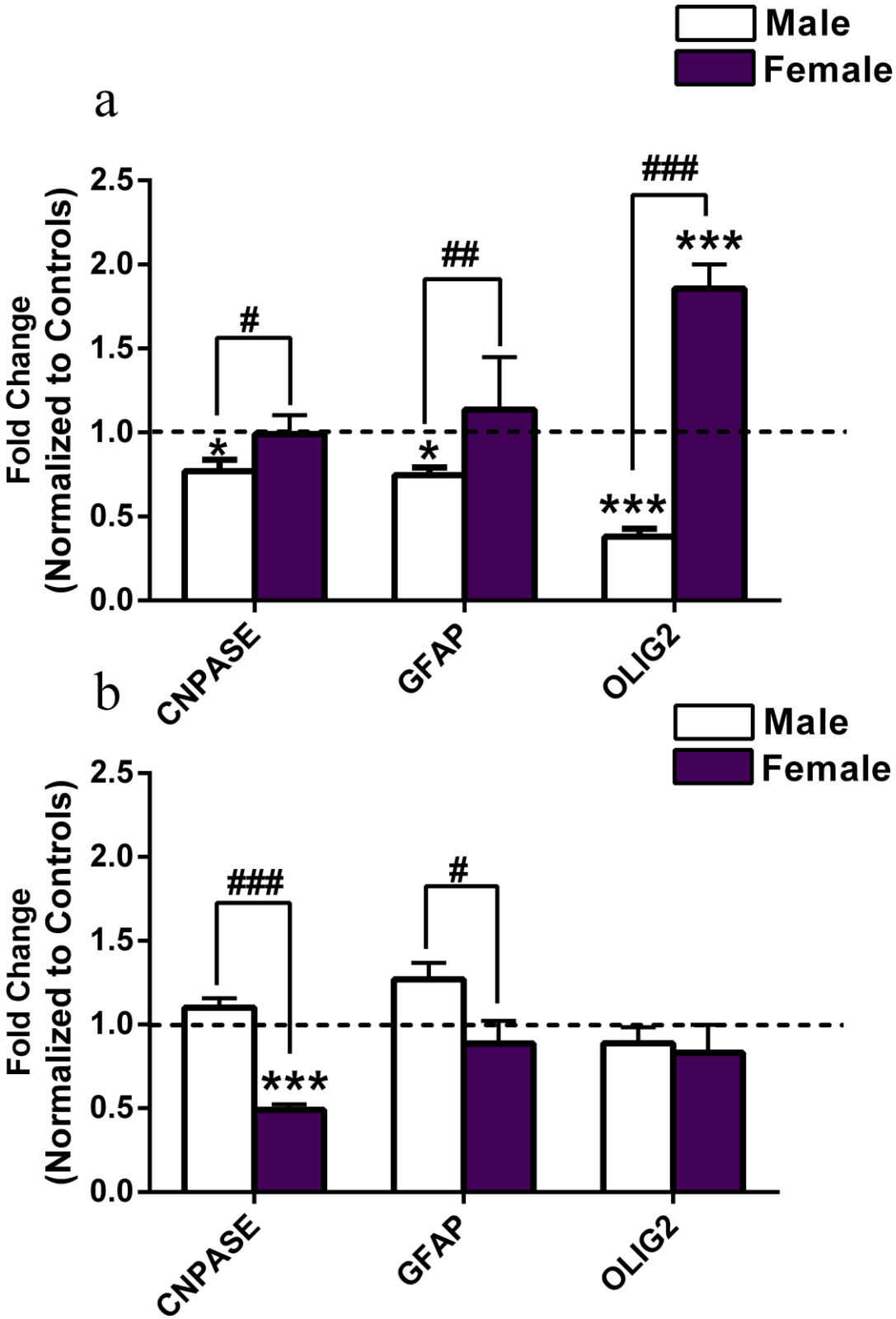

a
Male
Female
Fold Change
(Normalized to Controls)
2.5
2.0
1.5
1.0
0.5
0.0
#
##
###
*
*
***
***
CNPASE
GFAP
OLIG2
b
Male
Female
Fold Change
(Normalized to Controls)
2.5
2.0
1.5
1.0
0.5
0.0
###
#
***
CNPASE
GFAP
OLIG2

Figure 3. 7 Detection of glial-lineage markers in differentiated NSC by immunofluorescence staining following chronic ethanol exposure.

Expression of GFAP (top panel), OLIG2 (middle panel), and CNPASE (bottom panel) in untreated and ethanol-treated differentiated NSC of male and female cells of a) CD1 and b) BL6 embryos (N=3±SEM, for each group). Scale bars represent 50 μm. Fold changes are reported relative to untreated control (male CD1, female CD1, male BL6, and female BL6) groups (shown as dashed line). Significant differences from controls are shown with $p< 0.001$***, $p<0.05$*. Significant differences of ethanol effects on male and female groups are shown with $p<0.001$###, $p<0.01$##, and $p<0.05$#.

3.2. AIM 2. Investigating potential gene biomarkers in differentiating NSC following continuous ethanol exposure.

3.2.1. Chronic ethanol treatment altered the transcript expression of Dcc, Scn3a, and Sptbn2 genes in a sex-, and strain-specific manner.

To investigate the effects of chronic ethanol treatment on the expression of *Dcc*, *Scn3a*, and *Sptbn2*, we conducted a series of qRT-PCR experiment on ethanol-treated and untreated differentiated NSC from all experimental groups. We first analyzed the basal expression of these genes in our experimental groups. Our results showed that there were no significant differences in the basal expression of *Dcc*, *Scn3a*, and *Sptbn2* between male and female cells in both CD1 and BL6 strains (Fig.3.8A, $p>0.05$ for all three studied genes). However, untreated (control) female CD1cells expressed significantly higher levels of *Dcc* transcript in comparison with untreated (control) female BL6 cells (Fig.3.8A, $p<0.05$*). Also, untreated (control) female BL6 cells expressed significantly higher levels of *Scn3a* transcript in comparison with untreated (control) female CD1 cells (Fig.3.8A, $p<0.01$**). In addition, untreated (control) male CD1

($p<0.05$*) and female differentiated NSC ($p<0.001$**) expressed significantly higher levels of basal *Sptbn2* transcripts in comparison with their male and female BL6 counterparts (Fig3.8A).

In comparison with untreated (control) BL6 cells, chronic ethanol treatment significantly decreased the transcript expression of *Dcc* in male (but not female) BL6 cells (Fig.3.8B, $p<0.05$* and $p>0.05$, respectively). Similar treatment induced no significant effect in the transcript expression of *Scn3a* and *Sptbn2* in both male and female BL6 cells when compared to control counterparts (Fig.3.8B, $p>0.05$). In comparison with untreated (control) CD1cells, chronic ethanol treatment significantly decreased the transcript expression of *Dcc* in both male ($p<0.01$**) and female ($p<0.01$**) CD1 cells (Fig.3.8C). Similar treatment decreased the transcript expression of *Scn3a* in female (but not male) CD1 cells (Fig.3.8C, $p<0.05$* and $p>0.05$, respectively). Similarly, chronic ethanol treatment decreased the transcript expression of *Sptbn2* in female (but not male) CD1 cells (Fig.3.8C, $p<0.05$* and $p>0.05$, respectively). We observed no significant differences between male and female groups of both strains following ethanol exposure (Fig.3.8A and B, $p>0.05$). These results indicate that the effects of chronic ethanol treatment on the expression of cell lineage marker genes are in a sex-, and strain-specific manner in differentiating NSC. Also, the basal expression of these genes in untreated (control) cells did not differ significantly between male and female groups, while there were significant differences in the expression of *Dcc, Scn3a,* and *Sptbn2* genes in BL6 and CD1 strains.

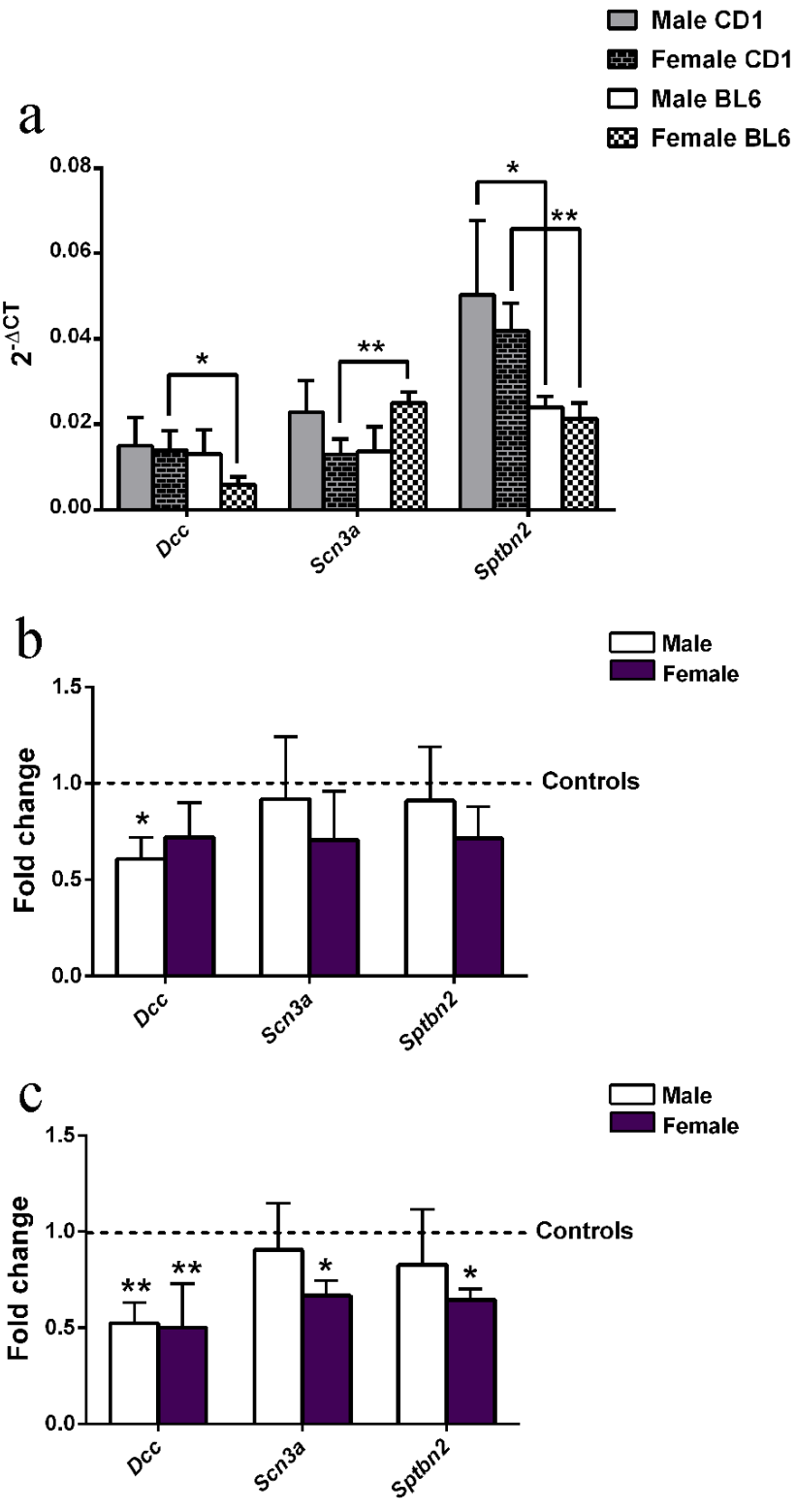

a
Male CD1
Female CD1
Male BL6
Female BL6
0.08
0.06
0.04
0.02
0.00
2-ΔCT
*
**
*
**
Dcc
Scn3a
Sptbn2
b
Male
Female
1.5
1.0
0.5
0.0
Fold change
Controls
*
Dcc
Scn3a
Sptbn2
c
Male
Female
1.5
1.0
0.5
0.0
Fold change
Controls
**
**
*
*
Dcc
Scn3a
Sptbn2

Figure 3. 8 Effect of chronic ethanol treatment on the expression of potential biomarker genes in the male and female differentiated NSC of CD1 and BL6 embryos.

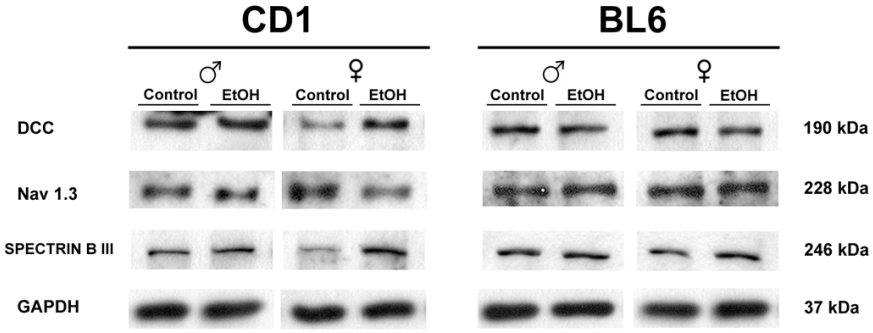
a) Comparison of the basal expression of *Dcc, Scn3a,* and *Sptbn2* transcript levels in differentiated NSC of male and female cells of CD1 and BL6 embryos (n=4±SEM, for each group). Significant differences between untreated (control) groups are shown with $p<0.01$** and $p<0.05$*. Effect of chronic ethanol treatment on the expression of *Dcc, Scn3a,* and *Sptbn2* transcript levels in differentiated NSC of male and female cells from b) BL6 and c) CD1 embryos (N=4±SEM, for each group). Fold changes are reported relative to untreated control (male CD1, female CD1, male BL6, and female BL6) groups (shown as dashed line). Significant differences from controls are shown with $p<0.01$** and $p<0.05$*. The transcript expression values were normalized to *Gapdh* expression.

3.2.2. Chronic ethanol treatment altered the protein expression of DCC, $Na_v1.3$, and Spectrin βIII in a sex- and strain-specific manner.

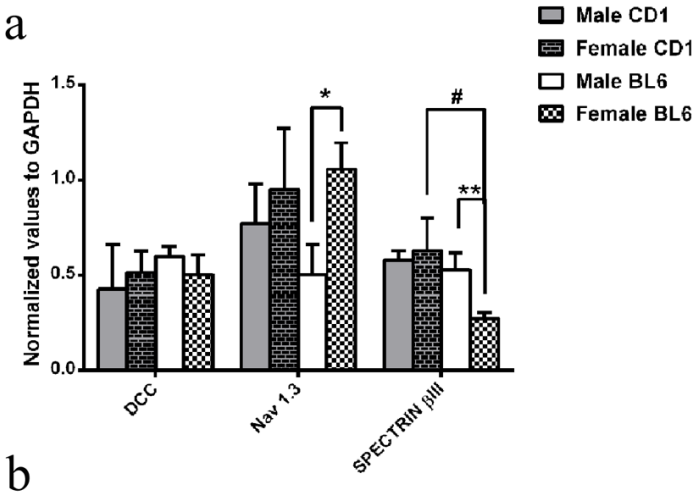
To investigate the effects of chronic ethanol treatment on the protein expression of DCC, $Na_v1.3$, and SPECTRIN βIII, we conducted a series of WB experiment on ethanol-treated and untreated differentiated NSC of all experimental groups. We first analyzed the basal expression of these proteins in our experimental groups. In the case of basal DCC expression, our results showed that there were no significant effect of strain ($p>0.05$) and sex ($p>0.05$) in the protein expression of DCC in untreated CD1 (male and female) and BL6 (male and female) cells (Fig.3.9A). In the case of $Na_v1.3$, the effect of sex was significant in BL6 cells and untreated (control) female BL6 cells expressed significantly higher levels of basal $Na_v1.3$ in comparison to untreated (control) male counterparts (Fig3.9A, $p<0.05$*). However, there were no significant effect of strain ($p>0.05$) and sex (in CD1 cells, $p>0.05$) in the protein expression of $Na_v1.3$ in

other untreated groups. In the case of SPECTRIN βIII, the effect of sex (in BL6 cells) and strain (between female CD1 and BL6) were significant. In this regard, untreated (control) female BL6 cells expressed significantly lower levels of basal SPECTRIN βIII in comparison to untreated (control) male counterparts (Fig3.9A, $p<0.01$**). Also, untreated (control) female BL6 cells expressed significantly lower levels of basal SPECTRIN βIII in comparison with untreated female CD1 cells (Fig.3.9A, $p<0.05$#).

Figure 3.9B shows that chronic ethanol treatment induced significant changes in the protein expression of potential biomarkers in our experimental groups. In comparison with untreated (control) male cells, chronic ethanol treatment induced no significant effect on the protein expression of DCC ($p>0.05$), $Na_v1.3$ ($p>0.05$), and SPECTRIN βIII ($p>0.05$) in the male CD1 differentiated NSC (Fig.3.9B). However, same treatment significantly decreased $Na_v1.3$ ($p<0.05$*) and increased DCC ($p<0.05$*) and SPECTRIN βIII ($p<0.05$*) protein levels in female CD1 cells when compared to their counterparts (Fig.3.9B). Also, there were no significant differences in protein expression between male and female groups in the case of CD1 cells (Fig3.9B, $p>0.05$). In comparison with untreated (control) BL6 cells, chronic ethanol treatment induced no significant effect on the protein expression of DCC (both sexes, $p>0.05$), $Na_v1.3$ (both sexes, $p>0.05$), and SPECTRIN βIII (both sexes, $p<0.05$) (Fig.3.9B). Also, there were no significant differences in protein expression between male and female cells in the case of BL6 cells (Fig3.9B, $p>0.05$). Overall, these findings indicate that the effects of chronic ethanol treatment on the protein expression of potential biomarkers in differentiating NSC are in a sex-, and strain-specific manner, namely in CD1 cells. Also, the basal expression of aforementioned proteins (except for DCC) in untreated (control) cells differs between the two tested strains as well as the two sexes.


CD1
BL6
♂
♀
Control
EtOH
DCC
Nav 1.3
SPECTRIN B III
GAPDH
190 kDa
228 kDa
246 kDa
37 kDa

a


Male CD1
Female CD1
Male BL6
Female BL6
Normalized values to GAPDH
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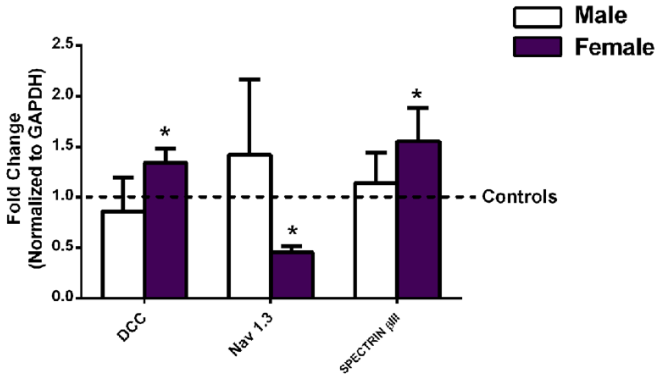
1.0
0.5
0.0
*
#
**
DCC
Nav 1.3
SPECTRIN βIII

b


Male
Female
Fold Change
(Normalized to GAPDH)
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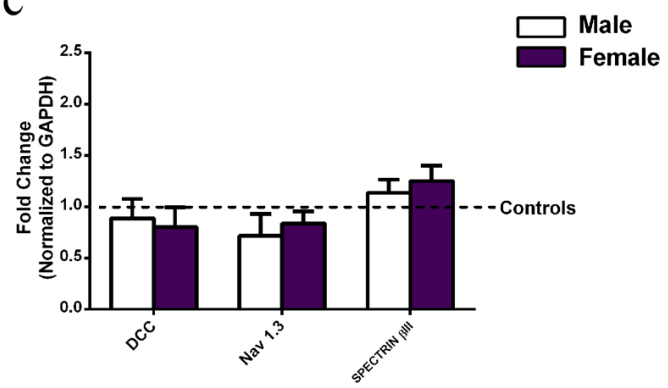
2.0
1.5
1.0
0.5
0.0
Controls
*
DCC
Nav 1.3
SPECTRIN βIII

c


Male
Female
Fold Change
(Normalized to GAPDH)
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2.0
1.5
1.0
0.5
0.0
Controls
DCC
Nav 1.3
SPECTRIN βIII

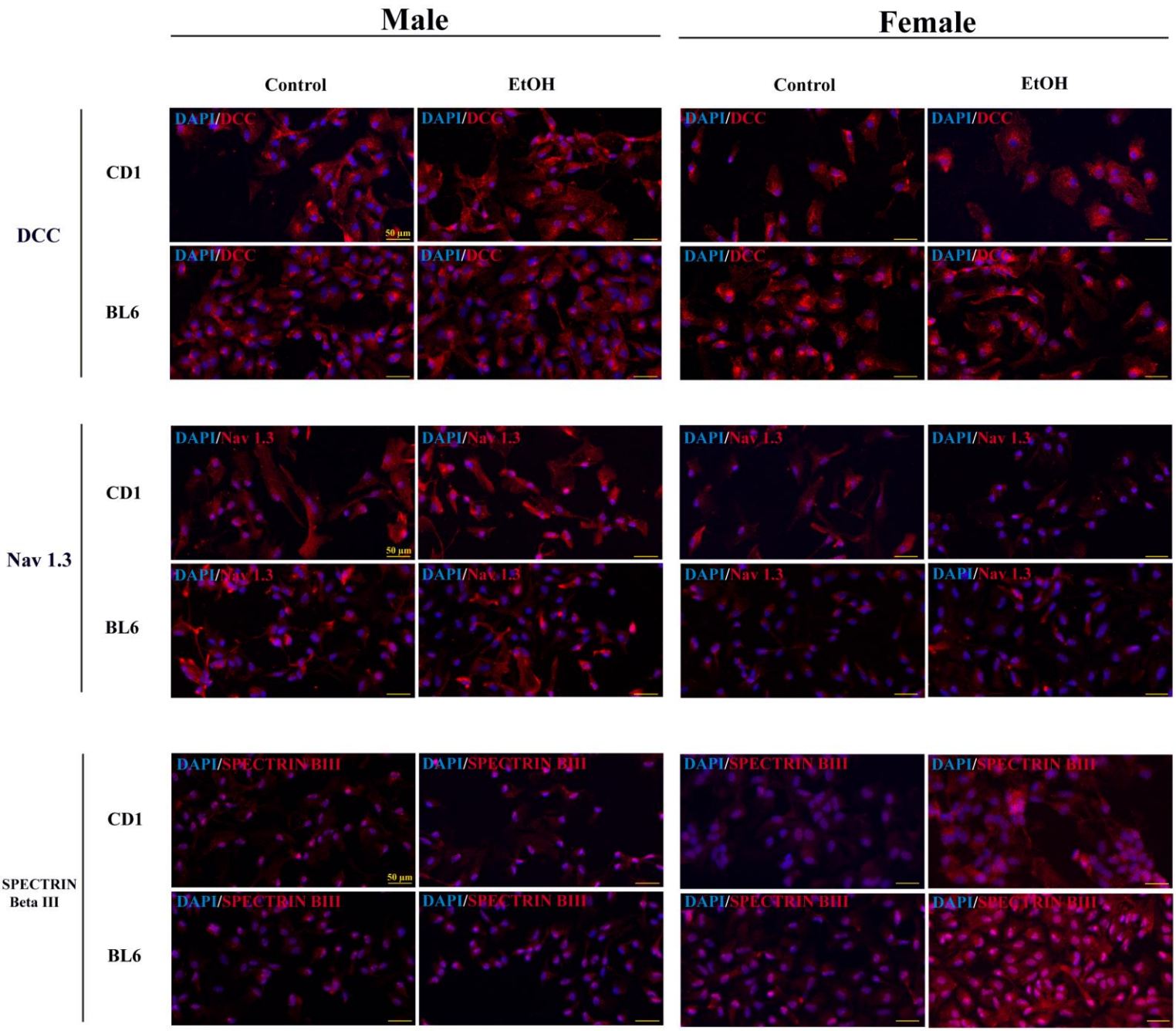
Figure 3. 9 Effect of chronic ethanol treatment on the expression level of potential biomarker proteins in the male and female differentiated NSC of CD1 and BL6 embryos.

a) Comparison of the basal protein expression of DCC, $Na_v1.3$, and SPECTRIN βIII in the differentiated NSC of male and female cells of CD1 and BL6 embryos (n=3±SEM, for each group). Significant differences between male and female untreated (control) groups are shown with $p<0.01$** and $p<0.05$*. Significant differences between strains (CD1 and BL6) are shown with $p<0.05$#, Effect of chronic ethanol treatment on the expression of DCC, Nav1.3, and SPECTRIN βIII in the differentiated NSC of male and female cells from b) CD1 and c) BL6 embryos (N=3±SEM, for each group). Fold changes are reported relative to untreated control (male CD1, female CD1, male BL6, and female BL6) groups (shown as dashed line). Significant differences from controls are shown with $p< 0.05$*. GAPDH was used as a loading control for western blots.

3.2.3. Immunofluorescence staining showed a sex- and strain-specific effect of chronic ethanol treatment on the protein expression of potential biomarkers.

To investigate the effects of chronic ethanol treatment on the expression of DCC, Nav1.3, and SPECTRIN βIII in differentiating NSC, we conducted a series of IF experiments on ethanol-treated and untreated differentiated NSC of all experimental groups. We analyzed the basal expression of potential biomarker proteins by WB in our experimental groups (Fig.3.9A). Chronic ethanol treatment induced significant differences in the expression of potential biomarkers in both CD1 and BL6 cells. In comparison with untreated (control) BL6 cells, ethanol treatment significantly increased SPECTRIN βIII in female BL6 cells ($p<0.01$**), but had no significant effect on the protein expression of DCC (both sexes, $p>0.05$), $Na_v1.3$ (both sexes, $p>0.05$), and SPECTRIN βIII (only male group, $p>0.05$) in other groups (Fig.3.10). In

comparison to untreated (control) female and male CD1 cells, chronic ethanol treatment significantly increased the protein expression of DCC in female CD1 cells and had no effect on male CD1 cells (Fig.3.10, $p<0.001$*** and $p>0.05$, respectively). In addition, there was significant effect of ethanol treatment on male and female CD1 cells in the expression of DCC (Fig.3.10, $p<0.05$#). In comparison to untreated (control) female and male CD1 cells, chronic ethanol treatment significantly decreased the protein expression of $Na_v1.3$ in female CD1 cells and had no effect on male CD1 cells (Fig.3.10, $p<0.05$* and $p>0.05$, respectively). In addition, there was a significant effect of ethanol treatment between sexes in the expression of $Na_v1.3$ (Fig.3.10, $p<0.05$#). In comparison to untreated (control) female and male CD1 cells, chronic ethanol treatment significantly increased the protein expression of SPECTRIN βIII in female CD1 cells and had no effect on male CD1 cells (Fig.3.10, $p<0.001$*** and $p>0.05$, respectively). Also, there was a significant effect of ethanol treatment between male and female CD1 cells in the expression of SPECTRIN βIII (Fig.3.10, $p<0.001$###). Collectively, these findings indicate that the effects of chronic ethanol treatment on the protein expression of potential biomarkers in differentiating NSC are in a sex-, and specific manner.


Male
Female
Control
EtOH
Control
EtOH
DCC
CD1
BL6
Nav 1.3
CD1
BL6
SPECTRIN Beta III
CD1
BL6
DAPI/DCC
DAPI/Nav 1.3
DAPI/SPECTRIN BIII
50 µm

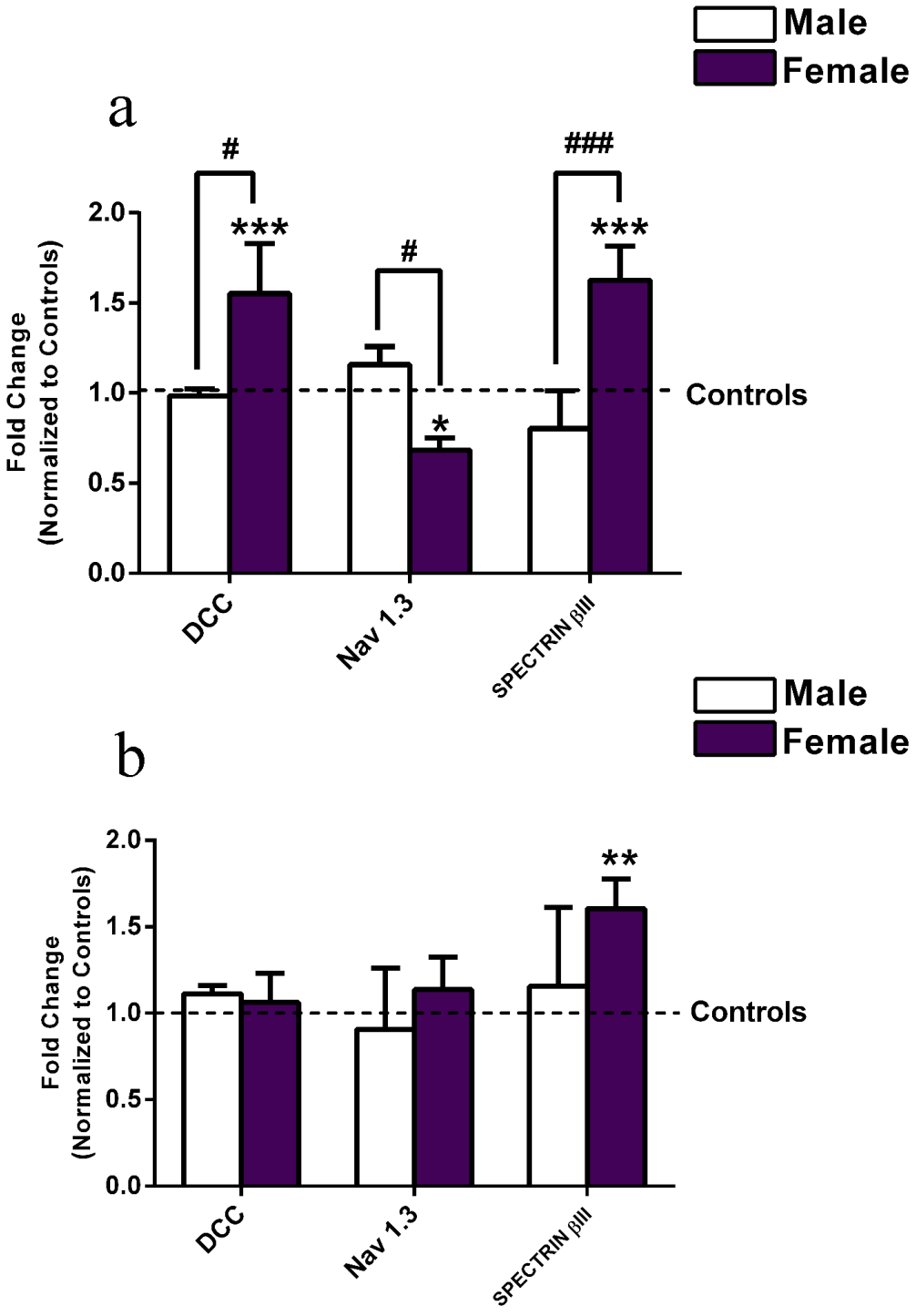

Male
Female
a
#
###
***
***
#
*
2.0
1.5
1.0
0.5
0.0
Fold Change
(Normalized to Controls)
Controls
DCC
Nav 1.3
SPECTRIN βIII
Male
Female
b
**
2.0
1.5
1.0
0.5
0.0
Fold Change
(Normalized to Controls)
Controls
DCC
Nav 1.3
SPECTRIN βIII

Figure 3. 10 Detection of potential biomarker proteins in the differentiated NSC by immunofluorescence staining following chronic ethanol exposure.

Expression of DCC (top panel), Nav1.3 (middle panel), and SPECTRIN βIII (bottom panel) in untreated and ethanol-treated differentiated NSC of male and female cells of a) CD1 and b) BL6 embryos (N=3±SEM, for each group). Scale bars represent 50 μm. Fold changes are reported relative to untreated control (male CD1, female CD1, male BL6, and female BL6) groups (shown as dashed line). Significant differences from controls are shown with$p<0.001$***, $p<0.01$**, and $p< 0.05$*. Significant differences of ethanol effects on male and female groups are shown with $p<0.001$### and $p<0.05$#.

CHAPTER FOUR: DISCUSSION

In the current work, we applied a previously used standard *in vitro* model system to investigate the effects of chronic ethanol exposure on NSC during differentiation (101, 142, 241). Since forebrain makes limbic system and cortical areas during development, we isolated NSC from forebrain as an anatomically determining region in the brain for the effects of ethanol (242, 243). We used 70 mM ethanol that not only was reported to alter DNA methylation and NSC differentiation (101, 127, 142), but also is a clinically and pharmacologically relevant dosage (244, 245). Also, we used 70 mM ethanol because previous studies reported that ethanol concentration is around 182-227 mg/dl (39.57 mM-49.35 mM) in the medium following evaporation in the incubator, which is relevant to moderate chronic ethanol consumption in humans (142, 246). Using such a controlled and valid *in vitro* model system, we discuss the results of chronic ethanol treatment on differentiating NSC with regard to: 1) sex-specific effects of ethanol on DNA methylation in CD1 and BL6 strains, 2) sex-specific effects of ethanol on NSC commitment in CD1 and BL6 strains, and 3) the validity of potential biomarkers for PAE.

4.1. Chronic ethanol altered components of DNA methylation machinery of NSC in a sex-specific manner in CD1 and BL6 strains

As mentioned in section 1.4, DNA methylation plays a critical role in during brain development and previous research suggested that specific changes in DNA methylation are considered as potential FASD biomarkers (118, 192). Our results revealed that chronic ethanol exposure during NSC differentiation alters global DNA methylation by decreasing the 5-mC levels in both male and female NSC from BL6, as well as female (and not male) cells of CD1. These results suggest that chronic ethanol exposure induces global hypomethylation in all experimental groups, except for male CD1 cells. Most of *in vivo* and *in vitro* models of PAE

revealed that ethanol induces global hypomethylation ((104, 247). However, some previous investigations reported that ethanol exposure induces global hypermethylation in animal studies (48, 112). The inconsistency between our results and latter studies are possibly attributed to different applied methods, ethanol concentrations, time of exposure, time of study, and many other factors. There is consensus opinion that ethanol disrupts one-carbon metabolism through interacting with SAM and folate and reduces the absorption of methyl donors in biological systems, thus inducing global hypomethylation in cells/tissues exposed to ethanol (247). We also observed a sex-specific effect of ethanol on the 5-mC levels of NSC from CD1 strain, while we did not detect such an effect in BL6 strain. In this regard, sex differences in DNA methylation under normal and pathological conditions (including PAE) were reviewed in section 1.5.1 and in the literature (146, 157, 186). However, this study is the first report about the sex-specific effects of ethanol on NSC from E14.5 embryos in the literature. A previously published study by our lab revealed that ethanol exposure increases global 5-mC in NSC (not sexually determined) from CD1, while the results of the current study indicate chronic ethanol decreases 5-mC levels only in female, but not in male NSC from CD1 (101). Interestingly, we found that there are differences in the basal 5-mC levels between male and female cells in both CD1 and BL6 cells suggesting that sex-specific effects of ethanol may be in part related to the basal differences in 5-mC levels. We also found that the effects of ethanol on global 5-mC is strain-specific. In this regard, evidence has shown that the effects of ethanol on many biological systems (including epigenetic modifications) are strain-specific and some strains are more resistant to the negative effects of PAE (248-252). Our results also revealed that ethanol treatment decreased the 5-hmC levels in female (but not male) NSC from BL6, while had no significant effects on both male and female CD1 cells suggesting that effects of chronic ethanol exposure on 5-hmC levels in

differentiating NSC is sex- and strain-specific. Although the effects of ethanol has been extensively reported on 5-mC and DNMT enzymes in the literature, but there are few studies that investigate the effects of ethanol on 5-hmC levels and TET enzymes (104, 247). In this regard, a study by Chen and colleagues demonstrated that PAE to pregnant mice is associated with decreased 5-mC, 5-hmC, and TET1 levels in the ammonic neuro-epithelium and intermediate zone of P7 mice embryos from BL6 strain (112). Results of the latter research are in line with our results that chronic ethanol exposure reduces 5-mC and 5-hmC levels in the BL6 strain. In addition, our results confirm the previous report of our lab about the effects of ethanol on 5-hmC levels in CD1 strain (101). Unlike basal 5-mC levels, we did not observe a significant difference in the basal levels of 5-hmC between male and female cells of both strains.

Since we observed the significant effects of ethanol treatment on the global 5-mC and 5-hmC levels in differentiating NSC, we investigated the effects of ethanol exposure on the transcription of genes involved in DNA methylation. First, we observed that there is no significant difference between CD1 and BL6 strain in the basal transcript expression of genes involved in DNA methylation, but female BL6 cells expressed lower transcript level of *Tet* genes in comparison to male cells, suggesting the sex-specific expression of *Tet* genes in BL6 strain. Chronic ethanol treatment increased *Dnmt1* expression in both CD1 and BL6 male NSC, while decreased *Dnmt1* expression in female NSC indicated the sex-specific impact of ethanol on *Dnmt1* expression. It is important to note that most of the reported studies on the effects of PAE (both *in vitro* and *in vivo*) did not consider the factor of sex differences, and reported their results only in males/combined male and female subjects (104, 247). There are inconsistencies between the results of previous studies on the effects of ethanol on *Dnmt1* expression (104). In this context, Mukhopadhyay *et al.* applied high dose of ethanol (200 mM) to embryonic fibroblasts

(obtained from male BL6 embryos at E13.5) for 48 h, and observed that ethanol reduced the expression of *Dnmt1* in these cells (99). Another study by Veazey *et al*. showed that treating neurospheres (isolated fromBL6 embryos at E12.5) with 70 mM ethanol for 72 h had no effect on the expression of *Dnmt1* (126). Also, *in vivo* studies revealed that ethanol exposure is associated with decreased (114), or increased (47) *Dnmt1* expression in animals. These results indicate that effects of ethanol on a variety of enzymes and molecular pathways depend on the dosage of ethanol, methodology, type of cells, duration of exposure, and other factors. Unlike *Dnmt1* expression, our results showed that ethanol exposure induced no effect on the expression of *Dnmt3a* and *Dnmt3b* (in both sexes of CD1 and BL6) in differentiated NSC. Similar to *Dnmt1*, inconsistent results were reported on the effects of ethanol on the transcript expression of *Dnmt3a* and *Dnmt3b* (104, 247). Using different methodologies, previous studies revealed that ethanol exposure decreases (101, 103), increases (99, 101, 126), or has no impact (129) on the expression of the *Mecp2* gene. Our results showed that chronic ethanol treatment had no effect on the expression of both *Mecp2e1* and *Mecp2e2* in all groups. We also observed a sex-specific effect of ethanol exposure on the transcript expression of *Tet* genes. Ethanol exposure decreased the transcript expression of *Tet2* and *Tet3* in male cells of BL6, and increased *Tet3* in female BL6 cells. However, ethanol had minimum effect on the expression of *Tet* genes in the CD1 strain and only reduced the transcript expression of *Tet3* in male cells. These results suggest that the effects of chronic ethanol on the transcript expression of *Tet* genes are different in differentiating NSC between CD1 and BL6 strains. As mentioned earlier, there are few studies on the effects of ethanol on *Tet* genes or proteins in the literature, and we found no study on the effects of ethanol on *Tet* genes' expression. Our results also revealed that ethanol exposure mostly affected the expression of genes in NSC of BL6 rather than CD1 cells. In addition,

ethanol exposure mostly affected the gene expression in male NSC cells rather than females in CD1 cells.

Unlike gene expression profiles, our results showed that there are significant differences between sexes and strains in the basal expression of proteins involved in DNA methylation in differentiating NSC. Sex-differences were only observed in the expression of TET1, DNMT1, and DNMT3A in CD1 strain, while the same effect was not observed in the BL6 strain. Despite the fact that we showed the transcript expression of *Tet3* in NSC of all groups, we were not able to detect TET3 protein in our samples when using WB method. To address this, previous research revealed that TET3 protein expression is important in neuronal differentiation in the brain. Authors of the latter study revealed that following the induction of neurogenesis in stem cells, there were undetectable levels of TET3, even after 8 days (253). Another study showed that TET1 and TET2 (and not TET3) regulate the production of 5-hmC and cell lineage differentiation in embryonic stem cells (254). Considering that we induced gliogenesis (and not neurogenesis) in NSC in our study and TET3 is abundant in neurons, the lack of TET3 protein expression was not surprising. Interestingly, BL6 NSC showed relatively higher expression of basal DNA methylation proteins than CD1 cells, except for DNMT1. These results suggest that there are differences in the basal expression of proteins, which regulate DNA methylation in BL6 and CD1 during differentiation of NSC. Our results also demonstrate that the effects of ethanol exposure on the expression of DNMT proteins are different in CD1 and BL6 strains. Ethanol decreased DNMT1 in male CD1 cells, while increased DNMT1 expression in BL6 cells. This strain-specific effect of ethanol may be related to different responses of CD1 and BL6 strains to ethanol dosage. Previous research showed that while 48 h exposure to ethanol 25 mM induced DNMT1, DNMT3A, and DNMT3B expression in embryonic fibroblasts (isolated from BL6

embryos at E13.5), exposure to 200 mM ethanol significantly decreased the expression of DNMT1, DNMT3A, and DNMT3B protein (99). There are also *in vitro* and *in vivo* studies that reported ethanol exposure decreased (114) or had no effect (98, 255) on DNMT1 expression. Although prior investigations demonstrated that ethanol exposure decreased DNMT3A expression, its reduction in our study was not significant (98, 114). Interestingly, evidence showed that treating BL6 mice (P7) with low dose ethanol (blood alcohol levels ~45 mM) increased the expression of DNMT3A in the hippocampus and cortex of P8 pups, but the same treatment with high ethanol dose (blood alcohol levels ~200 mM) decreased DNMT3A expression in both the hippocampus and cortex of P8 pups (114, 129). We also found that chronic ethanol exposure increased DNMT3B protein levels in CD1 female cells. There is no evidence on the effects of ethanol on DNMT3B expression in female subjects. However, a recent study revealed that acute ethanol exposure decreased *Dnmt3b* transcript and increased DNMT3B protein in embryonic fibroblasts (99). These results indicate that the effects of ethanol exposure on the expression of DNMT enzymes are associated with many factors namely, dose of ethanol used in the study. We also observed that ethanol exposure had no effect on the protein expression of MeCP2 in BL6 cells. However, the same treatment reduced MeCP2 levels in male differentiating NSC, while induced MeCP2 expression in female CD1 cells. These results suggest that ethanol differently affects MeCP2 expression in CD1 and BL6 strains and exhibits a sex-specific effect in CD1 strain. Previously published research from our lab showed that both acute (48 h) and chronic (8 days) ethanol exposure induced MeCP2 expression in differentiating NSC (CD1 strain), which were not sexually determined (101). Other reports revealed that ethanol increases (47, 129, 132) or decreases (103, 112, 255) MeCP2 expression in both *in vitro* and *in vivo* studies. In this regard, Bekdash and colleagues treated rats during gestational period

E7-21 with low ethanol dose (blood alcohol level ~ 32 mM), and showed that MeCP2 levels increased in the hypothalamus of adult male rats (47). Similarly, another study by Tunc-ozcan *et al*. treated rats during gestational period E8-21 with low ethanol dose (blood alcohol level ~27 mM), and showed that MeCP2 levels increased in the hippocampus of adult male rats (132). In contrast, treating BL6 mice with low dose ethanol (30 mM) during E5-16 resulted in a decrease in MeCP2 expression in the hippocampus of P7 pups (112). These results indicate that effects of ethanol on MeCP2 expression depend on several factors such as ethanol dose and duration of treatment, type of species, time of experiment, tissue of target and other factors. Most of the studies on the effects of ethanol on DNA methylation did not consider the sex and strain of animals. However, most studies showed that low dose ethanol increased MeCP2 in male subjects, while there is no evidence about the females in the literature. We also found that chronic ethanol exposure reduced the expression of TET1 and TET2 in male CD1 and female BL6 differentiating NSC, but observed effects were not statistically significant. These results indicate that chronic ethanol exposure had no significant effect on the protein expression of TET enzymes in differentiated NSC in both strain. A study on the effects of ethanol on TET1 expression revealed that treating pregnant BL6 mice at E5-16 reduced TET1 expression in the hippocampus of P7 pups (no sex determined) (112). However, reduced levels of TET1 in our study were not statistically significant.

Focusing on the effects of ethanol on the DNMT activities, our results showed that there are higher basal DNMT activities in female NCS of CD1 when compared to male CD1 and female BL6 cells indicating the differences in the basal DNMT activities between sexes in CD1 as well as strains. We also found that ethanol only decreased DNMT activities in differentiated NSC of female CD1, and had no effect on NSC of other groups. Previous investigations showed

that effects of ethanol on DNMT activities are inconsistent. In this context, Perkins *et al.* showed that treating rats (E1-P10) with relatively high ethanol dosage (blood alcohol concentration ~ 85mM) and found that ethanol increased DNMT activity in the hippocampus of P21 pups (combined sexes), while decreased the transcript expression of *Dnmt1*, *Dnmt3a*, and *Mecp2* (131). In another study, Swiss mice were treated (E9-E11) with high ethanol dose (3 g/kg) and authors found DNMT activities and 5-mC levels reduced in whole embryonic tissues suggesting that high concentrations of ethanol induces hypomethylation in the whole embryonic tissues (105). Similarly, treating cortical astrocytes with 75 mM ethanol decreased both 5-mC and DNMT activities, and DNMT3A protein expression in these cells. The authors also showed that there was no effect of ethanol on DNMT1 protein as well as transcript expression of *Dnmt1* and *Dnmt3a,* despite decreased *Dnmt3b* transcript expression levels (98). These results show that effects of ethanol on DNMT activities depend on a variety of factors such as mode of ethanol exposure, dose of ethanol and many other factors. Also, these results indicate that DNMT activities do not fully depend on the transcript or protein expression of DNMT enzymes. We also found that ethanol exposure had no effect on the TET activities in differentiated NSC of all groups.

Overall, the results of this study revealed that the effects of chronic ethanol treatment on the components of DNA methylation machinery are sex-specific and strain-specific in differentiated NSC obtained from the forebrain of E14.5 embryos. A recent study on the sex differences in the transcriptome of mouse embryonic stem cells (at E3.5) showed that hundreds of coding and non-coding RNAs are differentially expressed between male and female cells, including epigenetic and chromatin remodeling factors such as *Mecp2* and *Dnmt3b* (256). These findings confirm our results about sex-differences in the basal expression of DNA methylation-

related proteins. Focusing on the effects of ethanol, a recent study by McGrath and colleagues showed that treating adult mice with chronic ethanol, changes NSC differentiation in a brain region-dependent manner and sex is a determinant factor for the regional differences of NSC response to ethanol (257). We found that effects of ethanol on differentiated NSC in CD1 strain are associated with a decrease in DNA methylation, namely in the female cells. Discrepancy between the DNMT activity, DNMT protein, or transcript expression was reported previously (104, 247), and we observed similar results in our study. In the case of BL6 strain, although we observed a global hypomethylation (both 5-mC and 5-hmC), we observed an increase in DNMT1 protein (in male cells) and DNMT activity (in female cells) suggesting that unlike CD1 cells, BL6 cells show increased DNMT1 expression/activity in response to ethanol. Overall, our findings are in line with previous studies that chronic ethanol exposure alters components of DNA methylation machinery in a sex- and strain-specific manner.

4.2. Chronic ethanol altered cell lineage differentiation of NSC in a sex-specific manner in CD1 and BL6 strains.

Effects of PAE (both *in vitro* and *in vivo* studies) on NSC differentiation, cell fate commitment, and neurogenesis have been extensively reported in the literature (246, 261, 264). However, the effects of PAE on glial lineage (including both astrocytes and oligodendrocytes) in a sex- and strain-dependent manner has not been studied yet. To do this, we exposed differentiating NSC to a glio-inductive culture conditions (FBS 10% and DMEM without B27 or neurobasal media) to boost NSC differentiation toward gliogenesis (258-260). Our results revealed that while chronic ethanol induced minimal effects on the transcript expression of cell lineage markers in CD1 cells, same ethanol exposure altered expression of cell lineage markers in BL6 cell suggesting the strain-specific effects of ethanol on NSC differentiation. In addition,

there was no difference in the basal transcript expression of cell lineage markers between different sexes and strains in this study. We observed no significant effect of ethanol on the transcript expression of *Gfap* and *Cnp* in both strains. A study by Valles *et al*. showed that treating pregnant rats with low dose ethanol (blood alcohol levels 23 mM) leads to hypermethylation of the *Gfap* promoter and decreased transcript and protein expression of GFAP in astrocytes isolated from E21 embryos (119). However, at the protein level, we found that ethanol treatment decreased the expression of GFAP and CNPASE in male (but not female) CD1 NSC, while similar effect was not seen in BL6 NSC. These results suggest the sex-specific and strain-dependent effects of ethanol. In this regard, McGrath *et al*. showed that different effects of chronic ethanol on GFAP expression are not only sex-specific, but also depend on the affected region in the brain. Although the latter study used adult male and female BL6 mice, they found that effects of ethanol on GFAP expression differ in hippocampal dentate gyrus, hypothalamus, and sub-ventricular zone (257). Other studies had no focus on the sex or strain when evaluating the effects of ethanol on NSC differentiation or other model systems. These studies reported that ethanol exposure decreased (ethanol dose, 70 mM), increased (ethanol dose, 21 mM), or had no effect (ethanol doses, 34 and 52 mM) on GFAP protein or transcript expression in NSC (126, 127, 261). However, clinical and experimental studies (only on male cells or combined sexes) showed that PAE is associated with a variety of abnormalities in astrocytic lineage such as: 1) reduced number of glial progenitors, astrocytic proliferation and differentiation, 2) decreased production of neurotrophic factors and antioxidants, 3) increased release of pro-inflammatory cytokines (66). In addition, we observed the same pattern for *Olig2* transcripts (both strains) and OLIG2 protein expression (only CD1) with decreased expression in male cells and increased expression in female NSC suggesting the opposite and sex-specific effects of ethanol on NSC

during differentiation. Although there is no previous research on the sex- or strain-specific effects of ethanol on OLIG2 expression, both human and animal studies showed that PAE alters all classes of glial cells and their precursors including oligodendrocytes (66, 68). In this regard, a study by Navarro and Mandyam revealed that exposing male rats to ethanol vapor (blood ethanol concentration 27-54 mM) resulted in altered oligodendrocyte performance and prefrontal cortex structure by reducing the phosphorylation of OLIG2 in medial prefrontal cortex, and consequently decreasing the myelin formation (262). Another study on male mice revealed that chronic exposure to ethanol vapor (blood alcohol level 30-40 mM) during third trimester (P3-P10) resulted in oligodendrocyte (about 60%) and oligodendrocyte progenitors (around 75%) loss and persistent white matter injury in the corpus callosum P16 male mice (263). However, there are *in vitro* studies on the effects of ethanol on NSC suggesting that ethanol (100 mM) decreased the expression of GAL-C (oligodendrocyte marker), while ethanol (32-54 mM) had no effect on *Olig2* transcript expression (126, 261). It is important to note that many clinical and experimental studies revealed that PAE is associated with delayed, decreased, and disorganized myelination of cortical areas, thin myelin layer, and decreased oligodendrocyte differentiation (66, 68). However, the majority of these studies used male or combined sexes in their studies and the effects of ethanol on oligodendrocytes in female subjects have not been reported. We observed a significant difference between the basal expression of glial markers in our study that may partly be involved in the different responses of NSC to ethanol in CD1 and BL6. Also, ethanol exposure decreased the transcript expression of *Tuj1* in both male and female BL6 cells, and had no effect on CD1 NSC. Previous studies revealed that ethanol decreased (116), increased (126), or had no effect on the expression of mRNA and protein of neuronal markers such as

MAP2 and TUJ1 in differentiating NSC (127). However, we did not detect TUJ1 protein in our samples due to high population of glial lineage cells in our study.

Ample evidence indicates that PAE alters the proliferation, differentiation, and cell fate commitment of stem cells, and these effects are involved in the pathophysiology of FASD (111, 261, 264). In our study, we showed that chronic ethanol treatment altered the expression of glial markers in a sex- and strain-specific manner. In this study, we isolated NSC from E14.5 mice, which is equivalent of second trimester in pregnancy. Late first trimester and early second trimester is a time-window during which NSC make the most neurons in the brain (139). In addition, a transient increase in basic fibroblast growth factors (bFGFs) in the mouse cortex in E14.5 is considered as an important modulatory factor for the transition from neurogenesis to gliogenesis (265, 266). However, significant gliogenesis is observed around E18 in rodents (267). Evidence indicates that DNA methylation, histone modifications, and micro RNAs cooperate together during NSC differentiation and a variety of intrinsic and extrinsic factors may interfere in the process of DNA methylation (267). In our study, we only showed the effects of ethanol (as an extrinsic factor) on the components of DNA methylation machinery (as an intrinsic factor) during NSC differentiation, and did not focus on other epigenetic modifications. DNMT enzymes play a critical role in the process of NSC differentiation by both maintenance and *de novo* DNA methylation of genes responsible for NSC differentiation (268, 269). There has been evidences that DNA methylation has a prominent role in the initiation of astrogliogenesis (269). Deletion of DNMT1 in NSC has been reported to induce precocious astroglial differentiation through hypomethylation and activation of the JAK-STAT pathway (270). In our study, we observed that ethanol induced hypomethylation in differentiated NSC in most of the experimental groups, but we observed no increase in the expression of GFAP in our

study. Interestingly, it has been shown that ethanol effectively inhibits JAK-STAT pathway, thus decrease in the glial markers in male CD1 cells, may be associated with the inhibitory effects of ethanol on JAK-STAT pathway (271, 272). However, no study was conducted about the effects of ethanol on JAK-STAT in female subjects, and our result showed that ethanol exposure not only produced no effect on the glial markers (such as OLIG2 in female CD1 cells), but also increased them. More interestingly, we observed that effects of ethanol on CD1 strain are associated with a similar pattern of protein expression for OLIG2 and MeCP2. Previous research demonstrated that MeCP2 regulates the expression of both GFAP and OLIG2, but here in our study we observed that MeCP2 alterations are consistent with OLIG2 changes in CD1 strain (273, 274). Our results also provided evidence that sex-specific and strain-specific effects of ethanol are associated with different responses of components of DNA methylation machinery and cell lineage markers during NSC differentiation.

4.3. Chronic ethanol altered the expression of DCC, Na_v 1.3, and Spectrin βIII only in female and not male cells.

In our study, selection of *Dcc*, *Scn3a*, and *Sptbn2* genes as potential biomarkers for PAE was based on our unpublished RNA-seq study, in which we treated unseparated male and female NSC of the CD1 strain with chronic ethanol. Surprisingly, in the current study we found that effects of ethanol on both transcript and protein expression of *Dcc*, *Scn3a*, and *Sptbn2* were observed in the CD1 strain and, namely in the female cells. Indeed, chronic ethanol treatment had no effect on the transcription of these genes (except for *Dcc* in male cells) and protein expression (except for SPECTRIN βIII in female cells) in the BL6 strain. Intriguingly, the effects of ethanol mostly affected female differentiated NSC in CD1 rather than male cells. We observed a consistent trend in the *Dcc* transcription levels in all groups indicating that ethanol

decreases the expression of this gene. However, we found that the same treatment had no effect at the protein expression of DCC, except for female CD1 cells with an increased DCC expression. It is well known that DCC plays a crucial role during fetal development by regulation of neuronal migration. However, as mentioned earlier, we induced gliogenesis in differentiating NSC and few neurons were detected when immunofluorescent labeling was applied to the differentiated NSC. To explain this, evidence indicates that oligodendrocytes, astrocytes, and their progenitors express DCC at their surfaces during development and adulthood in mammals (275-277). In this regard, previous studies revealed that activation of DCC and neogenin (a member of DCC family) induces astrocytic differentiation and migration in developing mouse cortex and glioblastoma cells through activation of Yes-associated protein (YAP) (278, 279). In addition, it has been shown that oligodendrocytes respond to Netrin-1 during different stages of oligodendrocyte development. For example, DCC, as a Netrin-1 receptor, is expressed on oligodendrocyte precursor cells and repels these cells from ventral ventricular zone during their migration (280-282). Also, Netrin-1 promotes the maturation of oligodendrocytes by extending the processes through inhibition of RhoA and activation of fyn by DCC receptor (276, 283). Fyn is a member of the Src-family of kinases (SFK), which along with RhoA (a Rho member of GTPase) contribute to the process branching of oligodendrocytes. Interestingly, RhoA mediates chemo-attractive / repulsive effects of netrin-1 on neurons through a DCC-dependent manner. RhoA also controls the morphological changes in oligodendrocytes in response to DCC activation by netrin-1 (202, 276, 283). Thus, DCC contributes to the differentiation and maturation of glial cells in developing brain. In our study, decrease in the transcript expression of *Dcc* was associated with decreased transcription of *Olig2* in male BL6 and CD1 cells, but not female cells. Also, increased DCC protein expression in female CD1 NSC was associated with

increased OLIG2 expression in these cells suggesting that ethanol exposure affects the differentiation of NSC by altering DCC expression. In the section 1.7, we introduced *Scn3a* gene and $Na_v1.3$ protein and the role of voltage-gated sodium channels during brain development. Evidence indicates that *Scn3a* is not only expressed by neurons, but also in stem cells and oligodendrocyte lineage (284-286). In this regard, a recent research revealed that $Na_v1.3$ is present in differentiating NSC, but disappears at terminal stages of neuronal differentiation (285). It is important to note that $Na_v1.3$ is highly expressed during embryonic and fetal period and partly in postnatal brain (hippocampus and cerebellum), and is not detectable at P30 in rodents (287). It is expected that voltage-gated sodium channels contribute in electrical activities and action potential of neurons, but evidence indicates that these channels (including $Na_v1.3$) are involved in a variety of biological processes in the developing brain such as oligodendrocytes differentiation and synapse formation (288, 289). For instance, oligodendrocyte progenitors express higher transcript of *Scn3a* in comparison with more mature oligodendrocytes during differentiation and play a part in oligodendrocyte maturation (286). Our results showed that chronic ethanol treatment had no effects on the expression of $Na_v1.3$ protein or *Scn3a* transcript in the experimental groups, except for female CD1 NSC. These results suggest that ethanol has the minimum effect on the expression of Nav1.3 in differentiating NSC, and the effect is restricted to female cells. In the case of spectrin βIII, we found that chronic ethanol treatment increased spectrin βIII in female NSC of both CD1 and BL6 indicating the sex-specific effects of ethanol on the expression of this protein. Spectrin βIII was first discovered by Ohara and colleagues in 1998, and is expressed in adult human and rodent brain with highest expression in the cerebellum, olfactory bulb and cortex (220). It is well known that spectrin βIII plays a critical role in synapse formation, dendritic arborization, vesicle transportation, stabilization of

membrane proteins and axonal transport (219, 225, 226, 290). In our study, we observed the expression of protein and transcript of *Sptbn2* in our cells, which most of them expressed glial markers. To explain the expression of spectrin βIII in differentiating NSC, it should be noted that this protein plays a pivotal role in the building of cytoskeleton of majority of cells, namely cytoplasmic cytoskeleton associated with Golgi apparatus and cytoplasmic vesicles (219, 291). It has been reported that spectrin βIII contributes to the stabilization of T-type calcium channels, which are necessary for NSC survival (292, 293). In addition, glial cells were reported to express spectrin βIII in both normal and pathologic conditions (294-296). Evidence indicates that altered expression of spectrin βIII is associated with behavioral and cognitive dysfunction in both human and animals (297-299).

In the current study, we examined the protein and transcript expression of *Dcc*, *Scn3a*, and *Sptbn2* (as potential biomarkers for PAE) in male and female differentiating NSC of CD1 and BL6 strains. We found that these genes and their related proteins did not alter in both male and female cells of all groups suggesting that these candidates are not suitable biomarkers for the diagnosis of PAE. We only observed significant alterations in female cells, namely CD1 NSC. It is interesting to note that *Sptbn2* has been reported as a biomarker for Parkinson's disease and aspirin-intolerant asthma (300, 301). Also, it has been reported as one of the genes that may play a role in the pathophysiology of autistic spectrum disorders (302). However, in our study, spectrin βIII only increased in female cells, thus it may not be a suitable biomarker for male subjects. On the other hand, *Scn3a* may not be considered as a potential candidate for PAE, because we observed no significant alteration of this gene or its protein (Na_v 1.3) in the majority of our experimental groups. Also, it should be noted that a wide range of human disorders are associated with altered expression of *Scn3a*, which makes it a poor candidate as a PAE

biomarker (213, 303, 304). Although we observed down regulation of *Dcc* in most of our experimental groups, but protein levels of DCC were altered only in female CD1 NSC following ethanol exposure. In addition, abnormal DCC expression has been reported in several mental disorders such as depression, drug addiction, and schizophrenia, which makes it a poor candidate as a biomarker for PAE due to the lack of specificity (204, 305, 306).

4.4. Future directions

In the current study, we used a controlled standard *in vitro* model system to investigate the effects of chronic ethanol exposure on differentiating NSC. Although our results led to interesting findings about the role of sex and strain in response to extrinsic modulation of NSC differentiation, these results should be confirmed in both *in vivo* and post-mortem studies. Applying a reasonable and valid animal model of PAE will show us that to what extent sex-differences in DNA methylation machinery proteins may affect behavior, brain development, and function of animals in different stages of lifespan. Also, using an animal model of PAE and applying a reasonable and realistic dose of ethanol, we will be able to see how sex-differences in glial lineage expression may contribute to pathophysiology of PAE-related disorders in adulthood. Using human FASD brain samples, we will be able to confirm the sex-specific impact of PAE on human subjects, and how chronic ethanol exposure during pregnancy may affect the architecture and structure of cortical areas in subjects. Focusing on DCC, $Na_v1.3$, and spectrin βIII, we will be able to explore the role of these proteins in the FASD brain and find new molecular pathways, which may be involved in the pathophysiology of disease.

4.5. Summary

Results of the current study added significant solid evidence to the literature that chronic ethanol exposure during the differentiation of NSC affects their cell fate commitment at least in

part through altered DNA methylation. We found the aforementioned effects to be sex-specific and that the CD1 strain is more susceptible than the BL6 strain to the adverse effects of chronic ethanol exposure. Our results revealed that DNA methylation is not a reliable biomarker for FASD since the effects of ethanol are sex-specific and depend on the studied strain. We also found that our candidate biomarker genes may not be fully applicable for the diagnosis of PAE, but our findings unmasked the involvement of novel genes in the pathophysiology of PAE. Future studies on the role of Spectrin βIII and DCC will shed light on the effects of ethanol on the cell cytoskeleton and cellular migration during development.

CHAPTER FIVE: REFERENCES

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