November 2016

Effects of adolescent alcohol binge drinking on prefrontal myelin

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EFFECTS OF ADOLESCENT ALCOHOL BINGE DRINKING ON PREFRONTAL MYELIN

A Dissertation Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2016

Neuroscience and Behavior Graduate Program

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DEDICATION

To:
my amazing and loving husband, Michael Hany Riad;
my wonderful super-woman mother Wanda Ivette Rodríguez;
my awesome father Elmo Vargas;
my dear granny Zoraida Bermúdez.

Thank you all for your continuous and unconditional love and support.

I love you all very much.

ACKNOWLEDGMENTS

I would like to thank my wonderful advisor, Dr. Heather N. Richardson for giving me the opportunity to work with her and for all her guidance and support. Heather has been more than an advisor; she has been my mentor throughout this time, teaching me valuable lessons not only about science and career, but also about life. Thank you to my amazing committee, Dr. Elizabeth A. Connor, Dr. David Moorman, and Dr. R. Thomas Zoeller, for their insightful feedback, support, scientific guidance, and encouragement.

I am grateful of Lynn Bengston for her help with experiments, training, and written feedback. I thank current and past lab members: Kristie Fierro, Chrisanthi Karanikas, Dr. Yi-Ling Lu, Dr. Jesse McClure, and Andrea Silva-Gotay for their feedback, help, and support. They are lab mates and friends. I am also very thankful for all the undergraduate students that worked with me, especially, Sean McDougall, Aditi Dave, Divya Harpalani, Ryan Tressel, Callie Dacyczyn, Azinne Zarrabi, Kyle O'Donnell, and Frank Jackson.

I would like to extend my appreciation to Dr. Brian Whitcomb for his support and expert advice on statistical tests, Dr. Jerrold Meyer for his expert guidance on the oxidative stress experiments, Dr. Geng-Lin Li for collaborating with our lab for the electrophysiology experiments, Dr. Samuel Hazen for his assistance with the RT-qPCR experiments, Dr. Peter Chien and lab for lending us their spectrophotometer, Dr. Nicholas Gilpin and Dr. Maury Cole for their help with some experiments, and to Dr. Luke Remage-Healey for critical reading of our manuscript.

I would also like to thank animal care personnel, especially Ed Golembeski for all their help with the animals. Thank you also to the Neuroscience and Behavior program,

its director Dr. Rebecca Spencer, GOC chair Dr. Gerald Downes, and managers (Maggie DeGregorio, Vickie Rupp, Susan Capistran, Linda Witt), and NSB friends for being such a supportive group. A special thanks to NEAGEP (Dr. Sandy Petersen, Dr. Vanessa Hill, Dr. Heyda Martinez, and Dr. Marlina Duncan) for the NEAGEP fellowship and for providing a community of support and encouragement. This research was partially supported by a Dissertation Research Grant from the University of Massachusetts Amherst to WMV.

Lastly, I want to thank God for blessing me always, and my parents, Elmo and Wanda, my husband Michael, and my family and friends for their unconditional love and support. My mother is my motivation and role model. Michael shares with me a love for neuroscience and has been so kind, loving, and patient. I truly admire him. Thank you all for believing in me.

ABSTRACT

EFFECTS OF ADOLESCENT ALCOHOL BINGE DRINKING ON PREFRONTAL MYELIN

SEPTEMBER 2016

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Alcohol binge drinking is highly prevalent in teenagers and is associated with various harmful health effects and social problems. During adolescence, brain regions such as the prefrontal cortex (PFC) are still undergoing active development, characterized by increases in white matter volume. While the morphological details and the cellular and molecular sequences governing adolescent white matter development are not fully known, it is known that this development process is sensitive and can be disrupted. Although consumption of alcohol in a binge drinking pattern has been linked to lower white matter integrity in humans, it is important to determine if alcohol is causing this change or if predisposing factors can influence drinking. A rodent model of voluntary binge drinking was used to elicit high alcohol intake during a short developmental window in adolescence. Myelin was then assessed using several histochemical measures. Results showed that adolescent development is marked by an increase in myelinated fibers in the PFC that accompanied an increase in conduction velocity, and alcohol reduces prefrontal white matter and myelinated fiber density. In addition, heavy drinking was associated with long-term cognitive deficits. I also investigated sex differences in the effects of adolescent alcohol consumption on PFC myelination, showing that males

appear to be more vulnerable than females. The findings altogether increase our understanding of the developmental process of prefrontal myelination in adolescence and the maladaptive effects alcohol can have on this critical process.

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CHAPTER 1

GENERAL INTRODUCTION AND BACKGROUND

1.1 Alcohol, adolescent consumption pattern, and consequences

Alcohol (ethanol) is a small molecule that is soluble in water and is absorbed by the stomach and small intestine (Paton and McCune, 2015). Alcohol is considered to be a sedative that influences brain regions involved in reward and pleasure (Paton and McCune, 2015). However, it also acts as a stressor by activating the hypothalamic pituitary adrenal axis, producing physiological responses such as sweating and increases in blood pressure in humans (Wand and Dobs, 1991; Hundt et al., 2001; Thayer et al., 2006; Paton and McCune, 2015). In addition, alcohol induced-activation of the hypothalamic pituitary adrenal axis has been shown in rodent models (e.g., Richardson et al., 2008).

Although buying alcohol in the United States is illegal for any person under 21 years of age, adolescents (12-18 years) have both a vast experience with this substance and a great deal of active use (Johnston et al., 2009). In fact, alcohol is the most consumed drug among young people today (Witt, 2010). Approximately 47% of the underage adolescent population consumes alcohol, and they do so at higher rates than adults (Miller et al., 2006). Underage drinking has an estimated annual cost of roughly $60 billion for the US, including medical and property loss costs (Miller et al., 2006). More specifically, the adolescent population has a particularly high prevalence of binge drinking (Miller et al., 2007; Johnston et al., 2009), which is defined as an excessive pattern of alcohol intake that brings a person's blood alcohol level to 0.08 gram% or

higher; equivalent to 5 or more drinks for males and 4 or more drinks for females in a two-hour period (Wechsler and Nelson, 2001; NIAAA, 2005).

Binge drinking is associated with various harmful health effects and social problems such as injuries, poor academic performance, increased risky behavior, sexual/physical assault, death, etc. (NIAAA, 2005; Rehm et al., 2009; Schuckit et al., 2008). Binge drinking is one of the leading preventable causes of death in the United States (Rivara et al., 2004; NIAAA, 2005). Studies in humans indicate that heavy alcohol consumption may cause damage to the brain, especially in adolescents (Crego et al., 2009; Maurage et al., 2012), and may have negative long-term consequences, such as a higher risk of developing alcohol dependence in adulthood (Boden and Fergusson, 2011; McCambridge et al., 2011).

1.1.1 Adolescent voluntary alcohol binge drinking rodent model

The present dissertation used a recently developed operant rodent model that elicits voluntary alcohol binge drinking during early adolescence (Gilpin et al., 2012). In this model, adolescent Wistar rats without a genetic predisposition for alcohol abuse are trained to self-administer alcohol by pressing a lever. This is an ideal model to determine causality (whether alcohol drinking induces changes in the brain) while being able to accurately model human adolescent alcohol consumption. Particularly, it is a useful model to understand the effect of alcohol consumption on myelin, using a level of analysis and anatomical detail that cannot be accomplished using conventional neuroimaging methods in humans.

The traditional method for modeling excessive alcohol consumption in rodents uses forced or involuntary administration of alcohol via gavage (Yamaguchi et al., 2007),

injections (Markwiese et al., 1998; Ristuccia and Spear, 2008) or vapors (Becker and Lopez, 2004; O'Dell et al., 2004). However, rats that passively receive a drug can exhibit different behaviors and physiological responses from those who self-administer the drug. For example, rats that were injected with alcohol in adolescence showed reduced voluntary alcohol consumption later in adulthood (Gilpin et al., 2012). To avoid this limitation, operant self-administration models are used to elicit and control voluntary intake of drugs and control solutions in adolescent animals (Deroche-Gamonet, 2004). In addition, the operant self-administration model used for the research of this dissertation captures some of the characteristics of drinking behavior observed in human adolescents (e.g., moderate to heavy episodic drinking of sweetened alcohol with access to food and water at all times) (Windle et al., 2008; Gilpin et al., 2012).

Our model implements an alcohol exposure schedule in which the operant lever is only accessible for six 30-minute sessions (bouts) every night for a two-week period, lasting from postnatal days (PD) 28 to 42. These 30-minute bouts are evenly distributed across 8 hours, with 1-hour breaks between bouts, during which the levers retract (Karanikas et al., 2013). Access to food and water is *ad libitum*. In our laboratory, the combination of sweetened alcohol and an intermittent drinking schedule has been successful in promoting moderate (around 50 g/kg) to high (around 75 g/kg or more) alcohol intake in ~90-95% rats tested so far. While rats do not reach the blood alcohol level of 0.08 grams% in every session/bout, they do reach this level at least a few times during the two-week period (84 bouts in total) resulting in high cumulative intake within a short developmental window.

1.2 White matter and myelination

White matter is composed of neuronal fibers coated with myelin, a compacted cell membrane composed of lipids and proteins (Fields, 2008). Multiple layers of myelin are wrapped around axons of neurons in the brain, providing electrical insulation for action potentials (Fields, 2008). In the central nervous system (CNS), myelin is produced by oligodendrocytes, a class of glial cells capable of insulating multiple axons simultaneously (Fields, 2008; Kearns et al., 2015). This process, known as myelination, is critical for development as it augments the rate of neuronal communication (Giedd, 2004; Fields, 2008; Deoni et al., 2012). Indeed, past research in humans has shown a positive relationship between cognitive performance in tasks and increased myelination in the frontal cortex (Paus et al., 1999; Yurgelun-Todd et al., 2002; Blakemore and Choudhury, 2006; Silveri et al., 2006).

The molecular process of myelination in the CNS involves several steps (Baumann and Pham-Dinh, 2001; Simons and Trajkovic, 2006; Simons and Trotter, 2007; Simons and Nave, 2016). First, oligodendrocyte precursor cells (OPCs) proliferate, a process moderated by various growth factors secreted by neurons and astrocytes. Second, OPCs migrate into their final positions in brain regions that will eventually become rich with white matter. Next, axons and glia interchange signals that promote the differentiation of OPCs into myelinating oligodendrocytes. Finally, after OPC differentiation, myelin formation occurs and is composed of 5 main steps (Baumann and Pham-Dinh, 2001; Simons and Trajkovic, 2006; Simons and Nave, 2016). (1) Oligodendrocytes identify and make contact with the proper axon. (2) Oligodendrocytes transcribe, produce, and transport to the axons the proteins necessary for myelin

synthesis. (3) The myelin membrane grows/expands and wraps around the axons in a spiral fashion. (4) After wrapping, myelin undergoes compaction, leaving dense segments of myelin. (5) Finally, segments of axon are left unmyelinated, forming gaps along the axon known as the nodes of Ranvier.

There are numerous players involved in myelination, but their roles are not well known yet, and we are just starting to understand the multitude of interactions necessary for CNS myelination (Nave, 2010). Platelet-derived growth factor subunit A and fibroblast growth factor-2 regulate OPC proliferation and migration (Pringle et al., 1992; Baron et al., 2000; Mitew et al., 2014). OPC differentiation is orchestrated by several molecules: Notch 1 (Genoud et al., 2002), Olig2 (Lee et al., 2005), fibroblast growth factor-2 (Baron et al., 2000), leucine rich repeat and immunoglobulin domain containing-1 (Mi et al., 2005), and thyroid hormone 3 (T3) (Durand and Raff, 2000).

Thyroid hormone is in fact important for several stages of oligodendrocyte development (Rodriguez-Pena, 1999), in addition to oligodendrocyte differentiation (Barres et al., 1994; Gao et al., 1998), as mentioned above. T3 is also involved in regulating myelin regeneration (Dugas et al., 2012) and in oligodendrocyte survival as it protects oligodendrocytes from apoptosis (Jones et al., 2003; Schoonover et al., 2004). Recent work has shown that oligodendrocyte differentiation and myelin regeneration are regulated through the induction of the KLF9 gene by T3 (Dugas et al., 2012).

After OPC differentiation, Neuregulin-1 signaling ensures proper oligodendrocyte maturation and survival (Simons and Trajkovic, 2006). Axonal caliber is a strong stimulator for myelination initiation, and laminin-α2 is involved in the initial oligodendrocyte process extension toward the axon (Hu et al., 2006). Neuregulin-1 type

III binding to ErbB2/B3 is thought to be involved in the transcription, production, and transport to the axons of myelin components, such as myelin basic protein (MBP), proteolipid protein and other proteins important for myelin synthesis (Simons and Trajkovic, 2006). Finally, it is thought that the instructions for how much myelin to produce are relayed by Neuregulin-1 type III axonal expression (Simons and Trajkovic, 2006), particularly in mediating experience-dependent myelination (Makinodan et al., 2012).

1.2.1 Corpus callosum and forceps minor

The corpus callosum (CC) is the largest white matter tract in the mammal brain (Bloom and Hynd, 2005; Chao et al., 2009; Pfefferbaum et al., 2010; Elofson et al., 2013), and contains from 200 to 800 million fibers (Bloom and Hynd, 2005). The CC is important for facilitating the integration of diverse inputs and communicating information between the left and right hemispheres of the brain (Bloom and Hynd, 2005; Chao et al., 2009; Pfefferbaum et al., 2010; Elofson et al., 2013). This brain region has been divided into four main sub-regions (from anterior to posterior CC): the genu, body, isthmus, and splenium (Keshavan et al., 2002). Most of the CC sub-regions contain fiber tracts that connect different brain cortices (Standring, 2005; Moore and Puri, 2012). The forceps minor in the genu connects the frontal cortices, the body passes through the corona radiata and connects neocortical areas, and the forceps major in the splenium connects the occipital lobes. Just like white matter in general, the CC develops caudally first and then rostrally, from the splenium to the genu (Hynd et al., 1995; Bloom and Hynd, 2005; Vincze et al., 2008).

The genu is the anterior portion of the CC of the brain and consists of fibers from the left and right prefrontal cortex (Bloom and Hynd, 2005). The forceps minor is a small fiber bundle that interconnects the medial and lateral prefrontal cortices and crosses through the genu of the CC (Wing and Osborn, 1977; Raybaud and Girard, 2005; Standring, 2005; Kulkarni, 2007; Orrison, 2008; Jackson et al., 2011). The medial and lateral prefrontal cortices connected by the forceps minor are responsible for cognitive control (Taren et al., 2011) and have been implicated with risk-taking behavior (Crone et al., 2008).

1.2.2 White matter development during adolescence and sex differences

A large amount of white matter development studies have been carried out using imaging studies in humans. Two of the most common methods for measuring white matter are magnetic resonance imaging (MRI), which measures white matter volume (Karlsgodt et al., 2012) and, more recently, diffusion tensor imaging (DTI), which measures water movement within fibers to assess white matter integrity (Ladouceur et al., 2012). DTI is therefore helpful for determining the microstructure of white matter. The most common DTI measures are fractional anisotropy (FA) and mean diffusivity (MD) (Brenhouse and Andersen, 2011). FA is a measure of water diffusion restricted by direction (Bava et al., 2010), and a higher measure of FA means greater white matter integrity. MD, also called trace, indexes the average overall diffusion (Ladouceur et al., 2012), and a higher measure of MD is related with less white matter.

As mentioned above, myelination in the CNS is similar in many species as it progresses from caudal to rostral areas (Hynd et al., 1995; Bloom and Hynd, 2005; Doretto et al., 2011; Downes and Mullins, 2014). It is still occurring in the human brain

throughout adolescence up to the third decade of life, particularly with higher-level association regions such as the frontal cortex, which is fully myelinated last (Yakovlev and Lecours, 1967; Hynd et al., 1995; Baumann and Pham-Dinh, 2001). During adolescence, white matter volume in brain areas such as the frontal cortices increases usually in a linear fashion, while gray matter volume decreases (usually in an inverted-U manner) (Giedd et al., 1999; Paus et al., 1999; 2001; Sowell et al., 2003; Barnea-Goraly et al., 2005; Brenhouse and Andersen, 2011). This increase in white matter volume may be linked to increases in axonal diameter, increases in myelin thickness, or both (Paus, 2010; Karlsgodt et al., 2012). Similar to humans, myelination in rodents continues throughout adolescence and into early adulthood (Juraska and Markham, 2004; Markham et al., 2007; Doretto et al., 2011; Mengler et al., 2014; Willing and Juraska, 2015). There is a massive increase in myelination during postnatal days 15-30, a period corresponding to early adolescence (Salvati et al., 2000; Karlsgodt et al., 2012).

A great deal of research regarding white matter development during adolescence has investigated developmental sex differences in humans. Although both human males and females show general increases in white matter during adolescence, there are several developmental differences between the sexes. Male teenagers showed greater overall white matter volume and CC area than female teenagers (De Bellis et al., 2001). Males also showed higher fractional anisotropy than females in overall white matter (Herting et al., 2012), in frontal regions (Schmithorst et al., 2008), and in white matter regions (Silveri et al., 2006). Moreover, male adolescents had higher proportions of prefrontal white matter volume to overall white mater volume than female adolescents (Nagel et al., 2006). Although males show more white matter volume in general, white matter in

females develops earlier than in males (De Bellis et al., 2001; Asato et al., 2010; Wang et al., 2012). In fact, males showed a linear relationship between age and frontal white matter volume, while females showed a curvilinear relationship (Brain Development Cooperative Group, 2012). In addition, female teens showed a higher FA than male teens in the splenium of the CC (Schmithorst et al., 2008).

Similar to humans, male and female rodents show general white matter increases throughout adolescence, also with particular sex differences in white matter development. The increase in frontal cortex white matter volume between 35- and 90-day old male rats was greater (approximately 40%) than between 35- and 90-day old female rats (approximately 25%) (Markham et al., 2007). There were no differences between sexes in frontal white matter at postnatal day 35. Similar to frontal white matter, both males and females showed a similar increase in glial cell number with age in the dorsal mPFC. However, in the ventral mPFC, only males displayed an increase between age groups. There were no differences between sexes in glial cell number in the ventral or dorsal mPFC at postnatal day 35 (Markham et al., 2007). Similarly, males and females showed no differences in myelinated axon number in the splenium of the CC at postnatal day 25 (Juraska and Markham, 2004). Taken together, these results suggest there are sex-based differences in white matter development during adolescence.

1.3 Prefrontal cortex

The prefrontal cortex (PFC) is the brain region responsible for executive cognitive functions such as emotion regulation and impulse control (Clark et al., 2008; Alfonso-Loeches and Guerri, 2011). The PFC can be roughly divided into the medial prefrontal, orbitofrontal, and dorsolateral cortices (Hamid, 2014). An important aspect of the PFC is

that its development/maturation continues throughout adolescence (Spear, 2000; Gogtay et al., 2004; Crews et al., 2007; Markham et al., 2007; Casey et al., 2008; Guerri and Pascual, 2010). In humans, as mentioned above, frontal cortex development during adolescence is characterized by an increase in white matter volume and a decrease of gray matter volume (independent of white matter) (Giedd et al., 1999; Paus et al., 1999; 2001; Sowell et al., 2003; Barnea-Goraly et al., 2005), which are thought to enhance cognitive processing (Giedd, 2004). Similarly, rodent studies also show an increase in myelination during this period. For example, myelinated fibers projecting from the basolateral amygdala into layers II and V of the anterior cingulate and infralimbic subdivisions of the medial PFC increased in number during adolescent development (Cunningham et al., 2002). In addition, the myelinated fiber density of female Long Evans rats significantly increased from young-aged (4-6 months old) to middle-aged rats (18 months) (Yang et al., 2009), suggesting that myelination continues into adulthood.

The dynamic changes occurring in the developing PFC during adolescence (Casey et al., 2008) may make this brain region more vulnerable to toxic external stimuli, such as alcohol (Blakemore and Choudhury, 2006; Crews et al., 2007). In fact, the PFC is hypothesized to be particularly susceptible to alcohol exposure during adolescence (Clark et al., 2008; Nixon and McClain, 2010; Alfonso-Loeches and Guerri, 2011). In support of this, binge drinking during adolescence is associated with reduced white matter quality/integrity in the PFC (McQueeny et al., 2009), and with smaller PFC white matter volume in human adolescents (De Bellis et al., 2005). If alcohol interferes with myelination of axons in the PFC during adolescent development, it could permanently impair the normal development of executive functions. Indeed, binge drinking during

adolescence is related to greater susceptibility to cognitive deficits (Brown et al., 2000; Hartley et al., 2004; Guerri and Pascual, 2010).

1.3.1 Medial prefrontal cortex

The medial prefrontal cortex (mPFC) is an important brain region directly involved in higher order cognitive functions such as goal directed behavior and emotional processing (Fuster, 2000; 2001; Hoover and Vertes, 2007). The three major subdivisions of the mPFC in rodents are the anterior cingulate, prelimbic, and infralimbic cortices (Uylings and van Eden, 1990; Groenewegen and Uylings, 2000; Ongür and Price, 2000; Vertes, 2004). The anterior cingulate cortex is part of the dorsal mPFC and the infralimbic cortex is part of the ventral mPFC (Hoover and Vertes, 2007; Linley et al., 2013).

The subdivisions of the mPFC are thought to have specific functions (Vertes, 2004). The anterior cingulate division of the mPFC is important for planning motor behaviors, specifically eye movements in the rat (Hall and Lindholm, 1974), but also for motor reaction memory and temporal and spatial information (Dalley et al., 2004). In addition, the anterior cingulate cortex may be important for response flexibility (Seamans et al., 1995). The prelimbic cortex of the mPFC connects to the limbic system, plays a role in working memory, and is important for processing limbic-cognitive functions (Delatour and Gisquet-Verrier, 1996; Hoover and Vertes, 2007). The infralimbic cortex of the mPFC also connects to the limbic system and is important for regulation of autonomic ("visceromotor") activity such as respiration, heart rate, and blood pressure (Hardy and Holmes, 1988; Hoover and Vertes, 2007) and also for some cognitive functions (Kesner and Ragozzino, 2003). It is important to mention that the subdivisions

of the mPFC are interconnected as well. For example, all three subdivisions of the mPFC have projections to the basolateral amygdala, indicating all three subdivisions are involved in emotional responses and fear conditioning (Gabbott et al., 2005).

The mPFC is composed mostly of pyramidal cells, which mainly transmit glutamatergic/excitatory signals (De-May and Ali, 2013; Miguéns et al., 2015), and GABAergic interneurons, which represent roughly 20% of mPFC cells (De-May and Ali, 2013; Riga et al., 2014). There are five layers in the mPFC, with layers II, III, and V being some of the most studied. Layers II and III are often grouped together and are the main layers for processing information within the mPFC. Layer V is the main output layer of this region (Goodfellow et al., 2009; van Aerde and Feldmeyer, 2015).

The mPFC has numerous efferent and afferent projections, which can sometimes be unique to a particular mPFC subdivision. This contributes to the distinct functions observed in the different subdivisions of the mPFC. Summarizing mPFC efferents, as mentioned above, the mPFC projects heavily to the basolateral amygdala (Gabbott et al., 2005), sending glutamatergic projections to the local interneurons (Rosenkranz and Grace, 2001). The interneurons in the basolateral amygdala suppress sensory inputs to the cortex, and therefore play a role in the regulation of affective behaviors (Rosenkranz and Grace, 2001). The ventral mPFC also projects heavily to the ventral tegmental area, a connection involved in reward learning and motivation (Carr and Sesack, 2000). In addition, the mPFC projections to the striatum are abundant. Particularly, the infralimbic cortex projects heavily to the nucleus accumbens shell, while the dorsal part of the prelimbic cortex projects predominantly to the nucleus accumbens core (Heidbreder and Groenewegen, 2003), both of which are implicated in addictive behaviors and reward

learning (Hodge et al., 1995; Di Chiara et al., 2004; Russo et al., 2010). mPFC efferents, specifically those from the infralimbic subdivision to the striatum, have been shown to be necessary for the expression of habitual behaviors (Smith et al., 2012), whereas projections from the prelimbic subdivision to the striatum are activated during decision-making tasks (Friedman et al., 2015). The mPFC also projects to the thalamus, hypothalamus, spinal cord, and raphe nuclei (Heidbreder and Groenewegen, 2003).

Some of the main afferent projections to the mPFC are summarized below. In general, the dorsal mPFC receives mostly *sensorimotor* input from the cortex and thalamus, which suggests that the dorsal mPFC takes in abundant information from sensory modalities (Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007). In contrast, the ventral mPFC receives more *limbic* input from higher association cortices, suggesting that this subdivision is involved in the integration of information (Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007). Both dorsal and ventral mPFC receive projections from the basolateral amygdala, which may involve integrating emotional responses (Cunningham et al., 2008). In addition, the mPFC in general receives projections from the ventral tegmental area and is involved in the regulation of voluntary motor execution (Seamans and Yang, 2004). The dorsal and ventral mPFC also receive input from the hippocampus (Gabbott et al., 2002; Heidbreder and Groenewegen, 2003). Particularly, the infralimbic cortex receives the most abundant projections from this region (Hoover and Vertes, 2007). Other brain regions that send projections to the mPFC are the thalamus, hypothalamus, and raphe nuclei (Heidbreder and Groenewegen, 2003).

1.4 Effects of adolescent alcohol consumption on the prefrontal cortex

Human studies have shown correlations between alcohol consumption and alterations in the PFC. Adolescents with alcohol use disorders displayed a significantly reduced PFC size (De Bellis et al., 2005) and decreased blood flow in frontal regions (Norman et al., 2011), when compared to control adolescents. Interestingly, there are sex differences in the effects of alcohol on PFC. Adolescent female binge drinkers displayed less activation of frontal lobes than control adolescent females when completing a spatial working memory task. Conversely, in the same study, adolescent male binge drinkers showed higher activation of this region than adolescent male controls when completing the same task (Squeglia et al., 2011). In addition, males with alcohol use disorders showed larger PFC volume than healthy control males, while females with alcohol use disorders displayed smaller PFC volume than healthy control females (Medina et al., 2008). However, as is clearly noted and due to the limitations of the methodology, it is not understood whether a reduction in PFC size is a predisposing factor or a maladaptive consequence of alcohol use disorders (De Bellis et al., 2005).

Drug use and addiction are associated with deficits in PFC-dependent functions, such as executive control of behavior and impulse control (Bechara, 2005; Oscar-Berman and Marinkovic, 2007). In fact, behavioral similarities exist between drug addicts and patients with physical trauma to the PFC (Bechara, 2005), suggesting the dysfunction in the PFC is highly correlated with addictive behaviors. Moreover, alcohol-dependent women displayed significantly reduced activity in prefrontal regions during a spatial working memory task (Tapert et al., 2001). In addition, both male and female binge

drinkers showed a marked deficit in PFC-dependent executive functions, such as spatial working memory and pattern recognition (Weissenborn and Duka, 2003).

In rodents, adolescent alcohol exposure dampened the brain response to alcohol later in adulthood; particularly alcohol dampened the activation of the PFC and amygdala (Liu and Crews, 2015). This suggests that alcohol exposure during adolescence has long-term effects to the PFC. Concurrently, adolescent consumption of alcohol markedly reduced spike activity of PFC neurons in a dose-dependent manner, as observed via *in vivo* electrophysiological recordings (Tu et al., 2007). Interestingly, chronic intermittent alcohol exposure in adolescence did not induce significant acute deficits in certain PFC-dependent behaviors, such as set-shifting (Badanich et al., 2011), suggesting that behavioral repercussions may not be immediate but may occur later in adulthood. In contrast, adolescent alcohol exposure sensitizes brain areas involved in addictive behaviors, such as nucleus accumbens and ventral tegmental area, both of which displayed exacerbated activation to alcohol consumption later in adulthood (Liu and Crews, 2015).

1.5 Effects of adolescent alcohol consumption on frontal white matter

Human studies have shown correlations between adolescent alcohol consumption and reduction in frontal white matter integrity. For example, binge drinking adolescents showed a significant reduction in FA (white matter integrity) in major white matter frontal pathways when compared to controls (Jacobus et al., 2009; McQueeny et al., 2009). In addition, the number of hangover symptoms was significantly correlated with lower FA in the genu and body of the CC (McQueeny et al., 2009). Similarly, increased

alcohol consumption in adolescents was correlated with higher MD (lower white matter quality) in fiber tracts of frontal regions (Bava et al., 2012).

Other human studies have examined the effects on white matter in adolescents with alcohol use disorders, showing some inconsistencies and sex differences. For example, one study showed smaller PFC white matter volume in both male and female teenagers with alcohol use disorders (De Bellis et al., 2005). Similarly, another study showed that females with alcohol use disorders displayed smaller PFC white matter volumes than healthy control females (Medina et al., 2008). However, males with alcohol use disorders showed larger PFC white matter volumes than healthy control males, suggesting inconsistencies in sex differences in the effect of alcohol on PFC white matter (Medina et al., 2008). Interestingly, De Bellis et al. (2008) showed that adolescents with alcohol use and comorbid mental disorders showed increased FA, as compared to controls, in the rostral body and isthmus of the CC. This increase in FA may suggest an accelerated myelination, which could function as a compensatory response in adolescents with heightened risk for substance use and other comorbid mental disorders (De Bellis et al., 2008).

Very few studies have been conducted in *adolescent* rodents to investigate the effect of alcohol exposure on myelin. In one study, adolescent mice that were injected with 3 g/kg of alcohol showed down-regulation of myelin proteins and aberrant compaction of myelin in the PFC, when compared to saline controls (Montesinos et al., 2015). Koss et al. (2012) showed that male adolescent rats injected with 3 g/kg of alcohol had fewer mPFC glial cells later in adulthood than male controls, indicating long-term effects of alcohol on mPFC glial cell count. Females, however, did not display significant

differences in glial cell count between the alcohol and control groups in adulthood, which indicate sex differences exist in the interaction between alcohol exposure and glial cell count (Koss et al., 2012).

The majority of rodent studies investigating the effect of alcohol exposure on myelin have been conducted in adult animals. For example, adult mice treated with 10% v/v alcohol (replacing water) for 5 months showed a reduction of MBP immunoreactivity in the PFC, a downregulation of proteins and mRNAs involved in myelination in the cortex, an increase in oligodendrocyte cell death in the mPFC, and disruptions in myelin compaction in the CC and cortex when compared to controls (Alfonso-Loeches et al., 2012). In addition, adult rats exposed to chronic intermittent ethanol vapor exposure displayed reduced MBP expression in the mPFC compared to controls (Kim et al., 2015). Another study showed a reduction in CC thickness, area, and myelin thickness in adult rats exposed to alcohol for 1 year (3 bottle-choice paradigm), as compared to controls (He et al., 2007).

1.6 Objectives

Based on the current literature regarding adolescent alcohol consumption and white matter development discussed above, the present dissertation is composed of three aims. The first aim is to examine the morphological changes associated with developmental increases in myelination of the PFC and forceps minor of the CC during adolescence (Chapter 2). The second aim is composed of two sections: (1) Determine the factor(s) that could drive the previously observed relationship between increased alcohol consumption and decreased white matter, and (2) Determine the functional significance of alcohol consumption during adolescence at the behavioral level (Chapter 3). The third

aim of this dissertation is also composed of two sections: (1) Test for sex differences in the effects of adolescent alcohol consumption on PFC myelination, and (2) Identify a potential process that may be involved in how alcohol impacts prefrontal myelin structure (Chapter 4).

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CHAPTER 2

MYELINATION OF PREFRONTAL AXONS IS ACCOMPANIED BY INCREASED SPEED AND INTEGRITY OF CORTICAL NEUROTRANSMISSION IN RATS

In preparation.

Note: My work (WMV) for this manuscript focused on carrying out the histological experiments to study the morphology of myelin. We collaborated with Dr. Geng-Lin Li and his laboratory for the electrophysiological experiments, which were conducted by Sean McDougall. I have included both histological and electrophysiological experiments in the present chapter in order to provide the complete story of the manuscript.

2.1 Abstract

The anterior cingulate is a sub-region of the prefrontal cortex involved in emotional and cognitive processing. Cells in the anterior cingulate project to—and receive projections from—other cortical and subcortical structures via the anterior branches of the corpus callosum, specifically the forceps minor. Improvements in cognitive ability during adolescent development correspond with increases in frontal white matter—the fiber tracts comprised primarily of myelinated axons. Myelin is a lipid-rich coating wrapped around the axons of neurons, and myelination of axons during adolescence may serve to increase the speed of communication between neurons. Alternatively, the developmental increases in white matter may correspond with other functions such as the integrity of firing, e.g., the probability that action potentials successfully propagate along the axon to the terminal. We tested whether the

developmental increase in white matter corresponds with increased speed or with enhanced integrity of neural transmission by examining the microstructural changes in myelinated axons in the medial prefrontal cortex (mPFC) and the neurophysiological properties of these axons. We found a significant increase in the number of myelinated axons in mPFC from postnatal days (PD) 15-43, which corresponded with a significant increase in conduction velocity during PD 15-22. The relationship between response latency and transmission distance depended on the level of stimulation used, suggesting that different levels of stimulation preferentially activate separate fiber populations. High stimulation intensity produced a positive relationship between latency and distance in all age groups. Conversely, stimulation at threshold intensity produced no correlation between latency and distance, but did produce a positive relationship between conduction velocity and transmission distance. This indicates that for this population of fibers, increased conduction velocity in longer fibers may serve as a mechanism to keep response latency constant over different distances so signals may be received at the same time. Disruption in myelination of these axons in developing animals could impair the speed, integrity, and synchronization of neural signals, which could have long-term effects on cognitive processing in adulthood.

2.2 Introduction

Myelination of axons in the brain is a critical process that can increase the rate of neuronal communication (Giedd, 2004). Myelination continues from birth into adulthood in some brain regions in both rodents and humans. The brain undergoes a general posterior to anterior process of myelination, with the prefrontal cortex (PFC) in the anterior portion of the brain one of the last structures to finish myelinating (Aubert-

Broche et al., 2008). The PFC is a structure crucially involved in different aspects of executive function and emotional regulation. A positive correlation between myelin density in the PFC and performance on different cognitive tasks has been demonstrated in both humans and rats, so it is important to understand how prefrontal axons change structurally and functionally during development (Fuster, 2002). As will be described in Chapter 3, research in our lab has also demonstrated that myelin density in the anterior cingulate cortex (Cg1), a specific sub-region of the mPFC, is decreased by voluntary alcohol binge drinking in adolescent rats (Vargas et al., 2014). This cortical region has an important role in the affective perception of pain (Fuchs et al., 2014), stress response regulation (Law et al., 2009), working memory (Seamans et al., 1995), behavioral control (Takenouchi et al., 1999), and attention (Rushworth et al., 2003; Kaping et al., 2011). To fully understand the role of Cg1 myelin in complex cognitive processing or how this is affected by alcohol, we must first determine how myelination of axons changes within the Cg1 during adolescence. This was the objective of the present study.

In humans, myelination begins prenatally and continues into adolescence. In contrast, when rats are born, their central nervous systems show almost no myelin. Myelination begins shortly after birth, with the first myelinated fibers being observed in the forebrain by postnatal day (PD) 7 (Downes and Mullins, 2013). The corpus callosum starts to undergo a steady increase in myelination by PD 8, and continues to myelinate until PD 34 (Downes and Mullins, 2013).

Electron microscopy has been used to examine microstructural changes in myelin that occurred over the course of rats' adolescent development in the genu of the corpus callosum (Calabrese and Johnson, 2013) —the most anterior part of the corpus callosum

that includes the forceps minor (FM) (Fitsiori et al., 2011). The FM has axon connections with cells in the Cg1 (Higashi et al., 1991). These axons were almost devoid of myelin at birth, but showed evidence of early myelination by PD 12, showing a rapid increase in myelin between PD 12 and PD 24 animals (Calabrese and Johnson, 2013). Calabrese and Johnson also used diffusion tensor magnetic resonance imaging to quantify changes in functional anisotropy and diffusivity in different white matter structures of the rat brain over the course of adolescent development. These parameters can be used as markers of myelin development in white matter structures. They found that rats underwent a period of rapid myelination in most white matter structures between PD 10 and 20, and that measures of myelination were relatively stable after this point up to adulthood (PD 80). This corroborates other studies showing that most of the myelination process is completed by about PD 24 in the entire brain, including the PFC (Downes and Mullins, 2013). However, many of the studies measured the degree of myelination only in the white matter regions such as the corpus callosum of the forebrain. It is possible that changes in myelin within the gray matter could follow a different, and perhaps later, time course than that for white matter. For example, aging tends to promote a loss of myelin density in white matter, but not gray matter, structures in the brain of humans (Piguet et al., 2009). Similar age-related losses of myelin density in white matter structures have also been observed in rats (Yang et al., 2009).

Several studies have demonstrated that myelination does not progress to the same extent in all mature fibers (Tomassy et al., 2014). In particular, differential myelination can be used as a mechanism to keep conduction times synchronous despite differences in pathlength of individual fibers that target the same regions (Lang and Rosenbluth, 2003;

Seidl, 2014). Differential myelination has been observed in various regions of the brain: retinal ganglion cell axons traveling to the lateral geniculate nucleus (Stanford, 1987), connections between the ventrobasal nucleus of the thalamus and layer IV cells in the primary somatosensory cortex (Salami et al., 2003), and axonal projections from perirhinal cortex neurons to lateral amygdala (Pelletier and Paré, 2002). These studies show that axon populations can have different relationships between latency and transmission distance, even within the same anatomical area.

It is generally assumed that cognitive improvements that accompany normal myelin development in the PFC, or behavioral changes induced by drugs that affect myelin density, such as alcohol, result from changes in action potential conduction velocity. The goal of our study was to take the first step in answering this question by measuring action potential conduction velocity changes relative to distance in the PFC over the course of adolescent development. We targeted gray matter fiber connections between the FM and the Cg1, based on previous research implicating myelin in this region as being especially susceptible to interference from adolescent alcohol consumption (Vargas et al., 2014). This study provides the necessary baseline data to interpret future results on how environmental deviations from normal developmental circumstances (e.g. alcohol) affect action potential conduction velocity in this important region of the PFC.

2.3 Materials and Methods

2.3.1 Animals

A total of 45 male Wistar rats were used in the present study (eight for the histological portion and 37 for the electrophysiological portion of this study). For the histological studies, rats arrived from Charles River with dams on PD 11 (pre-adolescent group) or without dams on PD 39 (adolescent group). PD 11 animals were housed with mothers and PD 39 animals were housed in pairs. For the electrophysiological studies, animals were divided into four age groups: PD 8-15 (juvenile), PD 22-28 (pre-adolescent period), PD 43-52 (mid-adolescent period), and PD 81-93 (early adulthood). All animals were kept on a 12-hour cycle (lights on at 8am), with *ad libitum* food and water. All procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

2.3.2 Semi-thin tissue processing

Brains were collected in pre-adolescent (PD 15) and adolescent (PD 43) male rats following intracardial perfusions with 1% paraformaldehyde/ 1.25% glutaraldehyde. After 4 days of post-fixation, brains were sectioned coronally with a vibratome into 150-μm sections to isolate the PFC (between 3.20 mm - 1.85 mm away from Bregma) (Paxinos and Watson, 1998). Tissue was contrasted with the lipid cross-linker osmium tetraoxide and with the negative stain uranyl acetate to visualize phospholipids, nucleic acids, and myelin sheaths, dehydrated with various percentages of alcohols and propylene oxide, embedded in epoxy resin, and mounted on epoxy blocks. Digital images from the

150-μm sections were taken at 10x and the semi-thin (2.5 μm) coronal sections were collected using an RMC MT6000-XL ultra microtome and glass knives. Sections were then stained with 0.1M toluidine blue in borax, dehydrated with graded alcohols, and cover slipped for microscopic analysis.

2.3.3 Semi-thin microscopic analysis

Photomicrographs were taken in layer V of the mPFC using a Leica microscope (100x oil objective) attached to a DP71 Olympus camera. We focused on layer V because it is the main output layer of the mPFC (Goodfellow et al., 2009; van Aerde and Feldmeyer, 2013). In addition, PFC layers II/III display more heterogeneity than layers V/VI (Tomassy et al., 2014). Layer V was identified by locating its characteristic large pyramidal neurons with apical dendrites that project towards layer I of the cortex (Kiernan and Hudson, 1991; Swenson, 2006; Wang et al., 2006; Fénelon et al., 2011; Vostrikov and Uranova, 2011).

The number of myelinated axons and the thickness of myelin relative to axonal size (g-ratio) were quantified using Image J software (Rasband, 1997) (modified from (Michailov et al., 2004). Axons were identified by matching the characteristics (e.g. shape) that previous studies using ultra-thin sections and electron microscopy found (Peters et al., 2001; Chomiak and Hu, 2009; Liu et al., 2012). We quantified g-ratio in at least 100 randomly selected myelinated axons for each animal as an index of myelin thickness. For consistency purposes, we only selected axons that had its entire myelin sheath visible, and thus did not select any axon that had a partial myelin sheath visible. G-ratio was calculated by dividing the axon area by the area of the axon plus the myelin sheath combined. We used area-based g-ratio as opposed to the standard diameter-based

g-ratio (as Bakken and Stevens, 2011) because axons in the central nervous system are not perfectly circular in cross sections (Almeida et al., 2011; Perge et al., 2012).

2.3.4 Electrophysiology tissue processing

Following euthanasia, brains were rapidly removed and placed in ice-cold cutting solution, which contained 89.1 mM sucrose, 13.88 mM glucose, 87.27 mM NaCl, 2.48 mM KCl, 1.25 mM sodium phosphate monobasic monohydrate, 25 mM sodium bicarbonate, 7 mM MgCl2 ·6H2O, and 0.37 mM CaCl2. Coronal sections were cut at 300-µm thickness with a vibratome (Leica VT1200 S with Vibrocheck) while submerged in ice-cold cutting solution. The sections were then incubated in artificial cerebral spinal fluid (aCSF) at 33°C for 45 minutes, which contained 127 mM NaCl, 25 mM sodium bicarbonate, 25 mM glucose, 2.5 mM KCl, and 1.25 mM sodium phosphate monobasic monohydrate.

2.3.5 Recordings

Sections were then transferred with a pipet to a recording chamber, where they were perfused with aCSF for the duration of recording. While submerged in either cutting solution or aCSF, tissue was always continuously bubbled with 95% O2 and 5% CO2 gas. During recording, neurons were located using an Olympus BX51W1 microscope with 4x and 60x objectives. Cells in the Cg1 were then patched under whole-cell mode (amplifier was HEKA EPC10/2 USB with PatchMaster for data acquisition). Micropipettes had a bath resistance between 3-11MΩ (pipet puller Narishige PC-10), with an internal solution containing 130 mM KGlu, 10 mM KCl, 10 mM HEPES, 1 mM EGTA, 3 mM MgATP, and 0.5 mM NaGTP combined with Alexa 594 fluorescent dye. A

concentric bipolar stimulating electrode was used to excite fibers in the dorsal-medial region of the FM branches of the corpus callosum (stimulator used was Analog Stimulus Isolator Model 2200 from S-M Systems). For animals in which the FM was visibly myelinated, this region was easy to locate microscopically under 4x magnification. However, for all animals, including those that had no visible myelin under 4x magnification, the boundary of the FM white matter and the beginning of the cortex could easily be discerned under 60x magnification. The cortex could be determined from the presence of neurons, while the FM could be determined by the presence of fiber bundles. Excitatory postsynaptic currents (EPSCs) were recorded in the patched cells under voltage clamp at -80mV. Initially, a stimulation ramp protocol was run to see how the EPSC profile changed with increasing stimulation voltage, and then the cell was repeatedly stimulated with constant intensity stimulation, in order to establish a stable response.

2.3.6 Stimulation paradigms

Two different stimulation paradigms were used. The first used high intensity stimulation (10-200uA, 1ms) to generate maximal amplitude EPSCs. The EPSC onset was determined as the time point following the artifact when the current changed by 20 pA from baseline. A threshold stimulation paradigm was also used (10-200uA, 0.2ms). Threshold was determined by lowering the stimulation intensity to the minimum level at which EPSCs could still be evoked, accompanied by frequent failures. The EPSC onset was measured as the time point where the slope of the current changed from baseline following the EPSC artifact.

2.3.7 Analysis

Images of the patched cells were taken under 60x magnification for identification of cell type. Images taken under 4x magnification, showing the placement of the stimulation electrode relative to the recorded cell, were used to determine the transmission distance. This was estimated as the distance between the cell and the stimulating electrode. For each cell that showed a stable EPSC response, these distances were plotted against the EPSC onset time. Two different methods were used to determine the response latency. The first method used the time difference between the start of the stimulation artifact and the point following the post-artifact current change that fell below the baseline current of the cell prior to applying stimulation. The second method determined the latency between the start of the artifact and the peak of the resulting EPSC. One drawback of this method was that the EPSC peak did not necessary reflect the most accurate measure of EPSC onset because cell-to-cell EPSCs exhibited a wide range of different kinetics, i.e. the time difference between the baseline and the EPSC peak varied widely between cells. However, the second method also had advantages. One advantage was that calculations of the average response latencies and standard deviations in latency could easily be determined using an automated program in Igor Pro for a series of stimulations (usually $\geq$10 traces for each cell). Another advantage of this method was that it allowed for the determination of the response latency jitter for each cell.

Jitter is the variability in response latency observed throughout various stimulations and it is important because it can reliably discriminate between mono- and polysynaptic pathways (Doyle and Andresen, 2001). Jitter for each series of stimulations

was calculated as the standard deviation of the response latencies using ≥10 individual traces.

2.3.8 Statistical Analyses

ANOVAs and t-tests were used to assess the difference in conduction velocity and myelinated axon number between the different age groups. Pearson correlation analyses were used to assess relationships between latency and transmission distance, and between g-ratio and axon diameter. In addition, a multiple regression analysis was performed to examine whether axon diameter, age, and their interaction predicted g-ratio in the FM. Statistical significance was defined as $p \leq 0.05$ using two-tailed tests. Statistical analyses were performed using R statistical software package (R Core Team, 2014).

2.4 Results

We noted that young animals (PD 8-14) had minimal amounts of myelin visible in the FM. By PD 15, some myelination was visible on the lateral regions of the FM. By PD 22, the entire FM appeared to be myelinated. We therefore used histological methods (Fig 2.1) to explore the microstructural features underlying developmental increases in myelin. As shown in Fig 2.2, myelin was visible within the dorsal-lateral, but not the dorsal-medial region of the FM of pre-adolescent animals (PD 15). In adolescent animals (PD 43), the medial and lateral regions of the FM were visibly myelinated. Moreover, measurements of cross-sectional area of the FM in embedded tissue showed a significant increase in PD 43 animals, when compared to PD 15 animals ($t(4.06) = 4.95$, $p < 0.007$, Fig 2.3).

Microscopic analysis of semi-thin slices of embedded tissue from the Cg1 in the mPFC showed that the number of myelinated axons increases significantly between PD 15 and 43 ($t(6) = 14.17$, $p < 0.0001$, Fig 2.4), similar to the changes in the FM white matter seen over a comparable age range (Calabrese and Johnson, 2013). The number of pyramidal cells in the mPFC did not differ between groups ($t(6) = 0.31$, $p = 0.78$, not shown), indicating that the images were actually from the mPFC. Most importantly, no changes in pyramidal cell number, alongside an increase in myelinated axons, show that no new pyramidal neurons are being formed in the mPFC after PD 15. Therefore, it is very likely that the axons are already present at PD 15 without yet being myelinated. This is assuming that the myelinated axons counted originate from the pyramidal cells in the mPFC. We cannot rule out the possibility that these axons originate from cells in other cortical regions or from other brain regions, such as the amygdala which projects to the mPFC. The latter is less likely, however, because most brain regions are myelinated by this time (Baumann and Pham-Dinh, 2001) and layer V is mainly an output layer (Goodfellow et al., 2009; van Aerde and Feldmeyer, 2013).

Very few myelinated fibers could be identified in the PD 15 animals. However, a nearly seventy-fold increase in the number of myelinated fibers was observed when compared to tissue from PD 43 animals. Although there were not enough myelinated axons in the PD 15 animals required to measure their g-ratio (>100 axons required per animal), there was a positive relationship between the g-ratio and axon diameter in the mPFC of PD 43 animals (all regressions with $p < 0.05$, Fig 2.5).

In early experiments, we found that stimulation of the dorsal-medial portion of the FM reliably induced EPSCs in cells of the Cg1. The dorsal-medial portion of the FM is

the area of the FM closest to the cingulum, which may be the source of some of the fiber connections onto the recorded cells. Stimulation of this region of the FM was used throughout this study (Fig 2.6a).

The waveforms of maximal amplitude responses were often complex, while those evoked with threshold stimulation were often monophasic (Fig 2.6). This indicates that maximal stimulation tended to activate many fibers that synapse on the same cell, often showing different latencies despite traversing to the same final distance from the stimulation site. In contrast, the monophasic responses as well as frequent response failures obtained using threshold stimulation indicate that only one or possibly a few fibers were activated using this level of stimulation.

Using the change from baseline criteria to determine EPSC latency (Fig 2.7), a significant increase in conduction velocity was observed between PD 8-15 animals and older animals (≥ PD 22) using both maximal amplitude ($F(3, 113) = 3.93$, $p = 0.0104$, Fig 2.7a left) and threshold stimulation ($t(29) = 5.08$, $p < 0.0001$, Fig 2.7a right). Planned comparisons between the PD 8-15 group and all the other groups were significant (all paired t-tests $p < 0.05$, Fig 2.7a left). No significant increase in conduction velocity was observed after this age point. Threshold and maximal amplitude stimulation varied significantly in the relationship between latency and transmission distance. While there was a strong positive relationship between latency and distance for the maximal amplitude stimulation ($r = 0.73$, $p < 0.0001$, Fig 2.7b), no such correlation was observed with the threshold stimulation ($r = 0.16$, $p = 0.31$, Fig 2.7c). However, there was a moderately strong linear relationship between velocity and transmission distance for the threshold stimulation data ($r = 0.61$, $p < 0.0001$, Fig 2.7d).

The overall trends observed using peak latency to determine EPSC onset largely agreed with those seen using the baseline current criteria (Fig 2.8). However, larger standard errors were observed in the maximal stimulation data set and the latency was significantly larger between PD 8-15 and PD 43-52 groups ($t(52) = 3.53$, $p = 0.001$, Fig 2.8a left). A significant increase in latency jitter was observed in PD 43-52 animals, as compared to PD 8-15 animals, using maximal stimulation ($t(72) = 2.15$ $p = 0.035$, Fig 2.8d) and between PD 8-15 and PD 22-58 animals using threshold stimulation ($t(27) = 1.68$, $p = 0.032$, Fig 2.8e). No statistically significant differences in jitter were observed between PD 8-15 and either PD 22-28 or PD 81-93 groups using maximal stimulation (both $p > 0.05$).

2.5 Discussion

The objective of this study was to determine how conduction velocity changes in axonal fibers of the Cg1 over the course of adolescent development, and to identify whether this change was consistent with periods of significant myelination. This is important baseline information for future studies investigating conduction velocity in this region, particularly those examining the effects of substances, such as alcohol, that can interfere with the myelination process. We used electrophysiology to measure conduction velocity in rats from four age groups ranging from neonates to early adulthood, and histology of semi-thin sections of the mPFC to determine myelin changes between pre-adolescent and adolescent rats. A great degree of variability in conduction velocity was observed on a cell-to-cell basis between animals of the same age group, and even within the same animal. Still, a significant increase in conduction velocity was observed between the second and third postnatal weeks, which was consistent with the significant

myelination increase observed in the mPFC from PD 15 and 43. This conduction velocity increase also corresponded with a period of rapid myelin development (Calabrese and Johnson, 2013). However, different stimulation intensities produced differential relationships between response latency and transmission distance. These findings suggest that the same response latency may be maintained over different transmission distances. This has implications for substances that interfere with myelin development, since they could cause disruption and aberrant network activity.

The observed increase in conduction velocity between PD 15 and 22 corresponds with the increased myelination observed in our embedded and semi-thin tissue. The FM cross-sectional area increased from PD 15 to PD 43, and the number of mPFC myelinated axons increased dramatically at PD 43, after being almost non-existent in PD 15. This increase in mPFC myelinated axon number indicates that myelin is increasing because more axons are undergoing *de novo* myelination during adolescence, and the effect observed is not only due to myelin sheaths thickening on myelinated fibers already present. Therefore, the amount of myelinated axons plays an important role in adolescent myelin development in the PFC. These results confirmed previous research showing an increase in myelination during adolescence (Downes and Mullins, 2013).

Our data also provided new information regarding myelinated axons within the cortex (gray matter), particularly in the mPFC, by analyzing specific axonal populations at the microstructural level. The results showing an increase in mPFC myelinated axons in the adolescent group, with no group changes in pyramidal cell number, help explain that the increase in conduction velocity is likely due to an increase in myelinated axons (*de novo* myelination) rather than thickening of the sheath on axons that were already

myelinated. The observed positive relationship between g-ratio and axon diameter indicates that axons with larger diameters require less myelin to maintain an adequate conduction velocity, as has been previously suggested (Purves et al., 2008). This finding is consistent with previous literature illustrating the same relationship in other brain regions (Taveggia et al., 2007; Liu et al., 2012), but provides new information regarding microstructural measures of axonal myelin thickness relative to axonal size in the adolescent Cg1 of the mPFC.

These results are consistent with our predictions that increases in myelination should correspond with increases in average conduction velocity. However, it is important to remember that our current investigation examined changes in conduction velocity in the gray matter between the FM and cells in the cortex, not in the white matter itself. It is possible that subtle increases in myelination occur after this period of significant myelin development in these gray matter connections, but have small enough impacts on conduction velocity that we were not able to detect. There was a high degree of variability in conduction velocity between cells from animals of the same age, and even between cells within the same animal. As a result, smaller average increases in conduction velocity would likely be hard to detect.

While our results demonstrate a massive increase of myelinated mPFC fibers between PD 15 and PD 43, further work is still necessary to quantify changes in myelin density in gray matter over the entire course of adolescent development. Furthermore, it would be valuable to know whether the time course for myelin development is different for afferent versus efferent fibers between the FM and the Cg1. It is likely that some of the fibers that were observed during our tissue embedding procedure represent axonal

projections from Cg1 cells to the FM. These would not contribute to the EPSCs recorded electrophysiologically, and it is important to differentiate them in order to establish correlations between myelin density in the Cg1 and measures of conduction velocity. This is particularly important for future experimentation examining substances that affect myelination, such as alcohol. For example, in previous research in our laboratory we observed a reduction in overall myelin density in the Cg1 in response to adolescent binge drinking in rats (Vargas et al., 2014). However, we do not know if this myelin loss affected afferent and efferent projections equally. Expanded electrophysiology data over the entire course of adolescent development, in particular between PD 50 to PD 80, is necessary in order to more effectively probe the relationship between conduction velocity and myelin density. It is possible that a larger number of animals would be required to detect subtle changes in conduction velocity after the PD 15 to PD 22 period.

Regarding the threshold stimulation data, most of the cells recorded from this data set had shorter transmission distances for the younger animal group (PD 8-15), because their brains were physically smaller (Fig 2.8c). The observed correlation between distance and velocity holds even in the younger PD 8-15 animals (Fig 2.7d), before significant myelination has occurred. This indicates that myelin differences may not be the only factor that leads fibers that project over longer distances to have faster average velocities. These fibers may also have larger diameters, even when unmyelinated, which could account for the differences in velocity.

The question remains as to why high intensity stimulation produces a positive correlation between latency and transmission distance, while threshold stimulation produces a positive correlation between velocity and transmission distance. A similar

phenomenon was observed by Pelletier and Paré (2002) with reciprocal connections between the lateral amygdala and perirhinal cortex. The authors of this study suggest that different subpopulations of fibers, in their case afferent and efferent populations, were responsible for the different relationships. Using minimal stimulation should increase the probability that only direct monosynaptic connections are being activated, as triggering an EPSC through a purely polysynaptic connection will likely require more stimulation than through a monosynaptic connection, since the intermediary neuron would have to reach threshold. We found that the minimal amount of stimulation that could be applied to get an all-or-none EPSC response from a cell was typically about 10-30 uA for a period of 0.2 ms. For comparison, Kumar and Huguenard (2001) reported that to achieve minimal stimulation, defined for them as 1.2 times the threshold for a response, they typically applied 100-500 uA of current for a period of 0.05-0.3 ms (Kumar and Huguenard, 2001). They used a minimal stimulation paradigm in order to isolate purely monosynaptic responses, and the levels of stimulation that they used are comparable to our own. Presumably, cells that respond with this low amount of stimulation are triggered by monosynaptic inputs. In addition, an increase in response jitter, or variability in latency over the course of multiple stimulations, should indicate an increased contribution of polysynaptic inputs (Doyle and Andresen, 2001). This is because the signal must pass through an additional intermediary neuron, and the time it takes to do this can vary due to a number of factors (e.g. excitability, neurotransmitter release). The higher degree of latency jitter that we observed in older animals using both threshold and maximal amplitude stimulation may indicate a higher proportion of polysynaptic connections in our data for older animals, regardless of the type of stimulation used (Fig 2.9).

The two stimulation paradigms are likely preferentially activating different sub-populations of fibers. High intensity stimulation presumably activates many fibers that project onto the same cell at once. However, the measured latency is determined only by the fastest fiber. For threshold stimulation, the fiber that is physically closest to the stimulating electrode and has the lowest resistance to electrical activation will likely determine the measured velocity. It has been shown that larger diameter fibers have a lower electrical threshold for action potential initiation via artificial stimulation (Sundar and González-Cueto, 2006). However, other factors could influence which fiber determines the EPSC onset time. A fiber that synapses directly on the soma may have a shorter response latency than one that synapses on a distal dendrite. If the fiber synapses on a distal dendrite, it may also require more stimulation to generate an EPSC of sufficient magnitude to be detected by the recording electrode in the cell soma. The simplest explanation may be that most cells typically have at least one "fast" (i.e. large diameter and/or heavily myelinated) fiber connection from the FM that is typically activated when all fibers that connect to the cell are activated at once with high intensity stimulation (Fig 2.9a left). As a result, conduction velocity appears to be more or less independent of transmission distance. Threshold stimulation may randomly activate one of the many fibers that synapse on a single cell. There is a high degree of variability in individual fiber conduction velocity, as well as myelin thickness relative to axonal size (g-ratios ranging from 0.5 to 0.9) and fiber diameter (ranging from 0.5 to 3 μm^2), even within a single animal. As a result, the velocity of individual fibers that are randomly activated with threshold stimulation may show no strong relationship with the distance they travel, which explains why latency is not correlated with distance (Fig 2.9a right and

b). This also indicates that individual fibers may have mechanisms (i.e. increased myelin or diameter) that compensate for differences in pathlength, in order to keep response latencies constant over different transmission distances. Such functional organization could be important computationally in synchronizing cellular responses in different cortical layers.

A limitation of this study is that we have not identified the source of the fibers from the FM onto the cells from which we have recorded. While these fibers may originate from the contralateral mPFC, they could also originate from other areas of the brain. Little and Carter (2013) demonstrated the presence of reciprocal connections between pyramidal cells in the mPFC and the contralateral mPFC that extend throughout the different layers of the mPFC. However, they also showed that there are reciprocal connections between pyramidal cells in the mPFC and the basolateral amygdala, although these connections are localized predominately to the layer I-II boundary, and in our experiments data was sampled from cells across the different cortical layers. It is likely that the fibers stimulated during our study originate from the contralateral mPFC, although other origins are possible. Therefore, further histological work is necessary to determine the origin of these fibers. It is possible that the differences in the latency versus distance relationship in our data could be explained by different fiber populations originating from different anatomical areas.

This study will serve as an important baseline for future investigations that will determine how alcohol interferes with the normal myelination process in this region. The data in the present study suggest that alcohol could potentially reduce conduction velocity in mPFC axons by damaging existing myelin, rather than by preventing myelination. In

our previous study, animals binged on alcohol from PD 28-42 (Vargas et al., 2014), which is after the period of significant myelination during PD 15-22 (Calabrese and Johnson, 2013). However, the mPFC may be undergoing active myelination even if white matter such as the corpus callosum has been fully myelinated by this time (Downes and Mullins, 2013) as more myelinated fibers appear in the Cg1 gray matter by PD 43. Myelination is a gradual process in which the initial sheath becomes encapsulated by additional layers of myelin, and the layers transition from a loose arrangement to a compact structure (Calabrese and Johnson, 2013). If the Cg1 myelin sheaths are not fully compact by the binge drinking period, alcohol may interfere with the transition from a loose to a compacted myelin. The biochemical composition of myelin differs in adolescent and adults. Throughout adolescent development, the concentration of galactolipids, myelin basic protein, and myelin proteolipid protein increases, while the concentration of phosphatidylcholine and various other proteins decreases (Quarles et al., 2006). Alcohol could also interfere with these biochemical transitions in non-compact myelin, which could affect the final structure and functional properties of myelin. In future experimentation, it will be important to determine whether substances that interfere with myelination in the Cg1 disrupt the positive relationship between velocity and transmission distance observed with threshold stimulation in our study. If myelin is acting to increase the velocity of the longer fibers relative to the shorter ones, and a loss of myelin reduces or abolishes this relationship, then the threshold stimulation data may start to show a positive relationship between latency and distance similar to the maximal stimulation data.

2.6 Figures and Tables

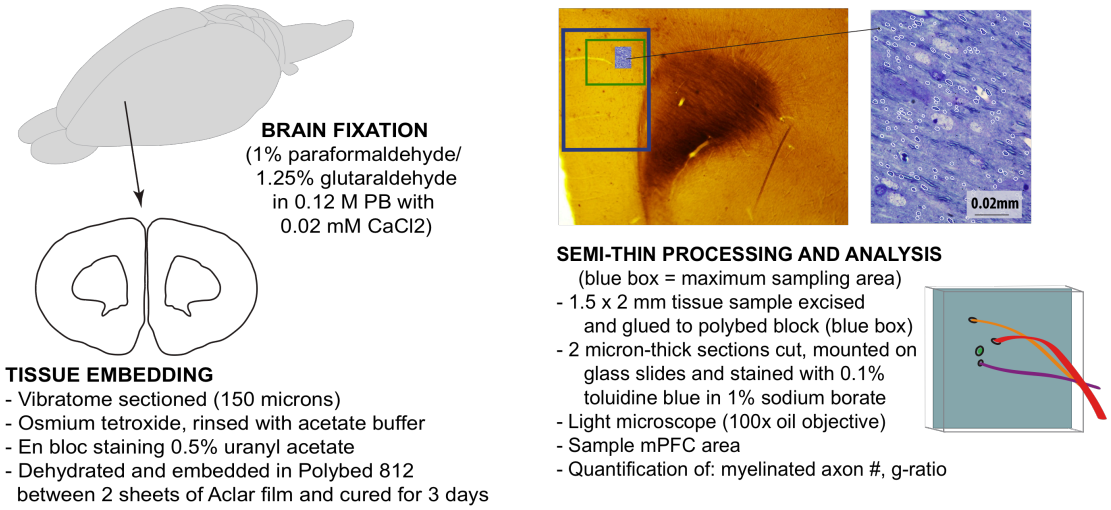


Figure 2.1. Microstructural myelin analysis and processing for semi-thin analysis. Left: Summary of brain fixation and tissue embedding for semi-thin sections. Brains were fixated in 1% paraformaldehyde/1.25% glutaraldehyde, sectioned on a vibratome into 150 µm-thick sections, and embedded in Polybed 812. Right: Summary of semi-thin section processing and analysis. The mPFC of the embedded tissue was further sectioned into 2.5 µm-thick semi-thin sections and stained with 0.1% toluidine blue. Semi-thin sections were analyzed microscopically.

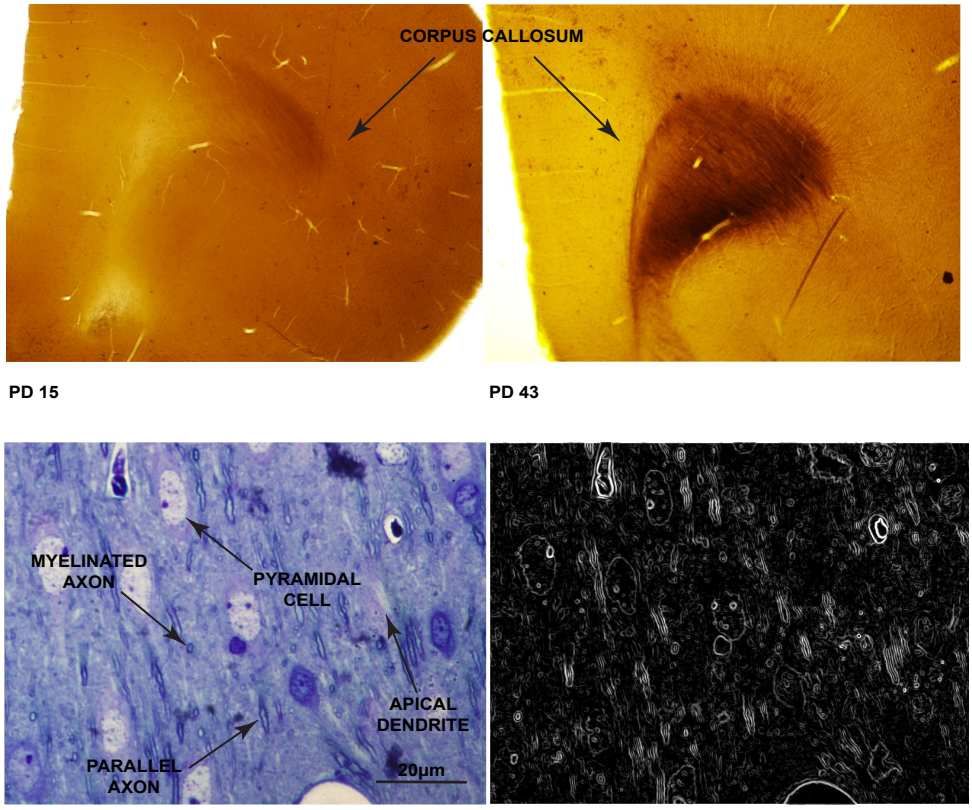


Figure 2.2. Representative photomicrographs of myelin development over adolescence in the FM and of semi-thin analysis. Top. Left: Embedded tissue of a PD 15 animal. No myelin was visible on the dorsal-medial region of the FM, although some was visible on the lateral portions. Right: Embedded tissue of a PD 43 animal. By PD 43, all of the FM was visibly myelinated. **Bottom.** Left: Image showing the anatomy of a semi-thin mPFC section. Right: Image used for the g-ratio analysis of a semi-thin mPFC section.

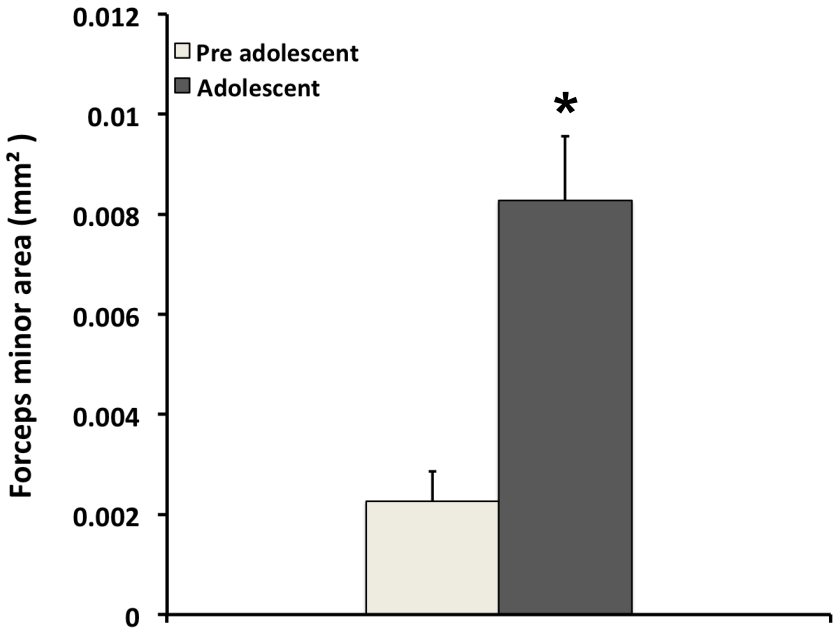


Figure 2.3. Significant increase in FM cross-sectional area in adolescence.
Adolescent (PD 43) rats (dark bar) showed a significant increase in FM cross sectional area in comparison to the pre-adolescent (PD 15) rats ($t(4.06) = 4.95$, $p < 0.007$). Data expressed as mean ± SEM.

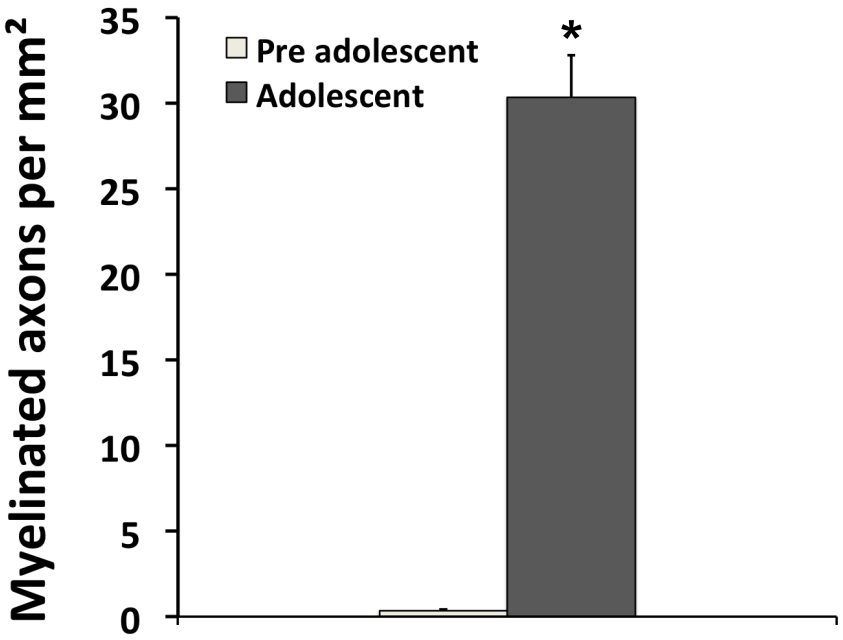


Figure 2.4. Significant increase in mPFC myelinated axons in adolescence. PD 43 rats showed a significant increase in mPFC myelinated axons in comparison to the PD 15 rats ($t(6) = 14.17$, $p < 0.0001$). Data expressed as mean ± SEM.

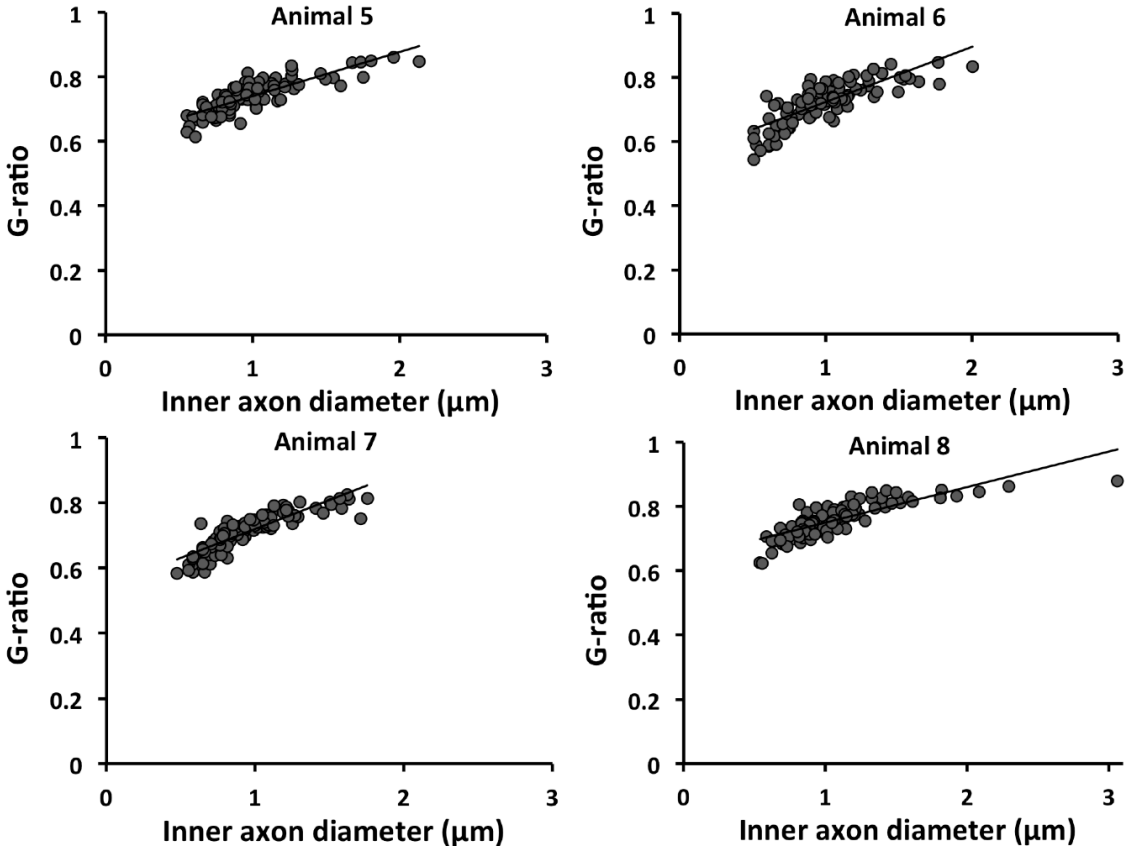


Figure 2.5. Significant positive correlation between the g-ratio and diameter of mPFC myelinated axons in adolescence. There was a positive relationship between the g-ratio and axon diameter in the mPFC of PD 43 animals (all regressions with $p < 0.05$).

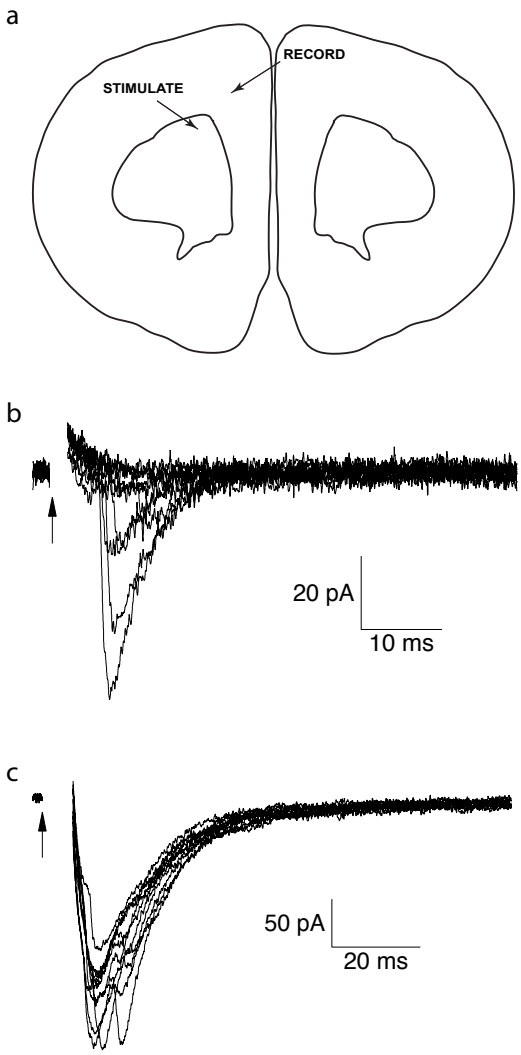
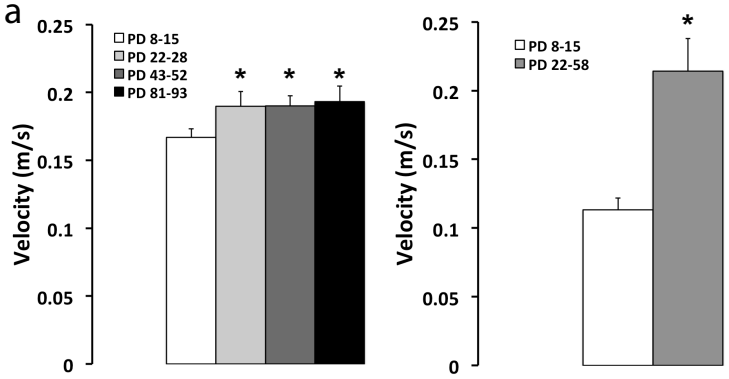
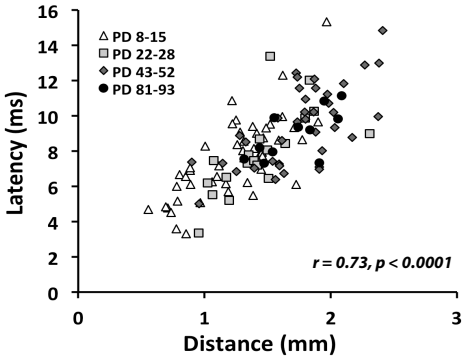


Figure 2.6. Electrophysiology overview and example recordings taken from a PD 15 rat. a. Schematic of a coronal section illustrating placement of the stimulating and recording electrodes, in the forceps minor and Cg1 cortex, respectively. **b.** A series of 15 superimposed traces taken from a single cell using constant amplitude threshold stimulation. Responses did differ in the maximum amplitude of their current, but were still all-or-none, seen by the frequent instances when stimulation failed to evoke any response from the cell. **c.** Recordings from the same cell taken with maximum amplitude stimulation. The latency of the EPSC onset was shifted earlier, and the maximum amplitude was increased. Arrow in b and c represents the stimulation.

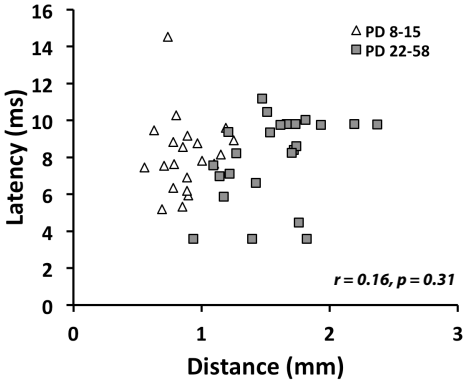
a


PD 8-15
PD 22-28
PD 43-52
PD 81-93
Velocity (m/s)
0.25
0.2
0.15
0.1
0.05
0
*
*
*
PD 8-15
PD 22-58
Velocity (m/s)
0.25
0.2
0.15
0.1
0.05
0
*

b


PD 8-15
PD 22-28
PD 43-52
PD 81-93
Latency (ms)
16
14
12
10
8
6
4
2
0
0
1
2
3
Distance (mm)
r = 0.73, p < 0.0001

c


PD 8-15
PD 22-58
Latency (ms)
16
14
12
10
8
6
4
2
0
0
1
2
3
Distance (mm)
r = 0.16, p = 0.31

d

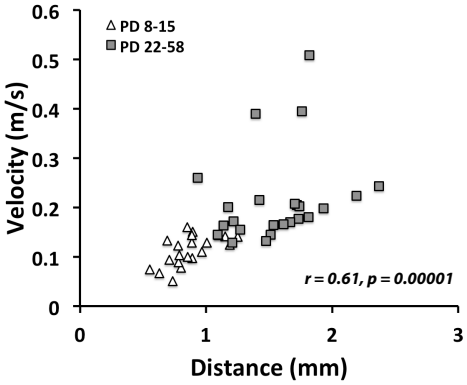
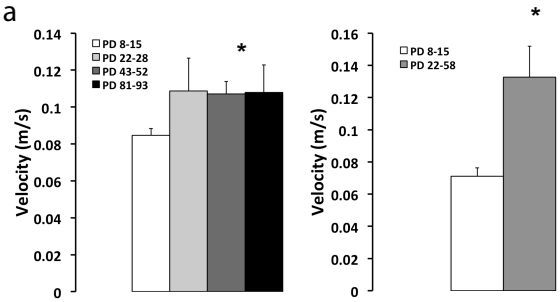
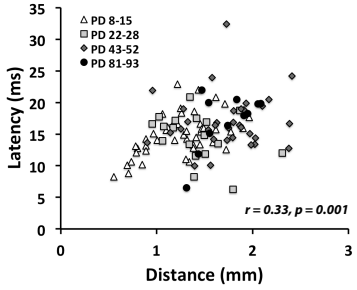

PD 8-15
PD 22-58
Velocity (m/s)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
Distance (mm)
r = 0.61, p = 0.00001

Figure 2.7. Significant increase in conduction velocity in adolescence, relationship between latency and distance was positive using maximal stimulation, and disappeared using threshold stimulation (using change from baseline current criteria). a. Left: There were significant differences between the age groups in average conduction velocity using maximal amplitude stimulation ($F(3, 113) = 3.93$, $p = 0.0104$). The PD 8-15 group (10 animals, 48 cells, white bar) showed a significant decrease in conduction velocity in comparison to each of the older groups: PD 22-28 (5 animals, 20 cells, light gray bar), PD 43-52 (7 animals, 38 cells, dark gray bar), and PD 81-93 (4 animals, 11 cells, black bar) (all t-tests with $p < 0.05$). Right: Average conduction velocity using threshold stimulation. The PD 22-58 (6 animals, 24 cells, gray bar) showed a significant increase in conduction velocity compared to the younger PD 8-15 group (5 animals, 26 cells, white bar) ($t(29) = 5.08$, $p < 0.0001$). **b.** A strong positive correlation was observed between latency and transmission distance for recordings taken with maximal amplitude stimulation ($r = 0.73$, $p < 0.0001$). Each dot represents data from a single cell, with colors white, light gray, dark gray, and black representing the age groups PD 8-15, PD 22-28, PD 43-52, and PD 81-93, respectively. **c.** There was no correlation between latency and transmission distance in recordings taken with threshold stimulation ($r = 0.16$, $p = 0.31$). **d.** There was a moderately strong correlation between velocity and distance using threshold stimulation ($r = 0.61$, $p < 0.0001$). This correlation holds even when only the youngest age group (PD 8-15, white dots) is considered ($r = 0.61$, $p = 0.0036$). Data expressed as mean ± SEM.

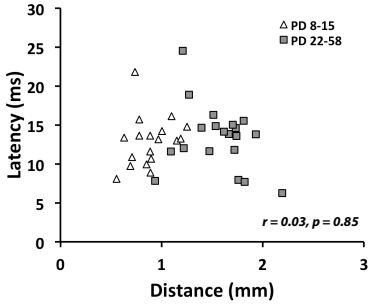
a


PD 8-15
PD 22-28
PD 43-52
PD 81-93
Velocity (m/s)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
PD 8-15
PD 22-58
Velocity (m/s)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*

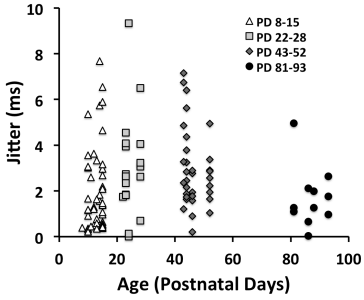
b


PD 8-15
PD 22-28
PD 43-52
PD 81-93
Latency (ms)
35
30
25
20
15
10
5
0
r = 0.33, p = 0.001
0
1
2
3
Distance (mm)

c


PD 8-15
PD 22-58
Latency (ms)
30
25
20
15
10
5
0
r = 0.03, p = 0.85
0
1
2
3
Distance (mm)

d


PD 8-15
PD 22-28
PD 43-52
PD 81-93
Jitter (ms)
10
8
6
4
2
0
0
20
40
60
80
100
Age (Postnatal Days)

e

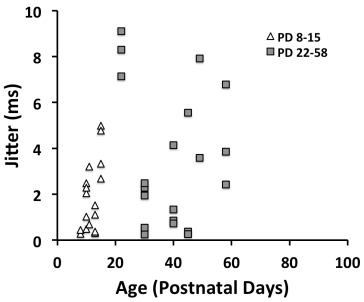

PD 8-15
PD 22-58
Jitter (ms)
10
8
6
4
2
0
0
20
40
60
80
100
Age (Postnatal Days)

Figure 2.8. Significant increase in conduction velocity in adolescence, relationship between latency and distance was positive using maximal stimulation, and disappeared using threshold stimulation (using peak latency criteria). a. Left: There were significant differences between the age groups in average conduction velocity for maximal stimulation ($F(3, 103) = 3.93$, $p = 0.011$); specifically the PD 43-52 group (dark gray bar) showed a significant increase in conduction velocity compared to the PD 8-15 group ($t(52) = 3.53$, $p = 0.001$). However, the increase observed when comparing the PD 8-15 group to either the PD 22-18 or the PD 81-93 group was only marginally significant ($t(19) = 1.7$, $p = 0.10$ and $t(12) = 2.14$, $p = 0.053$, respectively). Right: The PD 22-58 showed a significant increase in average conduction velocity for threshold stimulation compared to the younger PD 8-15 group ($t(22) = 3.98$, $p = 0.00064$). **b.** Linear regression showed a weak relationship between response latency and transmission distance for maximal stimulation ($r = 0.33$, $p = 0.001$). **c.** Similarly, there was no relationship between response latency and transmission distance for threshold stimulation ($r = 0.03$, $p = 0.85$). **d.** Conduction time jitter as a function of age for maximal amplitude. A statistically significant increase in conduction time jitter was observed between PD 8-15 and PD 43-52 animals in the maximum amplitude data set (PD 8-15 mean = 2.07; PD 43-52 mean = 2.95; $t(72) = 2.15$, $p = 0.035$). **e.** Conduction time jitter as a function of age for threshold stimulation. A statistically significant increase in conduction time jitter was observed between PD 8-15 and PD 22-58 animals in the threshold stimulation data set (PD 8-15 mean = 1.80; PD 22-58 mean = 3.49; $t(29) = 2.25$, $p = 0.032$). These increases in jitter correspond with age groups in which a statistically significant increase in average latency was also observed. Data expressed as mean ± SEM.

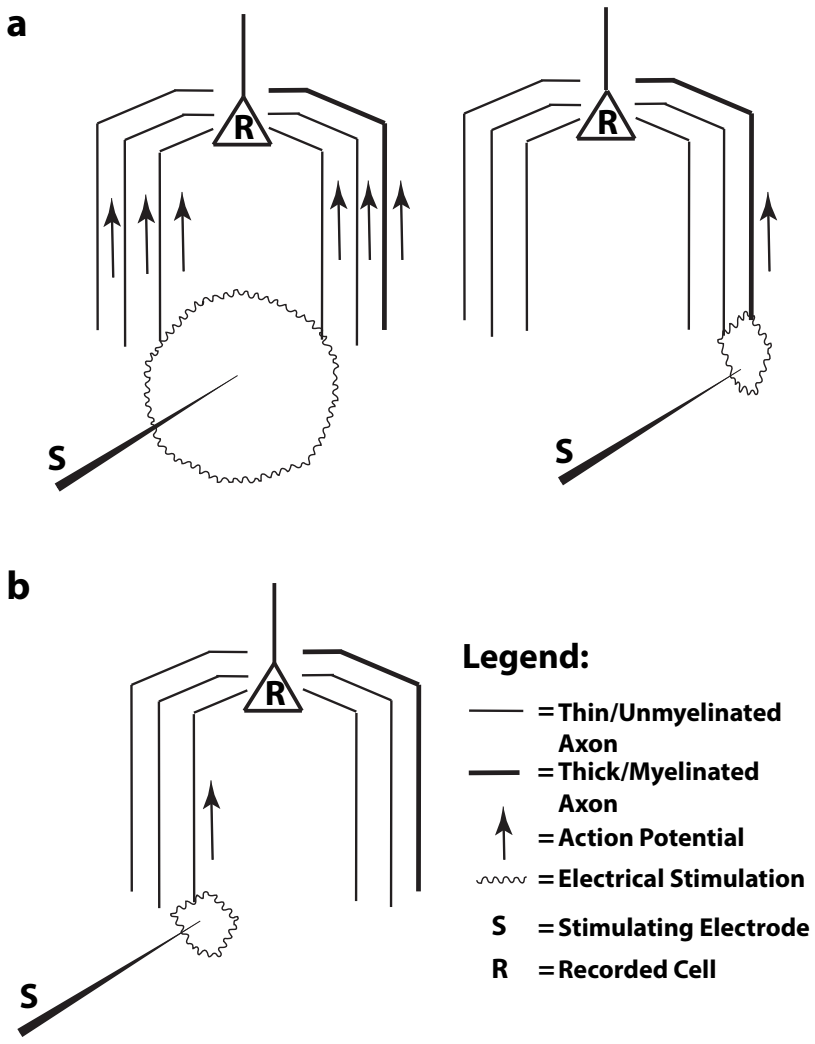


Figure 2.9. Proposed model to explain differences in latency versus distance relationship between maximal and threshold stimulation. a. Left: Maximal amplitude stimulation triggers action potentials in many fibers that synapse on the recorded cell, as indicated by complex EPSC waveforms in the current trace. At least one of these is a large diameter or myelinated fiber. Right: Threshold stimulation randomly activates a thick or myelinated fiber, resulting in short response latency. **b.** Threshold stimulation randomly activates a thin or unmyelinated axon, resulting in a long response latency.

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CHAPTER 3

ALCOHOL BINGE DRINKING DURING ADOLESCENCE OR DEPENDENCE DURING ADULTHOOD REDUCES PREFRONTAL MYELIN IN MALE RATS

Published in *Journal of Neuroscience* Oct. 2014

Vargas, W. M., Bengston, L., Gilpin, N. W., Whitcomb, B. W. & Richardson, H. N. (2014) Alcohol Binge Drinking during Adolescence or Dependence during Adulthood Reduces Prefrontal Myelin in Male Rats. *Journal of Neuroscience 34*, 14777–14782.

3.1 Abstract

Teen binge drinking is associated with low frontal white matter integrity and increased risk of alcoholism in adulthood. This neuropathology may result from alcohol exposure or reflect a pre-existing condition in people prone to addiction. Here we used rodent models with documented clinical relevance to adolescent binge drinking and alcoholism in humans to test whether alcohol damages myelinated axons of the prefrontal cortex. In Experiment 1, outbred male Wistar rats self-administered sweetened alcohol or sweetened water intermittently for 2 weeks during early adolescence. In adulthood, drinking behavior was tested under nondependent conditions or after dependence induced by 1 month of alcohol vapor intoxication/withdrawal cycles, and prefrontal myelin was examined 1 month into abstinence. Adolescent binge drinking or adult dependence induction reduced the size of the anterior branches of the corpus callosum, i.e., forceps minor (CC_{FM}), and this neuropathology correlated with higher relapse-like drinking in

adulthood. Degraded myelin basic protein in the gray matter medial to the CC_{FM} of binge rats indicated myelin was damaged on axons in the medial prefrontal cortex (mPFC). In follow-up studies we found that binge drinking reduced myelin density in the mPFC in adolescent rats (Experiment 2) and heavier drinking predicted worse performance on the T-maze working memory task in adulthood (Experiment 3). These findings establish a causal role of voluntary alcohol on myelin and give insight into specific prefrontal axons that are both sensitive to alcohol and could contribute to the behavioral and cognitive impairments associated with early onset drinking and alcoholism.

3.2 Introduction

Adolescence is a period of heightened vulnerability when teenagers engage in high risk activities like binge drinking (Romer, 2010) as the frontal lobes undergo developmental processes including axonal myelination (Barnea-Goraly, 2005). Myelination increases conductance speed in axons and enhances information processing and cognitive performance (Blakemore and Choudhury, 2006). If prefrontal fiber tracts are sensitive to alcohol exposure during this time of plasticity, drinking could significantly impair the social and mental health trajectories of teenagers because the prefrontal cortices are responsible for evaluating reward (Taren et al., 2011) and regulating risk-taking behavior (Crone et al., 2008).

Early onset of alcohol use predicts increased impulsivity (Stephens and Duka, 2008), cognitive performance impairments (Konrad et al., 2012), and alcoholism in adulthood (Grant and Dawson, 1998). Heavy episodic (binge) drinking is related to lower white matter integrity in the corpus callosum (CC) of teenagers (McQueeny et al., 2009) and in alcoholic adults (Pfefferbaum et al., 2006). The link between reduced white matter

and increased addiction vulnerability in humans suggests alcohol exposure may damage myelin. Alternatively, predisposing factors could explain the relationship between adolescent drinking and frontal white matter.

The present study tested the hypothesis that alcohol damages CC myelin tracts within the prefrontal cortex. Preclinical models of adolescent binge drinking (Gilpin et al., 2012) and adult dependence (Becker, 2013; Vendruscolo and Roberts, 2014) were used to elicit different *in vivo* alcohol exposures in outbred rats. Myelin was labeled 1 month into abstinence in adulthood to quantify white matter changes in the frontal lobes. We show that voluntary binge drinking reduces myelin density in the mPFC in adolescent rats, relates to working memory deficits in adulthood, and produces enduring prefrontal white matter loss comparable to that observed after alcohol dependence. Moreover, greater severity of prefrontal white matter neuropathology was correlated with higher levels of relapse-like drinking in adulthood. These findings give insight into myelinated prefrontal axons that are vulnerable to alcohol and may underlie adverse mental health outcomes associated with early alcohol use in humans.

3.3 Materials and Methods

3.3.1 Animals

Male Wistar rats were shipped with their mothers on postnatal day (PD) 18 from Charles River, weaned, and housed in triads beginning on PD21. All procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

3.3.2 Study Design

3.3.2.1 Experiment 1: Effect of binge drinking and dependence on prefrontal white matter

The experimental design is shown in Fig 3.1a. Rats (N = 27) were given differential exposure to alcohol during adolescence and adulthood (described briefly below; details in Gilpin et al., 2012). After operant training, PD28 rats were randomly assigned to control (n = 9) or binge (n = 18) treatment [see below, Adolescent alcohol exposure (experiments 1–3)]. A priori, a larger binge group was planned to account for anticipated variability in alcohol self-administration. After 5 weeks of abstinence (beginning on PD78), binge and control rats were tested for baseline drinking in adulthood. At PD130, groups were further divided after balancing for adolescent and adult drinking behaviors, and animals were either made dependent [1 month of intermittent alcohol vapors; target blood alcohol levels (BALs) were 0.15– 00.20 g/dl] or remained nondependent (1 month of ambient air control) and tested for relapse-like drinking, i.e., augmented drinking after short deprivation periods. Details on tail nick blood collection and Analox measurement of BALs are described in Gilpin et al. (2012). Brains available for this myelin study were from (1) control nondependent (n = 4), (2) control dependent (n = 5), (3) binge nondependent (n = 9), and (4) binge dependent (n = 9) rats that were perfused 1 month after vapor/air treatment ended. Brains were processed for quantification of prefrontal white matter loss and myelin damage, as described below.

3.3.2.2 Experiment 2: Effect of binge drinking on myelinated axons in the mPFC of adolescent rats

The experimental design is shown in Fig 3.3a. Rats (N = 25) underwent binge (n = 8) or control (n = 8) drinking from PD28–PD42, or remained naive to operant training and alcohol (n = 9). The day after the binge period ended, animals were perfused and brain tissue was processed and analyzed for myelin density (described below). Brain sections from a small proportion of these animals were used in a previous study (Karanikas et al., 2013).

3.3.2.3 Experiment 3: Effect of binge drinking on working memory (T-maze)

The experimental design is shown in Fig 3.3a. Animals (N = 27) underwent adolescent binge (n = 13) or control (n = 14) drinking from PD28–PD42. After the binge period, animals were tested on PD43, PD44, and PD48 for performance on the T-maze spontaneous alternation task as described below, and were tested again in adulthood after 6 weeks of abstinence (PD88 –PD89).

3.3.3 Adolescent alcohol exposure (Experiments 1–3)

Beginning on ~PD25, animals were trained to self-administer sweetened water (3% glucose/0.125% saccharin/tap water). On PD28, rats were either switched to sweetened alcohol (8–10% w/v ethanol/3% glucose/0.125% saccharin/tap water; binge) or remained on sweetened water (control). Overnight operant sessions consisted of six 30 min bouts divided by time-out periods when the levers were retracted and alcohol was unavailable. Time-out periods lasted 90 min in Experiment 1 (Gilpin et al., 2012) and were reduced to 60 min in Experiments 2 and 3 to maximize operant box usage in our

laboratory. Food and water were available *ad libitum* in the operant boxes throughout the binge exposure period.

3.3.4 Perfusions and brain sectioning (Experiments 1–2)

Animals were intracardially perfused the day after the two-week binge period ended (PD43, Experiment 2) or several months later after drinking behavior was tested in adulthood (PD196, Experiment 1, Gilpin et al., 2012). After 4 h post fixation and 24–48 h in 20% sucrose, brains were snap frozen using isopentane (2-methylbutane; Sigma) and dry ice, and stored at 80°C until sectioning. Thirty-five micrometer coronal sections were sliced on a freezing microtome and stored at 20°C in cryoprotectant (50% 0.1 M PBS, 30% ethylene glycol, and 20% glycerol).

3.3.5 Myelin labeling and microscopic analysis of prefrontal white matter (Experiment 1)

Black Gold II (BG-II) was used to impregnate myelin and label white matter in every eighth brain section (Schmued et al., 2008). For white matter microscopic analysis, brain sections were classified as (1) forceps minor (CC_{FM}), anterior to the CC joining across hemispheres, 2.10 –1.85 mm from bregma or (2) genu (CC_{Genu}), posterior to the joining of the left and right CC, 1.60 –1.35 mm from bregma (Paxinos and Watson, 1998; Fig 3.1b). Fifteen to 20 photomicrographs were taken using a Leica microscope (5x objective) attached to a DP71 Olympus camera and were digitally montaged for a single hemisphere. Two to four hemispheres were used for microscopic analysis of each anatomical classification for each animal. CC_{FM} and CC_{Genu} cross-sectional areas were quantified using ImageJ software (Rasband, 1997).

3.3.6 Degraded myelin basic protein labeling and microscopic analysis (Experiment 1)

Using a marker of degraded myelin basic protein (dMBP), we quantified myelin damage in the center and the borders of the CC_{FM} where these axons extend into the mPFC or the striatum Fig 3.2a. Free-floating sections were prepared using the immunohistochemistry standard protocol for dMBP (Millipore) primary antibody (1:1000), as described previously (Matsuo et al., 1997; Li and Stys, 2000), and Cy3 fluorescent secondary antibody (1:300, Jackson ImmunoResearch). Photomicrographs were taken 2.2 mm from bregma (20x objective) using constant imaging parameters, and were analyzed for intensity quantification using ImageJ software; the threshold function was used to highlight dMBP-positive regions. Percentage of dMBP-positive area within the total area was calculated.

3.3.7 Myelin labeling and microscopic analysis of myelin density in the mPFC (Experiment 2)

Every 10th section was labeled for BG-II (Schmued et al., 2008), and dorsal mPFC photomicrographs were taken 2.2 mm from bregma (5x objective). Aperio ImageScope software was used to quantify myelinated fiber density in cortical layers II–V by thresholding the images. Percentage of myelinated fiber area within the total area was calculated.

3.3.8 T-maze spontaneous alternation task (Experiment 3)

T-maze assays were conducted as previously described (Deacon and Rawlins, 2006), with the modification of a 70 s delay between trials, making this task mPFC

dependent (Delatour and Gisquet-Verrier, 1996; Lalonde, 2002). In total, animals underwent 10 trials during adolescence and 6 trials during adulthood.

3.3.9 Statistical Analyses

Overall effects of adolescent alcohol exposure and adult dependence on white matter cross-sectional area and dMBP intensity were analyzed using between-subjects two-way ANOVAs. One-way ANOVAs were used to analyze the effect of adolescent alcohol exposure on mPFC myelinated fiber density and on T-maze performance. Pearson correlation analyses were used to assess relationships between white matter and adolescent alcohol intake and adult baseline and relapse-like drinking behaviors, and to examine the relationship between adolescent alcohol intake and T-maze performance. Statistical significance was defined as ≤ 0.05 using two-tailed tests. Statistical analyses were performed using R statistical software package (RCoreTeam, 2013).

3.4 Results

Binge drinking during adolescence and/or dependence during adulthood reduced frontal white matter. This reduction persisted well into abstinence (over 5 months after adolescent binge ended and 1 month after vapor exposure ended). Binge rats had smaller CC_{FM} areas than control rats (adolescent treatment main effect, $F(1,21) = 10.50$, $p = 0.004$; Fig 3.1c). Likewise, alcohol-dependent rats had smaller CC_{FM} areas than nondependent rats (adult treatment main effect, $F(1,21) = 9.47$, $p = 0.006$, Fig 3.1c). No significant interaction between adolescent and adult treatments was detected ($p = 0.15$). The CC_{Genu}, which is located just posterior to the mPFC, was not significantly altered after a history of adolescent binge drinking and adult dependence, and there was no

interaction between these treatments (all ps > 0.05; Fig 3.1d). However, CC_{Genu} size was significantly inversely related to adolescent alcohol consumption (r = 0.64, p = 0.005; data not shown), suggesting that higher alcohol levels may be necessary to reduce CC_{Genu} size.

We next examined behavioral correlates of white matter loss. CC_{FM} size was not significantly correlated with baseline drinking levels (g/kg/30 min, r = 0.26, p = 0.21; Fig 3.1e). Conversely, smaller CC_{FM} size predicted higher drinking after short deprivation periods, indexed by percentage increase in intake relative to baseline (r = 0.47, p = 0.02; Fig 3.1f). No significant correlations were detected between CC_{Genu} size and any adult drinking behaviors.

A marker of dMBP was next used to index myelin health and identify the axonal population of the CC_{FM} axons damaged by alcohol. We detected significantly elevated dMBP at the dorso-medial border of the CC_{FM} (CC_{FM}mPFC) in rats with a history of adolescent binge drinking (adolescent treatment main effect, F(1,23) = 7.00, p = 0.01; Fig 3.2b). Dependence did not significantly increase dMBP in the CC_{FM}mPFC (p > 0.05; Fig 2b).

dMBP was detected in the CC_{FM}Center and CC_{FM}Striatum, but the intensity did not significantly differ between groups (ps > 0.05; Fig 3.2c,d). Based on the findings above, we next tested the hypothesis that binge drinking decreases myelinated axons in layers II–V of the mPFC of adolescent rats. Indeed, the day after the last binge session, mPFC myelinated axonal density was reduced in binge drinking rats compared with control and naive rats (F(2,21) = 8.43; p = 0.002; Fig 3.3b).

We next explored whether binge drinking related to performance on the mPFC-dependent, spontaneous alternation T-maze working memory task. There was no main effect of binge drinking on performance ($p > 0.05$), but the amount of alcohol consumed early in adolescence predicted poor performance on the T-maze in adulthood ($r = 0.69$, $p = 0.009$; Fig 3.3c). Conversely, this relationship between sweetened water drinking and T-maze performance was not observed in control rats ($r = 0.14$, $p = 0.63$; Fig 3.3c). There were no significant relationships between adolescent drinking and T-maze performance at the end of the adolescent treatment period for either group ($ps > 0.05$; data not shown).

Table 3.1 summarizes average daily alcohol intake over the two-week adolescent binge period for rats in Experiments 1–3. BALs ranged between 0.0 and 0.17 g/dl after 0.0 –2.20 g/kg alcohol consumption in single, 30 min self-administration bouts, which were randomly assessed from animals in the three experiments. These BALs are moderate and similar to what has been reported for adolescent rats consuming sweetened alcohol in the home cage (Walker et al., 2008; Gilpin et al., 2012; Broadwater et al., 2013).

3.5 Discussion

The current study examined the effect of adolescent and adult alcohol exposure on myelin in the frontal lobes of male rats. Similar to human studies, we found negative correlations between adolescent alcohol drinking and white matter. We also provide empirical evidence that alcohol reduces myelin density in adolescent rats and causes enduring white matter deficits in the mPFC. Alcohol treatment—adolescent binge drinking or adult dependence—reduced the size of the CC_{FM} and this neuropathology was predictive of higher levels of relapse-like drinking. Adolescent drinking predicted poor

performance on an mPFC-dependent task. Structural changes in white matter and degraded myelin health persisted well into abstinence in adulthood, suggesting that adolescent binge drinking produces irreversible changes in prefrontal circuitry.

The effect of alcohol on CC_{FM} axons may have broad implications for adolescent and adult mental health outcomes. This anterior fiber bundle connects the mPFC to the lateral prefrontal cortex and striatum, and is implicated in depression, multiple sclerosis, Tourette's syndrome, and chronic schizophrenia (Cader et al., 2006; Friedman et al., 2008; Jackson et al., 2011; Tadayonnejad et al., 2014). The structures interconnected by the CC_{FM} are also implicated in addiction (Everitt and Robbins, 2005; Volkow et al., 2005) and our current data suggest impaired connectivity may influence drinking risk in adulthood. Reduced CC_{FM} size predicted augmented drinking during relapse tests, but did not predict increased baseline drinking behavior. Importantly, relapse-like drinking behavior was normalized for each animal to his own baseline. This suggests that the link between CC_{FM} structure and relapse is not simply reflecting the effect of alcohol dose on CC_{FM} axons. Instead, these axons may help control the magnitude of increase in alcohol intake, or "over-doing it," after experiencing short periods of abstinence from drinking.

Adolescent alcohol drinking and adult alcohol dependence produced changes of similar magnitude in the CC_{FM}. This is remarkable considering the substantial difference in the duration and dose of alcohol exposure between the two treatment groups. Binge rats consumed alcohol voluntarily, reaching 0.08 g/dl BALs in only some of the drinking bouts over a two-week adolescent drinking period that took place early in adolescence, >5 months before the brains were processed in adulthood. Conversely, dependence was induced in adulthood by 1 month of exposure to daily cycles of alcohol vapor-induced

intoxication that maintained BALs between 0.15 and 0.20 g/dl for 14 h/d—an exposure that produces mild physical dependence (Richardson et al., 2008). The fact that the adolescent alcohol exposure duration and amount was much less than vapor exposure in adulthood suggests that the adolescent brain has heightened sensitivity to alcohol and the effects are enduring. The data support the hypothesis that the dynamic changes occurring in the developing prefrontal cortices during adolescence (Casey et al., 2008) increase susceptibility to potentially toxic external stimuli such as alcohol (Crews et al., 2007). The fact that dependence induction did not further reduce CC_{FM} size in the binge group suggests a ceiling effect of alcohol on degradation of this axonal bundle.

The lack of group changes in CC_{Genu} structure suggests that higher doses and a more prolonged exposure to alcohol, perhaps in combination with vitamin B1 deficiency, may be required to significantly impair white matter structure, as previously suggested (He et al., 2007; Pfefferbaum et al., 2008). This notion is supported by the significant inverse correlation between adolescent alcohol consumption and CC_{Genu} size observed in this study. It will be important in future studies to determine how prefrontal myelin is damaged by alcohol using other models of alcohol use disorders (Simms et al., 2008; Crabbe et al., 2009) and to extend these findings to females.

Finally, to assay functional integrity of the mPFC, we tested rats for spontaneous alternation on the T-maze using a 70 s delay to increase working memory demand (Delatour and Gisquet-Verrier, 1996; Lalonde, 2002). Higher binge drinking levels during adolescence predicted poorer performance on the T-maze task in adulthood. This relationship was not evident in control rats, suggesting that alcohol consumption produces enduring functional changes in mPFC circuitry. Nevertheless, without a

significant main effect, we cannot exclude the possibility that the correlation between alcohol and performance may reflect a relationship that is not causal in nature. The lack of a significant main effect could be due to variability in intake, as well as limitations in assaying a subtle cognitive impairment. In future studies, treatment differences in performance might be detected after increasing the retention interval, i.e., a larger delay between sample and choice phases (Deacon and Rawlins, 2006).

In conclusion, the present study provides causal evidence for alcohol-induced reductions in myelin in prefrontal circuits. To the best of our knowledge, this is the first study to show that adolescent voluntary binge drinking reduces the density of myelinated axons in the mPFC and has long-lasting effects on prefrontal white matter. Future work exploring the mechanisms by which alcohol damages prefrontal myelin may lead to new therapeutic strategies for the treatment of alcohol use disorders.

3.6 Figures and Tables

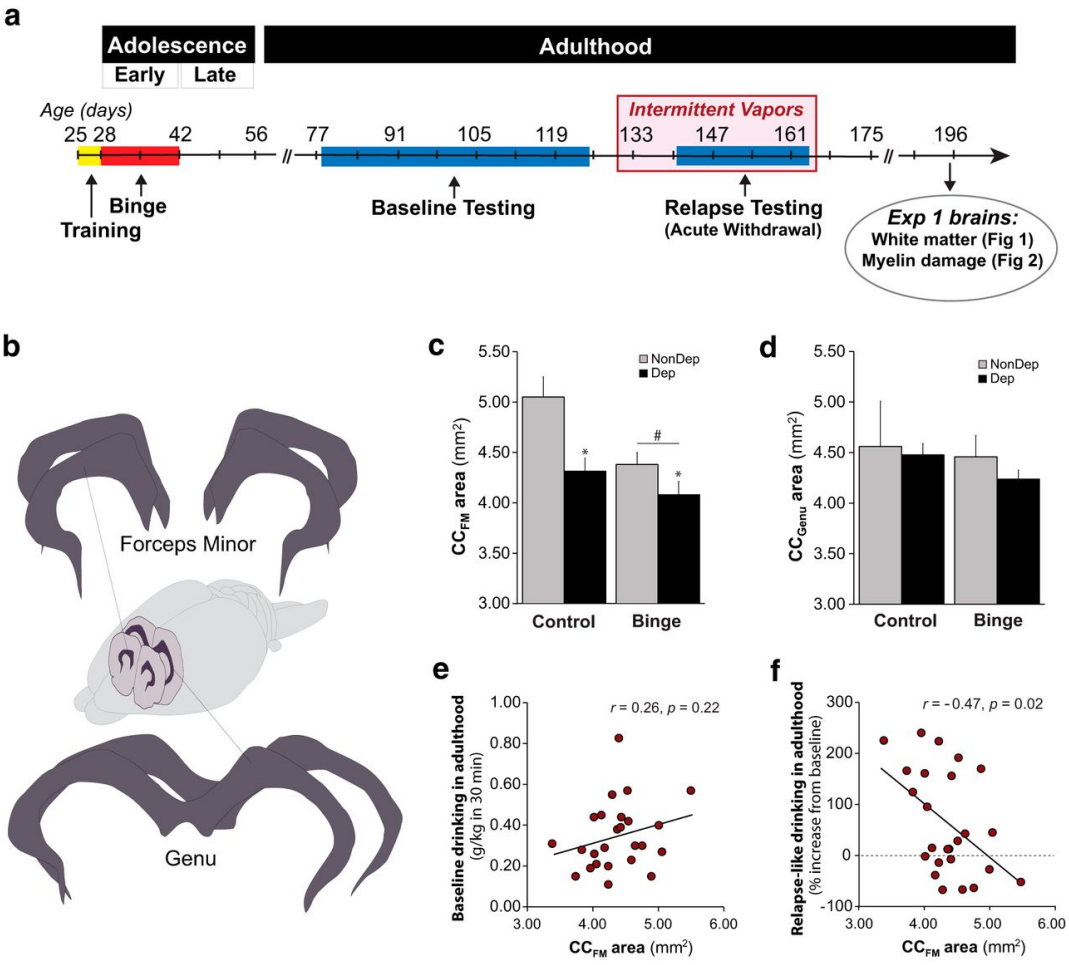

Figure 3.1. Alcohol causes reductions in prefrontal white matter that predict higher levels of relapse-like drinking in adulthood (Experiment 1). a. Schematic illustrating the alcohol exposure timeline during adolescence and adulthood. Male rats underwent voluntary, binge self-administration sessions with sweetened alcohol (binge) or sweetened water (control) during early adolescence. In adulthood, rats were tested for baseline alcohol drinking, then exposed to chronic alcohol vapors (dependent) or ambient air (nondependent), and tested for relapse drinking (details in Gilpin et al., 2012). **b.** Schematic illustrating the anatomical location of CC_{FM} and CC_{Genu} sections. **c, d.** Alcohol reduced cross-sectional area of the CC_{FM} (c; main effect of adolescent binge drinking, #p = 0.004; main effect of adult alcohol dependence, *p = 0.006), but not the CC_{Genu} (d; all ps > 0.05). **e, f.** CC_{FM} cross-sectional area did not predict baseline alcohol intake (e), but did predict the percentage increase from baseline levels after short abstinence periods, i.e., relapse-like drinking (f; p = 0.02). Data expressed as mean ± SEM (n = 4–9 rats/group).

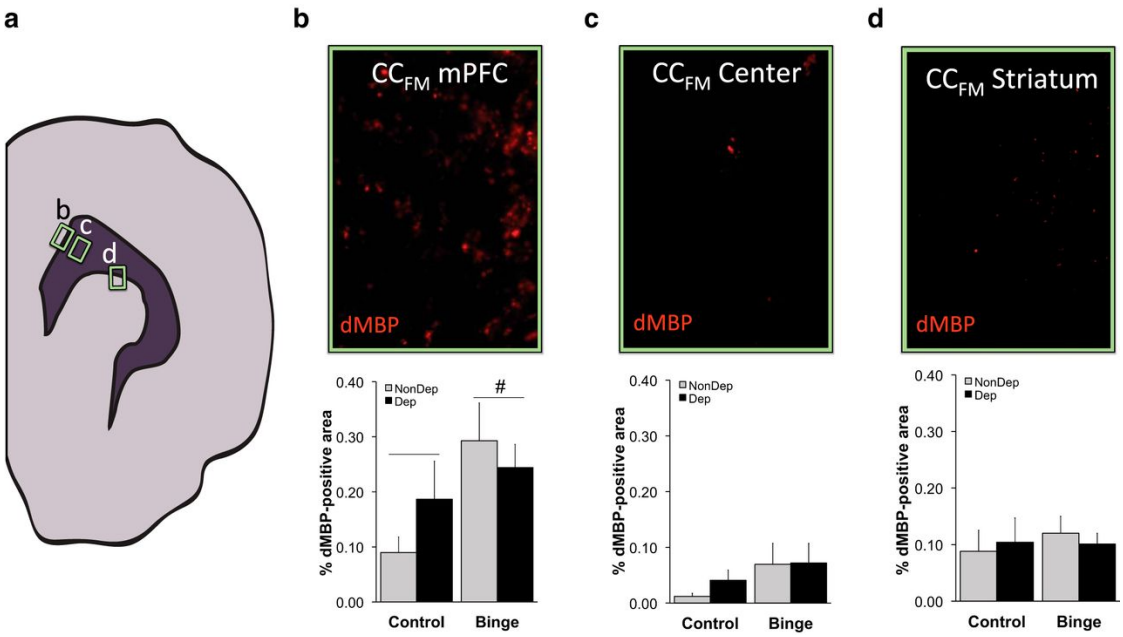

Figure 3.2. Adolescent alcohol causes enduring damage to mPFC myelin (Experiment 1). a. Schematic illustrating the anatomical regions analyzed. **b–d.** Adolescent alcohol increased myelin damage, indexed by dMBP, in the gray matter just medial to the CC_{FM} (CC_{FM}mPFC; main effect of adolescent binge drinking, #p = 0.02). Adult dependence did not significantly increase dMBP in the CC_{FM}mPFC (b; p > 0.05) and neither treatment significantly increased dMBP in the center of the medial branch of the CC_{FM} (CC_{FM}Center; c, p > 0.05) or striatum (CC_{FM}Striatum; d, p > 0.05). Data expressed as mean ± SEM (n = 4–9 rats/group).

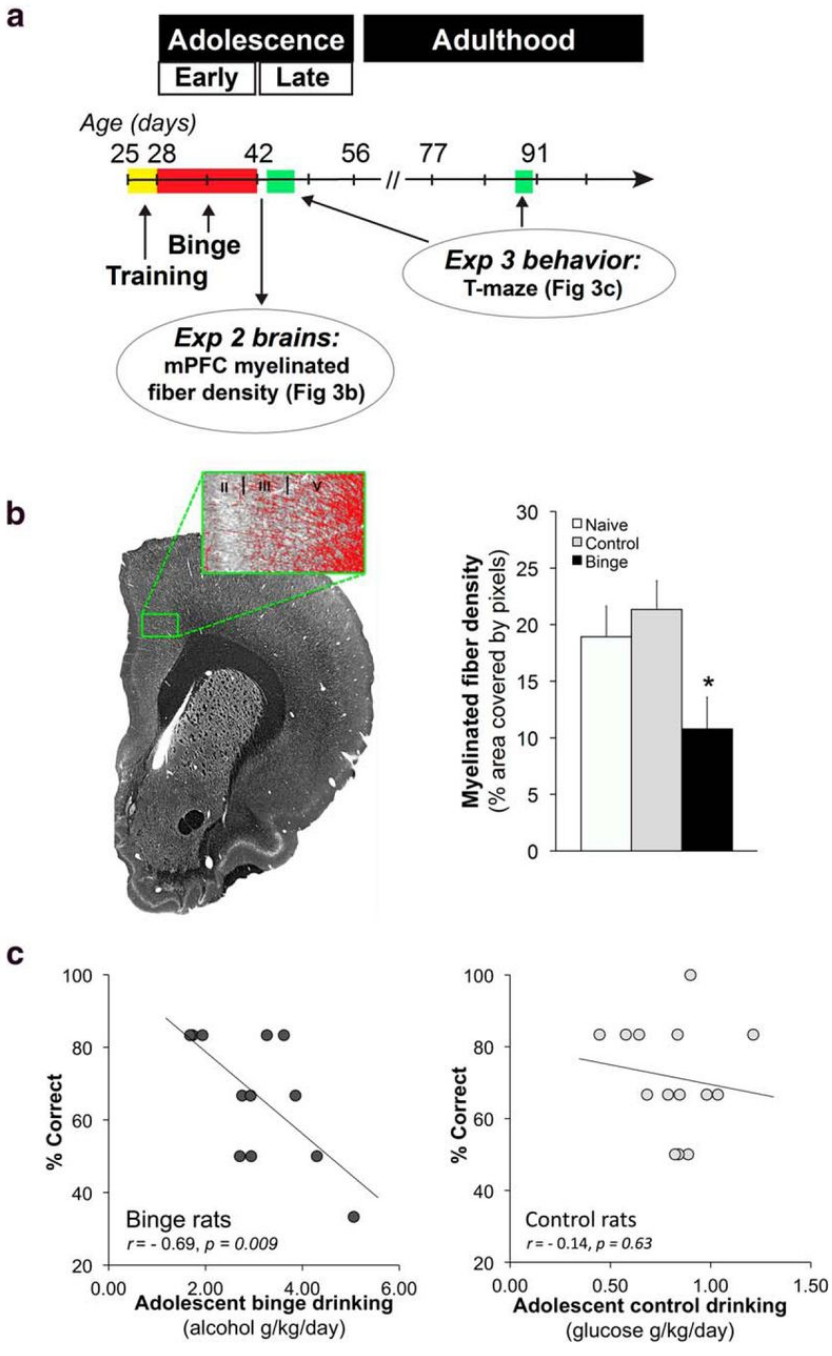
a
Adolescence
Adulthood
Early
Late
Age (days)
25 28 42 56 77 91
Binge
Training
Exp 3 behavior:
T-maze (Fig 3c)
Exp 2 brains:
mPFC myelinated
fiber density (Fig 3b)
b
II III V
Naive
Control
Binge
Myelinated fiber density
(% area covered by pixels)
30
25
20
15
10
5
0
*
c
% Correct
100
80
60
40
20
Binge rats
r = - 0.69, p = 0.009
0.00 2.00 4.00 6.00
Adolescent binge drinking
(alcohol g/kg/day)
Control rats
r = - 0.14, p = 0.63
0.00 0.50 1.00 1.50
Adolescent control drinking
(glucose g/kg/day)

Figure 3.3. Adolescent alcohol decreases myelinated fiber density in the mPFC and predicts poor T-maze performance in adulthood (Experiments 2 and 3). a. Schematic illustrating the timeline of alcohol exposure during adolescence and neural and behavioral measures. Male rats underwent voluntary, binge self-administration sessions with sweetened alcohol (binge) or sweetened water (control) during early adolescence. Rats were tested on the T-maze as adolescents and again in adulthood after 6 weeks of abstinence. **b.** Schematic illustrating the anatomical location of the myelinated fiber density measurement (left). Alcohol reduced myelinated fiber density in the mPFC (*$p = 0.002$). **c.** There was a significant negative correlation between daily adolescent consumption and percentage correct responses in the T-maze in adulthood in binge rats (left graph; $p = 0.009$). This relationship was not observed in control rats (right graph; $p > 0.05$) and no relationships were detected between drinking behavior and T-maze performance tested in adolescence (ps > 0.05; data not shown). Data expressed as mean ± SEM (b, n = 8–9 rats/group; c, n = 13–14 rats/group).

Table 3.1. Adolescent binge drinking (g/kg/day)

	Experiment 1	Experiment 2	Experiment 3
Groups	Mean ± SEM	Mean ± SEM	Mean ± SEM
Binge		3.21 ± 0.38	2.96 ± 0.31
Binge Non-Dep	4.36 ± 0.16		
Binge Dep	4.52 ± 0.15		

Table 3.1. Alcohol binge consumption during adolescence (g/kg/d) for the groups in each experiment. Data shown as mean ± SEM.

3.7 References

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Note: The effects observed in myelinated fiber density (Experiment 2) were specific to cingulate cortex of the mPFC. No significant changes were observed in ventral mPFC.

CHAPTER 4

EFFECTS OF ALCOHOL BINGE DRINKING ON MYELINATED AXONS, OXIDATIVE STRESS, AND MYELIN-ASSOCIATED GENES IN THE PREFRONTAL CORTEX OF ADOLESCENT RATS

4.1 Abstract

Adolescent binge drinking is associated with lower white matter volume in the frontal lobe of human adolescents. We recently showed that binge drinking reduces myelin density in the prefrontal cortex (PFC) of adolescent male rats. It is currently unknown if alcohol also reduces PFC myelin density in females as well. Here we used a preclinical model of adolescent binge drinking to determine the effect of alcohol on the PFC myelin of female rats. We also assessed if the alcohol-induced changes in males are associated with an increase in oxidative stress, a reduction in myelin related genes, or both. Thus, the present study also explored whether alcohol exposure alters oxidative stress levels and mRNA expression of myelin-related genes in the PFC. Outbred male and female Wistar rats underwent a voluntary two-week binge drinking model during early adolescence. Adolescent binge drinking in females did not reduce PFC myelin as was observed in males. In addition, oxidative stress levels were elevated in the PFC only in males. Binge drinking does not appear to affect myelin-related genes in the PFC of males or females. Future studies will examine the mechanisms underlying adolescent alcohol-induced alterations in myelinated axons in the PFC and differential sensitivity in males and females, as aberrant development of this circuitry during adolescence may have long-term impacts on several executive functions in adulthood.

4.2 Introduction

The adolescent population (12-18 years) has a particularly high prevalence of binge drinking (Johnston et al., 2009). In fact, almost 25% of high school students have reported binge drinking within the preceding month (Centers for Disease Control and Prevention, 2013). Alcohol consumption during adolescence can have detrimental effects on the developing brain, particularly on myelination. Heavy episodic (binge) drinking has been linked to lower white matter volume in human adolescents (De Bellis et al., 2005; Medina et al., 2008; McQueeny et al., 2009), and reduces myelin in the PFC of adolescent male rats (Vargas et al., 2014). It is unknown, however, if heavy alcohol consumption would have a similar effect on prefrontal myelin of adolescent female rats, and how alcohol might impact prefrontal myelin is also poorly understood.

Research suggests that women may be more vulnerable than men to the neurotoxic effects of alcohol in general (Alfonso-Loeches et al., 2013). However, research regarding sex differences in the effects of adolescent alcohol consumption on myelination in particular is limited and has shown some inconsistencies. In humans, some studies show that alcohol affects white matter equally in both male and female adolescents (De Bellis et al., 2005; McQueeny et al., 2009). However, other studies suggest that adolescent females may be more vulnerable to alcohol consumption than males (Medina et al., 2008).

The processes underpinning the observed alcohol-mediated reduction of PFC myelin are also unclear. One possible factor contributing to the reduced level of myelination after alcohol consumption is oxidative stress, the imbalance between the production of antioxidants and oxidants (Durackova, 2010; Xiao et al., 2015; Cabello-

Verrugio et al., 2016). Oxidative stress is induced by intraperitoneal injection of alcohol (Sultatos, 1988; Wu and Cederbaum, 2003; Wu et al., 2006), and has been linked to demyelination (Lynn et al., 2005).

Another possible explanation for the alcohol-induced myelinated fiber decrease is a reduction of genes important for myelination. Many myelin-related genes, such as proteolipid protein (PLP) and myelin basic protein (MBP) can be affected by alcohol consumption (Alfonso-Loeches et al., 2012) and are reduced in demyelination (Gregg et al., 2009). Several lipids and proteins are necessary for proper myelination (Simons and Trotter, 2007), with PLP and MBP being the most abundant proteins in myelin (Umemori et al., 1999; Baumann and Pham-Dinh, 2001; Quarles et al., 2006; Simons and Trajkovic, 2006; Simons and Trotter, 2007; Jahn et al., 2009). PLP is important for compaction of the extracellular apposition of the myelin membrane (Baumann and Pham-Dinh, 2001; Quarles et al., 2006; Aggarwal et al., 2011; Inouye and Kirschner, 2015) and for axonal integrity (Griffiths et al., 1998; Nave, 2010). MBP is essential for generation of myelin in the central nervous system, and it mediates adhesion of compact myelin layers by interacting with lipids in the myelin membrane (Boggs, 2006; Jahn et al., 2009). MBP is also important for myelin compaction (Baumann and Pham-Dinh, 2001; Simons and Nave, 2016). Myelin-associated glycoprotein (MAG), located in the periaxonal membrane of the myelin sheath (non-compact myelin), is important in the maintenance of myelinated axons, and is involved in the inhibition of neuronal regeneration (Quarles, 2007; Nave, 2010). Myelin-associated oligodendrocytic basic protein (MOBP), like MBP, is present in the major dense line of compact myelin (Baumann and Pham-Dinh, 2001; Montague et al., 2006). Although MOBP has been thought to have a similar role to

MBP in compaction of myelin, only MBP is considered to be the essential protein for myelination (Baumann and Pham-Dinh, 2001; Montague et al., 2006). MOBP may also be involved in myelin membrane-associated signaling, but the exact function of MOBP is still unknown (Montague et al., 2006).

The present study tested the hypothesis that adolescent alcohol consumption affects myelination differentially in male and female adolescent rats, and that these effects are explained by changes in oxidative stress and in myelin-associated gene expression. Our hypothesis is based on previous literature suggesting that females are more vulnerable to the effects of alcohol consumption. We used a preclinical model of adolescent binge drinking (Gilpin et al., 2012) to elicit voluntary binge alcohol drinking in outbred Wistar rats. In order to determine the effects of alcohol during adolescence, myelin and oxidative stress measures were taken after the binge drinking period. Results show that binge drinking affects males and females differently and increases oxidative stress in the medial prefrontal cortex (mPFC) of adolescent male rats. The present findings provide insight into understanding sex differences in the effects of alcohol on the brain. In addition, these findings provide some understanding on what could modulate the alcohol-mediated reductions in myelin. This understanding is foundational in determining a mechanism between alcohol consumption and myelin reduction.

4.3 Materials and Methods

4.3.1 Animals

Male and female Wistar rats were shipped from Charles River with their mothers on postnatal day (PD) 18 or 19. They were weaned and housed in same-sex triads starting on PD 21, with *ad libitum* food and water. All procedures were performed in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

4.3.2 Study Design

4.3.2.1 Experiment 1: Effect of binge drinking on prefrontal myelinated axons of female adolescent rats

The experimental design is shown in Fig 4.1. From PD 28–PD 42, female rats (N = 25) underwent binge (n = 8) or control (n = 8) drinking. Another group remained naive to operant training and alcohol consumption (n = 9). The three female groups were run alongside the male groups described earlier in the dissertation (Vargas et al., 2014, Chapter 3). Animals were perfused one day after the binge period ended (described below), and brain tissue was processed and analyzed for myelin density (described below).

4.3.2.2 Experiment 2: Effect of binge drinking on oxidative stress in the mPFC of adolescent rats

The experimental design is shown in Fig 4.1. From PD 28–PD 42, male and female rats (N = 52; 26 per sex) underwent binge (n = 9) or control (n = 9) drinking.

Another group remained naive to operant training and alcohol consumption (n = 8). Brains were extracted one day after the binge period ended. The mPFC, along with other brain regions, were dissected and homogenized, and the 2, 7-Dicholorofluorescein (DCF) and bicinchoninic acid (BCA) assays were conducted as described below.

4.3.2.3 Experiment 3: Effect of binge drinking on myelin-related gene mRNA in the mPFC of adolescent rats

The experimental design is shown in Fig 4.1. From PD 28–PD 42, male and female rats (N = 53; 27 males, 26 females) underwent binge (n = 9) or control (n = 9) drinking. Another group remained naive to operant training and alcohol (n = 8-9). Brains were extracted one day after the binge period ended. The mPFC was dissected and fresh frozen until RNA extraction and qPCR was performed, as described below.

4.3.3 Adolescent alcohol exposure (Experiments 1–3)

Animals were exposed to the voluntary adolescent binge drinking model described previously (Karanikas et al., 2013). Briefly, animals were trained to self-administer sweetened water (3% glucose/0.125% saccharin/tap water) starting on ~PD 23. On PD 28, rats remained on sweetened water (control) or were switched to sweetened alcohol (10% w/v ethanol/3% glucose/0.125% saccharin/tap water; binge). Overnight operant sessions consisted of six 30-min bouts with 1 hour timeout periods in which the levers were retracted and alcohol was unavailable. Food and water were always available in the operant boxes during the binge exposure period.

4.3.4 Perfusions and brain sectioning (Experiment 1)

The day after the two-week binge period ended, animals were intracardially perfused with 4% paraformaldehyde in 0.1 M borate buffer, pH 9.4. After post fixation in 4% paraformaldehyde for 4 hours and 24–48 h in 20% sucrose, brains were snap frozen with isopentane (2-methylbutane; Sigma) and dry ice, and stored at -80°C until sectioning. Coronal sections with a thickness of 35 μm were sliced on a freezing microtome and stored at -20°C in cryoprotectant (50% 0.1 M PBS, 30% ethylene glycol, and 20% glycerol).

4.3.5 Brain dissections/ Tissue preparation (Experiments 2-3)

The day after the two-week binge period ended, animals were lightly anesthetized with CO2 and decapitated, and their brains were collected. The mPFC was rapidly dissected from a ~1 mm-thick section cut from the front of the brain (Fig 4.1). For Experiment 2, the tissue was immediately homogenized (111.11 mg tissue/mL medium) in a medium made in 1:1:1:2 ratios of 0.5 M Sodium Phosphate, 700 mM KCl, 5 mM EDTA, and distilled MilliQ water, respectively. The homogenized tissue was then stored in -20°C. For Experiment 3, the dissected tissue was snap frozen on dry ice and stored at -80°C.

4.3.6 Myelin labeling and microscopic analysis of myelin density in the mPFC (Experiment 1)

Black Gold II was used to label every 10^{th} section (Schmued et al., 2008), and photomicrographs of the dorsal mPFC were taken 2.2 mm from bregma (5x objective). Myelinated fiber density in cortical layers II–V was quantified using Aperio ImageScope

software by thresholding the images. Percentage of myelinated fiber area within the total area was calculated.

4.3.7 2, 7-Dichlorofluorescein Assay (Experiment 2)

In order to determine oxidative stress in the mPFC, the DCF assay was used (HaMai et al., 2001). The DCF assay is one of the most commonly used methods to detect oxidative stress (Wang and Joseph, 1999; Pavelescu, 2015). In this assay, 2′,7′ dichlorofluorescin diacetate (DCFH-DA) is hydrolyzed to 2′,7′ dichlorofluorescin (DCFH) by intracellular esterases. The DCFH is rapidly oxidized by the presence of reactive oxygen species (ROS) to the highly fluorescent 2′,7′ dichlorofluorescein (DCF) (Bass et al., 1983; LeBel and Ischiropoulos, 1992; Lu et al., 2015). The DCFH oxidation rate is interpreted as the general oxidative stress level found in the neural tissue. Therefore, it provides a measurement for the overall ROS concentration rather than targeting a specific ROS (LeBel and Ischiropoulos, 1992; Chen et al., 2010; Pavelescu, 2015).

DCF stock (10 mM) was made by adding DCF (Sigma Aldrich, Catalogue No. 35848) to dimethyl sulfoxide (Fisher Scientific), and was diluted to 100 nM DCF for making standards. Homogenized tissue was centrifuged at 3,000 rpm for 10 minutes at 4°C, and 10.2 μL of tissue supernatant were added to 1.5 mL of homogenizing medium. 10mM DCFH-DA was made by adding DCFH-DA (Sigma Aldrich, Catalogue No. D6883) to dimethyl sulfoxide (Fisher Scientific). In duplicate, 200 μL of the diluted homogenates were pipetted into a 96-well microplate (Fisher Scientific). Then, under low-light conditions, 15.3 μL of DCFH-DA were added to each well. After a 30-minute

incubation at 37°C of the samples, fluorescence readings were taken using a SpectraMax M5 (Molecular Devices) multi-mode microplate reader with excitation at 485 nm and emission at 538 nm. A DCF standard curve (0-60 nM) was used to convert fluorescence readings into pmol DCF/mg of tissue.

4.3.8 Bicinchoninic acid Assay (Experiment 2)

The Pierce BCA assay kit (Thermo Scientific) was used to measure protein concentration in the mPFC (as previously described) (Smith et al., 1985; Walker et al., 2008). This assay depends on the conversion from Cu2+ to Cu+, which is detected by reacting the tissue with BCA. Briefly, 25 μL of the diluted tissue homogenates were pipetted in duplicate into a 96-well microplate (Fisher Scientific). Then, 200 μL of the working reagent (50 parts of BCA reagent A to 1 part of BCA reagent B) were added to each well. After a 30-minute incubation at 37°C, absorbance readings were taken using a SpectraMax M5 (Molecular Devices) multi-mode microplate reader with a maximum absorbance of 562 nm. A BCA standard curve (0-2,000 μg/mL), which uses bovine serum albumin, was used to convert absorbance readings into milligrams of tissue.

4.3.9 Reverse transcription and real time quantitative polymerase chain reaction (RT-qPCR) (Experiment 3)

RNA was extracted from 10-15 mg of mPFC tissue using the RNeasy Lipid Tissue kit (Qiagen). Its quantity and quality were estimated using a NanoDrop 1000 spectrophotometer (Thermo Fisher). cDNA was synthesized from 5-6.25 μL of total RNA from the mPFC using the SuperScript® III First-Strand Synthesis SuperMix for

qRT-PCR kit (Thermo Fisher Scientific). Quantitative PCR reactions (20 μL) were carried out in duplicates using the QuantiFast SYBR Green PCR kit (Qiagen) in a 96-well plate RealPlex machine (Eppendorf). Different amounts of cDNA were used for these reactions in order to obtain a very similar Ct score (around 20) between the different target and housekeeping genes, which were beta-actin (Actb) and general transcription factor IIB (Gtf2b). The amount of cDNA was: 2.5 μL for the MOBP and Gtf2b genes, 10^{-1} μL for the MAG gene, and 10^{-2} μL for the MBP, PLP, and Actb genes. The forward and reverse primers and their sequences, identical to those used from Gregg et al. (2009), are listed on Table 4.1. In addition, no-template controls were included in duplicates. Finally, the ΔΔCt method (Livak and Schmittgen, 2001) was used to normalize the obtained Ct scores to the average of the two housekeeping genes and control group, and therefore, to calculate relative gene expression changes. The formula below was used:

$2^{-\Delta\Delta}Ct$

where $\Delta\Delta Ct = (Ct_{\text{Target gene}} - Ct_{\text{Housekeeping gene}})_{\textbf{Alcohol}} - (Ct_{\text{Target gene}} - Ct_{\text{Housekeeping gene}})_{\textbf{Control average}}$

The actual steps used to calculate this formula were the following:

#1: Calculate ΔCt using the equation below.

$$Ct_{\text{Target gene}} - Ct_{\text{Housekeeping gene}}$$

#2: Calculate the average ΔCt of control animals.

$$\bar{x}\ \Delta Ct_{\text{Control animals}}$$

#3: Calculate ΔΔCt by subtracting both ΔCt values as shown below.

$$\Delta Ct_{\text{Alcohol animal}} - \Delta Ct_{\text{Control animals average}}$$

#4: Input ΔΔCt in the formula $2^{-\Delta\Delta}Ct$ (also shown above).

4.3.10 Statistical Analyses

Two-way ANOVAs were used to analyze the effect of adolescent alcohol exposure on mPFC myelinated fiber density, on oxidative stress levels, and on myelin-related gene expression in males and females. In addition, t-tests were conducted to examine any differences in alcohol or glucose consumption between the sexes. Differences in body weight between treatment groups were assessed by 1-way ANOVAs. Statistical significance was defined as $p \leq 0.05$ using two-tailed tests. Statistical analyses were performed using the R statistical software package (R Core Team, 2014).

4.4 Results

In Experiment 1, alcohol consumption during adolescence had differential effects on male and female rats. In myelinated fiber density, the overall ANOVA was significant ($F(5,44) = 3.21$, $p = 0.015$), with significant main effects of treatment ($p = 0.005$) and sex ($p = 0.037$), and a significant interaction between treatment and sex ($p = 0.01$). Thus, myelinated fiber density was reduced in alcohol binge male rats (see also Vargas et al., 2014 or Chapter 3), but was not significantly altered in females after adolescent binge drinking (Fig 4.2). Although there was a sex difference in myelinated fiber density after alcohol consumption, the average cumulative alcohol consumption was not different between the sexes ($t(10.71) = 0.032$, $p > 0.05$, Table 4.2). Similarly, there were no significant differences in glucose intake between treatment groups or sexes (all $p > 0.05$, Table 4.3). In addition, alcohol treatment did not affect the body weight of the animals ($p > 0.05$, Table 4.4).

Based on the findings above, we next tested the hypothesis that adolescent binge drinking increases oxidative stress in the mPFC of adolescent rats. The overall ANOVA of oxidative stress in the mPFC was not significant ($F(5,46) = 1.23$, $p = 0.309$), with no significant main effect of sex ($p = 0.969$) or interaction between treatment and sex ($p = 0.145$), but with a borderline significant main effect of treatment ($p = 0.076$) for males only (Fig 4.3a). The results therefore show trends of an increase in pmol DCF/mg of tissue in the mPFC in alcohol binge male rats, but no significant differences in females (Fig 4.3b). It is important to mention that when a sex subgroup analysis was performed (1-way ANOVA in males), the treatment effect observed had the same magnitude as the previous 2-way ANOVA, but the p-value was slightly lower, becoming statistically significant ($F(2,23) = 3.48$, $p = 0.048$). In Experiment 2, average cumulative alcohol consumption was higher in the binge females when compared to the binge males ($t(15.04) = -2.835$, $p < 0.05$, Table 4.2). However, even when females consumed more alcohol than males, the trend of increased oxidative stress was only observed in males. Glucose intake was not significantly different between treatment groups or sexes (all $p > 0.05$, Table 4.3). Similarly, the body weight of the animals was not affected by alcohol treatment ($p > 0.05$, Table 4.4).

We next used real time quantitative polymerase chain reaction to explore whether adolescent binge drinking decreases mRNA of some of the genes important for myelination of axons (Experiment 3). The overall ANOVA of the fold change of mRNA expression of the myelin-related genes was not significant, with no significant main effects or interactions, and no trends in males or females (Fig 4.4, all $p > 0.05$). This suggests that adolescent binge drinking does not seem to alter the mRNA expression of

genes related to myelination in males or females. The average cumulative alcohol consumption was not different between binge males and females ($t(12.64) = -1.492$, $p > 0.05$, Table 4.2). Likewise, there were no significant differences in glucose intake between treatment groups or sexes (all $p > 0.05$, Table 4.3). Finally, as observed in the experiments above, alcohol treatment had no effect on the body weight of the animals ($p > 0.05$, Table 4.4).

4.5 Discussion

The present study investigated sex differences in the effect of adolescent alcohol consumption on PFC myelin of rats. In addition, this study examined potential explanations for how alcohol may impact prefrontal myelin structure. We found that alcohol affects myelination differently in males and females. In contrast to males, adolescent females did not show a significant reduction in myelinated fiber density after alcohol consumption. Similarly, the alcohol group showed a subtle increase in measured oxidative stress in the mPFC of males, but not female rats, suggesting that oxidative stress may be involved in the alcohol-induced reduction of myelin.

Our results suggest sex differences exist in the effect of adolescent alcohol consumption on myelin. Particularly, the prefrontal myelin of adolescent males seems to be more vulnerable to voluntary alcohol binge drinking, when compared to females. Studies in humans have also found sex differences, but females tend to have higher levels of vulnerability compared to males. For example, Medina et al. (2008) found lower prefrontal white matter volume in females with alcohol use disorders, but higher prefrontal white matter volume in males with alcohol use disorders, compared to control

males (Medina et al., 2008). In addition, female rodents were more vulnerable to neuroinflammation after chronic alcohol consumption starting during adolescence (Alfonso-Loeches et al., 2013). Conversely, there are human studies that have found alcohol affected both sexes similarly and thus found no sex differences. For example, De Bellis et al. (2005) found that both males and females with adolescent-onset alcohol use disorders showed smaller prefrontal cortex white matter volume (De Bellis et al., 2005). Similarly, binge drinking male and female teenagers showed impaired white matter integrity in the corpus callosum (McQueeny et al., 2009). These inconsistencies may be due to the varied alcohol exposures, species, and myelin measures used in each study.

The mechanism by which alcohol disrupts prefrontal myelin remains unknown (Alfonso-Loeches et al., 2012). It is well known that alcohol induces oxidative stress. However, this has not been shown particularly in the brain until recently, when studies showed the induction of oxidative stress using chronic alcohol administration through gavage (Reddy et al., 2013; Teixeira et al., 2014) or by replacement of drinking water (Kharchenko, 2015). We show trends of an increase in oxidative stress, particularly induced by adolescent binge alcohol consumption. This suggests that oxidative stress and inflammation may be involved in the myelin disruptions caused by voluntary binge alcohol consumption. In fact, Alfonso-Loeches et al. (2012) provide evidence for a role for alcohol-induced inflammation in myelin disruption (Alfonso-Loeches et al., 2012). Oxidative stress has also been linked to demyelination. Specifically, myelin and oligodendrocytes have been shown to be highly vulnerable to oxidative stress (Bongarzone et al., 1995; Dewar et al., 2003; Yoshioka et al., 2014), and reactive oxygen species result in oligodendrocyte death (Smith et al., 1999). In addition, oligodendrocyte

precursor cells are very sensitive to ROS and, in their earlier stages, can be easily damaged (Husain and Juurlink, 1995).

Based on the lack of group changes in the mRNA of myelin-related genes, we suggest that reductions in myelin-related genes do not seem to be involved in alcohol-induced damage to myelin, at least in this particular binge drinking model. In this model, alcohol exposure duration and amount is less than other models of alcohol use disorders and dependence (Simms et al., 2008; Crabbe et al., 2009; Gilpin et al., 2012). In fact, some of the genes we tested (PLP, MBP, MAG) were down-regulated in the hippocampus of adult rats after alcohol injection (Lee et al., 2010), and in the cortex, hippocampus, and corpus callosum after chronic alcohol consumption (Alfonso-Loeches et al., 2012). In addition, these mRNA transcripts of MBP, PLP, MAG, and MOBP are directly linked to myelin, as they were reduced in the mPFC of adolescent rats (Gregg et al., 2009) and in the cerebellum of adult mice (Groebe et al., 2009) after cuprizone treatment, a widely used model in rodents to study demyelination (Denic et al., 2011; Silvestroff et al., 2012). Moreover, human studies have shown that postmortem alcoholic brains had a reduction in most myelin-related genes in comparison to controls (Lewohl et al., 2000; Liu et al., 2004). We can therefore assume that, although myelin-related gene transcription is reduced in alcoholism or after chronic alcohol consumption, adolescent binge drinking does not seem to reduce transcription of these genes in the mPFC.

In conclusion, the present investigation provides evidence of sex differences in alcohol-induced effects in prefrontal myelin. To the best of our knowledge, this is the first study to investigate the effect of adolescent voluntary binge drinking on the density of myelinated axons in the mPFC of female rats. Therefore, this research contributes to

the understanding of the inconsistencies in the sex differences alcohol literature across time. In addition, this study suggests there may be subtle alcohol-induced increases in oxidative stress in the prefrontal cortex of adolescent males. Future work is necessary to further explore oxidative stress as a mechanism by which alcohol disrupts prefrontal myelin and to precisely determine whether sex differences exist in this process. These findings will be foundational and an important step in creating new treatment of alcohol use disorders.

4.6 Figures and Tables

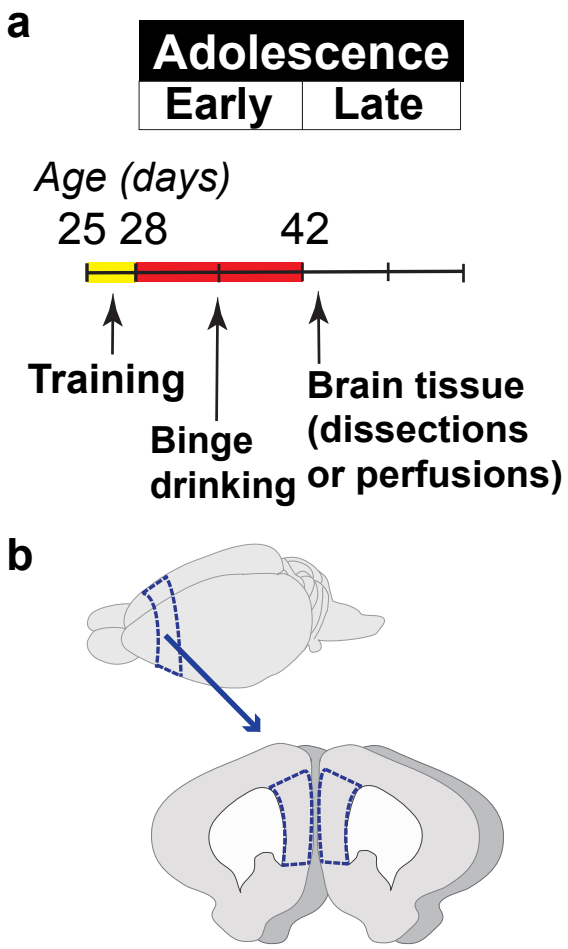

Figure 4.1. Study design. a. Timeline of Experiments 1-3. Rats were trained from postnatal day (PD) 22-27 and underwent binge drinking from PD 28-42. Brains were collected for perfusions (Exp. 1) or dissections (Exp. 2 and 3) on PD 43. **b.** Schematic showing the main area of interest: the medial prefrontal cortex (mPFC). The mPFC was dissected from the fresh frozen brains for assays of reactive oxidative species (Exp 2) and myelin genes (Exp 3). Myelinated fiber density was analyzed in perfused tissue sections within the same region (Exp 1).

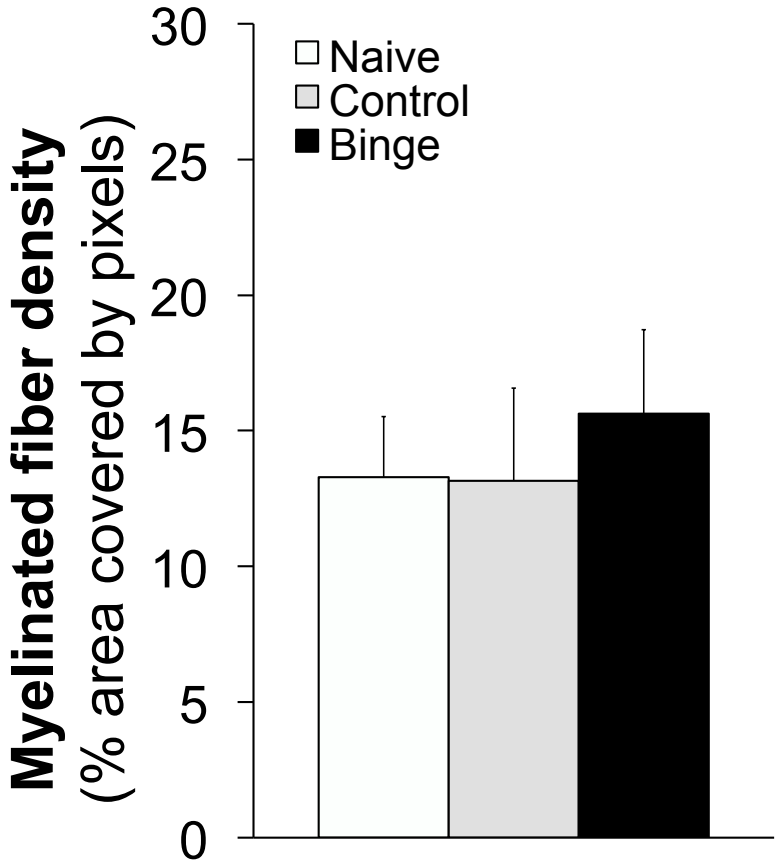

Figure 4.2. Effect of adolescent binge drinking on myelinated fiber density in females. Alcohol binge drinking during adolescence did not significantly reduce myelinated fiber density in the medial prefrontal cortex (mPFC) of adolescent female rats ($p > 0.05$). This was a different effect from adolescent binge mPFC of male rats, which showed a significant reduction in myelinated fiber density (Vargas et al. 2014, or refer to Chapter 3). Data expressed as mean ± SEM (n=8–9 rats/group).

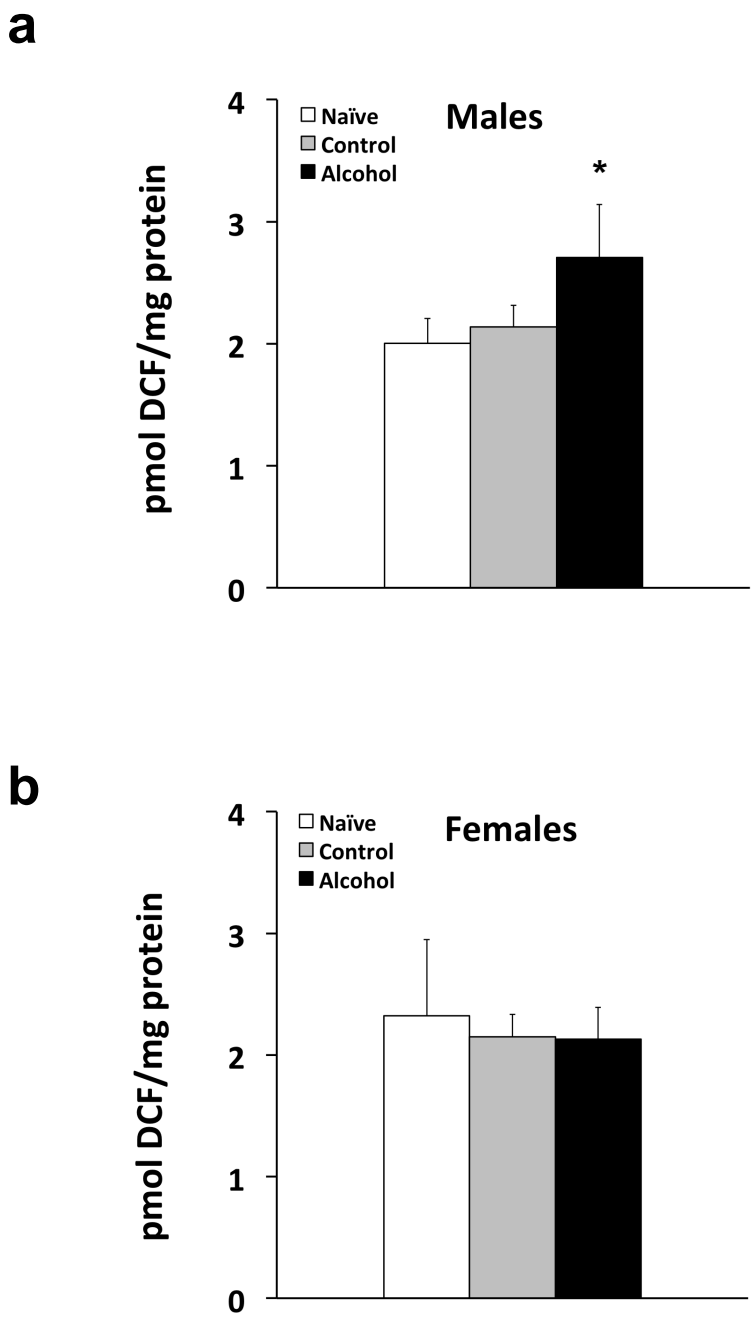

Figure 4.3. Effect of adolescent binge drinking on oxidative stress in the medial prefrontal cortex. a. Adolescent male alcohol rats showed trends of an increase in oxidative stress in the mPFC ($p = 0.076$). **b.** No significant changes in oxidative stress were observed in the mPFC of adolescent female alcohol rats. Data expressed as mean ± SEM (n=8–9 rats/group).

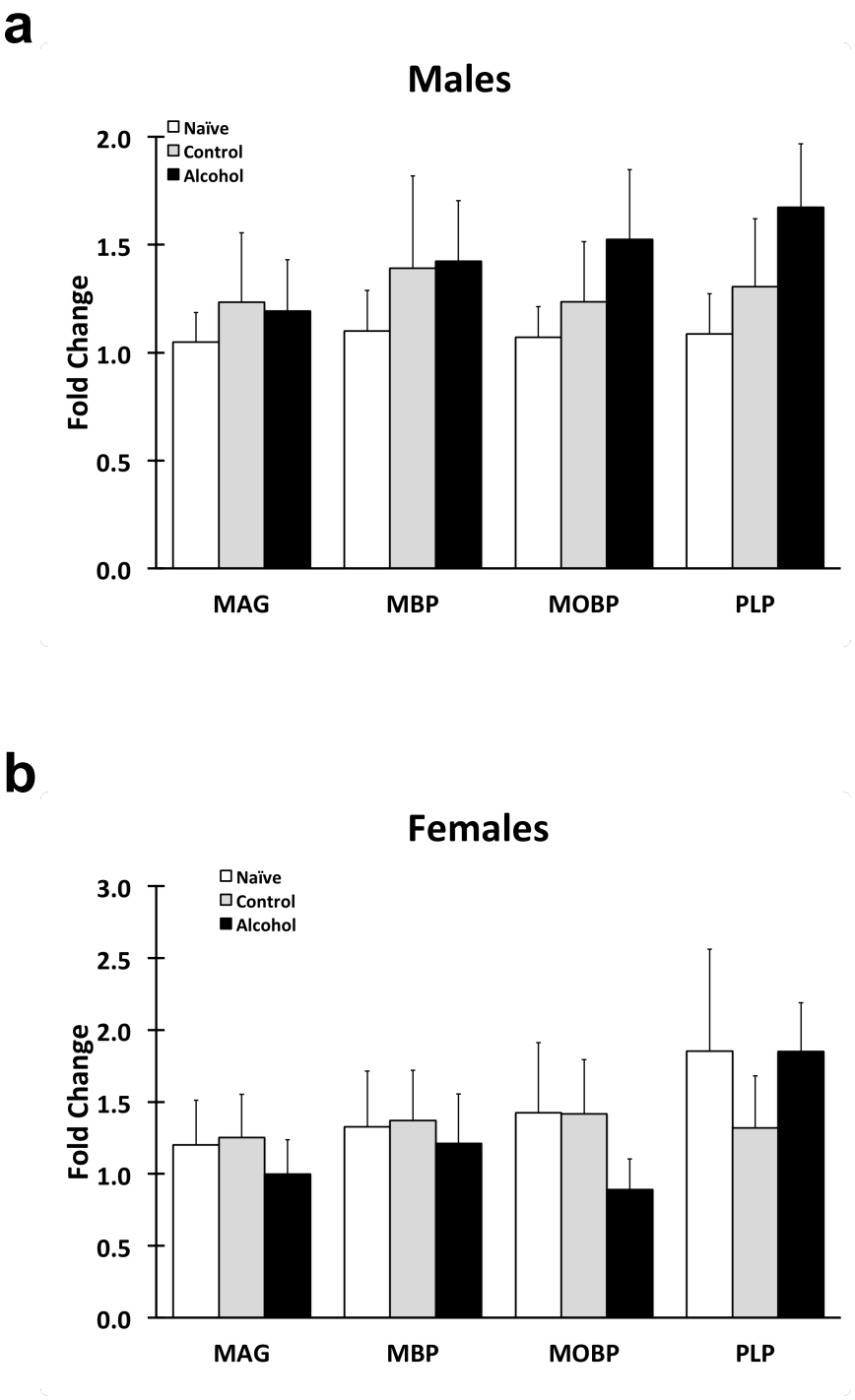

Figure 4.4. Effect of adolescent binge drinking on myelin gene expression in the medial prefrontal cortex of male and female rats. Alcohol did not significantly change myelin gene expression in the mPFC of **(a)** male or **(b)** female adolescent rats. Data expressed as mean ± SEM (n=8–9 rats/group).

Table 4.1. Primer sequences used in Experiment 3

Gene name	Forward primer	Reverse primer
MAG	ATCCTTCTGGAATCGCACTG	CCCTCTCTGTCTCGTTCACA
MBP	ACAGGAAACGGGGACTTAGG	TGGGCTCTGAGAGGAAACAG
MOBP	TACAAGGAAGTCCGGCTCAC	CTGGAGGAAGGAAGGGTTTC
PLP	GCATCACCTATGCCCTGACT	AGCATTCCATGGGAGAACAC
Actb	AGGGAAATCGTGCGTGACAT	AAGGAAGGCTGGAAGAGAGC
Gtf2b	TGCGATAGCTTCTGCTTGTC	TCAGATCCACGCTCGTCTC

Table 4.1. Primer sequences used in the RT-qPCR (Experiment 3). These same primers were used in Gregg et al., 2009.

Table 4.2. Summary of adolescent alcohol consumption (g/kg)

Groups	Experiment 1	Experiment 2	Experiment 3
Binge Male	48.35 ± 7.00	42.29 ± 4.67	39.11 ± 4.19
Binge Female	48.11 ± 3.75	62.74 ± 6.06	51.08 ± 7.41

Table 4.2. Alcohol consumption during the two-week binge drinking period during early adolescence (cumulative total g/kg) for the groups in each experiment. Data shown as mean ± SEM.

Table 4.3. Summary of adolescent glucose intake (g/kg)

Groups	Experiment 1	Experiment 2	Experiment 3
Control Male	17.44 ± 1.28	15.30 ± 1.90	11.82 ± 1.11
Control Female	17.18 ± 1.73	19.16 ± 1.91	15.21 ± 2.11
Binge Male	16.41 ± 2.36	13.02 ± 1.43	11.77 ± 1.25
Binge Female	18.00 ± 1.74	18.87 ± 1.84	15.33 ± 2.22

Table 4.3. Glucose intake during the two-week binge drinking period during early adolescence (cumulative total g/kg) in each experiment. Data shown as mean ± SEM.

Table 4.4. Summary of body weight (g) on PD 42 for experiments 1–3

Groups	Experiment 1	Experiment 2	Experiment 3
Naive Male	214.4 ± 6.1	201.6 ± 12.0	203.0 ± 8.0
Control Male	212.8 ± 8.7	211.3 ± 12.0	213.3 ± 9.9
Binge Male	216.4 ± 5.3	201.6 ± 12.9	197.7 ± 9.9
Naive Female	164.3 ± 4.2	162.5 ± 6.4	155.4 ± 4.8
Control Female	180.6 ± 5.5	162.8 ± 8.0	159.8 ± 5.8
Binge Female	172.9 ± 3.1	162.9 ± 5.4	152.8 ± 2.4

Table 4.4. Body weight (g) of animals on postnatal day 42 in each experiment. Data shown as mean ± SEM.

4.7 References

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CHAPTER 5

GENERAL DISCUSSION

5.1 Main Findings

The present dissertation consisted of three main aims. First, it was important to understand the developmental increases in myelination of the prefrontal cortex (PFC) and forceps minor of the corpus callosum by examining the morphological changes in myelin in these regions. Results in Chapter 2 showed an age-related increase in myelinated axons in the PFC during early adolescence, but no changes in myelin thickness. This suggests that unmyelinated axons may be undergoing *de novo* myelination during this time.

The second aim established the relationship between decreased white matter and increased alcohol consumption, and identified the behavioral effects of alcohol consumption during adolescence. Chapter 3 results showed that binge rats displayed myelin damage and a reduction in myelinated fiber density and white matter area in the PFC. This implies that alcohol exposure may reduce and damage myelin, as opposed to predisposition factors, such as low myelin, increasing alcohol consumption. In addition, results showed a negative relationship between performance in a working memory PFC-dependent task and the amount of alcohol consumption during adolescence, suggesting alcohol may be related to deficits in cognitive performance.

The last aim determined potential sex differences in alcohol-induced PFC myelin changes, and contributed to our understanding of how alcohol alters myelin in the PFC. Chapter 4 results showed that unlike males, binge drinking females did not show evidence of reduced myelin in the PFC, indicating sex differences exist in the interaction between alcohol consumption and PFC myelin. In addition, trends of an increase in

oxidative stress were observed for male, but not female, binge rats, indicating that oxidative stress/inflammation may be related to myelin changes after alcohol consumption.

5.2 Proposed Model

Based on the results of the present studies, and in combination with previous literature, I have created a working model to help increase our understanding of the effects of alcohol exposure on prefrontal myelin, particularly the potential mechanisms underlying these effects, and of the functional significance of prefrontal myelination during adolescence. Refer to Fig 5.1 for a schematic illustrating the model. I propose that intermittent alcohol exposure may reduce myelin in white and gray matter: (1) by preventing oligodendrocyte maturation via reduction of glutamate levels, or (2) by promoting injury/damage and death of mature oligodendrocytes via oxidative stress/inflammation. It is also possible that it may be a combination of these two potential mechanisms. Also, I deduce that this reduction in myelin in white and gray matter areas is accompanied by a compensatory response meant to promote remyelination.

5.2.1 Potential mechanism #1: Reduction of glutamate levels by alcohol, preventing oligodendrocyte maturation

It is known that chronic alcohol exposure affects glutamate. Particularly, administration of 2 g/kg of alcohol significantly reduced glutamate levels in the nucleus accumbens, and this reduction persisted for two hours (Ferrer et al., 2007). In addition, it is known that intermittent and chronic alcohol exposure interferes with glutamatergic transmission (Stuber et al., 2008; Zhao et al., 2015). Ion channels, most notably NMDA

(N-methyl-D-aspartate) receptors, displayed significantly reduced ion currents in response to alcohol exposure (Lovinger et al., 1989). NMDA receptors are involved in excitatory neurotransmission, synaptic plasticity, cognitive function, and some behaviors. Alcohol inhibits NMDA receptors in the PFC, (Weitlauf and Woodward, 2008), nucleus accumbens, and ventral tegmental area (Ding et al., 2012), and in a dose-dependent manner in the hippocampus (Lovinger et al., 1989). NMDA inhibition potentially contributes to some of the perturbations in neural excitation, plasticity, and behavior associated with exposure to alcohol. Chronic alcohol exposure is thought to affect plasticity by increasing calcium/calmodulin dependent kinase type II (CaMKII) activity, impacting long-term potentiation of NMDA receptors (Zhao et al., 2015).

Withdrawal from alcohol is marked by drastically increased extracellular concentrations of glutamate (Rossetti and Carboni, 1995). The exact mechanism by which withdrawal induces increases in glutamate levels is still poorly understood. However, deficits in glutamate clearance (Ding et al., 2013) and glutamate transport and reuptake proteins (Schreiber and Freund, 2000) have been observed. Excessive excitatory synaptic transmission, correlated with increased glutamate levels, has been observed in the ventral tegmental area 24 hours following chronic voluntary alcohol exposure (Stuber et al., 2008). This indicates that while alcohol intoxication acutely inhibits excitatory synaptic transmission, chronic alcohol exposure and withdrawal exacerbates excitatory synaptic transmission. The result is aberrantly high levels of excitation in the brain, particularly in areas involved in addictive behaviors and drug seeking behavior.

In addition to being influenced by alcohol exposure, recent research has demonstrated that glutamate is involved in CNS myelination. Martinez-Lozada et al.

(2014) explained a potential mechanism underlying the relationship between glutamate and CNS myelination. Sodium-dependent glutamate transporters in oligodendrocytes are activated by glutamate secreted from, for example, an unmyelinated axon. The glutamate transporter, in turn, produces a transient increase in intracellular calcium, which leads to phosphorylation of the actin-binding/-stabilizing domain of calcium/calmodulin dependent kinase type IIβ (CaMKIIβ). CaMKIIβ is a kinase that comes from a highly conserved family of serine/threonine kinases (Waggener et al., 2013) and is associated with the actin skeleton in differentiating oligodendrocytes (Martinez-Lozada et al., 2014). After its phosphorylation, CaMKIIβ detaches from the actin cytoskeleton, allowing actin phosphorylation and cytoskeleton remodeling in oligodendrocytes. Finally, the actin cytoskeleton stabilizes when CaMKIIβ binds actin again after phosphorylation. Cycles of binding and unbinding of CaMKIIβ to actin result in a reorganization of the actin cytoskeleton, promoting oligodendrocyte maturation (Martinez-Lozada et al., 2014).

To summarize this first potential mechanism in my proposed model, intermittent alcohol exposure is thought to reduce glutamate levels, preventing oligodendrocyte maturation and ultimately myelination. In contrast, alcohol withdrawal is thought to increase glutamate transmission via interfering with its reuptake/clearance. Glutamate, in turn, promotes maturation of oligodendrocytes, thereby promoting myelination via sodium-dependent glutamate transporters and CaMKIIβ. The alcohol-induced reduction of glutamate would thereby perturb myelin in white and gray matter.

5.2.2 Potential mechanism #2: Induction of oxidative stress/inflammation by alcohol leading to mature oligodendrocyte injury and death

Alcohol exposure, especially binge-like alcohol exposure, is correlated with brain degeneration and inflammation (Crews et al., 2006). However, the mechanism by which consumption of alcohol induces damage to the brain is poorly understood. Inflammation, generated via oxidative stress and free radical damage, is thought to play a role in this process. There are several key players involved in the inflammatory response to alcohol consumption.

Central to alcohol's capability to promote an inflammatory response is the widely expressed transcription factor nuclear factor-kappa B (NF-kB) (Crews et al., 2006). This transcription factor is necessary for activating the inflammatory response, and it is important for cell proliferation, growth, and adhesion (Surh et al., 2001). Normally, NF-kB is inactive and bound to IkappaB (IkB) kinase. Alcohol consumption acts on a member of the toll-like receptor family, TLR4, to phosphorylate and degrade IkB (Murakami and Ohigashi, 2007). This in turn disinhibits NF-kB, allowing for nuclear translocation and subsequent activation of inflammatory genes. NF-kB is then free to bind on the promoter region of the pro-inflammatory genes cyclooxygenase (COX-2 isoform) and inducible nitric oxide synthase (iNOS). Both COX-2 and iNOS promoter regions contain NF-kB binding sites, and thus could be dually activated by the same stressor, i.e. alcohol.

COX-2 mRNA increases Prostaglandin E2 activity, which in turn promotes an inflammatory response. In parallel, NF-kB activation, coupled with NADPH oxidase, promotes iNOS mRNA expression and iNOS enzyme activity, enhancing the generation of nitric oxide (Wu et al., 2008; Koppula et al., 2012). This in turn generates reactive

oxygen species (ROS), capable of denaturing and degrading proteins, DNA, and various other biological components (Murakami and Ohigashi, 2007). These ROS subsequently activate and promote inflammation, as mentioned above. These series of cellular and molecular events form a bridge between alcohol consumption, inflammatory response, and subsequent degradation of brain areas.

The role of oxidative stress in demyelination was reviewed by Smith et al. (1999). Many in-vitro studies have shown that ROS can affect myelination in a variety of manners. For example, ROS production can result in oligodendrocyte death, which leads to demyelination (Smith et al., 1999). In fact, apoptosis in oligodendrocytes was mediated by ROS (Yeo et al., 2012). It is known that oligodendrocytes are much more vulnerable to ROS than astrocytes because oligodendrocytes produce low levels of antioxidants. Indeed, oligodendrocyte death occurs at a much lower dose of ROS than that required for astrocyte death (Griot et al., 1990).

Another way ROS production affects myelination is by directly affecting the lipids and proteins that make up myelin (Konat and Wiggins, 1985). Incubating myelin with ROS caused myelin decompaction and peroxidation of myelin basic protein (MBP), lipids, and proteolipid protein (PLP) (Bongarzone et al., 1995). In addition, some matrix metalloproteinases, neutral endoproteinases that degrade all components of the extracellular matrix, have been shown to degrade MBP when released as a consequence of ROS production (Chandler et al., 1995; Smith et al., 1999).

To summarize the second potential mechanism in my proposed model, intermittent alcohol exposure is thought to induce oxidative stress/inflammation through the TLR and NF-kB pathway, promoting injury to oligodendrocytes and apoptosis.

Interference with the function of oligodendrocytes will likely affect the expression of myelin-related genes, such as MBP or PLP. In addition, intermittent alcohol exposure is expected to downregulate myelin-related genes, similar to the downregulation of myelin-related genes observed in mice after chronic alcohol exposure (Alfonso-Loeches et al., 2012). The alcohol-induced disruption in oligodendrocyte function and/or the down-regulation of myelin proteins will lead to reduction of myelin in white and gray matter.

5.2.2.1 Compensatory response to inflammation: remyelination

As mentioned above, the inflammatory response degrades myelin through mature oligodendrocyte death, peroxidation of MBP and PLP, etc. However, there are compensatory and regenerative effects of inflammation that can also promote remyelination, and occur through activation of a different member of the toll-like receptor family, TLR2, (Choi et al., 2014). During the inflammatory response, macrophages are activated and secrete the pro-myelinating cytokine endothelin-2 (Yuen et al., 2013). Activation of endothelin-2 receptors promotes OPC migration and differentiation (Gadea et al., 2009), which in turn increase production of MBP mRNA expression (Jung et al., 2011). OPCs express receptors for endothelin-2, which when activated promote remyelination and when blocked inhibit remyelination (Yuen et al., 2013).

5.2.3 Functional significance of prefrontal myelination during adolescence

The present working model will also aid in the understanding of the functional significance of adolescent myelination, at both the physiological and behavioral levels. During adolescent development, myelin in white and gray matter increases and

corresponds with an augmentation in the conductance speed for fibers in the mPFC (Chapter 2). In addition, it is well known that myelination increases conduction velocity of fibers (Purves et al., 2008). Therefore, I conclude that increasing the conduction speed along axons in the mPFC is a functional outcome of increased myelination of these axons during adolescence.

It is also important to determine if alcohol causes structural and physiological changes in PFC myelin and whether these changes affect behavior. Indeed, reduced frontal white matter correlated with increased relapse-like drinking in adulthood (Chapter 3). Myelination of the PFC correlates with an improvement in performance of cognitive tasks (Fuster, 2002). I found that adolescent drinking reduced myelin in the mPFC, and the amount of alcohol consumed was inversely related to performance in a working memory PFC-dependent task (Chapter 3). Finally, adolescent alcohol consumption augmented relapse-like drinking in adulthood (Gilpin et al., 2012). Therefore, based on the evidence outlined above, I can also conclude that myelination in the PFC has functional significance at the behavioral level as well.

5.3 Concluding Remarks

The results of the experiments in this dissertation have confirmed adolescent development is characterized by an increase in myelin in the PFC. Results have demonstrated that consumption of alcohol during adolescence reduces white matter and myelin involved in the prefrontal circuits, and is associated with long-term cognitive deficits. In addition, the present work suggests that alcohol consumption during adolescence induces subtle increases in oxidative stress in the PFC. This suggests that inflammation may be involved in the myelin reduction induced by alcohol consumption.

The present results identify that there are sex differences in the interaction between adolescent alcohol consumption and prefrontal myelin, with males appearing more vulnerable than females.

Therefore, this dissertation contributes to our understanding of the developmental increases in prefrontal myelination during adolescence and identifies a specific effect of alcohol consumption on adolescent myelin development. Future work investigating the cellular and molecular mechanisms underlying alcohol-induced reduction in prefrontal myelin and the sex differences observed could lead to novel treatment and prevention strategies for alcohol use disorders.

5.4 Figures and Tables

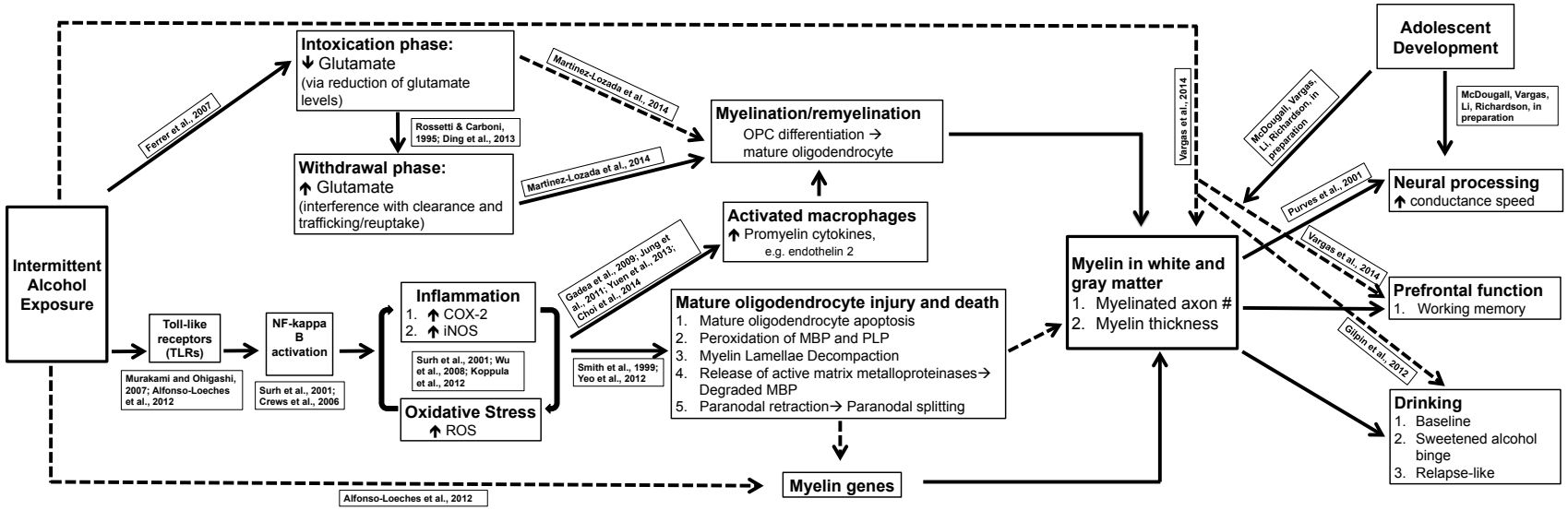

Figure 5.1. Schematic illustrating the proposed model. Solid lines signify inducing and dotted lines signify inhibiting. Abbreviations: NMDA, N-methyl-D-aspartate; COX-2, cyclooxygenase-2; iNOS, nitric oxide synthase; ROS, reactive oxygen species; OPC, oligodendrocyte precursor cells; MBP, myelin basic protein; PLP, proteolipid protein; ↑, increase; ↓, decrease.

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