



Virginia Commonwealth University  
VCU Scholars Compass

---

Theses and Dissertations

Graduate School

---

2020

## The immediate and persistent effects of binge ethanol exposure on myelin protein expression in DBA/2J mice

Aminatta Z. Tejan-Kamara  
*Virginia Commonwealth University*

Follow this and additional works at: <https://scholarscompass.vcu.edu/etd>

---

© The Author

---

Downloaded from  
<https://scholarscompass.vcu.edu/etd/6245>

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact [libcompass@vcu.edu](mailto:libcompass@vcu.edu).

© Aminatta Z. Tejan-Kamara

All Rights Reserved

**The immediate and persistent effects of binge ethanol exposure on myelin  
protein expression in DBA/2J mice**

A thesis submitted in partial fulfillment of the requirements for the degree of  
Master of Science in Pharmacology & Toxicology at Virginia Commonwealth  
University

By

Aminatta Zainab Tejan-Kamara

Bachelor of Science, Mathematics, The College of William and Mary, May 2017

Advisor: Jennifer Wolstenholme, Ph.D.  
Assistant Professor  
Department of Pharmacology and Toxicology

Virginia Commonwealth University  
Richmond, Virginia  
April 2020

## **Acknowledgments**

I would like to acknowledge every member of the Wolstenholme lab for their assistance and constant support throughout this academic year. Thank you all for your continuous encouragement, expertise, and teachings. I appreciated it tremendously. Additionally, I acknowledge the Miles' lab for their scientific insight and guidance. In particular, I would like to thank Dalton Huey for offering immense guidance with my Western Blot experiments and Guy Harris for providing me with numerous helpful techniques that greatly aided me while completing my immunohistochemistry experiments. I acknowledge my committee members Dr. Bigbee and Dr. Sim-Selley for their willingness to assist me in all aspects of this project. I also acknowledge Dr. DeLorenzo and Dr. Akbarali for graciously allowing me to use their laboratory spaces during this academic year. Most importantly, I must acknowledge and sincerely thank Dr. Wolstenholme for constantly reminding me to "claim my space" in science and challenging me to "think bigger," in order to foster greater curiosity in both this project and beyond. I am grateful for the thoughtful instruction, ever-present patience and strong commitment she provided as I endeavored to complete this project. I also acknowledge my family for being my inspiration to pursue higher education, as they reminded me that no task is insurmountable when it is met with consistent diligence and faith. Lastly, but not without significance, I would like to acknowledge and thank my partner Michael for being an unwavering source of emotional support throughout this year.

# Table of Contents

<b>List of Figures</b>	<b>5</b>
<b>List of Abbreviations</b>	<b>6</b>
<b>Abstract</b>	<b>7</b>
<b>Chapter 1: INTRODUCTION</b>	<b>9</b>
Ethanol Mechanism of Action	11
Alcohol and Adolescent Neurodevelopment	12
Persistent Neural Effects of Ethanol	19
Sex differences and Alcohol	20
Myelin related proteins and Alcohol	23
<b>Chapter 2: METHODS</b>	<b>27</b>
Animals	27
Estrus and Testes Assessment	29
Tissue harvest	34
Myelin Basic Protein (MBP) immunostaining	35
Myelin Proteolipid (PLP) immunostaining	37
Image acquisition and quantification	38
Western Blots	39
Statistical Analysis	43
<b>Chapter 3: RESULTS</b>	<b>45</b>
Optimization and specificity of MBP antibody	45
MBP immunoreactivity in adolescent PFC tissue (PND 43)	47
MBP immunoreactivity in adult PFC tissue (PND 66)	51
Fluorescence-based Western blot assays for MBP: PFC tissue (PND 43)	54
Fluorescence-based Western blot assays for MBP: PFC tissue (PND 84)	58
Fluorescence-based Western blot assays for PLP: PFC tissue (PND 43)	62
Fluorescence-based Western blot assays for PLP: PFC tissue (PND 84)	64
Sex Differences Between Treatment Groups	66
<b>Chapter 4: DISCUSSION</b>	<b>74</b>
MBP immunoreactivity in adolescent PFC tissue (PND 43)	74
MBP immunoreactivity in adult PFC tissue (PND 66)	75
Fluorescence-based Western blot assays for MBP: PFC tissue (PND 43)	78
Fluorescence-based Western blot assays for MBP: PFC tissue (PND 84)	78

PLP immunoreactivity in C57BL/6J (untreated tissue)	80
Fluorescence-based Western Blot assays for PLP : PFC tissue (PND 43)	81
Fluorescence-based Western Blot assays for PLP : PFC tissue (PND 84)	82
Sex Differences	84
Study Limitations	88
Future Directions	92
Conclusion	94
<b>Appendix</b>	<b>96</b>
Supplemental Methods	96
Tissue harvest	96
Cryosectioning	96
Myelin Basic Protein (MBP) immunostaining	97
Myelin Proteolipid (PLP) immunostaining	99
PLP antibody with Mouse on Mouse (M.O.M) Immunodetection reagents	100
Image acquisition and quantification for MBP immunoreactivity	104
Western Blots	105
Supplemental Western Blot Data for MBP and PLP protein expression	110
<b>References</b>	<b>119</b>

## List of Figures

Figure	Page
Figure 1: Adolescent binge ethanol dosing paradigm	29
Figure 2: Estrus Chart	31
Figure 3: MBP immunostaining in corpus callosum	46
Figure 4: MBP Immunoreactivity in adolescent PFC tissue (PND 43)	49
Figure 5: Quantification of MBP Immunoreactivity at PND 43	50
Figure 6: MBP Immunoreactivity in adult PFC tissue (PND 66)	52
Figure 7: Quantification of MBP Immunoreactivity at PND 66	53
Figure 8: Western blot analysis of MBP relative abundance- PND 43	57
Figure 9: Western Blot analysis for relative MBP abundance- PND 84	61
Figure 10: Western Blot Analysis of PLP relative abundance-PND 43	63
Figure 11: Western Blot Analysis of PLP relative abundance-PND 84	65
Figure 12: Analysis of MBP and PLP expression in control animals	68
Figure 13: Puberty onset in DBA/2J females	71
Figure 14: Puberty onset in DBA/2J males	72
Figure 15: Mean PND age at Puberty Onset	73
Supplemental Figure 1: Specificity of PLP antibody in C57BL/6J tissue	99
Supplemental Figure 2: PLP Immunoreactivity +/- M.O.M. reagents	103
Supplemental Figure 3: MBP relative abundance (PND 43)	112
Supplemental Figure 4: MBP relative abundance (PND 84)	114
Supplemental Figure 5: PLP relative abundance (PND 43)	116
Supplemental Figure 6: PLP relative abundance (PND 84)	118

## List of Abbreviations

PFC	Prefrontal cortex
mPFC	Medial Prefrontal cortex
BG	Basal ganglia
FA	Fractional Anisotropy
DTI	Diffusion tensor imaging
DA	Dopamine
GABA	Gamma-amino-butyric acid
MBP	Myelin basic protein
PLP	Proteolipid protein
NMDA	N-methyl-D-aspartate
NP	Neuropsychological
PND	Postnatal development
CM	Control Male
CF	Control Female
EM	Ethanol Male
EF	Ethanol Female
C57	C57BL/6J
DBA	DBA/2J
AIE	Adolescent intermittent ethanol exposure
AUD	Alcohol Use Disorder
PAM	Positive allosteric modulator
NAM	Negative allosteric modulator
ACC	Anterior Cingulate Cortex
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline + Triton X 100
TBS	Tris buffered saline
TBS-T	Tris buffered saline + Triton X 100
OCT	Optimal Cutting Temperature
BSA	Bovine Serum Albumin
ROI	Region of Interest
OPC	Oligodendrocyte progenitor cells
fMRI	functional Magnetic Resonance Imaging
COMT	Catechol-O-methyltransferase

## **Abstract**

Binge drinking is the most lethal of drinking patterns, responsible for 77% of all U.S. alcohol misuse costs. Adolescents have increased sensitivity to ethanol's rewarding properties and decreased sensitivity to ethanol's aversive effects likely allowing for their increased binge drinking, as compared to adults. Ethanol consumption during adolescent neurodevelopment can lead to immediate and lasting neurobiological-consequences. We have previously shown that adolescent intermittent binge ethanol exposure decreases myelin-related gene expression in DBA/2J mice. Here, we hypothesize intermittent binge ethanol exposure in adolescent DBA/2J mice will decrease myelin protein expression in the prefrontal cortex (PFC) immediately following binge ethanol and this may persist into adulthood. Adolescent DBA/2J mice (PND 29-42) were intermittently dosed with 4g/kg ethanol or water through oral gavage. Markers of puberty onset and sexual maturation were tracked in males and females to assess if ethanol affects sexual development. To assess immediate and persistent effects of binge ethanol exposure, PFC was harvested in adolescence (PND 43) and adulthood (PND 66), cryosectioned and immunostained for myelin basic protein (MBP). Western blotting was conducted to provide a complementary semi-quantitative technique to further assess the effects of binge ethanol exposure on MBP and PLP protein expression. We found that binge ethanol did not significantly alter myelin protein expression, specifically for MBP and PLP. However, we noted that ethanol males displayed a slight trend towards decreased protein expression in adolescence. In addition, we found that adult control females had significantly more PLP protein expression as compared to control males in adulthood (PND 84). Thus, we added to our previous mRNA findings with a potential trend towards decreased MBP and PLP expression in ethanol males at the protein level and found sex differences. Further work to verify these trends is needed. Together, these findings help elucidate the potential effects of ethanol on white matter integrity in the PFC and bolster our earlier mRNA findings at the protein level, which may provide the

framework for future studies to uncover a mechanism by which ethanol acts to disrupt white matter.

## **Chapter 1: INTRODUCTION**

Throughout history, alcohol has served as the multifaceted drug of choice for numerous Americans (Olson and Gerstein 1985). The consumption of alcohol leads to general central nervous system (CNS) depression, in part, because of increased levels of the major inhibitory neurotransmitter GABA and decreased levels of the major excitatory neurotransmitter glutamate (Costardi et al 2015). This altered neurochemistry may lead to decreased social inhibition and reduced anxiety (Banerjee 2014; Valdez et al 2002). These social incentives coupled with alcohol's relative availability, increase its abuse potential (Simons et al 2009). The excessive use of alcohol leads to the development of physiological dependence on the drug, making treatment and long-term abstinence difficult for affected individuals (Kendall et al 2011). Additionally, the psychosocial ramifications that occur with the progression of the disease compound the effects of this disorder; there are concerns for maintenance of relationships, job status and overall mental health when alcoholism is unmanaged (Kendall et al 2011). Thus, alcohol abuse is complex and multifaceted because of the interplay of the biological, social and psychological consequences.

In 2013, the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) combined alcohol abuse and alcohol dependence into a single diagnosis of Alcohol Use Disorder or AUD with eleven specified symptoms (Mewton et al 2010). From the National Institute on Alcohol Abuse and Alcoholism (NIAAA), a 2018 National Survey on Drug Use and Health (NSDUH) revealed approximately 5.8% of adults (ages eighteen years or older) had AUD with 7.6% of this group being male and 5.3% female (NIAAA, 2018). For the adolescent age group (ages 12-17), NSDUH revealed 1.6 % of adolescents had an AUD with 1.4% and 1.9% being males and females respectively (NIAAA 2018). The public health concerns related to alcohol misuse are vast. The NIAAA noted that alcohol was the third leading cause of preventable death in America with approximately 88,000 alcohol-related deaths occurring annually (NIAAA 2018).

Regarding the financial strains of alcohol misuse, the United States spent 249 billion dollars in 2010 alone; approximately three-quarters of this total cost was related to binge drinking (NIAAA 2018). Binge drinking is a drinking pattern that raises an individual's blood alcohol concentration (BAC) to a level greater than or equal to 0.08 grams percent; this BAC level is typically achieved after five drinks in males and four drinks in females within a two hour timeframe (CDC

2019). The CDC states that 90% of American individuals consume alcohol in the form of binge drinking. This statistic is of significance because binge drinking is the most lethal drinking pattern (CDC, 2019). Binge drinking is most common in younger individuals including the ages 18-34 years old (majority of adolescents), with this pattern of drinking being two times more likely in men than women (CDC, 2019).

### **Ethanol Mechanism of Action**

Ethanol is a psychotropic depressant that can result in major neurobiological alterations when consumed in excess (Costardi et al 2015). These molecular consequences stem from ethanol's structure and subsequent mechanism of action. Ethanol is a small polar compound with structural features that confers amphiphilicity. This dual hydrophobic and hydrophilic quality allows ethanol to enter the hydrophobic blood-brain-barrier where it precipitates a generally depressive effect on the CNS. Ethanol's amphiphilicity makes for a promiscuous drug of abuse. Ethanol binds to CNS receptor systems including but not limited to gamma-aminobutyric acid (GABA) receptors, NMDA receptors, acetylcholine receptors, and serotonin receptors and alters these neuronal systems as a result (Costardi et al 2015). Ethanol functions, in part, by enhancing the actions of the

major CNS inhibitory neurotransmitter GABA by acting as a positive allosteric modulator (PAM) and allowing the endogenous chemical to exert a greater inhibitory or depressive effect on the CNS (Olsen and Liang 2017). Ethanol also inhibits the major CNS excitatory neurotransmitter, glutamate, by functioning as a negative allosteric modulator (NAM) and suppressing glutamatergic activity (Mihov 2016). Thus, this coupled action leads to an overall depressed CNS susceptible to neurobiological harm if chronic alcohol abuse continues.

### **Alcohol and Adolescent Neurodevelopment**

Adolescence is defined as a transitional period with severe social pressures and notable neurodevelopmental changes (Spear 2013). Studies have revealed both regressive and progressive development occurring within the adolescent brain through extensive synaptic pruning and increased myelination (Giedd et al, 1996; Sowell et al 1999; Spear 2013). Myelin is the lipid rich substance that insulates the axonal processes of the neurons thereby augmenting the conduction of electrical transmission within the nerve cell during the process of saltatory conduction; the bundles of myelinated axons constitute the white matter in the CNS (Filley 2005; Morell and Quarles 1999). Thus, the presence of myelin leads to increased conduction velocity that enhances communication and processing within the CNS.

These ongoing developmental changes in the adolescent brain may affect the behavior of this demographic because of the neural alterations taking place (Spear 2000).

In particular, the prefrontal cortex (PFC) is one of the last regions to develop due to the caudal to rostral growth pattern of the human brain (Arain et al 2013). This delayed maturation of PFC accounts for the increased risk taking seen in adolescents; the PFC is an associative region implicated in impulse control and higher order executive functioning (Wilson et al 2010). Thus, adolescents are more likely to engage in risk taking behavior (Arain et al 2013). Adolescents' excessive consumption of alcohol demonstrates their proclivity for risky behavior (Spear 2000). In addition, adolescents' propensity to consume alcohol in excess is likely attributed to their increased sensitivity to the rewarding aspects of alcohol and decreased sensitivity to the aversive properties (Spear 2005).

Regarding adolescent binge drinking, loosely defining this age range from 12 years old to approximately 25 years old, there was an estimated 4.3 million people within this group that engaged in binge drinking (NIAAA, 2018). The concern for this illegal underage alcohol use not only highlights the need for more

stringent underage drinking laws, but may lead to potential neurobiological consequences.

These neurobiological consequences may lead to lasting, deleterious effects including possible memory deficits, emotional abnormalities due to alterations in the amygdala, attention impairments, decreased hypothalamic-pituitary axis activity and decreased white matter in the frontal cortex (Oscar-Berman and Marinković 2007; Spear 2014). The decrease in myelination will yield downstream effects on brain maturation (Spear and Swartzwelder 2014). Numerous human and rodent studies have sought to further elucidate the mechanisms by which ethanol affects adolescent neurodevelopment.

Epidemiological findings revealed earlier onset of alcohol consumption is a strong predictor of a later AUD diagnosis (Grant et al 1998; Spear 2000). This finding gives credence to the proposed theory that adolescence is a critical period (Spear 2000). The presumed lack of temperance among the adolescent population coupled with the significant neural alterations occurring during this time warrant the need for further investigation in adolescent neuropathology. However, seminal studies assessing the neurobiological consequences of ethanol mainly incorporated adults in the experimental models (Charness, 1993; Pfefferbaum et al., 1997).

Adolescent studies revealed the potential consequences of early onset drinking patterns. For example, an adolescent MRI human study revealed that subjects with adolescent onset AUD had smaller PFC regions and decreased white matter volume in this brain region, compared to controls (De Bellis et al 2005). The investigators also found thalamus, brainstem, cerebellar volumes did not differ between control subjects and those with AUD, suggesting that the PFC region may be particularly vulnerable following ethanol exposure in adolescence (De Bellis et al 2005). Additional studies have determined other notable findings with respect to adolescent neurobiology and alcohol.

McQueeny et al found that adolescent binge drinkers (with no prior AUD diagnosis) displayed a comparatively lower fractional anisotropy (FA) ratio in the numerous white matter areas including the corpus callosum, limbic, brainstem and cortical projection fibers compared to controls. FA is defined as a scalar value that ranges between zero and one, to describe the degree of anisotropy in a given diffusion process, thus it serves as a robust metric for quantifiably assessing the integrity of white matter (McQueeny et al 2009). This finding provided preliminary evidence that because the adolescent brain is not fully formed, drugs of abuse such as alcohol may result in undesirable consequences, including but not limited to

decreased white matter in various brain regions. Indeed, the introduction of alcohol into the adolescent CNS is associated with deleterious effects that may culminate into potential neuropsychological (NP) impairments. Tapert et al investigated twenty-two NP variables through interviews and two hour NP battery of tests. They determined five factors including attention, memory, visuospatial, language, and intrusion resistance accounted for a majority of the variance in NP scores between groups. In particular, attention performance was significantly related to substance use patterns throughout the 4 year period, with recent adolescent abusers having the worst performance in the attention NP test and non-abusers having the best performance. This NP impairment suggests that ethanol may lead to certain cognitive deficits.

Adolescent drinking studies in learning and memory have found evidence of differential effects between binge drinking and control groups (Schweinsburg et al 2010; Sneider et al 2012). In a human study employing a virtual human analogue of the Morris Water Maze task, binge drinking and controls (light drinking) “emerging adults” (ages ranging between 21-23) demonstrated a poorer performance on verbal memory tasks compared to controls with no notable differences in spatial memory between the two groups (Sneider et al 2012). Binge

drinkers also displayed worse learning as compared to the controls. A functional magnetic resonance imaging (fMRI) study assessing learning and memory through verbal encoding tasks, found that control subjects (nondrinkers) showed significant hippocampal activation during novel encoding, while binge drinkers showed more responses in the frontal region during verbal encoding, suggesting use of working memory but not long-term memory (Schweinsburg et al 2010). Thus, these findings support the associated potential learning and memory impairments involved with binge drinking. Indeed, the neurobiological compromise of the white matter manifests into altered cognitive functioning for the persons that use this drug of abuse.

However, the adolescent human model is not without ethical restriction and complex variability investigating the mechanisms of unclear neurocognitive sequelae (Kottekod et al 1988; Mandal et al 2011; Tsay 2015). Rodent model organisms, while with their own ethical guidelines (Frussa-Filho 2015; Meerburg et al 2008; Nattrass et al 2018; Nuffield Council on Bioethics 2005), allow for controlled experimental study of the various consequences of alcohol (Coleman et al 2011; Richardson et al 2014; Wolstenholme et al 2017). Preclinical rodent studies have improved the understanding of the underlying molecular mechanisms

that are causing the neurocognitive deficits. For example, adolescent binge ethanol treatment in C57BL/6 (C57) mice (5g/kg/day i.g., postnatal development (PND) days 28-37) altered gene expression of neurotransmitters in the adult (PND 60-88) brain (Coleman et al 2011). In particular, mRNA gene expression of cholinergic neurotransmitters were reduced after ethanol; the cholinergic neurotransmitters are implicated in cognitive processing in the forebrain. The loss of cholinergic neurotransmitters and forebrain structures after ethanol treatment could uncover the molecular mechanism of adult reversal learning deficits after adolescent alcohol exposure.

Additionally, binge-like ethanol administration in adolescent rats increases neuroimmune activation in the PFC that precipitates neuroinflammation and demyelination processes in the PFC that will have long-term consequences on adult cognitive processing (Pascual et al 2014). These experimental findings support the prevailing theory that the adolescent brain is particularly sensitive to the neurotoxic effects of ethanol. Crews et al provided seminal findings that further support the vulnerability of the adolescent brain and its susceptibility to ethanol-induced neurotoxicity (Crews et al 2000). Binge ethanol exposure in both adolescent and adult rats led to damage in the olfactory bulb mainly in the

glomeruli, however there is differential sensitivity in ages; with juvenile rats exhibiting increased damage to the frontal cortical region, and piriform cortices as compared to adult rats. These brain regions are implicated in memory and integrated cognitive processes, thus their damage in adolescence could result in long-term consequences in adulthood (Crews et al 2019).

### **Persistent Neural Effects of Ethanol**

The underlying mechanisms of persistent neurobiological alterations in the presence of ethanol are still under investigation (Crews et al 2019; Ehlers et al 2010). However, the potential for white matter recovery following alcohol abstinence further complicates the role alcohol plays in neurotoxicity and demyelination (Miller and Fyffe-Maricich et al 2010; Pfefferbaum et al 2014; Zahr et al 2015). There is a growing preponderance of evidence that alcohol persistently impacts the CNS. Ethanol causes the release of dopamine through the mesocorticolimbic reward pathway (Brodie 1999). Dopamine (DA) is a neurochemical that plays a significant role in the PFC, enabling higher order cognitive functioning, including but not limited to attention and behavioral flexibility (Puig 2014). Repeated binge ethanol exposure during adolescence negatively alters dopaminergic neurotransmitters in the medial PFC (mPFC) by

hyper-methylation of the promoter region of Catechol-O-methyltransferase (COMT), a DA degradative enzyme (Trantham-Davidson et al 2017). This hypermethylation of COMT leads to decreased regulation of DA that may disrupt PFC development and cognitive control. These negative alterations are possible contributors to the limited behavior control and cognitive deficits observed in adults that consumed alcohol during adolescence. The N-methyl-D-aspartate (NMDA) receptor system implicated in synaptic plasticity and dendritic morphology contains the GluN2B subunit, which is an ethanol sensitive component. In a preclinical rodent study, adolescent intermittent ethanol (AIE) exposure (PND 30-45) altered GluN2B associated proteins in the hippocampus of adult (PND 70) Sprague-Dawley rats (Swartzwelder et al 2016). Therefore, ethanol-induced changes to these proteins are possible molecular contributors to the persistent changes observed in adult hippocampus following AIE.

### **Sex differences and Alcohol**

While the prevalence of alcohol use has been consistently observed to be greater in men (Grant et al 1997; Warner et al 1995; Wilsnack et al 2009) the gender gap is closing (Keyes 2007). In general, females were more likely to engage in heavy drinking activity and under report this behavior (Keyes 2007;

Wagoner et al 2013). These differences in alcohol use may be attributed to the morphological differences in the brains of males and females.

Sexual dimorphism of white matter is still an area requiring further investigation, with the degree of influence from sexual hormones as compared to sex chromosomes on differential white matter microstructure remaining somewhat unclear. A diffusion tensor imaging (DTI) study with men, women, and women with genetic complete androgen insensitivity syndrome (CAIS) found that men displayed higher FA ratio in thalamus, basal ganglia and brainstem regions compared to both women groups while women and women with CAIS had relatively similar FA ratios in the aforementioned regions; genetic CAIS women (46,XY karyotype) appear phenotypically female and typically possess elevated luteinizing hormone (LH), estradiol and testosterone levels with a mutant nonfunctional androgen receptor (van Hemmen et al 2017). The CAIS women and 46, XX karyotype women possessed no significant differences in estradiol levels, while both groups of women had higher estradiol levels than the men. These findings suggest that the differences in white matter microstructures between sexes are possibly attributed to sexual hormones more so than genetics.

The differences in white matter microstructure are further illustrated with the introduction of ethanol. In a human magnetic resonance spectroscopy study, brain metabolism in both control males and females along with alcoholic males and females was assessed with the N-acetylaspartate, a marker for neuronal activity (Schweinsburg et al 2003). Alcoholic males and females displayed decreased N-acetylaspartate in the frontal lobe, but female subjects (alcoholic and control) had significantly lower concentrations of N-acetylaspartate, suggesting that the differences in white matter may affect brain metabolism. Preclinical animal studies have shown that males have greater density of myelin compared to females (Cerghet et al 2009; Roughton et al 2013; Yang et al 2008). Thus, it is logical that females have been observed to be more vulnerable to the negative effects of alcohol use (Johnston et al 2009; Schulte et al 2009).

However, there is limited evidence of ethanol's effects in both males and females' white matter integrity. Preclinical studies have sought to further elucidate the role of sex as a biological variable. Richardson et al have shown that self-administration of alcohol leads to decreased myelin density in the mPFC in male rats (Richardson et al 2014). In a follow up study including male and female rats, ethanol-drinking patterns were similar between the sexes. However, ethanol

self-administration did not significantly change myelin density in the anterior cingulate division of mPFC in females but this effect was seen in males (Richardson et al 2019). These sex differences in myelin alteration may be correlated with the sex differences in pubertal onset and sexual maturation. Adolescence encompasses the period of sexual maturation i.e. puberty. Females generally mature faster than males and there are noted sex differences because of the hormonal increases during puberty (Spear 2000). Therefore, it is possible that sexual maturation and puberty onset may serve as a marker when assessing effects of ethanol treatment (Dees and Skelley 1990; Dees et al 2000).

Our laboratory has shown that adolescent binge ethanol exposure decreases myelin related gene expression in the adolescent PFC of DBA/2J (DBA) mice with males having a more robust decrease than females in four myelin-related genes including myelin basic protein (*Mbp*) and proteolipid (*Plp*) (Wolstenholme et al 2017). These findings prompt the question of the existence of alterations at the protein level between males and females that may lead to these differential effects in myelin density.

### **Myelin related proteins and Alcohol**

The myelin sheath found in the CNS that surrounds the axonal processes of neurons is a product of the glial cells, namely, oligodendrocytes (del-Rio Hortega 1921). Glial cells are structural support cells that greatly enhance the neurotransmission within the CNS. The unique myelin-related proteins in the CNS have been well characterized (Davison 1989). In particular, MBP and PLP are two major structural myelin related proteins present in mature oligodendrocytes (Boggs 2006; Nadon et al 1998).

These myelin-related proteins have been shown to decrease in the frontal cortex and hippocampus, after continued drug abuse (Lee et al 2010; Smith et al 2014). MBP is highly abundant in the CNS myelin and belongs to a conformationally adaptable protein family with 4 classic isoforms (Smith 2012). The exact functions of the MBP isoforms have not been fully determined. However, studies reveal that two of the isoforms (18.5 kDa and 14.0 kDa) predominate in compact myelin (Akiyama et al 2002; Boggs and Harauz 2013; Devine-Beach et al 1989) suggesting that these isoforms possess a structural role along with PLP to maintain the myelin in the CNS, while the other isoforms (21.5 kDa and 17.0 kDa) are re-expressed in remyelination lesions in multiple sclerosis

models (Akiyama et al 2002; Capello et al 1997) suggesting a possible role in remyelination.

PLP is ubiquitous in the CNS. It plays a significant role in the maturation of oligodendrocytes and acts to form and maintain the lipid-rich, multilamellar structure of myelin (Nadon et al 1998; Yamaguchi et al 1996). Mutations in the *Mbp* and *Plp* genes that encode these myelin related proteins are associated with demyelination (Duncan and Radcliff 2016; Yamaguchi et al 1996). However, more studies are needed to assess alterations of myelin at the protein level. The abundance of these two major structural proteins and their ability to cause potential myelin alterations in the CNS make them appropriate targets for experimental study.

We have previously determined that binge ethanol exposure decreases myelin related gene expression in DBA/2J (DBA) mice (Wolstenholme et al 2017). Additionally, we conducted a smaller scale pilot study assessing the protein expression of MBP and PLP. PLP showed a significant decrease and MBP showed a downward trend (Wolstenholme and Rahmipour; unpublished work ca 2015). Thus, the prior studies provided the framework for the proposed project to assess

myelin related alterations in the PFC through the assessment of MBP and PLP protein expression.

We hypothesize that adolescent binge ethanol exposure will decrease MBP and PLP protein expression in the PFC of adolescent DBA mice. In aim one, we assessed the immediate effects of binge ethanol exposure on adolescent DBA mice (PND 43) through immunohistochemistry and Western Blots. We hypothesized ethanol treated animals would show decreased myelin-related protein expression. In aim two, we assessed the persistent effects of adolescent binge ethanol exposure on adult DBA mice after three weeks of abstinence. We hypothesized that alterations in myelin-related protein expression would persist into adulthood with ethanol treated animals showing greater decreases in myelin-related protein expression. In aim three, we specifically analyzed sex differences in both adolescents and adults following binge ethanol exposure, and we tracked pubertal onset/pubertal progression in order to assess if ethanol affects sexual maturation. We hypothesized that ethanol treated males would display a decrease in myelin-related proteins compared to their female counterparts. Together, the goal of these studies will be to further elucidate the structural effects of binge ethanol

exposure in adolescents, and in doing so, provide further scientific evidence of the immediate and lasting effects of ethanol consumption.

## **Chapter 2: METHODS**

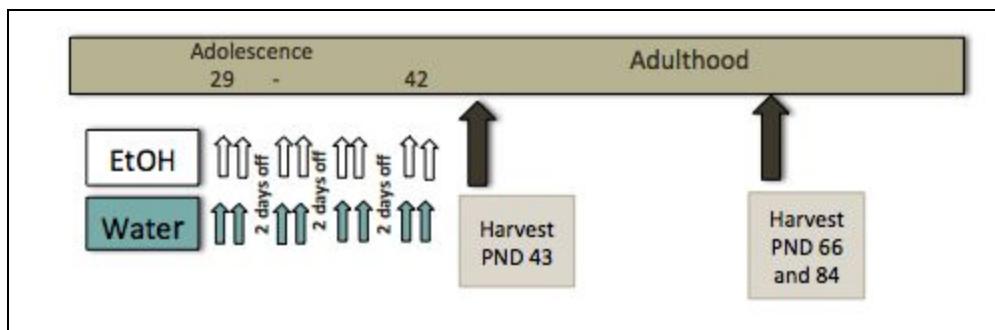
### **Animals**

Two cohorts of male and female DBA/2J mice were purchased from Jackson Laboratories East (Bar Harbor, ME) and housed in the animal vivarium on the eighth floor of VCU Kontos Medical Sciences Building. The mice arrived at 20-21 days postnatal development (PND 20-21) and acclimated to the vivarium for one week. The animals were kept on a 12 hour-12 hour light-dark cycle with food and water available ad libitum throughout the course of the experiment.

The experimenter administered 0.2 milliliters of a 0.1% saccharin solution (0.045 grams saccharin, 45 mL sterile filtered tap water) to each mouse from PND 26/27-28 to habituate the mice to the oral gavage procedure. The mice were then divided into ethanol treatment and control groups ( $n = 7-8$  per group, 3-5 mice per cage) and counterbalanced by body weight.

From PND 29-42, mice followed an intermittent dosing schedule (**Figure 1**) with ethanol treated animals receiving a 4g/kg dose of a 25% w/v ethanol solution in sterile filtered tap water and vehicle animals receiving sterile filtered tap water for two consecutive days followed by two days of abstinence (dosing days included 29-30, 33-34, 37-38, 41-42) (Wolstenholme et al, 2017). Ethanol solutions were

remade for each two-day dosing period. For each dosing day, animals were weighed, re-tail marked and administered the required volume of either ethanol or water; body weights were recorded daily on dosing days throughout the experiment. All conducted experiments were in accordance with the Institutional Animal Care and Use Committee of Virginia Commonwealth University.



**Figure 1.** Adolescent binge ethanol dosing paradigm.  
DBA/2J mice intermittently dosed from PND 29-42,  
specifically mice dosed for two consecutive days,  
followed by two days of abstinence during the early to  
mid adolescent period. The adolescent harvest time  
point is postnatal day 43, while the adult time points  
are postnatal days 66 and 84.

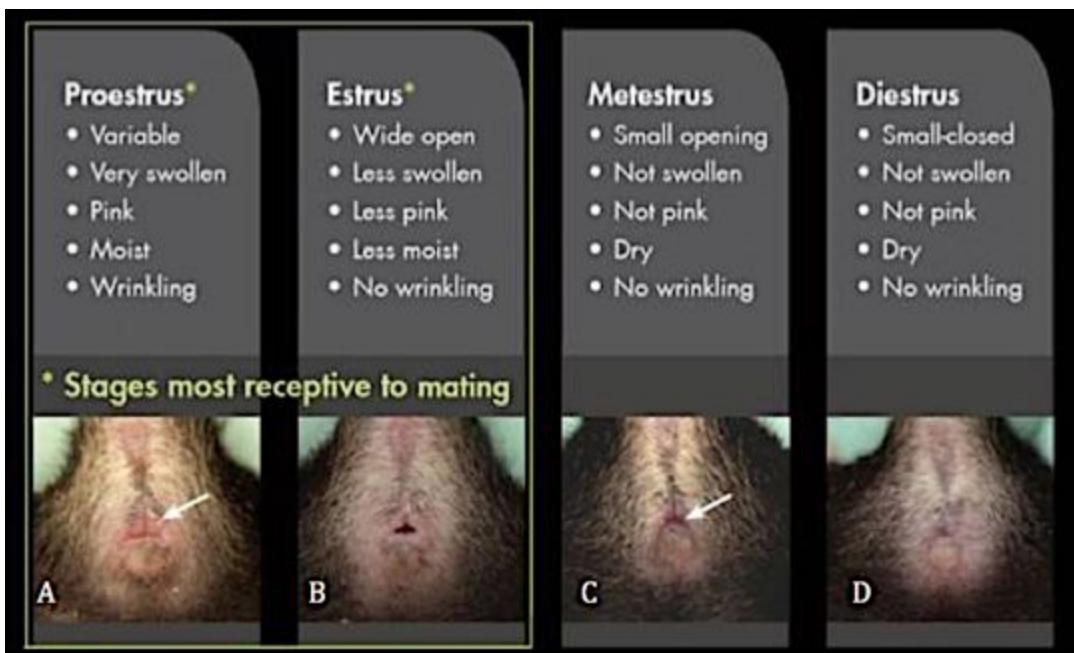
### **Estrus and Testes Assessment**

For the second cohort of animals (25 females and 24 males) puberty onset and progressive sexual maturation were tracked throughout the second adolescent binge ethanol study into adulthood (PND 50/51). These assessments were

completed to assess if ethanol affects sexual development, to assess the association between sexual maturation and myelin-related protein expression, and to assess potential sex differences in PFC development. Human studies have found conflicting evidence regarding the introduction of ethanol and sexual maturation (Damgaard et al, 2007; Kjersgaard et al 2018). Similarly, rodent studies have not come to a consensus regarding ethanol consumption and sexual maturation (Dare et al 2002; Priddy et al 2017).

However, the fact remains that adolescence encompasses a definitive period of sexual maturation i.e. puberty (Spear 2000). Thus, markers for onset of the estrus cycle in females and testes descent in males were used in order to further analyze sex differences following binge ethanol exposure.

Females were placed on the top of a metal cage and allowed a brief period of habituation. The experimenter then held the animal with her non-dominant hand, such that the hind paws were at the edge of the cage and the tail was gently pulled up orthogonal to the experimenter's non-dominant hand (Ekambaram et al 2017). The animal's vaginal area was assessed for pinkness, swelling and presence of a vaginal opening (VO). The Jackson Laboratory estrus cycle chart (**Figure 2**) was used as a reference to assist with visual assessment (Byers et al 2012; Jax.org).



**Figure 2.** This graphic depicts a portion of the Estrus chart available from Jackson Laboratory based on the Byers et al 2012 manuscript; the strain represented in the graphic is a C57BL/6J mouse.

**A. Proestrus B. Estrus C. Metestrus D. Diestrus**

The source of lighting during estrus assessment remained constant and the experimenter ensured that the lighting permitted precise observations. We opted to use visual estrus assessment over vaginal cytology because the procedure is less stressful (Champlin et al 1973) and our laboratory frequently conducts behavioral assays to assess anxiety-like phenotypes (e.g. anxiety-like behavior in the light-dark box, Wolstenholme, et al 2017). Thus, we did not seek to further stress the mice with the vaginal lavage procedure involved with vaginal cytology.

Various experimenters in the field have espoused visual estrus cycle staging as a gentler and reliable form of assessment (Byers et al 2012; Champlin et al 1973). DBA/2J mice have been reported as a more difficult strain for estrus staging (Champlin et al 1973) however, the main assessment involved evaluating the animals for the VO event which was relatively clear across all animals (Byers et al 2012; Ekambaram et al 2017). Throughout the collection of estrus data, a confidence assessment was taken that described the experimenters' overall confidence in staging the animals. The confidence level ranged from "low," "medium," to "high." The experimenters' confidence increased with subsequent assessments.

Male mice were habituated to the metal cage in the same fashion as females. The experimenter then lifted the animal's tail and gently pressed just above the hind paw to check for the presence of testicular swelling. Males were then raised to the eye level of the experimenter and peripheral assessment of testicular "drop" was completed. Finally, the male mouse was checked for the presence of a darkened area surrounding the genitalia.

Similarly to the estrus assessments, a confidence assessment was taken to describe the experimenters' confidence level when tracking testes descent. The scoring rubric included a numeric score of 3 which corresponds to a "Yes" indicating full testes descent i.e. bilateral testicular swelling, a medium to large testicular drop from side view, and a medium to large darkened area around the genital area. The animal received a numeric score of 2 corresponding to "yes part/maybe" indicating the animal was in partial testes descent with bilateral testicular swelling, a small to medium testicular drop from side view and a medium sized darkened area surrounding the genitalia. A numeric score of 1 represents "no" indicating the animal had no distinguishable testicular swelling, no testicular drop from side view and a small sized darkened area surrounding the external genitalia. All animals were handled with care throughout these assessments.

## **Tissue harvest**

In experiment 1, for immunostaining, PFC was harvested either twenty-four hours following the last ethanol binge dose (PND 43, immediate time point) or three weeks following the last ethanol binge dose (PND 66, persistent time point). Animals were organized to ensure that ethanol treated animals and control animals were counterbalanced during the terminal transcardial perfusions. Animals were sedated with isoflurane and transcardially perfused with 4% paraformaldehyde (40 g paraformaldehyde, 9 g NaCl, 800 mL MilliQ water, pH 7.4) for ten minutes; the flow rate of the perfusion pump was 0.827 ml/min.

Following the perfusion, animals were decapitated, the skull was removed and the brain was extracted. Brain tissue was then placed in 40 mL vials of paraformaldehyde stored at 4 °C for a twenty-four hour post fixation period. Tissue was then transferred into 40 mL of 30% sucrose cryoprotectant solution (780 g sucrose; 0.01 M phosphate buffered saline (PBS) solution, for detailed recipe for PBS solution see Appendix) for forty-eight hours to ensure the integrity of the morphology remained intact (i.e. prevent ice crystals from forming inside the brain tissue and disrupting the cellular components of the brain tissue) (Hossain and Osuamkpe 2007). Tissue was placed in isopentane (beaker of isopentane was

placed in a bucket of dry ice) and frozen. Frozen tissue was stored at -80 °C until cryosectioning (see Appendix for detailed cryosectioning methods).

In experiment 2, for Western blotting, mice were sacrificed through terminal cervical dislocation and a rapid decapitation procedure. Brain tissue was harvested at postnatal development days 43 and 84. Brains were placed into a chilled beaker of 0.9% saline solution (9 g NaCl, 700 mL deionized water, 300 mL MilliQ water) for thirty seconds. Brains were then oriented with the ventral side facing up, and placed on a chilled dissecting block. The olfactory bulb was removed, then a wedge-shaped cut was made; this wedge-shaped tissue dissection included the medial prefrontal cortex (mPFC, 1.40 mm distance from bregma to 0.50 mm), anterior cingulate cortex (ACC), sensory cortex, and motor cortex. Tissue dissections were stored at -20°C until tissue homogenization.

### **Myelin Basic Protein (MBP) immunostaining**

64 animals were selected from both the immediate harvest time point (PND 43) and the persistent harvest time point (PND 66, n= 8 per sex/treatment, 4 groups per time point). A series of 5-8 25 um coronal sections (with region of interest (ROI) prefrontal-cortex present) per animal were placed into a 12-well plate

(Corning, Corning, NY, catalogue # 3513) with three 12-well plates used for both the persistent and immediate time points, 6 plates total).

Sections were washed in 0.01 M PBS twice, for five minutes each wash. Sections were then blocked in PBS solution with 0.5% Triton (PBS-T) (5% normal goat serum, Jackson ImmunoResearch, West Grove, PA, catalogue # 005-000-121; 0.5% Triton X 100; 0.01 M PBS solution) for thirty minutes at room temperature with gentle agitation. Sections were incubated in MBP anti-rat primary antibody solution with 400 ul per well (1:1000, Abcam, Cambridge, UK, catalogue # 7349; 3.2 mL PBS-T; 3% normal goat serum) overnight at 4 °C with gentle agitation. Sections were subsequently washed three times in PBS for ten minutes each with gentle agitation. Sections were then incubated in the Invitrogen Alexa Fluor 594 goat anti-rat secondary antibody solution (1:1000, Thermo Fisher, Waltham, MA, catalogue # A11007; 0.5% Triton X 100; 3.2 mL 0.01 M PBS) for two hours with gentle agitation and protected from light. Sections were then washed in PBS three times for ten minutes each wash and stored in PBS at 4 °C until mounting. Each animals' sections were placed in a separate indent free container containing PBS and transferred to a Fisherbrand Superfrost microscope slide (Fisher Scientific, Waltham, MA, catalogue # 12-55-0-143).

Sections were organized into columns with the most rostral section first and the most caudal section last. Excess PBS was pipetted from the microscope slide and slides were left to dry for a short period. Vectashield Vibrance Antifade mounting medium with DAPI counterstain for CNS nuclei (VECTOR Laboratories, Burlingame, CA, catalogue # H-1800) was used to mount sections further, with 50 ul added to each slide. Slides were coverslipped with Corning Cover Glass rectangle coverslips (Corning, Corning, NY, catalogue # 2980-245) and sealed with clear nail polish. Sections for one ethanol male animal were destroyed during the tissue mounting portion of the experiment, leading to an N = 63 for the immunohistochemistry study. See Appendix for detailed methods on MBP immunostaining.

### **Myelin Proteolipid (PLP) immunostaining**

The initial intent of this project was to assess immediate and persistent effects of binge ethanol exposure on myelin-related proteins MBP and PLP protein expression in the PFC (PLP anti-mouse, Millipore, Burlington, MA, catalogue # MAB388). Due to the fact that using an anti-mouse antibody on mouse brain tissue could lead to non-specific binding to the endogenous proteins expressed in the murine brain section (see Appendix for PLP immunostaining on C57BL/6J

untreated tissue), we used the Basic Mouse-on-Mouse (M.O.M) immunodetection kit (VECTOR Laboratories, Burlingame, CA, catalogue # BMK-2202) to block the Invitrogen Alexa Fluor 594 goat anti-mouse secondary antibody (Thermo Fisher, Waltham, MA, catalogue # A32742) from detecting the endogenous immunoglobulins through a Avidin-Biotin Complex (ABC) system (Xing et al 2019). We conducted a -/+ PLP MOM kit experiment with negative controls and varying concentrations (PLP 1:250, PLP 1:500). See Appendix for detailed methods for -/+ PLP MOM kit experiment and images.

### **Image acquisition and quantification**

Images were acquired using the Olympus DP74 microscope (Olympus Life Science, Tokyo, Japan) with cellSense Standard software (Olympus Life Science, Tokyo, Japan). Each section had a representative image taken of both hemispheres in the Cy3 light at the 10X objective (scale bar was present on each 10X image). Images for quantification were taken at the 20X objective with a constant exposure of 24.9 milliseconds and the Super Fluorescence (SFL) setting for all images. Sections ranged from 1.40 mm distance from bregma to 0.50 mm from bregma; no section imaged at the 20X objective included the corpus callosum (see Appendix

for detailed supplemental methods on image acquisition and quantification including image exclusion criteria). Following the quantification, three mice from the persistent time point including one control male and two ethanol males were removed because of excess damage to the region of interest leading to N = 60 for the immunohistochemistry study.

### **Western Blots**

Fifty-three dissected PFC tissue samples (control and ethanol treated animals, immediate and persistent time points, n= 6-8 per group, 4 groups per time point) were sonicated with a chilled volume of 150 ul RIPA buffer (2.5 mL of 1M Tris, pH 8.0; 1.5 mL of 5M NaCl; 0.5 mL of Triton x 100; 0.5 mL of 0.5M EDTA; 0.25 g sodium deoxycholate, 50 mL MilliQ water) with HALT protease inhibitor (1:100 concentration in RIPA buffer, Thermo Fisher, Waltham, MA, catalogue # 78440) in order to lyse the cells and extract protein from the samples (Peach et al 2015). Sample lysates were stored at -20 °C until used in order to prevent premature denaturation of protein samples. The BCA protein assay was performed using Pierce BCA Assay kit (Thermo Fisher, Waltham, Massachusetts, catalogue #

23227; see Appendix for more detailed BCA assay methods) in order to calculate protein concentration (Walker 1994).

After sample preparation, the samples along with the Chameleon™ Kit Prestained ladder/molecular weight marker to estimate the unknown protein quantities (LICOR, Lincoln, NE, catalogue # P/N: 928-90000) were separated through sodium-dodecyl sulfate polyacrylamide gel electrophoresis with NuPage 4-12% Bis-Tris Protein gels (Thermo Fisher, Waltham, MA, catalogue # NP0329BOX) in 0.01 M MES SDS running buffer at 150 V for approximately 45 minutes (see Appendix for detailed methods on gel electrophoresis and sample preparation) using the Thermo Fisher mini gel tank apparatus and Bio-Rad PowerPac Basic power supply (Bio-Rad Laboratories, Hercules, CA, catalogue # 1645050).

PVDF membranes were soaked in 100% methanol for 5 minutes, followed by four washes in MilliQ water. Membranes were then soaked in transfer buffer (50 mL of NuPage transfer buffer, Thermo Fisher, Waltham, MA, catalogue # NP00061; 200 mL of 100% methanol, Sigma-Aldrich, St. Louis, MO, catalogue # 322415; 749 mL of MilliQ water; 1 mL of antioxidant, Thermo Fisher, Waltham, MA, catalogue # NP0005). Western blot transfer was performed with the Bio-Rad

PowerPac supply set to 27 V for a one hour transfer period at 4 °C. Following transfer, membranes were air-dried for at least one hour and rehydrated in 100% methanol for one minute. Membranes were then washed in MilliQ-water between three and four times followed by a two minute wash in 0.01 M tris-buffered saline (TBS) wash (see Appendix for detailed TBS recipe). Membranes were subsequently blocked with Intercept TBS Blocking buffer (LICOR, Lincoln, NE, catalogue # P/N: 927- 60001) for one hour with gentle agitation (plate shaker was used to provide gentle agitation) at room temperature.

Membranes were then probed with primary antibodies MBP anti-rat (1:1000; Abcam, Cambridge; United Kingdom, catalogue # 7439; 7.5 mL Intercept Blocking buffer; 0.2% Tween-20; 0.02% w/v sodium azide), Beta-actin anti-mouse (1:10,000; Abcam, United Kingdom; catalogue # 8266; 7.5 mL Intercept Blocking buffer; 0.2% Tween-20; 0.02% w/v sodium azide) and kept on a gentle plate rocker for overnight incubation at 4 °C. Following overnight incubation, membranes were briefly rinsed in TBS with 0.1% Tween-20 (TBS-T) followed by a five minute TBS-T wash repeated four times (all washes were completed at room temperature with gentle agitation provided by the plate shaker).

Membranes were then probed with IRDye 800 CW Goat anti-Rat Secondary Antibody (1:30,000, LICOR, Lincoln, NE, catalogue # P/N: 926-32219; 0.2% Tween-20; 0.02% SDS solution; 7.5 mL Intercept Blocking buffer) and IR-Dye 680 RD Goat anti-Mouse Secondary Antibody (1:30,000; LICOR, Lincoln, NE; catalogue # P/N: 926-68070; 0.2% Tween-20; 0.02% SDS solution). Membranes were light protected and incubated in secondary antibody solution for one hour at room temperature with gentle agitation. After the secondary antibody incubation period, membranes were briefly rinsed in TBS-T followed by a five minute TBS-T wash with gentle agitation; the TBS-T wash was repeated for a total of four times. Membranes were then rinsed in the TBS buffer and stored at 4 °C until imaging. Membranes were imaged using the LICOR detection system and Odyssey software and quantified using ImageJ software (NIH, Bethesda Maryland). For detailed methods on membrane imaging, see Appendix.

Following the imaging, membranes were stripped of antibodies using NewBlot IR Stripping buffer for NIR Western Blots (LICOR; Lincoln, NE; catalogue # P/N: 928-40028), blocked in Intercept Blocking buffer for fifteen minutes and reprobed with anti-PLP antibody (1:5,000; 0.2% Tween-20; 0.02% w/v sodium azide) followed by IRDye 800 CW Goat anti-Mouse Secondary

Antibody (1:30,000, LICOR, Lincoln, NE, catalogue # P/N: 926-32210; 0.2% Tween-20; 0.02% SDS solution). LICOR immunodetection system and ImageJ software were used for analysis and quantification of PLP relative abundance (see Appendix for detailed methods on membrane stripping and reprobing for PLP-antibody).

Following the imaging steps, one ethanol female (immediate time point) was not included in protein quantification because the signal of the target protein was absent (despite the presence of the loading control protein in the corresponding lane). In the persistent time point, one ethanol male, one ethanol female, and one control female were not included in protein quantification for similar reasons. Thus, N = 49 (n = 5-8 per group, 4 groups per time point).

### **Statistical Analysis**

Based on our findings that *Mbp* and *Plp* mRNA levels were robustly decreased in adolescent ethanol treated animals compared to controls (PND 43) and that these alterations in gene expression did not persist into adulthood (PND 66), we formed the a priori hypotheses that ethanol will decrease myelin protein expression and this decrease in myelin protein expression will persist into adulthood. We conducted separate two-way ANOVA statistical analyses for each

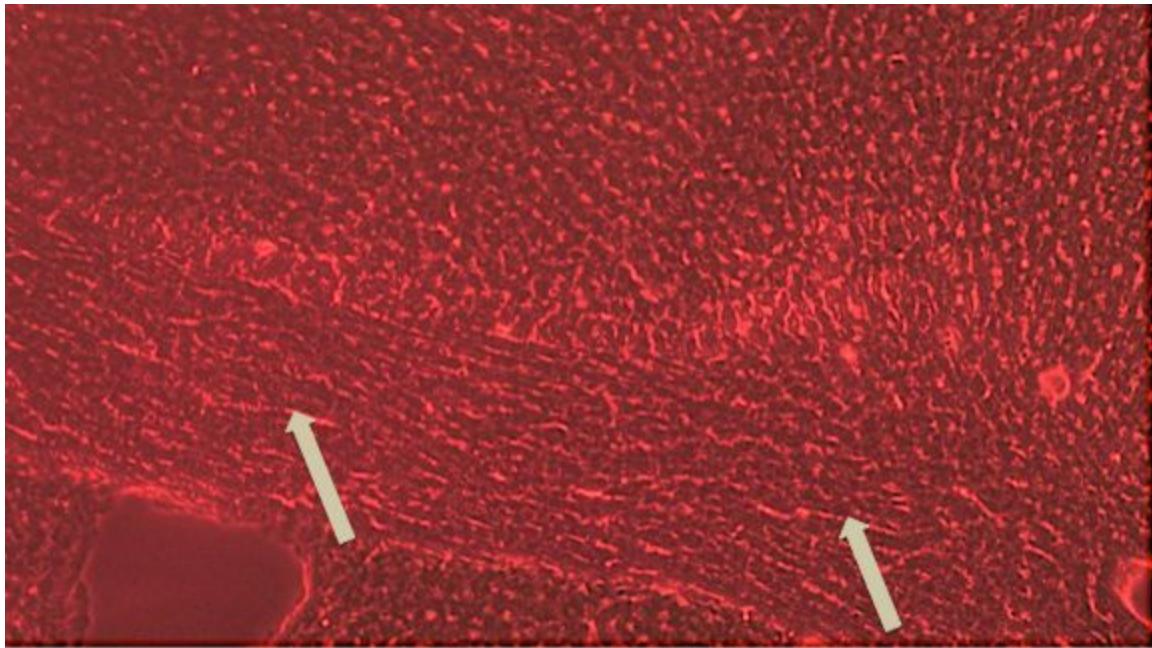
time point in order to specifically assess the potential for recovery. In order to ensure there was no significant variability in the loading control protein expression, two-way ANOVAs with sex and treatment as factors were completed for actin area values for both immediate (PND 43) and persistent (PND 84) samples; no post-hoc tests were required.

To assess potential sex differences and potential treatment effects of ethanol in the MBP and PLP protein expression measured from the fluorescent-based Western blots, two-way ANOVA with sex and treatment as factors were performed with alpha level set to 0.05. No post hoc tests were required. To specifically assess sex differences, we conducted two-way ANOVA with sex and time point as factors for control animals only; Student-Newman-Keuls post hoc analyses were completed as needed. The alpha level was set to 0.05 for all statistical tests. All statistical testing and analysis was done using SigmaPlot 14 (Systat software, Chicago, IL), GraphPad Prism8 (GraphPad Software, San Diego, CA) and JMP Pro 14 (SAS Institute, Cary, NC).

## **Chapter 3: RESULTS**

### **Optimization and specificity of MBP antibody**

We first titrated our MBP antibody (1:1000, 1:1500) on C57BL/6J (untreated tissue) and included a negative control (Alexa Fluor 594 1:1000, no primary antibody) in order to determine the optimal concentration and ensure that we were accurately localizing our target of interest. We determined that MBP at 1:1000 concentration was optimal compared to the 1:1500 concentration, as MBP 1:1000 displayed a stronger MBP signal (without excess saturation) than the more dilute concentration which displayed a lower fluorescent intensity. We used the corpus callosum (DBA/2J strain; treated tissue) as an anatomical landmark to ensure we were visualizing specific myelin staining (**Figure 3**).



**Figure 3. MBP immunostaining in corpus callosum.**

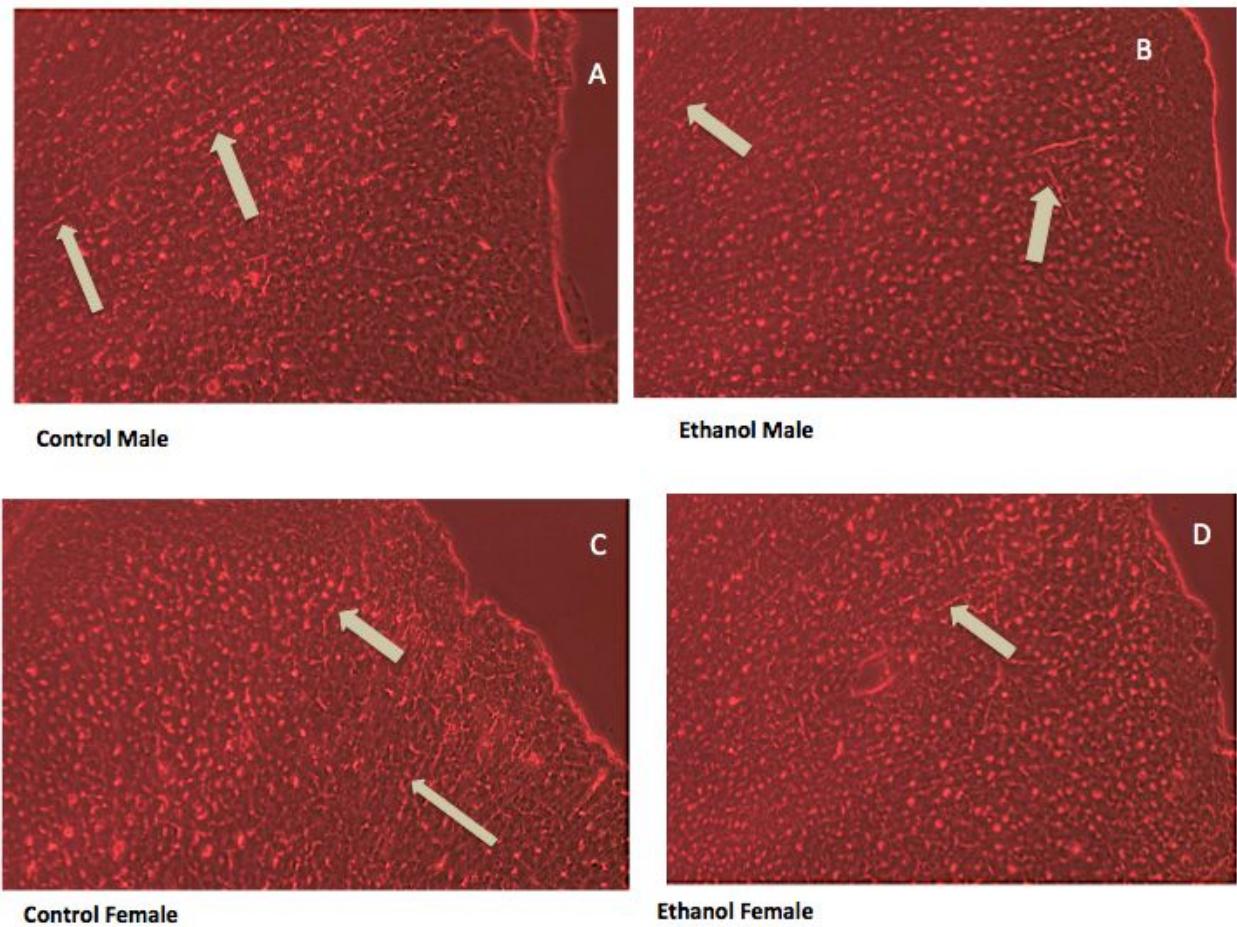
Images for quantification were taken at 20X objective with corpus callosum used as an anatomical landmark. Corpus callosum was excluded from field of view for actual image acquisition. Arrows indicate myelinated axons

### **MBP immunoreactivity in adolescent PFC tissue (PND 43)**

We used an MBP antibody as a protein marker for myelin to assess changes in MBP expression through semi-quantitative immunohistochemistry. Our experimental design included four groups (i.e. control males, control females, ethanol males, and ethanol females). All mice in this study were intermittently dosed with a 4g/kg of ethanol or water from PND 29-42. We sought to assess potential alterations immediately following adolescent binge ethanol exposure. Thus, we harvested PFC tissue twenty four hours after the last ethanol binge and immunostained the tissue with the MBP antibody.

The corpus callosum was used as anatomical landmark, but was excluded from 20X images used for quantification. We visually observed slight variability of MBP immunostaining in the PFC (**Figure 4**). The mean values of the uncorrected integrated densities were used to compare relative MBP immunoreactivity (**Figure 5**). We performed a two-way ANOVA with sex and treatment as factors across all four groups to assess for any statistically significant differences across the four groups from the immediate time point. Two-way ANOVA on adolescents harvested at PND 43 revealed no main effect of treatment on MBP protein expression ( $F(1,31) = 0.582$ ,  $p = 0.452$ ) nor was there a main effect of sex ( $F(1,31)$

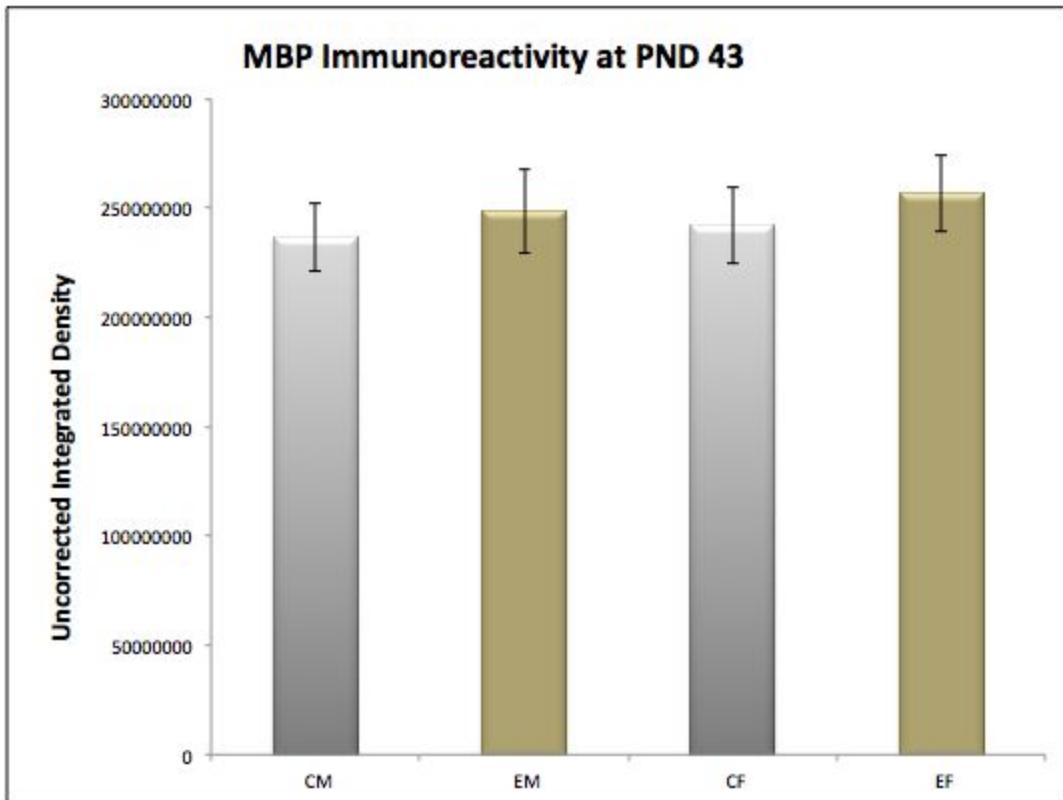
= 0.151, p = 0.701). There was no interaction effect between sex and treatment ( $F(1,31) = 0.00572$ , p= 0.940) thus no post-hoc tests were conducted. The significance level was set to 0.05. We concluded there were no notable alterations in MBP protein levels at PND 43.



**Figure 4. MBP Immunoreactivity in adolescent PFC tissue (PND 43)**

Images of 25  $\mu$ m coronal sections of tissue (left brain hemisphere with mPFC, motor cortex in field of view) displaying MBP immunostaining taken with 20X objective lens. There is a slight observable difference between groups. Arrows indicate myelinated axons.

( $p > 0.05$ ) for mean +/- SEM group values,  $n = 8$ /group.



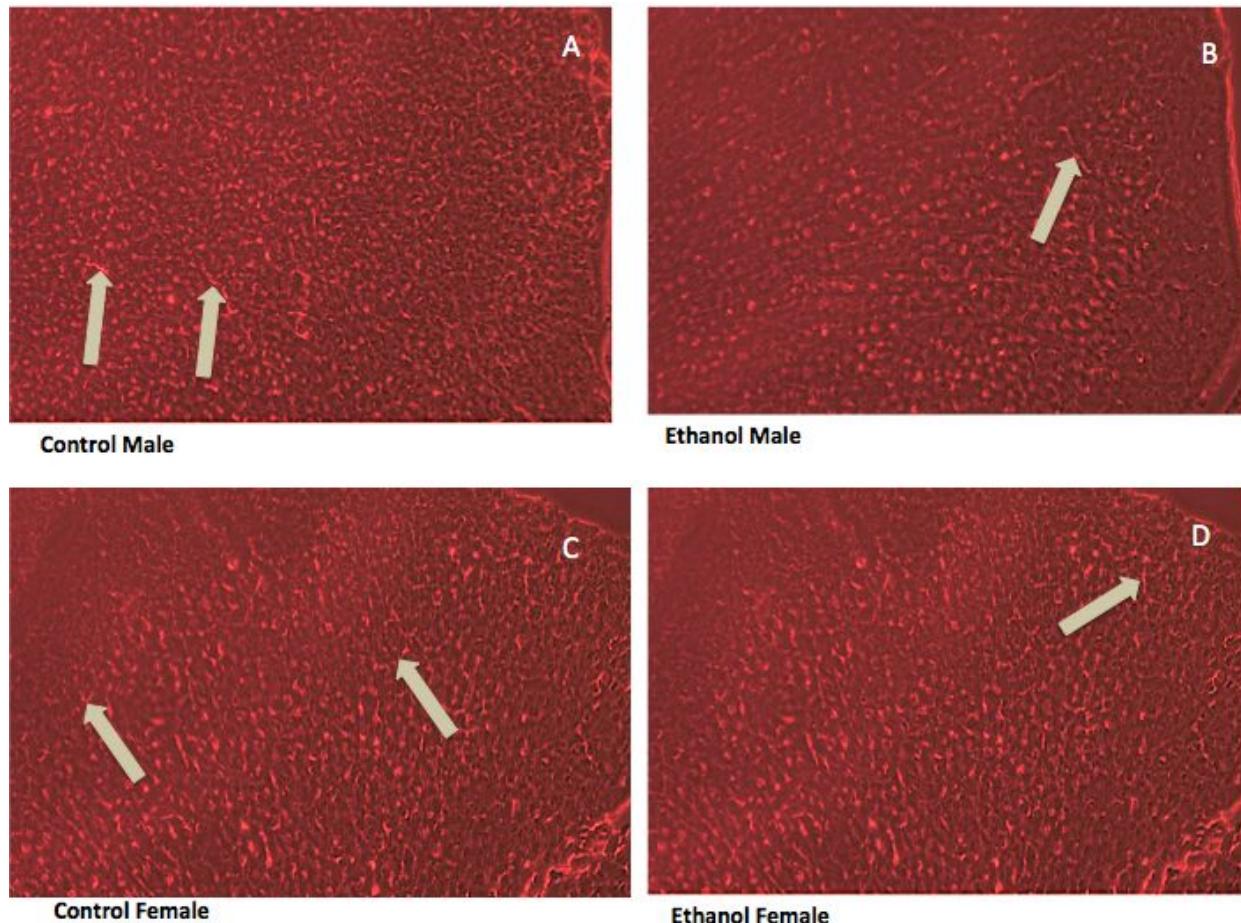
**Figure 5. Quantification of MBP Immunoreactivity at PND 43**

Mean  $\pm$  SEM; n = 8/group. The MBP immunoreactivity was quantified using ImageJ software, uncorrected integrated densities (without background subtracted) displayed for all four groups. There appears to be no differences in MBP immunoreactivity. We confirmed with statistical analyses that no statistically significant differences exist between the four groups ( $p > 0.05$ ).  
 CM- Control Male; EM- Ethanol Male; CF- Control Female; EF- Ethanol Female.

### **MBP immunoreactivity in adult PFC tissue (PND 66)**

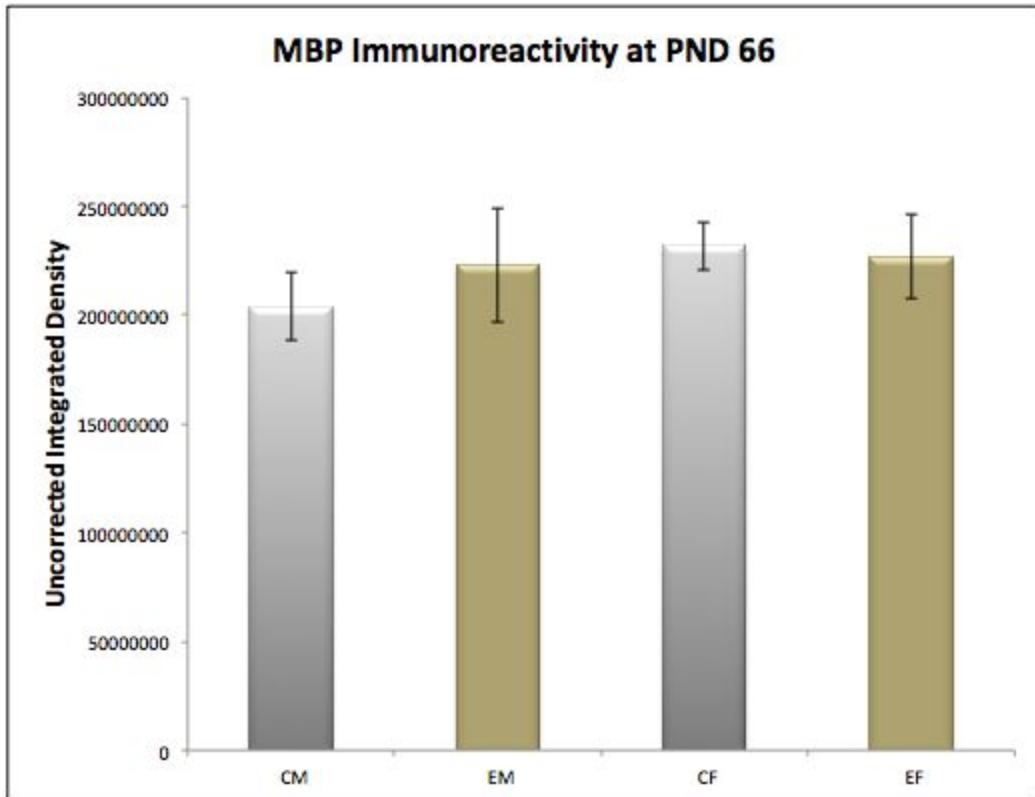
To assess the persistent effects of binge ethanol exposure, we proceeded to immunostain PFC tissue at PND 66, three weeks after the last ethanol binge, with the MBP antibody. Here again, we visually observed slight variability in MBP immunostaining among the four groups (control males, ethanol males, control females, ethanol females) in the persistent time point (**Figure 6**). We quantified the uncorrected integrated density values (without background subtracted) and averaged the values for each group (**Figure 7**).

However, we found no statistically significant differences between groups. Two-way ANOVA on adults harvested at PND 66 revealed no main effect of treatment ( $F(1,27) = 0.181$ ,  $p = 0.675$ ) nor did it reveal a main effect of sex ( $F(1,27) = 0.00641$ ,  $p = 0.937$ ). There was no interaction effect present ( $F(1,27) = 0.0235$ ,  $p = 0.880$ ). Alpha level to determine significance was set to 0.05. We determined that binge ethanol exposure results in no significant alterations in MBP immunoreactivity in the PFC at PND 66 (**Figure 7**).



**Figure 6. MBP Immunoreactivity in adult PFC tissue (PND 66)**

Images of 25 um coronal sections of DBA/2J PFC brain tissue (left hemisphere with mPFC, motor cortex in field of view) displaying MBP staining pattern. There appears to be slight variability between ethanol and control animals, however, there were no statistically significant differences between groups ( $p > 0.05$ ,  $n = 7-8/\text{group}$ ). All images taken with 20X objective lens. Arrows indicate myelinated axons.



**Figure 7. Quantification of MBP Immunoreactivity at PND 66**

Mean  $\pm$  SEM; n = 6-8/group. The MBP immunoreactivity was quantified using ImageJ software, uncorrected integrated densities (without background subtracted) displayed for all four groups. There were no statistically significant differences between groups ( $p > 0.05$ ). CM- Control Male; EM- Ethanol Male; CF- Control Female; EF- Ethanol Female.

### **Fluorescence-based Western blot assays for MBP: PFC tissue (PND 43)**

Fluorescence-based Western blot experiments were performed as an additional quantitative measure of MBP relative abundance (see Appendix for MBP immunostained membranes with adolescent PFC protein). The relative abundance of MBP protein was normalized to beta-actin (loading control protein). We visually observed MBP at four molecular weights (14 kDa; 17 and 18.5 kDa quantified as a doublet band; 21.5 kDa) across the different treatment groups (**Figure 8**) and quantified the total MBP immunoreactivity ranging from 14 kDa to 21.5 kDa and the separate immunoreactivity levels at the four molecular weights (**Figure 8**).

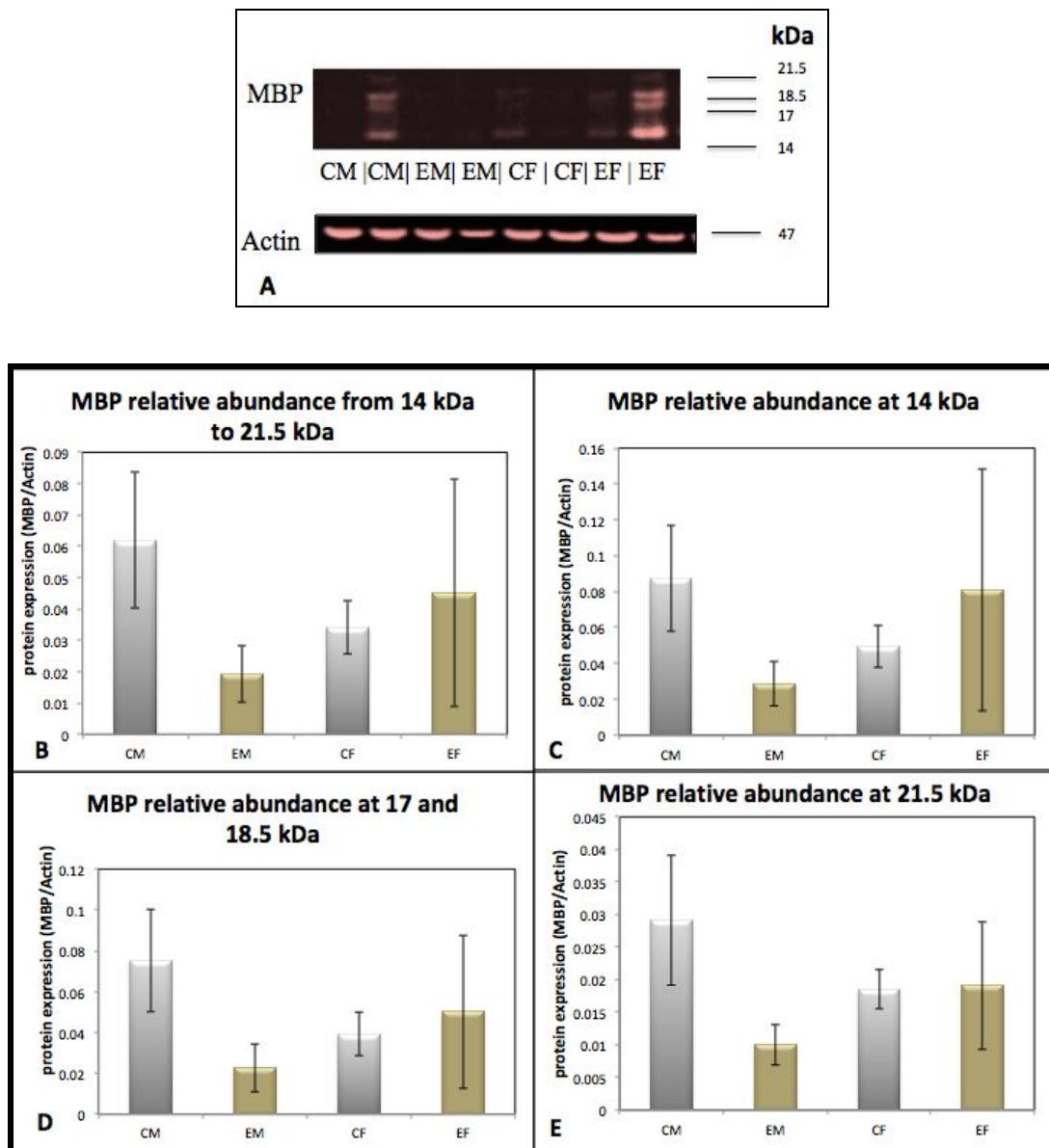
We observed that ethanol males displayed a slight but not significant (n.s.) trend towards decreased MBP protein expression as compared to control males at all molecular weights, while the ethanol females appeared to show a slight trend towards increased MBP protein expression or stayed relatively similar to control females. Separate two-way ANOVA tests were completed to assess for significant differences in total MBP relative abundance and at the various molecular weights visualized on the fluorescent PVDF membrane (**Figure 8**).

### Beta-actin: molecular weight approximated to 47 kDa

We first completed a two-way ANOVA with sex and treatment as factors to confirm there were no statistically significant differences in our loading control mean values (beta-actin, molecular weight 47 kDa). Two-way ANOVA revealed no statistically significant differences i.e. no main effects of sex ( $F(1,27) = 1.793$ ,  $p = 0.193$ ), treatment ( $F(1,27) = 2.512$ ,  $p = 0.126$ ) and no interaction effect ( $F(1,27) = 0.00165$ ,  $p = 0.968$ ). From these statistical findings, we determined the visually observed variability in MBP relative abundance was not due to inconsistent sample loading.

We then performed a two-way ANOVA with sex and treatment as factors in order to assess for statistically significant differences between treatment groups for MBP relative abundance. For total MBP protein relative abundance (molecular weights ranging from 14 kDa to 21.5 kDa) two-way ANOVA revealed no main effect of sex ( $F(1,27) = 0.00218$ ,  $p = 0.963$ ) nor was there a main effect of treatment ( $F(1,27) = 0.748$ ,  $p = 0.396$ ). No interaction effect between sex and treatment was present ( $F(1,27) = 2.150$ ,  $p = 0.156$ ). We proceeded to assess the relative MBP abundance at the various molecular weights. The two-way ANOVA assessing the relative abundance for MBP at 14 kDa revealed no main effect of

sex ( $F(1,27) = 0.0552$ ,  $p = 0.816$ ) nor was there a main effect of treatment ( $F(1,27) = 0.204$ ,  $p = 0.655$ ). There was no interaction effect ( $F(1,27) = 2.277$ ,  $p = 0.1444$ ). We visually observed two bands at 17 kDa, 18.5 kDa molecular weights and quantified these bands as a doublet. The two-way ANOVA for mean values at 17 and 18.5 kDa showed no main effect of sex ( $F(1,27) = 0.0413$ ,  $p = 0.871$ ) nor was there a main effect of treatment ( $F(1,27) = 1.016$ ,  $p = 0.323$ ). No interaction effect was present ( $F(1,27) = 2.372$ ,  $p = 0.137$ ). When assessing relative MBP abundance at 21.5 kDa, two-way ANOVA revealed there was no main effect of sex nor was there a main effect of treatment ( $F(1,27) = 0.0134$ ,  $p = 0.909$ ), ( $F(1,27) = 1.943$ ,  $p = 0.176$ ) and no interaction effect ( $F(1,27) = 2.189$ ,  $p = 0.152$ ). Chameleon™ Kit Prestained ladder/molecular weight marker was loaded with 2.5 ul of 700 nm channel and 2.5 ul of 800 nm channel in order to estimate the unknown protein quantities (LICOR, Lincoln, NE, catalogue # P/N: 928-90000), and observed MBP molecular weight values were the cited predicted values in the literature (Akiyama et al 2002; Boggs 2006; Devine-Beach et al 1990).



**Figure 8. Western blot analysis of MBP relative abundance at PND 43**

Panel A Representative portion of Western blot. CM-control male; EM- ethanol male; CF-control female; EF-ethanol female. Panel B depicts relative abundance of MBP at molecular weights ranging from 14 kDa to 21.5 kDa, Panel C depicts relative abundance of MBP at molecular weight 14 kDa, Panel D depicts the relative abundance of MBP at molecular weight 17 and 18.5 kDa (two bands that were quantified as a doublet band), Panel E depicts relative abundance of MBP at molecular weight at 21.5 kDa.

### **Fluorescence-based Western blot assays for MBP: PFC tissue (PND 84)**

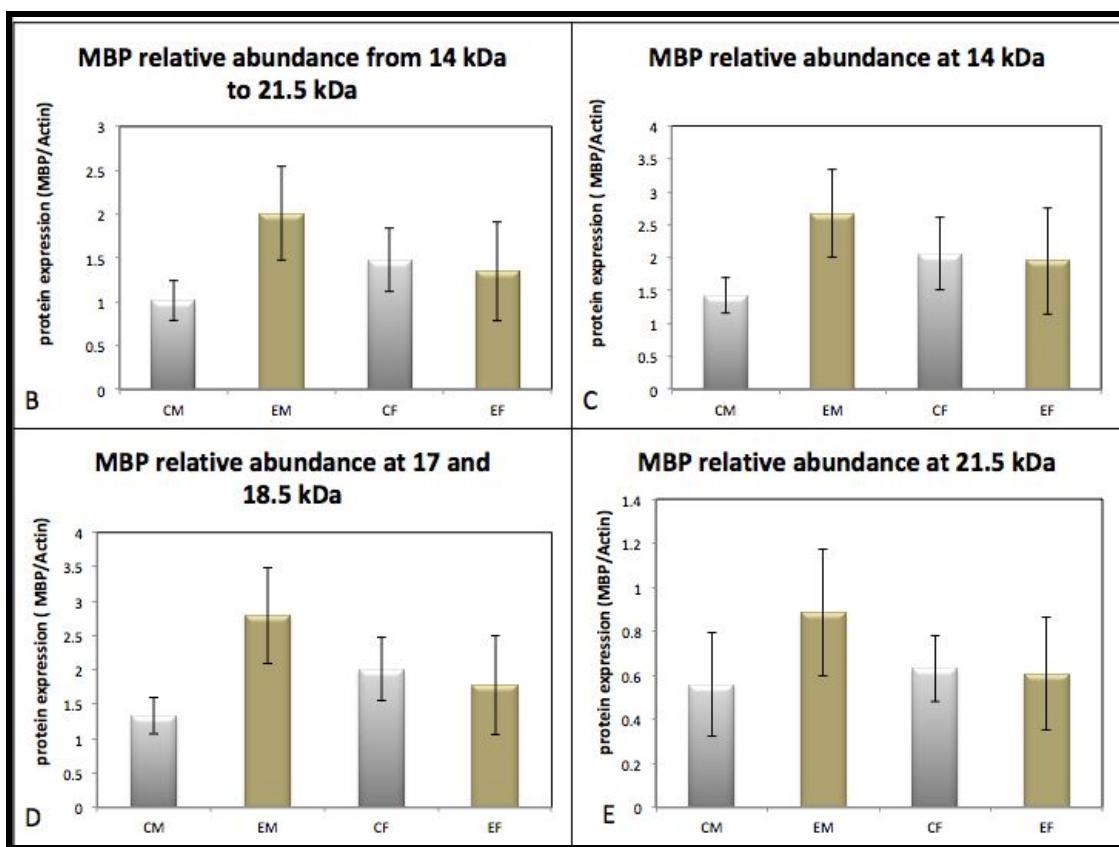
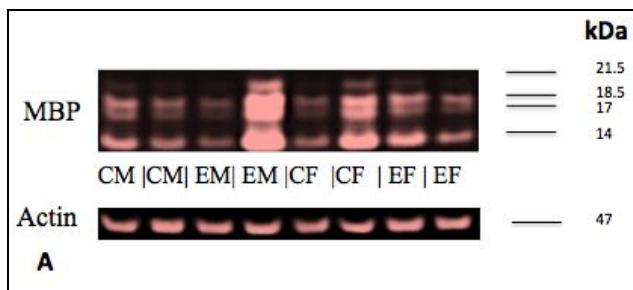
We conducted fluorescence-based Western blot experiments using PFC sample lysates from adult PFC tissue (see Appendix for MBP immunostained membranes with adult PFC protein). Similar to the Western blot experiments on adolescent tissue sample lysates, we included beta-actin loading control and normalized all quantified MBP relative amounts to actin. The blot displayed MBP at four different molecular weights (14 kDa; 17 kDa and 18.5 kDa quantified as a doublet band; 21.5 kDa). We quantified total MBP as well as the MBP amounts at each of the four molecular weights (**Figure 9**). Ethanol males appear to show a slight but not significant trend towards increased relative MBP abundance as compared to the control males, while females appear to be relatively similar between ethanol and control groups. To determine if statistical differences were present, we performed separate two-way ANOVA statistical tests with sex and treatment as factors on total MBP relative abundance and at each of the distinct molecular weights. Significance level was set to 0.05 for all statistical tests reported hereafter.

### Beta-actin: molecular weight approximated to 47 kDa

Similar to the Western blot assays completed with immediate time point sample lysates (PND 43), we completed a two-way ANOVA with sex and treatment as factors on the actin mean values in order to confirm that no statistically significant differences were present. Two-way ANOVA revealed no main effect of sex ( $F(1,20) = 0.0512$ ,  $p = 0.824$ ), no main effect of treatment ( $F(1,20) = 0.0563$ ,  $p = 0.815$ ), and no interaction effect ( $F(1,20) = 0.00165$ ,  $p = 0.968$ ). Thus, we confirmed that the observed variability seen in our blots were unlikely due to inconsistent sample loading.

The two-way ANOVA for total MBP relative abundance (molecular weight ranging from 14 to 21.5 kDa) revealed no statistically significant differences between groups. There was no main effect of sex ( $F(1,20) = 0.0521$ ,  $p = 0.822$ ) nor was there a main effect of treatment ( $F(1,20) = 1.024$ ,  $p = 0.326$ ). No interaction effect was noted ( $F(1,20) = 1.752$ ,  $p = 0.203$ ). Two-way ANOVA for mean values at 14 kDa revealed no main effect of sex ( $F(1,20) = 0.00578$ ,  $p = 0.940$ ), no main effect of treatment ( $F(1,20) = 0.948$ ,  $p = 0.344$ ), and no interaction effect ( $F(1,20) = 1.338$ ,  $p = 0.263$ ). The two-way ANOVA analyzing the mean values at molecular weights 17 kDa and 18.5 kDa (quantified as a doublet band) revealed similar

findings. There were no main effects of sex ( $F(1,20) = 0.0984$ ,  $p = 0.758$  ), treatment ( $F(1,20) = 1.256$ ,  $p = 0.278$ ), nor was there an interaction effect ( $F(1,20) = 2.384$ ,  $p = 0.141$ ) at molecular weights 17 and 18.5 kDa. There were no statistically significant findings at 21.5 kDa. The two-way ANOVA revealed no main effect of sex ( $F(1,20) = 0.181$  ,  $p = 0.676$ ), no effect of treatment ( $F(1,20) = 0.404$  ,  $p = 0.533$ ), and no interaction effect ( $F(1,20) = 0.532$  ,  $p = 0.476$ ) at molecular weight 21.5 kDa.



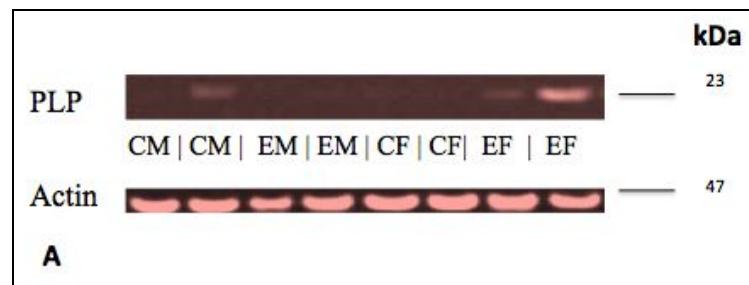
**Figure 9. Western Blot analysis of MBP relative abundance at PND 84**

Representative portion of Western blot from adult time point. CM-control male; EM-ethanol male; CF-control female; EF-ethanol female

Mean  $\pm$  SEM; n = 5-8/group. Two-way ANOVAs showed no significant alterations in MBP protein expression. Panel B displays MBP relative abundance from molecular weights ranging from 14 kDa to 21.5 kDa, Panel C displays MBP relative abundance at 14 kDa abundance, Panel D displays MBP relative abundance at 17, 18.5 kDa (two bands quantified as a doublet band), Panel E displays MBP relative abundance at 21.5 kDa

### **Fluorescence-based Western blot assays for PLP: PFC tissue (PND 43)**

We performed fluorescence-based Western blot experiments on adolescent PFC sample lysates (see Appendix for PLP immunostained membranes with adolescent PFC protein) and assessed PLP immunoreactivity (**Figure 10**). PLP relative abundance was normalized to actin loading control protein and quantified (**Figure 10**). Two-way ANOVA with sex and treatment as factors were completed to assess for any statistically significant differences between samples. Two way ANOVA revealed no main effect of sex ( $F(1,27) = 0.954$ ,  $p = 0.339$ ) and no main effect of treatment ( $F(1,27) = 0.219$ ,  $p = 0.644$ ) on PLP protein expression (molecular weight 23 kDa). No interaction effect was present ( $F(1,27) = 0.867$ ,  $p = 0.362$ ). Significance level for all statistical tests is 0.05. The Chameleon protein ladder was used to approximate molecular weight for PLP; the observed molecular weight is consistent with the range for the anti-PLP antibody used in these Western Blot assays.

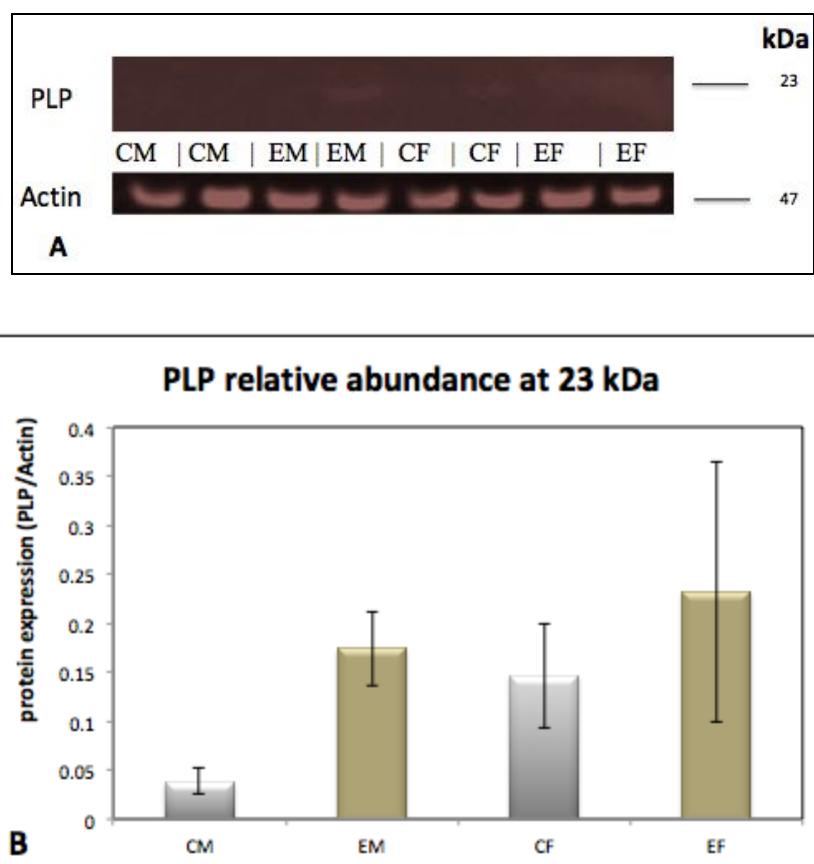


**Figure 10. Western Blot Analysis of PLP relative abundance at PND 43**

Panel A Representative portion of Western Blot from adolescent time point. PLP relative abundance was observed to be lower as compared to MBP. PLP was observed at 23 kDa predicted value. Panel B Mean  $\pm$  SEM; n = 6-8/group. Two-way ANOVA showed no significant changes in PLP relative abundance. CM-control male; EM-ethanol male; CF-control female; EF-ethanol female.

### **Fluorescence-based Western blot assays for PLP: PFC tissue (PND 84)**

We conducted additional Western blot experiments using adult PFC sample lysates (see Appendix for PLP immunostained membranes with adult PFC protein) to assess potential alterations in PLP immunoreactivity (**Figure 11**). All relative PLP amounts were normalized to actin. Two-way ANOVA was performed (with sex and treatment as factors) to determine potential significant differences. There was no effect of sex, no effect of treatment and no interaction effect on PLP (molecular weight 23 kDa) protein expression ( $F(1,20) = 1.362$ ,  $p = 0.259$ ), ( $F(1,20) = 2.447$ ,  $p = 0.136$ ), ( $F(1,20) = 0.129$ ,  $p = 0.724$ ). Significance level for all statistical tests was set to 0.05.



**Figure 11. Western Blot Analysis of PLP relative abundance at PND 84**

Panel A Representative portion of Western Blot from adult time point (PND 84). PLP relative abundance was observed to be lower as compared to MBP. The statistical analyses completed for actin revealed no statistically significant differences, thus the decreased signal was unlikely due to inconsistent sample loading. PLP was also observed at 23 kDa predicted value. Panel B Mean  $\pm$  SEM; n = 5-8/group. Two-way ANOVA revealed insignificant differences between treatment groups. CM-control male; EM-ethanol male; CF-control female; EF-ethanol female

## **Sex Differences Between Treatment Groups**

The third aim of this project was to analyze potential sex differences following adolescent binge ethanol exposure. We formed the a priori hypothesis that males would show a decrease in myelin-related protein expression as compared to females, based on our earlier microarray findings (Wolstenholme et al 2017).

We performed multiple two-way ANOVAs with sex and treatment as factors and found no main effects of sex from both our immunohistochemistry and Western blot experiments. However, our western blot protein quantification graphically revealed the appearance of slight but not significant trends across our myelin proteins MBP and PLP such that adolescent ethanol males displayed a trend towards decreased protein expression as compared to control males (**Figure 8** and **Figure 10**); this trend shifted such that adult ethanol males displayed a slight trend towards increased myelin protein expression (**Figure 9** and **Figure 11**). We also noted that our ethanol females tended to visually display similar levels of myelin protein expression as compared to control females in both adolescence and adulthood. Thus, we sought to determine if the relative basal expression of myelin proteins differed between sexes and across time. We performed two-way ANOVAs

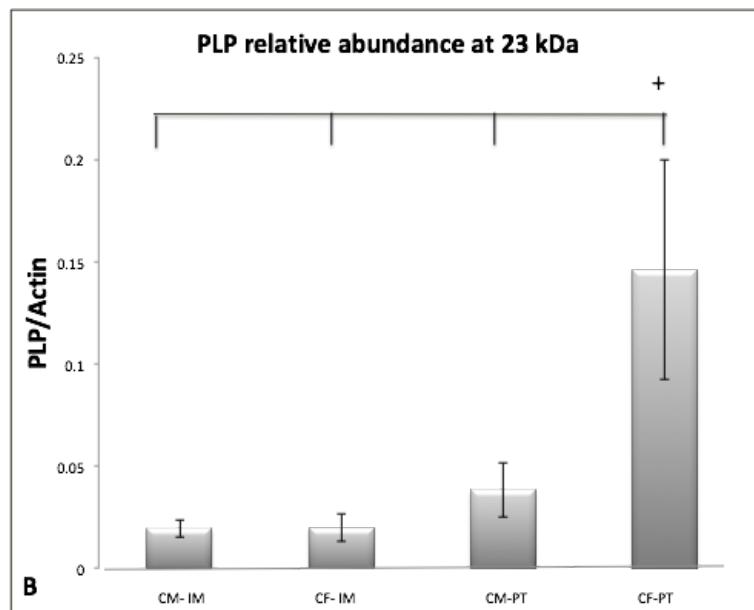
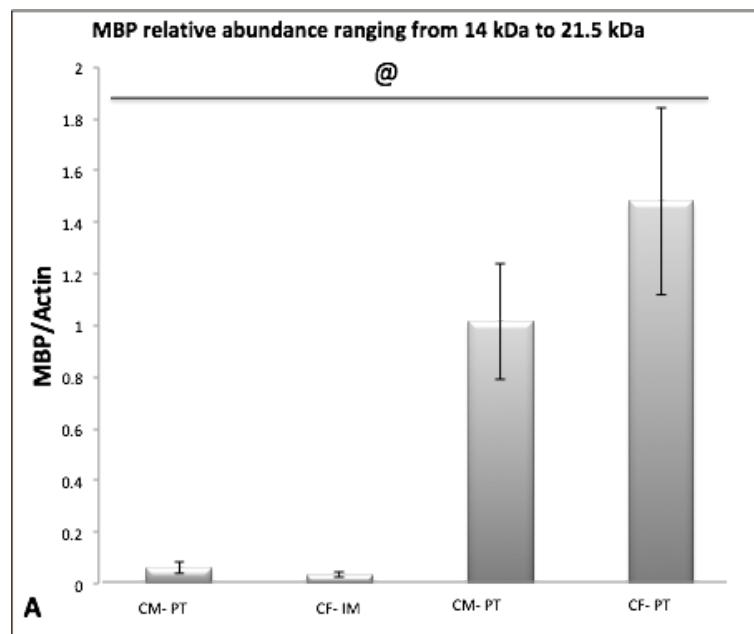
with time point (immediate, PND 43; persistent, PND 84) and sex (male, female) as factors on only control animals (**Figures 12**).

#### Sex Differences from MBP Western Blot assays for control animals

There were no significant differences in sex for total (molecular weight ranging from 14 kDa to 21.5 kDa) MBP immunoreactivity ( $F(1,25) = 1.612$ ,  $p = 0.218$ ), 14 kDa relative abundance ( $F(1,25) = 1.469$ ,  $p = 0.238$ ), 21.5 kDa ( $F(1,25) = 0.0638$ ,  $p = 0.803$ ), bands at 17 and 18.5 kDa were quantified as a doublet ( $F(1,25) = 2.238$ ,  $p = 0.149$ ). However, a main effect of time point was noted ( $F(1,25) = 47.968$ ,  $p < 0.05$ ) where total MBP expression was greater in adults (**Figure 12A**).

#### Sex Differences from PLP Western Blot assays for control animals

In control animals, we found an interaction effect between sex and time point with respect to PLP relative expression ( $F(1,25) = 5.982$ ,  $p = 0.023$ ). Student-Newman-Keuls post hoc analyses revealed control females at the persistent time point expressed significantly more PLP protein than their adult control male counterparts (**Figure 12 B**). In addition, we noted that adult control females expressed significantly more PLP protein than adolescent control females ( $p < 0.05$ ). Significance level for all reported results was set to 0.05.



**Figure 12. Analysis of MBP and PLP expression in control animals**

Mean  $\pm$  SEM; n = 5-8/group

Panel A- control MBP (molecular weight ranging from 14 kDa to 21.5 kDa)

mean values (PND 43 and PND 84). @ p < 0.05 , main effect of time point present i.e. MBP expression is greater in adulthood.

Panel B- control PLP mean values (PND 43 and PND 84). + p < 0.05, interaction effect between time point and sex (p < 0.05).

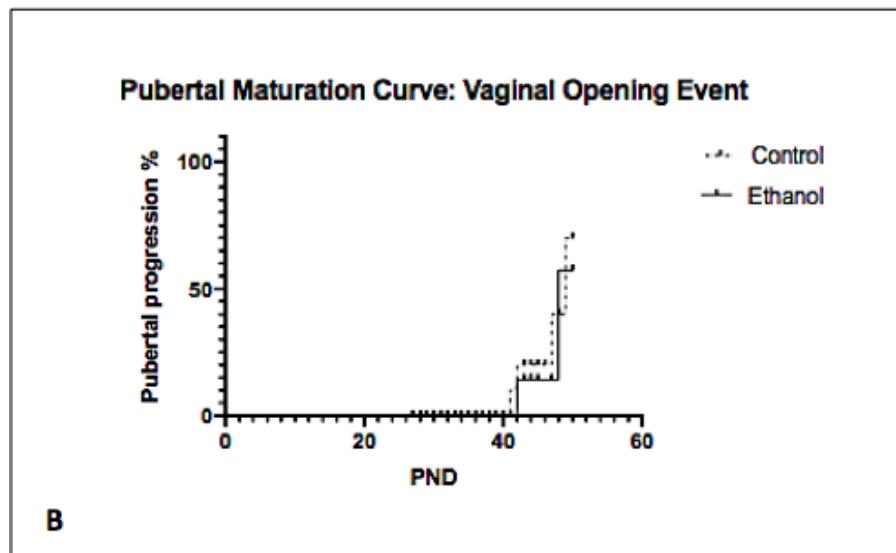
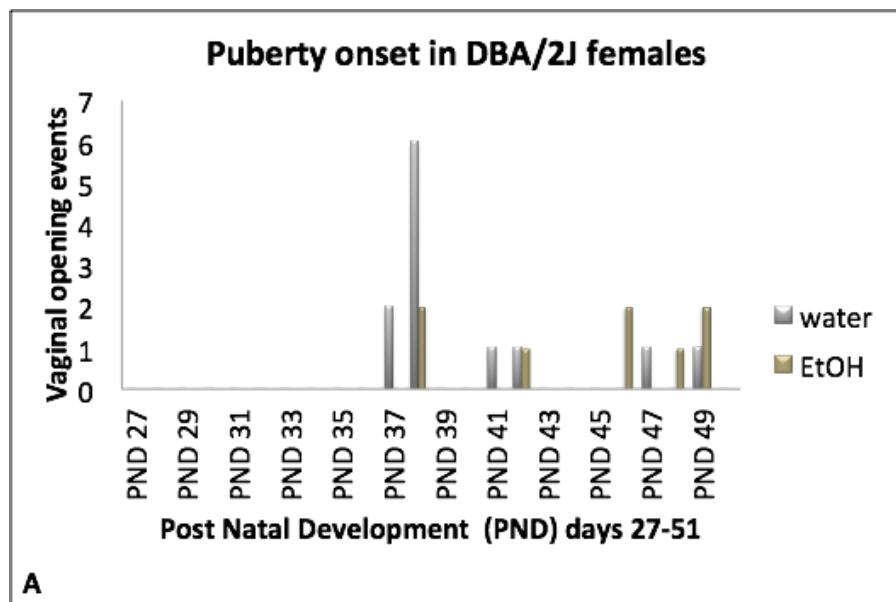
Adult females express significantly more myelin protein than adult males and adolescent females. CM-PT- control male, persistent time point; CF- control female, persistent time point; CM-IM- control male, immediate time point; CF-IM- control female persistent time point.

### Puberty Onset and Progression Assessments

A notable study found that females experienced sexual maturation at a relatively faster rate than males, and males displayed significantly decreased myelin density following binge ethanol exposure (Richardson et al 2019). These findings of faster sexual maturation in females were consistent with prior literature (Vetter-O'Hagen and Spear 2012). Richardson et al noted differential rates in sexual maturation could be a factor in how susceptible myelin density is to ethanol insults. However, the aforementioned study did not directly assess a logical follow up question, that is, if ethanol can affect sexual maturation in males and females. We sought to assess if ethanol treatment delays sexual maturation and analyze potential sex differences within the groups.

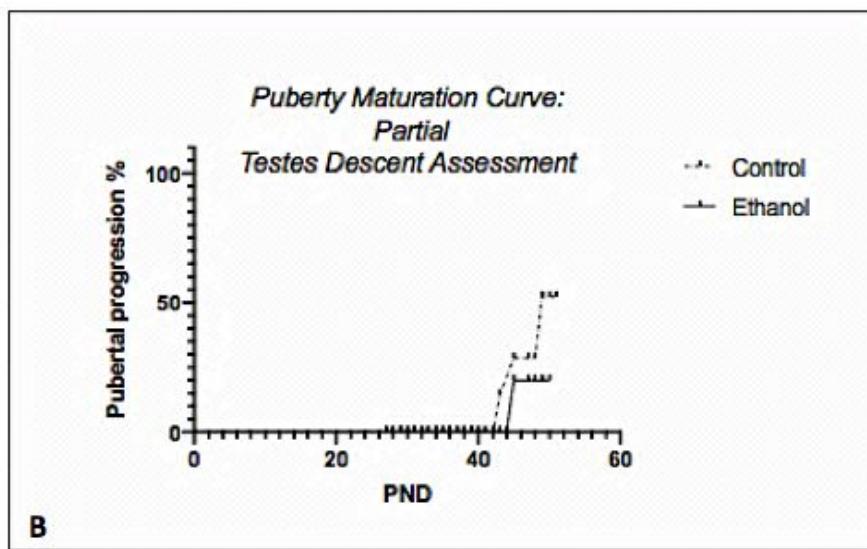
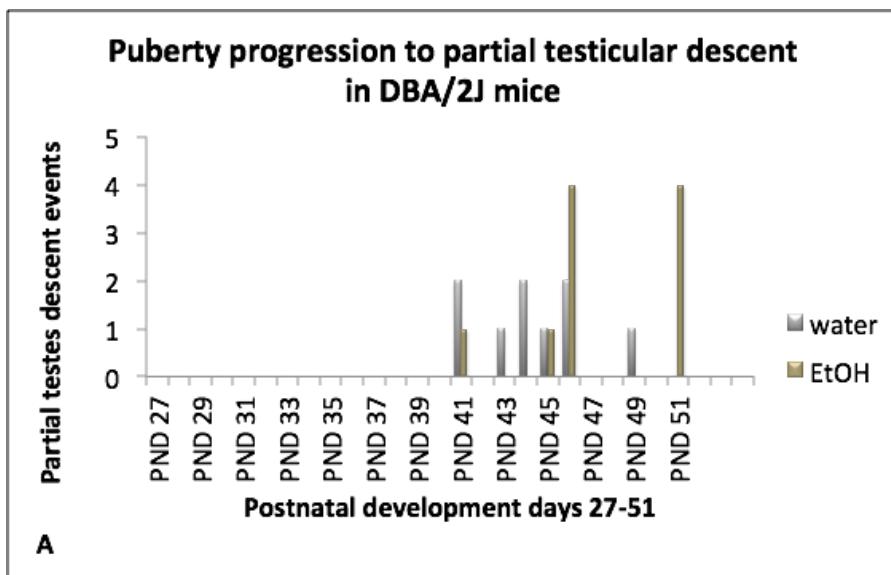
For our second cohort of mice, we performed routine visual assessments for external signs of puberty onset in both our ethanol and control animals in order to assess if ethanol affects sexual maturation (**Figures 13-14**). We then performed two-way ANOVA with sex and treatment as factors to assess the mean PND age at puberty onset (**Figure 15**). The two-way ANOVA was completed with sex and treatment as factors in order to assess potential treatment effects. We visually observed males displayed external signs of puberty slightly later than their female

counterparts (**Figures 13-14**). The two-way ANOVA confirmed this observation was in fact statistically significant (**Figure 15**). There was a main effect of sex ( $F(1,39) = 10.092, p < 0.05$ ) such that females displayed significantly lower mean PND ages at puberty onset as compared to males (**Figure 15**). We also found a main effect of treatment such that ethanol treated animals displayed significantly higher mean PND ages as compared to control animals (**Figure 15**,  $F(1,39) = 6.875, p < 0.05$ ), although no interaction effect was found.



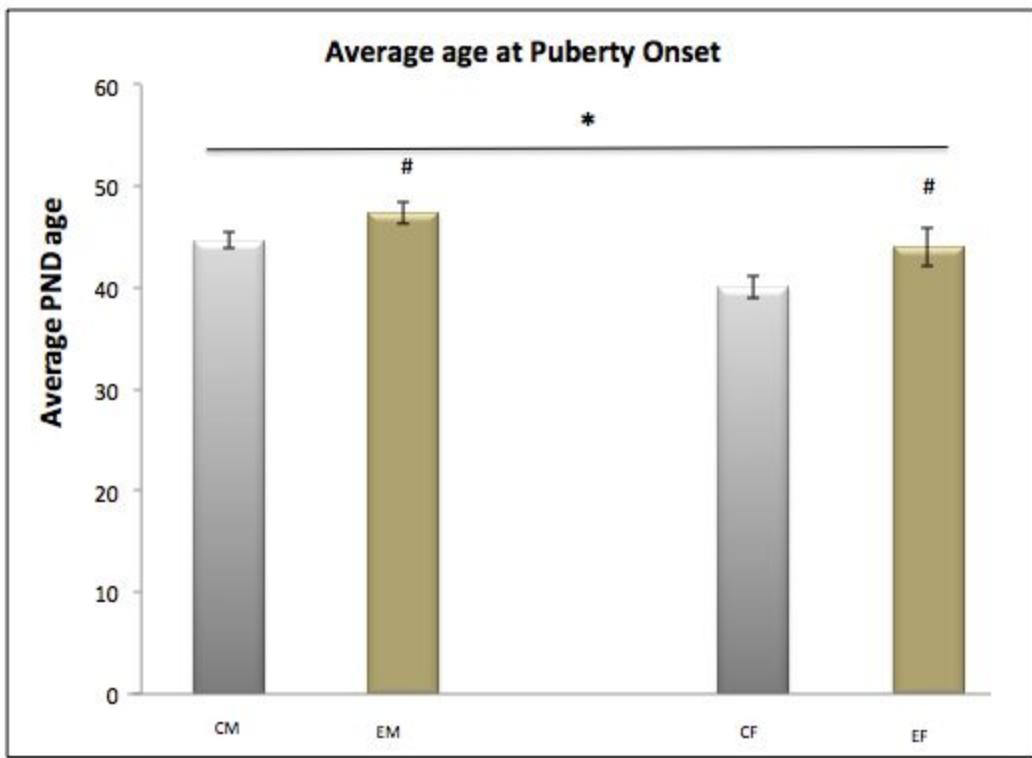
**Figure 13. Puberty Onset in DBA/2J females**

Panel A displays the total number of females that experienced a vaginal opening (VO) per day of assessment from PND 27-51, Panel B displays the puberty maturation curve for females



**Figure 14. Puberty Onset in DBA/2J males**

Panel A displays the total number of males that achieved partial testes descent per day of assessment (from PND 27-51), Panel B displays the puberty maturation curve



**Figure 15. Mean PND age at Puberty Onset.**

Mean  $\pm$  SEM; n = 8-11/group. Main effect of sex (\* $p < 0.05$ ) with females having significantly lower PND age at puberty onset as compared to males by two-way ANOVA. Main effect of treatment by two-way ANOVA (# $p < 0.05$ ) with ethanol treated animals having significantly higher PND age at puberty onset.

## **Chapter 4: DISCUSSION**

Alterations in white matter and myelin-related protein expression following ethanol consumption is an area under active investigation. The molecular mechanisms of ethanol that result in these alterations are still being determined (Lewohl 2000; Lewohl 2005; Wolstenholme et al 2017). The main objective of this thesis was to rigorously assess the effects of adolescent binge ethanol exposure on myelin-related (MBP and PLP) protein expression in the PFC, and in doing so, better understand the potential molecular consequences of excessive alcohol consumption.

### **MBP immunoreactivity in adolescent PFC tissue (PND 43)**

In experiment one, we used an adolescent binge ethanol exposure paradigm where all animals were intermittently dosed with 4 g/kg ethanol or water through PND 29-42. We harvested PFC tissue twenty four hours following the last ethanol binge (PND 43, adolescent immediate time point) to assess changes in MBP expression using semi-quantitative immunohistochemistry. Our experimental design included four groups (i.e. control males, control females, ethanol males, ethanol females) to allow for the study of potential sex differences and treatment

effects following binge ethanol exposure. Ultimately, we found no significant alterations in MBP immunoreactivity following binge ethanol exposure between our control and ethanol treated groups.

### **MBP immunoreactivity in adult PFC tissue (PND 66)**

We also aimed to assess if potential alterations in myelin protein expression persist into adulthood. Our experimental design remained the same for this second cohort of mice, with a 4g/kg dose of either ethanol or water from PND 29-42. The second cohort of mice were abstinent from binge ethanol exposure for three weeks following the dosing paradigm and were harvested at PND 66 (persistent time point). There were no statistically significant differences between the ethanol treated animals and the animals given vehicle.

It is well established that adolescent binge ethanol exposure is linked with reduced white matter in the frontal cortex (Bava et al 2013; Richardson et al 2014; Richardson et al 2019). Others have used immunohistochemistry with free-floating sections, specifically assessing degraded myelin basic protein (dMBP) following binge ethanol and found significant increases in dMBP at the dorsal medial border of corpus callosum forceps minor (Richardson et al 2014). Thus, we considered

the areas in our study that resulted in no statistically significant findings in myelin protein expression following binge ethanol exposure.

We first considered that our method of quantification was not as rigorous. Here, we solely used the uncorrected integrated density values without background correction. However, reliable and accurate quantification should utilize the corrected total cell fluorescence (CTCF) i.e. a corrected integrated density value that removes background noise (“noise”) (El-Sharkawey, in submission; Verdaasdonk 2014). Thus, it is possible the manner in which we quantified our data was not representative of the potential changes in MBP immunoreactivity. These potentially unrepresentative mean values may not truly convey the consequences of binge ethanol on white matter integrity. Therefore, if significant alterations were present, our method of data acquisition did not readily reveal them.

Another technical consideration is the thickness in our sections (25 microns), other published findings have used 35 um thick sections (Richardson et al 2014; Richardson et al 2019) in their immunostaining experiments, thus it is possible that the thinner sections were more susceptible to damage during tissue processing, making consistent data acquisition more challenging. The concern for slightly

inconsistent data acquisition prompted a standardized imaging acquistion process that avoided damaged areas, however, this method was subject to some experimenter bias which could have impacted the final results (experimenter was blinded to sex, treatment and time point at the time of imaging acquistion and quantification).

Biological considerations are also warranted. This study formed an a priori hypothesis based on our prior mRNA findings where adolescent binge ethanol decreased myelin-related gene expression. We relied on our previous findings in order to hypothesize that binge ethanol will decrease myelin protein expression. However, we erred in heavily relying on the central dogma of molecular biology as a mechanism rather than a potential flow of biological information. There are potential epigenetic regulatory mechanisms at play as well. These mechanisms could have potentially altered the transcription levels, and resulted in downstream effects in protein expression, which may have impacted our results. For example, H3K9me, an epigenetically modified version of histone 3 protein, is notable for its presence on oligodendrocyte progenitor cells (OPCs) and mature oligodendrocytes (Liu et al 2015). Our laboratory has found evidence of slight alterations to the H3K9 mark following binge ethanol exposure (Wolstenholme et al 2017). While

H3K9 methylation was not significantly reduced by ethanol, future studies may consider assessing the methylation of this mark in conjunction with myelin protein expression.

### **Fluorescence-based Western blot assays for MBP: PFC tissue (PND 43)**

In experiment two, fluorescence-based Western blotting was completed to employ a semi-quantitative technique in conjunction with the immunostaining from experiment one. Animals (four groups ie. control males, control females, ethanol males, ethanol females) followed the same adolescent binge ethanol dosing paradigm, and sample lysates (PND 43) were prepared and multiplexed on a fluorescent-PVDF membrane with beta-actin; there were no statistically significant differences in beta-actin mean values ( $p > 0.05$ ). We found no statistically significant differences in adolescent MBP expression between group means. We noted, as evidenced by the error bars, there was reasonably large variability within the treatment groups. Thus, we could not rule out the potential for tissue dissection variability. Variable tissue dissections would have altered the white matter present and possibly increased our sample-to-sample variation.

### **Fluorescence-based Western blot assays for MBP: PFC tissue (PND 84)**

Similarly, sample lysates (PND 84) were prepared and multiplexed on a fluorescent-PVDF membrane with beta actin; no statistically significant differences were present in beta actin mean values ( $p>0.05$ ). Protein expression was analyzed by quantifying total MBP peak intensities and the four distinct bands visualized on the blot to assess any difference in trends across the observed molecular weights (14 kDa; 17 and 18.5 kDa quantified as a doublet; 21.5 kDa). We note that these observed molecular weights were approximated with the Chameleon protein ladder and we consulted with the literature to confirm that these observed molecular weights matched the predicted molecular weights for the various isoforms.

Prior studies have observed two bands at the major classic isoforms 18.5 kDa and 14 kDa (Karthigasan et al 1996, Lewohl 2005). These isoforms of MBP are abundant in compact myelin. Thus, they have a suggested role in the formation and stabilization of myelin (Akiyama et al 2002; Karthigasan et al 1996). However, the minor isoforms (17 kDa, 21.5 kDa) may have a role in remyelination (Capello et al 1997). While further work is required to fully understand the functions of these various isoforms, we speculate that our slight but not significant trends toward decreased myelin protein expression (n.s.) seen in adolescent ethanol males

as compared to the slight (n.s.) trend towards increased myelin protein expression seen in adult ethanol males suggests possible recovery of myelin protein following abstinence. This potential recovery may involve the minor MBP isoforms, but further work is required to assess this possibility. In general, we observed similar slight (n.s.) trends across the different isoforms for MBP, specifically in the ethanol males following adolescent binge ethanol (**Figure 8**) that appeared to shift during adulthood (**Figure 9**). The ethanol females appeared to display relatively similar levels of myelin protein expression as compared to control females in adulthood (**Figure 8**, **Figure 9**), suggesting potential differences in PFC development.

#### **PLP immunoreactivity in C57BL/6J (untreated tissue)**

We were unable to study PLP protein levels through immunostaining due to concerns for nonspecific binding of endogenous murine immunoglobulins (see Appendix for **Supplemental Figures 1-2**). However, others have been able to successfully conduct immunohistochemistry studies with this proteolipid protein antibody (Buccinna, et al 2009). Thus, future experiments could employ the use of another PLP antibody that has been validated for reproducible, specific and selective binding of the target of interest. Additionally, others have improved

non-specific binding in PLP antibodies in murine studies through the use of an avidin-biotin complex (ABC) method and confirmed specificity with isotype controls (Goodpaster et al 2014). While our exact antibody was not used in the aforementioned study, their experimentation on numerous mouse based primary antibodies was rigorous and they replicated specific anti-mouse binding with minimal background. Thus, it is possible that repeat experiments using the ABC method along with isotype negative controls could allow for a better evaluation of PLP immunoreactivity.

### **Fluorescence-based Western Blot assays for PLP : PFC tissue (PND 43)**

Our experimental design remained the same (four groups i.e. control males, ethanol males, control females, ethanol females), dosed with 4g/kg of ethanol or water from PND 29-42, with tissue harvested at PND 43. Sample lysates were prepared, and the stripped fluorescent-PVDF membrane was blocked and subsequently probed with PLP antibody. All PLP mean values were normalized to beta-actin; beta-actin statistical analyses revealed no significant differences between means ( $p > 0.05$ ). We found no statistically significant differences in PLP relative abundance between groups. However, we observed that the mean relative abundance of PLP displayed the same slight (n.s.) trend seen in MBP. Specifically,

we noted that adolescent ethanol males appeared to show a small but not significant trend towards decreased PLP protein expression as compared to their control male counterparts (**Figure 10**). Ethanol females appeared to show a slight trend towards increased PLP protein expression (**Figure 11**). Here again, we believe these slight (n.s.) trends could be suggestive of possible differences in PFC development.

#### **Fluorescence-based Western Blot assays for PLP : PFC tissue (PND 84)**

Similar to the Western Blot assays for PLP at PND 43, the sample lysates (PND 84) from the adolescent binge ethanol exposure study were prepared, and the stripped fluorescent-PVDF membrane was then blocked and probed with PLP antibody. All PLP mean values were normalized to beta-actin; no statistically significant were different in mean actin values i.e.  $p > 0.05$ . While we found no significant differences in relative PLP abundance between our four groups. We noted that the adult ethanol animals displayed a trend towards increased PLP protein expression as compared to control animals.

Both experiments revealed a lack of persistent myelin protein alterations following adolescent binge ethanol exposure. This finding seen across our immunohistochemistry and Western blot assays may be evidence of white matter

recovery (Pfefferbaum et al 1995; Shear et al 1994; Lewohl et al 2005). However studies have found conflicting evidence on how ethanol exposure affects myelin related protein expression, specifically MBP and PLP, in the frontal cortex (Lewohl et al 2005; Samantaray et al 2015). In a human study, controls and uncomplicated alcoholics had no significant differences in myelin-related protein expression in the superior frontal gyrus and the primary cortex (Lewohl et al 2005). This result is notable because Lewohl et al previously found MBP showed decreased expression at the mRNA level in the superior frontal gyrus (Lewohl 2000). Thus, ethanol's molecular mechanisms may not be fully understood by step-wise analysis of mRNA encoded myelin-proteins.

Eukaryotic transcription and translation processes are physically separated by the nuclear membrane (Berg et al 2002), specifically transcription occurs inside the cell's nucleus, while translation occurs in the cytoplasm. This spatial separation introduces a temporal component, as transcription and translation will not occur in tandem as is the case for prokaryotes (Berg 2002). Thus, eukaryotes are able to regulate gene expression in a more intricate fashion, as the regulatory mechanisms between transcription and translation are distinct. We speculate that the different regulatory mechanisms in transcription and translation may be a possible

explanation for why our prior findings of significant decreases in myelin-related mRNA expression were not explicitly found at the myelin-protein level.

At the level of transcription, there is the possibility for epigenetic regulatory mechanisms that could affect mRNA expression. The potential alterations in mRNA expression may be short lived i.e. faster mRNA turnover rates whereas MBP and PLP have notably slower turnover rates (Sabri et al 1974). Thus, these differences in life spans may explain why we did not see the prior significant decreases in mRNA expression following binge ethanol manifest into significant decreases at the myelin-protein level. The potential for post-translational modifications should also be considered. MBP and PLP are notable for their longer life spans and both proteins undergo extensive post-translational modifications e.g. phosphorylation and prenylation to confer increased stability (Messier and Bizzozero 2000; Zhang et al 2012). Thus, it is logical that the increased stability of these myelin proteins made them less vulnerable to significant decreases in expression following adolescent binge ethanol. The slight (n.s.) trends towards decreased protein expression in adolescent ethanol males coupled with the small trend towards increased protein expression in adult ethanol animals, suggests that

ethanol may affect myelin protein expression, however future studies are needed to temporally assess when these potential alterations are occurring.

### **Sex Differences**

We noted no main effects of sex in the statistical analyses of our ethanol and control animals from our Western Blot assays and immunohistochemistry experiments. However, we observed that adolescent ethanol males displayed a small but not significant trend towards decreased myelin protein expression, specifically in MBP and PLP, as compared to control males (**Figure 8, Figure 10**); ethanol females were relatively similar to control females during adolescence (**Figure 8, Figure 10**). In adulthood, ethanol males appeared to show a slight trend towards increased MBP and PLP expression; adult females displayed relatively similar levels of MBP and PLP expression in ethanol and control groups (**Figure 9, Figure 11**).

We speculate that these slight trends may be suggestive of possible neurodevelopmental differences between the sexes. Prior studies have noted that adolescent males displayed decreased myelin density in the mPFC following binge ethanol (Richardson et al 2014) while adolescent females did not display notable myelin decreases following binge ethanol, suggesting sex differences (Richardson

et al 2019). These prior findings coupled with our noted trends within the adolescent and adult time points led to the assessment of basal myelin protein expression in control animals to further assess possible sex differences. Specifically, we performed a two-way ANOVA with sex and time point (PND 43, adolescent versus PND 84, adult) as factors to assess potential sex differences in basal MBP and PLP expression. Ultimately, we found that total basal MBP protein expression was significantly lower in adolescence (main effect of time point,  $p < 0.05$ , **Figure 12A**), reiterating that adolescence is a critical neurodevelopmental period. We also found an interaction effect between sex and time point in basal PLP expression where adult females had significantly greater levels of PLP expression as compared to adult males and adolescent females (**Figure 12B**). This increased protein expression in adult females further suggests possible neurodevelopmental differences between the sexes.

The adolescent transitional period encompasses the sexual maturation period i.e. puberty (Vetter-O'Hagen and Spear 2012). Prior studies have found that females may experience sexual maturation relatively faster than males (Richardson et al 2019) and earlier studies have found that introducing ethanol may delay sexual maturation in females (Dees and Skelley 1990; Emanuele et al 2002). Thus,

we sought to assess if ethanol affects sexual maturation in both sexes. We assessed all ethanol and control animals for external signs of puberty onset and observed that females tended to display signs slightly earlier than males (**Figure 13** versus **Figure 14**). We also observed that ethanol animals appeared to show external signs of puberty onset relatively slower than control animals in both sexes (**Figure 13-14**).

To specifically assess for statistical differences in mean PND age at puberty onset, we performed a two-way ANOVA with sex and treatment as factors. We found that ethanol animals displayed external signs of sexual maturation at relatively later PND ages (**Figure 15**). We also found that females displayed external signs of puberty onset at an earlier age than males (**Figure 15**). These findings coupled with our findings from relative basal MBP and PLP expression may imply that males are more susceptible to ethanol insults as compared to females. However, further work is required to temporally assess this directly.

Our lab previously found that binge ethanol exposure leads to reduced myelin-related gene expression, in genes including *Mbp* and *Plp* immediately after the adolescent period (PND 43) (Wolstenholme et al 2017). Specifically, microarray data found that binge ethanol exposure significantly decreased *Mbp* and

*Plp* gene expression in adolescent ethanol males compared to control males. Thus, we may have replicated this finding at the myelin-related protein level as we have noted slight but not significant trends for males to have decreased MBP and PLP protein expression immediately following binge ethanol exposure. In addition, our western blot data at the persistent time point (PND 84), displays a small trend toward increased relative myelin protein abundance (n.s.) in ethanol males as compared to control males. These slight trends in myelin protein expression following binge ethanol may indicate possible recovery of myelin protein expression following abstinence which is consistent with prior studies (Pfefferbaum 1995; Shear 1994) as well as our microarray results at the mRNA level (Wolstenholme et al 2017). When we consider our significant findings in basal myelin protein expression as well as puberty onset, we note that potential myelin protein recovery may vary between the sexes and possess a temporal component. We speculate that these findings may provide the framework for follow up studies to temporally assess when myelin related protein expression recovers after binge ethanol exposure and how these potential structural alterations in myelin protein differ between the sexes.

## **Study Limitations**

In experiment one, quantification of MBP protein was completed using the uncorrected integrated densities and images were not background corrected. The rationale for this method quantification was due to the fact that the image acquisition included vast fluctuations in background, thus the calculated CTCF was not used. These fluctuations in background were, in part, due to variable staining across sections but also damaged sections following tissue processing. The experimenter avoided regions of excessive damage, highly fluorescent areas that were not characteristic of our target protein, and areas with excess mounting media to ensure that data would not be skewed in any particular direction. However, in excluding these areas, consistent quantification of the immunostained sections was not completed as rigorously. Thus, only 3 representative sections per animal were used. In general, quantification of immunostained images (despite experimenter being blinded to sex, treatment and time point) were subject to a small degree of bias that could have affected the data and subsequent results.

Regarding the PLP immunoreactivity studies, the PLP anti-mouse antibody displayed non-specific binding despite the use of a M.O.M immunodetection kit, which led us to only assess MBP immunoreactivity through semi-quantitative

immunohistochemistry. Experiment 1 would have benefited from testing other sources for a PLP antibody or from analyzing other myelin related protein markers such as myelin associated glycoprotein (MAG), or myelin associated oligodendrocyte basic protein (MOBP). Both of the aforementioned myelin proteins were notable in that the ethanol males showed a robust decrease in mRNA levels for the genes *Mobp* and *Mag* (Wolstenholme et al 2017). Thus, more conclusions of ethanol exposure on myelin-related protein could be formulated because of the assessment of other proteins that play different functional roles in myelin formation and maintenance.

In experiment two, MBP and PLP protein expression were studied using tissue samples from prior studies collected several years ago. While these samples were maintained at proper temperature conditions, it is possible that fresher tissue may have yielded less variable results. In addition, when studying MBP levels, the same PVDF membranes were first probed with MBP, stripped, and reprobed with PLP. We compared our observed molecular weights to the predicted values for each protein and normalized these values to a loading control. We visually observed that PLP displayed a lower signal compared to MBP at both time points (PND 43, **Figures 8 vs Figure 10**; PND 84, **Figures 9 vs Figure 11**, see Appendix

for supplemental Western data). Fluorescent PVDF membranes are considered more robust than nitrocellulose membranes and are recommended for protocols involving antibody stripping (Bass et al 2016). However, it is possible that the decreased PLP signal may be attributed to the stripping reagents. This possibility would affect our western blotting results.

These western blot experiments also did not include a negative control, presence of a negative control (i.e. a sample lysate from a cell line devoid of myelin) may have allowed for improved comparison of the samples and confirm that our commercial antibodies were not displaying non-specific binding that would skew our results. Our collaborators in the Dr. Miles' laboratory routinely use these antibodies under the same conditions and have validated their specificity. Additionally, they have found MBP and PLP perform at similar molecular weights as we have reported. An additional study limitation for our Western Blot assays was the potential for tissue dissection variability. While we attempted to optimize our sample selection through the BCA assay, we must consider the potential for human error. Thus, some PFC samples may have possessed more white matter than others, this could also account for the sample-to-sample variation observed in our fluorescent-blots.

Our analysis of sex differences included the tracking of pubertal onset/progression in the second cohort of animals to assess if ethanol treatment affects sexual maturation. However, an improved experimental design would include these assessments in all animals being tested. In addition, future studies should consider other measures for comparisons between sexes. For example, we subjectively determined “partial puberty progression in males” (corresponding to the numeric score of 2) and used this metric to track the number of males in a pubertal state. However, this method was subject to a high level of bias, coupled with experimenter inexperience (although the experimenter’s confidence in assessments improved over time). Thus, future male puberty assessments should consider assessing for external signs of the balano-preputial separation, others have reported published findings using this assessment to correlate with histological slides in preclinical studies with rats (Gaytan et al 1988, Korebrot et al 1977)

### **Future Directions**

Immunohistochemistry in combination with fluorescence-based Western blots with fresher PFC tissue should be repeated to assess if these thesis findings are replicable at the protein level. PLP anti-mouse antibody experiments using a

commercial M.O.M kit should be repeated to assess if non-specific binding is still present, however future studies should include an isotype control to further validate the antibody's specificity. Experimenters should also consider including an antigen retrieval step with future PLP immunoreactivity studies, while our antigen is robust against tissue fixation, this additional step may yield more improved and consistent staining results. A comparative experiment using the PLP anti-mouse antibody with the ABC system in the M.O.M kit and a biotin-free system should also be performed to see if there is a difference in the level background observed (Goodpaster et al 2014). Western blot experiments should include negative controls and a comparative analysis should be done looking at MBP and PLP protein levels following a stripping protocol versus MBP and PLP being probed on separate blots. These experiments will provide further information on the technical effects of stripping reagents. Western blot experiments should consider including another myelin-related protein target e.g. myelin associated protein MAG to assess if protein levels after binge ethanol follow similar patterns of alteration in myelin-related gene expression.

Future studies should also consider analyzing protein levels at various time points within adulthood to assess when this potential recovery is occurring e.g.

PND 42, PND 50, PND 66, PND 84. These experiments will assess the protein level changes. In addition, future studies should consider analyzing the OPC and mature oligodendrocyte myelin ultrastructures through electron microscopy, as assessing these myelin producing cells for potential alterations after binge ethanol will provide further information, upstream of myelin protein expression. Future work should incorporate behavioral assays such as the novel object recognition task (Wolstenholme et al 2017) to assess potential PFC-mediated, behavioral effects of altered myelin-related protein expression, as they will be correlated with the molecular results. Additional behavioral assays should assess social recognition as preclinical studies have implicated PFC in social cognition (Avale et al 2011; Finlay et al 2015). The behavioral assays coupled with the molecular studies may uncover a mechanism by which ethanol acts to alter myelin related protein. These future studies may allow for the uncovering of a mechanism of myelin recovery following binge ethanol exposure and will have excellent translational merit.

## **Conclusion**

In these studies, we found a slight (n.s.) trend towards decreased myelin-related (MBP, PLP) protein expression in adolescent ethanol males that appeared to recover in adulthood. These findings are notable because we

previously showed adolescent binge ethanol exposure leads significantly decreased myelin-related gene expression (including *Mbp*, *Plp*) (Wolstenholme et al 2017). While we did not find significant alterations in myelin-related protein expression following binge ethanol, we found that basal MBP expression is significantly lower during adolescence and that basal levels of PLP expression are significantly greater in adult females as compared to adolescent females and adult males. Additionally, we found that ethanol delays sexual maturation and males generally display signs of puberty onset later than females. Taken together, we may have found potential structural alterations in myelin protein expression following binge ethanol exposure that may not necessarily manifest into decreased myelin protein expression along with possible evidence of differences in PFC development between the sexes. This assessment of myelin-related protein expression will lay the foundation for follow up studies that will further investigate how ethanol mechanistically affects myelin in the CNS.

## **Appendix**

### **Supplemental Methods**

#### **Tissue harvest**

PBS recipe- 1.82 L of 1X phosphate buffered saline (PBS) solution from 10X stock, 1.8 L MilliQ water, 200 mL 10X PBS stock solution, VitaScientific, Beltsville, MD, catalogue # QBIC20044; 10X PBS recipe includes 1.78 g of 100 mM  $\text{Na}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 0.24 g of 27 mM  $\text{KH}_2\text{PO}_4$ , 8 g of 1.37 M NaCl, 0.2 g of 27 mM; 400 mL MilliQ water, total volume of 3 L)

#### **Cryosectioning**

Cryostat machine (Leica CM 1950, Leica Biosystems, Buffalo Grove, IL) was used for all cryosectioning. Cryostat object temperature and chamber temperature were set to -16°C and -19°C respectively. Frozen tissue was placed in the cryostat machine for approximately twenty minutes to calibrate to set temperatures. Tissue was then oriented to the dorsolateral view and cerebellum was removed using a cryostat blade (Leica Biosystems, Buffalo Grove, IL, catalogue # 14035838382). Optimal cutting temperature (OCT) compound (Fisher Scientific,

Waltham, MA, catalogue # 4583) was then applied to tissue on cryostat chuck and placed in the cryostat machine to readjust to temperatures. Coronal sections with 25 micron thickness were collected and placed serially into 12 well plates containing a PBS and sodium azide solution (1X PBS; 0.02% w/v sodium azide, Sigma Aldrich, St Louis, MO, catalogue # S2002-25G). Tissue was sectioned from the area just past the olfactory bulb to approximately -2.30 mm from bregma. The 12-well plate was then labeled with animal identification number, study name, section thickness and date. All 12 well plates were wrapped with parafilm and stored at 4°C.

### **Myelin Basic Protein (MBP) immunostaining**

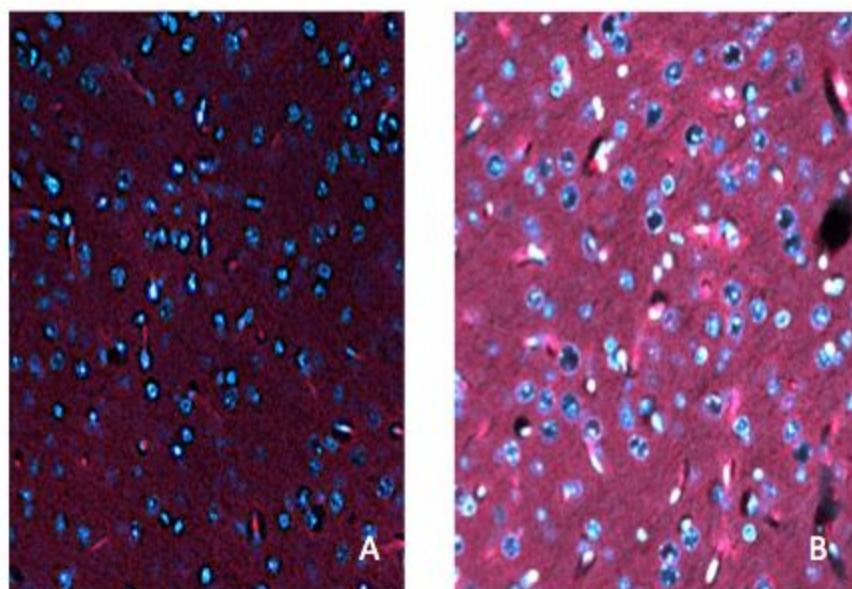
Sections were washed in 1X PBS twice, for five minutes each wash. Sections were then blocked in 1X-PBS-T (5% normal goat serum, Jackson ImmunoResearch, West Grove, PA, catalogue # 005-000-121; 0.5% Triton X 100; 1X PBS solution) for thirty minutes at room temperature with gentle agitation. Sections incubated in MBP anti-rat primary antibody solution with 400 ul per well (1:1000, Abcam, Cambridge, UK, catalogue # 7349; 3.2 mL 1X PBS-T; 3% normal goat serum) overnight at 4 °C with gentle agitation. Sections were subsequently washed three times in 1X PBS for ten minutes each with gentle

agitation. Sections were then incubated in the Invitrogen Alexa Fluor 594 goat anti-rat secondary antibody solution (1:1000, Thermo Fisher, Waltham, MA, catalogue # A11007; 0.5% Triton X 100; 3.2 mL 1X PBS) for two hours with gentle agitation and protected from light. Sections were then washed in 1X PBS three times for ten minutes each wash and stored in 1X PBS at 4 °C until mounting. Each animals' sections were placed in a separate indent free container containing PBS and transferred to a Fisherbrand Superfrost microscope slide (Fisher Scientific, Waltham, MA, catalogue # 12-55-0-143).

Sections were organized into columns with the most rostral section first and the most caudal section last. Excess 1X PBS was pipetted from the microscope slide and slides were left to dry for a short period. Vectashield Vibrance Antifade mounting medium with DAPI (VECTOR Laboratories, Burlingame, CA, catalogue # H-1800) was used to mount sections further, with 50 ul added to each slide. Slides were coverslipped with Corning Cover Glass rectangle coverslips (Corning, Corning, NY, catalogue # 2980-245) and sealed with clear nail polish. Sections for one ethanol male animal were destroyed during the tissue mounting portion of the experiment, leading to an N = 63 for the immunohistochemistry study.

## **Myelin Proteolipid (PLP) immunostaining**

We previously determined at 1:250 was the optimal concentration with a strong fluorescent signal (without excess saturation) and minimal background. However, we conducted follow up negative control experiments using secondary antibody only (1:1000) on C57BL/6J (untreated) coronal sections and observed high amounts of non-specific binding (**Supplemental Figure 1**)



**Supplemental Figure 1. Specificity of PLP Antibody in C57BL/6J brain tissue**

Panel **A** shows PLP 1:250 in red , Panel **B** shows negative control Alexa Fluor 594 goat anti-mouse; DAPI counterstaining of nuclei in blue

### **PLP antibody with Mouse on Mouse (M.O.M) Immunodetection reagents**

Due to the fact that using a goat anti-mouse secondary antibody may lead to the binding of endogenous mouse immunoglobulins, we purchased a M.O.M kit and conducted a PLP (+/-) M.O.M kit experiment with proper negative controls (sections stained with only secondary antibody without M.O.M. kit reagents, sections stained with only secondary antibody and M.O.M. kit reagents). Ultimately, both negative control slides revealed similar levels of non-specific binding (**Supplemental Figure 2**). The concern for inaccurate localization of PLP immunoreactivity and quantification led us to only assess MBP immunoreactivity through immunohistochemistry.

### **Plate 1- M.O.M reagents with PLP antibody solutions**

Three sections (C57BL/6J untreated practice tissue) per well were placed into four wells of a 12-well plate (two plates were used, M.O.M reagents were used on sections in plate 1 and no M.O.M reagents were used on sections in plate 2). All sections were washed in 0.01 M PBS twice, for five minutes each wash. Sections were then blocked in a blocking buffer (PBS with 0.5% Triton X 100; 3% normal goat serum) for thirty minutes with gentle agitation at room temperature. Sections in plate 1 were then incubated in the M.O.M IgG Blocking Reagent (90 ul M.O.M

IgG Blocking Reagent, 2.5 mL PBS) for 1 hour at room temperature. Sections in plate 1 were washed in PBS twice, for two minutes each wash. Following this step, sections incubated in the M.O.M Diluent (600 ul M.O.M protein concentrate stock into 7.5 mL PBS) for five minutes.

Primary antibody solutions (1:250 PLP anti-mouse, 600 ul M.O.M protein concentrate stock, 7.5 mL PBS-T; 1:500 PLP anti- mouse, 600 ul M.O.M protein concentrate stock, 7.5 mL PBS-T) were made, with 400 ul of each solution placed into separate wells (2 wells total). The negative control wells were each filled with 400 ul PBS-T. The 12 well plate was stored at 4 °C for overnight incubation with gentle agitation. All sections from plate 1 were incubated in the prepared M.O.M Biotinylated Anti-Mouse IgG Reagent (10 ul M.O.M. biotinylated Anti-Mouse IgG reagent in 2.5 mL PBS) for ten minutes. Sections from plate 1 were washed in PBS-T twice for five minutes each wash.

Sections from plate 1 all four wells were then incubated in 400 ul of secondary antibody solution (1:1000 secondary antibody; 600 ul M.O.M protein concentrate stock, 7.5 mL PBS-T) with gentle agitation at room temperature (light protected) for two hours. Sections were washed in PBS twice for five minutes each

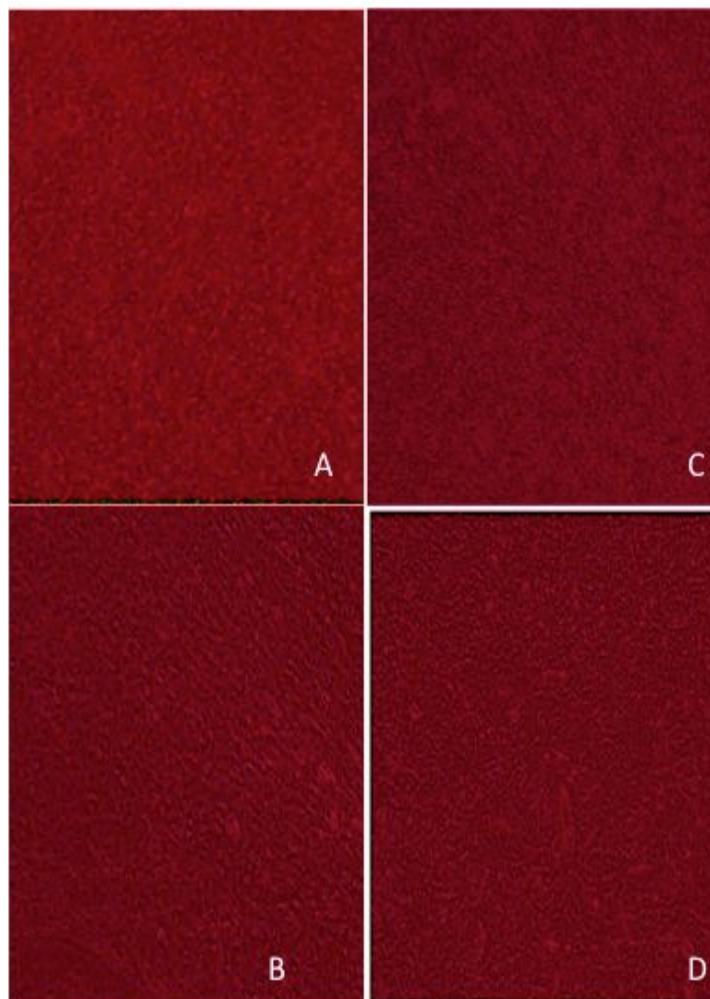
wash. Sections were then mounted onto slides and coverslipped in the same manner as the MBP immunostained sections.

Plate 2- PLP antibody solutions without M.O.M reagents

Three sections (C57BL/6J untreated practice tissue) per well were placed into four wells of a 12-well plate (two plates were used, M.O.M reagents were used on sections in plate 1 and no M.O.M reagents were used on sections in plate 2). All sections were washed in PBS twice, for five minutes each wash. Sections were then blocked in a blocking buffer (PBS-T, 3% normal goat serum) for thirty minutes with gentle agitation at room temperature.

Sections in plate 2 had 400 ul of primary antibody solution placed into each of the two wells (PLP 1:250, PLP 1:500, PBS-T, 3% normal goat serum) and the negative control sections had 400 ul of PBS-T placed into the remaining two wells, plate 2 was stored at 4°C for overnight incubation with gentle agitation. Sections from plate 2 were washed in PBS-T three times, ten minutes per wash. Sections from plate 2 were incubated in a secondary antibody solution (1:1000 secondary antibody, PBS-T, 3% normal goat serum) for two hours (protected from light) on a gentle rocker. Sections from plate 2 were washed in PBS three times for ten

minutes each wash. Sections were then mounted onto slides and coverslipped in the same manner as the MBP immunostained sections.



**Supplemental Figure 2. PLP immunoreactivity +/- M.O.M kit reagents**

Panel **A** displays PLP 1:250 concentration with no M.O.M reagents; Panel **B** displays PLP negative control (secondary antibody, 1:1000 only; no M.O.M reagents); Panel **C** displays PLP 1:250 with M.O.M. reagents; Panel **D** displays PLP negative control with M.O.M reagents (secondary antibody, 1:1000 only). There are minimal differences in PLP immunostained sections with and without M.O.M reagents. Images taken at 10X objective.

### **Image acquisition and quantification for MBP immunoreactivity**

Images were acquired using the Olympus DP74 microscope (Olympus Life Science, Tokyo, Japan) with cellSense Standard software (Olympus Life Science, Tokyo, Japan). Each section had a representative image taken of both hemispheres in the Cy3 light at the 10X objective (scale bar burned in). Images for quantification were taken at the 20X objective with a constant exposure of 24.9 milliseconds and the Super Fluorescence (SFL) setting for all images. DAPI counterstain of the CNS nuclei (light 1 on Olympus DP74) was observed to ensure proper localization of myelinated axonal processes that were characteristic of MBP staining (Ortiz et al 2019; Pajooohesh-Ganji and Miller 2019) Consistent image acquisition was completed with two images taken for each hemisphere of a section and at least 12 images were taken per mouse. Sections ranged from 1.40 mm distance from bregma to 0.50 mm from bregma; no section imaged included the corpus callosum.

Exclusion criterion for quantification included sections with severe damage to the PFC region. Only 10X images were taken of these damaged sections for data records. Following image acquisition, 3 representative sections were chosen for each of the 63 animals and Allen Mouse Brain atlas ([brain-map.org](http://brain-map.org)) was

referenced to ensure that region of interest was present in the field of view. All images were quantified using ImageJ software with raw integrated density and mean grey value measurements taken for each image.

The uncorrected integrated densities for each section (two 20X images per hemisphere, four 20X images per section) were summed followed by the addition of each section to determine the total integrated density for each animal. Experimenter was blinded to sex, treatment, and time point of the animals during the image acquisition and image quantification process. Following the quantification, three mice from the persistent time point including one control male and two ethanol males were removed because of excess damage to the region of interest leading to N = 60 for the immunohistochemistry study.

### **Western Blots**

The BCA protein assay was performed using Pierce BCA Assay kit (Thermo Fisher, Waltham, Massachusetts, catalogue # 23227) in order to calculate protein concentration (Walker 1994). The expected color change to light green occurred once the working reagent was added to all wells. The plate reader measured absorbance at 450 nm and 630 nm respectively. The replicate values from the 450 nm wavelengths were averaged and the mean standard values versus their

respective concentrations were plotted to generate a standard curve on Microsoft excel; the standard curve equation was used to determine the concentration of protein based on serial dilutions of a standard BSA reagent and 10 ug of total protein was calculated for sample and to be loaded into the gel (with loading dye and reducing reagent).

After sample preparation, Thermo Fisher mini gel tank chambers (Thermo Fisher, Waltham, MA, catalogue #A25977) were each filled with 200 mL of 1X MES SDS Running Buffer (50 mL 20X MES Bolt™ MES SDS Running Buffer, Thermo Fisher, Waltham, MA, catalogue # B000202; 950 mL MilliQ water). Following this step, 500 ul of NuPage Antioxidant (Thermo Fisher, Waltham, MA, catalogue # NP0005) was added to each chamber to prevent reoxidation of protein samples during the gel electrophoresis and transfer steps. NuPage 4-12% Bis-Tris Protein gels (Thermo Fisher, Waltham, MA, catalogue # NP0329BOX) were loaded with protein samples. Following the sample loading, the Chameleon™ Kit Prestained ladder/molecular weight marker was loaded with 2.5 ul of 700 nm channel and 2.5 ul of 800 nm channel in order to estimate the unknown protein quantities (LICOR, Lincoln, NE, catalogue # P/N: 928-90000). Gel electrophoresis was run at 150 V for approximately 45 minutes using the Thermo Fisher mini gel

tank apparatus and Bio-Rad PowerPac Basic power supply (Bio-Rad Laboratories, Hercules, CA, catalogue # 1645050).

1X tris-buffered saline (TBS) recipe- (100 mL 10X TBS buffer solution in 900 mL MilliQ-water; 10X TBS recipe includes 2.4 g of 200 mM Tris Base, 8.8 g of 1500 mM NaCl).

### **LICOR immunodetection imaging and ImageJ quantification**

Membranes were imaged using the LICOR detection system and Odyssey software with the LICOR scan preset to “membrane” and intensities set to 4 and 8 for the 700 nm and 800 nm channels respectively. All LICOR imaging scans were saved with the Analysis folder containing 700 and 800 TIFF files. TIFF files from the Analysis folder were downloaded into ImageJ (NIH, Bethesda Maryland) and analysis of signal intensity was used by measuring the area of the peak intensities; the band intensity corresponded to peak height and area of each of the peaks were recorded. Additionally, the area of the loading control peak intensities were recorded and all target signal peak areas were normalized to the loading control protein (beta-actin).

### **Stripping procedure for fluorescent-PVDF membranes**

Following the imaging, membranes were stripped of antibodies using 1X dilution of 5X NewBlot IR Stripping buffer for NIR Western Blots (LICOR; Lincoln, NE; catalogue # P/N: 928-40028), membranes incubated in stripping solution for twenty minutes at room temperature with gentle agitation. Following the stripping period, membranes were washed in 1X TBS three times. Membranes were then subsequently probed with the appropriate LICOR secondary antibodies and light protected during a one hour incubation at room temperature with gentle agitation. Membranes were then rinsed in 1X TBS-T four times for five minutes each (with gentle agitation) followed by a brief rinse in 1X TBS. Using the LICOR infrared detection system and Odyssey software, membranes were scanned to detect any presence of primary antibody.

Once membranes were sufficiently stripped of previous target protein, membranes were blocked with Intercept Blocking buffer for 15 minutes followed by overnight incubation with PLP anti-mouse primary antibody (1:5,000; 0.2% Tween-20; 0.02% w/v sodium azide). Membranes were then rinsed with 1X TBS-T followed by four washes in 1X TBS-T (five minutes each wash) at room temperature with gentle agitation. Membranes were then probed with IRDye 800

CW Goat anti-Mouse Secondary Antibody (1:30,000; LICOR, Lincoln, NE, catalogue # P/N: 926-32210; 0.2% Tween-20; 0.02% SDS) and incubated in the secondary solution for one hour (light-protected). Membranes were then washed with 1X-TBS-T and stored in 1X TBS prior to imaging with LICOR detection system and Odyssey software. Imaging and subsequent quantification were completed in the same manner as the MBP anti-rat blots. PLP anti-mouse blots were normalized to actin area values from initial ImageJ quantification of MBP and actin peak intensities.

### **Supplemental Western Blot Data for MBP and PLP protein expression**

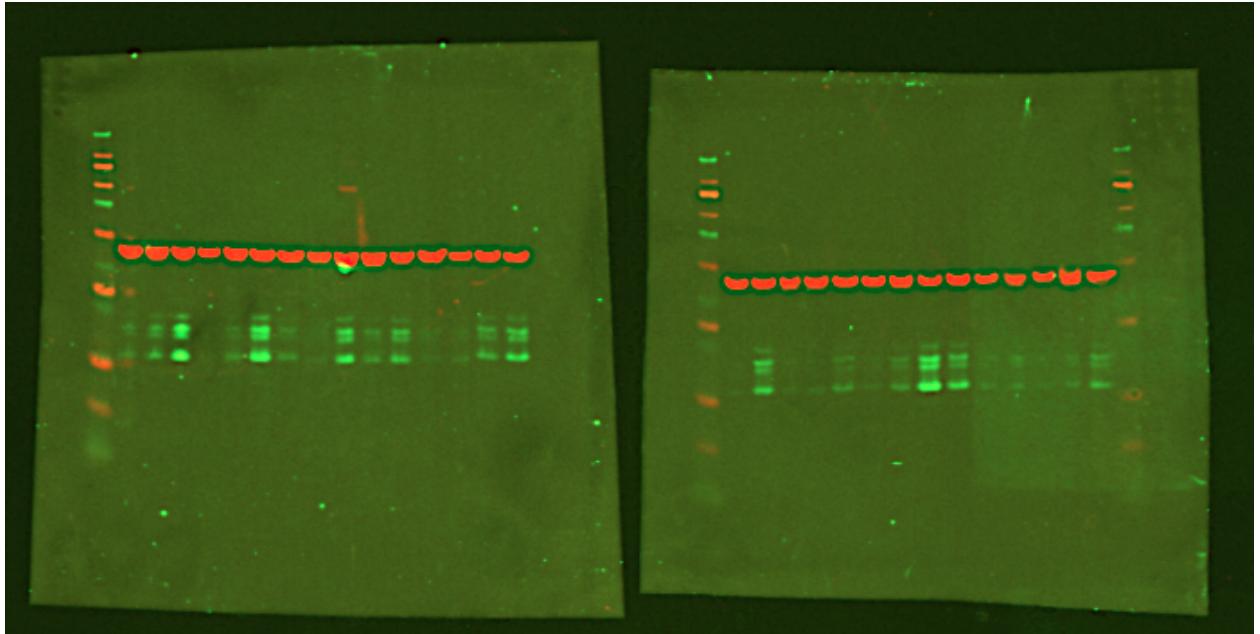
Here, we present the fluorescent-PVDF membranes with MBP and PLP expression for the adolescent (PND 43) and adult (PND 84) time points. We note that two membranes with adolescent sample lysates (PND 43) were first probed with MBP as well beta-actin antibodies followed by a stripping procedure and reprobed with PLP antibody. Similarly, to assess MBP and PLP relative abundance at the persistent time point, two membranes with adult sample lysates (PND 84) were first probed with MBP and beta-actin antibodies, stripped and reprobed with PLP.

We first present the ordering of the PFC sample lysates, including the molecular weight marker (MWM); we bold the **MWM** term when appropriate to indicate a greater volume of the Chameleon protein ladder present. Following the ordering of the samples, we present the images for MBP and PLP relative abundance. We start with MBP immunoreactivity at the adolescent time point (PND 43, **Supplemental Figure 3**) followed by the adult time point (PND 84, **Supplemental Figure 4**), we end with PLP immunoreactivity at both time points(PND 43, **Supplemental Figure 5** and PND 84, **Supplemental Figure 6**). We note that the ordering of the samples are presented such that the samples on the

left column correspond to the left membrane, likewise for the right column and right membrane.

**Supplemental Figure 3: MBP relative abundance following adolescent binge ethanol (PND 43)**

lane	sample	lane	sample
1	MWM	1	<b>MWM</b>
2	73 EF	2	35 CM
3	11 CM	3	7 CM
4	12 CM	4	15 EM
5	75 EF	5	22 EM
6	79 CF	6	62 CF
7	32 CM	7	42 CF
8	59 CF	8	46 EF
9	4 EM	9	74 EF
10	64 CF	10	55 CF
11	23 EM	11	37 EM
12	63 CF	12	8 CM
13	66 EF	13	68 EF
14	26 EM	14	27 EM
15	10 CM	15	56 CF
16	16 EM	16	MWM

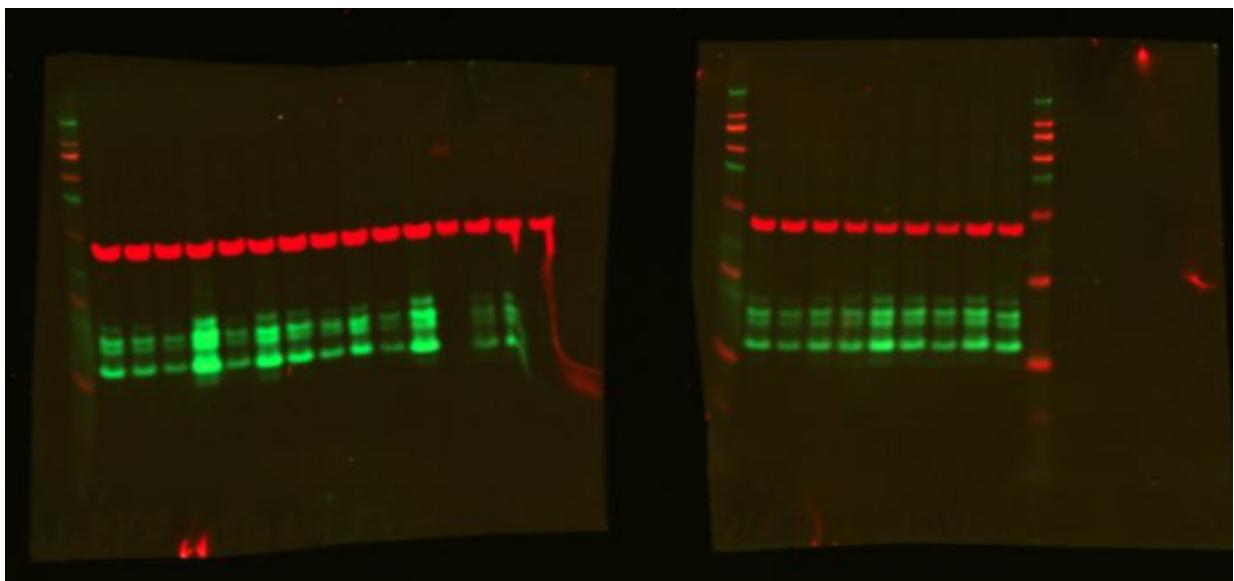


**Supplemental Figure 3. MBP relative abundance following adolescent binge ethanol (PND 43)**

Adolescent PFC sample lysates immunostained with anti-PLP antibody. We see sample-to sample variation that is not due to inconsistent sample loading. Two-way ANOVA with sex and treatment as factors revealed no significant differences.

**Supplemental Figure 4: MBP relative abundance following adolescent binge ethanol (PND 84)**

lane	Left gel sample	Right gel lane	sample
1	MWM	1	MWM
2	11.2 CM	2	2.5
3	6.3 CM	3	3.1
4	5.1 EM	4	9.4
5	10.3 EM	5	8.3
6	4.3 CF	6	14.1
7	17.1 CF	7	19.1
8	12.1 EF	8	7.2
9	20.1 EF	9	6.4
10	4.2 CF	10	18.4
11	18.1 EM	11	<b>MWM</b>
12	15.4 CM		
13	9.2 EF		
14	13.4 CM		
15	14.2 EF		
16	19.4 CF		



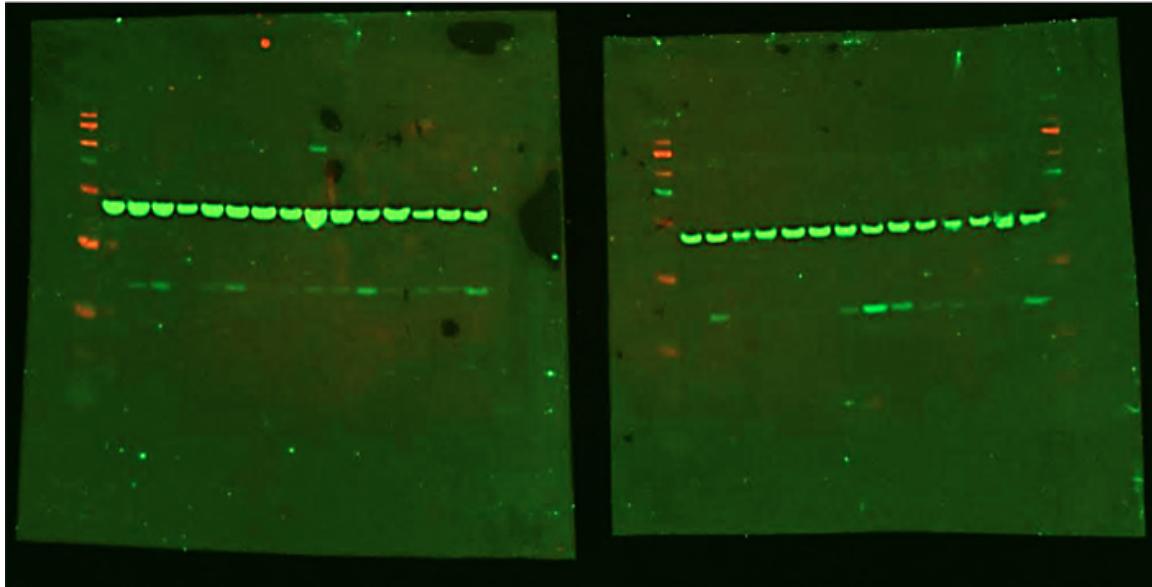
**Supplemental Figure 4. MBP relative abundance following adolescent binge ethanol (PND 84)**

Adult PFC sample lysates immunostained with anti-PLP antibody. We see sample-to-sample variation that is not due to inconsistent sample loading. Two-way ANOVA with sex and treatment as factors revealed no significant differences.

**Supplemental Figure 5: PLP relative abundance following adolescent binge ethanol (PND 43)**

Left gel      Right gel

lane	sample	lane	sample
1	MWM	1	<b>MWM</b>
2	73 EF	2	35 CM
3	11 CM	3	7 CM
4	12 CM	4	15 EM
5	75 EF	5	22 EM
6	79 CF	6	62 CF
7	32 CM	7	42 CF
8	59 CF	8	46 EF
9	4 EM	9	74 EF
10	64 CF	10	55 CF
11	23 EM	11	37 EM
12	63 CF	12	8 CM
13	66 EF	13	68 EF
14	26 EM	14	27 EM
15	10 CM	15	56 CF
16	16 EM	16	MWM

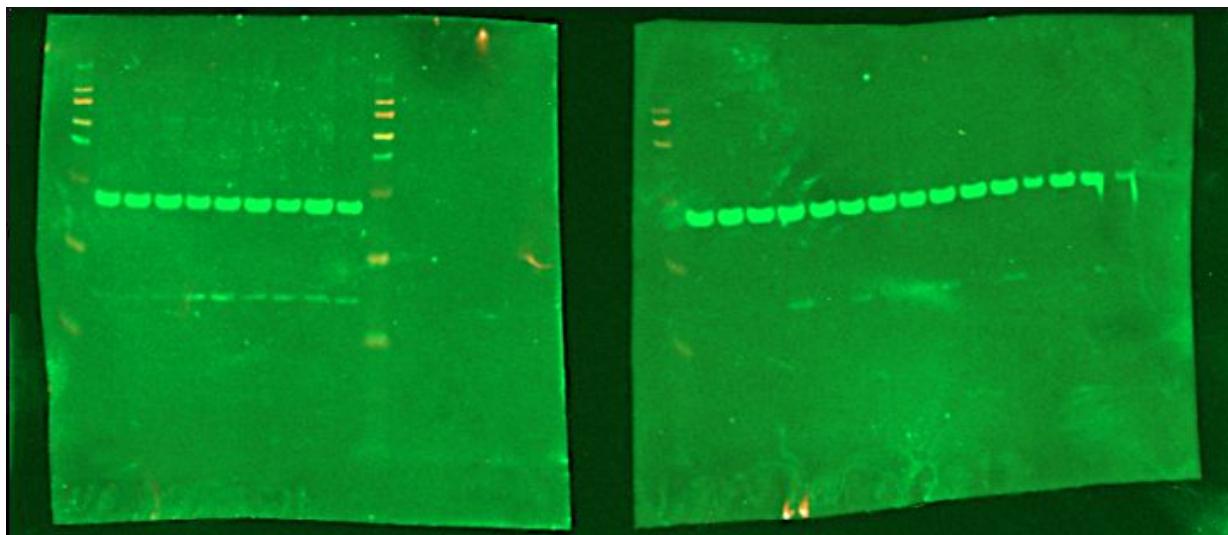


**Supplemental Figure 5. PLP relative abundance following adolescent binge ethanol (PND 43)**

Adolescent PFC sample lysates immunostained with anti-PLP antibody. We see sample-to-sample variation that is not due to inconsistent sample loading. Two-way ANOVA with sex and treatment as factors revealed no significant differences.

**Supplemental Figure 6: PLP relative abundance following adolescent binge ethanol (PND 84)**

lane	Left gel sample	Right gel lane	sample
1	MWM	1	MWM
2	2.5	2	11.2 CM
3	3.1	3	6.3 CM
4	9.4	4	5.1 EM
5	8.3	5	10.3 EM
6	14.1	6	4.3 CF
7	19.1	7	17.1 CF
8	7.2	8	12.1 EF
9	6.4	9	20.1 EF
10	18.4	10	4.2 CF
11	<b>MWM</b>	11	18.1 EM
		12	15.4 CM
		13	9.2 EF
		14	13.4 CM
		15	14.2 EF
		16	19.4 CF



**Supplemental Figure 6. PLP relative abundance following adolescent binge ethanol (PND 84)**

Adult PFC sample lysates immunostained with anti-PLP antibody. We see sample-to-sample variation that is not due to inconsistent sample loading. Two-way ANOVA with sex and treatment as factors revealed no significant differences.

## **References**

1. Akiyama, Kyoichi et al. "Study of Expression of Myelin Basic Proteins (MBPs) in Developing Rat Brain Using a Novel Antibody Reacting with Four Major Isoforms of MBP." *Journal of Neuroscience Research*, 2002, doi:10.1002/jnr.10188.
2. "Alcohol Facts and Statistics." *National Institute on Alcohol Abuse and Alcoholism*, U.S. Department of Health and Human Services, 18 Feb. 2020, [www.niaaa.nih.gov/publications/brochures-and-fact-sheets/alcohol-facts-and-statistics](http://www.niaaa.nih.gov/publications/brochures-and-fact-sheets/alcohol-facts-and-statistics).
3. Arain, Mariam, et al. "Maturation of the Adolescent Brain." *Neuropsychiatric Disease and Treatment*, 2013, p. 449., doi:10.2147/ndt.s39776.
4. Auta, James, et al. "Chronic Alcohol Exposure Differentially Alters One-Carbon Metabolism in Rat Liver and Brain." *ENeuro*, 2017, doi:10.1111/acer.13382.
5. Banerjee, Niladri. "Neurotransmitters in Alcoholism: A Review of Neurobiological and Genetic Studies." *Indian Journal of Human Genetics*, 2014, doi:10.4103/0971-6866.132750.
6. Barnea-Goraly, Naama et al. "White Matter Development during Childhood and Adolescence: A Cross-Sectional Diffusion Tensor Imaging Study." *Cerebral Cortex*, 2005, doi:10.1093/cercor/bhi062.
7. Bass, J. J., et al. "An Overview of Technical Considerations for Western Blotting Applications to Physiological Research." *Scandinavian Journal of Medicine & Science in Sports*, vol. 27, no. 1, 2016, pp. 4–25., doi:10.1111/sms.12702.
8. "Binge Drinking Is a Serious but Preventable Problem of Excessive Alcohol Use." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 30 Dec. 2019, [www.cdc.gov/alcohol/fact-sheets/binge-drinking.htm](http://www.cdc.gov/alcohol/fact-sheets/binge-drinking.htm).
9. Blanca, Maria et al. "Datos No Normales: ¿Es El ANOVA Una Opción Válida?" *Psicothema*, 2017, doi:10.7334/psicothema2016.383.
10. Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: W H Freeman; 2002. Section 28.2, Eukaryotic Transcription and Translation Are Separated in Space and Time.
11. Boggs, J. M. "Myelin Basic Protein: A Multifunctional Protein." *Cellular and Molecular Life Sciences*, 2006, doi:10.1007/s00018-006-6094-7.
12. Brodie, Mark S. "Ethanol Directly Excites Dopaminergic Ventral Tegmental Area Reward Neurons." *Alcoholism: Clinical and Experimental Research*, 1999, doi:10.1111/j.1530-0277.1999.tb04082.x.
13. Buccinnà, Barbara, et al. "Alterations of Myelin-Specific Proteins and Sphingolipids Characterize the Brains of Acid Sphingomyelinase-Deficient Mice, an Animal Model of Niemann-Pick Disease Type A." *Journal of Neurochemistry*, doi:10.1111/j.1471-4159.2009.05947.x.
14. Byers, Shannon L et al. "Mouse Estrous Cycle Identification Tool and Images." *PLoS ONE*, 2012, doi:10.1371/journal.pone.0035538.

15. Capello, Elisabetta, et al. "Multiple Sclerosis: Re-Expression of a Developmental Gene in Chronic Lesions Correlates with Remyelination." *Annals of Neurology*, vol. 41, no. 6, 1997, pp. 797–805., doi:10.1002/ana.410410616.
16. Cerghet, Mirela et al. "Sexual Dimorphism in the White Matter of Rodents." *Journal of the Neurological Sciences*, 2009, doi:10.1016/j.jns.2009.06.039.
17. Champlin, A.K., et al. "Determining the Stage of the Estrous Cycle in the Mouse by the Appearance of the Vagina." *Biology of Reproduction*, 1973, doi:10.1093/biolreprod/8.4.491.
18. Charness, Michael E. "Brain Lesions in Alcoholics." *Alcoholism: Clinical and Experimental Research*, vol. 17, no. 1, 1993, pp. 2–11., doi:10.1111/j.1530-0277.1993.tb00718.x
19. Coleman,, Leon G et al. "Adolescent Binge Drinking Alters Adult Brain Neurotransmitter Gene Expression, Behavior, Brain Regional Volumes, and Neurochemistry in Mice." *Alcoholism: Clinical and Experimental Research*, 2011, doi:10.1111/j.1530-0277.2010.01385.x.
20. Costardi, João Victor Vezali, et al. "A Review on Alcohol: from the Central Action Mechanism to Chemical Dependency." *Revista Da Associação Médica Brasileira*, vol. 61, no. 4, 2015, pp. 381–387., doi:10.1590/1806-9282.61.04.381.
21. Crews, Fulton et al. "Binge Ethanol Consumption Causes Differential Brain Damage in Young Adolescent Rats Compared with Adult Rats." *Alcoholism: Clinical and Experimental Research*, 2000, doi:10.1111/j.1530-0277.2000.tb01973.x.
22. Crews, Fulton T., et al. "Mechanisms of Persistent Neurobiological Changes Following Adolescent Alcohol Exposure: NADIA Consortium Findings." *Alcoholism: Clinical and Experimental Research*, vol. 43, no. 9, 2019, pp. 1806–1822., doi:10.1111/acer.14154.
23. Damgaard, Ida N., et al. "Cryptorchidism and Maternal Alcohol Consumption during Pregnancy." *Environmental Health Perspectives*, vol. 115, no. 2, 2007, pp. 272–277., doi:10.1289/ehp.9608.
24. Davis, E. M et al. "Associations between Early Alcohol and Tobacco Use and Prolonged Time to Puberty in Boys." *Child: Care, Health and Development*, 2015, doi:10.1111/cch.12160.
25. Davison, A. "Basic Neurochemistry: Molecular, Cellular, and Medical Aspects." *Journal of Neurology, Neurosurgery & Psychiatry*, vol. 52, no. 8, 1989, pp. 1021–1021., doi:10.1136/jnnp.52.8.1021-a.
26. Dare, W. N., et al. "The Effect of Ethanol on Spermatogenesis and Fertility in Male Sprague-Dawley Rats Pretreated with Acetylsalicylic Acid." *The Nigerian Postgraduate Medical Journal*, vol. 9, no. 4, 2002, pp. 194–198.
27. De Bellis, Michael, et al. "Prefrontal Cortex, Thalamus, and Cerebellar Volumes in Adolescents and Young Adults with Adolescent-Onset Alcohol Use Disorders and Comorbid Mental Disorders." *Alcoholism: Clinical and Experimental Research*, vol. 29, no. 9, 2005, pp. 1590–1600., doi:10.1097/01.alc.0000179368.87886.76.
28. Dees, W. Les, et al. "Alcohol Ingestion Inhibits the Increased Secretion of Puberty-Related Hormones in the Developing Female Rhesus Monkey\*." *Endocrinology*, vol. 141, no. 4, 2000, pp. 1325–1331., doi:10.1210/endo.141.4.7413.

29. Dees, Les, and Carl W. Skelley. "Effects of Ethanol during the Onset of Female Puberty." *Neuroendocrinology*, vol. 51, no. 1, 1990, pp. 64–69., doi:10.1159/000125317.
30. Del-Rio Hortega, Pio et al. "La Glía De Escasa Radiaciones (Oligodendroglia)." *Boletín De La Real Sociedad Española De Historia Natural*, 1921.
31. Devine-Beach, Kathleen, et al. "Myelin Basic Protein Gene Transcription Identification of Proximal and Distal Cis-Acting Regulatory Elements ." *The Journal of Biological Chemistry*, vol. 265, Aug. 1990.
32. Ehlers, Cindy L et al. "Adolescent Ethanol Exposure: Does It Produce Long-Lasting Electrophysiological Effects?" *Alcohol*, 2010, doi:10.1016/j.alcohol.2009.09.033.
33. Ekambaram, Gnanagurudasan et al. "Comparative Study on the Estimation of Estrous Cycle in Mice by Visual and Vaginal Lavage Method." *Journal of Clinical and Diagnostic Research*, 2017, doi:10.7860/JCDR/2017/23977.9148.
34. Elofson, Jonathan et al. "Alcohol Use and Cerebral White Matter Compromise in Adolescence." *Addictive Behaviors*, doi:10.1016/j.addbeh.2013.03.001.
35. Emanuele, Nicholas, et al. "EtOH Disrupts Female Mammalian Puberty: Age and Opiate Dependence." *Endocrine*, vol. 18, no. 3, 2002, pp. 247–254., doi:10.1385/endo:18:3:247.
36. Filley, C. M. "White Matter and Behavioral Neurology." *Annals of the New York Academy of Sciences*, vol. 1064, no. 1, 2005, pp. 162–183., doi:10.1196/annals.1340.028.
37. Frussa-Filho, Roberto et al. "The plus-Maze Discriminative Avoidance Task: An Ethical Rodent Model for Concomitant Evaluation of Learning, Memory, Anxiety, Motor Activity and Their Interactions." *Rodent Model as Tools in Ethical Biomedical Research*, 2015, doi:10.1007/978-3-319-11578-8\_19.
38. Gaytan, F, et al. "Balano-Preputial Separation as an External Sign of Puberty in the Rat: Correlation with Histologic Testicular Data." *Andrologia*, 1988.
39. Giedd, Jay et al. "Quantitative MRI of the Temporal Lobe, Amygdala, and Hippocampus in Normal Human Development: Ages 4–18 Years." *Journal of Comparative Neurology*, 1996, doi:10.1002/(SICI)1096-9861(19960304)366:2<223::AID-CNE3>3.0.CO;2-7.
40. Goodpaster, Tracy et al. "A Flexible Mouse-On-Mouse Immunohistochemical Staining Technique Adaptable to Biotin-Free Reagents, Immunofluorescence, and Multiple Antibody Staining." *Journal of Histochemistry and Cytochemistry*, 2014, doi:10.1369/0022155413511620.
41. Grant, Bridget F. "Prevalence and Correlates of Alcohol Use and DSM-IV Alcohol Dependence in the United States: Results of the National Longitudinal Alcohol Epidemiologic Survey." *Journal of Studies on Alcohol*, vol. 58, no. 5, 1997, pp. 464–473., doi:10.15288/jsa.1997.58.464.
42. Grant, Bridget F et al. "Age of Onset of Drug Use and Its Association with DSM-IV Drug Abuse and Dependence: Results from the National Longitudinal Alcohol Epidemiologic Survey." *Journal of Substance Abuse*, 1998, doi:10.1016/S0899-3289(99)80131-X.
43. Heilig,, M et al. "Reprogramming of MPFC Transcriptome and Function in Alcohol Dependence." 2017, doi:10.1111/gbb.12344.

44. Hossain, A. M., and C. O. Osuamkpe. "Sole Use of Sucrose in Human Sperm Cryopreservation." *Archives of Andrology*, vol. 53, no. 2, 2007, pp. 99–103., doi:10.1080/01485010701225675.
45. Johnston, L. D., O'Malley, P. M., Bachman, J. G., & Schulenberg, J. E. (2009). Monitoring the Future national survey results on drug use, 1975–2008: Volume II, College students and adults ages 19–50 (NIH Publication No. 09-7403). Bethesda, MD: National Institute on Drug Abuse.
46. Karthigasan, J., et al. "Immunolocalization of 17 and 21.5 KDa MBP Isoforms in Compact Myelin and Radial Component." *Journal of Neurocytology*, vol. 25, no. 1, 1996, pp. 1–7., doi:10.1007/bf02284781.
47. Kendall, Tim, et al. "Quality, Bias and Service User Experience in Healthcare: 10 Years of Mental Health Guidelines at the UK National Collaborating Centre for Mental Health." *International Review of Psychiatry*, vol. 23, no. 4, 2011, pp. 342–351., doi:10.3109/09540261.2011.607432.
48. Keyes, Katherine M et al. "Evidence for a Closing Gender Gap in Alcohol Use, Abuse, and Dependence in the United States Population." *Drug and Alcohol Dependence*, 2008, doi:10.1016/j.drugalcdep.2007.08.017.
49. Kjersgaard, Camilla, et al. "Lifestyle in Pregnancy and Cryptorchidism in Sons: a Study within Two Large Danish Birth Cohorts." *Clinical Epidemiology*, Volume 10, 2018, pp. 311–322., doi:10.2147/clep.s150657.
50. Korenbrot,, C.C et al. "Preputial Separation as an External Sign of Pubertal Development in the Male Rat1." *Biology of Reproduction*, 1977, doi:10.1095/biolreprod17.2.298.
51. Kotegoda, S. R et al. "Ethics in Human Research." *The Ceylon Medical Journal*, 1988, doi:10.4103/2229-5070.72105.
52. Lee, Dong Hoon et al. "Ethanol down Regulates the Expression of Myelin Proteolipid Protein in the Rat Hippocampus." *Anatomy & Cell Biology*, 2010, doi:10.5115/acb.2010.43.3.194.
53. Lewohl, Joanne et al. "Gene Expression in Human Alcoholism: Microarray Analysis of Frontal Cortex." *Alcoholism: Clinical and Experimental Research*, 2000, doi:10.1111/j.1530-0277.2000.tb01993.x.
54. Lewohl, Joanne M et al. "Expression of MBP, PLP, MAG, CNP, and GFAP in the Human Alcoholic Brain." *Alcoholism: Clinical and Experimental Research*, 2005, doi:10.1097/01.alc.0000179406.98868.59.
55. Liu, Gaoxia et al. "Effect of Protease Inhibitors on the Quantitative and Qualitative Assessment of Oral Microorganisms." *FEMS Microbiology Letters*, 2010, doi:10.1111/j.1574-6968.2010.02100.x.
56. Mandal, Jharna, et al. "Ethics in Human Research." *Tropical Parasitology*, vol. 1, no. 1, 2011, p. 2., doi:10.4103/2229-5070.72105.
57. McDougall, Sean et al. "Myelination of Axons Corresponds with Faster Transmission Speed in the Prefrontal Cortex of Developing Male Rats." *ENeuro*, 2018, doi:10.1523/ENEURO.0203-18.2018.

58. McQueeny, Tim et al. "Altered White Matter Integrity in Adolescent Binge Drinkers." *Alcoholism: Clinical and Experimental Research*, 2009, doi:10.1111/j.1530-0277.2009.00953.x.
59. Meerburg, Bastiaan G et al. "Perspective: The Ethics of Rodent Control." *Pest Management Science*, 2008, doi:10.1002/ps.1623.
60. Messier, Amber M., and Oscar A. Bizzozero. *Neurochemical Research*, vol. 25, no. 4, 2000, pp. 449–455., doi:10.1023/a:1007551723752.
61. Mewton, L., et al. "The Epidemiology of DSM-IV Alcohol Use Disorders amongst Young Adults in the Australian Population." *Alcohol and Alcoholism*, vol. 46, no. 2, 2010, pp. 185–191., doi:10.1093/alcalc/agq091.
62. Mihov, Yoan et al. "Negative Allosteric Modulators of Metabotropic Glutamate Receptors Subtype 5 in Addiction: a Therapeutic Window." *The International Journal of Neuropsychopharmacology*, 2016, doi:10.1093/ijnp/pyw002.
63. Miller, R. H., and S. L. Fyffe-Maricich. "Restoring the Balance between Disease and Repair in Multiple Sclerosis: Insights from Mouse Models." *Disease Models & Mechanisms*, vol. 3, no. 9-10, 2010, pp. 535–539., doi:10.1242/dmm.001958.
64. Morell, Pierre, and Richard Quarles. "Myelin Formation, Structure and Biochemistry." *Basic Neurochemistry, Molecular, Cellular, and Medical Aspects*, Lippincott, Williams & Wilkins, 1999.
65. Nadon, Nancy L et al. "Myelin Proteolipid Protein: Function in Myelin Structure Is Distinct from Its Role in Oligodendrocyte Development." *Developmental Neuroscience*, doi:10.1159/000017354.
66. Natrass, Nicoli, et al. "Animal Welfare and Ecology in the Contested Ethics of Rodent Control in Cape Town." *Journal of Urban Ecology*, vol. 5, no. 1, 2019, doi:10.1093/jue/juz008.
67. Nuffield Council on BioEthics. "The Ethics of Research Involving Animals." *The Ethics of Research Involving Animals*, 2005, doi:10.1136/jme.28.1.56-b.
68. Olsen, Richard W., and Jing Liang. "Role of GABA<sub>A</sub> Receptors in Alcohol Use Disorders Suggested by Chronic Intermittent Ethanol (CIE) Rodent Model." *Molecular Brain*, vol. 10, no. 1, 2017, doi:10.1186/s13041-017-0325-8.
69. Olson, Steve, and Dean R. Gerstein. *Alcohol in America: Taking Action to Prevent Abuse*. National Academic Press, 1985.
70. Ortiz, Fernando C., et al. "Neuronal Activity in Vivo Enhances Functional Myelin Repair." *JCI Insight*, vol. 4, no. 9, 2019, doi:10.1172/jci.insight.123434.
71. Oscar-Berman, Marlene, and Ksenija Marinković. "Alcohol: Effects on Neurobehavioral Functions and the Brain." *Neuropsychology Review*, vol. 17, no. 3, 2007, pp. 239–257., doi:10.1007/s11065-007-9038-6.
72. Pajooresh-Ganji, Ahdeah, and Robert H. Miller. "Targeted Oligodendrocyte Apoptosis in Optic Nerve Leads to Persistent Demyelination." *Neurochemical Research*, vol. 45, no. 3, 2019, pp. 580–590., doi:10.1007/s11064-019-02754-z.
73. Pascual, María et al. "Neuroimmune Activation and Myelin Changes in Adolescent Rats Exposed to High-Dose Alcohol and Associated Cognitive Dysfunction: A Review with

- Reference to Human Adolescent Drinking.” *Alcohol and Alcoholism*, 2014, doi:10.1093/alcalc/agt164.
74. Peach, Mandy, et al. “Solubilization of Proteins: The Importance of Lysis Buffer Choice.” *Methods in Molecular Biology Western Blotting*, 2015, pp. 49–60., doi:10.1007/978-1-4939-2694-7\_8.
75. Pfefferbaum, Adolf et al. “Longitudinal Changes in Magnetic Resonance Imaging Brain Volumes in Abstinent and Relapsed Alcoholics.” *Alcoholism: Clinical and Experimental Research*, 1995, doi:10.1111/j.1530-0277.1995.tb01598.x.
76. Pfefferbaum, Adolf et al. “White Matter Microstructural Recovery with Abstinence and Decline with Relapse in Alcoholism: Interaction with Normal Aging Revealed with Longitudinal DTI.” *The Lancet Psychiatry*, 2014, doi:10.1016/S2215-0366(14)70301-3.
77. Pfefferbaum, Adolf et al. “Dynamic Responses of Selective Brain White Matter Fiber Tracts to Binge Alcohol and Recovery in the Rat.” *PLoS ONE*, 2015, doi:10.1371/journal.pone.0124885.
78. Priddy, Brittany M., et al. “Sex, Strain, and Estrous Cycle Influences on Alcohol Drinking in Rats.” *Pharmacology Biochemistry and Behavior*, vol. 152, 2017, pp. 61–67., doi:10.1016/j.pbb.2016.08.001.
79. Puig, M. Victoria et al. “Dopamine Modulation of Learning and Memory in the Prefrontal Cortex: Insights from Studies in Primates, Rodents, and Birds.” *Frontiers in Neural Circuits*, 2014, doi:10.3389/fncir.2014.00093.
80. Richardson, Heather et al. “Alcohol Binge Drinking during Adolescence or Dependence during Adulthood Reduces Prefrontal Myelin in Male Rats.” *Journal of Neuroscience*, 2014, doi:10.1523/JNEUROSCI.3189-13.2014.
81. Richardson, Heather et al. “Sex Differences in the Effect of Alcohol Drinking on Myelinated Axons in the Anterior Cingulate Cortex of Adolescent Rats.” *Brain Sciences*, 2019, doi:10.3390/brainsci9070167.
82. Roughton, K, et al. “Irradiation to the Young Mouse Brain Impaired White Matter Growth More in Females than in Males.” *Cell Death & Disease*, vol. 4, no. 10, 2013, doi:10.1038/cddis.2013.423.
83. Sabri, M. I., et al. “Turnover of Myelin and Other Structural Proteins in the Developing Rat Brain.” *Biochemical Journal*, vol. 142, no. 3, 1974, pp. 499–507., doi:10.1042/bj1420499.
84. Samantaray, Supriti et al. “Chronic intermittent ethanol induced axon and myelin degeneration is attenuated by calpain inhibition.” *Brain research* vol. 1622 (2015): 7-21. doi:10.1016/j.brainres.2015.06.014
85. Schulte, Marya T., et al. “Gender Differences in Factors Influencing Alcohol Use and Drinking Progression among Adolescents.” *Clinical Psychology Review*, vol. 29, no. 6, 2009, pp. 535–547., doi:10.1016/j.cpr.2009.06.003.
86. Schweinsburg, Alecia D et al. “A Preliminary Study of Functional Magnetic Resonance Imaging Response during Verbal Encoding among Adolescent Binge Drinkers.” *Alcohol*, 2010, doi:10.1016/j.alcohol.2009.09.032.

87. Schweinsburg, Brian C., et al. "Effects of Alcoholism and Gender on Brain Metabolism." *American Journal of Psychiatry*, vol. 160, no. 6, 2003, pp. 1180–1183., doi:10.1176/appi.ajp.160.6.1180.
88. Shear, Paula K et al. "Volumetric Magnetic Resonance Imaging Quantification of Longitudinal Brain Changes in Abstinent Alcoholics." *Alcoholism: Clinical and Experimental Research*, 1994, doi:10.1111/j.1530-0277.1994.tb00899.x.
89. Simons, Jeffrey et al. "Alcohol Abuse and Dependence Symptoms: A Multidimensional Model of Common and Specific Etiology." *Psychology of Addictive Behaviors*, 2009, doi:10.1037/a0016003.
90. Smith, Graham S.T et al. "Classic 18.5- and 21.5-KDa Myelin Basic Protein Isoforms Associate with Cytoskeletal and SH3-Domain Proteins in the Immortalized N19-Oligodendroglial Cell Line Stimulated by Phorbol Ester and IGF-1." *Neurochemical Research*, 2012, doi:10.1007/s11064-011-0700-2.
91. Smith, Hilary R et al. "Regionally-Specific Alterations in Myelin Proteins in Nonhuman Primate White Matter Following Prolonged Cocaine Self-Administration." *Drug and Alcohol Dependence*, 2014, doi:10.1016/j.drugalcdep.2014.01.015.
92. Sneider, Jennifer T et al. "Differential Effects of Binge Drinking on Learning and Memory in Emerging Adults." *Journal of Addiction Research & Therapy*, 2012, doi:10.4172/2155-6105.s7-006.
93. Sowell, Elizabeth R et al. "Localizing Age-Related Changes in Brain Structure between Childhood and Adolescence Using Statistical Parametric Mapping." *NeuroImage*, 1999, doi:10.1006/nimg.1999.0436.
94. Spear, Linda. "Modeling Adolescent Development and Alcohol Use in Animals." *Alcohol Research and Health*, 2000.
95. Spear, Linda. "The Adolescent Brain and Age-Related Behavioral Manifestations." *Neuroscience and Biobehavioral Reviews*, 2000, doi:10.1016/S0149-7634(00)00014-2.
96. Spear, Linda Patia. "Adolescent Neurodevelopment." *Journal of Adolescent Health*, vol. 52, no. 2, 2013, doi:10.1016/j.jadohealth.2012.05.006.
97. Spear, Linda Patia, and H. Scott Swartzwelder. "Adolescent Alcohol Exposure and Persistence of Adolescent-Typical Phenotypes into Adulthood: A Mini-Review." *Neuroscience & Biobehavioral Reviews*, vol. 45, 2014, pp. 1–8., doi:10.1016/j.neubiorev.2014.04.012.
98. Swartzwelder, H. Scott et al. "Changes in the Adult GluN2B Associated Proteome Following Adolescent Intermittent Ethanol Exposure." *PLoS ONE*, 2016, doi:10.1371/journal.pone.0155951.
99. Tapert, Susan et al. "Neuropsychological Correlates of Adolescent Substance Abuse: Four-Year Outcomes." *Journal of the International Neuropsychological Society*, doi:10.1017/S1355617799566010.
100. "The Jackson Laboratory." *The Jackson Laboratory*, wwwjax.org/.
101. Trantham-Davidson, Heather et al. "Binge-Like Alcohol Exposure during Adolescence Disrupts Dopaminergic Neurotransmission in the Adult Prefrontal Cortex." *Neuropsychopharmacology*, 2017, doi:10.1038/npp.2016.190.

102. Tsay, Cynthia. "Revisiting the Ethics of Research on Human Subjects." *AMA Journal of Ethics*, vol. 17, no. 12, 2015, pp. 1105–1107., doi:10.1001/journalofethics.2015.17.12.fred1-1512.
103. Valdez, Glenn R., et al. "Increased Ethanol Self-Administration and Anxiety-Like Behavior During Acute Ethanol Withdrawal and Protracted Abstinence: Regulation by Corticotropin-Releasing Factor." *Alcoholism: Clinical and Experimental Research*, vol. 26, no. 10, 2002, pp. 1494–1501., doi:10.1111/j.1530-0277.2002.tb02448.x.
104. van Hemmen, J et al. "Sex Differences in White Matter Microstructure in the Human Brain Predominantly Reflect Differences in Sex Hormone Exposure." *Cerebral Cortex*, 2017, doi:10.1093/cercor/bhw156.
105. Verdaasdonk,, Jolien Suzanne et al. "Determining Absolute Protein Numbers by Quantitative Fluorescence Microscopy." *Methods in Cell Biology*, 2014, doi:10.1016/B978-0-12-420138-5.00019-7.
106. Vetter-O'Hagen, Courtney S., and Linda Spear. "Hormonal and Physical Markers of Puberty and Their Relationship to Adolescent-Typical Novelty-Directed Behavior." *Developmental Psychobiology*, 2012, doi:10.1002/dev.20610.
107. Wagoner, Kimberly G et al. "Free Alcohol Use and Consequences: Gender Differences among Undergraduates." *American Journal of Health Behavior*, 2012, doi:10.5993/AJHB.36.4.2.
108. Walker, John M. *Basic Protein and Peptide Protocols*. Humana Press, 1994.
109. Warner, Lynn A. "Prevalence and Correlates of Drug Use and Dependence in the United States." *Archives of General Psychiatry*, vol. 52, no. 3, 1995, p. 219., doi:10.1001/archpsyc.1995.03950150051010.
110. Wilsnack, Richard et al. "Gender and Alcohol Consumption: Patterns from the Multinational GENACIS Project." *Addiction*, 2009, doi:10.1111/j.1360-0443.2009.02696.x.
111. Wolstenholme, Jennifer T., et al. "Intermittent Ethanol during Adolescence Leads to Lasting Behavioral Changes in Adulthood and Alters Gene Expression and Histone Methylation in the PFC." *Frontiers in Molecular Neuroscience*, vol. 10, 2017, doi:10.3389/fnmol.2017.00307.
112. Wolstenholme, Jennifer et al. "Transgenerational Bisphenol A Causes Deficits in Social Recognition and Alters Postsynaptic Density Genes in Mice." *Endocrinology*, 2019, doi:10.1210/en.2019-00196.
113. Xing, Weirong. "Conditional Disruption of the Osterix Gene in Chondrocytes during Early Postnatal Growth Impairs Secondary Ossification in the Mouse Tibial Epiphysis." *Bone Research*, 2019, doi:10.1038/s41413-019-0064-9.
114. Yamaguchi, Yoshihide. "Myelin Proteolipid Protein (PLP), but Not DM-20, Is an Inositol Hexakisphosphate-Binding Protein." *Journal of Biological Chemistry*, 1996, doi:10.1074/jbc.271.44.27838

115. Yang, Shu. "Sex Differences in the White Matter and Myelinated Nerve Fibers of Long-Evans Rats." *Brain Research*, 2008, doi:10.1016/j.brainres.2008.03.052.
116. Zahr, Natalie M., et al. "Dynamic Responses of Selective Brain White Matter Fiber Tracts to Binge Alcohol and Recovery in the Rat." *Plos One*, vol. 10, no. 4, 2015, doi:10.1371/journal.pone.0124885.
117. Zhang, Chunchao, et al. "Myelin Basic Protein Undergoes a Broader Range of Modifications in Mammals than in Lower Vertebrates." *Journal of Proteome Research*, vol. 11, no. 10, 2012, pp. 4791–4802., doi:10.1021/pr201196e.