CONSEQUENCES OF MATERNAL PRECONCEPTION NICOTINE AND ALCOHOL: A RODENT MODEL OF THE LONG-TERM EFFECTS ON OFFSPRING BRAIN AND BEHAVIOR

SERENA JENKINS
Master of Science, **University of Lethbridge, 2017**

A thesis submitted
in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

NEUROSCIENCE

Department of Neuroscience
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

CONSEQUENCES OF MATERNAL PRECONCEPTION NICOTINE AND ALCOHOL: A RODENT MODEL OF THE LONG-TERM EFFECTS ON OFFSPRING BRAIN AND BEHAVIOR

SERENA JENKINS

Date of Defence: September 1, 2021

Dr. R. Gibb Thesis Supervisor	Professor	Ph.D.
Dr. B. Kolb Thesis Examination Committee Member	Professor	Ph.D.
Dr. O. Kovalchuk Thesis Examination Committee Member	Professor	Ph.D.
Dr. C. Gonzalez Internal External Examiner Department of Kinesiology Faculty of Arts and Science	Professor	Ph.D.
Dr. A. Lodha External Examiner University of Calgary Calgary, Alberta	Professor	M.D.
Dr. S. Pellis Chair, Thesis Examination Committee	Professor	Ph.D.

DEDICATION

This thesis is dedicated to my future children; I hope that everything I have learned about brain development these past six years will be to your benefit. I also dedicate this work to my husband, who has equally taken this knowledge to heart.

ABSTRACT

This thesis explores the impacts of maternal preconception experience on the development of brain and behavior in the next generation. Chapter 1 provides a review of the literature on prenatal and preconception exposure to common recreational drugs of abuse, nicotine and alcohol. The review covers behavioral, neuroanatomical, molecular/epigenetic, and physiological impacts of developmental drug exposure. Chapter 2 discusses the results of an experiment that investigated the early impacts of maternal preconception nicotine and environmental enrichment. Chapter 3 covers how maternal preconception nicotine and enrichment continue to affect development in adolescent and adult offspring. Chapter 4 discusses an experiment into the impacts of maternal preconception alcoholism on offspring development throughout life. Chapter 5 presents a brief general conclusion. The overall goal of this thesis is to highlight how maternal experiences prior to conception can have just as significant an impact on offspring development as much more thoroughly studied prenatal experiences.

ACKNOWLEDGMENTS

First, I must acknowledge my doctoral supervisor, Dr. Robbin Gibb. Robbin, since you agreed to welcome me into your lab sight unseen six years ago, I have grown into what I hope will be a highly successful, productive scientist. You provided me with opportunities to be an "independent" researcher, to teach and mentor, and develop all of the essential skills of a good scientist. I owe so much to everything you have taught me and enabled me to discover.

Next, I must acknowledge the members of my supervisory committee. Bryan, you have been a comforting source of knowledge and encouragement since we met. One of the things I will miss most about no longer being a student is seeing you everything Tuesday, Wednesday, and Thursday. Claudia and Olga, thank you for your thoughtful suggestions (and compliments!) during each of my committee meetings, my thesis proposal defence, and my Comprehensive Exam.

To Sergio Pellis, thank you for agreeing to chair all of my formal academic events. It's been a pleasure to have you at an arm's length all these years.

I must also acknowledge some specific colleagues who have shaped the past four years in innumerable ways. Loni, your friendship has been one of my favorite things about my time at the U of L. Having you sit a few feet behind (or ahead of) me has saved me time and time again. It saddens me that we haven't been in the office together for the past year a half due to the pandemic. I hope that we find a way to continue working together as long as we can; I have faith that we will. Claire, I have adored you since we met six years ago, but our friendship has grown so much since the pandemic hit and it was just you and I in the lab for so long. When I'm not on campus, I keep a mental list of

things to tell you when I see you next. You've been a fantastic sounding board this past year and I hope I've been the same for you. I must also thank all of the students that have helped me over the years. From coming in early in the morning to weigh animals and help run behavior, to spending hours and hours engaged in monotonous scoring, you have all contributed to this final thesis.

Thank you to all of the support staff in the CCBN, the Animal Services Staff, and the Animal Welfare Committee for helping to make my research possible and ensuring it's quality.

To my husband, I have been a student for our entire nine-year relationship. You have always stood by me and supported me, even when my habit of procrastination caused you more stress than it did me. You supported me when I wanted to move to Lethbridge to pursue Graduate Studies, even though that meant leaving behind everything we knew. As I transition into this new stage of my career, you have shown me nothing but understanding and patience. You always want what is best for me and give me hell when I settle for anything less. I don't know what these past years of my life would have looked like if I had spent them with anyone but you. I only hope that I can begin to reciprocate everything you have done for me.

Lastly, I would like to acknowledge my parents, who only occasionally made comments about how I was "still in school" and always pushed me to strive for better and achieve my potential.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 2: Distinct sex-dependent effects of maternal preconception nicotine and enrichment on the early development of rat offspring brain and behavior

Chapter 3: Long-term consequences of maternal preconception exposure to nicotine and environmental enrichment: Sex- and age-dependent effects on anxiety, motor control, spatial learning, and the brain

Chapter 4: Maternal preconception alcoholism: Effects on offspring behavioral development and dendritic spine density from neonatal to adult life

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
ADHD	Attention-deficit hyperactivity disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ARBD	Alcohol-related birth defects
ARND	Alcohol-related neurodevelopmental delay
AVP	Arginine vasopressin
BAC	Blood alcohol content
BDNF	Brain-derived neurotrophic factor
CRF	Corticotropin releasing factor
DALY	Disability-adjusted life-year
DAT	Dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
EPM	Elevated plus maze
F	Filial
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorder
fMRI	Functional magnetic resonance imaging
G	Gestational day
HPA	Hypothalamic-pituitary-adrenal
IGF1	Insulin-like growth factor
ILK	Integrin-linked kinase
IQ	Intelligence quotient
LTP	Long-term potentiation
MWT	Morris water task
nAChR	Nicotinic acetylcholine receptor
NET	Norepinephrine transporter
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NRT	Nicotine replacement therapy
OR	Odds ratio
P	Postnatal day
PFC	Prefrontal cortex
SIDS	Sudden infant death syndrome
SIUDS	Sudden intrauterine unexplained death syndrome
FST	Forced swim task

CHAPTER 1

Does mother know best? Consequences of maternal prenatal and preconception exposure to recreational drugs of abuse

Abstract

The use of common recreational drugs during pregnancy is high; 10% of woman continue to either smoke tobacco or consume alcohol throughout their pregnancy, despite countless targeted public health campaigns regarding the danger of both substances. Both prenatal nicotine and alcohol are associated with a range of consequences that impact development throughout life. Furthermore, recent evidence is emerging to suggest that maternal substance abuse prior to pregnancy can shape offspring development, even in the complete absence of gestational exposure. This review covers current literature on the effects of prenatal and preconception nicotine and alcohol use on the development of offspring, considering the behavioral, neuroanatomical, molecular/epigenetic, and physiological consequences. We focus on studies that have been conducted using humans and rodent models.

The use of drugs to alter one's physiological and psychological state is prevalent globally. The two most widely consumed drugs are alcohol (18.4% heavy episodic users) and tobacco (15.2% daily smokers), followed by cannabis, amphetamine, opioids, and cocaine (Peacock et al., 2017). Tobacco and alcohol account for the greatest number of substance-related deaths (111 and 33 per 100,000 people, respectively) and have the highest attributable disability-adjusted life-years (DALYs; 171 million and 85 million, respectively), which represents the total years of life lost due to premature mortality or lived with disability (Peacock et al., 2017). Furthermore, alcohol and tobacco dependence often co-occur, as 80 – 95% of alcoholics are also smokers (Hendrickson, Guildford, & Tapper, 2013). It is likely no coincidence that the two most abused substances are legal throughout most of the world and subjected only to restrictions based on where and by whom they can be bought and used. Related to the legality of nicotine and alcohol, young people (18 – 24 years) consider these two substances to be among the least harmful drugs of abuse, with tobacco being rated the least harmful, followed by cannabis, and then alcohol (Cheeta et al., 2018). Amazingly, these ratings are in stark contrast to ratings from experts in the relevant areas of research; young people underestimate the danger associated with tobacco and especially alcohol, which experts rank as being more harmful than even heroin, cocaine, and methamphetamine (Cheeta et al., 2018).

Direct exposure to these common drugs of abuse has a variety of negative side effects on the user, but these drugs are also considerably harmful when used maternally during critical developmental time periods. This review presents an overview of the research into prenatal and preconception exposure to commonly used recreational drugs, namely nicotine and alcohol. We focus on studies conducted with human populations and

rodents, which each confer distinct benefits. Human studies are directly translatable and are essential to enacting change to public health recommendations. However, human studies are generally correlational and highly confounded by innumerable external factors, and it can often be impossible to make definitive statements regarding underlying causes and mechanisms. For example, many of the consequences associated with prenatal drug use are confounded by the demographic characteristics of the mother, including education, age, and socioeconomic status (Winzer-Serhan, 2008). Animal studies circumvent these issues by using highly controlled experimental conditions that allow direct associations between cause and effect. Animal studies also permit the use of informative methods that are impossible or unethical to use with human subjects.

1.1 Nicotine

A recent Canadian survey revealed that 13% of adults of reproductive age self-classify as nicotine users, with 9% identifying as daily users (Health Canada, 2019). Although the rate of smoking among youth is encouragingly low (5%), the recent popularization of "vaping", or the use of electronic cigarettes, has led to an increase in the number of adolescents addicted to nicotine (Health Canada, 2019). The commonality of smoking or nicotine use has decreased in recent years, but currently the rate of quitting closely matches the rate of uptake by children and adolescents, so the decline in the number of users has stagnated (Benowitz, 2010).

Nicotine primarily acts on nicotinic acetylcholine receptors (nAChRs), which are pentameric ligand-gated cation (primarily Na+) channels that are located throughout the brain and appear as early in development as the formation of the neural tube (Blood-Siegfried & Rende, 2010; Hendrickson, Guildford, & Tapper, 2013). Most nAChRs are

located pre-synaptically, where they influence overall neuronal excitability and trigger voltage-gated Ca+ channels (Dwyer, McQuown, & Leslie, 2009). Nicotine is a nAChR agonist and competes with endogenous acetylcholine, thereby interfering with the role of acetylcholine in neuronal proliferation, maturation, pathfinding, and differentiation (Dwyer, McQuown, & Leslie, 2009; Blood-Siegfried & Rende, 2010). Depending on the timing of developmental nicotine exposure, nicotine can interfere with the growth and migration of neurons, synapse formation, and cell populations in the hippocampus, cerebellum, and neocortex (Blood-Siegfried & Rende, 2010). nAChRs also regulate the prenatal development of catecholamine neurons; therefore, developmental nicotine has a substantial influence on the dopaminergic system (Dwyer, McQuown, & Rende, 2009).

1.1.1 Prenatal Nicotine

Prenatal exposure to nicotine, either through cigarette smoking or nicotine replacement therapy (NRT), remains prevalent despite years of public health outreach regarding the harm to the fetus. Ten percent of women who smoke continue to smoke throughout their pregnancy (Al-Sahab, Saqib, Hauser, & Tamim, 2010). Complete cessation of nicotine intake is recommended for all women who are pregnant or breastfeeding, with NRT suggested as an alternative in the event that cessation is unsustainable (CAN-ADAPTT, 2011). However, although NRTs do not contain most of the 4000 compounds contained within cigarettes and generally have a lower concentration of nicotine, nicotine alone is still highly teratogenic as it crosses the placenta and concentrates in the fetal blood and amniotic fluid at a concentration 15% higher than in the mother (Blood-Siegfried & Rende, 2010; Bruin, Gerstein, & Holloway, 2010). Furthermore, maternal smoking physically damages the placenta, resulting in

impaired nutrient and oxygen delivery to the fetus (Blood-Siegfried & Rende, 2010). What follows is an overview of the human and rodent research into the consequences of prenatal nicotine exposure. The majority of human studies concerned maternal smoking, whereas animal studies are predominantly conducted using isolated nicotine that is either injected, consumed orally, or administered using an osmotic minipump.

1.1.1.1 Behavioral Consequences

The main behavioral consequences associated with prenatal nicotine exposure in human and rodent models include attention-deficit hyperactivity disorder (ADHD), mental health problems and other social impairments, and cognitive deficits.

Perhaps the most robust behavioral consequence of prenatal smoking/nicotine exposure is an increased prevalence of ADHD in childhood and adolescence (Gutvirtz, Wainstock, Landau, & Sheiner, 2019). One retrospective study conducted with a population from when prenatal smoking was common (33% of mothers) found that after controlling for maternal demographic characteristics and other confounds, there was a strong dose-dependent relationship between maternal smoking and the presence of childhood hyperactivity, with the strength of the relationship increasing with the number of cigarettes smoked per day (Keyes, Smith, & Susser, 2014). The risk of a child being diagnosed with ADHD is significantly higher if the mother smoked during pregnancy (adjusted odds ratio (OR) 2.64), or if the mother was a smoker but reported abstaining during pregnancy (adjusted OR 2.47). Additionally, among mothers who did not smoke during their pregnancy, the prevalence of ADHD was increased slightly (adjusted OR 1.16) if the mother was exposed to environmental tobacco smoke (Han et al., 2015). ADHD diagnosis can also be predicted by prenatal levels maternal serum cotinine, the

main metabolite of nicotine; after controlling for confounds, the highest levels of cotinine increased the likelihood of an ADHD diagnosis by 3.34X relative to the lowest levels of cotinine (Sourander et al., 2019).

Rodent studies have generally confirmed the relationship between maternal nicotine and ADHD symptoms, suggesting that nicotine itself is exerting the teratogenic effect as opposed to other compounds in cigarette smoke and that the relationship is causal. Studies frequently report increased hyperactivity following prenatal nicotine, although there is some variability based on how the nicotine was administered (Polli & Kohlmeier, 2020). In addition to hyperactivity. prenatal nicotine in rodents is also associated with deficits in attention and increased impulsivity, (Schneider et al., 2011; Polli et al., 2020), therefore corresponding to all the major diagnostic features of ADHD. Studies from the research group led by Bhide use a perinatal paradigm that exposes dams to nicotine before and during pregnancy, as well as during lactation. These studies also corroborate the relationship between developmental nicotine and ADHD symptomology, including hyperactivity, inattention, and impulsivity (Zhu et al., 2012, 2017; Zhang et al., 2018).

Furthermore, evidence from both human and animal studies suggests that the symptoms of ADHD that emerge following prenatal nicotine exposure are indistinguishable from ADHD diagnoses that are not associated with prenatal nicotine. One retrospective study of children with an ADHD diagnosis found there to be no difference between the clinical features of the condition based on whether the mother smoked during pregnancy (Biederman et al., 2012). Rodent studies have determined that the symptoms of ADHD that arise following prenatal nicotine exposure are reversed by a

single dose of oral methylphenidate (Zhu et al., 2012, 2017). Methylphenidate, commonly sold under the brand name Ritalin, is a central nervous system stimulant that increases the level of extracellular dopamine by preventing dopamine transport (Schrantee et al., 2016). The findings of Zhu and colleagues (2012, 2017) suggest that the mechanism driving prenatal nicotine-induced hyperactivity, inattention, and impulsivity is the same as non-nicotine-associated ADHD diagnoses.

Mental health concerns and issues with emotional regulation are also commonly associated with prenatal smoking/nicotine. Maternal smoking increases the prevalence of conduct disorders and externalizing behaviors in children and adolescents, including aggression, antisocial behavior, and criminal behavior (Höök, Cederblad, & Berg, 2006; Tiesler & Heinrich, 2014; Holbrook 2016). Adolescents exposed to nicotine in utero are also more likely to abuse drugs themselves and show heightened dependency and withdrawal symptoms (Jacobsen et al., 2006). There is also some evidence that prenatal nicotine predisposes children and adolescents to deficits in emotional regulation, although not all studies support this (Tiesler & Heinrich, 2014). Maternal smoking has also been identified as a risk factor for schizophrenia; mothers classified as heavy smokers during their pregnancy based on serum cotinine levels have children with a 38% increased risk of developing schizophrenia (Niemelä et al., 2016). In rodent models, prenatal nicotine is frequently associated with increased anxiety and depressive behaviors (Polli & Kohlmeier, 2020), and the effects are often sexually dimorphic. For example, some studies report increased anxiety and compulsivity in male offspring only (Vaglenova et al., 2004; Polli et al., 2020), whereas another study found that prenatal nicotine results in increased depressive tendencies in female offspring only (Zhang et al., 2019). However, a

study that used the perinatal exposure paradigm discussed above reported decreased anxiety in both males and females, which the authors propose may represent decreased inhibition towards novel and potentially risky situations (Martin et al., 2020). In line with this speculation, other studies have demonstrated that prenatal nicotine increases risk-taking (Buck et al., 2019), drug self-administration (Polli & Kohlmeier, 2020), and induces desensitization to nicotine in offspring rats (Slotkin, Tate, Cousins, & Seidler, 2006).

The final major category of behavioral impairments associated with prenatal nicotine exposure relates to cognitive behavior and academic achievement. The prevalence of learning disorders, language problems, and intellectual disabilities is twice as high among children of smoking mothers (Gutvirtz et al., 2019). Children exposed to prenatal nicotine frequently have lower intelligence quotient (IQ) and academic achievement than their non-exposed peers; however, the relationship weakens after controlling for factors related to maternal demography, and a large amount of the shared variance is accounted for by low birthweight (Winzer-Serhan, 2008; Holbrook, 2016). In animal models, prenatal nicotine induces impairments in long-term memory determined using multiple assessments of learning and memory (Polli & Kohlmeier, 2020). Offspring rats exposed to prenatal nicotine also exhibit deficits in executive function, including poor behavioral inhibition (Bryden et al., 2016) and working memory (Parameshwaran et al., 2012; Polli et al., 2020). However, many rodent studies do report a decrease in birthweight and/or postnatal growth (Vaglenova et al., 2004, 2008; Schneider et al., 2011, 2012; Zhu et al., 2012). Therefore, although maternal nicotine/smoking is commonly

associated with impaired cognitive functioning, the exact etiology of these impairments remains unclear.

Prenatal nicotine exposure has also been associated with disorders of movement. In humans, the incidence of movements disorders, including epilepsy, tremors, ataxia, and dystonia, is twice as high among children born to smoking mothers compared to children from non-smoking mothers (Gutvirtz et al., 2019). In rodents, prenatal nicotine delays the emergence of sensorimotor reflexes necessary for properly orienting the body in space (Ajarem & Ahman, 1998; Vaglenova et al., 2008; Schneider et al., 2011), weakens grip strength (Schneider et al., 2011), and results in a decrease in activity in young offspring (LeSage, Gustaf, Dufek, & Pentel, 2006).

1.1.1.2 Neuroanatomical Consequences

The action of endogenous acetylcholine on nAChRs is vital for brain development. In both humans and rodents, nAChRs appear very early in development and follow a caudal-rostral gradient, with the specific timing of appearance varying by receptor subtype (Dwyer, McQuown, & Leslie, 2009). Acetylcholine is necessary for the proper development of the central nervous system, including neuron proliferation, growth, and apoptosis (Dwyer, Broide, & Leslie, 2008; Winzer-Serhan, 2008; Dwyer, McQuown, & Leslie, 2009). Nicotine is a high-affinity agonist of nAChRs and competes with acetylcholine for binding sites, where it disrupts the neural processes normally ascribed to acetylcholine. Therefore, prenatal exposure to nicotine has severe detrimental effects of the developing brain beginning in the initial weeks (or days in the rodent) of gestation. Prenatal nicotine is particularly harmful to the development of the hippocampus, cerebellum, and neocortex.

The volume of the hippocampus is increased in adolescents (mean age 14 years) gestationally exposure to nicotine and/or alcohol even after controlling for extraneous factors; unfortunately, the authors did not perform the analysis separated by drug, but a large proportion of the prenatally exposed group had tobacco exposure (80%), whereas only 54% had alcohol exposure. Furthermore, these adolescents show an impairment in tests of learning and recall (Riggins et al., 2012). In the rodent hippocampus, nicotine exposure throughout pregnancy decreases neuron size, decreases the thickness of neuronal layers, and increases cell density in CA3 and the dentate gyrus, with a similar yet less pronounced trend in CA1 (Roy, Seidler, & Slotkin, 2002). Others have found that prenatal nicotine in the form of a time-release implant does not impact the number of cells or the volume of the various hippocampal subfields (Chen et al., 2006). Prenatal nicotine also impacts neuronal architecture in the hippocampus. In adolescent offspring, dendritic spine density is increased on granule cells in the dentate gyrus and on basilar and terminal dendrites of pyramidal cells in CA1 and CA3, but decreased on apical dendrites of pyramidal cells in CA3. Granule and pyramidal cells in all regions also show a decrease in distal dendritic branching (Roy & Sabherwal, 1998). Such changes to neuronal structure indicate that prenatal nicotine is altering structural plasticity in the hippocampus, which can have cascading effects on how the brain responds to later experience.

In the neocortex, neuroimaging studies with young children (6 – 8 years) exposed to maternal smoking in utero reveal reduced cortical volume, specifically grey matter volume, and reduced thickness of the superior frontal, superior parietal, and precentral gyri after controlling for maternal characteristics, birth weight, and multiple comparisons

(El Marroun et al., 2014). Interestingly, none of these effects were significant among children born to mothers who smoked but then quit upon learning of their pregnancy (El Marroun et al., 2014), suggesting the importance of direct exposure to tobacco smoke/nicotine while in utero. Prenatal nicotine also reduces head circumference, cortical grey matter volume, and parenchymal volume in slightly older children (mean age of 12 years) after adjusting for maternal characteristics including the use of other substances (alcohol, cannabis, or cocaine; Rivkin et al., 2008). In rodents, perinatal nicotine reduces the volume of the cingulate cortex by 24%, although the striatum, sensorimotor cortex, corpus callosum, and lateral ventricles remain unchanged; the reduction in the cingulate cortex may be related to impaired attention in ADHD (Zhu et al., 2012). In the somatosensory cortex forelimb and hindlimb areas of juvenile rat offspring exposed to prenatal nicotine, layer V shows a decreased ratio of medium pyramidal neurons to small non-pyramidal neurons and an increased number of glia (Roy, Seidler, & Slotkin, 2002). Prenatal nicotine also changes neuronal architecture in the prefrontal cortex (PFC) of offspring. In weanling offspring, prenatal nicotine increases dendritic branching and length in orbitofrontal (OFC; AID) and medial PFC (Cg3) in both males and females (Muhammad et al., 2012). In adult offspring, the effects are sexually dimorphic; males have increased arborization of basilar dendrites in AID and Cg3, but decreased branching of apical dendrites in Cg3, whereas females show decreased arborization in both apical and basilar dendrites in Cg3. Furthermore, both males and females have increased spine density in AID, and among apical spines in Cg3, males have increased density and females decreased density (Mychasiuk, Muhammad, Gibb, & Kolb, 2013). Again, these changes collectively demonstrate the ability of prenatal nicotine to affect structural

plasticity, and in the case of the PFC, that these changes are dependent on offspring age and sex.

The volume of the cerebellum is impacted by both maternal smoking and the presence of an ADHD diagnosis. Children with ADHD and exposed to prenatal nicotine had the smallest cerebellar volume, followed by unexposed children with ADHD, and then unexposed controls. Therefore, prenatal nicotine and behavioral impairments associated with ADHD have distinct yet additive effects on cerebellar volume (de Zeeuw et al., 2012). Autopsy of fetal and neonatal brains from cases of sudden intrauterine unexplained death syndrome (SIUDS) and sudden infant death syndrome (SIDS), respectively, showed numerous abnormalities in cerebellar Purkinje cells and a strong association between cerebellar structure and maternal smoking, suggesting a potential mechanism behind unexplained perinatal death (Lavezzi, Corna, Repetti, & Matturri, 2013). In rodents, early postnatal exposure to nicotine during the third trimester-equivalent reduces the number of Purkinje cells in the cerebellar vermis (Chen, Parnell, & West, 1998). Interestingly, studies in rodents that expose the dam to nicotine throughout the entire pregnancy (i.e. first and second trimester equivalent) have found no change in the number or density of Purkinje cells or the volume of the cerebellar vermis (Chen & Edwards, 2003; Chen et al., 2006). Therefore, rodent studies identify that the timing of nicotine exposure determines the teratogenic effects on the cerebellum. Indeed, others have identified that the third trimester is the most sensitive period for nicotine exposure (Holbrook et al., 2016).

1.1.1.3 Molecular and Epigenetic Consequences

Prenatal nicotine changes the expression of nAChRs in the brains of offspring. In fetal offspring, α2, α4, α7, and β2 subunits were all upregulated, whereas there was no change in the expression of the remaining subunits (Lv et al., 2008). Upregulation of subunit mRNA persists into postnatal life as well. Offspring continue to show elevated expression of α4 and β2 mRNA on postnatal day (P)14, although levels returned to control levels by P28 (Shacka & Robinson, 1998). Upregulation of nAChRs would not only impact how the brain responds to nicotine, but also impact the response to endogenous acetylcholine.

Prenatal nicotine exposure exerts a widespread influence over developing neurotransmitter systems, including acetylcholine, serotonin, and catecholamines dopamine, epinephrine, and norepinephrine (Oliff & Gallardo, 1999; Blood-Siegfried & Rende, 2010). Following perinatal nicotine exposure, offspring rats have increased dopamine in the frontal cortex (intracellular + extracellular) but decreased dopamine turnover in the frontal cortex and striatum (extracellular only), suggesting that although dopamine was available in vesicles, it was not being released into the synapse where it could broken down into 3,4-dihydroxyphenylacetic acid (DOPAC), the main metabolic of dopamine; the authors suggest this deficit in synaptic dopamine is driving the hyperactivity they observed in these animals (Zhu et al., 2012). Another study confirmed aberrations to the dopaminergic system in the frontal cortex and striatum, including altered release and reuptake by dopamine transporter (DAT), along with changes in the expression and function of nAChR subunits, which exemplifies the crucial role of nAChR activity in the proper functioning of the dopaminergic system (Buck et al., 2019).

These authors also report hyperactivity, risk-taking, and increased preference for nicotine in adolescence. Regarding serotonin, prenatal nicotine impacts receptor binding differently depending on brain region, receptor subtype, and offspring sex. Overall trends show increased serotonin binding in the cortex and brainstem, and increased binding in the striatum of male offspring but decreased binding in the striatum of female offspring (Slotkin et al., 2006). Prenatal nicotine also increases the density of serotonin transporter in the forebrain of juvenile offspring, which would effectively reduce the availability of serotonin in the synaptic cleft (Muneoka et al., 2001). Serotonin is essential in the regulation of mood, and therefore dysfunction of the serotonergic system is implicated in mental health issues associated with prenatal nicotine exposure. Serotonergic system dysfunction is also implicated in SIDS, which is a significant concern following maternal smoking (Blood-Siegfried & Rende, 2010; Holbrook, 2016).

Several studies have shown that prenatal nicotine in rodents results in memory deficits and ADHD symptomology by impairing glutaminergic signaling in the hippocampus by altering the expression and functionality of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor subunits (Vaglenova et al., 2008; Wang et al., 2011; Parameshwaran et al., 2012; Kalejaiye & Gondré-Lewis, 2017; Polli et al., 2020). Prenatal nicotine also alters the activity of AMPA receptors in the laterodorsal tegmentum of the midbrain, an area implicated in addiction and drug dependency (Polli & Kohlmeier, 2018). Perinatal nicotine exposure using the paradigm described above (preconception + prenatal + lactation) reduces the ratio of inhibitory GABAnergic neurons to excitatory non-GABAnergic neurons in the frontal cortex resulting in an overall increase in excitability,

which may partially explain behavioral effects such as hyperactivity and decreased anxiety/increased risk-taking (Martin et al., 2020).

Epigenetic effects are also observed following prenatal nicotine. In humans, DNA methylation patterns can predict the presence of prenatal exposure to nicotine with 81% accuracy (Ladd-Acosta et al., 2016), suggesting that maternal smoking results in a highly predictable set of epigenetic modifications that are specific to tobacco exposure. Detailed reviews of the epigenetics of maternal smoking in humans can be found elsewhere (Knopik, Maccani, Francazio, & McGeary, 2012; Nielsen, Larsen, & Nielsen, 2016). Briefly, maternal smoking induces abnormal DNA methylation and expression of microRNAs in the placenta, umbilical cord blood, and offspring brain, which may be responsible for the teratogenic effects of nicotine. Changes in DNA methylation may also be responsible for the increased prevalence low birthweight, obesity, insulin resistance, Type II diabetes, and hypertension seen in individuals prenatally exposed to nicotine (Rogers, 2019). Prenatal nicotine also induces epigenetic alterations in rodents, including decreased DNA methylation throughout the frontal cortex and striatum (Buck et al., 2019), and reduced expression of glutamate receptor associated genes in the prefrontal cortex (Polli et al., 2020).

1.1.1.4 Physiological Consequences

Prenatal nicotine exposure is robustly associated with dose-dependent low birthweight caused by intrauterine growth restriction, which likely results from nicotine-induced placental damage and resistance to blood flow (Winzer-Serhan, 2008; Bruin, Gerstein, & Holloway, 2010; Holbrook, 2016). Low birthweight is also observed in rodent models of prenatal nicotine exposure (Vaglenova et al., 2004, 2008; Schneider et

al., 2011, 2012; Zhu et al., 2012). Low birthweight subsequently increases the susceptibility to metabolic syndrome later in life, including obesity, Type II diabetes, insulin resistance, and hypertension (Rogers, 2019). Furthermore, evidence suggests that the relationship between prenatal nicotine and metabolic syndrome persists even after adjusting for birthweight, suggesting that prenatal nicotine also has direct effects on the development of metabolic syndrome (Bruin, Gerstein, & Holloway, 2010). The use of electronic cigarettes has also been associated with some components of the metabolic syndrome, specifically glucose levels, the development of a prediabetic state, and the risk of obesity and hypertension (Górna, Napierala, & Florek, 2020). In rodents, prenatal nicotine induces hypercholesterolemia in adult offspring, an effect that is exacerbated by the offspring consuming a high fat diet, and alters the expression of genes related to cholesterol metabolism (Zhu et al., 2018). Prenatal nicotine also increases serum corticosterone and glucose but decreases insulin-like growth factor (IGF1) in adult offspring and upregulates genes for glucose and lipid metabolism (Hu et al., 2020). Conversely, in fetal offspring, triglyceride and cholesterol levels are reduced, and genes related to glucose and lipid metabolism are downregulated (Hu et al., 2020). Pre- and perinatal nicotine in rodent models is associated with increased adiposity, glucose intolerance, insulin resistance, and multiple alterations in the structure and function of the pancreas (Somm et al., 2009).

Perhaps the most serious consequence of maternal smoking is an increased risk of SIDS. The risk of SIDS is increased 2.7X for infants born to smoking mothers, and 23 – 34% of SIDS cases are attributable to prenatal nicotine (Dietz et al., 2010). The exact cause of SIDS remains elusive, but nicotine impacts several neural systems that may

provide a link between prenatal nicotine use and increased risk of SIDS. Potential underlying mechanisms include an impaired response to hypoxic conditions caused by a nicotine-induced decrease in catecholamine release or impaired functioning of raphe neurons in the brainstem (Cerpa et al., 2015; Holbrook, 2016). As previously mentioned, cerebellar abnormalities have also been implicated (Lavezzi et al., 2013). Additionally, maternal smoking and maternal exposure to second-hand smoke during the first trimester both significantly increase the risk of neural tube defects (OR 2.2 – 3.4 depending on dose, and 2.6, respectively), even after controlling for confounding variables including folate intake (Suarez et al., 2008).

1.1.2 Preconception Nicotine

The consequences of maternal nicotine exposure prior to the onset of pregnancy are severely understudied. Only a select few studies examining nicotine exposure exclusively prior to conception have been conducted, with several of these works using a transgenerational exposure model, in which nicotine is administered to the filial (F)0 generation and the effects are examined in the F2 or F3 generations. The following sections will provide an overview of what we currently know about preconception nicotine exposure based on this limited collection of studies.

1.1.2.1 Behavioral Consequences

The predominant behavioral consequences of maternal preconception nicotine that have been explored relate to cognitive behavior and symptoms related to ADHD, two behavioral deficits also commonly observed following prenatal nicotine. Chronic injections of nicotine (1.0mg/kg) to adolescent male and female rats results in cognitive impairments in adult offspring, manifested as difficulty in learning complex patterns

(Renaud & Fountain, 2016). Of note, however, is that this study exposed both prospective parents to nicotine, so it is impossible to separate maternal and paternal effects. Chronic exposure of adult female rats to moderate levels of nicotine in drinking water (15mg/L) for seven weeks prior to conception impairs the emergence of locomotor behavior in developing rat offspring; 10-day-old pups have impaired postural support, delayed movement initiation, and increased stereotypy, and therefore decreased exploration of an open field (Torabi et al., 2021). Transgenerational maternal nicotine also impacts offspring behavior for multiple generations. Transgenerational transmission refers to exposure that occurs in one generation but impacts the development of future generations in the absence of repeated exposures (Yohn, Bartolomei, & Blendy, 2015). Perinatal nicotine administration in the F0 generation induces hyperactivity in the F2 and F3 matrilineal generations, but not the F2 patrilineal generation; therefore, the authors speculate that mitochondrial DNA, which is inherited exclusively from the mother, may be the target of epigenetic modification (Zhu et al., 2014). In another transgenerational study, authors report that prenatal exposure in the F0 generation increases activity, risk-taking, and nicotine-self-administration in the F2 generation, and that the effects were reversed by methylphenidate (Buck et al., 2019).

1.1.2.2 Neuroanatomical Consequences

No studies have investigated how exclusively preconception nicotine impacts neuroanatomical development of offspring.

1.1.2.3 Molecular and Epigenetic Consequences

In rodents, prenatal nicotine exposure in the F0 generation alters the expression and activity of nAChRs in the frontal cortex and striatum of the F2 generation (Buck et

al., 2019). Furthermore, due to the central role of nAChRs in the dopaminergic system (Faure, Tolu, Valverde, & Naudé, 2014), such transgenerational nicotine also induces dysfunction in the release and transport of dopamine in the frontal cortex and striatum (Buck et al., 2019), which likely drives many of the behavioral aberrations associated with developmental nicotine.

Transgenerational nicotine also induces epigenetic modifications in the subsequent generations. Prenatal nicotine exposure in the F0 generation alters DNA methylation in F2 offspring frontal cortex, striatum (decreased; Buck et al., 2019), and gonads (increased in testes, decreased in ovaries; Liu et al., 2020). This experience also increases protein levels in the lung (both sexes) and trachea (males only), conferring an asthmatic phenotype (see next section; Liu et al., 2020).

1.1.2.4 Physiological Consequences

Pre- and perinatal nicotine exposure result in the transgenerational transmission of altered metabolism and glucose homeostasis. Fetal rat offspring of sires exposed to prenatal nicotine exhibit decreased serum glucose, triglycerides, and cholesterol, along with downregulated genes related to lipid and glucose metabolism, and these effects are generally stable into adulthood (Hu et al., 2020). Adult male offspring whose mothers were exposed to perinatal nicotine exhibit elevated leptin and cholesterol, hypertension, and increased fasting insulin and insulin response to a glucose challenge, suggesting insulin resistance (Holloway et al., 2007). However, these males were not overweight compared to control males, which goes against the typical association of metabolic syndrome and obesity. Prenatal nicotine in an F0 generation of rats alters lung development and functioning in F1 and F2 offspring, suggesting transgenerational

transmission of asthma risk (Rehan et al., 2012; Liu et al., 2020). Furthermore, the asthmatic phenotype is sexually dimorphic, as only male F2 offspring show increased tracheal constriction in response to acetylcholine administration, but both males and females have increased respiratory system resistance (Rehan et al., 2012; Liu et al., 2020). Additionally, the effect of prenatal nicotine on asthma risk is additive, in that the consequences are further exacerbated if there is repeat exposure to nicotine in the F1 generation (Liu et al., 2020). Lastly, retrospective human studies reveal that children with grandmothers who smoked during pregnancy have greater than twice the risk (2.2X) of developing childhood cancers than children with non-smoking grandmothers; amazingly, this risk was greater than for children whose mothers smoked during pregnancy (1.8X; Ortega-Garcia et al., 2010).

1.2 Alcohol

Alcohol is the most commonly used recreational drug in Canada by a substantial margin; in 2017, 78.2% of the general population aged 15 years and older reported having consumed alcohol in the past year (Canadian Centre on Substance Use and Addiction, 2019). The prevalence of "heavy drinking", defined as consuming at least four alcoholic drinks on one occasion at least once a month during the last year, is also high, with the highest prevalence observed among individuals of reproductive age (18 – 34 years; Statistics Canada, 2021). Between 2015 and 2019, between 22% and 25% of females and 32% and 36% of males in this age bracket qualified as heavy drinkers (Statistics Canada, 2021), representing a significant proportion of the population of prospective parents. Globally, approximately 10% of women consume alcohol during their pregnancy (Popova et al., 2017). Therefore, it is vital to understand the

consequences of maternal alcohol consumption on the development of the next generation.

1.2.1 Prenatal Alcohol

The consequences of prenatal alcohol exposure were first published in the late 1960's by a French pediatrician who noticed physical abnormalities and behavioral and cognitive deficits in the children of alcoholic parents (Lemoine et al., 1968; Petrelli, Weinberg, & Hicks, 2018). More often credited with the identification of these effects, however, are Drs. Kenneth Jones and David Smith with their 1973 paper *Recognition of the fetal alcohol syndrome in early infancy*. It was with this seminal work that Jones and Smith coined the term fetal alcohol syndrome (FAS) to describe the collection of symptoms that follow prenatal alcohol use. In the years since, others have observed that FAS is not a single condition, but that a significant amount of variation exists for the severity and specifics of the symptoms of prenatal alcohol. Therefore, now it is more accepted to use the umbrella term fetal alcohol spectrum disorder (FASD) to encompass the range of prenatal alcohol-related pathologies: full FAS, partial FAS, alcohol-related neurodevelopmental disorder (ARND), and alcohol-related birth defects (ARBD; Petrelli, Weinberg, & Hicks, 2018; Mattson, Bernes, & Doyle, 2019). FASDs are generally diagnosed using four characteristic features: craniofacial abnormalities, growth restriction, neurodevelopmental impairment, and confirmed maternal alcohol consumption during gestation (Haycock, 2009; Petrelli, Weinberg, & Hicks, 2018). Alcohol easily crosses the placenta and accumulates in the amniotic fluid as the fetal elimination rate of alcohol is only 3 – 4% of that of the mother (Gupta, Gupta, & Shirasaka, 2016). FASD is common; one estimate using elementary school children in

Canada indicates that 2 – 3% of this population meets the criteria for FASD (Popova et al., 2019).

1.2.1.1 Behavioral Consequences

FASD is associated with a range of neuropsychological impairments, the most common including impairments in executive function, learning and memory, hyperactivity and attention deficits, anxiety and depression, and issues with sensory processing. There are also a number of secondary disabilities that are observed in increased frequency following prenatal alcohol: mental health issues, trouble with the law, problems in school, and vulnerability to alcohol and drug abuse (Rasmussen, 2005; Gupta, Gupta, & Shiarsaka, 2016).

Executive function is a set of cognitive skills that are involved in cognitive flexibility, goal setting, attentional control, and information processing (Anderson, 2002). Children, adolescents, and adults with FASD show impairments in executive function (Rasmussen, 2005; Mattson, Bernes, & Doyle, 2019). Interestingly, most impairments in executive function are evident even without a diagnosis of full FAS including craniofacial abnormalities (Rasmussen, 2005), suggesting that neurobehavioral impairments should be central to the diagnosis of FASD (Rasmussen, 2005; Mattson, Bernes, & Doyle, 2019). In humans, FASD impairs performance on the Stroop task (response inhibition), Wisconsin Card Sorting Task (shifting), tower task (planning), tests of verbal and non-verbal fluency, trail making test (cognitive flexibility), and digit span recall (working memory; Rasmussen, 2005; Gautam et al., 2014; Mattson, Bernes, & Doyle, 2019; Rockhold et al., 2021). Some assessments of rodent behavior have been developed to measure components of executive function, including working memory,

cognitive flexibility, and inhibition (Bizon, Foster, Alexander & Glisky, 2012). Using such tests, authors have reported that prenatal alcohol exposure in rodents impairs executive function similarly as in humans. Rodents exposed to prenatal alcohol show impairments in reversal learning (Mihalick et al., 2001; Marquardt, Sigdel, Caldwell, & Brigman, 2014; Marquardt & Brigman, 2016), response inhibition (Olguin, Thompson, Young, & Brigman, 2021), and passive avoidance (Schneider, Moore, & Adkins, 2011; Marquardt & Brigman, 2016).

Hippocampus-dependent learning and memory is also impaired following prenatal alcohol. In humans, children show impairments in the ability to encode, recall, and discriminate information (Mattson, Bernes, & Doyle, 2019). Children with FASD also typically have lowered IQ and impaired school performance (Mattson, Bernes, & Doyle, 2019), although these deficits are more likely related to impaired executive function (Schneider, Moore, & Adkins, 2011). Hippocampus-dependent learning and memory has been extensively studied using rodent models. Spatial learning and memory are particularly well studied, using a variety of tasks including the Morris water task (MWT), radial arm maze, Barnes maze, T-maze, and Y-maze (Berman & Hannigan, 2000). Assessments of object recognition are also common, as well as tests of classical conditioning, such as fear conditioning. Generally, these tests show prenatal alcohol-induced deficits, although the specifics vary depending on the timing of exposure and the age of the offspring at testing (Marquardt & Brigman, 2016).

FASD is highly comorbid with ADHD; estimates place the co-occurrence of the two conditions between 50 and 95% (Clark, Lutke, Minnes, & Ouellette-Kuntz, 2004; Schneider, Moore, & Adkins, 2011; Mattson, Bernes, & Doyle, 2019). However, a recent

meta-analysis found no systematic link between low-to-moderate prenatal alcohol and increased prevalence of ADHD symptoms (San Martin Porter, Maravilla, Betts, & Alati, 2019), suggesting that the dose of prenatal alcohol is critical. Furthermore, the rate of co-occurrence may be partially a consequence of the similarity between the behavioral phenotypes of FASD and ADHD (Mattson, Bernes, & Doyle, 2019). However, FASD does appear to be the leading cause of ADHD; individuals with FASD have a 7.6X increased risk of developing ADHD compared to the general population (Burd, 2016). Numerous studies into prenatal alcohol exposure with humans and rodents report hyperactivity and problems with attention (O'Malley & Nanson, 2002). In humans, children with FASD have problems with several components of attention, including establishing, organizing, sustaining, and shifting attention (Mattson, Bernes, & Doyle, 2019). Data also suggest that they particularly struggle when attention-demanding tasks are fast-paced and overstimulating (Schneider, Moore, & Adkins, 2011). Hyperactivity and attention have also been observed in rodents exposed to prenatal alcohol, albeit inconsistently. Activity level is frequently assessed with a measure of open field exploration, and attention using a reaction time task. Some authors report increased activity level and/or impaired attention (Brys, Pupe, & Bizarro, 2014; Marquardt & Brigman, 2016; Muñoz-Villegas, 2017), whereas others find no significant difference for either trait (Brolese et al., 2014; Marquardt & Brigman, 2017; Olguin et al., 2021).

Prenatal alcohol is associated with mental health concerns (Rasmussen, Andrew, Zwaigenbaum, & Tough, 2008), most notably anxiety and depression. A recent systematic review found that nearly 70% of studies investigating the link between prenatal alcohol and anxiety/depression report a positive association, a higher rate of

association that any other mental health issue (Easey, Dyer, Timpson, & Munafò, 2019). Nearly all (92%) of adults with FASD have a comorbid mental health diagnosis, with almost half (47%) reporting a diagnosis of depression (Clark et al., 2004). The two most common assessments of anxiety and depression in rodents are the elevated plus maze (EPM) and the forced swim task (FST), respectively. Typically, prenatal alcohol is associated with increased anxiety and depression in animal models (Marquardt & Brigman, 2016). However, others have found sex-dependent changes in anxiety, with male offspring exhibiting increased anxiety but females having decreased anxiety (Wieczorek et al., 2015; Lam et al., 2019). There has also been inconsistency in the expression of depressive behavior; Wieczorek et al. (2015) found that prenatal alcohol decreased immobility in the FST among male offspring, which would usually be interpreted as a decrease in depressive-like tendencies. However, they speculated that perhaps the male animals had a cognitive impairment that prevented them from recognizing the situation as perilous.

Individuals with FASD present with a variety of social deficits and problems with adaptive functioning, which includes the set of skills necessary for independent living. Children with FASD have impaired socialization, experience problems getting along with others, get teased by their peers, and have reduced play with others (Rasmussen et al., 2008; Kully-Martens et al., 2012). They are also prone to aggressive and delinquent behavior and conduct disorders (Easey et al., 2019). Moreover, issues with socialization and adaptive functioning appear to worsen with age, as the demand of adult responsibilities (e.g. employment, living independently) puts additional strain on compromised social competency (Rasmussen et al., 2008; Kully-Martens et al., 2012;

Mattson, Bernes, & Doyle, 2019). In adolescents and adults, prenatal alcohol is associated with inappropriate sexual behavior and alcohol and drug use (Rasmussen et al., 2008; Mattson, Bernes, & Doyle, 2019). In rodents, prenatal alcohol has varied effects on social interaction, vocalization, and aggression depending on the timing of alcohol exposure and the sex of the offspring (Marquardt & Brigman, 2016). The most common manner of assessing social behavior in rodents is through the analysis of social play; rodents are highly social and engage in very well-characterized rough and tumble play behavior (Pellis, Pellis, & Bell, 2010). Prenatal alcohol in rodents changes the manner in which adolescents (de Ávila et al., 2020) and adults (Hamilton et al., 2014) engage in social play. Furthermore, non-exposed adult rats reared with an alcohol-exposed cage-mate exhibit similar deficits in social behavior, suggesting that the impairments in social interaction induced by alcohol are so significant as to lead to long-term alterations in the development of the healthy control animal (Rodriguez et al., 2016).

The final category of behavior we will discuss in this review relates to motor skills. Gross and fine motor competency in developing children is impaired by prenatal alcohol in a dose-dependent manner. One systematic review reported that when the mother consumed more than four drinks/day, most studies found a positive association between alcohol and motor impairment, whereas if the mother had less than ten drinks/week, most studies found no association (Bay & Kesmodel, 2010). In adults, motor deficits include impairments in balance, fine motor control, and hand-eye coordination (Mattson, Bernes, & Doyle, 2016). In rodents, developing pups exposed to prenatal alcohol have weakened grip strength (Brys, Pupe, & Bizarro, 2014), delayed appears of rotating and righting reflexes (Kleiber, Wright, & Singh, 2011), and impaired

coordination (El Shawa, Abbott, & Huffman, 2013), and adolescent animals have impairments in balance and sensorimotor integration (Abbott et al., 2016).

1.2.1.2 Neuroanatomical Consequences

Prenatal alcohol induces widespread disruptions to brain development. Structural imaging studies have elucidated much about the gross morphological difference between alcohol-exposed and typically developing brains. Prenatal alcohol decreases the volume of white matter and subcortical grey matter throughout most of the brain (Gautam et al., 2015), with the notable exception of the amygdala (Treit et al., 2017). Global cortical thickness is also affected, with some studies reporting decreased thickness (Treit et al., 2017; Zhou et al., 2018) and others increased thickness (Sowell et al., 2008). One possible explanation for these conflicting findings relates to the composition of the FASD groups in these studies. Among the individuals included in the FASD group, 67% met the criteria for a full FAS diagnosis in the Sowell et al. (2008) study, whereas only 14% and 9% met the full criteria in the Treit et al. (2017) and Zhou et al. (2018) studies, respectively. Therefore, changes in cortical thickness may be linked with the severity of the condition. The cortex of children and adolescents with FASD also exhibits decreased gyrification (Hendrickson et al., 2017). Both volume estimates and gyrification are correlated with the intensity of behavioral deficits (Gautam et al., 2015; Hendrickson et al., 2017). Next, we focus on a few specific brain regions that are particularly affected by prenatal alcohol exposure: frontal/prefrontal cortex, hippocampus, and cerebellum.

The frontal cortex of the fetal human exposed to alcohol is reduced in volume, with 23% of alcohol-exposed fetuses falling beneath the 10th percentile (Wass, Persutte, & Hobbins, 2001). Using a computerized game and a wearable neuroimaging device to

measure blood oxygenation and volume changes, Kable and Coles (2017) identified that children with FASD experience less activation (decreased oxyhemoglobin and increased deoxyhemoglobin) in the PFC in response to positive emotions. Deoxygenation of the PFC is associated with executive function impairments in children with FASD, but not in healthy or clinical controls, suggesting that impairments in executive function seen in FASD may be uniquely associated with altered hemodynamics in the PFC (Kable, Coles, & Mattson, 2020). Impaired behavioral inhibition, specifically the speed of inhibition, is predicted by the surface area of the anterior cingulate cortex, which is reduced in adolescents with FASD (Migliorini et al., 2015). In rodents, the effect of prenatal alcohol on cortical thickness in the frontal cortex is dependent on age; the cortex is thickened during the first five days post-natal but thinned from P10 into adulthood (Zimatkin & Bon, 2017). Prenatal ethanol reduces the number of neurons in the medial PFC, and the neuron loss significantly predicts impairment on an assessment of executive function (Mihalick et al., 2001). Cells in the frontal cortex are not only less abundant, but also have a reduced soma (Zimatkin & Bon, 2017). Pyramidal cells in the medial PFC have reduced spine density although there is no change in dendritic complexity (Whitcher & Klintsova, 2008).

Prenatal alcohol exposure reduces the volume of the hippocampus in human children (Dodge et al., 2020) and adults (Coles et al., 2011), and in both groups the reduction in volume, especially of the right hippocampus, partially explains observable memory deficits. In rodents, several authors have demonstrated that prenatal alcohol results in a long-term reduction in hippocampal cell number in the CA fields and dentate gyrus (Berman & Hannigan, 2000), although others have reported no change or even an

increase in cell density in the dentata gyrus (Gil-Mohapel, Boehme, Kainer, & Christie, 2010). Prenatal alcohol, especially during the third-trimester equivalent, also impacts neurogenesis in the subgranular zone of the dentate gyrus; neurogenesis is thought to be a critical component of hippocampus-dependent learning and memory (Gil-Mohapel et al., 2010). Furthermore, the effects on neurogenesis are dependent on age. In adolescent/young adult offspring, there are no differences in the proliferation of new cells, but the transition of proliferative cells to immature neurons is impaired (Olateju et al., 2018). However, in aged animals, there is a significant deficit in hippocampal neurogenesis (Gil-Mohapel et al., 2014). Prenatal alcohol induces transient changes in neuronal morphology in the different hippocampal subregions, including decreased soma size in granule cells and reduced dendritic branching and spine density in pyramidal cells (Jakubowska-Dogru, Elibol, Dursun, & Yürüker, 2017). Others have also reported drastic decreases in spine density throughout development, which may reflect impaired synaptic plasticity (Berman & Hannigan, 2000; Gil-Mohapel et al., 2010).

The cerebellum shows obvious morphological damage in 75% of adolescents and adults with FAS (Bookstein, Streissguth, Connor, & Sampson, 2006). Cerebellar volume is reduced in adolescents and young adults with FASD and shrinks in volume as a function of age, whereas it becomes larger with age in healthy controls (Inkelis, Moore, Bischoff-Grethe, & Riley, 2020). Volume deficits in the cerebellum are attributable to reductions in specific lobules, and are graded by the severity of FASD, with full FAS showing greater reductions than milder diagnoses. These authors speculate that this gradation corresponds to the range of behavioral deficits relating to emotional regulation, coordination, and postural support (Sullivan et al., 2020). In rodents, exposure to alcohol

during gestation or the first postnatal week (i.e. third trimester equivalent in humans) reduces the number of cerebellar Purkinje cells proportional to maternal blood alcohol content (BAC), with a particularly sensitive period between 4 and 6-days-old; if maternal BAC is high and exposure occurs during this sensitive window, reduction in Purkinje cell number can be as high at 72% compared to controls (Pierce, Williams, & Light, 1999). The pattern of cell loss in the different lobules of the cerebellum follows a similar time course; the data form an inverted-U with all lobules displaying apoptosis following a single ethanol dose on P4, and no lobules displaying significant levels of apoptosis on P0 or 10 (Idrus & Napper, 2012). Therefore, it is evident from the animal studies that the early- to mid-third trimester equivalent is the most vulnerable time for cerebellar development.

Functional connectivity within the brain is also impacted by fetal alcohol, indicating that both the neural structures and the networks associating such structures are affected. Resting state functional magnetic resonance imaging (fMRI) shows that individuals with FASD have altered functional connectivity within multiple networks, including the default mode network, executive control network, and attentional networks (Fan et al., 2017; Ware, Long, & Lebel, 2021). Furthermore, functional connectivity in these networks predicts cognitive performance; there is a positive association between connectivity and cognition in healthy controls, but a negative association for children with FASD (Ware, Long, & Lebel, 2021). Changes in white matter tracts are already apparent in newborns exposed to prenatal alcohol, when the process of myelination is still underway. FASD is associated with lower axial diffusivity throughout the brain, which may be due to alcohol-induced axonal damage, and changes in callosal and projection

tracts relative to healthy controls (Taylor et al., 2015). Children with FASD exhibit a 12% decrease in inter-hemispheric functional connectivity in the region of the corpus callosum aligned with the paracentral gyrus between the frontal and parietal lobes, which correlates with reasoning ability (Wozniak et al., 2011). In the rat, prenatal alcohol leads to sex-dependent changes in the functional connectivity between the cortex, hippocampus, thalamus, striatum, midbrain, and cerebellum, with some networks displaying decreased connectivity and others increased (Rodriguez et al., 2016). In mice, coordinated activity between the OFC and dorsolateral striatum is associated with impairments in reversal learning (behavioral flexibility = executive function); animals exposed to prenatal alcohol have sustained activity in these regions and take longer to shift strategies, whereas in healthy controls, the two areas disengage and enable behavioral flexibility (Marquardt, Cavanagh, & Brigman, 2020).

1.2.1.3 Molecular and Epigenetic Consequences

Prenatal alcohol influences the activity of neurotransmitter systems throughout the offspring brain, especially dopamine and serotonin. Dopamine levels in the nucleus accumbens are doubled in adolescent rat offspring exposed to prenatal ethanol and associated with hyperactivity and risk-taking (Muñoz-Villegas, Rodríguez, Giordano, & Juárez, 2017). Prenatal alcohol also increases excitatory glutaminergic activity on dopaminergic neurons in the dorsomedial striatum and ventral tegmental area, leading to elevated alcohol and amphetamine preference and sustained hyperactivity in adult offspring (Hausknecht et al., 2015; Cheng et al., 2018). Therefore, alterations in the dopaminergic system appear to predict alcohol-linked changes in activity level and susceptibility to drug use. Conversely, serotonergic activity is consistently reduced

followed prenatal alcohol. There is a 20% reduction in the number of serotonergic neurons in the brainstem of fetal offspring exposed to alcohol, and this reduction persists when offspring reach adolescence (Sari & Zhou, 2004) and early adulthood (Sliwowska, Song, Bodnar, & Weinberg, 2014). Furthermore, the survival of serotonergic neurons following prenatal alcohol is mediated by female gonadal hormones, suggesting an explanatory link between prenatal alcohol, disorders of mental health, and sex differences (Sliwowska et al., 2014). Neurotrophins, molecules essential for the survival and proliferation of neurons as well as some behavioral processes, are also dysregulated by prenatal alcohol. Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are two neurotrophins that are consistently impacted by prenatal alcohol in various brain regions throughout the development of offspring (Carito et al., 2019). Interestingly, moderate doses of prenatal ethanol, but not low doses, reduce the level of BDNF in the hippocampus and cortex of 1-week-old offspring rats (Feng, Yan, & Yan, 2005). In the rat cerebellum, prenatal alcohol induces oxidative stress, indicated by increased levels of reactive oxygen species and lipid peroxide, decreases the levels of integrin-linked kinase (ILK), important for cell migration, differentiation, and survival, decreases the expression of AMPA receptor subunits, suggesting impaired synaptic plasticity, and increases caspase 3 expression, indicating elevated apoptosis (Bhattacharya et al., 2018).

Prenatal alcohol also induces epigenetic modifications throughout the brain. Detailed reviews of these changes can be found elsewhere (Haycock, 2009; Vaiserman, 2013; Kleiber et al., 2014; Lussier, Weinberg, & Kobor, 2017; Kaminen-Ahola, 2020). Epigenetic modifications are factors that regulate gene expression without changing the sequence of DNA base pairs and include DNA methylation, histone modification, and

regulatory noncoding RNA. All these means of epigenetic modification of implicated in FASD (see reviews). A new study used DNA methylation as an epigenetic clock to determine biological age of individuals with FASD and found that FASD induces accelerated epigenetic aging (Okazaki et al., 2021).

1.2.1.4 Physiological Consequences

Two of the diagnostic features of FAS are craniofacial abnormalities resulting in the characteristic facial phenotype and growth restriction (Petrelli, Weinberg, & Hicks, 2018). Sentinel facial features include shortened palpebral fissure, indistinct philtrum, and a thin upper lip (Cook et al., 2016). Interestingly, acute alcohol treatment during the first trimester in mice also results in craniofacial abnormalities, including changes to the morphology of the eyes and snout and reduced skull size (Petrelli, Weinberg, & Hicks, 2018; Draghici et al., 2021). Prenatal alcohol results in pre- and postnatal growth restriction. Low birthweight is commonly observed following gestational alcohol in humans (Carter et al., 2016) and rodents (Draghici et al., 2021), and may explain the increased risk of metabolic syndrome and impaired glucose metabolism (Hales & Barker, 2001; Chen & Nyomba, 2003; Xia et al., 2014; Nguyen, Steane, Moritz, & Akison, 2019; Kable, Mehta, & Coles, 2021). Long-term growth restriction is also common, although catch-up growth can occur in both humans (Carter et al., 2016) and rodents (Kaminen-Ahola et al., 2010). Furthermore, the severity of growth restriction predicts the severity of cognitive impairment; children with pre- and postnatal growth restriction show the most drastic cognitive impairments, followed by children with prenatal growth restriction followed by postnatal catch-up growth, then children with no growth restriction (Carter et al., 2016). Lastly, prenatal alcohol alters activity of the hypothalamic-pituitary-adrenal

(HPA) axis, contributing to increased stress reactivity, anxiety, and depression. Furthermore, the effects of prenatal alcohol on the HPA axis are sexually dimorphic, further explaining sex differences in disorders related to emotional regulation (Weinberg, Sliwowska, Lan, & Hellemans, 2008). In humans, children with FASD show elevated salivary cortisol levels compared to healthy controls (Keiver, Bertram, Pritchard, & Clarren, 2015) and a flattened diurnal rhythm, showing less change between morning and evening cortisol levels (McLachlan et al., 2016). In rodents, prenatal alcohol decreases basal HPA activity but increases HPA activation in response to stress (Xia et al., 2014; Wieczorek et al., 2015). Furthermore, prenatal alcohol increased the expression of glucocorticoid receptors in the hippocampus of female rat offspring but has no impact on receptor expression in males (Lam et al., 2019).

1.2.2 Preconception Alcohol

Preconception alcohol refers to the consumption of alcohol any time prior to fertilization of the oocyte, including intermittent, frequent, or binge consumption. The most frequently proposed mechanism for the transmission of preconception alcohol exposure in the absence of any direct exposure to the offspring is epigenetic inheritance, the transmission of reversible changes in gene expression from one generation to the next (Yohn, Bartolomei, & Blendy, 2015; Chastain & Sarker, 2017). Epigenetic inheritance can be classified as germline-dependent, inherited from epigenetic marks on the germline, or germline-independent, inherited from behavioral transmission or altered maternal physiology that induced epigenetic modifications (Chastain & Sarker, 2017).

1.2.2.1 Behavioral Consequences

Limited research to date has indicated that maternal preconception alcohol does not impair the quality of maternal care in rodents. Jabbar and colleagues (2016) exposed adult females to free-access of 6.7% ethanol in drinking water for 30 days, followed by a three-week period of sobriety prior to mating with non-alcohol-exposed males. The resulting litters were not cross-fostered, and analysis of maternal care between P1 and P6 demonstrated no alcohol-induced changes in time spent on pups, time spent licking and grooming, or time spent engaged in arch-back nursing. To further demonstrate the equality of maternal care, these authors further showed that the stress reactivity of these offspring was the same regardless of if they were reared by their dam or cross-fostered to a non-alcohol-exposed dam, demonstrating that the alcohol treatment and not maternal care was responsible (Jabbar et al. 2016).

Behavioral consequences of maternal preconception alcohol exposure for the next generation offspring currently relate to social behavior, anxiety-like behavior, and behaviors associated with ADHD. The juvenile offspring (P25-30) of male and female rats exposed to 20% alcohol during early and late adolescence exhibit a drastic decrease in social play behavior (Asimes et al., 2018). Social play in rodents is essential for proper brain development and social competency in adulthood (Pellis, Pellis, & Bell, 2010); therefore, an alcohol-induced reduction in play would have substantial and long-term impacts on functioning.

The above study exposed both parents to ethanol during the preconception period. Others have examined offspring outcomes following maternal-only exposure. Females exposed to 6.7% ethanol for 30 days as described above rear male offspring that exhibit

increased anxiety-like behavior as assessed by an open field task and EPM, whereas the female offspring show no effect (Jabbar et al., 2016). Females exposed to 25% ethanol via intragastric intubation for 10 days six weeks prior to conception have offspring that display hyperactivity (open field), impaired attention (reduced spontaneous alternation in Y-maze), and impulsivity (indifference to aversive foot-shock; Choi et al. 2012).

Intergenerational effects of maternal ethanol have also been observed. Tunc-Ozcan and colleagues (2016) exposed female rats (F0) to ethanol from gestational day (G)8 – G20, then bred the resulting female offspring (F1) with non-alcohol-exposed males to produce a second generation (F2). F2 offspring from this maternal lineage displayed impaired hippocampus-dependent contextual fear memory as adults, and the male F2 offspring were hypoactive. Interestingly, F2 offspring from the paternal lineage (i.e. F1 males exposed to prenatal alcohol) showed no behavioral impairment (Tunc-Ozcan et al., 2016).

Others have examined how alcohol exposure during the entire reproductive cycle impacts offspring. Brancato and colleagues (2018, 2020) used to two-bottle choice paradigm to expose female rats to 20% ethanol for 12wks throughout pre-gestation, gestation, and lactation. Females either had continuous ("habitual") or intermittent ("binge") access to ethanol during this 12wk period. When the dam was habitually exposed to 20% ethanol, juvenile offspring (3-4wks) exhibited hypoactivity (open field), increased anxiety (EPM), increased depressive symptomology (FST), and decreased social interaction compared to non-ethanol exposed offspring (Brancato et al., 2018). However, these offspring did not show an elevated vulnerability to alcohol abuse, unlike the offspring of dams exposed to ethanol following an intermittent ("binge") schedule.

Juvenile offspring of binge-drinking dams were not hypoactive or overly anxious compared to controls, but they showed depressive tendencies and an even greater impairment in social interaction (Brancato et al., 2018). These results are made even more interesting by the finding that the binge-drinking dams consumed significantly more ethanol in a 24hr period than the habitual drinkers.

Using this same two-bottle choice paradigm, Brancato and colleagues (2020) continued to show that the early adult offspring (P55 and up) of the dams exposed to habitual ethanol continue to display hypoactivity and have impaired novel object recognition, whereas early adult offspring of intermittent drinkers show impaired recognition of novelty and spatial learning and memory deficits (MWT). Importantly, the authors also demonstrate that a positive experience, postnatal rearing in a complex environment, is capable of mitigating many of these effects. It is critical to note that these studies exposed the dam to alcohol throughout pregnancy and lactation, so it is impossible to disentangle how exposure during the various developmental time periods contributed to the offspring's behavioral phenotype.

1.2.2.2 Neuroanatomical Consequences

To date, no studies have examined how maternal preconception alcohol impacts offspring neuroanatomy.

1.2.2.3 Molecular and Epigenetic Consequences

Preconception alcohol impairs the functioning of the HPA axis, which is central to the body's physiological response to stress. Male rat offspring of parents exposed to a binge-drinking paradigm in adolescence have lower baseline corticosterone levels, although there are no differences in the expression of genes involved in the negative

feedback of the HPA axis (glucocorticoid receptor, corticotropin releasing factor, CRF, and arginine vasopressin, AVP; Asimes et al., 2018). In response to an immune challenge, offspring of dams chronically exposed to 6.7% ethanol three weeks prior to conception display a drastic increase in plasma corticosterone and adrenocorticotropin hormone (ACTH) and in hypothalamic CRF, but lowered levels of hypothalamic β-endorphin (Jabbar et al., 2016).

Maternal alcohol exposure for 10 days six weeks prior to conception induces a 50% increase in norepinephrine transporter (NET) expression in the frontal cortex and a 30% decrease in DAT expression in the striatum, although this change did not reach significance (Choi et al., 2012).

Adolescent binge-drinking by male and female rats induces widespread changes in hypothalamic gene expression in subsequent offspring, notably in genes related to neurogenesis and synaptic plasticity, chromatin remodeling, regulation of transcription and translation, and obesity and reproductive functioning (Przybycien-Szymanska et al., 2014), and uniquely modifies DNA methylation patterns in the hypothalamus depending on which parent was exposed to alcohol (Asimes, et al., 2017). Four weeks of maternal exposure to 6.7% ethanol three weeks prior to conception induces multiple changes in the expression of HPA axis regulatory genes, including: 1) elevated *Crf* mRNA in hypothalamus; 2) elevated *Crfr1* mRNA in the hippocampus and amygdala; and 3) reduced *Pomc* mRNA in the hypothalamus. These authors found no difference in the expression of glucocorticoid or mineralocorticoid receptor mRNA (*gr* and *mr*) throughout the brain (Jabber et al., 2016).

1.2.2.4 Physiological Consequences

Mild exposure to ethanol (5%) for two weeks prior to conception increases the incidence of fetal abnormalities, such as toe deformities and delayed eye development (Lee et al., 2020). Studies have reported conflicting effects of maternal preconception alcohol on offspring body weight. Fetal offspring (G14) of dams exposed to 60 days of ethanol immediately prior to conception (3g/kg/day) are significantly smaller (Livy, Maier, & West, 2004). However, Lee and colleagues (2020) report that P0 offspring of ethanol-exposed mice are nearly twice as large as control offspring but display growth retardation in the second and third weeks of life. Conversely, Asimes and colleagues (2018) report no effect of adolescent binge-drinking on offspring weight at P0 or P7, but that ethanol treated offspring were smaller in adolescence (P36). The adult offspring of female rats exposed to prenatal ethanol (i.e. grandmaternal exposure) are significantly heavier than controls (Harper et al., 2014).

Chronic alcohol exposure prior to conception is associated with impaired glucose metabolism (Jung, et al., 2011). Maternal exposure to 6.7% ethanol for four weeks three weeks prior to breeding increases susceptibility to diabetes in adult offspring; offspring present with hyperglycemia, hyperinsulinemia, increased inflammatory cytokines and cell death in the pancreas, and impaired insulin activity in the liver (Al-Yasari et al., 2021). Furthermore, grandmaternal gestational ethanol exposure induces hypoglycemia and hyperinsulinemia in second generation male and female offspring, suggesting that ethanol may impact the germline of the developing first generation while in utero (Harper et al., 2014).

1.3 General Conclusions

Prenatal use of common recreational drugs of abuse remains a concern despite decades of targeted public health education. Human and rodent models have elucidated a number of behavioral, neuroanatomical, molecular/epigenetic, and physiological consequence of gestational drug exposure, with prenatal alcohol and nicotine exposure sharing many common side effects. Interestingly, nAChRs appear to have a role in the pharmacological dependence of both nicotine and alcohol (Hendrickson, Guildford, & Tapper, 2013), indicating a shared molecular mechanism. The consequences of prenatal nicotine and alcohol are thoroughly researched and have been a topic of investigation for decades, yet we continue to discover further nuances regarding the mechanisms and long-term effects of prenatal drug use. Conversely, the effects of maternal preconception nicotine and alcohol use are very poorly understood. Research is only just beginning to demonstrate that maternal drug use that occurs exclusively prior to conception can induce side effects in the subsequent offspring that often mimic the consequences of prenatal exposure. This area of investigation is likely to be a prominent topic of research for years to come and will contribute to bettering public health recommendations regarding pregnancy planning and improve public knowledge regarding the teratogenicity of legal, socially acceptable drugs of abuse.

1.4 Remainder of Thesis

This thesis will begin to address this gap in the literature by discussing two experiments that investigated the impacts of maternal preconception nicotine (Chapters 2 and 3) and alcohol (Chapter 4). Both of these experiments used a rodent model to chronically expose adult females to the appropriate drug, thereby mimicking substance

abuse in humans. Importantly, substance use ceased prior to mating, such that there was no gestational exposure. Overall, we predicted that the abuse of nicotine or alcohol prior to conception would result in impairments in offspring development that mirror the consequences seen following prenatal exposure, although potentially less pronounced due to the absence of direct fetal exposure. We also predicted that the effects would be apparent throughout the lifespan of the offspring, as in also seen with prenatal exposure. We examined the impacts on offspring in terms of behavior and brain development. Our behavioral findings contribute to the currently limited collection of studies that explore this topic with respect to behavior, and our neuroanatomical findings provide the first evidence of how these maternal experiences impact brain development.

References

Abbott, C. W., Kozanian, O. O., Kanaan, J., Wendel, K. M., & Huffman, K. J. (2016). The impact of prenatal ethanol exposure on neuroanatomical and behavioral development in mice. *Alcoholism: Clinical and Experimental Research, 40*, 122 – 133. DOI:10.1111/acer.12936

Ajarem, J. S., & Ahmad, M. (1998). Prenatal nicotine exposure modifies behavior of mice through early development. *Pharmacology, Biochemistry, and Behavior, 59*, 313 – 318. DOI:10.1016/s0091-3057(97)00408-5

Al-Sahab, B., Saqib, M., Hauser, G., & Tamim, H. (2010). Prevalence of smoking during pregnancy and associated risk factors among Canadian women: A national survey. *BMC Pregnancy and Childbirth, 10*, 24. DOI:10.1186/1471-2393-10-24

Al-Yasari, A., Jabbar, S., Cabrera, M. A., Rousseau, B., & Sraker, D. K. (2021). Preconception alcohol exposure increases the susceptibility to diabetes in the offspring. *Endocrinology, 162*, 1 – 18. DOI:10.1210/endocr/bqaa188

Anderson, P. (2002). Assessment and development executive function (EF) during childhood. *Child Neuropsychology, 8*, 71 – 82. DOI:10.1076/chin.8.2.71.8724

Asimes, A., Kim, C. K., Cuarenta, A., Auger, A. P., & Pak, T. R. (2018). Binge drinking and intergenerational implication: Parental preconception alcohol impacts offspring development in rats. *Journal of the Endocrine Society, 2*, 672 – 686. DOI:10.1210/js.2018-00051

Asimes, A., Torcaso, A., Pinceti, E., Kim, C. K., Zeleznik-Le, N. J., & Pak, T. R. (2017). Adolescent binge-pattern alcohol exposure alters genome-wide DNA methylation patterns in the hypothalamus of alcohol-naïve male offspring. *Alcohol, 60*, 179 – 189. DOI;10.1016/j.alcohol.2016.10.010

Bay, B., & Kesmodel, U. S. (2010). Prenatal alcohol exposure – a systematic review of the effects on child motor function. *Acta Obstetricia et Gynecologica Scandinavica, 90*, 210 – 226. DOI:10.1111/j.1600-0412.2010.01039.x

Benowitz, N. L. (2010). Nicotine Addiction. *New England Journal of Medicine, 362*, 2295 – 2303. DOI:10.1056/nejmra0809890

Berman, R. F., & Hannigan, J. H. (2000). Effects of prenatal alcohol exposure on the hippocampus: Spatial behavior, electrophysiology, and neuroanatomy. *Hippocampus, 10*, 94 – 100. DOI:10.1002/(sici)1098-1063(2000)

Bhattacharya, D., Majrashi, M., Ramesh, S., Govindarajulu, M., Bloemer, J., Fujihashi, A., Crump, B.-R., Hightower, H., Bhattacharya, S., Moore, T., Suppiramaniam, V., & Dhanasekaran, M. (2018). Assessment of the cerebellar neurotoxic effects of

nicotine in prenatal alcohol exposure in rats. *Life Sciences, 194*, 177 – 184. DOI:10.1016/j.lfs.2017.12.010

Biederman, J., Petty, C. R., Bhide, P. G., Woodworth, Y., & Faraone, S., (2012). Does exposure to maternal smoking during pregnancy affect the clinical features of ADHD? Results from a controlled study. *The World Journal of Biological Psychiatry, 13*, 60 – 64. DOI:10.3109/15622975.2011.562243

Bizon, J. L., Foster, T. C., Alexander, G. E., & Glisky, E. L. (2012). Characterizing cognitive aging of working memory and executive function in animal models. *Frontiers in Aging Neuroscience, 4*, 19. DOI:10.3389/fnagi.2012.00019

Blood-Siegfried, J., & Rende, E. K. (2010). The long-term effects of prenatal nicotine exposure on neurologic development. *Journal of Midwifery and Women's Health, 55*, 143 – 152. DOI:10.1016/j.jmwh.2009.05.006

Bookstein, F. L., Streissguth, A. P., Connor, P. D., & Sampson, P. D. (2006). Damage to the human cerebellum from prenatal alcohol exposure: The anatomy of a simple biometrical explanation. *The Anatomical Record (Part B: New Anatomy), 289B*, 195 – 209. DOI:10.1002/ar.b.20114

Brancato, A., Castelli, V., Cavallaro, A., Lavanco, G., Plescia, F., & Cannizzaro, C. (2018). Pre-conceptional and peri-gestational maternal binge alcohol drinking produces inheritance of mood disturbances and alcohol vulnerability in adolescent offspring. *Frontiers in Psychiatry, 9*, 150. DOI:10.3389/fpsyt.2018.00150

Brancato, A., Castelli, V., Lavanco, G., & Cannizzaro, C. (2020). Environmental enrichment during adolescence mitigates cognitive deficits and alcohol vulnerability due to continues and intermittent perinatal alcohol exposure in adult rats. *Frontiers in Behavioral Neuroscience, 14*, 583122. DOI:10.3389/fnbeh.2020.583122

Brolese, G., Lunardi, P., Broetto, N., Engelke, D. S., Lírio, F., Batassini, C., Tramontina, A. C., & Gonçalves, C.-A. (2014). Moderate prenatal alcohol exposure alters behavior and neuroglial parameters in adolescent rats. *Behavioral Brain Research, 269*, 175 – 184. DOI:10.1016/j.bbr.2014.04.023

Bruin, J. E., Gerstein, H. C., & Holloway, A. C. (2010). Long-term consequences of fetal and neonatal nicotine exposure: A critical review. *Toxicological Sciences, 116*, 364 – 374. DOI:10.1093/toxsci/kfq103

Bryden, D. W., Burton, A. C., Barnett, B. R., Cohen, V. J., Hearn, T. N., Jones, E. A., Kariyil, R. J., Kunin, A., Kwak, S. I., Lee, J., Lubinski, B. L., Rao, G. K., Zhan, A., & Roesch, M. R. (2016). Prenatal nicotine exposure impairs executive control signals in medial prefrontal cortex. *Neuropsychopharmacology, 41*, 716 – 725. DOI:10.1038/npp.2015.197

Brys, I., Pupe, S., & Bizarro, L. (2014). Attention, locomotor activity, and developmental milestones in rats prenatally exposed to ethanol. *International Journal of Developmental Neuroscience, 38*, 161 – 168. DOI:10.1016/j.ijdevneu.2014.08.007

Buck, J. M., Sanders, K. N., Wagemen, C. R., Knopik, V. S., Stitzel, J. A., & O'Neill, H. C. (2019). Developmental nicotine exposure precipitates multigenerational maternal transmission of nicotine preference and ADHD-like behavioral, rhythmometric, neuropharmacological, and epigenetic anomalies in adolescent mice. *Neuropharmacology, 149*, 66 – 82. DOI:10.1016/j.neuropharm.2019.02.006

Burd, L. (2016). FASD and ADHD: Are they related and how? *BMC Psychiatry, 16*, 325. DOI:10.1186/s12888-016-1028-x

CAN-ADAPTT Canadian Smoking Cessation Guideline. (2011). *Specific Populations: Pregnant and Breastfeeding Women*. Center for Addiction and Mental Health: Toronto, ON.

Canadian Centre on Substance Use and Addiction. (2019). Canadian Drug Summary: Alcohol. Ottawa: Canadian Centre on Substance Use and Addiction.

Carito, V., Ceccanti, M., Ferraguti, G., Coccurello, R., Ciafre, S., Tirassa, P., & Fiore, M. (2019). NGF and BDNF alterations by prenatal alcohol exposure. *Current Neuropharmacology, 17*, 308 – 317. DOI:10.2174/1570159x15666170825101308

Carter, R. C., Jacobson, J. L., Molteno, C. D., Dodge, N. C., Meintjes, E. M., & Jacobson, S. W. (2016). Fetal alcohol growth restriction and cognitive impairment. *Pediatrics, 138*, e20160775

Cerpa, V. J., Aylwin, M. L. O., Beltrán-Castillo, S., Bravo, E. U., Llona, I. R., Richerson, G. B., & Eugenin, J. L. (2015). The alteration of neonatal raphe neurons by prenatal-perinatal nicotine: Meaning for sudden infant death syndrome. *American Journal of Respiratory Cell and Molecular Biology, 53*, 489 – 499. DOI:10.1165/rcmb.2014-0329OC

Cheeta, S., Halil, A., Kenny, M., Sheehan, E., Zamyadi, R., Williams, A. L., & Webb, L. (2018). Does perception of drug-related harm change with age? A cross-sectional online survey of young and older people. *BMJ Open, 8*, e021109. DOI:10.1136/bmjopen-2017-021109

Chen, L., & Nyomba, B. L. G. (2003). Effects of prenatal alcohol exposure on glucose tolerance in the rat offspring. *Metabolism, 52*, 454 – 462. DOI:10.1053/meta.2003.50073

Chen, W.-J., A., & Edwards, R. B. (2003). Prenatal nicotine does not cause Purkinje cell loss in the developing rat cerebellar vermis. *Neurotoxicology and Teratology, 25*, 633 – 637. DOI:10.1016/s0892-0362(03)00048-5

Chen, W.-J. A., King, K. A., Lee, R. E., Sedtal, C. S., & Smith, A. M. (2006). Effects of nicotine exposure during prenatal or perinatal period on cell numbers in adult rat hippocampus and cerebellum. *Life Science, 79*, 2221 – 2227. DOI:10.1016/j.lfs.2006.07.019

Chen, W.-J. A., Parnell, S. E., & West, J. R. (1998). Neonatall alcohol and nicotine exposure limits brain growth and depletes cerebellar Purkinje cells. *Alcohol, 15*, 33 – 41. DOI:10.1016/s0741-8329(97)00084-0

Cheng, Y., Wang, X., Wei, X., Xie, X., Melo, S., Miranda, R. C., & Wang, J. (2018). Prenatal exposure to alcohol induces functional and structural plasticity in dopamine D1 receptor-expressing neurons of dorsomedial striatum. *Alcoholism: Clinical and Experimental Research, 42*, 1493 – 1502. DOI:10.1111/acer.13806

Choi, I., Kim, P., Joo, S. H., Kim, M. K., Park, J. H., Kim, H. J., Ryu, J. H., Cheong, J. H., & Shin, C. Y. (2012). Effects of preconceptional ethanol consumption on ADHD-like symptoms in Spragu-Dawley rat offsprings. *Biomolecules and Therapeutics, 20*, 226 – 233. DOI:10.4062/biomolther.2012.20.2.226

Clark, E., Lutke, J., Minnes, P., & Ouellette-Kuntz, H. (2004). Secondary disabilities among adults with fetal alcohol spectrum disorder in British Columbia. *Journal of FAS International, 2*, e13.

Coles, C. D., Goldstein, F. C., Lynch, M. E., Chen, X., Kable, J. A., Johnson, K. C., & Hu, X. (2011). Memory and brain volume in adults prenatally exposed to alcohol. *Brain and Cognition, 75*, 67 – 77. DOI:10.1016/j.bandc.2010.08.013

Cook, J. L., Green, C. R., Lilley, C. M., Anderson, S. M., Baldwin, M. E., Chudley, A. E., Conry, J. L., LeBlanc, N., Loock, C. A., Lutke, J., Mallon, B. F., McFarlane, A. A., Temple, V. K., & Rosales, T. for the Canada Fetal Alcohol Spectrum Disorder Research Network. (2016). Fetal alcohol spectrum disorder: A guideline for diagnosis across the lifespan. *Canadian Medical Association Journal, 188*, 191 – 197. DOI:10.1503/cmaj.151425

De Ávila, M. A. P., Gonçalves, R. M., Nascimento, E. C. C., Cabral, L. D. M., Vilela, F. C., & Giusti-Paiva, A. (2020). Prenatal exposure to alcohol impairs social play behavior in adolescent male mice. *Neurotoxicology, 79*, 142 – 149. DOI:10.1016/j.neuro.2020.05.007

De Zeeuw, P., Zwart, F., Schrama, R., van Engeland, H., & Durston, S. (2012). Prenatal exposure to cigarette smoke or alcohol and cerebellum volume in attention-

deficit/hyperactivity disorder and typical development. *Translational Psychiatry, 2*, e84. DOI:10.1038/tp.2012.12

Dietz, P. M., England, L. J., Shapiro-Mendoza, C. K., Tong, V. T., Farr, S. L., Callaghan, W. M. (2010). Infant morbidity and mortality attributable to prenatal smoking in the U.S. *American Journal of Preventative Medicine, 39*, 45 – 52. DOI:10.1016/j.amepre.2010.03.009

Dodge, N. C., Thomas, K. G. F., Meintjes, E. M., Molteno, C. D., Jacobson, J. L., & Jacobson, S. W. (2020). Reduced hippocampal volumes partially mediate effects of prenatal alcohol exposure on spatial navigation on a virtual water maze task in children. *Alcoholism: Clinical and Experimental Research, 44*, 844 – 855. DOI:10.1111/acer.14310

Draghici, D., Barr, K., Hardy, D. B., Allman, B. L., & Willmore, K. E. (2021). Effects of advanced maternal age and acute prenatal alcohol exposure on mouse offspring growth and craniofacial phenotype. *Alcoholism: Clinical and Experimental Research, 00*, 1 – 15. DOI:10.1111/acer.14631

Dwyer, J. B., Broide, R. S., & Leslie, F. M. (2008). Nicotine and brain development. *Birth Defects Research (Part C), 84*, 30 – 44. DOI:10.1002/bdrc.20118

Dwyer, J. B., McQuown, S. C., & Leslie, F. M. (2009). The dynamic effects of nicotine on the developing brain. *Pharmacology & Therapeutics, 122*, 125 – 139. DOI:10.1016/j.pharmthera.2009.02.003

Easey, K. E., Dyer, M. L., Timpson, N. J., & Munafò, M. R. (2019). Prenatal alcohol exposure and offspring mental health: A systematic review. *Drug and Alcohol Dependence, 197*, 344 – 353. DOI:10.1016/j.drugalcdep.2019.01.007

El Marroun, H., Schmidt, M. N., Franken, I. H. A., Jaddoe, V. W. V., Hofman, A., van der Lugt, A., Verhulst, F. C., Tiemeier, H., & White, T. (2014). Prenatal tobacco exposure and brain morphology: A prospective study in young children. *Neuropsychopharmacology, 39*, 792 – 800. DOI:10.1039/npp.2013.273

El Shawa, H., Abbott, C. W., & Huffman, K. J. (2013). Prenatal ethanol exposure disrupts intraneocortical circuitry, cortical gene expression, and behavior in a mouse model of FASD. *The Journal of Neuroscience, 33*, 18893 – 18905. DOI:10.1523/jneurosci.3721-13.2013

Fan, J., Taylor, P. A., Jacobson, S. W., Molteno, C. D., Gohel, S., Biswal, B. B., Jacobson, J. L., & Meintjes, E. M. (2017). Localized reductions in resting-state connectivity in children with prenatal alcohol exposure. *Human Brain Mapping, 38*, 5217 – 5233. DOI:10.1002/hbm.23726

Faure, P., Tolu, S., Valverde, S., & Naudé, J. (2014). Role of nicotinic acetylcholine receptors in regulating dopamine neuron activity. *Neuroscience, 282,* 86 – 100. DOI:10.1016/j.neuroscience.2014.05.040

Feng, M.-J., Yan, S.-E., & Yan, Q.-S. (2005). Effects of prenatal alcohol exposure on brain-derived neurotrophic factor and its receptor tyrosine kinase B in offspring. *Brain Research, 1042*, 125 – 132. DOI:10.1016/j.brainres.2005.02.017

Gautam, P., Lebel, C., Narr, K. L., Mattson, S. N., May, P. A., Adnams, C. M., Riley, E. P., Jones, K. L., Kan, E. C., & Sowell, E. R. (2015). Volume changes and brain-behavior relationships in white matter and subcortical grey matter in children with prenatal alcohol exposure. *Human Brain Mapping, 36,* 2318 – 2329. DOI:10.1002/hbm.22772

Gautam, P., Nuñez, S. C., Narr, K. L., Kan, E. C., & Sowell, E. R. (2014). Effects of prenatal alcohol exposure on the development of white matter volume and change in executive function. *NeuroImage: Clinical, 5*, 19 – 27. DOI:10.1016/j.nicl.2014.05.010

Gil-Mohapel, J., Boehme, F., Kainer, L., & Christie, B. R. (2010). Hippocampal cell loss and neurogenesis after fetal alcohol exposure: Insights from different rodent models. *Brain Research Reviews, 64,* 283 – 303. DOI:10.1016/j.brainresrev.2010.04.011

Gil-Mohapel, J., Titterness, A. K., Patten, A. R., Taylor, S., Ratzlaff, A., Ratzlaff, T., Helfer, J., & Christie, B. R. (2014). Prenatal ethanol exposure differentially affects hippocampal neurogenesis in the adolescent and aged brain. *Neuroscience, 273*, 174 – 188. DOI:10.1016/j.neuroscience.2014.05.012

Górna, I., Napierala, M., & Florek, E. (2020). Electronic cigarette use and metabolic syndrome development: A critical review. *Toxics, 8,* 105. DOI:10.3390/toxics8040105

Gupta, K. K., Gupta, V. K., & Shirasaka, T. (2016). An update on fetal alcohol syndrome – Pathogenesis, risks, and treatment. *Alcoholism: Clinical and Experimental Research, 40*, 1594 – 1602. DOI:10.1111/acer.13135

Gutvirtz, G., Wainstock, T., Landau, D., & Sheiner, E. (2019). Maternal smoking during pregnancy and long-term neurological morbidity of the offspring. *Addictive Behaviors, 88,* 86 – 91. DOI:10.1016/j.addbeh.2018.08.013

Hales, C. N., & Barker, D. J. P. (2001). The thrifty phenotype hypothesis. *British Medical Bulletin, 60,* 5 – 20. DOI:10.1093/bmb/60.1.5

Hamilton, D. A., Barto, D., Rodriguez, C. I., Magcalas, C., Fink, B. C., Rice, J. P., Bird, C. W., Davies, S., & Savage, D. D. (2014). Effects of moderate prenatal ethanol

exposure and age on social behavior, spatial response perseveration errors and motor behavior. *Behavioral Brain Research, 269*, 44 – 54. DOI:10.1016/j.bbr.2014.04.029

Han, J.-Y., Kwon, H.-J., Ha, M., Paik, K.-C., Lim, M.-H., Lee, S. G., Yoo, S.-J., & Kim, E.-J. (2015). The effects of prenatal exposure to alcohol and environmental tobacco smoke on risk for ADHD: A large population-based study. *Psychiatry Research, 225*, 164 – 168. DOI:10.1016/j.psychres.2014.11.009

Harper, K. M., Tunc-Ozcan, E., Graf, E. N., & Redei, E. E. (2014). Intergenerational effects of prenatal ethanol on glucose tolerance and insulin response. *Physiological Genomics, 46*, 159 – 168. DOI:10.1152/physiolgenomics.00181.2013

Hausknecht, K., Haj-Dahmane, S., Shen, Y.-L., Vezina, P., Dlugos, C., & Shen, R.-Y. (2015). Excitatory synaptic function and plasticity is persistently altered in ventral tegmental area dopamine neurons after prenatal ethanol exposure. *Neuropsychopharmacology, 40*, 893 – 905. DOI:10.1038/npp.2014.265

Haycock, P. C. (2009). Fetal alcohol spectrum disorders: The epigenetic perspective. *Biology of Reproduction, 81*, 607 – 617. DOI:10.1095/biolreprod.108.074690

Health Canada. (2019). *Canadian tobacco and nicotine survey (CTNS): Summary of results for 2019*. Government of Canada. https://www.canada.ca/en/health-canada/services/canadian-tobacco-nicotine-survey/2019-summary.html#n4

Hendrickson, L. M., Guildford, M. J., & Tapper, A. R. (2013). Neuronal nicotine acetylcholine receptors: Common molecular substrates of nicotine and alcohol dependence. *Frontiers in Psychiatry, 4*, 29. DOI:10.3389/fpsyt.2013.00029

Hendrickson, T. J., Mueller, B. A., Sowell, E. R., Mattson, S. N., Coles, C. D., Kable, J. A., Jones, K. L., Boys, C. J., Lim, K. O., Riley, E. P., & Wozniak, J. R. (2017). Cortical gyrification is abnormal in children with prenatal alcohol exposure. *NeuroImage: Clinical, 15*, 391 – 400. DOI:10.1016/j.nicl.2017.05.015

Holbrook, B. D. (2016). The effects of nicotine of human fetal development. *Birth Defects Research (Part C), 108*, 181 – 192. DOI:10.1002/bdrc.21128

Holloway, A. C., Cuu, D. Q., Morrison, K. M., Gerstein, H. C., & Tarnopolsky, M. A. (2007). Transgenerational effects of fetal and neonatal exposure to nicotine. *Endocrine, 31*, 254 – 259. DOI:10.1007/s12020-007-0043-6

Höök, B., Cederblad, M., & Berg, R. (2006). Prenatal and postnatal maternal smoking as risk factors for preschool children's mental health. *Acta Pædiatrica, 95*, 671 – 677. DOI:10.1080/08035250500538965

Hu, W., Wang, G., He, B., Hu, S., Luo, S., Wen, Y., Chen, L., & Wang, H. (2020). Effects of prenatal nicotine exposure on hepatic glucose and lipid metabolism in offspring rats and its hereditability. *Toxicology, 432,* 152378. DOI:10.1016/j.tox.2020.152378

Idrus, N. M., & Napper, R. M. A. (2012). Acute and long-term Purkinje cell loss following a single ethanol binge during the early third trimester equivalent in the rat. *Alcoholism: Clinical and Experimental Research, 36,* 1365 – 1373. DOI:10.1111/j.1530-0277.2012.01743.x

Inkelis, S. M., Moore, E. M., Bischoff-Grethe, A., & Riley, E. P. (2020). Neurodevelopment in adolescents and adults with fetal alcohol spectrum disorders (FASD): A magnetic resonance region of interest analysis. *Brain Research, 1732,* 146654. DOI:10.1016/j.brainres.2020.146654

Jabbar, S., Chastain, L. G., Gangisetty, O., Cabrera, M. A., Sochacki, K., & Sarkar, D. K. (2016). Preconception alcohol increases offspring vulnerability to stress. *Neuropsychopharmacology, 41,* 2782 – 2793. DOI:10.1038/npp.2016.92

Jacobsen, J. K., Slotkin, T. A., Westerveld, M., Menci, W. E., & Pugh, K. R. (2006). Visuospatial memory deficits emerging during nicotine withdrawal in adolescents with prenatal exposure to active maternal smoking. *Neuropsychopharmacology, 31,* 1550 – 1561. DOI:10.1038/sj.npp.1300981

Jakubowska-Dogru, E., Elibol, B., Dursun, I., & Yürüker, S. (2017). Effects of prenatal binge-like ethanol exposure and maternal stress on postnatal morphological development of hippocampal neurons in rats. *International Journal of Developmental Neuroscience, 61,* 40 – 50. DOI:10.1016/j.ijdevneu.2017.06.002

Jones, K. L., & Smith, D. W. (1973). Recognition of the fetal alcohol syndrome in early infancy. *The Lancet, 302,* 999 – 1001. DOI:10.1016/s0140-6736(73)91092-1

Jung, K.-I., Ju, A., Lee, H.-M., Lee, S.-S., Song, C.-H., Won, W.-Y., Jeong, J.-S., Hong, O.-K., Kim, J.-H., & Kim, D.-J. (2011). Chronic ethanol ingestion, type 2 diabetes mellitus, and brain-derived neurotrophic factor (BDNF) in rats. *Neuroscience Letters, 487,* 149 – 152. DOI:10.1016/j.neulet.2010.10.011

Kable, J. A., Coles, C. D., & Mattson, S. N. (2020). Neurodevelopmental outcomes associated with prefrontal cortex deoxygenation in children with fetal alcohol spectrum disorder. *Developmental Neuropsychology, 45,* 1 – 16. DOI:10.1080/87565641.2020.1712604

Kable, J. A., & Coles, C. D. (2017). Prefrontal cortical responses in children with prenatal related neurodevelopmental impairment: A functional near-infrared spectroscopy study. *Clinical Neurophysiology, 128,* 2099 – 2109. DOI:10.1016/j.clinph.2017.08.009

Kable, J. A., Mehta, P. K., & Coles, C. D. (2021). Alterations in insulin levels in adults with prenatal alcohol exposure. *Alcoholism: Clinical and Experimental Research, 45*, 500 – 506. DOI:10.1111/acer.14559

Kalejaiye, O. O., & Gondré-Lewis, M. C. (2017). Enhanced susceptibility of CA3 hippocampus to prenatal nicotine exposure. *Journal of Developmental Origins of Health and Disease, 8*, 155 – 160. DOI:10.1017/s2040174416000775

Kaminen-Ahola, N., Ahola, A., Flatscher-Bader, T., Wilkins, S. J., Anderson, G. J., Whitelaw, E., & Chong, S. (2010). Postnatal growth restriction and gene expression changes in a mouse model of fetal alcohol syndrome. *Birth Defects Research (Part A), 88*, 818 – 826. DOI:10.1002/bdra.20729

Kaminen-Ahola, N. (2020). Fetal alcohol spectrum disorders: Genetic and epigenetic mechanisms. *Prenatal Diagnosis, 40*, 1185 – 1192. DOI:10.1002/pd.5731

Keiver, K., Bertram, C. P., Orr, A. P., & Clarren, S. (2015). Salivary cortisol levels are elevated in the afternoon and at bedtime in children with prenatal alcohol exposure. *Alcohol, 49*, 79 – 87. DOI:10.1016/j.alcohol.2014.11.004

Keyes, M. K., Smith, G. D., & Susser, E. (2014). Associations of prenatal maternal smoking with offspring hyperactivity: Causal or confounded? *Psychological Medicine, 44*, 857 – 867. DOI:10.1017/s0033291713000986

Kleiber, M. L., Diehl, E. J., Laufer, B. I., Mantha, K., Chokroborty-Hoque, A., Alberry, B., & Singh, S. M. (2014). Long-term genomic and epigenomic dysregulation as a consequence of prenatal alcohol exposure: A model for fetal alcohol spectrum disorders. *Frontiers in Genetics, 5*, 161. DOI:10.3389/fgene.2014.00161

Kleiber, M. L., Wright, E., & Singh, S. M. (2011). Maternal voluntary drinking in C57BL/6J mice: Advancing a model for fetal alcohol spectrum disorder. *Behavioral Brain Research, 223*, 376 – 387. DOI:10.1016/j.bbr.2011.05.005

Knopik, V. S., Maccani, M. A., Francazio, S., & McGeary, J. E. (2012). The epigenetics of maternal cigarette smoking during pregnancy and effects on child development. *Development and Psychopathology, 24*, 1377 – 1390. DOI:10.1017/s0954579412000776

Kully-Martens, K., Denys, K., Treit, S., Tamana, S., & Rasmussen, C. (2012). A review of social skills deficits in individuals with fetal alcohol spectrum disorders and prenatal alcohol exposure: Profiles, mechanisms, and interventions. *Alcoholism: Clinical and Experimental Research, 36*, 568 – 576. DOI:10.1111/j.1530-0277.2011.01661.x

Ladd-Acosta, C., Shu, C., Lee, B. K., Gidaya, N., Singer, A., Schieve, L. A., Schendel, D. E., Jones, N., Daniels, J. L., Windham, G. C., Newschaffer, C. J., Croen, L. A., Feinberg, A. P., & Fallin, M. D. (2016). Presence of an epigenetic signature of prenatal cigarette smoke exposure in childhood. *Environmental Research, 144*, 139 – 148. DOI:10.1016/j.envres.2015.11.014

Lam, V. Y. Y., Raineki, C., Wang, L. Y., Chiu, M., Lee, G., Ellis, L., Yu, W., & Weinberg, J. (2019). Role of corticosterone in anxiety- and depressive-like behavior and HPA regulation following prenatal alcohol exposure. *Progress in Neuropsychopharmacology, Biology, and Psychiatry, 90*, 1 – 15. DOI:10.1016/j.pnpbp.2018.10.008

Lavezzi, A. M., Corna, M. F., Repetti, M. L., & Matturri, L. (2013). Cerebellar Purkinje cell vulnerability to prenatal nicotine exposure in sudden unexplained perinatal death. *Folia Neuropathologica, 51*, 290 – 301. DOI:10.5114/fn.2013.39718

Lee, Y. J., Kim, J. Y., Lee, D. Y., Park, K. J., Kim, G. H., Kim, J., E., Roh, G. S., Lim, J. Y., Koo, S., Lim, M. K., Park, H. Y., & Kim, W.-H. (2020). Alcohol consumption before pregnancy causes detrimental fetal development and maternal metabolic disorders. *Scientific Reports, 10*, 10054. DOI:10.1038/s41598-020-66971-1

Lemoine, P., Harousseau, P., Borteryu, J. P., & Menuet, J. C. (1968). Les enfants de parents alcooliques: Anomalies observee a propos de 127 cas. *Ouest Medicale, 21*, 476 – 482.

LeSage, M. G., Gustaf, E., Dufek, M. B., & Pentel, P. R. (2006). Effects of maternal intravenous nicotine administration on locomotor behavior in pre-weanling rats. *Pharmacology, Biochemistry, and Behavior, 85*, 575 – 583. DOI:10.1016/j.pbb.2006.10.012

Liu, J., Yu, C., Doherty, T. M., Akbari, O., Allard, P., & Rehan, V. K. (2020). Perinatal nicotine exposure-induced transgenerational asthma: Effects of reexposure in F1 generation. *The FASEB Journal, 34*, 11444 – 11459. DOI:10.1096/fj.201902386r

Livy, D. J., Maier, S. E., & West, J. R. (2004). Long-term alcohol exposure prior to conception results in lower fetal body weights. *Birth Defects Research (Part B), 71*, 135 – 141. DOI:10.1002/bdrb.20007

Lussier, A. A., Weinberg, J., & Kobor, M. S. (2017). Epigenetics studies of fetal alcohol spectrum disorder: Where are we now? *Epigenomics, 9*, 291 – 311. DOI:10.2217/epi-2016-0163

Lv, J., Mao, C., Zhu, L., Zhang, H., Pengpeng, H., Xu, F., Liu, Y., Zhang, L., & Xu, Z. (2008). The effect of prenatal nicotine on expression of nicotine receptor subunits in the fetal brain. *NeuroToxicology, 29*, 722 – 726. DOI:10.1016/j.neuro.2008.04.015

Marquardt, K., & Brigman, J. L. (2016). The impact of prenatal alcohol exposure on social, cognitive, and affective behavioral domains: Insights from rodent models. *Alcohol, 51*, 1 – 15. DOI:10.1016/j.alcohol.2015.12.002

Marquardt, K., Cavanagh, J. F., & Brigman, J. L. (2020). Alcohol exposure in utero disrupts cortico-striatal coordination required for behavioral flexibility. *Neuropharmacology, 162*, 107832. DOI:10.1016/j.neuropharm.2019.107832

Marquardt, K., Sigdel, R., Caldwall, K., & Brigman, J. L. (2014). Prenatal ethanol exposure impairs executive function in mice into adulthood. *Alcoholism: Clinical and Experimental Research, 38*, 2962 – 2968. DOI:10.1111/acer.12577

Martin, M. M., McCarthy, D. M., Schatschneider, C., Trupiano, M. X., Jones, S. K., Kalluri, A., & Bhide, P. G. (2020). Effects of developmental nicotine exposure on frontal cortical GABA-to-non-GABA neuron ratio and novelty-seeking behavior. *Cerebral Cortex, 30*, 1830 – 1842. DOI:10.1093/cercor/bhz207

Mattson, S. N., Bernes, G. A., & Doyle, L. R. (2019). Fetal alcohol spectrum disorders: A review of the neurobehavioral deficits associated with prenatal alcohol exposure. *Alcoholism: Clinical and Experimental Research, 43*, 1046 – 1062. DOI:10.1111/acer.14040

McLachlan, K., Rasmussen, C., Oberlander, T. F., Loock, C., Pei, J., Andrew, G., Reynolds, J., & Weinberg, J. (2016). Dysregulation of the cortisol diurnal rhythm following prenatal alcohol exposure and early life adversity. *Alcohol, 53*, 9 – 18. DOI:10.1016/j.alcohol.2016.03.003

Migliorini, R., Moore, E. M., Glass, L., Infante, M. A., Tapert, S. F., Lyons Jones, K., Mattson, S. N., & Riley, E. P. (2015). Anterior cingulate cortex surface area relates to behavioral inhibition in adolescents and adults with and without heavy prenatal alcohol exposure. *Behavioral Brain Research, 292*, 26 – 35. DOI:10.1016.j.bbr.2015.05.037

Mihalick, S. M., Crandall, J. E., Langlois, J. C., Krienke, J. D., & Dube, W. V. (2001). Prenatal ethanol exposure, generalized learning impairment, and medial prefrontal cortical deficits in rats. *Neurotoxicology and Teratology, 23*, 453 – 462. DOI:10.1016/s0892-0362(01)00168-4

Muneoka, K., Ogawa, T., Kamei, K., Mimura, Y., Kato, H., & Takigawa, M. (2001). Nicotine exposure during pregnancy is a factor which influences serotonin transporter density in the rat brain. *European Journal of Pharmacology, 411*, 279 – 282. DOI:10.1016/s0014-2999(00)00925-0

Muhammad, A., Mychasiuk, R., Nakahashi, A., Hossain, S. R., Gibb, R., & Kolb, B. (2012). Prenatal nicotine exposure alters neuroanatomical organization of the developing brain. *Synapse, 66*, 950 – 954. DOI:10.1002/syn.21589

Muñoz-Villegas, P., Rodríguez, V. M., Giordano, M., & Juárez, J. (2017). Risk-taking, locomotor activity, and dopamine levels in the nucleus accumbens and medial prefrontal cortex in male rats treated prenatally with alcohol. *Pharmacology, Biochemistry, and Behavior, 153*, 88 – 96. DOI:10.1016/j.pbb.2016.12.011

Mychasiuk, R., Muhammad, A., & Gibb, R., & Kolb, B. (2013). Long-term alterations to dendritic morphology and spine density associated with prenatal exposure to nicotine. *Brain Research, 1499*, 53 – 60. DOI:10.1016/j.brainres.2012.12.021

Nielsen, C. H., Larsen, A., & Nielsen, A. L. (2016). DNA methylation alterations in response to prenatal exposure to maternal cigarette smoking: A persistent epigenetic impact on health from maternal lifestyle? *Archives of Toxicology, 90*, 231 – 245. DOI:10.1007/s00204-014-1426-0

Niemelä, S., Sourander, A., Surcel, H.-M., Hinkka-Yli-Salomäki, S., McKeague, I. W., Cheslack-Postava, K., & Brown, A. S. (2016). Prenatal nicotine exposure and risk of schizophrenia among offspring in a national birth cohort. *American Journal of Psychiatry, 173*, 799 – 806. DOI:10.1176/appi.ajp.2016.15060800

Nguyen, T. M. T., Steane, S. E., Moritz, K. M., & Akison, L. K. (2019). Prenatal alcohol exposure programmes offspring disease: Insulin resistance in adult males in a rat model of acute exposure. *The Journal of Physiology, 597*, 5619 – 5637. DOI:10.1113/jp279051

Okazaki, S., Otsuka, I., Shinko, Y., Horai, T., Hirata, T., Yamaki, N., Sora, I., & Hishimoto, A. (2021). Epigenetic clock analysis in children with fetal alcohol spectrum disorder. *Alcoholism: Clinical and Experimental Research, 45*, 329 – 337. DOI:10.1111/acer.14532

Olateju, O. I., Spocter, M. A., Patzke, N., Ihunwo, A. O., & Manger, P. R. (2018). Hippocampal neurogenesis in the C57BL/6J nice at early adulthood following prenatal alcohol exposure. *Metabolic Brain Disease, 33*, 397 – 410. DOI:10.1007/s11011-017-0156-4

Olguin, S. L., Thompson, S. M., Young, J. W., & Brigman, J. L. (2021). Moderate prenatal alcohol exposure impairs cognitive control, but not attention, on a rodent touchscreen continuous performance task. *Genes, Brain, and Behavior, 20*, e12652. DOI:10.1111/gbb.12652

Oliff, H. S., Gallardo, K. A. (1999). The effect of nicotine on developing brain catecholamine systems. *Frontiers in Bioscience, 4*, d883 – 897. DOI:10.2741/oliff

O'Malley, K. D., & Nanson, J. (2002). Clinical implications of a link between fetal alcohol spectrum disorder and attention-deficit hyperactivity disorder. *Canadian Journal of Psychiatry, 47*, 349 – 354. DOI:10.1177/070674370204700405

Ortega-Garcia, J. A., Martin, M., Lopez-Fernandez, M. T., Fuster-Soler, J. L., Donat-Colomer, J., Lopez-Ibor, B., Claudio, L., & Ferris-Tortajada, J. (2010). Transgenerational tobacco smoke exposure and childhood cancer: An observational study. *Journal of Pediatrics and Child Health, 46*, 291 – 295. DOI:10.1111/j.1440-1754.2010.01710.x

Parameshwaran, K., Buabeid, M. A., Karuppagounder, S. S., Uthayathas, S., Thiruchelvam, K., Shonesy, B., Dityatev, A., Escobar, M. C., Dhanasekaran, M., & Suppiramaniam, V. (2012). Developmental nicotine exposure induced alterations in behavior and glutamate receptor function in hippocampus. *Cellular and Molecular Life Sciences, 69*, 829 – 841. DOI:10.1007/s00018-011-0805-4

Peacock, A., Leung, J., Larney, S., College, S., Hickman, M., Rehm, J., Giovino, G. A., West, R., Hall, W., Grittiths, P., Ali, R., Gowing, L., Marsden, J., Ferrari, A. J., Grebely, J., Farrell, M., & Degenhardt, L. (2018). Global statistics on alcohol, tobacco and illicit drug use: 2017 status report. *Addiction, 113*, 1905 – 1926. DOI:10.1111/add.14234

Pellis, S. M., Pellis, V. C., & Bell, H. C. (2010). The function of play in the development of the social brain. *American Journal of Play, 2*, 278 – 296.

Petrelli, B., Weinberg, J., & Hicks, G. G. (2018). Effects of prenatal alcohol exposure (PAE): Insights into FASD using mouse models of PAE. *Biochemistry and Cell Biology, 96*, 131 – 147. DOI:10.1139/bcb-2017-0280

Pierce, D. R., Williams, D. K., & Light, K. E. (1999). Purkinje cell vulnerability to developmental ethanol exposure in the rat cerebellum. *Alcoholism: Clinical and Experimental Research, 23*, 1650 – 1659. DOI:10.1111/j.1530-0277.1999.tb04057.x

Polli, F. S., Ipsen, T. H., Caballero-Puntiverio, M., Østerbøg, T. B., Aznar, S., Andreasen, J. T., & Kohlmeier, K. A. (2020). Cellular and molecular changes in hippocampal glutamate signaling and alterations in learning, attention, and impulsivity following prenatal nicotine exposure. *Molecular neurobiology, 57*, 2002 – 2020. DOI:10.1007/s12035-019-01854-9

Polli, F. S., & Kohlmeier, K. A. (2018). Prenatal nicotine exposure alters postsynaptic AMPA receptors and glutamate neurotransmission within the laterodorsal tegmentum (LDT) of juvenile mice. *Neuropharmacology, 137*, 71 – 85. DOI:10.1016/j.neuropharm.2018.04.024

Polli, F. S., & Kohlmeier, K. A. (2020). Prenatal nicotine exposure in rodents: Why are there so many variations in behavioral outcomes? *Nicotine and Tobacco Research, 22*, 1694 – 1710. DOI:10.1093/ntr/ntz196

Polli, F. S., Scharff, M. B., Ipsen, T. H., Aznar, S., Kohlmeier, K. A., & Andreasen, J. T. (2020). Prenatal nicotine exposure in mice induces sex-dependent anxiety-like behavior, cognitive deficits, hyperactivity, and changes in the expression of glutamate receptor associated-genes in the prefrontal cortex. *Pharmacology, Biochemistry, and Behavior, 195*, 172951. DOI:10.1016/j.pbb.2020.172951

Popova, S., Lange, S., Poznyak, V., Chudley, A. E., Shield, K. D., Reynolds, J. N., Murray, M., & Rehm, J. (2019). Population-based prevalence of fetal alcohol spectrum disorder in Canada. *BMC Public Health, 19*, 845. DOI:10.1186/s12889-019-7213-3

Popova, S., Lange, S., Probst, C., Gmel, G., & Rehm, J. (2017). Estimation of national, regional, and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: A systematic review and meta-analysis. *The Lancet Global Health, 5*, E290 – E299. DOI:10.1016/s2214-109x(17)30021-9

Przybycien-Szymanska, M. M., Rao, Y., S., Prins, S. A., & Pak., T. R. (2014). Parental binge alcohol abuse alters F1 generation hypothalamic gene expression in the absence of direct fetal alcohol exposure. *PLoS ONE, 9*, e89320. DOI:10.1371/journal.pone.0089320

Rehan, V. K., Liu, J., Naeem, E., Tian, J., Sakurai, R., Kwong, K., Akbari, O., & Torday, J. S. (2012). Perinatal nicotine exposure induces asthma in second generation offspring. *BMC Medicine, 10*, 129. DOI:10.1186/1741-7015-10-129

Renaud, S. M., & Fountain, S. B. (2016). Transgenerational effects of adolescent nicotine exposure in rats: Evidence for cognitive deficits in adult female offspring. *Neurotoxicology and Teratology, 56*, 47 – 54. DOI:10.1016/j.ntt.2016.06.002

Rasmussen, C., Andrew, G., Zwaigenbaum, L., & Tough, S. (2008). Neurobehavioral outcomes of children with fetal alcohol spectrum disorders: A Canadian perspective. *Pediatrics and Child Health, 13*, 185 – 191. DOI:10.1093/pch/13.3.185

Rasmussen, C. (2005). Executive functioning and working memory in fetal alcohol spectrum disorder. *Alcoholism: Clinical and Experimental Research, 29*, 1359 – 1367. DOI:10.1097/01.alc.0000175040.91007.d0

Riggins, T., Cacic, K., Buckingham-Howes, S., Scaletti, L. A., Salmeron, B. J., & Black, M. M. (2012). Memory ability and hippocampal volume in adolescents with prenatal drug exposure. *Neurotoxicology and Teratology, 34*, 434 – 441. DOI:10.1016/j.ntt.2012.05.054

Rivkin, M. J., Davis, P. E., Lemaster, J. L., Cabral, H. J., Warfield, S. K., Mulkern, R. V., Robson, C. D., Rose-Jacobs, R., & Frank, D. A. (2008). Volumetric MRI study of brain in children with intrauterine exposure to cocaine, alcohol, tobacco, and marijuana. *Pediatrics, 121*, 741 – 750. DOI:10.1542/peds.2007-1399

Rockhold, M. N., Krueger, A. M., de Water, E., Lindgren, C. W., Sandness, K. E., Eckerle, J. K., Schumacher, M. J., Fink, B. A., Boys, C. J., Carlson, S. M., Fuglestad, A. J., Mattson, S. J., Jones, K. L., Riley, E. P., & Wozniak, J. R. (2021). Executive and social functioning across development in children and adolescents with prenatal alcohol exposure. *Alcoholism: Clinical and Experimental Research, 45*, 457 – 469. DOI:10.1111/acer.14538

Rodriguez, C. I., Davies, S., Calhoun, V., Savage, D. D., & Hamilton, D. A. (2016). Moderate prenatal alcohol exposure alters functional connectivity in the adult rat brain. *Alcoholism: Clinical and Experimental Research, 40*, 2134 – 2146. DOI:10.1111/acer.13175

Rodriguez, C. I., Magcalas, C. M., Barto, D., Fink, B. C., Rice, J. P., Bird, C. W., Davies, S., Pentkowski, N. S., Savage, D. D., & Hamilton, D. A. (2016). Effects of sex and housing on social, spatial, and motor behavior in adult rats exposed to moderate levels of alcohol during prenatal development. *Behavioral Brain Research, 313*, 233 – 243. DOI:10.1016/j.bbr.2016.07.018

Rogers, J. M. (2019). Smoking and pregnancy: Epigenetics and developmental origins of the metabolic syndrome. *Birth Defects Research, 111*, 1259 – 1269. DOI:10.1002/bdr2.1550

Roy, T. S., & Sabherwal, U. (1998). Effects of gestational nicotine exposure on hippocampal morphology. *Neurotoxicology and Teratology, 20*, 465 – 473. DOI:10.1016/s0892-0362(97)00137-2

Roy, T. S., Seidler, F. J., & Slotkin, T. A. (2002). Prenatal nicotine exposure evokes alterations of cell structure in hippocampus and somatosensory cortex. *The Journal of Pharmacology and Experimental Therapeutics, 300*, 124 – 133. DOI:10.1124/jpet.300.1.124

San Martin Porter, M., Maravilla, J. C., Betts, K. S., & Alati, R. (2019). Low-moderate prenatal alcohol exposure and offspring attention-deficit hyperactivity disorder: systematic review and meta-analysis. *Archives of Gynecology and Obstetrics, 300*, 269 – 277. DOI:10.1007/s00404-019-05204-x

Sari, Y., & Zhou, F. C. (2004). Prenatal alcohol exposure causes long-term serotonin neuron deficit in mice. *Alcoholism: Clinical and Experimental Research, 28*, 941 – 948. DOI:10.1097/01.alc.0000128228.08472.39

Schneider, T., Ilott, N., Brolese, G., Bizarro, L., Asherson, P. J. E., & Stolerman, I. P. (2011). Prenatal exposure to nicotine impairs performance of the 5-choice serial reaction time task in adult rats. *Neuropsychopharmacology, 36,* 1114 – 1125. DOI:10.1038/npp.2010.249

Schrantee, A., Tamminga, H. G. H., Bouziane, C., Bottelier, M. A., Bron, E. E., Mutsaerts, H.-J., M. M., Zwinderman, A. H., Groote, I. R., Rombouts, S. A. R. B., Lindauer, R. J. L., Klein, S., Niessen, W. J., Opmeer, B. C., Boer, F., Lucassen, P. J., Anderson, S. L., Geurts, H. M., & Reneman, L. (2016). Age-dependent effects of methylphenidate on the human dopaminergic system in young vs adult patients with attention-deficit/hyperactivity disorder. *JAMA Psychiatry, 73,* 955 – 962. DOI:10.1001/jamapsychiatry.2016.1572

Shacka, J. J., & Robinson, S. E. (1998). Exposure to prenatal nicotine transiently increases neuronal nicotinic receptor subunit α7, α4, and β2 messenger RNAs in the postnatal rat brain. *Neuroscience, 84,* 1151 – 1161. DOI:10.1016/s0306-4522(97)00564-2

Sliwowska, J. H., Song, H. J., Bodnar, T., & Weinberg, J. (2014). Prenatal alcohol exposure results in long-term serotonin neuron deficits in female rats: Modulatory role of ovarian steroids. *Alcoholism: Clinical and Experimental Research, 38,* 152 – 160. DOI:10.1111/acer.12224

Slotkin, T. A., Skavicus, S., Card, J., Stadler, A., Levin, E. D., & Seidler, F. J. (2015).

Slotkin, T. A., Tate, C. A., Cousins, M. M., & Seidler, F. J. (2006). Prenatal nicotine exposure alters the responses to subsequent nicotine administration and withdrawal in adolescence: Serotonin receptors and cell signaling. *Neuropsychopharmacology, 31,* 2462 – 2475. DOI:10.1038/sj.npp.1300988

Somm, E., Schwitzgebel, V. M., Vauthay, D. M., Aubert, M. L., & Hüppi, P. S. (2009). Prenatal nicotine exposure and the programming of metabolic and cardiovascular disorders. *Molecular and Cellular Endocrinology, 304,* 69 – 77. DOI:10.1016/j.mce.2009.02.026

Sourander, A., Sucksdorff, M., Chudal, R., Surcel, H.-M., Hinkka-Yli-Salomäki, Gyllenberg, D., Cheslack-Postava, K., & Brown, A. S. (2019). Prenatal cotinine levels and ADHD among offspring. *Pediatrics, 143,* e20183144. DOI:10.1542/peds.2018-3144

Sowell, E. R., Mattson, S. N., Kan, E., Thompson, P. M., Riley, E. P., & Toga, A. W. (2008). Abnormal cortical thickness and brain-behavior correlation patterns in individuals with heavy prenatal alcohol exposure. *Cerebral Cortex, 18,* 136 – 144. DOI:10.1093/cercor/bhm039

Statistics Canada. (2021). Table 13-10-0096-11 Heavy drinking by age group. https://doi.org/10.25318/1310009601-eng

Suarez, L., Felkner, M., Brender, J. D., Canfield, M., & Hendricks, K. (2008). Maternal exposures to cigarette smoke, alcohol, and street drugs and neural tube defect occurrence in offspring. *Maternal and Child Health Journal, 12*, 394 – 401. DOI:10.1007/s10995-007-0251-y

Sullivan, E. V., Moore, E. M., Lane, B., Pohl, K. M., Riley, E. P., & Pfefferbaum, A. (2020). Graded cerebellar lobular volume deficits in adolescents and young adults with fetal alcohol spectrum disorders (FASD). *Cerebral Cortex, 30*, 4729 – 4746. DOI:10.1093/cercor/bhaa020

Taylor, P. A., Jacobson, S. W., van der Kouwe, A., Molteno, C. D., Chen, G., Wintermark, P., Alhamud, A., Jacobson, J. L., & Meintjes, E. M. (2015). A DTI-based tractography study of effects on brain structure associated with prenatal alcohol exposure in newborns. *Human Brain Mapping, 36*, 170 – 186. DOI:10.1002/hbm.22620

Tiesler, C. M. T., & Heinrich, J. (2014). Prenatal nicotine exposure and child behavior problems. *European Child and Adolescent Psychiatry, 23*, 913 – 929. DOI:10.1007/s00787-014-0615-y

Torabi, R., Jenkins, S., Harker, A., Whishaw, I. Q., Gibb, R., & Luczak, A. (2021). A neural network reveals motoric effects of maternal preconception exposure to nicotine on rat pup behavior: A new approach for movement disorders diagnosis. *Frontiers in Neuroscience, 15*, 686767. DOI:10.3389/fnins.2021.686767

Treit, S., Chen, Z., Zhou, D., Baugh, L., Rasmussen, C., Andrew, G., Pei, J., & Beaulieu, C. (2017). Sexual dimorphism of volume reduction but not cognitive deficit in fetal alcohol spectrum disorders: A combined diffusion tensor imaging, cortical thickness and brain volume study. *NeuroImage: Clinical, 15*, 284 – 297. DOI:10.1016/j.nicl.2017.05.006

Tunc-Ozcan, E., Harper, K. M., Graf, E. N., & Redei, E. E. (2016). Thyroxine administration prevents matrilineal intergenerational consequences of *in utero* ethanol exposure in rats. *Hormones and Behavior, 82*, 1 – 10. DOI:10.1016/j.yhbeh.2016.04.002

Vaglenova, J., Birru, S., Pandiella, N. M., & Breese, C. R. (2004). An assessment of the long-term developmental and behavioral teratogenicity of prenatal nicotine exposure. *Behavioral Brain Research, 150*, 159 – 170. DOI:10.1016/j.bbr.2003.07.005

Vaglenova, J., Parameshwaran, K., Suppiramaniam, V., Breese, C. R., Pandiella, N., & Birru, S. (2008). Long-lasting teratogenic effects of nicotine on cognition: Gender

specificity and role of AMPA receptor function. *Neurobiology of Learning and Memory, 90*, 527 – 536. DOI:10.1016/j.nlm.2008.06.009

Vaiserman, A. M. (2013). Long-term health consequences of early-life exposure to substance abuse: An epigenetic perspective. *Journal of Developmental Origins of Health and Disease, 4*, 269 – 279. DOI:10.1017/s2040174413000123

Wang, H., Dávila-García, M. I., Yarl, W., & Gondré-Lewis, M. C. (2011). Gestational nicotine exposure regulates expression of AMPA and NMDA receptors and their signaling apparatus in developing and adult rat hippocampus. *Neuroscience, 188*, 168 – 181. DOI:10.1016/j.neuroscience.2011.04.069

Ware, A. L., Long, X., & Lebel, C. (2021). Functional connectivity of the attention networks is altered and relates to neuropsychological outcomes in children with prenatal alcohol exposure. *Developmental Cognitive Neuroscience, 48*, 100951. DOI:10.1016/j.den.2021.100951

Wass, T. S., Persutte, W. H., & Hobbins, J. C. (2001). The impact of prenatal alcohol exposure on frontal cortex development in utero. *American Journal of Obstetrics and Gynecology, 185*, 737 – 742. DOI:10.1067/mob.2001.117656

Weinberg, J., Sliwowska, J. H., Lan, N., & Hellemans, K. G. C. (2008). Prenatal alcohol exposure: Foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome. *Journal of Neuroendocrinology, 20*, 470 – 488. DOI:10.1111/j.1365-2826.2008.01669.x

Whitcher, L. T., & Klintsova, A. Y. (2008). Postnatal binge-like alcohol exposure reduces spine density without affecting dendritic morphology in rat mPFC. *Synapse, 62*, 566 – 573. DOI:10.1002/syn.20532

Wieczorek, L., Fish, E. W., O-Leary-Moore, K., Parnell, S. E., & Sulik, K. K. (2015). Hypothalamic-pituitary-adrenal axis and behavioral dysfunction following early binge-like prenatal alcohol exposure in mice. *Alcohol, 49*, 207 – 217. DOI:10.1016/j.alcohol.2015.01.005

Winzer-Serhan, U. H. (2008). Long-term consequences of maternal smoking and developmental chronic nicotine exposure. *Frontiers in Bioscience, 13*, 636 – 649. DOI:10.2741/2708

Wozniak, J. R., Mueller, B. R., Muetzel, R. L., Bell, C. J., Hoecker, H. L., Nelson, M. L., Chang, P.-N., & Lim, K. O. (2011). Inter-hemispheric functional connectivity disruption in children with prenatal alcohol exposure. *Alcoholism: Clinical and Experimental Research, 35*, 849 – 861. DOI:10.1111/j.1530-0277.2010.01415.x

Xia, L. P., Shen, L., Kou, H., Zhang, B. J., Zhang, L., Wu, Y., Li, X. J., Xiong, J., Yu, Y., & Wang, H. (2014). Prenatal ethanol exposure enhances the susceptibility to

metabolic syndrome in offspring rats by HPA axis-associated neuroendocrine metabolic programming. *Toxicology Letters, 226*, 98 – 105. DOI:10.1016/j.toxlet.2014.01.023

Yohn, N. L., Bartolomei, M. S., & Blendy, J. A. (2015). Multigenerational and transgenerational inheritance of drug exposure: The effects of alcohol, opiates, cocaine, marijuana, and nicotine. *Progress in Biophysics and Molecular Biology, 118*, 21 – 33. DOI:10.1016/j.pbiomolbio.2015.03.002

Zhang, C., Fan, S.-J., Sun, A.-B., Liu, Z.-Z., & Liu, L. (2019). Prenatal nicotine exposure induces depression-like behavior in adolescent female rats via modulating neurosteroid in the hippocampus. *Molecular Medicine Reports, 19*, 4185 – 4194. DOI:10.3892/mmr.2019.10105

Zhang, L., Spencer, T. J., Biederman, J., & Bhide, P. G. (2018). Attention and working memory deficits in a perinatal nicotine exposure mouse model. *PLoS ONE, 13*, e0198064. DOI:10.1371/journal.pone.0198064

Zhou, D., Rasmussen, C., Pei, J., Andrew, G., Reynolds, J. N., Beaulieu, C. (2018). Preserved cortical asymmetry despite thinner cortex in children and adolescents with prenatal alcohol exposure and associated conditions. *Human Brain Mapping, 39*, 71 – 88. DOI:10.1002/hbm.23818

Zhu, C., Guo, Y., Luo, H., Wu, Y., Magdalou, J., Chen, L., & Wang, H. (2018). Synergistic effects of prenatal nicotine exposure and post-weaning high fat diet on hypercholesterolaemia in rat offspring of different sexes. *Basic and Clinical Pharmacology and Toxicology, 124*, 730 – 740. DOI:10.1111/bcpt.13187

Zhu, J., Fan, F., McCarthy, D. M., Zhang, L., Cannon, E. N., Spencer, T. J., Biederman, J., & Bhide, P. G. (2017). A prenatal nicotine exposure mouse model of methylphenidate responsive ADHD-associated cognitive phenotypes. *International Journal of Developmental Neuroscience, 58*, 26 – 34. DOI:10.1016/j.ijdevneu.2017.01.014

Zhu, J., Lee, K. P., Spencer, T. J., Biederman, J., & Bhide, P. G. (2014). Transgenerational transmission of hyperactivity in a mouse model of ADHD. *The Journal of Neuroscience, 34*, 2768 – 2773. DOI:10.1523/jneurosci.4402-13.2014

Zhu, J., Zhang, X., Xu, Y., Spencer, T. J., Biederman, J., & Bhide, P. G. (2012). Prenatal nicotine exposure mouse model showing hyperactivity, reduced cingulate cortex volume, reduced dopamine turnover, and responsiveness to oral methylphenidate treatment. *The Journal of Neuroscience, 32*, 9410 – 9418. DOI:10.1523.jneurosci.1041-12.2012

Zimatkin, S. M., & Bon, E. I. (2016). Dynamics of histological changes in the frontal cortex of the brain in rats subjected to antenatal exposure to alcohol. *Neuroscience and Behavioral Physiology, 47*, 11 – 15. DOI:10.1007/s11055-017-0407-1

CHAPTER 2

Distinct sex-dependent effects of maternal preconception nicotine and enrichment on the early development of rat offspring brain and behavior

Abstract

Developmental nicotine exposure has harmful effects on offspring. Whereas much is known about the consequences of prenatal nicotine exposure, relatively little is understood about how maternal preconception nicotine impacts the next generation. Positive experiences, such as environmental enrichment/complexity, have great potential to improve developmental outcomes and even treat and prevent drug addiction. Therefore, the current study aimed to identify how maternal exposure to moderate levels of nicotine prior to conception impacts offspring development, and if the presumably negative consequence of nicotine could be reversed by concurrent exposure to an enriched environment. We treated female Long Evans rats with nicotine in their drinking water (15mg/L) for seven weeks while residing in either standard or enriched conditions. Both experiences occurred entirely prior to mating. Females reared their own litters, and offspring were tested in two assessments of early development: negative geotaxis and open field. Offspring were euthanized at weaning (P21), and their brains were processed with Golgi-Cox solution to allow quantification of dendritic spine density. Results indicate that neither maternal nicotine or enrichment had an impact on maternal care, but male offspring were impaired at negative geotaxis and open field due to nicotine, female offspring showed altered open field exploration due to enrichment, and offspring of both sexes had increased spine density in OFC due to maternal enrichment. Therefore, this experiment provides novel insights into the negative consequence of maternal preconception nicotine on early development of rat pups, and the insufficiency of maternal enrichment to mitigate the deficits.

2.1 Introduction

Nicotine dependence remains a significant health challenge in Canada; in 2019, 13% of men and women aged 20 years and older reported as current cigarette smokers, with 9% identifying as daily smokers (Health Canada, 2019). The prevalence of cigarette smoking is much lower among today's youth (15 – 19-years-old) at 5%, although 15% of youth use vaping products at least occasionally (Health Canada, 2019). Preconception health planning advises that women of reproductive age cease all forms of nicotine intake for their own health and their health of their fetus. However, only 50% of women who use nicotine products prior to pregnancy achieve complete abstinence during pregnancy (Kim, 2020). In addition, nearly half of pregnancies are unplanned, meaning that a significant proportion of women unintentionally smoke immediately prior to and during the initial stages of their pregnancy. Therefore, it is critical to understand how nicotine exposure during the preconception and periconception life stages impacts the development of the next generation.

Much is known about the consequences of prenatal nicotine use. Offspring of mothers who consumed nicotine during pregnancy have low birthweight (Winzer-Serhan, 2008; Bruin, Gerstein, & Holloway, 2010), increased prevalence of birth defects (Holbrook, 2016), altered brain development (Dwyer, 2008; Muhammad et al., 2012; Holbrook, 2016), elevated risk of developing metabolic syndrome (Rogers, 2019), and increased prevalence of attention-deficit hyperactivity disorder (ADHD; Winzer-Serhan, 2008; Zhu et al., 2012; Keyes, Smith, & Susser, 2014), learning and memory impairments (Winzer-Serhan, 2008), substance abuse, conduct disorders (Holbrook, 2016), and anxiety disorders (Winzer-Serhan, 2008). However, relatively little is

understood about how preconception nicotine impacts development. Preconception nicotine refers to any exposure to nicotine prior to the onset of pregnancy, such that there is no direct exposure of the fetus to nicotine. Previously, we showed that postnatal day (P)10 offspring exposed to the maternal preconception nicotine paradigm used in the current study have reduced locomotor activity due to reduced postural support and increased stereotypy (Torabi et al., 2021). One study examined the offspring of female and male Long Evans rats that were exposed to chronic intraperitoneal injections of 1.0mg/kg nicotine during adolescence. These authors found that the female offspring of the nicotine-exposed parents were impaired at learning complex serial patterns, representing a cognitive deficit (Renaud & Fountain, 2016). Another study exposed females of the F0 generation to prenatal nicotine, then bred the resulting F1 offspring to generate an F2 generation to explore the transgenerational consequences of nicotine. They found that male F2 offspring were insulin resistant and had elevated blood pressure if their mother was exposed to prenatal nicotine (Holloway et al., 2007). A different study used a similar experimental design and found that the F2 and F3 offspring of F1 dams exposed prenatally to nicotine display hyperactivity, but that the descendants of F1 sires exposed to prenatal nicotine do not (Zhu et al., 2014). Others have used a nicotine exposure paradigm that covers the whole reproductive cycle, from three weeks prior to pregnancy, throughout pregnancy, and in some cases during lactation. Using this paradigm, authors have reported: 1) hyperactivity in adult offspring (Martin et al., 2020); 2) decreased anxiety in adult offspring (Martin et al., 2020); 3) altered ratio of excitatory to inhibitory neurons in the prefrontal cortex (PFC; Martin et al., 2020); 4) impaired spontaneous alternation in the Y-maze in adult male offspring (Zhu et al., 2017; Zhang et

al., 2018); 5) impaired attention in adult offspring (Zhu et al., 2017; Zhang et al., 2018); and 6) increased impulsiveness in adult male offspring (Zhu et al., 2017). Therefore, it is abundantly clear that although we have ample evidence of the negative consequences of prenatal nicotine, our understanding of how indirect maternal preconception nicotine impacts development is severely lacking. Interestingly, the topic of paternal preconception nicotine is relatively well explored (Vassoler, Byrnes, & Pierce, 2014; Yohn, Bartolomei, & Blendy, 2015; Dai et al., 2017; McCarthy et al., 2018, 2020; Goldberg & Gould, 2019; Goldberg et al., 2019; Zhang et al., 2020)

The current study investigated how maternal exposure to chronic nicotine immediately prior to conception impacts the preweaning development of offspring. We were also interested in how the environment in which drug use occurred moderates the effect of the nicotine. Enhancing a rodent's environment provides increased opportunities for social interaction, exercise, and sensory stimulation. In this way, it can be translated to increased affluence and educational and extracurricular opportunities in human populations. Ample research demonstrates that complex, enriching environments are beneficial, with several studies examining the benefits of preconception complex environments. Pups of dams exposed to enriched environments during adolescence have accelerated acquisition of complex motor behavior (Caporali et al., 2014), improved spatial learning, improved discriminative behavior (Cutuli et al., 2015), and improved coping strategies following stressful situations (Cutuli et al., 2017). Preconception enrichment also improved maternal care behaviors (Cutuli et al., 2015, 2017). It appears that an increase in brain-derived neurotrophic factor (BDNF) is largely responsible for these benefits of maternal enrichment; authors have found increased BDNF in the

cerebellum, striatum (Caporali et al., 2014), and hippocampus (Cutuli et al., 2015) in offspring following preconception enrichment. An increase in nerve growth factor (NGF) in the cerebellum has also been reported (Caporali et al., 2014). Parental enrichment can also reverse the negative consequences of preconception stress (Leshem & Schulkin, 2011; Gapp et al., 2016) and morphine dependency (Pooriamehr, Sabahi, & Miladi-Gorji, 2017), and enrichment shows potential as a therapy for drug addiction (Solinas, Thiriet, Chauvet, & Jaber, 2010; Mesa-Gresa, Ramos-Campos, & Redolat, 2013). Therefore, we hypothesized that preconception enrichment concurrent with nicotine exposure could mitigate any observable consequences of drug use.

To investigate how these two maternal preconception experiences affect brain development of the next generation, we used Golgi-Cox-stained tissue to analyze dendritic spine density in the PFC and parietal cortex. Changes in spine density, as well as spine morphology, are necessary mechanisms of synaptic plasticity that affect how the brain responds to later experience (Carlisle & Kennedy, 2005). However, it is not always possible to directly link changes in spine density with observed behavioral alterations, so caution must be used when attempting to correlate behavior and neuronal structure. Currently no studies have examined how preconception nicotine or enrichment alters spine density; however, both experiences can modify spine density during other developmental stages (Muhmmad et al., 2012; Gibb, Gonzalez, & Kolb, 2014; Mychasiuk, Muhammad, & Kolb, 2014).

Therefore, this study contributes to the literature in three novel ways: 1) identify how consumption of moderate levels of nicotine in adult rats immediately prior to conception impacts offspring development in early life; 2) identify if maternal

preconception enrichment during nicotine exposure mitigates the effects of drug use; and 3) explore how maternal preconception nicotine and enrichment impact the development of offspring brain in terms of dendritic spine density.

2.2 Methods

2.2.1 Animals

All procedures were approved by the University of Lethbridge Animal Welfare Committee and the Canadian Council on Animal Care. Parental animals were born in-house (3rd generation) from animals brought in from Charles River Laboratories. A total of 45 female Long Evans rats were bred with 45 male Long Evans between January 2017 and August 2018. Parents came from 11 different litters to achieve sufficient genetic diversity. Thirty-two breeding pairs led to successful pregnancies, resulting in 351 pups. All parents and offspring generated were handled daily and cages were cleaned twice weekly. All animals were maintained in a temperature- (21ºC) and humidity-controlled (50%) breeding room for the duration of the experiment and provided food and water *ad libitum* unless otherwise specified.

2.2.2 Maternal Experience

Female rats were randomly assigned to one of four treatment groups: sucralose/pair-housed (n = 10), nicotine/pair-housed (n = 10), sucralose/enriched (n = 12), and nicotine/enriched (n = 13). Females were divided into their housing conditions on P45 (adolescence). Pair-housed females were housed with one other female from the same drug condition in standard laboratory-style cages (24cm x 45cm x 20.5cm). Standard cages included a black PVC pipe for shelter and paper towel strips for nesting (Figure 2.1A). Enriched females were housed in large, multi-level “condos” (1.2m x 0.6m

x 1.8m) with 6 or 7 females from the same drug condition per condo and many stimulating objects that were changed weekly (e.g. plastic shelters, infant teething toys). Condos were of metal construction, had 3 levels connected by ramps, and climbable walls (Figure 2.1B). Six weeks into the housing treatment, baseline water consumption was assessed for each cage by weighing the water bottles at the same time each day for one week and dividing the change in volume by the number of animals in the cage. Nicotine administration began following baseline assessment (i.e. seven weeks into housing treatment), and continued for seven weeks while females remained in their respective housing condition. Seven weeks is the approximate length of the spermatogenic cycle in male Long-Evans rats (Jamerson, Wulser, & Kimler, 2004), and was chosen to mirror the paternal studies conducted using the same paradigm (unpublished data). Nicotine (Sigma, (-)-nicotine hydrogen tartrate salt) was administered as 15mg per liter of drinking water; this concentration is comparable to that used in other studies on oral administration of nicotine in rats (Nesil, Kanit, Collins, & Pogun, 2011). The drinking water was sweetened with 1% sucralose as rats have demonstrated an aversion to the bitterness of nicotine (Collins, Pogun, Nesil, & Kanit, 2012). Animals in both the nicotine and control conditions received sucralose in their water. Water bottles were weighed daily at the same time to measure water intake for the cage over a 24hr period. It is worth noting that animals were not singly housed during dosing; nicotine administration occurred for seven weeks, and single-housing for such an extended time would cause significant distress to the animals and be counter-indicative to enriched housing. Therefore, we can only calculate *average* nicotine intake for each treatment group, not each animal. Nicotine intake (mg/kg body weight) was calculated as follows:

$$\text{L water consumed on average} \times \frac{\text{15mg nicotine}}{\text{L water}} \times \frac{1}{\text{kg body weight of average animal in group}}$$

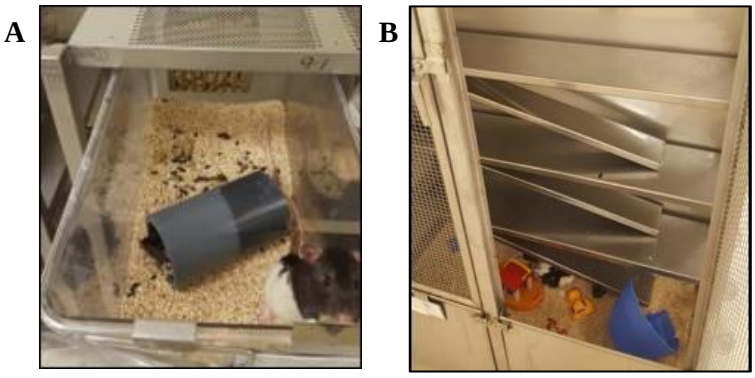

Figure 2.1: Depiction of standard, pair-housed housing condition (A) and enriched housing condition (B).

2.2.3 Breeding

Following the final day of nicotine exposure, all females in both housing conditions were supplied with plain, unsweetened water and pair-housed with a naïve male Long-Evans rat in a standard cage for 10 days. During this time, rats were not disturbed aside from twice weekly cage cleaning. Males were then removed, and females were pair-housed with a female from the same drug and housing treatment group. Females were weighed daily from this point to confirm and monitor pregnancy. Females were separated from their cage-mate once three weeks had elapsed since being paired with the males in preparation for delivery. Thirty-two dams became pregnant and delivered successfully: 1) sucralose/pair-housed n = 8 (55 females, 33 males); 2) nicotine/pair-housed n = 6 (39 females, 39 males); 3) sucralose/enriched n = 10 (47

females, 56 males); and 4) nicotine/enriched $n = 8$ (36 females, 46 males). Dams and their litters were left undisturbed until P7, at which time pups were sexed and daily weighing commenced.

2.2.4 Offspring Behavior

2.2.4.1 Maternal Care

Maternal care was recorded on P8, P12, and P16 for two 15min sessions, one in the morning (9:00am) and one in the afternoon (3:00pm). Recordings took place in the animal's home-cage in the housing room, although the cage was relocated within the room to a filming location that was separated from other animals. After the cage was relocated, the dam and litter were given 5min to return to their natural behavior before filming began. A camera on a tripod was directed at the side of the home-cage. Videos were double scored by two assistants blind to the treatment groups for nursing behaviors (arch-back nursing, blanket-posture nursing, and passive-posture nursing), licking and grooming, and non-contact. Morning and afternoon sessions were summed for the analysis.

2.2.4.2 Negative Geotaxis

Pups were assessed in negative geotaxis on P9 and P10. Negative geotaxis is a test of sensorimotor development and monitors the maturation of the vestibular system (Motz & Alberts, 2005). Each day, pups were individually placed facing downwards on a 40º incline constructed of Plexiglas® and covered in rubber mesh and filmed for 1min (Figure 2.2). The purpose of the task was for the pup for correct its orientation by rotating around the long axis of the body. The pup was replaced in the original position if it fell from the platform or touched the table surface. Videos were double scored by two

assistants blind to the treatment groups for the length of time the pup spent rotated above the horizontal plane and the number of falls from the platform.

Figure 2.2: Apparatus and filming of negative geotaxis, an assessment of early sensorimotor development in rodents.

2.2.4.3 Open Field

Pups were assessed for open field exploration on P10 – 13 and P15. Each day, pups were individually placed in the center of a Plexiglas® box that measured 20cm x 30cm and had a grid of 10 x 15 2cm x 2cm squares drawn on the floor surface (Figure 2.3). Pups were filmed for 1min by an overhead camera while they explored the field. The field was cleaned with 1% Virkon® between each animal. Videos were double scored by two assistants blind to the treatment groups for the number of novel squares and the total number of squares entered by each front paw. Novel squares were unique squares entered by the pup, up to a maximum of 150 (i.e. 10 x 15). Total squares were equivalent to the number of forelimb movements from one square to another. The proportion of time spent in the inner and outer regions of the open field was also

tabulated. Inner squares constituted the interior 6 x 11 rectangle, and outer squares consisted of the 2-square wide perimeter of the open field.

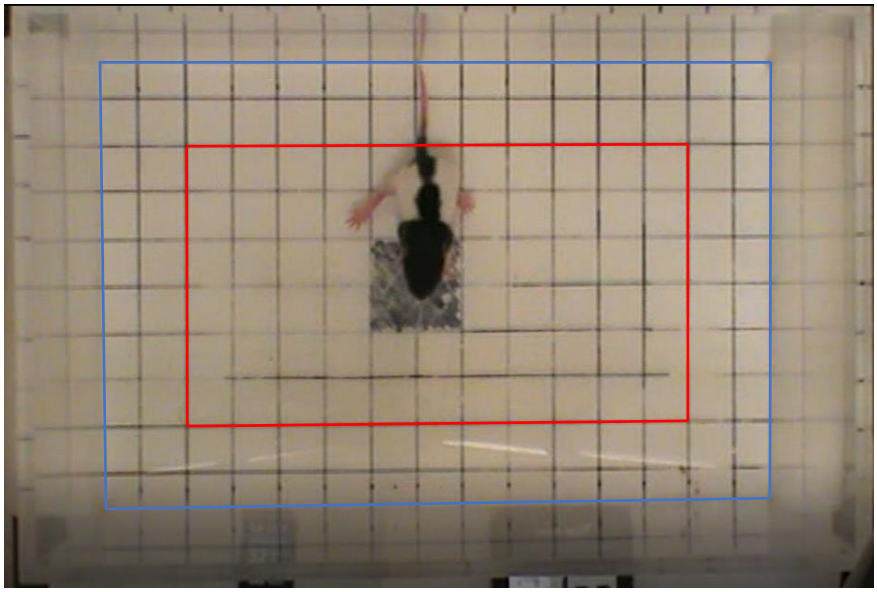

Figure 2.3: Arrangement of the open field. The inner squares are outlined in red, and the outer squares are outlined in blue.

2.2.5 Offspring Neuroanatomy

2.2.5.1 Euthanasia and Perfusion

A random subset of pups (n = 63) sampled across all litters (5 – 8 litters/treatment/sex) was euthanized on P21; remaining pups were used in other analyses and are discussed in Chapter 3. Pups were administered an overdose of pentobarbital sodium (i.p.; 300mg/kg) and in the absence of hindlimb reflexes, intracardially perfused with ~50mL of 0.9% saline solution. Pups were then decapitated, and their brains were removed, weighed, and placed in Golgi-Cox solution in dark (light-blocking) bottles for two weeks. The Golgi-Cox solution was replaced with 30% sucrose for at least two weeks prior to the brains being sectioned on a vibratome at 200µm. Every section of

brain tissue was placed on gelatin-coated (2%) slides, then stained as per Gibb & Kolb (1998).

2.2.5.2 Spine Density

Basilar dendritic spine density was estimated for pyramidal cells in the orbitofrontal cortex (OFC; layer III AID), medial PFC (layer V Cg3), and parietal cortex (layer III Par1) according to Zilles (1985). Dendrites were traced at 1250x using a camera lucida if they satisfied the following criteria: 1) at least 30μm long; 2) terminal; 3) at least third branch order; 4) seemingly complete (untruncated); and 5) unobstructed by blood vessels, cells, or staining artefacts. Preferably five but no less than three dendrites per area were drawn for each hemisphere to ensure an accurate representation of the spine density for the area; the 3 – 5 dendrites per hemisphere per area were averaged and this average was the unit of analysis. Drawings were scanned and analyzed by an assistant blind to the treatment groups using the Cell Counter plugin for ImageJ (https://imagej.nih.gov/ij/plugins/cell-counter.html). Spine density was expressed as the number of spines per 10μm length of dendrite.

2.2.6 Statistical Analysis

All statistical analysis was completed using IBM SPSS 27. For the analysis of behavioral data, scores for males and females within a litter were averaged to yield one male value and one female value per litter to control for litter effects. Therefore, 32 values were included in the analysis of female pup behavior, and 31 in the analysis of male pup behavior (one sucralose/pair-housed litter contained no male pups). Data was similarly averaged for pup body and brain weight, except not all litters had a representative male and female. Neuroanatomical data did not have to be averaged in this

manner as only five male and five female brains were drawn for each treatment group, each from a different litter. An *a priori* decision was made to analyze female and male offspring data separately due to the frequency of sex-dependent effects following nicotine exposure (Peters & Tang, 1982; Pauly, Sparks, Hauser, & Pauly, 2004; Polli et al., 2020). Data was analyzed using factorial or mixed analysis of variance (ANOVA) or analysis of covariance (ANCOVA) as appropriate. The Bonferroni correction was used to control for multiple comparisons. Significance level was $\alpha = .05$.

2.3 Results

2.3.1 Maternal Nicotine Consumption

The Greenhouse-Geisser value of epsilon was used to correct for departures from sphericity ($\varepsilon = .329$). Mixed ANOVA revealed a significant difference in the daily volume of water consumed throughout the nicotine exposure (Figure 2.4). There was a significant Week x Drug interaction, $F(2.304, 55.301) = 7.036$, $p = .001$, $\eta_P^2 = .227$; sucralose dams drank more water than nicotine dams during week 1, $F(1\ 24) = 17.924$, $p < .001$, $\eta_P^2 = .428$, week 2, $F(1, 24) = 16.915$, $p < .001$, $\eta_P^2 = .431$, week 4, $F(1, 24) = 9.715$, $p = .040$, $\eta_P^2 = .288$, week 5, $F(1, 24) = 14.240$, $p = .008$, $\eta_P^2 = .371$, and week 7, $F(1, 24) = 22.420$, $p < .001$, $\eta_P^2 = .483$ of nicotine dosing. There was no difference in water intake between sucralose and nicotine dams during the baseline assessment, $F(1, 24) = 0.946$, $p = 1.000$, $\eta_P^2 = .038$, week 3, $F(1, 24) = 1.936$, $p = 1.000$, $\eta_P^2 = .075$, or week 6, $F(1, 24) = 6.939$, $p = .120$, $\eta_P^2 = .224$, of dosing. There was also a significant Drug x Housing interaction, $F(1, 24) = 31.989$, $p < .001$, $\eta_P^2 = .571$; enriched sucralose dams consumed, on average, more water than pair-housed sucralose dams, $F(1, 24) =$

32.444, $p < .001$, $\eta_P^2 = .575$, but there was no effect of housing among the nicotine-treated dams, $F(1, 24) = 5.302$, $p = .060$, $\eta_P^2 = .181$.

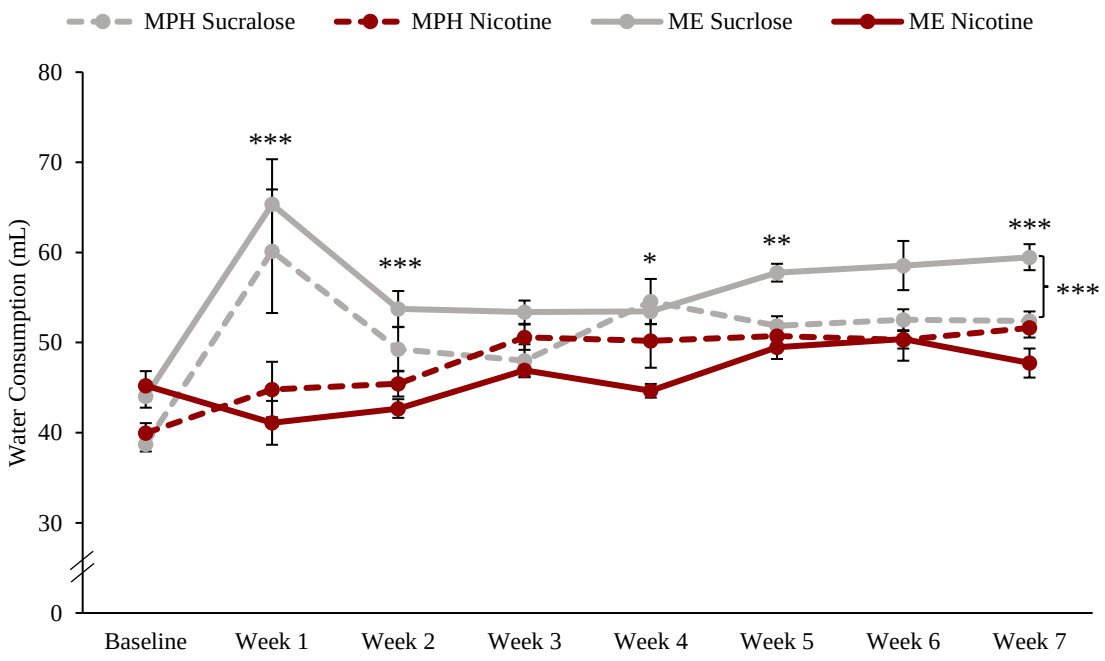

Figure 2.4: Average weekly water consumption (mL) of dams based on drug (sucralose or nicotine) and housing condition (MPH = maternal pair-housed, ME = maternal enrichment). Sucralose dams consumed more water than nicotine dams during week 1, 2, 4, 5, and 7. Overall, ME sucralose dams consumed more water than MPH sucralose dams. Data shown as mean ± *SE*. * $p \leq .05$; ** $p \leq .01$, *** $p \leq .001$

Two-way ANOVA revealed that nicotine had a significant impact on weight gain throughout the dosing paradigm, $F(1, 24) = 16.581$, $p < .001$, $\eta_P^2 = .413$; nicotine-treated dams gained significantly less weight than sucralose dams between baseline water assessment and week 7 of dosing. Nicotine-treated dams also consumed sucralose in their drinking water, meaning this was not a sucralose-induced weight increase in the drug

control group. There was no main effect of housing, $F(1, 24) = 2.321$, $p = .141$, $\eta_P^2 = .088$, nor a Drug x Housing interaction, $F(1, 24) = 2.707$, $p = .113$, $\eta_P^2 = .101$ (Figure 2.5).

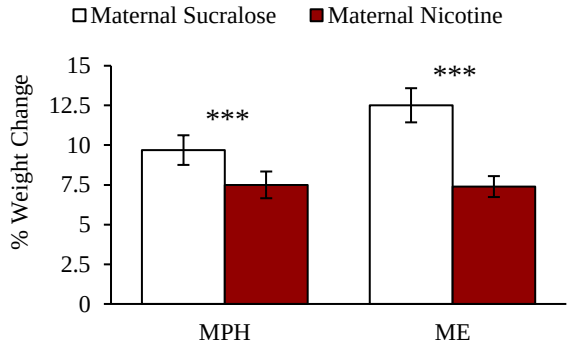

Figure 2.5: Percent increase in weight between baseline and the end of nicotine dosing. Sucralose dams gained significantly more weight than nicotine dams regardless of housing condition. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment. *** $p \leq .001$.

Mixed ANOVA revealed that the week of dosing had no influence on the quantity of nicotine consumed; therefore, a one-way ANOVA with housing as the between-subjects factor was used. There was a significant main effect of housing, $F(1, 12) = 30.630$, $p < .001$, $\eta_P^2 = .719$, such that pair-housed dams consumed more nicotine per kg of body weight than enriched dams (Figure 2.6). On average, nicotine exposed dams consumed between approximately 2.3 and 2.5mg/kg/day. We did not assess plasma nicotine or cotinine levels in this experiment, but previous work suggests that the dose of nicotine consumed here corresponds with that of a habitual smoker, which was our intention (Schneider, Bizarro, Asherson, & Stolerman, 2010).

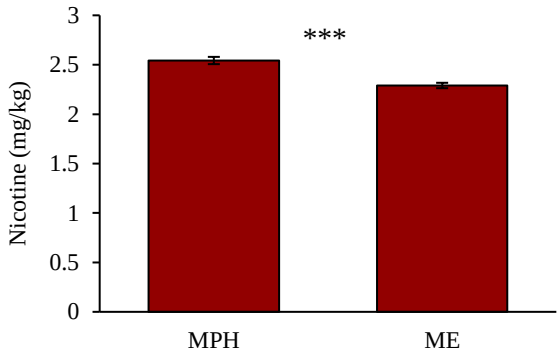

Figure 2.6: Overall quantity of nicotine consumed (mg) per kg of body weight. Pair-housed dams consumed significantly more nicotine than enriched dams. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment. *** $p \leq .001$.

2.3.2 Litter Characteristics

Thirty-two of 45 mating pairs resulted in a successful litter: eight sucralose/pair-housed (80% successful), six nicotine/pair-housed (60% successful), 10 sucralose/enriched (83% successful), and eight nicotine/enriched (62% successful). There were no significant main effects of maternal drug, $F(1, 28) = 0.480$, $p = .494$, $\eta_P^2 = .017$, or maternal housing, $F(1, 28) = 1.501$, $p = .231$, $\eta_P^2 = .051$, nor an interaction for the number of pups in a litter (Table 2.1). There was no difference in the percent male offspring per litter due to maternal drug, $F(1, 28) = 3.266$, $p = .081$, $\eta_P^2 = .104$, there was a nearly significantly effect of maternal housing, $F(1, 28) = 4.101$, $p = .052$, $\eta_P^2 = .128$, and no interaction, $F(1, 28) = 1.929$, $p = .176$, $\eta_P^2 = .064$ (Table 2.1).

Table 2.1: Summary data for litter size and percent male offspring per treatment group.

	Litter Size		Percent Male	
	M	*SE*	*M*	*SE*
Pair-Housed				
Sucralose	11.00	1.31	32.96	7.19
Nicotine	13	0.73	49.79	4.42
Enriched				
Sucralose	10.30	1.36	50.94	4.41
Nicotine	10.25	1.64	53.14	3.74

2.3.3 Maternal Care

There were no significant effects of drug treatment or housing condition on the quality of maternal care on P8, P12, or P16 (Figure 2.7). A 3x2x2 Mixed ANOVA with pup age as a within-subject factor and dam drug and housing condition as between-subjects factors revealed that there was no overall effect of nicotine exposure, $F(1, 28) = 1.490$, $p = .232$, $\eta_P^2 = .051$, or enriched housing, $F(1, 28) = 0.355$, $p = .556$, $\eta_P^2 = .013$, on the combined time spent nursing (any position), nor was there a significant Drug x Housing interaction, $F(1, 28) = 0.370$, $p = .548$, $\eta_P^2 = .013$. This was also observed for the time spent licking and grooming pups; dams spent an equivalent amount of time grooming their pups regardless of drug treatment, $F(1, 28) = 0.036$, $p = .852$, $\eta_P^2 = .001$, and housing condition, $F(1, 28) = .828$, $p = .544$, $\eta_P^2 = .013$, and there was no Drug x Housing interaction, $F(1, 28) = 0.048$, $p = .828$, $\eta_P^2 = .002$. Finally, there was no significant main effect of nicotine, $F(1, 28) = 0.895$, $p = .352$, $\eta_P^2 = .031$, or enrichment, $F(1, 28) = 0.362$, $p = .552$, $\eta_P^2 = .013$, nor a Drug x Housing interaction, $F(1, 28) = 0.056$, $p = .814$, $\eta_P^2 = .002$, on the time spent in non-contact with pups. There was a significant

main effect of age for time spent not in contact with pups, $F(2, 56) = 4.593$, $p = .014$, $\eta_P^2 = .141$; Bonferroni *post hoc* tests revealed that dams spent significantly more time in contact with their pups on P16 compared to both P8, $t(28) = 2.827$, $p = .026$, and P12, $t(28) = 2.847$, $p = .024$. There were no other significant main effects of age or interactions involving age for any measure (p's > .05).

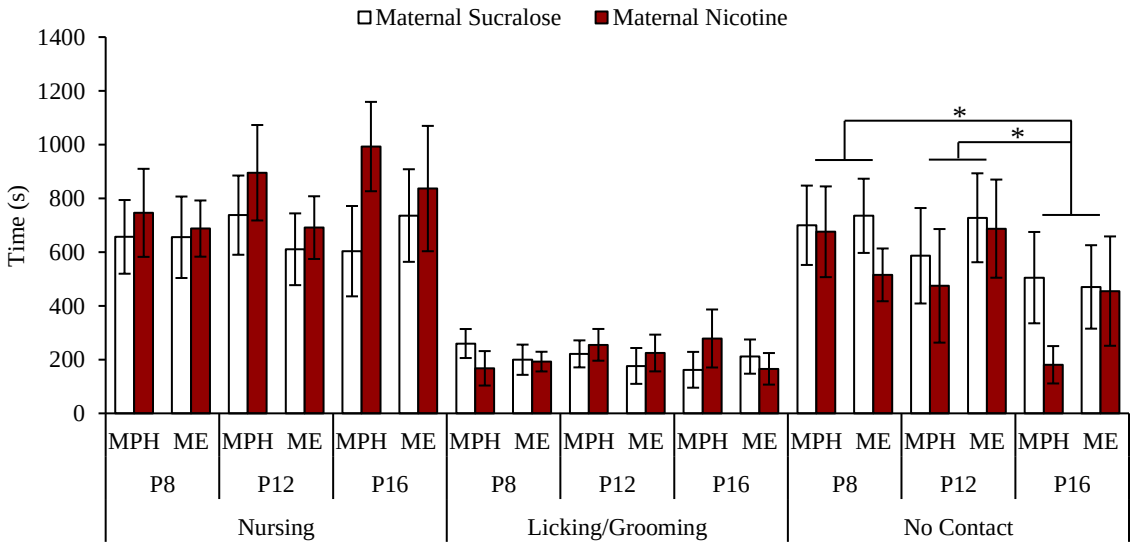

Figure 2.7: Time spent engaged in maternal care behaviors across three days of recording. All dams spent more time in contact with their pups on P8 and P12 relative to P16. There were no effects of maternal drug or housing. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment. * $p \leq .05$

2.3.4 Negative Geotaxis

A 2x2x2 Mixed ANOVA with pup age (P9 or P10) as a within-subject factor and maternal drug and housing condition as between-subjects factors revealed a significant main effect of maternal drug on the length of time (s) spent rotated above the horizonal

plane for male pups, $F(1, 27) = 6.919$, $p = .014$, $\eta_P^2 = .204$; regardless of age or maternal housing condition, male pups of dams exposed to preconception nicotine spent significantly less time rotated upright than male pups of sucralose-exposed dams. However, there was no main effect of maternal housing, $F(1, 27) = 0.362$, $p = 552$, $\eta_P^2 = .013$, or a Drug x Housing interaction, $F(1, 27) = 1.366$, $p = .253$, $\eta_P^2 = .048$, for male pups. For the time spent rotated upright in female pups, there were no significant main effects of maternal drug, $F(1, 28) = 2.592$, $p = .119$, $\eta_P^2 = .085$, or housing, $F(1, 28) = 2.149$, $p = .154$, $\eta_P^2 = .071$, nor was there a Drug x Housing interaction, $F(1, 28) = 0.906$, $p = .349$, $\eta_P^2 = .031$ (Figure 2.8A).

There was also a significant main effect of maternal drug for the number of falls off the platform for male pups; preconception nicotine-exposed male pups had to be replaced on the inclined plane more times than their sucralose-exposed counterparts, $F(1, 27) = 5.393$, $p = .028$, $\eta_P^2 = .166$. There was no main effect of housing for male pups, $F(1, 27) = 0.193$, $p = .664$, $\eta_P^2 = .007$, or a Drug x Housing interaction, $F(1, 27) = 2.414$, $p = .132$, $\eta_P^2 = .082$. There were no significant main effects of maternal drug, $F(1, 28) = 1.479$, $p = .234$, $\eta_P^2 = .050$, or housing, $F(1, 28) = 3.337$, $p = .078$, $\eta_P^2 = .106$, nor a Drug x Housing interaction, $F(1, 28) = 0.100$, $p = .754$, $\eta_P^2 = .004$, for female offspring (Figure 2.8B).

There was a significant main effect of age for both females, $F(1, 28) = 22.488$, $p < .001$, $\eta_P^2 = .445$, and males, $F(1, 27) = 36.141$, $p < .001$, $\eta_P^2 = .572$, for time spent rotated upright; pups of both sexes spent significantly longer in an upright position on P10 than on P9. The effect was also observed for the number of falls off the platform; females, $F(1, 28) = 17.599$, $p < .001$, $\eta_P^2 = .386$, and males, $F(1, 27) = 34.318$, $p < .001$, $\eta_P^2 = .560$,

fell off the platform significantly fewer times on P10 than on P9. There were no significant interactions with age for either variable in male or female pups (p's > .05).

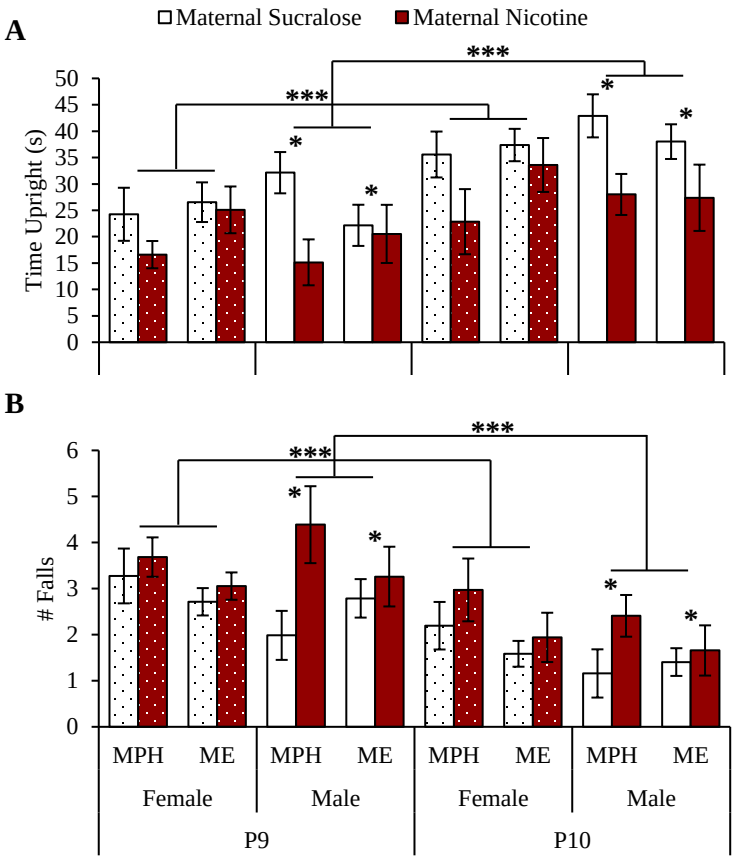

Figure 2.8: (A) Amount of time spent rotated upright and (B) the number of falls off the platform during negative geotaxis. There were significant main effects of age for both females and males; P10 offspring spent more time rotated upright and fell off the platform fewer times than P9 offspring. Male offspring were impaired by maternal nicotine in that they spent less time rotated upright and fell off the platform more times than male offspring of sucralose dams. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enriched; P = postnatal day. * $p \leq .05$; *** $p \leq .001$.

2.3.5 Open Field

Three-way mixed ANOVA with pup age as a within-subject factor and maternal drug and housing as between-subject factors was conducted for female and male offspring for the number of novel squares and total squares entered in the open field. Greenhouse-Geisser-corrected degrees of freedom are presented to adjust for departures from sphericity. For female offspring novel squares, there was a significant Age x Housing interaction, $F(2.9111, 78.585) = 4.367$, $p = .007$, $\eta_P^2 = .139$. Bonferroni *post hoc* tests revealed that female pups of enriched dams entered significantly fewer unique squares than females born to pair-housed dams on P13, $F(1, 27) = 12.890$, $p = .001$, $\eta_P^2 = .323$, and P15, $F(1, 27) = 5.186$, $p = .031$, $\eta_P^2 = .161$, but that there was no difference on P10, $F(1, 27) = 0.265$, $p = .611$, $\eta_P^2 = .010$, P11, $F(1, 27) = 0.101$, $p = .753$, $\eta_P^2 = .004$, or P12, $F(1, 27) = 0.214$, $p = .647$, $\eta_P^2 = .008$ (Figure 2.9A). There were no main effects of maternal drug for female pups, $F(1, 27) = 2.061$, $p = .163$, $\eta_P^2 = .071$, nor any significant interaction with maternal drug (p's > .05). For male offspring, there was a significant main effect of maternal drug for the number of novel squares, $F(1, 26) = 4.245$, $p = .049$, $\eta_P^2 = .140$; regardless of age, pups born to nicotine-exposed dams entered significantly fewer unique squares than pups born to sucralose dams (Figure 2.9A). There was also a significant main effect of age, $F(3.072, 79.881) = 93.975$, $p < .001$, $\eta_P^2 = .783$, such that pups entered a greater number of unique squares as they grew older. There was no main effect of maternal housing, $F(1, 26) = 0.463$, $p = .502$, $\eta_P^2 = .018$, nor any significant interactions (p's > .05).

For the total number of squares entered, female offspring once again had a significant Age x Housing interaction, $F(2.846, 76.830) = 3.098$, $p = .034$, $\eta_P^2 = .103$

(Figure 2.9B). However, for total squares there was only a significant difference between maternal pair-housed and maternal enrichment on P13, $F(1, 27) = 10.853$, $p = .003$, $\eta_P^2 = .287$; there was no effect of maternal housing on P10, $F(1, 27) = 0.668$, $p = .421$, $\eta_P^2 = .024$, P11, $F(1, 27) = 0.676$, $p = .481$, $\eta_P^2 = .024$, P12, $F(1, 27) = 0.034$, $p = .854$, $\eta_P^2 = .001$, or P15, $F(1, 27) = 3.608$, $p = .068$, $\eta_P^2 = .118$. There was no main effect of maternal drug for female offspring total squares, $F(1, 27) = 0.282$, $p = .600$, $\eta_P^2 = .010$, nor any significant interactions with drug (p's > .05). For male offspring total squares, only the main effect of age was significant, $F(2.889, 75.371) = 43.563$, $p < .001$, $\eta_P^2 = .626$; male pups entered significantly more total squares as they aged (Figure 2.9B). There was no main effect of maternal drug, $F(1, 26) = 1.310$, $p = .263$, $\eta_P^2 = .048$, or maternal housing, $F(1, 26) = 0.032$, $p = .860$, $\eta_P^2 = .001$, nor any significant interactions (p's > .05).

We also analyzed the percent of novel and total squares that resided in the inner portion of the open field as an early indicator of anxiety. We only analyzed this measure for P15 data, as this is the age when most pups have opened their eyes. For female offspring, two-way ANOVA with maternal drug and housing as between-subjects factors revealed no significant main effects of drug, $F(1, 28) = 3.217$, $p = .084$, $\eta_P^2 = .103$, or housing, $F(1, 28) = 0.263$, $p = .612$, $\eta_P^2 = .009$, nor an interaction, $F(1, 28) = 0.161$, $p = .691$, $\eta_P^2 = .006$, for the percent inner novel squares. Likewise for percent inner total squares, there were no effects of drug, $F(1, 28) = 0.509$, $p = .482$, $\eta_P^2 = .018$, housing, $F(1, 28) = 0.042$, $p = .839$, $\eta_P^2 = .002$, or an interaction, $F(1, 28) = 0.053$, $p = .820$, $\eta_P^2 = .002$. For male offspring percent inner novel squares, there were no main effects of drug, $F(1, 27) = 2.512$, $p = .125$, $\eta_P^2 = .085$, or housing, $F(1, 27) = 0.156$, $p = .696$, $\eta_P^2 = .006$, nor an interaction, $F(1, 27) = 0.416$, $p = .524$, $\eta_P^2 = .015$. Similarly, there were no effects

of drug, $F(1, 7) = 2.741$, $p = .109$, $\eta_P^2 = .092$, housing, $F(1, 27) = 0.025$, $p = .876$, $\eta_P^2 = .001$, or an interaction, $F(1, 27) = 0.014$, $p = .908$, $\eta_P^2 = .001$, for the percent inner total squares (Figure 2.9C).

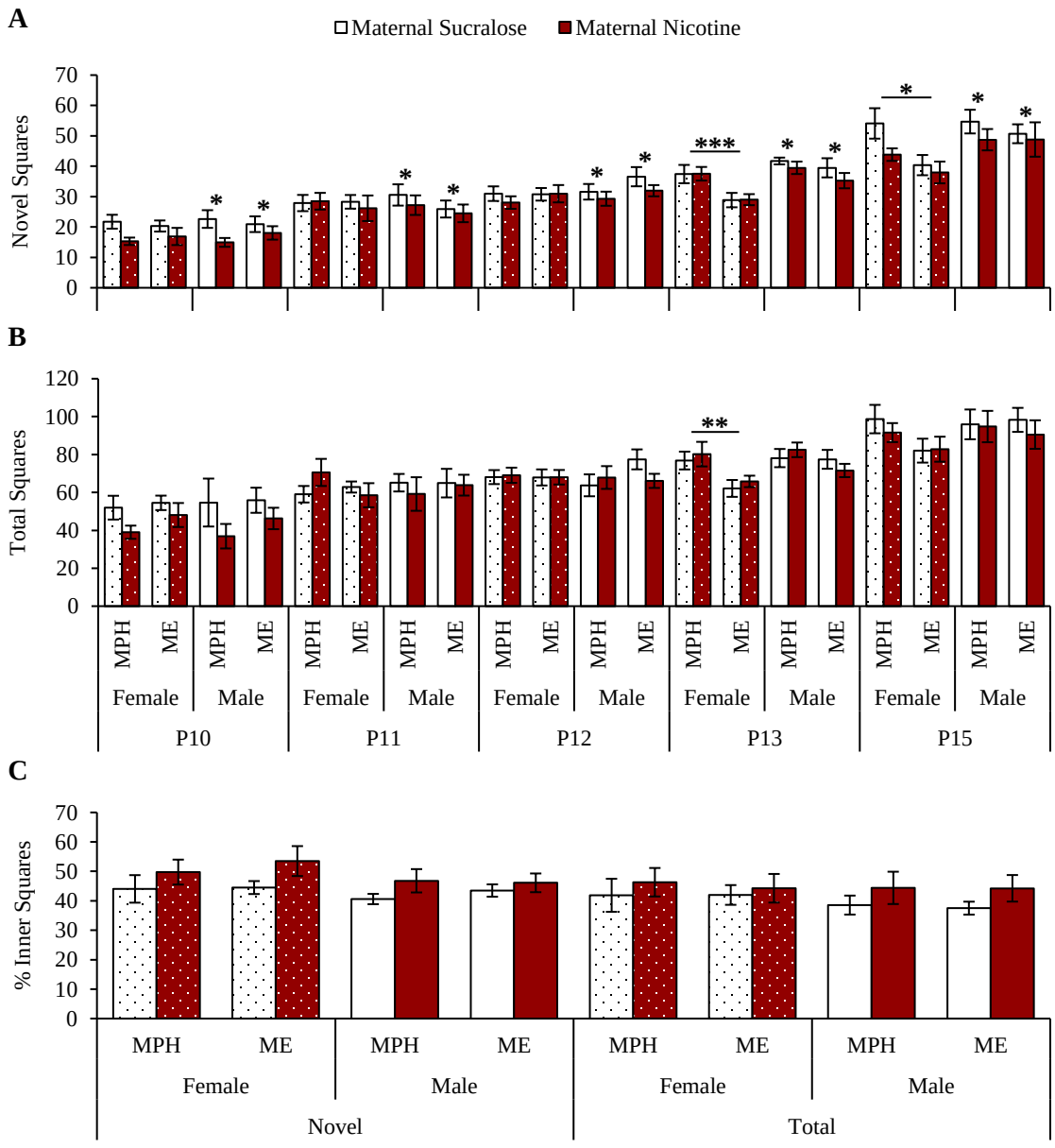

Figure 2.9: Exploratory and locomotor behavior of developing pups in the open field. (A) Female pups (patterned) born to enriched dams entered fewer novel squares on P13 and

P15 than females born to pair-housed dams. Overall, male pups (solid) born to nicotine-exposed dams entered fewer novel squares than males born to sucralose dams. (B) For total squares, female pups from enriched dams entered fewer total squares only on P13. (C) There were no differences among the percent of novel or total squares that were located within the inner portion of the open field. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment; P = postnatal day. * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$

2.3.6 Pup Weight

A 2x2 factorial ANOVA of female offspring P21 body weight revealed no significant main effects of maternal drug, $F(1, 24) = 0.817$, $p = .375$, $\eta_P^2 = .033$, or maternal housing, $F(1, 24) = 3.228$, $p = .085$, $\eta_P^2 = .119$, nor a Drug x Housing interaction, $F(1, 24) = 3.432$, $p = .076$, $\eta_P^2 = .125$. Likewise, there were no effects of drug, $F(1, 22) = 0.194$, $p = .664$, $\eta_P^2 = .009$, housing, $F(1, 22) = 1.618$, $p = .217$, $\eta_P^2 = .068$, or an interaction, $F(1, 22) = 1.121$, $p = .301$, $\eta_P^2 = .048$, for male P21 body weight (Figure 2.10A).

Factorial ANCOVA of female P21 brain weight revealed that body weight was a significant covariate, $F(1, 23) = 33.285$, $p < .001$, $\eta_P^2 = .591$, but there was no main effect of drug, $F(1, 23) = 0.891$, $p = .355$, $\eta_P^2 = .037$, or housing, $F(1, 27) = 0.028$, $p = .869$, $\eta_P^2 = .001$, nor a Drug x Housing interaction, $F(1, 23) = 0.004$, $p = .953$, $\eta_P^2 < .001$, for brain weight adjusted by body weight. Male P21 body weight was also a significant covariate of brain weight, $F(1, 21) = 19.552$, $p < .001$, $\eta_P^2 < .001$, but there were again no significant main effects of drug, $F(1, 21) = 0.017$, $p = .899$, $\eta_P^2 = .001$, or housing, $F(1,$

21) = 1.717, p = .204, η_P^2 = .076, nor an interaction, $F(1, 21)$ = 0.042, p = .840, η_P^2 = .002 (Figure 2.10B).

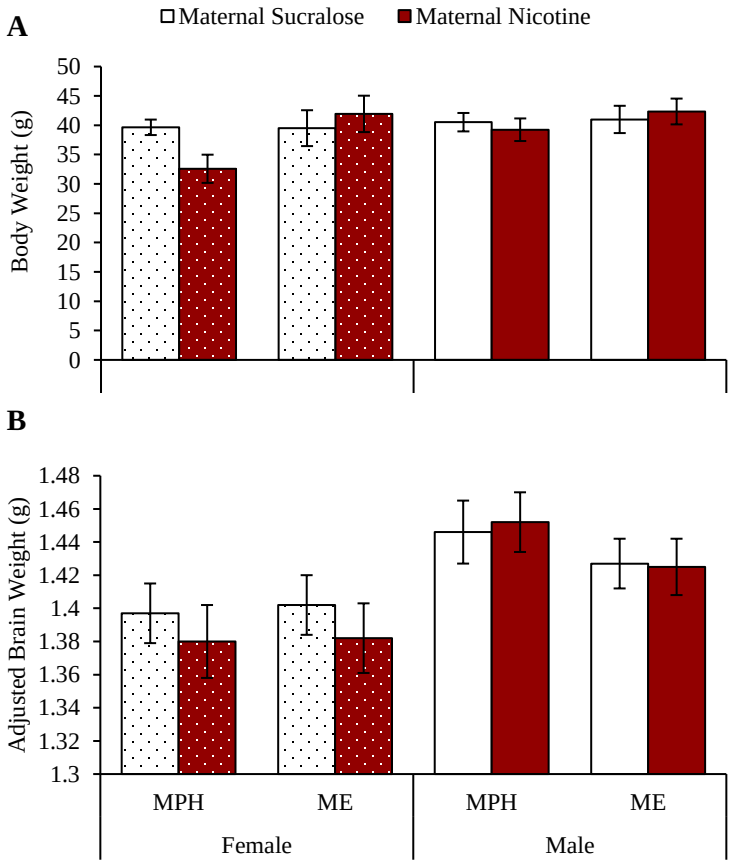

Figure 2.10: (A) P21 body weight and (B) brain weight adjusted by body weight for offspring. There were no significant differences. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment.

2.3.7 Pup Spine Density

Three-way ANOVA with maternal drug and housing and pup hemisphere as between-subject factors revealed no significant main effects or interactions with

hemisphere, so right and left spines densities were averaged, and a two-way ANOVAs were used.

2.3.7.1 AID

There was a significant main effect of maternal housing for both female, $F(1, 16) = 6.998$, $p = .018$, $\eta_P^2 = .304$, and male, $F(1, 16) = 7.676$, $p = .013$, $\eta_P^2 = .327$, offspring; for both sexes of offspring, maternal enrichment increased basilar dendritic spine density compared to maternal pair-housing (Figure 2.11). There were no significant main effects of maternal drug for females, $F(1, 16) = 0.516$, $p = .483$, $\eta_P^2 = .031$, or males, $F(1, 16) = 0.729$, $p = .406$, $\eta_P^2 = .044$. There was no interaction between maternal drug and housing for female offspring, $F(1, 16) = 0.382$, $p = .545$, $\eta_P^2 = .023$, but a trending interaction for male offspring, $F(1, 16) = 4.043$, $p = .062$, $\eta_P^2 = .202$.

2.3.7.2 Cg3

There were no significant main effects of maternal drug, $F(1, 16) = 0.036$, $p = .852$, $\eta_P^2 = .002$, or maternal housing, $F(1, 16) = 2.342$, $p = .145$, $\eta_P^2 = .128$, nor a Drug x Housing interaction, $F(1, 16) = 0.394$, $p = .539$, $\eta_P^2 = .024$, for female offspring. Likewise, there were no main effects, $F(1, 16) = 2.264$, $p = .152$, $\eta_P^2 = .124$ for maternal drug and $F(1, 16) = 1.772$, $p = .202$, $\eta_P^2 = .100$ for maternal housing, or an interaction, $F(1, 16) = 0.004$, $p = .948$, $\eta_P^2 = .000$, for male offspring (Figure 2.11).

2.3.7.3 Par1

There were no significant main effects of maternal drug, $F(1, 16) = 3.800$, $p = .069$, $\eta_P^2 = .192$, or maternal housing, $F(1, 16) = 0.426$, $p = .523$, $\eta_P^2 = .026$, for female offspring, nor a significant interaction, $F(1, 16) = 0.465$, $p = .505$, $\eta_P^2 = .028$. In male offspring, there was a significant Drug x Housing interaction, $F(1, 16) = 5.768$, $p = .029$,

η_P^2 = .265, but follow-up tests lacked sufficient power to detect any significant simple effects; there was a nearly significant increase in spine density due to maternal enrichment among sucralose offspring, $F(1, 16) = 3.247$, $p = .090$, $\eta_P^2 = .169$, and a slight yet insignificant decrease among nicotine offspring, $F(1, 16) = 2.543$, $p = .130$, $\eta_P^2 = .137$. There was no main effect of maternal drug, $F(1, 16) = 0.006$, $p = .940$, $\eta_P^2 = .000$, or maternal housing, $F(1, 16) = 0.022$, $p = .885$, $\eta_P^2 = .001$, for male offspring.

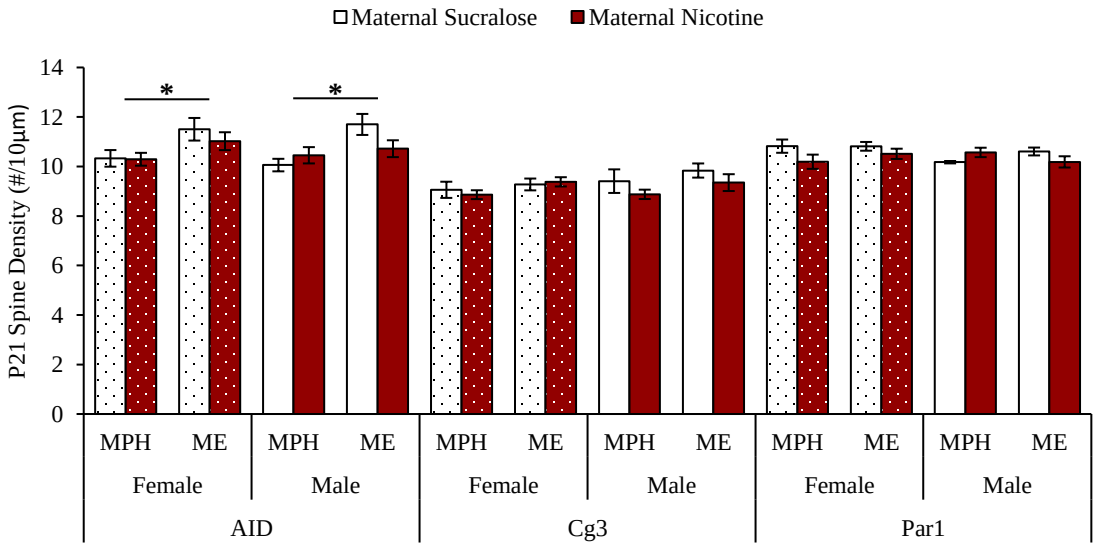

Figure 2.11: P21 basilar spine density in the OFC (AID), medial PFC (Cg3), and parietal cortex (Par1). There was a significant increase in density due to maternal enrichment in AID for both sexes. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment. * $p \leq .05$

2.4 Discussion

This work demonstrates that chronic maternal preconception nicotine and enrichment impact the early development of offspring brain and behavior without altering

the quality of maternal care. Specifically, we report: 1) No effect of maternal nicotine or enrichment on maternal care; 2) male pups have stunted sensorimotor development as assessed by negative geotaxis, but there is no effect for female pups; 3) exploratory behavior of female pups is reduced by maternal enrichment as a function of age, and male pup exploratory behavior is reduced by maternal nicotine regardless of age; 4) no effects on P21 body weight or adjusted brain weight; and 5) an increase in OFC dendritic spine density in offspring of both sexes due to maternal enrichment.

2.4.1 Maternal Nicotine Intake and Pregnancy Success

Pair-housed dams exposed to nicotine consumed significantly more nicotine (2.54mg/kg) than enriched nicotine dams (2.29mg/kg). This was true despite there being no difference in the volume of water consumed due to the enriched dams being slightly larger (~4%) than the pair-housed dams throughout the dosing paradigm. The enriched sucralose dams were similarly larger than the pair-housed sucralose dams.

Dams in the nicotine treatment groups had reduced fertility (60 and 62% successful) compared to sucralose dams (80 and 83% successful). Impaired fertility is a common consequence of nicotine exposure; cigarette smoking is associated with increased infertility, lowered fecundity, and increased spontaneous abortion risk (Mai et al., 2014). Cigarette smoke reduces the quality of the oocyte (Mai et al., 2014) and interferes throughout the early stages of pregnancy, from ovulation to implantation (Talbot & Lin, 2011). Therefore, it is not surprising that the nicotine-treated dams were less successful at becoming and remaining pregnant. Interestingly, offspring prenatally exposed to nicotine continue to display decreased fecundity in terms of the length of time required to become pregnant and levels of ovarian hormones (Holloway, Kellenberger, &

Petrik, 2006). Future work should investigate how preconception nicotine affects fertility in subsequent generations.

2.4.2 Neither Maternal Experience Impacted Maternal Care

We found that neither the negative experience, chronic exposure to nicotine, nor the positive experience, enriched housing, had any impact on the quality of maternal care. Regardless of their treatment condition, dams spent an equal length of time engaged in nursing, licking and grooming, and contact with pups. This is the ideal result for the current study, as it allows us to reasonably eliminate behavioral transmission as a potential driving force behind the alterations in pup development in favor of physiological or epigenetic causes (Bohacek & Mansuy, 2013; Chastain & Sarkar, 2017). However, this result is somewhat unexpected given the current literature into these experiences, especially enrichment. Several authors have reported that enriched environments, including prior to conception, benefit maternal behavior (Cutuli et al., 2015, 2017; Sparling, Barbeau, Boileau, & Konkle, 2020), although the benefits are less pronounced than when the enrichment occurs prenatally (Sparling et al., 2020). The impacts of nicotine on maternal care are less clear. To our knowledge, there are no studies into preconception nicotine and maternal care. Following simultaneous prenatal nicotine and ethanol, some have reported minor deficits in care accompanied by a decrease in oxytocin (McMurray et al., 2008). Nicotine exposure during lactation has been found to have no impact on maternal care (Heath, Horst, & Picciotto, 2010) or even slightly improve maternal care (Chirico, et al., 2017). Therefore, the current finding is valuable in contributing to a presently limited understanding of how preconception experiences impact maternal care. In line with other studies, we did not observe that

either experience impacted the average litter size or the percent of male offspring per litter (Caporali et al., 2014; 2015; Cutuli et al., 2015; Zhu et al., 2017).

2.4.3 Maternal Preconception Nicotine Impaired Sensorimotor Development in Male Offspring

The male pups born to dams exposed to preconception nicotine were impaired at negative geotaxis compared to sucralose-exposed male pups, regardless of age or maternal housing condition; nicotine male pups spent less time rotated upright and fell off the platform more times on both P9 and P10. Interestingly, there was no effect of maternal treatment for female pups. Prenatal exposure to nicotine in mice impairs the immediate development of motor coordination in offspring; nicotine-exposed offspring are slower to right themselves when placed on their backs and are slower to rotate upright in negative geotaxis (Ajarem & Ahmed, 1998; Schneider et al., 2011). Both studies only examined male offspring, so it is unclear if a similar sex difference as in the present study would be apparent. Also, these authors only examined the latency to rotate upright for negative geotaxis, not the total time rotated upright. Similarly, Vaglenova et al., (2008) demonstrated that prenatal nicotine delayed the emergence of rotating in negative geotaxis in both males and females; whereas all control pups were rotating within 45s by P7, nicotine pups did not reach this criterion until P9 or later. Conversely, using both male and female offspring, others have found that prenatal nicotine has no impact on the righting reflex or latency to turn in negative geotaxis (Lacy, Morgan, & Harrod, 2014). To our knowledge, ours is the first study to investigate the effects of maternal preconception nicotine on sensorimotor development in rodents. It is noteworthy that the consequences mirror those of prenatal nicotine. Nicotine may perturb sensorimotor

development through interfering with cerebellar development, which continues during the first two weeks of life in rodents, or by overstimulating the acetylcholinergic system through activation of nicotinic acetylcholine receptors (nAChR; Blood-Siegfried & Rende, 2010).

We did not observe any effect of maternal enrichment on sensorimotor development in the present study. Similarly, Caporali and colleagues (2014) found no effect of preconception maternal enrichment on the age at which the rotating reflex initially appears in offspring, with it occurring on approximately P6 for both the offspring of standard-housed and enriched dams. One study found that maternal enrichment beginning seven days prior to pregnancy and continuing until parturition reduced the length of time rotated upright in negative geotaxis (Mychasiuk et al., 2012), a seemingly negative consequence. These authors speculated that enrichment slowed maturation in order to facilitate brain plasticity in later life. Indeed, there is considerable evidence that enrichment improves plasticity throughout development (Baroncelli et al., 2009; Gibb, Gonzalez, & Kolb, 2014).

2.4.4 Maternal Preconception Nicotine Reduced Open Field Exploration in Male Pups, and Maternal Preconception Enrichment Reduced Exploration in Older Female Pups

Maternal preconception nicotine exposure resulted in an overall decrease in the exploratory behavior of developing male pups, regardless of pup age or maternal housing condition. However, male pups did not differ with respect to the total number of squares entered in the open field, suggesting that maternal nicotine affected only the initial exploration of the field (i.e. the number of novel squares) and not the total amount of

locomotion. There was no effect of maternal nicotine in female pups. Furthermore, nicotine did not significantly affect the proportion of novel or total squares that were located within the inner portion of the open field on P15, suggesting no difference in thigmotaxis. Developmental nicotine is frequently associated with hyperactivity, and more broadly with ADHD in humans and animal models (Keyes, Smith, & Susser, 2013). However, the majority of studies examine locomotor behavior in adolescent or adult animals, with very few studies analyzing differences in the emergence of locomotion as we have shown here. As we have previously reported, P10 pups exposed to the current maternal preconception nicotine paradigm display hypoactivity relative to controls due to a lack of postural support, immature warm-up sequence, and increased stereotypy (Torabi et al., 2021). One study of prenatal nicotine examined offspring on P19 and found a decrease in activity and stereotypy compared to saline-treated animals, but only during the initial 5min of being placed in the open field (LeSage Gustaf, Dufek, & Pentel, 2006), after which time nicotine animals were indistinguishable from controls. Conversely, when offspring were nearly a week older on P25, Vaglenova and colleagues (2004) found male offspring prenatally exposed to nicotine to be hyperactive, whereas female offspring continued to be hypoactive. Therefore, this limited body of research into the early locomotor consequences of developmental nicotine suggests a sex- and age-dependency, with early hypoactivity being followed by hyperactivity beginning in the juvenile period and continuing into adulthood. Our work (Torabi et al., 2021) suggests that the early deficiency is due to immaturity of the motor and coordination systems, potentially due to nicotine-induced abnormalities in the development of the cerebellum (de Zeeuw et al., 2012). Others have shown that hyperactivity in adolescence and adulthood is associated

with dysregulation of the dopaminergic system following prenatal exposure to nicotine (Zhu et al., 2012; Buck et al., 2019).

Maternal preconception enrichment decreased female offspring exploration of the open field in an age-dependent manner; whereas there was no difference in the number of novel or total squares on the initial days of testing, by P13 females born to enriched dams entered significantly fewer novel and total squares than females born to pair-housed dams. There was no effect of maternal enrichment for male offspring. Likewise, there was no effect of enrichment on the proportion of novel or total squares that were located within the inner portion of the open field on P15. Preconception enrichment has been found to not impact the emergence of locomotory behaviors in offspring, including pivoting, crawling, or quadrupedal movement; these authors found that only complex motor behaviors such as ladder climbing, bridge crossing, or hanging from a wire were improved by enrichment (Caporali et al., 2014). These authors did not assess the degree of locomotion in these animals; instead, they calculated the proportion of animals that had achieved each developmental milestone by each P-day. For example, they found that regardless of maternal preconception housing, 10% of pups were capable of quadrupedal locomotion by P11, 75% by P12, and 100% by P13 (Caporali et al., 2014). It is noteworthy that in the current study P13 is the first day that the exploratory behavior of female pups diverged based on maternal housing. Therefore, it appears that the influence of maternal preconception enrichment is not apparent until after pups have achieved competency in quadrupedal locomotion. After this time, maternal enrichment decreases both exploration of new areas of the open field and total movement. The reason for this decrease is unclear, but it likely does not relate to a developmental deficiency. One study

into prenatal enrichment found that adolescent female offspring (P35), but not males, had decreased activity in an open field due to enrichment (Maruoka et al., 2009), which mirrors the current finding. Others report increased exploration of an open field between P10 and P15 in the offspring of dams exposed to enriched environments from seven days prior to pregnancy until birth (Mychasiuk et al., 2012). Therefore, the timing of the maternal enrichment may be vital in determining if offspring locomotion experiences any developmental benefit.

2.4.5 Maternal Enrichment Increased Basilar Dendritic Spine Density in the OFC of Female and Male Offspring

Basilar dendritic spine density was increased in the OFC (AID) of offspring of both sexes as a result of maternal preconception enrichment. There were no significant effects of maternal preconception nicotine, although a nicotine-induced decrease was trending for female offspring medial PFC (Cg3). There was some indication of an interaction between maternal nicotine and enrichment; in the parietal cortex of male pups, there was trend towards increased spine density due to enrichment in the pups of sucralose dams, but not of nicotine dams. Changes to dendritic spine density, as well as dendritic length and complexity, are important mechanisms of neuronal plasticity that allow the brain to respond to experience (Hamilton & Kolb, 2005). Both positive experiences, such as environment complexity, and aversive experiences, such as exposure to drugs of abuse, induce structural plasticity. Currently, no other studies have examined the effects of preconception nicotine or enrichment on dendritic spine density. However, several studies examine these effects following prenatal exposure or exposure at other developmental stages. Pups (P21) exposed to prenatal nicotine show a sex-dependent

change in spine density in the parietal cortex (Par1), with density decreasing in males and increasing in female, as well as an increase in density in the nucleus accumbens (NAc; Muhammad et al., 2021). Prenatal nicotine also modifies spine density in several areas of the adult offspring brain: 1) increases in OFC (AID); 2) increases in male medial PFC (Cg3) but decreases in female Cg3; 3) increases in Par1; and 4) decreases in NAc (Mychasiuk, Muhammad, Gibb, & Kolb, 2013). Prenatal enrichment increases spine density in Par1 of adult animals (Gibb, Gonzalez, & Kolb, 2014). Adolescent enrichment following prenatal nicotine exposure increases spine density in the Cg3 (females only) but decreases spine density in the NAc (Mychasiuk et al., 2014). These authors also found that adolescent enrichment increased spine density in Par1, but only in the absence of prenatal nicotine exposure, and decreased spine density in AID of male animals, but only in combination with prenatal nicotine. Another study used adult animals to investigate the interaction between nicotine exposure and enriched housing. In two experiments, they first exposed adult rats to nicotine then housed them in either standard or enriched conditions, then in a second experiment they housed a different set of animals in one of the two conditions prior to treatment with nicotine. In both experiments, both nicotine and enriched housing increased spine density in the NAc, regardless of the order of the experience. In Par1, they found that enriched housing increased spine density, but nicotine had no impact (Hamilton & Kolb, 2005).

2.4.6 Limitations and Future Directions

The present study used robust methods to examine the combined impacts of maternal preconception nicotine and enrichment. Oral administration of nicotine is an effective, commonly used route of administration that is noninvasive and causes no

distress to the animals. Physical (i.e. larger cage, toys) and social (i.e. multiple cage-mates) enrichment is the most common form of enrichment in rodents and is well documented to be beneficial to development. However, a shortcoming of the current study is the inability to disentangle the effects of these experiences from the side effects of withdrawal from both experiences. Enriched females were removed from their housing condition and nicotine-treated females were taken off their drug the day prior to mating; therefore, withdrawal symptoms from both experiences may have impacted the early stages gestation. Alternatives could include ceasing treatments in advance of mating, to allow withdrawal to pass, or to continue treatments during initial gestation, which would not be ideal if interested in purely preconception exposure.

Future work in this area should focus on the neuropharmacological mechanisms driving the effects of preconception maternal nicotine. Promising directions include examining the impacts on neurotransmission, especially acetylcholine and nAChR expression, and levels of BDNF and other neurotrophins. Immediate next steps include the completion of the Golgi-Cox analysis to obtain data pertaining to neuronal size and complexity.

2.4.7 Concluding Remarks

This work demonstrated that chronic (7 weeks) exposure to moderate (2.3 – 2.5mg/kg) levels of nicotine prior to conception does indeed impact the development of preweaning offspring rats, even in the absence of deficits in maternal care. Male offspring of nicotine exposed dams displayed stunted sensorimotor development, although females were unaffected in such a way. Open field exploration as also impaired by maternal nicotine in male pups. In female pups, maternal enrichment decreased open field

exploration but only after locomotor competency had been achieved. Spine density in the OFC was increased by maternal enrichment, but no other brain area was significantly impacted. Therefore, this study contributes to our understanding of these experiences in three novel ways: 1) preconception nicotine is detrimental to the early development male offspring behavior and is not remediated by preconception enrichment; 2) the effects of preconception nicotine are sex-dependent, impacting male offspring to a greater extent than female offspring; and 3) maternal preconception enrichment, but not nicotine, impacts the density of basilar dendritic spines in the areas we examined, providing the first evidence of how enrichment prior to conception impacts this measure of neuroanatomy.

References

Ajarem, J. S., & Ahmad, M. (1998). Prenatal nicotine exposure modifies behavior of mice through early development. *Pharmacology, Biochemistry, and Behavior, 59*, 313 – 318. DOI:10.1016/s0091-3057(97)00408-5

Baroncelli, L., Braschi, C., Spolidoro, M., Begenisic, T., Sale, A., & Maffei, L. (2009). Nurturing brain plasticity: Impact of environmental enrichment. *Cell Death and Differentiation, 17*, 1092 – 1103. DOI:10.1038/cdd.2009.193

Blood-Siegfried, J., & Rende, E. K. (2010). The long-term effects of prenatal nicotine exposure on neurologic development. *Journal of Midwifery and Women's Health, 55*, 143 – 155. DOI:10.1016/j.jmwh.2009.05.006

Bohacek, J., & Mansuy, I. M. (2013). Epigenetic inheritance of disease and disease risk. *Neuropsychopharmacology, 38*, 220 – 236. DOI:10.1038/npp.2012.110

Bruin, J. E., Gerstein, H. C., & Holloway, A. C. (2010). Long-term consequences of fetal and neonatal nicotine exposure: A critical review. *Toxicological Sciences, 116*, 364 – 374. DOI:10.1093/toxsci/kfq103

Buck, J. M., Sanders, K. N., Wagemen, C. R., Knopik, V. S., Stitzel, J. A., & O'Neill, H. C. (2019). Developmental nicotine exposure precipitates multigenerational maternal transmission of nicotine preference and ADHD-like behavioral, rhythmometric, neuropharmacological, and epigenetic anomalies in adolescent mice. *Neuropharmacology, 149*, 66 – 82. DOI:10.1016/j.neuropharm.2019.02.006

Caporali, P., Cutuli, D., Gelfo, F., Laricchiuta, D., Foti, F., De Bartolo, P., Angelucci, F., & Petrosini, L. (2015). Interaction does count: A cross-fostering study on transgenerational effects of pre-reproductive maternal enrichment. *Frontiers in Behavioral Neuroscience, 9*, 320. DOI:10.3389/fnbeh.2015.00320

Caporali, P., Cutuli, D., Gelfo, F., Laricchiuta, D., Foti, F., De Bartolo, P., Mancini, L., Angelucci, F., & Petrosini, L. (2014). Pre-reproductive maternal enrichment influences offspring developmental trajectories: Motor behavior and neurotrophin expression. *Frontiers in Behavioral Neuroscience, 8*, 195. DOI:10.3389/fnbeh.2014.00195

Carlisle, H. J., & Kennedy, M. B. (2005). Spine architecture and synaptic plasticity. *Trends in Neurosciences, 28*, 182 – 187. DOI:10.1016/j.tins.2005.01.008

Chastain, L. G., & Sarkar, D. K. (2017). Alcohol effects on the epigenome in the germline: Role in the inheritance of alcohol-related pathology. *Alcohol, 60*, 53 – 66. DOI:10.1016/j.alcohol.2016.12.007

Chirico, D., Romano, E., Famele, M., Draisci, R., Mancinelli, R., Pascucci, T., & Adriani, W. (2017). Forced but not free-choice nicotine during lactation alters maternal behavior and noradrenergic system of pups: Impact on social behavior of adolescent isolated male rats. *Neuroscience, 361*, 6 – 18. DOI:10.1016/j.neuroscience.2017.08.007

Collins, A. C., Pogun, S., Nesil, T., & Kanit, L. (2012). Oral nicotine self-administration in rodents. *Journal of Addiction Research and Therapy, S2*, 004. DOI:10.4172/2155-6105.S2-004

Cutuli, D., Berretta, E., Pasqualini, G., De Bartolo, P., Caporali, P., Laricchiuta, D., Sampedro-Piquero, P., Gelfo, F., Pesoli, M., Foti, F., Begega, A., & Petrosini, L. (2017). Influence of pre-reproductive maternal enrichment on coping response to stress and expression of c-fos and glucocorticoid receptors in adolescent offspring. *Frontiers in Behavioral Neuroscience, 11*, 73. DOI:10.3389/fnbeh.2017.00073

Cutuli, D., Caporali, P., Gelfo, R., Angelucci, F., Laricchiuta, D., Foti, F., De Bartolo, P., Bisicchia, E., Molinari, M., Vecchioli, S. F., & Petrosini, L. (2015). Pre-reproductive maternal enrichment influences rat maternal care and offspring developmental trajectories: Behavioral performances and neuroplasticity correlates. *Frontiers in Behavioral Neuroscience, 9*, 66. DOI:10.3389/fnbeh.2015.00066

Dai, J., Wang, Z., Xu, W., Zhang, M., Zhu, Z., Zhao, X., Zhang, D., Nie, D., Wang, L., & Qiao, Z. (2017). Paternal nicotine exposure defines different behavior in subsequent generation via hyper-methylation of *mmu-miR-15b*. *Scientific Reports, 7*, 7286. DOI:10.1038/s41598-017-07920-3

De Zeeuw, P., Zwart, F., Schrama, R., van Engeland, H., & Durston, S. (2012). Prenatal exposure to cigarette smoke or alcohol and cerebellum volume in attention-deficit/hyperactivity disorder and typical development. *Translational Psychiatry, 2*, e84. DOI:10.1038/tp.2012.12

Dwyer, J. B., Broide, R. S., & Leslie, F. M. (2008). Nicotine and brain development. *Birth Defects Research (Part C), 84*, 30 – 44. DOI:10.1002/bdrc.20118

Gapp, K., Bohacek, J., Grossmann, J., Brunner, A. M., Manuella, F., Nanni, P., & Mansuy, I. M. (2016). Potential of environmental enrichment to prevent transgenerational effects of paternal trauma. *Neuropsychopharmacology, 41*, 2749 – 2758. DOI:10.1038/npp.2016.87

Gibb, R., Gonzalez, C., & Kolb, B. (2014). Prenatal enrichment and recovery from perinatal brain damage: Effects of maternal complex housing. *Frontiers in Behavioral Neuroscience, 8*, 223. DOI:10.3389/fnbeh.2014.00223

Gibb, R., & Kolb, B. (1998). A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *Journal of Neuroscience Methods, 79*, 1 – 4. DOI:10.1016/s0165-0270(97)00163-5

Goldberg, L. R., & Gould, T. J. (2019). Multigenerational and transgenerational effects of paternal exposure to drugs of abuse on behavioral and neural function. *European Journal of Neuroscience, 50*, 2453 – 2466. DOI:10.1111/ejn.14060

Goldberg, L. R., Zeid, D., Kutlu, M. G., Cole, R. D., Lallai, V., Sebastian, A., Albert, I., Fowler, C. D., Parikh, V., & Gould, T. J. (2019). Paternal nicotine enhances fear memory, reduces nicotine administration, and alters hippocampal genetic and neural function in offspring. *Addiction Biology*, e12859. DOI:10.1111/adb.12859

Hamilton, D. A., & Kolb, B. (2005). Differential effects of nicotine and complex housing on subsequent experience-dependent structural plasticity in the nucleus accumbens. *Behavioral Neuroscience, 119*, 355 – 365. DOI:10.1037/0735-7044.119.2.355

Health Canada. (2019). *Canadian tobacco and nicotine survey (CTNS): Summary of results for 2019*. Government of Canada. https://www.canada.ca/en/health-canada/services/canadian-tobacco-nicotine-survey/2019-summary.html#n4

Heath, C. J., Horst, N. K., & Picciotto, M. R. (2010). Oral nicotine consumption does not affect maternal care of early development in mice but results in modest hyperactivity in adolescence. *Physiology and Behavior, 101*, 764 – 769. DOI:10.1016/j.physbeh.2010.08.021

Holbrook, B. D. (2016). The effects of nicotine on human fetal development. *Birth Defects Research (Part C), 108*, 181 – 192. DOI:10.1002/bdrc.21128

Holloway, A. C., Cuu, D. Q., Morrison, K. M., Gerstein, H. C., & Tarnopolsky, M. A. (2007). Transgenerational effects of fetal and neonatal exposure to nicotine. *Endocrinology, 31*, 254 – 259. DOI:10.1007/s12020-007-0043-6

Holloway, A. C., Kellenberger, L. D., & Petrik, J. J. (2006). Fetal and neonatal exposure to nicotine disrupts ovarian function and fertility in adult female rats. *Endocrine, 30*, 213 – 216. DOI:10.1385/endo:30:2:213

Jamerson, P. A., Wulser, M. J., & Kimler, B. F. (2004). Neurobehavioral effects in rat pups whose sires were exposed to alcohol. *Developmental Brain Research, 149*, 103 – 111. DOI:10.1016/j.devbrainres.2003.12.010

Keyes, K. M., Smith, G. D., & Susser, E. (2014). Associations of prenatal maternal smoking with offspring hyperactivity: Causal or confounded? *Psychological Medicine, 44*, 857 – 867. DOI:10.1017/S0033291713000986

Kim, S. (2020). Changes in multiple and different tobacco product use behaviors in women before and during pregnancy: An analysis of longitudinal population assessment of tobacco and health data. *American Journal of Preventative Medicine, 59,* 588 – 592. DOI:10.1016/j.amepre.2020.05.006

Lacy, R. T., Morgan, A. J., & Harrod, S. B. (2014). IV prenatal nicotine exposure increases the reinforcing efficacy of methamphetamine in adult rat offspring. *Drug and Alcohol Dependence, 141,* 92 – 98. DOI:10.1016/j.drugalcdep.2014.05.010

LeSgae, M. G., Gustaf, E., Dufek, M. B., & Pentel, P. R. (2006). Effects of maternal intravenous nicotine administration on locomotor behavior in pre-weanling rats. *Pharmacology, Biochemistry, and Behavior, 85,* 575 – 583. DOI:10.1016/j.pbb.2006.10.012

Leshem, M., & Schulkin, J. (2011). Transgenerational effects of infantile adversity and enrichment in male and female rats. *Developmental Psychobiology, 54,* 169 – 186. DOI:10.1002/dev.20592

Mai, Z., Lei, M., Yu, B., Du, H., & Liu, J. (2014). The effects of cigarette smoke extract on ovulation, oocyte morphology and ovarian gene expression in mice. *PLoS ONE, 9,* e95945. DOI:10.1371/journal.pone.0095945

Martin, M. M., McCarthy, D. M., Schatschneider, C., Trupiano, M. X., Jones, S. K., Kalluri, A., & Bhide, P. G. (2020). Effects of developmental nicotine exposure on frontal cortical GABA-to-non-GABA neuron ratio and novelty-seeking behavior. *Cerebral Cortex, 30,* 1830 – 1842. DOI:10.1093/cercor/bhz207

Maruoka, T., Kodomari, I., Yamauchi, R., Wada, E., & Wada, K. (2009). Maternal enrichment affects prenatal hippocampal proliferation and open-field behaviors in female offspring mice. *Neuroscience Letters, 454,* 28 – 32. DOI:10.1016/j.neulet.2009.02.052

McCarthy, D. M., Lowe, S. E., Morgan, T. J., Cannon, E. N., Biederman, J., Spencer, T. J., & Bhide, P. G. (2020). Transgenerational transmission of behavioral phenotypes produced by exposure of male mice to saccharin and nicotine. *Scientific Reports, 10,* 11974. DOI:10.1038/s41598-020-68883-6

McCarthy, D. M., Morgan, T. J., Lowe, S. E., Williamson, M. J., Spencer, T. J., Biederman, J., & Bhide, P. G. (2018). Nicotine exposure of male mice produces behavioral impairment in multiple generations of descendants. *PLoS Biology, 16,* e2006497. DOI:10.1371/journal.pbio.2006497

McMurray, M. S., Williams, S. K., Jarrett, T. M., Cox, E. T., Fay, E. E., Overstreet, D. H., Walker, C. H., & Johns, J. M. (2008). Gestational ethanol and nicotine exposure: Effects on maternal behavior, oxytocin, and offspring ethanol intake in

the rat. *Neurotoxicology and Teratology, 30,* 475 – 486. DOI:10.1016/j.ntt.2008.07.001

Mesa-Gresa, P., Ramos-Campos, M., & Redolat, R. (2013). Enriched environments for rodents and their interaction with nicotine administration. *Current Drug Abuse Reviews, 6,* 191 – 200.

Motz, B. A., & Alberts, J. R. (2005). The validity and utility of geotaxis in young rodents. *Neurotoxicology and Teratology, 27,* 529 – 533. DOI:10.1016/j.ntt.2005.06.005

Muhammad, A., Mychasiuk, R., Nakahashi, A., Hossain, S. R., Gibb, R., & Kolb, B. (2012). Prenatal nicotine exposure alters neuroanatomical organization of the developing brain. *Synapse, 66,* 950 – 954. DOI:10.1002/syn.21589

Mychasiuk, R., Muhammad, A., Gibb, R., & Kolb, B. (2013). Long-term alterations to dendritic morphology and spine density associated with prenatal exposure to nicotine. *Brain Research, 1499,* 53 – 60. DOI:10.1016/j.brainres.2012.12.021

Mychasiuk, R., Muhammad, A., & Kolb, B. (2014). Environmental enrichment alters structural plasticity of the adolescent brain but does not remediate the effects of prenatal nicotine exposure. *Synapse, 68,* 293 – 305. DOI:10.1002/syn.21737

Nesil, T., Kanit, L., Collins, A. C., & Pogun, S. (2011). Individual differences in oral nicotine intake in rats. *Neuropharmacology, 61,* 189 – 201. DOI:10.1016/j.neuropharm.2011.03.027

Pauly, J. R., Sparks, J. A., Hauser, K. F., & Pauly, T. H. (2004). In utero nicotine exposure causes persistent, gender-dependent changes in locomotor activity and sensitivity to nicotine in C57B1/6 mice. *International Journal of Developmental Neuroscience, 22,* 329 – 337. DOI:10.1016/j.ijdevneu.2004.05.009

Peters, D. A. V., & Tang, S. (1982). Sex-dependent biological changes following prenatal nicotine exposure in the rat. *Pharmacology, Biochemistry, and Behavior, 17,* 1077 – 1082. DOI:10.1016/0091-3057(82)90497-x

Polli, F. S., Scharff, M. B., Ipsen, T. H., Aznar, S., Kohlmeier, K. A., Andreasen, J. T. (2020). Penatal nicotine exposure in mice induces sex-dependent anxiety-like behavior, cognitive deficits, hyperactivity, and changes in the expression of glutamate receptor associated-genes in the prefrontal cortex. *Pharmacology, Biochemistry, and Behavior, 195,* 172951. DOI:10.1016/j.pbb.2020.172951

Pooriamehr, A., Sabahi, P., & Miladi-Gorji, H. (2017). Effects of environmental enrichment during abstinence in morphine dependent parents on anxiety, depressive-like behaviors and voluntary morphine consumption in rat offspring. *Neuroscience Letters, 656,* 37 – 42. DOI:10.1016/j.neulet.2017.07.024

Renaud, S. M., & Fountain, S. B. (2016). Transgenerational effects of adolescent nicotine exposure in rats: Evidence for cognitive deficits in adult female offspring. *Neurotoxicology and Teratology, 56,* 47 – 54. DOI:10.1016/j.ntt.2016.06.002

Rogers, J. M. (2019). Smoking and pregnancy: Epigenetics and developmental origins of the metabolic syndrome. *Birth Defects Research, 111,* 1259 – 1269. DOI:10.1002/bdr2.1550

Schneider, T., Bizarro, L., Asherson, P. J. E., & Stolerman, I. P. (2010). Gestational exposure to nicotine in drinking water: Teratogenic effects and methodological issues. *Behavioral Pharmacology, 21,* 206 – 216. DOI:10.1097/fbp.0b013e32833a5bb5

Schneider, T., Ilott, N., Brolese, G., Bizarro, L., Asherson, P. J. E., & Stolerman, I. P. (2011). Prenatal exposure to nicotine impairs performance of the 5-choice serial reaction time task in adult rats. *Neuropsychopharmacology, 36,* 1114 – 1125. DOI:10.1038/npp.2010.249

Solinas, M., Thiriet, N., Chauvet, C., & Jaber, M. (2010). Prevention and treatment of drug addiction by environment enrichment. *Progress in Neurobiology, 92,* 572 – 592. DOI:10.1016/j.pneurobio.2010.08.002

Sparling, J. E., Barbeau, K., Boileau, K., & Konkle, A. T. M. (2020). Environmental enrichment and its influence on rodent offspring and maternal behaviors, a scoping style review of indices of depression and anxiety. *Pharmacology, Biochemistry, and Behavior, 197,* 172997. DOI:10.1016/j.pbb.2020.172997

Talbot, P., & Lin, S. (2011). The effect of cigarette smoke on fertilization and pre-implantation development: Assessment using animal models, clinical data, and stem cells. *Biological Research, 44,* 189 – 194. DOI:10.4067/s0716-97602011000200011

Torabi, R., Jenkins, S., Harker, A., Whishaw, I. Q., Gibb, R., & Luczak, A. (2021). A neural network reveals motoric effects of maternal preconception exposure to nicotine on rat pup behavior: A new approach for movement disorders diagnosis. *Frontiers in Neuroscience, 15,* 686767. DOI:10.3389/fnins.2021.686767

Vaglenova, J., Birru, S., Pandiella, N. M., & Breese, C. R. (2004). An assessment of the long-term developmental and behavioral teratogenicity of prenatal nicotine exposure. *Behavioral Brain Research, 150,* 159 – 170. DOI:10.1016/j.bbr.2003.07.005

Vaglenova, J., Parameshwaran, K., Suppiramaniam, V., Breese, C. R., Pandiella, N., & Birru, S. (2008). Long-lasting teratogenic effects of nicotine on cognition: Gender

specificity and role of AMPA receptor function. *Neurobiology of Learning and Memory, 90*, 527 – 536. DOI:10.1016/j.nlm.2008.06.009

Vassoler, F. M., Byrnes, E. M., & Pierce, R. C. (2014). The impact of exposure to addictive drugs on future generations: Physiological and behavioral effects. *Neuropharmacology, 76*, 269 – 275. DOI:10.1016/j.neuropharm.2013.06.016

Winzer-Serhan, U. H. (2008). Long-term consequences of maternal smoking and developmental chronic nicotine exposure. *Frontiers in Bioscience, 13*, 636 – 649. DOI:10.2741/2708

Yohn, N. L., Bartolomei, M. S., & Blendy, J. A. (2015). Multigenerational and transgenerational inheritance of drug exposure: The effects of alcohol, opiates, cocaine, marijuana, and nicotine. *Progress in Biophysics and Molecular Biology, 118*, 21 – 33. DOI:10.1016/j.pbiomolbio.2015.03.002

Zhang, L., Spencer, T. J., Biederman, J., & Bhide, P. G. (2018). Attention and working memory deficits in a perinatal nicotine exposure mouse model. *PLoS ONE, 13*, e0198064. DOI:10.1371/journal.pone.0198064

Zhang, M., Zhang, D., Dai, J., Cao, Y., Xu, W., He, G., Wang, Z., Wang, L., Li, R., & Qiao, Z. (2020). Paternal nicotine exposure induces hyperactivity in next-generation via down-regulating the expression of DAT. *Toxicology, 431*, 152367. DOI:10.1016/j.tox.2020.152367

Zhu, J., Fan, F., McCarthy, D. M., Zhang, L., Cannon, E. N., Spencer, T. J., Biederman, J., & Bhide, P. G. (2017). A prenatal nicotine exposure mouse model of methylphenidate responsive ADHD-associated cognitive phenotypes. *International Journal of Developmental Neuroscience, 58*, 26 – 34. DOI:10.1016/j.ijdevneu.2017.01.014

Zhu, J., Lee, K. P., Spencer, T. J., Biederman, J., & Bhide, P. G. (2014). Transgenerational transmission of hyperactivity in a mouse model of ADHD. *The Journal of Neuroscience, 34*, 2768 – 2773. DOI:10.1523/jneurosci.4402-13.2014

Zhu, J., Zhang, X., Xu, Y., Spencer, T. J., Biederman, J., & Bhide, P. G. (2012). Prenatal nicotine exposure mouse model showing hyperactivity, reduced cingulate cortex volume, reduced dopamine turnover, and responsiveness to oral methylphenidate treatment. *The Journal of Neuroscience, 32*, 9410 – 9418. DOI:10.1523/jneursci.1041-12.2012

Zilles, K. (1985). *The Cortex of the Rat: A Stereotaxic Atlas*. Springer-Verlag: Berlin, Germany.

CHAPTER 3

Long-term consequences of maternal preconception exposure to nicotine and environmental enrichment: Sex- and age-dependent effects on anxiety, motor control, spatial learning, and the brain

Abstract

Nicotine is a potent teratogen that disrupts long-term development. Research is beginning to demonstrate that nicotine exposure prior to the onset of pregnancy alters development, but there is still much to discover about this phenomenon. This work sought to examine how chronic (7wks) maternal exposure to moderate levels of nicotine in drinking water (15mg/L) immediately prior to conception impacts offspring development in adolescence and adulthood. Furthermore, we also examined how the effects of maternal nicotine are moderated by maternal environment; dams were reared in either standard or enriched conditions throughout nicotine dosing. Dams reared their own litters, and offspring completed a behavioral test battery in adolescence and adulthood to assess the expression of activity, anxiety, complex motor skills, and spatial learning and memory. Following behavioral testing, offspring were euthanized, and their brains were processed for Golgi-Cox analysis of spine density. Results indicate that maternal preconception nicotine and enrichment have a variety of impacts on offspring development, including changes to anxiety-like behavior, complex motor control, and spatial learning. Maternal enrichment decreased brain weight of adult male offspring, but only those born to dams not exposed to nicotine. Maternal enrichment also decreased basilar spine density in parietal cortex of adult male offspring. This work is significant because it is one of the first studies to show that maternal nicotine exposure that occurs exclusively prior to conception impacts offspring development, and the first to demonstrate the combined effects of maternal preconception nicotine and enrichment.

3.1 Introduction

Nicotine is a potent teratogen that disrupts development throughout life (Blood-Siegfried & Rende, 2010; Bruin, Gerstein, & Holloway, 2010; Holbrook, 2016). Prenatal nicotine is associated with a range of complications that persist into adolescence and adulthood including: 1) attention-deficit hyperactivity disorder (ADHD; Schneider et al., 2011; Polli et al., 2020); 2) increased anxiety (Vaglenova et al., 2004, 2008; Polli et al., 2020); 3) impaired learning and memory (Vaglenova et al., 2004, 2008); 4) impaired executive function (Bryden et al., 2016); 5) elevated risk of developing metabolic syndrome (Rogers, 2019); and 6) disrupted reproductive function (Holloway, Kellenberger, & Petrik, 2006). However, relatively little is known about if maternal exposure to nicotine prior to but not during pregnancy impacts offspring in a similar way. Several studies have used an exposure schedule that encompasses preconception, gestation, and at times lactation (Zhu et al., 2012, 2017; Zhang, Spencer, Biederman, & Bhide, 2018; Martin et al., 2020), but these studies do not allow conclusions regarding the relative contribution of nicotine exposure at the various times. Very few studies have examined nicotine exposure solely prior to conception, and each study used a unique approach to preconception exposure. One study exposed both parents to nicotine during adolescence and found cognitive impairments in adult female offspring (Renaud & Fountain, 2016). Others have explored the transgenerational effects of maternal nicotine exposure, and found hyperactivity (Zhu et al., 2014) and insulin resistance (Holloway et al., 2007) in the second and even third generation following prenatal nicotine.

The goal of this study was to explore how maternal nicotine exposure immediately prior to conception impacts long-term development of offspring behavior

and brain. Female Long-Evans rats were exposed to moderate levels of nicotine in their drinking water for seven weeks prior to mating with non-drug-exposed males, and offspring completed a behavioral test battery in adolescence and adulthood. We were also interested in if a positive experience could mitigate the effects of maternal drug use. We paired maternal preconception nicotine exposure with environmental enrichment/complexity; enrichment prior to conception induces multiple benefits for the next generation (Caporali et al., 2014; Cutuli et al., 2015, 2107) and enrichment can be used to reverse or treat drug addiction (Solinas, Thiriet, Chauvet, & Jaber, 2010; Mesa-Gresa, Ramos-Campos, & Redolat, 2013; Pooriamehr, Sabahi, & Miladi-Gorji, 2017). The combination of social and environmental enrichment that we used here is intended to translate to human living conditions that are rich in opportunities for social interaction, physical activity, and learning experiences. Female rats were exposed to nicotine while residing in standard or enriched conditions to explore the interaction between nicotine and enrichment on offspring development.

Our offspring behavioral test battery included assessments of activity (open field), anxiety (elevated plus maze), and learning and memory (Morris water task), as these behaviors are all consistently impacted by prenatal nicotine exposure. We also included a measure of fine motor control, Whishaw tray reaching. The effects of nicotine on motor performance are mixed. Nicotine interacts with nicotinic acetylcholine receptors (nAChR) to stimulate the cholinergic system, which is associated with release of dopamine in the striatum; in this way, nicotine has shown potential as a treatment of Parkinson's disease (Quik et al., 2009; Quik, Perez, & Bordia, 2012; Barreto, Iarkov, & Moran, 2015). Nicotine is also associated with enhanced fine motor skills in humans,

including handwriting, finger tapping, and pegboard performance (Heishman, Kleykamp, & Singleton, 2010), as well as in rats (Gonzalez, Gharbawie, Whishaw, & Kolb, 2005). However, developmental nicotine can impair fine motor control (Lee & Picciotto, 2019). Therefore, we investigated how maternal preconception nicotine affects the control of fine forelimb movements in adult offspring. To explore how maternal preconception nicotine and enrichment affect offspring brain development, we used Golgi-Cox-stained tissue to examine dendritic spine density in the prefrontal cortex (PFC) and parietal cortex. Both experiences are associated with changes in dendritic spine density (Robinson & Kolb, 2004; Hamilton & Kolb, 2005; Mychasiuk, Muhammad, Gibb, & Kolb, 2013b; Mychasiuk, Muhammad, & Kolb, 2014), although it is currently unknown how they affect spine density when experienced by the mother prior to conception.

Therefore, our research predictions were as follows: 1) maternal preconception nicotine exposure will impact offspring behavioral development by inducing hyperactivity, increasing anxiety, impairing learning and memory, but potentially improving fine motor control; 2) maternal preconception enrichment will mitigate or prevent the negative consequences of nicotine exposure; 3) both maternal nicotine and enrichment will result in changes in dendritic spine density throughout the brain; and 4) female and male offspring will be differentially impacted by maternal preconception experience.

3.2 Methods

3.2.1 Animals

All procedures were conducted in accordance with the University of Lethbridge Animal Welfare Committee and the Canadian Council on Animal Care. Forty-five female

Long Evans rats were bred with 45 male Long-Evans rats between January 2017 and August 2018. Parents were born in-house (3rd generation) from animals originally brought in from Charles River Laboratories and came from 11 different litters to ensure genetic diversity. Of the 45 breeding pairs, 32 pairs resulted in successful pregnancies, for a total of 351 pups. One third of these pups (*n* = 116) were euthanized as part of a different experiment and were discussed in Chapter 2. Final animal numbers for the following procedures were as follows: 1) Pair-housed/Sucralose, 8 litters (37 females and 22 males); 2) pair-housed/nicotine, 6 litters (26 females and 25 males); 3) enriched/sucralose, 10 litters (32 females and 38 males); and 4) enriched/nicotine, 8 litters (24 females and 31 males). All parents and offspring generated were handled daily, housed in a temperature- and humidity-controlled (21°/50%) breeding room, and provided food and water *ad libitum* unless otherwise specified.

3.2.2 Maternal Experience

The detailed procedures relating to the maternal preconception experiences are described in Chapter 2. Females (*n* = 45) were randomly assigned to a housing condition in adolescence (P45): 1) maternal pair-housed (*n* = 22), or 2) maternal enrichment (*n* = 23). Pair-housed females resided in standard laboratory style cages (24cm x 45cm x 20.5cm) with one other female, whereas enriched females resided in "condos" (1.2m x 0.6m x 1.8m) with 5 – 6 other females. Seven weeks into the housing treatment, half of the females in each housing condition began receiving 15mg of nicotine hydrogen tartrate salt (Sigma) per 1L of drinking water sweetened with 1% sucralose (Nesil, Kanit, Collins, & Pogun, 2011; Collins, Pogun, Nesil, & Kanit, 2012). Drug control females also received 1% sucralose. Nicotine intake was monitored by recording the volume of water

consumed within a 24hr period and calculating the mg of nicotine consumed per kg of body weight. Following the final day of nicotine administration, all females were provided plain drinking water and placed in a standard cage with a male Long-Evans rat for 10 days. Males were removed, and females were pair-housed with a female from the same treatment condition until 21 days had elapsed since breeding began, after which time they were singly housed in preparation for delivery. Offspring were reared by their respective dam and weaned on P22, then housed in same-sex, same-treatment groups of 3 – 5 animals per cage (38.5cm x 49cm x 21cm) for the duration of the experiment.

3.2.3 Offspring Behavior

3.2.3.1 Activity Box

Offspring were tested in activity box, an automated test of open field exploration, on P35 (adolescence) and P90 (adulthood). At each age, animals were individually placed in the chamber (41cm x 41cm x 30.5cm) of a VersaMax Legacy Open Field (Omnitech Electronics, Inc) and activity was monitored for 10min. The variables of interest were distance travelled (cm), time spent moving (s), and percent of time spent in the center of the chamber.

3.2.3.2 Elevated Plus Maze

Offspring were tested in elevated plus maze on P36 (adolescence) and P91 (adulthood). At each age, animals were filmed on the elevated plus maze apparatus for 5min (Figure 3.1). The apparatus was constructed of black opaque Plexiglas®. The maze was raised 1m off the floor and contained two “open” arms and two “closed” arms. Both open and closed arms were 40cm long and 10cm wide; however, closed arms had 40cm high walls along three sides. The distal 20cm of the open arms were considered a distinct

zone, the “end of the open arms”, and were marked with small white ticks. Where the four arms converged in the center of the maze was “center square”, and this was the starting point for each animal. Videos were double-scored by two blinded researchers for the number of entries into each zone of the maze and the total time spent in each zone.

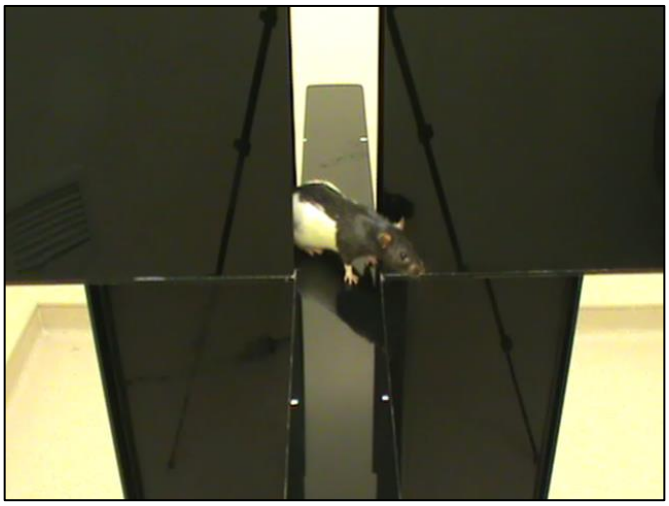

Figure 3.1: Testing apparatus for elevated plus maze, assessed when offspring were P36 and P91.

3.2.3.3 Whishaw Tray Reaching

Offspring were tested in the Whishaw tray reaching task from P92 – 113. The testing chambers were boxes that measured 20cm x 27cm x 19.5cm. The front of the cage was made up of 3mm metal bars that were spaced 10mm apart; the other three walls and ceiling of the boxes were solid, clear Plexiglas®. The floor of the chamber was wire mesh and lifted 5cm off the table such that any food that was dropped could not be retrieved. Immediately outside the front of the cage sat a food trough, 7mm deep and 5cm wide, that contained food pieces (ProStock® poultry feed) that were approximately 5mm

long. All throughout the training and testing period, animals were food restricted to 90% of their initial weight to increase motivation to reach. For the first week of the task, rats were habituated to the testing chambers for 30min each day, during which time the food trough was empty. Training began with 1hr sessions each day for three days, which was then reduced to 30min for four days, then 10min for seven days. During training, a research assistant was present in the room to encourage the rats to reach through the bars with their forepaws, discourage use of their tongue to reach the food, and to refill the food trough as it was emptied. All animals successfully reached within the first two days of training. On the final day of the task, rats were filmed for 5min while in the testing chambers. Videos were double scored by blinded assistants for the number of reach attempts for each forepaw and the number of attempts that led to the animal successfully consuming the food piece ("hits"). Reach success was calculated as the number of hits divided by the number of attempts.

Figure 3.2: Testing Apparatus for Whishaw tray reaching, assessed in adult offspring.

3.2.3.4 Morris Water Task

Offspring were tested in a place version of the Morris water task (Morris, 1984) from P114 – 118. The pool had a diameter of 1.5m, white interior walls, and was filled with water (24°) that was tinted white using non-toxic tempera paint to a depth of 35cm. Submerged 5cm beneath the surface of the water was a clear Plexiglas® platform with a surface measuring 12cm x 12cm. The pool was conceptually divided into four quadrants (NE, SE, SW, NW), with the hidden platform located in the center of the NW quadrant. The pool itself contained no distinguishing features, but the walls of the testing room contained unique visual cues. Animals were trained to locate the hidden platform using four trials per day for five days. Each trial started from one of the four cardinal directions, and the order of starting locations was pseudo-randomized so that the same order was not repeated. For each trial, the animal was placed in the pool, close to and facing the wall, released, and given up to 60s to locate the platform. If it did not locate the platform in this time, a research assistant would use their hand to direct it. Regardless of whether the animal found the platform on their own, they would be given 5s to visually scan the room before being returned to the holding cage until the next trial (5 – 10min). Trials were recorded by a ceiling-mounted camera and tracking software from HVS Image. For the training trials, we analyzed latency to locate the platform (s) and distance to the platform (m); latency and distance were averaged across the four trials per day. Following the training trials on the fifth day, the platform was removed from the pool and each animal was placed in the pool for one 30s probe trial. The main variable of interest for the probe trial was the percent of time spent in the target, previously rewarded quadrant (NW), the opposite quadrant (SE), and the two adjacent quadrants (average of NE and SW).

3.2.4 Offspring Neuroanatomy

3.2.4.1 Euthanasia and Perfusion

A subset of offspring was euthanized on P45 and P120, evenly sampled across litters and treatment conditions. Animals were administered an intraperitoneal injection of pentobarbital sodium (300mg/kg) and in the absence of hindlimb reflexes were intracardially perfused with ~200mL of 0.9% saline in distilled water. Animals were then decapitated, and the brains were promptly removed, weighed, and placed in Golgi-Cox solution in dark (light-blocking) bottles for two weeks. Golgi-Cox was then replaced with 30% sucrose for at least two weeks before brains were sectioned at 200μm on a vibratome. Sections were plated on 2% gelatin-coated slides and stained according to Gibb and Kolb (1998).

3.2.4.2 Spine Density

For P120 animals, spine density was estimated for basilar dendrites in medial PFC (layer V Cg3), orbitofrontal cortex (OFC; layer III AID), and parietal cortex (layer III Par1) according to Zilles (1985; Figure 3.3). Dendrites were traced by a research assistant blind to the experimental conditions using a camera lucida at 1250X magnification if they satisfied the following criteria: 1) at least 30μm in length; 2) terminal, 3) at least third branch order; 4) untruncated; and 5) unobstructed by blood vessels, cells, or staining artefacts. Five dendrites per area per hemisphere were drawn, and the average density was used in the analysis. Drawings were scanned and analyzed using the Cell Counter plugin for ImageJ (https://imagej.nih.gov/ij/plugins/cell-counter.html) by a different assistant also blinded to the experimental groups. Density was expressed as the number of spines per 10μm of dendrite length.

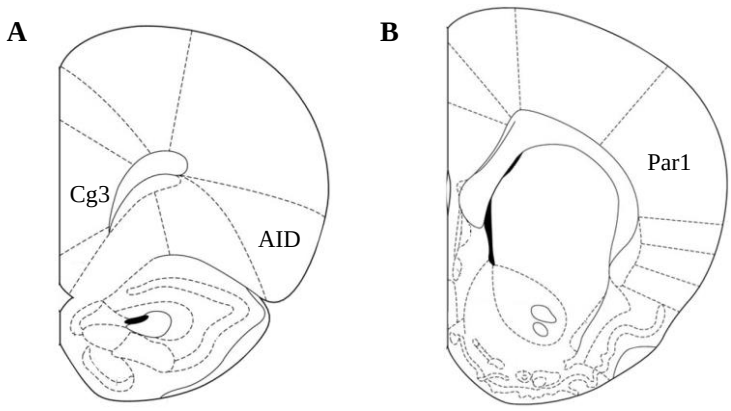

Figure 3.3: Representation of the areas sampled for dendritic spine density. (A) Dendrites were traced in Cg3 and AID from Bregma 4.2 – 2.7mm (Bregma 3.7mm is shown) (B) Dendrites were traced in Par1 from Bregma 2.2 – -0.4mm (Bregma 1.7mm is shown).

3.2.5 Statistical Analysis

All statistical analysis was conducted using IBM SPSS 27. An *a priori* decision was made to analyze female and male data separately due to the frequency of sex differences following developmental nicotine exposure (Peters & Tang, 1982; Pauly, Sparks, Hauser, & Pauly, 2004; Polli et al., 2020). To control for potential litter effects and satisfy the assumption of independence in general linear models, behavioral data for females and males within a litter was averaged to yield one female datapoint and male datapoint per litter. Body and brain weights were similarly averaged. Spine density was estimated for five or six females and males per treatment group, each from different litters. Data was analyzed using factorial analysis of variance (ANOVA), mixed ANOVA, or analysis of covariance (ANCOVA) as appropriate. The Bonferroni correction was used to control for multiple comparisons as needed. The level of alpha was set as .05.

3.3 Results

3.3.1 Maternal Nicotine Consumption

The effect of the maternal treatments on the water consumption and weight gain of the dams is discussed in detail in Chapter 2. In brief, nicotine-treated dams consumed less water than sucralose dams during most of the seven weeks of dosing, although there was no difference during the baseline assessment (see Figure 2.4 in Chapter 2). Nicotine-treated dams also gained significantly less weight between the baseline assessment and the end of dosing (see Figure 2.5). On average, pair-housed nicotine dams consumed 2.54 ± 0.04 mg nicotine/kg body weight, and enriched nicotine dams consumed 2.29 ± 0.03 mg/kg (see Figure 2.6), values that correspond to habitual cigarette smokers (Schneider, Bizarro, Asherson, & Stolerman, 2010).

3.3.2 Offspring Behavior

3.3.2.1 Activity Box

Two-way ANOVAs with maternal drug and housing as between-subject factors revealed no significant main effects or interactions for any measure examined in adolescent or adult activity box for female or male offspring (Table 3.1; Figure 3.4).

Table 3.1: Summary of statistical findings for activity box.

	Drug			Housing			Drug x Housing		
	F	p	η_P^2	F	p	η_P^2	F	p	η_P^2
P35 Female									
Distance (cm)	0.252	.620	.009	0.001	.971	.000	2.403	.133	.082
Movement (s)	0.285	.598	.010	0.027	.871	.001	2.760	.108	.093
Center (%)	0.001	.973	.000	0.355	.556	.013	0.001	.971	.000
P35 Male									
Distance (cm)	0.798	.380	.030	1.191	.285	.044	0.175	.679	.007
Movement (s)	0.380	.543	.014	0.796	.380	.030	0.324	.574	.012
Center (%)	0.907	.350	.034	1.184	.287	.044	0.605	.444	.023
P90 Female									
Distance (cm)	3.360	.077	.107	0.360	.553	.013	1.970	.171	.066
Movement (s)	1.137	.295	.039	0.092	.764	.003	0.886	.355	.031
Center (%)	1.310	.262	.045	0.004	.951	.000	1.695	.204	.057
P90 Male									
Distance (cm)	0.212	.649	.008	0.025	.875	.001	0.583	.452	.022
Movement (s)	0.136	.716	.005	0.009	.926	.000	1.309	.263	.048
Center (%)	0.036	.851	.001	0.730	.401	.027	0.704	.409	.026

P35: Model df = 1, Female Error df = 27, Male Error df = 26
P90: Model df = 1, Female Error df = 28, Male Error df = 26

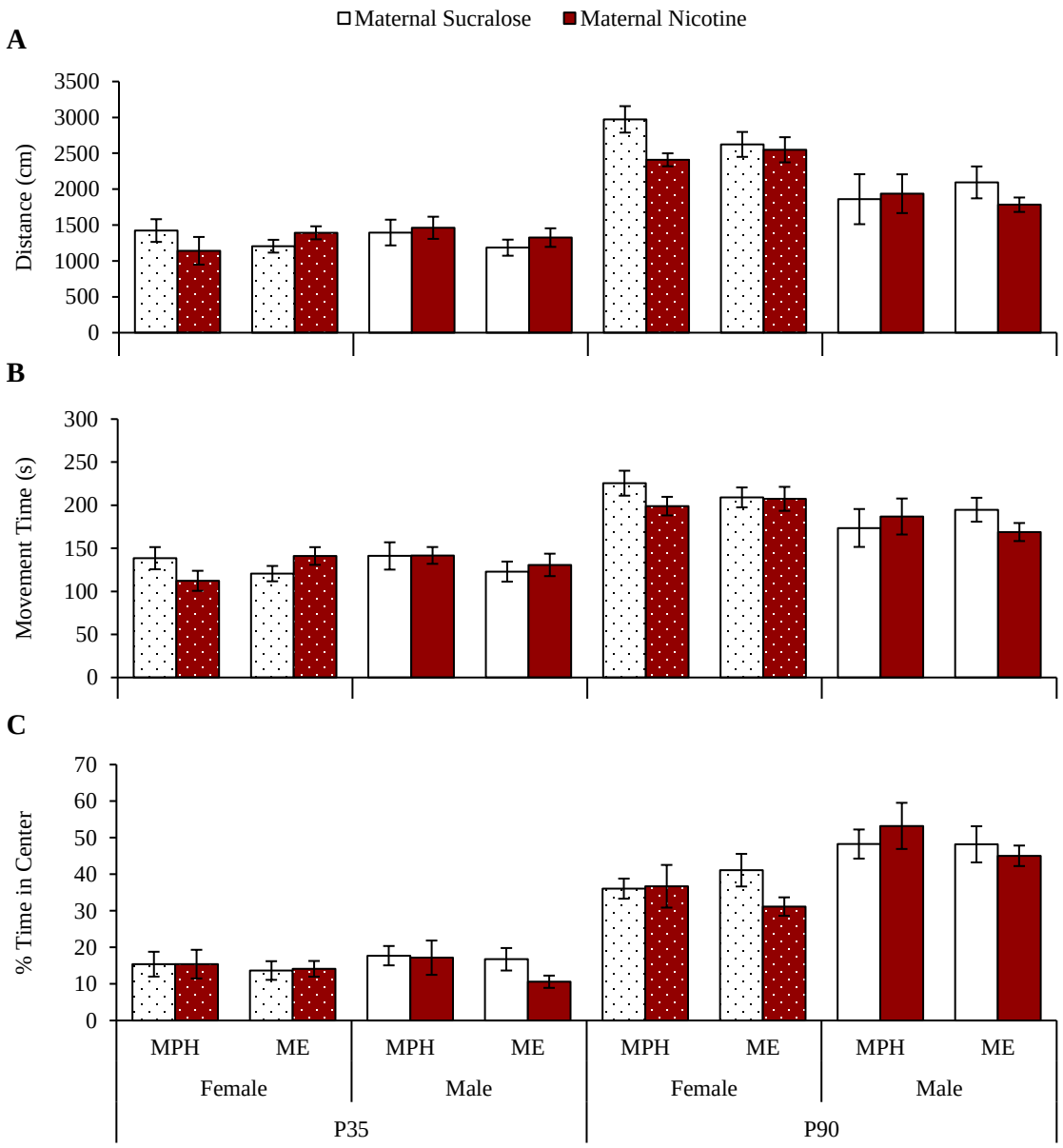

Figure 3.4: Activity level of female (patterned) and male (solid) offspring in adolescence (P35) and adulthood (P90). There were no significant effects of maternal drug or housing on (A) the total distance travelled in the open field (cm), (B) the length of time spent moving (s), or (C) the percent of time spent in the center of the open field, a measure of anxiety. Data is shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment, P = postnatal day.

3.3.2.2 Elevated Plus Maze

In adolescent (P36) female offspring, two-way ANOVA revealed a significant main effect of maternal drug for the length of time spent in the end of the open arms of the elevated plus maze; nicotine females spent significantly longer in the end of the open arms than sucralose females (Figure 3.5B). There were no other significant main effects or interactions. There were no significant effects for adult (P90) female offspring (Table 3.2; Figure 3.5A-D).

In adolescent (P36) male offspring, two-way ANOVA revealed significant main effects of maternal housing for the length of time in the open arms and the end of the open arms, and the total number of arm entries. Enriched males spent significantly less time in the open and in the end of the open arms and had fewer arm entries than pair-housed males (Figure 3.5A, B, D). There were no significant main effects of maternal drug or interactions. There were no significant effects for adult (P90) male offspring (Table 3.2; Figure 3.5A-D).

Table 3.2: Summary of statistical findings for elevated plus maze.

	Drug			Housing			Drug x Housing		
	F	p	η_P^2	F	p	η_P^2	F	p	η_P^2
P36 Female									
Open (s)	3.428	.075	.113	1.244	.275	.044	1.459	.238	.051
End Open (s)	**4.648**	**.040**	**.147**	2.464	.128	.084	1.559	.223	.055
Closed (s)	1.735	.199	.060	1.632	.212	.057	0.535	.471	.019
Entries	0.028	.867	.001	3.631	.067	.119	0.125	.726	.005
P36 Male									
Open (s)	0.418	.523	.015	**4.757**	**.038**	**.150**	0.689	.414	.025
End Open (s)	0.218	.644	.008	**8.992**	**.006**	**.250**	0.904	.350	.032
Closed (s)	0.604	.444	.022	2.563	.121	.087	0.581	.453	.021
Entries	0.731	.400	.026	**6.483**	**.017**	**.194**	0.055	.817	.002
P91 Female									
Open (s)	0.000	.996	.000	0.207	.653	.008	0.007	.934	.000
End Open (s)	0.004	.952	.000	0.038	.848	.001	0.329	.571	.012
Closed (s)	0.331	.571	.012	0.334	.568	.012	0.970	.334	.035
Entries	0.014	.907	.001	0.039	.845	.001	0.236	.631	.009
P91 Male									
Open (s)	0.285	.598	.011	2.418	.132	.088	0.002	.962	.000
End Open (s)	0.092	.764	.004	0.307	.584	.012	0.394	.536	.016
Closed (s)	0.211	.650	.008	3.093	.091	.110	0.106	.748	.004
Entries	0.146	.706	.006	1.076	.309	.041	1.659	.210	.062

P36: Model df = 1, Female Error df = 27, Male Error df = 27
P91: Model df = 1, Female Error df = 27, Male Error df = 25
Boldface = $p \leq .05$

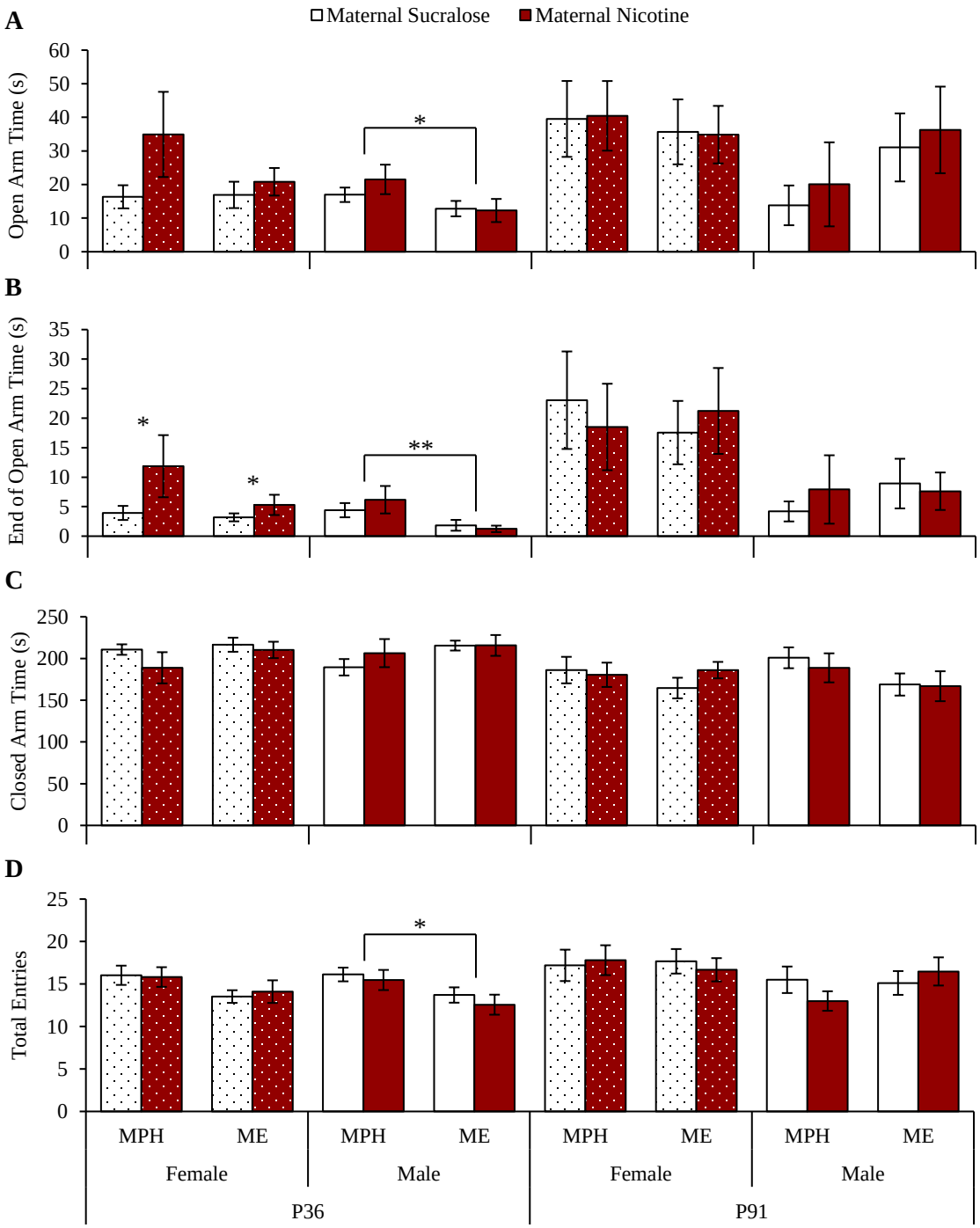

Figure 3.5: Assessment of anxiety-like behavior exhibited by female (patterned) and male (solid) offspring based on performance in the elevated plus maze in adolescence (P36) and adulthood (P91). Male adolescent offspring born to enriched mothers spent significantly less time in the open arms (A) and end of the open arms (B). Female

adolescent offspring preconceptionally exposed to nicotine spent significantly longer in the end of the open arms (B). There were no differences for the time spent in the closed arms (C). Male adolescent offspring of enriched dams had fewer arm entries overall (D). There were no differences for adult offspring (A-D, right panels). Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment, P = postnatal day.

* $p \leq .05$; ** $p \leq .01$.

3.3.2.3 Whishaw Tray Reaching

Two-way ANOVA with maternal drug and housing as between-subject factors revealed no significant main effects or interactions for the number of reach attempts of female or male offspring. In females, there was no difference between sucralose and nicotine offspring, $F(1, 28) = 0.074$, $p = .788$, $\eta_P^2 = .003$, or pair-housed and enriched offspring, $F(1, 28) = 2.882$, $p = .101$, $\eta_P^2 = .093$, nor was there a significant interaction, $F(1, 28) = 0.273$, $p = .606$, $\eta_P^2 = .010$. Likewise, in male offspring there was no effect of maternal drug, $F(1, 26) = 0.079$, $p = .780$, $\eta_P^2 = .003$, maternal housing, $F(1, 26) = 0.118$, $p = .734$, $\eta_P^2 = .005$, nor a Drug x Housing interaction, $F(1, 26) = 1.845$, $p = .186$, $\eta_P^2 = .066$ (Figure 3.6A). Therefore, there was no difference in the motivation to reach, and reach attempts was a suitable covariate for reach success.

Two-way ANCOVA revealed that the number of reach attempts was a significant covariate for both female, $F(1, 27) = 27.593$, $p < .001$, $\eta_P^2 = .505$, and male, $F(1, 25) = 18.834$, $p < .001$, $\eta_P^2 = .430$, offspring. In females, there was a significant main effect of maternal nicotine on reach success (%) adjusted by the number of reach attempts, $F(1, 27) = 4.379$, $p = .048$, $\eta_P^2 = .137$; sucralose offspring were more successful than nicotine

offspring. There was no main effect of maternal housing, $F(1, 27) = 0.231$, $p = .635$, $\eta_P^2 = .008$, and a nearly significant Drug x Housing interaction, $F(1, 27) = 3.663$, $p = .066$, $\eta_P^2 = .119$. In males, there was no significant main effect of maternal drug, $F(1, 26) = 2.113$, $p = .159$, $\eta_P^2 = .079$, or maternal housing, $F(1, 26) = 1.521$, $p = .229$, $\eta_P^2 = .057$, nor a Drug x Housing interaction, $F(1, 26) = 0.004$, $p = .949$, $\eta_P^2 = .000$ (Figure 3.6B).

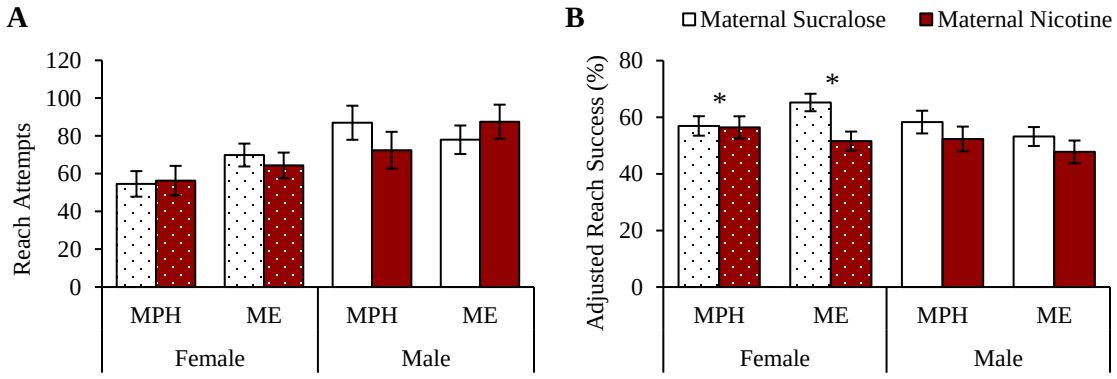

Figure 3.6: Assessment of complex forelimb control according to Whishaw tray reaching. (A) There were no changes in the motivation to reach in either female (patterned) or male (solid) offspring. (B) Female offspring reach success was significantly impaired by maternal nicotine, but there were no effects in males. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment. * $p \leq .05$

3.3.2.4 Morris Water Task

The Greenhouse-Geisser values of epsilon were used to correct for departures from sphericity; adjusted degrees of freedom are presented. For female offspring, three-way mixed ANOVA with training day as a within-subject factor and maternal drug and housing as between-subject factors revealed a significant main effect of training for the

latency to locate the hidden platform, $F(3.338, 93.477) = 130.2$, $p < .001$, $\eta_P^2 = .823$ (Figure 3.7A). Females located the platform significantly faster between days 1 and 2, $F(1, 28) = 168.2$, $p < .001$, $\eta_P^2 = .857$, and between days 2 and 3, $F(1, 28) = 7.492$, $p = .044$, $\eta_P^2 = .211$, but not between days 3 and 4, $F(1, 28) = 1.386$, $p = .996$, $\eta_P^2 = .047$, or days 4 and 5, $F(1, 28) = 2.519$, $p = .496$, $\eta_P^2 = .083$. There were no significant interactions with training (p's > .05), nor was there a main effect of maternal drug, $F(1, 28) = 0.014$, $p = .907$, $\eta_P^2 = .000$, or maternal housing, $F(1, 28) = 0.268$, $p = .609$, $\eta_P^2 = .009$, nor a Drug x Housing interaction, $F(1, 28) = 1.565$, $p = .221$, $\eta_P^2 = .053$. For male offspring, there was a significant Training x Drug x Housing interaction for the latency to locate the hidden platform, $F(2.939, 76.419) = 3.046$, $p = .035$, $\eta_P^2 = .105$ (Figure 3.7B). Among males born to sucralose dams, there was a significant effect of maternal housing on training days 2, $F(1, 26) = 4.312$, $p = .048$, $\eta_P^2 = .142$, and 3, $F(1, 26) = 5.450$, $p = .028$, $\eta_P^2 = .173$; enriched males located the platform significantly faster than pair-housed males. There was no effect of maternal housing on days 1, $F(1, 26) = 2.199$, $p = .150$, $\eta_P^2 = .078$, 4, $F(1, 26) = 2.182$, $p = .152$, $\eta_P^2 = .077$, or 5, $F(1, 26) = 0.199$, $p = .659$, $\eta_P^2 = .008$. There were no differences between the latency of enriched and pair-housed males of nicotine dams on any day of training (p's > .05).

Three-way mixed ANOVA also revealed a significant main effect of training for female offspring's distance to locate the platform, $F(2.865, 80.223) = 148.0$, $p < .001$, $\eta_P^2 = .841$ (Figure 3.7C). Females located the platform with significantly less distance between days 1 and 2, $F(1, 28) = 158.7$, $p < .001$, $\eta_P^2 = .850$, and days 2 and 3, $F(1, 28) = 15.253$, $p < .001$, $\eta_P^2 = .353$, but there was no difference between days 3 and 4, $F(1, 28) = 1.907$, $p = .712$, $\eta_P^2 = .064$, or days 4 and 5, $F(1, 28) = 1.116$, $p = 1.000$, $\eta_P^2 = .038$. There

were no significant interactions with training (p's > .05), or a main effect of maternal drug, $F(1, 28) = 0.141$, $p = .710$, $\eta_P^2 = .005$, or maternal housing, $F(1, 28) = 0.889$, $p = .354$, $\eta_P^2 = .031$, or a Drug x Housing interaction, $F(1, 28) = 0.390$, $p = .537$, $\eta_P^2 = .014$. There was also a significant main effect of training for male offspring's swim distance, $F(2.800, 72.800) = 129.7$, $p < .001$, $\eta_P^2 = .833$ (Figure 3.7D). Distance to locate the platform significantly decreased between days 1 and 2, $F(1, 26) = 157.7$, $p < .001$, $\eta_P^2 = .858$, days 2 and 3, $F(1, 26) = 11.033$, $p = .012$, $\eta_P^2 = .298$, and days 4 and 5, $F(1, 26) = 10.337$, $p = .012$, $\eta_P^2 = .284$, but not between days 3 and 4, $F(1, 26) = 4.721$, $p = .156$, $\eta_P^2 = .154$. The Training x Drug x Housing interaction observed for latency was no longer significant for distance, $F(2.800, 72.800) = 2.463$, $p = .073$, $\eta_P^2 = .087$, although the data for distance did follow a similar trend. There were no other significant interactions with training (p's > .05), and no significant main effect of maternal drug, $F(1, 26) = 1.139$, $p = .296$, $\eta_P^2 = .042$, or maternal housing, $F(1, 26) = 1.329$, $p = .259$, $\eta_P^2 = .049$, or a Drug x Housing interaction, $F(1, 26) = 3.703$, $p = .065$, $\eta_P^2 = .125$.

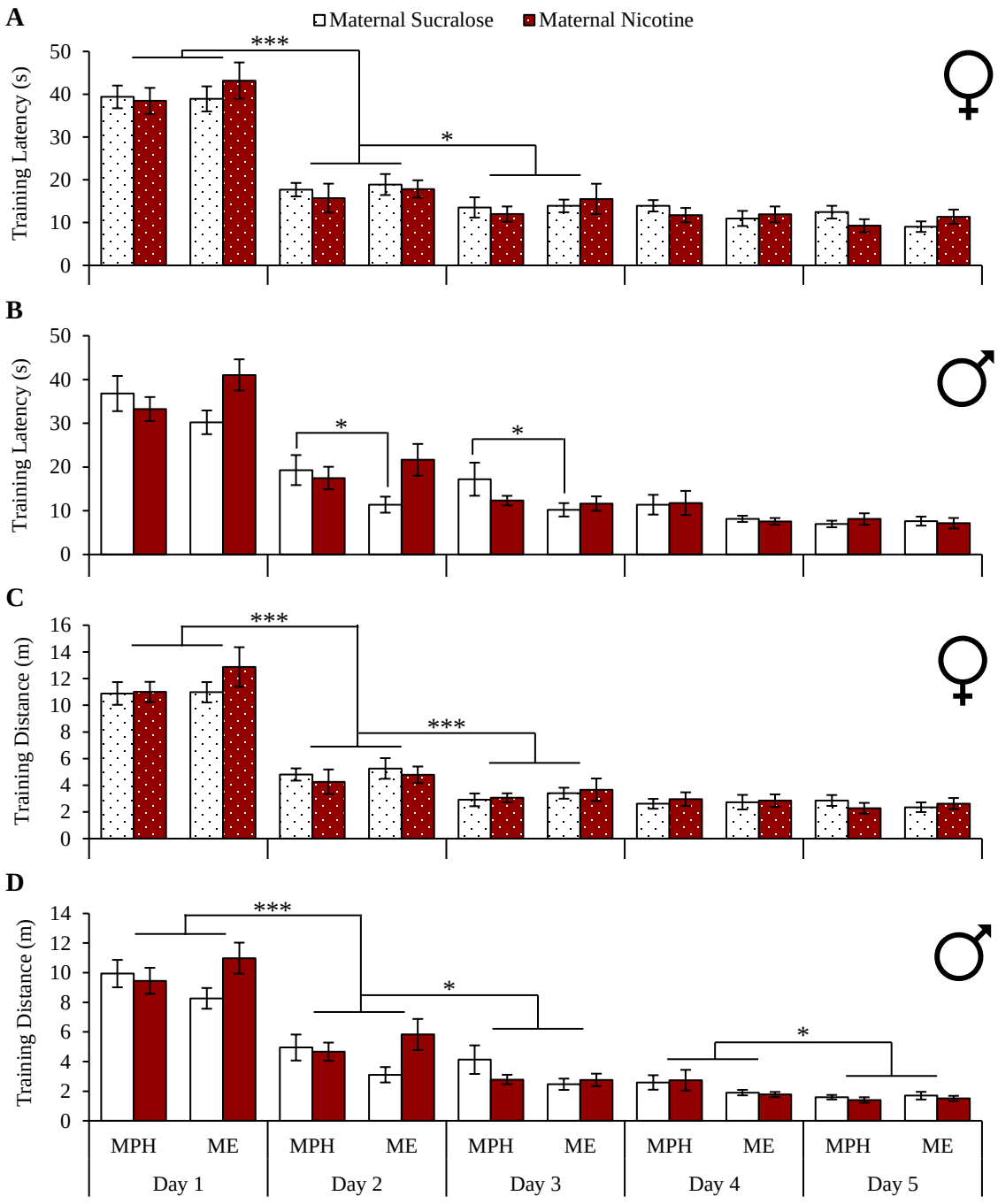

Figure 3.7: Performance in Morris water maze across the five days of training. Female (patterned) offspring located the platform in significantly less time (A) and with less distance (C) across training. Male (solid) offspring exhibited a Training x Drug x Housing interaction for latency (B), and a significant main effect of training for distance

(D). Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment. * $p \leq .01$; *** $p \leq .001$.

For the probe trial, two-way ANOVA revealed no significant main effects or interactions for the percent of time spent in the target, adjacent, or opposite quadrants for either female or male offspring (Table 3.3; Figure 3.8).

Table 3.3: Summary of statistical findings for the Morris water task probe trial.

	Drug			Housing			Drug x Housing		
	F	*p*	η_P^2	*F*	*p*	η_P^2	*F*	*p*	η_P^2
Female									
Target (%)	1.173	.288	.040	0.502	.484	.018	0.749	.394	.026
Adjacent (%)	1.615	.214	.055	1.677	.206	.057	0.031	.862	.001
Opposite (%)	0.139	.712	.005	0.019	.890	.001	0.899	.351	.031
Male									
Target (%)	0.793	.381	.030	0.425	.520	.016	0.486	.492	.018
Adjacent (%)	1.268	.270	.047	0.474	.497	.018	0.032	.859	.001
Opposite (%)	0.022	.882	.001	0.006	.941	.000	1.906	.179	.068

Model *df* = 1, Female Error *df* = 28, Male Error *df* = 26

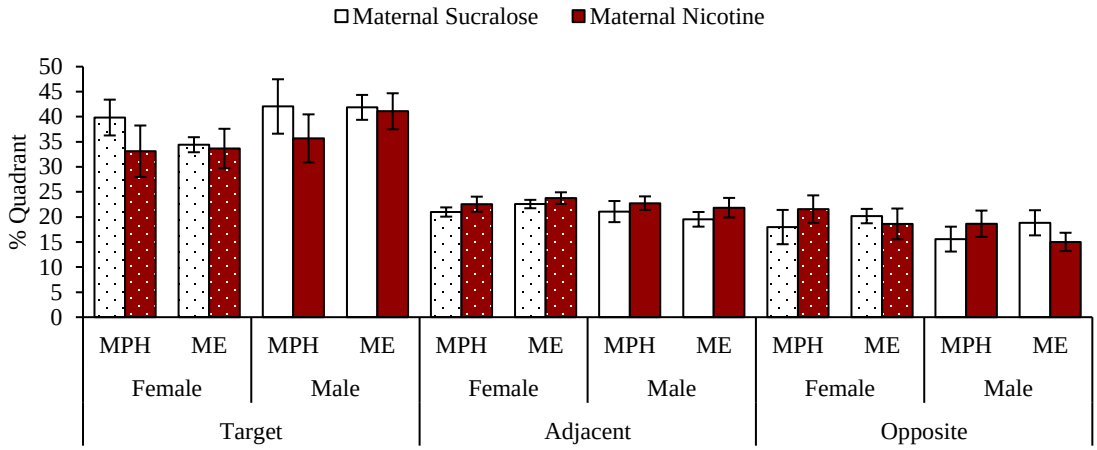

Figure 3.8: Offspring performance during the Morris water task probe trial. There was no effect for female (patterned) or male (solid) offspring for the percent of time spent in any quadrant of the pool. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment.

3.3.3 Offspring Neuroanatomy

On P45, females were randomly selected from 28 of 32 litters to be euthanized, with each treatment group represented by six to eight litters. Males were randomly selected from 24 of 32 litters, with each litter represented by five to seven litters. On P120, females were selected from 29 litters (six to eight litters per treatment) and males from 27 litters (six to eight litters per treatment). Although body weight was collected for all offspring, only the body weights of offspring that also contributed brain weight data are included in the analysis.

3.3.3.1 Offspring Body and Brain Weight

For adolescent female offspring body weight, two-way ANOVA revealed no significant effects of maternal drug, $F(1, 24) = 1.065$, $p = .312$, $\eta_P^2 = .042$, maternal

housing, $F(1, 24) = 0.000$, $p = .992$, $\eta_P^2 = .000$, or an interaction, $F(1, 24) = 0.269$, $p = .609$, $\eta_P^2 = .011$ (Figure 3.9A). Two-way ANCOVA indicated that female P45 body weight was a significant covariate of brain weight, $F(1, 23) = 27.183$, $p < .001$, $\eta_P^2 = .542$, but there were no significant main effects of drug, $F(1, 23) = 0.019$, $p = .893$, $\eta_P^2 = .001$, or housing, $F(1, 23) = 0.001$, $p = .972$, $\eta_P^2 = .000$, nor a Drug x Housing interaction, $F(1, 23) = 0.175$, $p = .679$, $\eta_P^2 = .008$, for P45 brain weight adjusted for body weight (Figure 3.9B). Likewise, for male adolescent body weight, two-way ANOVA revealed no significant effects due to maternal drug, $F(1, 20) = 0.556$, $p = .464$, $\eta_P^2 = .027$, housing, $F(1, 20) = 0.964$, $p = .338$, $\eta_P^2 = .046$, or the interaction, $F(1, 20) = 0.653$, $p = .428$, $\eta_P^2 = .032$ (Figure 3.9A). Male P45 body weight was a significant covariate for brain weight, $F(1, 18) = 19.837$, $p < .001$, $\eta_P^2 = .524$, but there were again no significant main effects of drug, $F(1, 18) = 1.562$, $p = .227$, $\eta_P^2 = .080$, or housing, $F(1, 18) = 1.099$, $p = .308$, $\eta_P^2 = .058$, nor a Drug x Housing interaction, $F(1, 19) = 0.001$, $p = .980$, $\eta_P^2 = .000$, for P45 brain weight adjusted by body weight (Figure 3.9B).

In adult female offspring, two-way ANOVA indicated that body weight did not differ according for maternal drug, $F(1, 25) = 1.071$, $p = .311$, $\eta_P^2 = .041$, maternal housing, $F(1, 25) = 0.146$, $p = .705$, $\eta_P^2 = .006$, or the interaction of the two variables, $F(1, 25) = 0.437$, $p = .515$, $\eta_P^2 = .017$ (Figure 3.9A). In adulthood, body weight was no longer a significant covariate of brain weight for female offspring, $F(1, 24) = 2.742$, $p = .111$, $\eta_P^2 = .103$. Similar to adolescence, there were no significant effects of maternal drug, $F(1, 24) = 0.941$, $p = .342$, $\eta_P^2 = .038$, housing, $F(1, 24) = 0.011$, $p = .916$, $\eta_P^2 = .000$, or an interaction, $F(1, 24) = 2.494$, $p = .127$, $\eta_P^2 = .094$, for female P120 brain weight adjusted by body weight (Figure 3.9B). For adult male body weight, two-way

ANOVA showed no significant main effects of drug, $F(1, 23) = 0.741$, $p = .398$, $\eta_P^2 = .031$, or housing, $F(1, 23) = 0.481$, $p = .495$, $\eta_P^2 = .020$, nor a Drug x Housing interaction, $F(1, 23) = 0.258$, $p = .616$, $\eta_P^2 = .011$ (Figure 3.9A). Two-way ANCOVA revealed that body weight was a significant covariate of brain weight, $F(1, 22) = 6.350$, $p = .019$, $\eta_P^2 = .224$, and there was a significant Drug x Housing interaction for brain weight adjusted by body weight, $F(1, 23) = 7.021$, $p = .015$, $\eta_P^2 = .242$; for male offspring of sucralose-treated dams, maternal enrichment decreased brain weight, $F(1, 22) = 9.590$, $p = .005$, $\eta_P^2 = .304$, although there was no effect of housing among males of nicotine-treated dams, $F(1, 22) = 0.434$, $p = .517$, $\eta_P^2 = .019$ (Figure 3.9B).

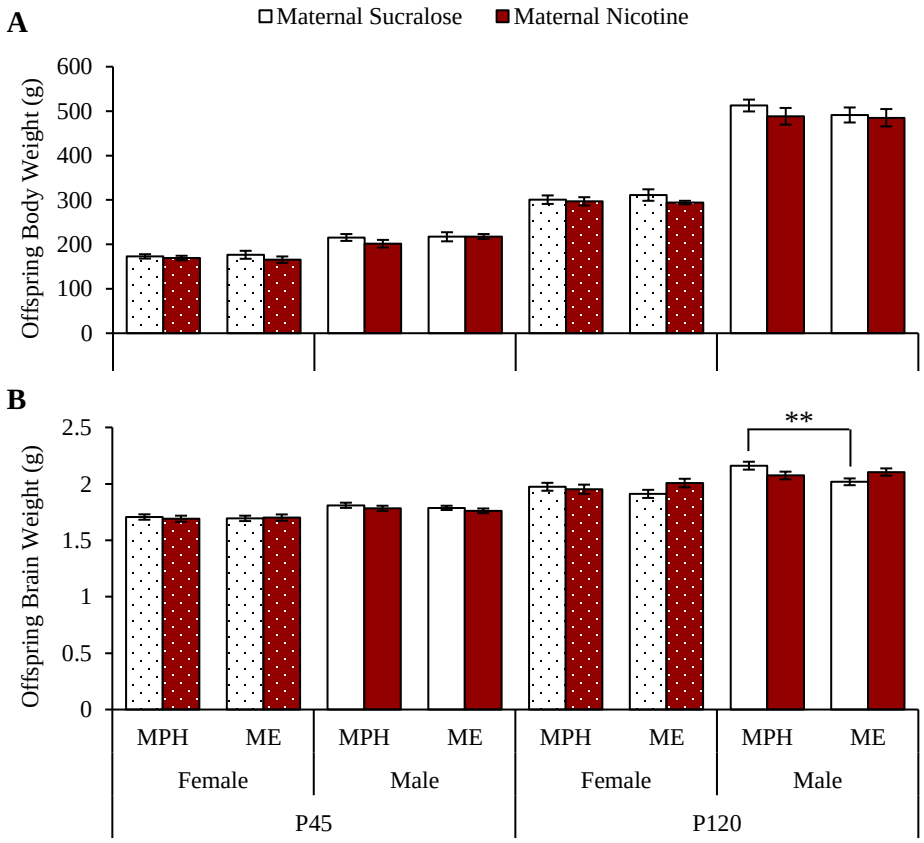

Figure 3.9: Female (patterned) and male (solid) offspring body weight (A) and adjusted brain weight (B) in adolescence (P45) and adulthood (P120). There were no differences in body weight due to maternal drug or housing. In adult male offspring, maternal enrichment decreased brain weight of males born to sucralose-exposed dams. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment, P = postnatal day. ** $p \leq .01$.

3.3.3.2 Offspring Spine Density

Three-way ANOVA for each sex with maternal drug, maternal housing, and offspring hemisphere was initially used; however, there were no significant main effects or interactions with hemisphere so right and left densities were averaged and two-way ANOVA was used.

There were no significant main effects of maternal drug or housing nor a Drug x Housing interaction for basilar spine density in OFC (AID) or medial PFC (Cg3) in female or male offspring (Table 3.4). In males, there was a significant main effect of maternal housing for basilar spine density in the parietal cortex (Par1); maternal enrichment significantly reduced spine density (Figure 3.10). There were no other significant effects for Par1 (Table 3.4).

Table 3.4: Summary of statistical findings for P120 offspring basilar spine density.

	Drug			Housing			Drug x Housing		
	F	p	η_P^2	F	p	η_P^2	F	p	η_P^2
Female									
AID	2.669	.120	.129	0.002	.964	.000	0.357	.558	.019
Cg3	4.003	.059	.167	0.099	.756	.005	2.827	.108	.124
Par1	0.044	.836	.002	0.257	.617	.012	0.425	.521	.020
Male									
AID	0.184	.673	.011	0.518	.481	.030	0.223	.642	.013
Cg3	0.766	.392	.037	0.297	.592	.015	0.067	.798	.003
Par1	1.245	.277	.056	**6.550**	**.018**	**.238**	1.320	.264	.059

AID: Model df = 1, Female Error df = 18, Male Error df = 17
Cg3: Model df = 1, Female Error df = 20, Male Error df = 20
Par1: Model df = 1, Female Error df = 21, Male Error df = 21
Boldface = $p \leq .05$

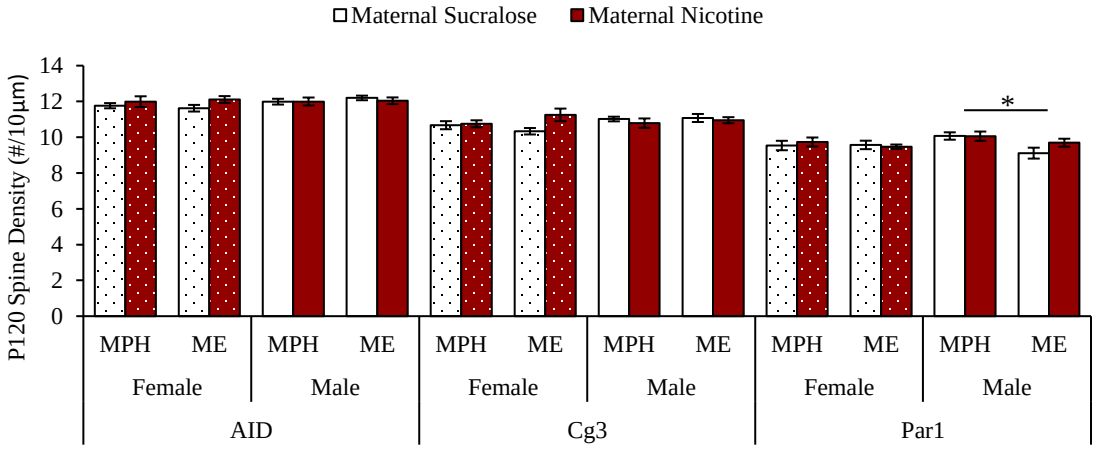

Figure 3.10: Basilar spine density (#/10µm) in the OFC (AID), medial PFC (Cg3), and parietal cortex (Par1) for female (patterned) and male (solid) offspring. There was a significant main effect of housing for male offspring in Par1 such that maternal enrichment decreased spine density. Data shown as mean ± *SE*. MPH = maternal pair-housed; ME = maternal enrichment. * $p \leq .05$.

3.4 Discussion

The current work highlights the impacts of maternal preconception nicotine and environmental enrichment on the long-term behavioral and neural development of offspring. Overall, we demonstrate complex interactions between maternal drug and environment, and offspring age and sex. Specifically, we found: 1) no change in activity level due to either maternal experience; 2) decreased anxiety in adolescent female offspring due to maternal nicotine, and increased anxiety in adolescent male offspring due to maternal enrichment, but no change in adult offspring anxiety; 3) impaired fine motor control in adult female offspring due to maternal nicotine; 4) impaired spatial learning in adult male offspring dependent on the combined effect of maternal nicotine and enrichment; 5) decreased brain weight of adult male offspring of sucralose-treated dams raised in enriched conditions; and 6) decreased spine density in the parietal cortex of male offspring due to maternal enrichment.

Previously, we showed that these maternal experiences do not impact the quality of maternal care (Chapter 2), allowing us to speculate that the effects observed in the offspring are not due to behavioral transmission (Weaver et al., 2004). Therefore, it is likely that the mechanism driving the effects of maternal preconception nicotine and enrichment is epigenetic transmission, the passing of epigenetic signatures from one generation to the next (Bohacek & Mansuy, 2013; Hanson & Skinner, 2016; Jawaid & Mansuy, 2019). It is also possible that nicotine exposure had a direct effect on the reproductive health of the females. Nicotine disrupts placental functioning when consumed during gestation (Zdravkovic, Genbacev, McMaster, & Fisher, 2005); however, the half-life of nicotine is relatively short (~2hr; Tutka, Mosiewicz, & Wielosz,

2005) so it is uncertain if a sufficient quantity of nicotine would be available in these animals at the time of breeding to have such an impact. It is also well established that nicotine impacts female fertility (Holloway, Kellenberger, & Petrik, 2006), such as by reducing the quality of the oocyte (Zenzes & Bielecki, 2004; Mai et al., 2014). Reduced oocyte quality could directly impact the health and development of the resulting offspring.

3.4.1 Main Effects of Maternal Preconception Nicotine: Decreased Anxiety and Impaired Fine Motor Skills in Females

We observed a significant decrease in anxiety in adolescent female offspring due to maternal preconception nicotine; females born to nicotine treated dams spent more time in the end of the open arms of the elevated plus maze than sucralose females. There was no effect of nicotine for adolescent male offspring, or adult offspring of either sex. Exposure to nicotine during preconception, gestation, and lactation decreased P60 offspring anxiety (more time in the open arms of elevated plus maze) and altered the relative proportion of inhibitory to excitatory neurons in the frontal cortex and PFC; these authors speculated that a relative decrease in "inhibitory tone" increased novelty-seeking in the nicotine-treated offspring (Martin et al., 2020). Conversely, others have reported an increase in anxiety in similarly aged male offspring following prenatal nicotine exposure, and no effect in females (Vaglenova et al., 2004, 2008; Polli et al., 2020). Therefore, it appears that the effect of nicotine on adolescent to young adult offspring anxiety depends on the timing of nicotine exposure as well as the sex of the offspring. Our finding that preconception nicotine had no impact on adult offspring anxiety is in agreement with

what other have found following prenatal nicotine (Vaglenova et al., 2008; Zhang, Spencer, Biederman, & Bhide, 2018).

We did not observe hyperactivity in the offspring due to maternal nicotine exposure; offspring showed no change in activity level in adolescence or adulthood. This result was surprising given the frequently cited association between developmental nicotine and hyperactivity/ADHD (Keyes, Smith, & Susser, 2014). Furthermore, hyperactivity is observed following transgenerational maternal exposure to nicotine (Zhu et al., 2014). One possible explanation for the absence of hyperactivity in the current work is that we assessed activity level at the incorrect time of day. We recorded activity during the early to late morning; one study into prenatal nicotine found that the most drastic divergence in activity levels occurred during the dark cycle, whereas as there was little to no difference during the light cycle (Zhu et al., 2012). However, other studies examining prenatal nicotine have found hyperactivity even during the light cycle (Polli et al., 2020). The transgenerational study cited above only examined the dark cycle (Zhu et al., 2014). Therefore, it is possible that we may have observed hyperactivity following preconception nicotine if we had examined the offspring during the dark cycle, and that perhaps prenatal nicotine is more capable of inducing hyperactivity even during the light cycle. Studies that examine activity level of adult offspring following developmental nicotine exposure frequently report no change (Zhang, Spencer, Biederman, & Bhide, 2018; Martin et al., 2020), suggesting that the presence of a hyperactive phenotype depends not only on the timing of nicotine and the time of day, but also on the age of the offspring.

Female offspring of nicotine treated dams were impaired at Whishaw tray reaching, indicating a deficit in the control of fine forelimb movements, although there was no effect in males. The association between nicotine and motor behavior is complex. On one hand, acute nicotine administration is linked with enhanced fine motor control in humans (Heishman, Kleykamp, & Singleton, 2010) and rats (Gonzalez, Gharbawie, Whishaw, & Kolb, 2005). Conversely, perinatal nicotine hinders success at the single pellet reaching task in male offspring (Lee & Picciotto, 2019). Furthermore, although nicotine appears to benefit previously learned motor behaviors, it impairs the learning of new motor skills (Gonzalez et al., 2005). Therefore, the effect of nicotine on motor skill depends on the timing of the exposure and the familiarity of the task, as well as the sex of the individual. The findings from the present experiment most closely align with those following perinatal (prenatal + lactation) exposure, except that we observed the opposite sex effect. In our work, females were impaired and males were unaffected, whereas in the study by Lee & Picciotto (2019), males were impaired and females were unaffected.

There were no effects of maternal preconception nicotine on basilar spine density in either the PFC or the parietal cortex. This finding contradicts what others have reported following nicotine exposure during other developmental periods. In adult offspring, prenatal nicotine increases spine density in the OFC (AID) and parietal cortex (Par1) but decreases density in the medial PFC (Cg3) and nucleus accumbens (Mychasiuk et al., 2013b; Mychasiuk, Muhammad, Carroll, & Kolb, 2013a; Mychasiuk, Muhammad, & Kolb, 2014). Therefore, further investigation is required, such as examining spine density in more areas of the offspring brain following preconception nicotine, including the nucleus accumbens.

3.4.2 Main Effects of Maternal Preconception Enrichment: Increased Anxiety and Decreased Spine Density in the Parietal Cortex in Males

Adolescent male offspring of dams reared in complex housing exhibited increased anxiety in the elevated plus maze, spending less time in the open arms and the ends of the open arms and having fewer arm entries than males born to standard-housed dams. This was the opposite result than we expected. Environmental enrichment is typically associated with decreased anxiety (Lopes, Céspedes, & Viana, 2018), including when experienced prior to conception (Pooriamehr et al., 2017). To our knowledge, one other study has reported that environmental enrichment increases anxiety as assessed by the elevated zero maze; enriched animals spent more time in the sheltered quadrants of the maze compared to impoverished animals. These authors speculated that the different outcomes following enrichment could be due to variations in the enrichment protocol (Dickson & Mittleman, 2021). Interestingly, these authors used an impoverished control group as opposed to a standard-housed control; animals were singly housed in a plastic cage with only aspen bedding (Dickson & Mittleman, 2021). A separate study into the effects of social isolation on anxiety found that single housing for a similar length of time (12wks vs. 10wks beginning post-weaning) decreased anxiety compared to pair-housing (Thorsell et al., 2006). Therefore, it is unclear if enrichment increased anxiety or isolation decreased anxiety in the Dickson and Mittleman (2021) study. However, the majority of research suggests that enriched housing decreases anxiety relative to standard-housed and impoverished animals (Simpson & Kelly, 2011). Clearly, more work is required to delineate the effects of housing condition on the expression of anxiety-like behavior, including maternal preconception housing condition.

Maternal preconception enrichment decreased basilar spine density in the parietal cortex of adult male offspring; there were no differences in either area of the PFC. Conversely, basilar spine density in Par1 is increased in adult males and females following prenatal enrichment, although there is no change in apical spine density (Gibb, Gonzalez, & Kolb, 2014). Direct environmental enrichment in adolescent rats also increases basilar spine density in both sexes (Mychasiuk, Muhammad, & Kolb, 2014). Similarly, direct exposure to an enriched environment for three weeks during adulthood increases spine density (apical and basilar) in the somatosensory cortex (Johansson & Belichenko, 2002). Another study demonstrated that environmental enrichment that begins in either infancy or adulthood not only increases the density of basilar spines in layer II/III of somatosensory cortex, but is also increases spine turnover, a mechanism important for structural plasticity (Jung & Herms, 2014). Therefore, our finding of decreased basilar spine density in Par1 disagrees with the currently well-established association between enrichment and increased spine density. However, no studies have examined how enrichment prior to conception impacts spine density, so the timing of the enrichment may be of critical importance. Further work is needed to identify the mechanisms responsible for these disparate findings. Additionally, it is necessary to acknowledge that although the change in spine density we report here indicates a change in synaptic plasticity in the parietal cortex, it very likely does not directly correlate with any of the behavioral changes we observed due to maternal enrichment.

3.4.3 Interactions Between Maternal Preconception Nicotine and Enrichment: Spatial Learning and Brain Weight in Males

Maternal preconception enrichment improved spatial learning, but only among males born to sucralose treated dams. Maternal nicotine prevented the benefit conferred by enrichment in male offspring, and there were no effects of enrichment or nicotine for female offspring. Direct exposure to enrichment improves spatial learning by reducing thigmotaxis; animals spend less time in proximity with the walls of the pool, likely due to a decrease in anxiety, and therefore locate the hidden platform more efficiently (Harris, D'Eath, & Healy, 2009). Maternal preconception enrichment also improves offspring performance in the Morris water task in terms of both the latency and the strategy used to locate the platform during training. These authors similarly found no benefit of enrichment on performance during the probe trial (Cutuli et al., 2015). Enrichment is capable of reversing the negative consequences of early life stress when considering learning and memory (McCreary & Metz, 2016), however it was ineffective at countering the effects of nicotine. One possible explanation for this is that rather than enrichment reversing the effect of nicotine, nicotine may have reversed the effect of enrichment; indeed, there was no main effect of nicotine or impairment due to nicotine in the pair-housed offspring. Such an adversarial relationship between nicotine and enrichment has been previously reported; prenatal nicotine can block the effects of adolescent enrichment on neuronal structure, suggesting that nicotine restricts plasticity (Mychasiuk, Muhammad, & Kolb, 2014). The ability of environmental enrichment to improve learning and memory has been attributed to increased hippocampal BNDF and other neurotrophins following enrichment (Simpson & Kelly, 2011; Cutuli et al., 2015; Dandi

et al., 2018); the effects of nicotine on BDNF levels are more variable (Machaalani & Chen, 2018).

Maternal enrichment decreased brain weight among adult male offspring born to sucralose treated dams; there was no effect of enrichment among nicotine males, nor any effects of nicotine on brain weight. There were also no differences for female brain weight. Direct exposure to enriched environments is frequently associated with increased brain weight and decreased body weight (Mohammed et al., 2002). Conversely, prenatal enrichment has been shown to have no impact on offspring brain weight (Mychasiuk et al., 2012; Gibb, Gonzalez, & Kolb, 2014). However, paternal preconception enrichment does decrease brain weight of both male and female offspring at the age of weaning (Mychasiuk et al., 2012). To our knowledge, no studies into maternal preconception enrichment have explored the impact on offspring brain weight. However, the combined findings of the present work and past literature suggests that enrichment has dynamic effects on brain weight that are highly dependent on the timing and recipient of the enrichment. No studies have examined the effects of maternal or paternal preconception nicotine on brain weight. Prenatal nicotine decreases brain weight in adult offspring of both sexes (Mychasiuk et al., 2013).

There is a slight possibility that a Drug x Housing interaction could be related to the different quantity of nicotine ingested by the nicotine dams housed in pair-housed and enriched conditions. Enriched nicotine dams consumed significantly less nicotine (2.29mg/kg/day) than pair-housed nicotine dams (2.54mg/kg/day). However, it is unlikely that this is the case as both interactions (for Morris water task and brain weight) involved there being an effect of housing in the sucralose offspring but not the nicotine

offspring. If there was an effect of the different dose of nicotine, we may expect to see differences between the two nicotine groups, with the enriched groups being less impacted than the pair-housed group, which was not the case. Therefore, we can be reasonably confident that the doses of nicotine consumed by each group were similar enough to exert the same effects.

3.4.4 Limitations and Future Directions

The main limitation of this work is discussed in Chapter 2: Females were removed from both the nicotine and enrichment conditions 24hrs prior to mating, so it is possible that withdrawal from these experiences interfered with the early stages of pregnancy, even though maternal care was not impacted. One additional shortcoming that we have already discussed pertains to our assessment of activity level; testing activity during the animal's active phase may have been more fruitful and better contribute to the link between nicotine and hyperactivity. Another limitation relates more broadly to the chosen behavioral tests. It is possible that the tests we used were not sensitive enough to detect potentially very subtle effects of maternal preconception nicotine and enrichment. Therefore, the current work may have benefited from using more sensitive variations of these tests, such as single pellet reaching instead of Whishaw tray reaching or a different version of the Morris water task that used fewer training trials.

Future work should expand on the findings presented here to gain a more comprehensive understanding of the long-term consequences of maternal preconception nicotine and enrichment. For example, additional behaviors such as working memory, depressive-like traits, and vulnerability to drug administration could be explored. Seeing as the mechanism driving the changes observed in offspring is likely epigenetic in nature,

measures of gene expression should also be included. Lastly, analysis of how preconception nicotine and enrichment affect dam reproductive health would be informative in parsing out the mechanism behind these effects.

3.4.5 Concluding Remarks

In line with our predictions, we observed an interaction between maternal preconception nicotine and enrichment in determining offspring outcomes. However, it appears that rather than enrichment moderating the effects of nicotine, nicotine moderated the effects of enrichment. We also observed distinct main effects of nicotine and enrichment that were highly sexually dimorphic. However, the directions of the observed effects were generally contradictory to our predictions. We observed that nicotine decreased anxiety and that enrichment increased anxiety, both opposite effects than we anticipated. We did observe a nicotine-induced impairment in fine motor control, adding to the complexity surrounding nicotine and motor behavior. In line with our predictions, maternal enrichment improved spatial learning in male offspring, but only if the male was born to a dam not exposed to nicotine, suggesting that concurrent exposure to nicotine prevented the transmission of improved spatial learning abilities. Finally, maternal enrichment decreased the density of basilar dendritic spines in the partial cortex of adult male offspring. Therefore, the present work contributes the following novel findings to our current understanding of maternal preconception nicotine and enrichment: 1) both preconception nicotine and enrichment individually impact the development of the next generation, and nicotine is capable of blocking the effects of enrichment, an effect that has been shown with these two experiences at other times during development; 2) similarly to nicotine exposure during other developmental stages, the effects of

preconception nicotine are highly sexually dimorphic; and 3) preconception enrichment affects basilar spine density, representing the first finding regarding neuronal morphology and this preconception experience.

References

Barreto, G. E., Iarkov, A., & Moran, V. E. (2015). Beneficial effects of nicotine, continine and its metabolites as potential agents for Parkinson's disease. *Frontiers in Aging Neuroscience, 6*, 340. DOI:10.3389/fnagi.2014.00340

Blood-Siegfried, J., & Rende, E. K. (2010). The long-term effects of prenatal nicotine exposure on neurologic development. *Journal of Midwifery and Women's Health, 55*, 143 – 155. DOI:10.1016/j.jmwh.2009.05.006

Bohacek, J., & Mansuy, I. M. (2013). Epigenetic inheritance of disease and disease risk. *Neuropsychopharmacology, 38*, 220 – 236. DOI10.1038/npp.2012.110

Bruin, J. E., Gerstein, H. C., & Holloway, A. C. (2010). Long-term consequences of fetal and neonatal nicotine exposure: A critical review. *Toxicological Sciences, 116*, 364 – 374. DOI:10.1093/toxsci/kfq103

Caporali, P., Cutuli, D., Gelfo, F., Laricchiuta, D., Foti, F., De Bartolo, P., Mancini, L., Angelucci, F., & Petrosini, L. (2014). Pre-reproductive maternal enrichment influences offspring developmental trajectories: Motor behavior and neurotrophin expression. *Frontiers in Behavioral Neuroscience, 8*, 195. DOI:10.3389/fnbeh.2014.00195

Collins, A. C., Pogun, S., Nesil, T., & Kanit, L. (2012). Oral nicotine self-administration in rodents. *Journal of Addiction Research and Therapy, S2*, 004. DOI:10.4172/2155-6105.S2-004

Cutuli, D., Berretta, E., Pasqualini, G., De Bartolo, P., Caporali, P., Laricchiuta, D., Sampedro-Piquero, P., Gelfo, F., Pesoli, M., Foti, F., Begega, A., & Petrosini, L. (2017). Influence of pre-reproductive maternal enrichment on coping response to stress and expression of c-fos and glucocorticoid receptors in adolescent offspring. *Frontiers in Behavioral Neuroscience, 11*, 73. DOI:10.3389/fnbeh.2017.00073

Cutuli, D., Caporali, P., Gelfo, R., Angelucci, F., Laricchiuta, D., Foti, F., De Bartolo, P., Bisicchia, E., Molinari, M., Vecchioli, S. F., & Petrosini, L. (2015). Pre-reproductive maternal enrichment influences rat maternal care and offspring developmental trajectories: Behavioral performances and neuroplasticity correlates. *Frontiers in Behavioral Neuroscience, 9*, 66. DOI:10.3389/fnbeh.2015.00066

Dandi, E., Kalamari, A., Touloumi, O., Lagoudaki, R., Nousiopoulou, E., Simeonidou, C., Spandou, E., & Tata, D. A. (2018). Beneficial effects of environmental enrichment on behavior, stress reactivity and synaptophysin/BDNF expression in the hippocampus following early life stress. *International Journal of Developmental Neuroscience, 67*, 19 – 32. DOI:10.1016/j.ijdevneu.2018.03.003

Dickson, P. E., & Mittleman, G. (2021). Environmental enrichment influences novelty reactivity, novelty preference, and anxiety via distinct genetic mechanisms in C57BL/6J and DBA/2J mice. *Scientific Reports, 11*, 3928. DOI:10.1038/s41598-021-83574-6

Gibb, R. L., Gonzalez, C. L. R., & Kolb, B. (2014). Prenatal enrichment and recovery from perinatal cortical damage: Effects of maternal complex housing. *Frontiers in Behavioral Neuroscience, 8*, 223. DOI:10.3389/fnbeh.2014.00223

Gibb, R., & Kolb, B. (1998). A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *Journal of Neuroscience Methods, 79*, 1 – 4. DOI:10.1016/s0165-0270(97)00163-5

Gonzalez, C. L. R., Gharbawie, O. A., Whishaw, I. Q., & Kolb, B. (2005). Nicotine stimulates dendritic arborization in motor cortex and improves concurrent motor skill but impairs subsequent motor learning. *Synapse, 55*, 183 – 191. DOI:10.1002/syn.20106

Hamilton, D. A., & Kolb, B. (2005). Differential effects of nicotine and complex housing on subsequent experience-dependent structural plasticity in the nucleus accumbens. *Behavioral Neuroscience, 119*, 355 – 365. DOI:10.1037/0735-7044.119.2.355

Hanson, M. A., & Skinner, M. K. (2016). Developmental origins of epigenetic transgenerational inheritance. *Environmental Epigenetics, 2*, 1 – 9. DOI:10.1093/eep/dvw002

Harris, A. P., D'Eath, R. B., & Healy, S. D. (2009). Environmental enrichment enhances spatial cognition in rats by reducing thigmotaxis (wall hugging) during testing. *Animal Behavior, 77*, 1459 – 1464. DOI:10.1016/j.anbehav.2009.02.019

Heishman, S. J., Kleykamp, B. A., & Singleton, E. G. (2010). Meta-analysis of the acute effects of nicotine and smoking on human performance. *Psychopharmacology, 210*, 453 – 469. DOI:10.1007/s00213-010-1848-1

Holbrook, B. D. (2016). The effects of nicotine on human fetal development. *Birth Defects Research (Part C), 108*, 181 – 192. DOI:10.1002/bdrc.21128

Holloway, A. C., Cuu, D. Q., Morrison, K. M., Gerstein, H. C., & Tarnopolsky, M. A. (2007). Transgenerational effects of fetal and neonatal exposure to nicotine. *Endocrine, 31*, 254 – 259. DOI:10.1007/s12020-007-0043-6

Holloway, A. C., Kellenberger, L. D., & Petrik, J. J. (2006). Fetal and neonatal exposure to nicotine disrupts ovarian function and fertility in adult female rats. *Endocrine, 30*, 213 – 216. DOI:10.1385/endo:30:2:213

Jawaid, A., & Mansuy, I. M. (2019). Inter- and transgenerational inheritance of behavioral phenotypes. *Current Opinion in Behavioral Sciences, 25*, 96 – 101. DOI:10.1016/j.cobeha.2018.12.004

Johansson, B. B., & Belichenko, P. V. (2002). Neuronal plasticity and dendritic spines: Effect of environmental enrichment on intact and postischemic rat brain. *Journal of Cerebral Blood Flow and Metabolism, 22*, 89 – 96. DOI:10.1097/00004647-200201000-00011

Jung, C. K. E., & Herms, J. (2014). Structural dynamics of dendritic spines are influenced by an environmental enrichment: An in vivo imaging study. *Cerebral Cortex, 24*, 377 – 384. DOI:10.1093/cercor/bhs317

Keyes, K. M., Smith, G. D., & Susser, E. (2014). Associations of prenatal maternal smoking with offspring hyperactivity: Causal or confounded. *Psychological Medicine, 44*, 857 – 867. DOI:10.1017/s0033291713000986

Lee, A. M., & Picciotto, M. R. (2019). Perinatal nicotine exposure impairs learning of a skilled forelimb reaching task in male but not female adult mice. *Behavioral Brain Research, 367*, 176 – 180. DOI:10.1016/j.bbr.2019.04.007

Lopes, D. A., Céspedes, I. C., & Viana, M. B. (2018). Effects of environmental enrichment on anxiety measurements: A review. *Research & Reviews: Neuroscience, 2*, 1 – 6.

Machaalani, R., & Chen, H. (2018). Brain-derived neurotrophic factor (BDNF), its tyrosine kinase receptor B (TrkB) and nicotine. *NeuroToxicology, 65*, 186 – 195. DOI:10.1016/j.neuro.2018.02.014

Mai, Z., Lei., M., Yu, B., Du, H., & Liu, J. (2014). The effects of cigarette smoke extract on ovulation, oocyte morphology and ovarian gene expression in mice. *PLoS One, 9*, e95945. DOI:10.1371/journal.pone.0095945

Martin, M. M., McCarthy, D. M., Schatschneider, C., Trupiano, M. X., Jones, S. K., Kalluri, A., & Bhide, P. G. (2020). Effects of developmental nicotine exposure on frontal cortical GABA-to-non-GABA neuron ratio and novelty-seeking behavior. *Cerebral Cortex, 30*, 1830 – 1842. DOI:10.1093/cercor/bhz207

McCreary, J. K., & Metz, G. A. S. (2016). Environmental enrichment as an intervention for adverse health outcomes of prenatal stress. *Environmental Epigenetics, 2*, 1 – 12. DOI:10.1093/eep/dvw013

Mesa-Gresa, P., Ramos-Campos, M., & Redolat, R. (2013). Enriched environments for rodents and their interaction with nicotine administration. *Current Drug Abuse Reviews, 6*, 191 – 200.

Mohammed, A. H., Zhu, S. W., Darmopil, S., Hjerling-Leffler, J., Ernfors, P., Winblad, B., Diamond, M. C., Eriksson, P. S., & Bogdanovic, N. (2002). Environmental enrichment and the brain. *Progress in Brain Research, 138*, 109 – 133. DOI:10.1016/s0079-6123(02)38074-9

Morris, R. (1984). Developments of a water maze procedure for studying spatial learning in the rat. *Journal of Neuroscience Methods, 11*, 47-60. DOI:10.1016/0165-0270(84)90007-4

Mychasiuk, R., Muhammad, A., Carroll, C., & Kolb, B. (2013a). Does prenatal nicotine exposure alter the brain's response to nicotine in adolescence? A neuroanatomical analysis. *European Journal of Neuroscience, 38*, 2491 – 2503. DOI:10.1111/ejn.12245

Mychasiuk, R., Muhammad, A., Gibb, R., & Kolb, B. (2013b). Long-term alterations to dendritic morphology and spine density associated with prenatal exposure to nicotine. *Brain Research, 1499*, 53 – 60. DOI:10.1016/j.brainres.2012.12.021

Mychasiuk, R., Muhammad, A., & Kolb, B. (2014). Environmental enrichment alters structural plasticity of the adolescent brain but does not remediate the effects of prenatal nicotine exposure. *Synapse, 68*, 293 – 305. DOI:10.1002/syn.21737

Mychasiuk, R., Zahir, S., Schmold, N., Ilnytskyy, S., Kovalchuk, O., & Gibb, R. (2012). Parental enrichment and offspring development: Modifications to brain, behavior, and the epigenome. *Behavioral Brain Research, 228*, 294 – 298. DOI:10.1016/j.bbr.2011.11.036

Nesil, T., Kanit, L., Collins, A. C., & Pogun, S. (2011). Individual differences in oral nicotine intake in rats. *Neuropharmacology, 61*, 189 – 201. DOI:10.1016/j.neuropharm.2011.03.027

Pauly, J. R., Sparks, J. A., Hauser, K. F., & Pauly, T. H. (2004). In utero nicotine exposure causes persistent, gender-dependent changes in locomotor activity and sensitivity to nicotine in C57B1/6 mice. *International Journal of Developmental Neuroscience, 22*, 329 – 337. DOI:10.1016/j.ijdevneu.2004.05.009

Peters, D. A. V., & Tang, S. (1982). Sex-dependent biological changes following prenatal nicotine exposure in the rat. *Pharmacology, Biochemistry, and Behavior, 17*, 1077 – 1082. DOI:10.1016/0091-3057(82)90497-x

Polli, F. S., Scharff, M. B., Ipsen, T. H., Aznar, S., Kohlmeier, K. A., Andreasen, J. T. (2020). Prenatal nicotine exposure in mice induces sex-dependent anxiety-like behavior, cognitive deficits, hyperactivity, and changes in the expression of glutamate receptor associated-genes in the prefrontal cortex. *Pharmacology, Biochemistry, and Behavior, 195*, 172951. DOI:10.1016/j.pbb.2020.172951

Pooriamehr, A., Sabahi, P., & Miladi-Gorji, H. (2017). Effects of environmental enrichment during abstinence in morphine dependent parents on anxiety, depressive-like behaviors and voluntary morphine consumption in rat offspring. *Neuroscience Letters, 656*, 37 – 42. DOI:10.1016/j.neulet.2017.07.024

Quik, M., Huang, L. Z., Parameswaran, N., Bordia, T., Campos, C., & Perez, X. A. (2009). Multiple roles for nicotine in Parkinson's disease. *Biochemical Pharmacology, 78*, 677 – 685. DOI:10.1016/j.bcp.2009.05.003

Quik, M., Perez, X. A., & Bordia, T. (2012). Nicotine as a potential neuroprotective agent for Parkinson's disease. *Movement Disorders, 27*, 947 – 957. DOI:10.1002/mds.25028

Renaud, S. M., & Fountain, S. B. (2016). Transgenerational effects of adolescent nicotine exposure in rats: Evidence for cognitive deficits in adult female offspring. *Neurotoxicology and Teratology, 56*, 47 – 54. DOI:10.1016/j.ntt.2016.06.002

Robinson, T. E., & Kolb, B. (2004). Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology, 47*, 33 – 46. DOI:10.1016/j.neuropharm.2004.06.025

Rogers, J. M. (2019). Smoking and pregnancy: Epigenetics and developmental origins of the metabolic syndrome. *Birth Defects Research, 111*, 1259 – 1269. DOI:10.1002/bdr2.1550

Schneider, T., Bizarro, L., Asherson, P. J. E., & Stolerman, I. P. (2010). Gestational exposure to nicotine in drinking water: Teratogenic effects and methodological issues. *Behavioral Pharmacology, 21*, 206 – 216. DOI:10.1097/fbp.0b013e32833a5bb5

Schneider, T., Ilott, N., Brolese, G., Bizarro, L., Asherson, P. J. E., & Stolerman, I. P. (2011). Prenatal exposure to nicotine impairs performance of the 5-choice serial reaction time task in adult rats. *Neuropsychopharmacology, 36*, 1114 – 1125. DOI:10.1038/npp.2010.249

Simpson, J., & Kelly, J. P. (2011). The impact of environmental enrichment in laboratory rats – Behavioral and neurochemical aspects. *Behavioral Brain Research, 222*, 246 – 264. DOI:10.1016/j.bbr.2011.04.002

Solinas, M., Thiriet, N., Chauvet, C., & Jaber, M. (2010). Prevention and treatment of drug addiction by environment enrichment. *Progress in Neurobiology, 92*, 572 – 592. DOI:10.1016/j.pneurobio.2010.08.002

Thorsell, A., Slawecki, C. J., Khoury, A. E., Mathe, A. A., & Ehlers, C. L. (2006). The effects of social isolation on neuropeptide Y levels, exploratory and anxiety-related

behaviors in rats. *Pharmacology, Biochemistry, and Behavior, 83*, 28 – 34. DOI:10.1016/j.pbb.2005.12.005

Tutka, P., Mosiewicz, J., & Wielosz, M., (2005). Pharmacokinetics and metabolism of nicotine. *Pharmacological Reports, 57,* 143 – 153.

Vaglenova, J., Birru, S., Pandiella, N. M., & Breese, C. R. (2004). An assessment of the long-term developmental and behavioral teratogenicity of prenatal nicotine exposure. *Behavioral Brain Research, 150*, 159 – 170. DOI:10.1016/j.bbr.2003.07.005

Vaglenova, J., Parameshwaran, K., Suppiramaniam, V., Breese, C. R., Pandiella, N., & Birru, S. (2008). Long-lasting teratogenic effects of nicotine on cognition: Gender specificity and role of AMPA receptor function. *Neurobiology of Learning and Memory, 90*, 527 – 536. DOI:10.1016/j.nlm.2008.06.009

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature Neuroscience, 7,* 847 – 854. DOI:10.1038/nn1276

Zdravkovic, T., Genbacev, O., McMaster, M. T., & Fisher, S. J. (2005). The adverse effects of maternal smoking on the human placenta: A review. *Placenta, 26*, Supplement, S81 – S86. DOI:10.1016/j.placenta.2005.02.003

Zensez, M., & Bielecki, R. (2004). Nicotine-induced disturbances of meiotic maturation in cultured mouse oocytes: Alterations of spindle integrity and chromosome alignment. *Tobacco Induced Diseases, 2*, 151 – 161. DOI:10.1186/1617-9625-2-3-151

Zhang, L., Spencer, T. J., Biederman, J., & Bhide, P. G. (2018). Attention and working memory deficits in a perinatal nicotine exposure mouse model. *PLoS ONE, 13*, e0198064. DOI:10.1371/journal.pone.0198064

Zhu, J., Fan, F., McCarthy, D. M., Zhang, L., Cannon, E. N., Spencer, T. J., Biederman, J., & Bhide, P. G. (2017). A prenatal nicotine exposure mouse model of methylphenidate responsive ADHD-associated cognitive phenotypes. *International Journal of Developmental Neuroscience, 58,* 26 – 34. DOI:10.1016/j.ijdevneu.2017.01.014

Zhu, J., Lee, K. P., Spencer, T. J., Biederman, J., & Bhide, P. G. (2014). Transgenerational transmission of hyperactivity in a mouse model of ADHD. *The Journal of Neuroscience, 34*, 2768 – 2773. DOI:10.1523/jneurosci.4402-13.2014

Zhu, J., Zhang, X., Xu, Y., Spencer, T. J., Biederman, J., & Bhide, P. G. (2012). Prenatal nicotine exposure mouse model showing hyperactivity, reduced cingulate cortex volume, reduced dopamine turnover, and responsiveness to oral methylphenidate

treatment. *The Journal of Neuroscience, 32*, 9410 – 9418. DOI:10.1523/jneursci.1041-12.2012

Zilles, K. (1985). *The Cortex of the Rat: A Stereotaxic Atlas*. Springer-Verlag: Berlin, Germany.

CHAPTER 4

Maternal preconception alcoholism: Effects on offspring behavioral development and dendritic spine density from neonatal to adult life

Abstract

The consequences of fetal exposure to alcohol are well-documented and collectively referred to as fetal alcohol spectrum disorder (FASD). However, it is becoming increasingly apparent that alcohol exposure prior to conception can also have detrimental effects on future progeny, even in the complete absence of direct fetal exposure. Here we investigated the effects of chronic high levels of ethanol exposure in Long-Evans rat dams during the preconception period on the development of the subsequent generation. Female Long-Evans (P90) were exposed to ethanol in their drinking water beginning at 2.5% v/v and escalating to 20% for a total of seven weeks. Females were then mated with non-alcohol-exposed males, and the behavioral and brain development of their offspring was compared to those offspring of dams not exposed to any drug. Results indicate that maternal preconception alcohol did not impair maternal care, although it did alter locomotor development, induce a decrease in anxiety-like behavior in adolescent offspring, and an apparent spatial learning advantage in adult female offspring. Maternal alcohol also induced sex- and region-specific changes in basilar dendritic spine density of adult offspring and decreased basilar dendritic spine density in the parietal cortex of the dams themselves following a long period of abstinence. Therefore, we can conclude that maternal preconception alcohol exposure induces sex- and age-dependent influences over the development of the next generation, providing further evidence of the importance of maternal behavior before pregnancy.

4.1 Introduction

Alcohol is the most widely consumed drug of abuse by individuals of reproductive age, with up to 25% of females and 36% of males between the ages of 18 and 34 years reporting as “heavy drinkers” (Statistics Canada, 2021). Alcohol is a potent teratogen, the effects of which have been studied in-depth for four decades (Gupta, Gupta, & Shirasaka, 2016; Haycock, 2009). Prenatal alcohol exposure results in fetal alcohol spectrum disorders (FASDs), the severity of which depends on the timing and dose of alcohol use and varies from alcohol-related neurodevelopmental delay (ARND) to full fetal alcohol syndrome (FAS; Haycock, 2009). The predominant consequences of FASDs include facial abnormalities, growth restriction, structural brain abnormalities, neurodevelopmental impairments, and mental health concerns (Koren, Nulman, Chudley, & Loocke, 2003; Sokol, Delaney-Black, & Nordstrom, 2003; Chudley et al., 2005; Cook et al., 2016; Easey, Dyer, Timpson, & Munafò, 2019).

Much less is understood about the consequences of alcohol exposure at earlier stages of development, such as during the preconception period. The preconception period encompasses a wide window of developmental time, as it can refer to any time prior to conception, including immediately prior, during adolescence, or even during gestation of the parent. Several studies to date have examined the effects of parental alcohol exposure during these variable time windows on the subsequent generation. Here we focus on maternal or biparental exposure, but several excellent reviews of paternal exposure are available (Finegersh, Rompala, Martin, & Homanics, 2015; Goldberg & Gould, 2019; Nieto & Kosten, 2019; Rompala & Homanics, 2019). Interestingly, there

has been a relative scarcity of research into the impacts of maternal preconception alcohol exposure alone (Vassoler, Byrnes, & Pierce, 2014; Yohn, Bartolomei, & Blendy, 2015).

Several exposure paradigms have been employed to examine the influence of maternal or biparental preconception alcohol, including: 1) administration of moderate levels of ethanol (5%) in liquid diet for two weeks immediately prior to mating (Lee et al., 2020); 2) free access to moderate levels of ethanol (6.7%) in drinking water for three weeks two weeks prior to mating (Jabbar et al, 2016); 3) binge-pattern exposure (3 days on, 2 days off, 3 days on) during early and late adolescence via oral gavage (3g/kg, 20% v/v; Asimes et al., 2017; 2018); 4) administration of 25% v/v ethanol via intragastric intubation for 10 days six weeks prior to mating (Choi et al., 2012); and 5) gestational exposure of F1 offspring to ethanol-containing liquid diet, mated to generate F2 offspring (Tunc-Ozcan, Harper, Graf, & Redei, 2016). The consequences of maternal/biparental preconception ethanol exposure are equally variable and include behavioral abnormalities (Choi et al., 2012; Jabbar et al., 2016; Tunc-Ozcan et al., 2016; Asimes, et al., 2018), hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Jabbar et al., 2016; Asimes et al., 2018), alterations in neurotransmitter systems (Choi et al., 2012), changes to hypothalamic gene expression (Przybycien-Szymanska et al., 2014; Asimes et al., 2017), altered growth patterns (Livy, Maier, & West, 2004; Harper et al., 2014; Asimes et al., 2018; Lee et al., 2020), and impaired glucose metabolism (Harper et al., 2014; Al-Yasari et al., 2021).

The current study used an ethanol exposure paradigm that mimicked alcohol dependency, wherein female Long-Evans rats consumed up to 20% ethanol in their drinking water for seven consecutive weeks immediately prior to mating (Jamerson,

Wulson, & Kimler, 2004). Seven weeks is the approximate length of the spermatogenic cycle in male rats (Jamerson, Wulson, & Kimler, 2004), and was chosen to mirror the complementary studies on paternal exposure conducted in our lab (unpublished). Females were mated with non-alcohol-exposed males the day following cessation of ethanol treatment, and the resulting offspring completed a behavioral test battery beginning in early life. This test battery includes measures of early sensorimotor and locomotor development, activity, anxiety, fine motor skill, and spatial learning and memory, and provides a thorough picture of behavioral competency throughout development. Offspring were euthanized at one of three ages, weaning (post-natal day (P)21), adolescence (P45), or adulthood (P120). To date, no studies have examined how maternal preconception alcohol impacts brain structure. Here we used Golgi-Cox staining to estimate dendrite spine density in the prefrontal cortex (PFC), parietal cortex, and hippocampus. Alcohol during other stages of development has been shown to impact spine density throughout the brain, including the PFC and hippocampus (Whitcher & Klintsova, 2008; Hamilton, Whitcher, & Klintsova, 2010; Risher et al., 2015). Therefore, we investigated how maternal preconception alcohol impacts spine density in these areas, as they are both heavily implicated in our behavioral test battery. We also examined spine density in the parietal cortex as a within-subject control to deduce if changes in density are global or area-specific.

4.2 Methods

4.2.1 Animals

All procedures were approved by the University of Lethbridge Animal Welfare Committee and the Canadian Council on Animal Care. Parental animals were born in-

house (3rd generation) from animals brought in from Charles River Laboratories and came from eight different litters to achieve sufficient genetic diversity. A total of 15 nulliparous female Long Evans rats were bred with 15 male Long Evans between October and December 2018. One additional female in the alcohol condition began the experiment but refused to drink the alcohol and so was not bred. Eleven breeding pairs led to successful pregnancies, resulting in 153 pups; however, one alcohol-exposed dam was not producing milk, so her litter was euthanized. Therefore, experimental offspring came from five alcohol dams (*n* = 70) and five control dams (*n* = 71). All parents and offspring generated were handled daily and cages were cleaned twice weekly. All animals were maintained in a temperature- (21ºC) and humidity-controlled (50%) breeding room and provided food and water *ad libitum* unless otherwise specified for the duration of the experiment.

4.2.2 Maternal Experience

Parental animals were pair-housed in standard laboratory-style cages (24cm x 45cm x 20.5cm) with a same-treatment cage-mate from adolescence. Baseline water consumption was monitored for one week prior to beginning ethanol treatments. Females in the alcohol condition (*n* = 8) began receiving ethanol in their drinking water on P90. Ethanol was introduced at a concentration of 2.5% v/v, which increased by 2.5% every second day until a final concentration of 20% was achieved, which was maintained until a total of seven weeks had elapsed (Jamerson, Wulson, & Kimler, 2004). Females in the control condition (*n* = 8) received plain drinking water for the duration of the experiment. For both groups, the water bottles were weighed at the same time each day to monitor

volume intake, and ethanol intake was calculated as g/kg of body weight. Females in both groups were provided free access to standard laboratory rat chow and weighed daily.

4.2.3 Breeding

Females in both conditions were paired with a male Long-Evans rat for breeding 24hrs after alcohol-exposed females were removed from their treatment. Breeding pairs were left undisturbed for 10 days aside from twice weekly cage cleaning. Males were then removed and singly housed, and females were re-caged with their previous cage-mate. Females were weighed daily from this point to monitor pregnancy, and singly housed 21 days after being paired with the males in preparation for delivery. The day of parturition was considered P1. Dams were left undisturbed with their litters aside from twice weekly cage cleaning until P7, at which time daily handling of the pups commenced. Pups were weaned on P22 and housed in same-sex, same-treatment groups of 3-5 in cages measuring 38.5cm x 49cm x 21cm.

4.2.4 Behavior

4.2.4.1 Maternal Care

Maternal care was recorded on P8, P12, and P16 for two 15min sessions (09:00 and 15:00). Recordings took place in the animals' home-cage in the housing room, although the cage was relocated within the room to a filming location that was separated from the other animals. After the cage was relocated, the dam and litter were given 5min to return to their natural behavior before filming began. A camera on a tripod was directed at the side of the home-cage. Videos were double scored by two assistants blind to the treatment groups for nursing behaviors (arch-back nursing, blanket-posture nursing, and passive-

posture nursing), licking and grooming, and non-contact. Morning and afternoon sessions were summed for the analysis.

4.2.4.2 Negative Geotaxis

Pups were assessed in negative geotaxis on P9 and P10. Negative geotaxis is an assessment of sensorimotor development and monitors the maturation of the vestibular system (Motz & Alberts, 2005). Each day, pups were individually placed facing downwards on a 40º incline constructed of Plexiglas® and covered in rubber mesh and filmed for 1min (Figure 4.1). The purpose of the task was for the pup to correct its orientation by rotating around the long axis of the body until it faced an upwards direction. The pup was replaced in the original position if it fell from the platform or touched the table surface. Videos were double scored by two assistants blind to the treatment groups for the length of time the pup spent rotated above the horizontal plane and the number of falls from the platform.

Figure 4.1: Apparatus and filming of negative geotaxis, an assessment of early sensorimotor development in rodents.

4.2.4.3 Open Field

Pups were assessed for open field exploration on P10 – 13 and P15. Each day, pups were individually placed in the center of a Plexiglas® box that measured 20cm x 30cm and had a grid of 10 x 15 2cm x 2cm squares drawn on the floor surface (Figure 4.2). Pups were filmed for 1min by an overhead camera while they explored the field. The field was cleaned with 1% Virkon® between each animal. Videos were double scored by two assistants blind to the treatment groups for the number of novel squares and the total number of squares entered by each front paw. Novel squares were unique squares entered by the pup, up to a maximum of 150 (i.e. 10 x 15). Total squares were equivalent to the number of forelimb movements from one square to another. The proportion of time spent in the inner and outer regions of the open field was also tabulated. Inner squares constituted the interior 6 x 11 rectangle, and outer squares consisted of the 2-square wide perimeter of the open field.

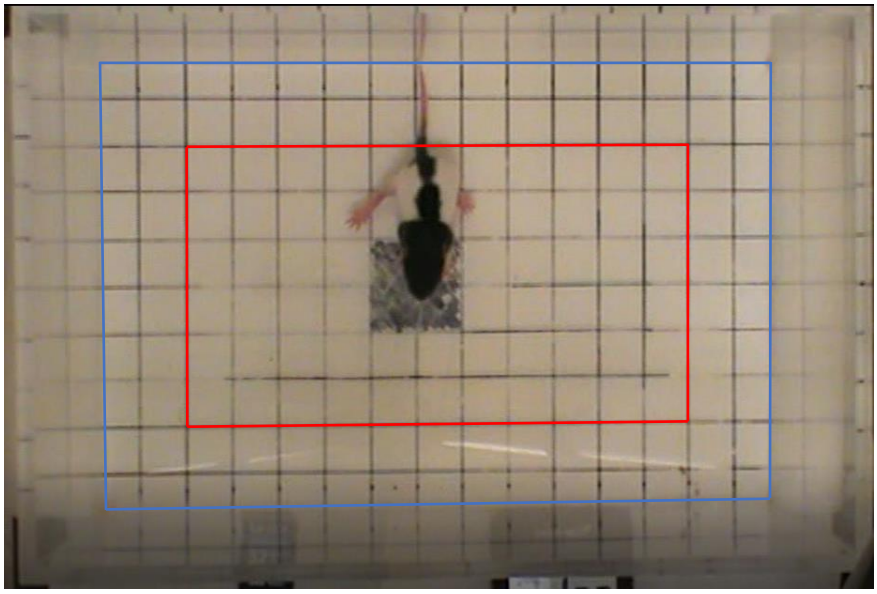

Figure 4.2: Arrangement of the open field. The inner squares are outlined in red, and the outer squares are outlined in blue.

4.2.4.4 Activity Box

Offspring were tested in activity box, an automated test of open field exploration, on P35 (adolescence) and P90 (adulthood). At each age, animals were individually placed in the chamber of a VersaMax Legacy Open Field (Omnitech Electronics, Inc) and activity was monitored for 10min. The chamber was constructed of clear Plexiglas and measured 41cm x 41cm x 30.5cm with a lid to prevent escape. No experimenters were present in the room during recording. The chamber was cleaned with 1% Virkon® between animals. The variables of interest were distance travelled (cm), time spent moving (s), proportion of time spent in the center versus the margins of the chamber (%), and number of rears.

4.2.4.5 Elevated Plus Maze

Offspring were tested in elevated plus maze on P36 (adolescence) and P91 (adulthood). At each age, animals were filmed on the elevated plus maze apparatus for 5min (Figure 4.3). The apparatus was constructed of black opaque Plexiglas®. The maze was raised 1m off the floor and consisted of two "open" arms and two "closed" arms. Both open and closed arms were 40cm long and 10cm wide; closed arms also had 40cm high walls along three sides. The distal 20cm of the open arms were considered a distinct zone, the "end of the open arms". Where the four arms converged in the center of the maze was "center square", and this was the starting point for each animal. Videos were double scored by two assistants blind to the treatment groups for the total time spent in each zone of the maze and the total number of arm entries.

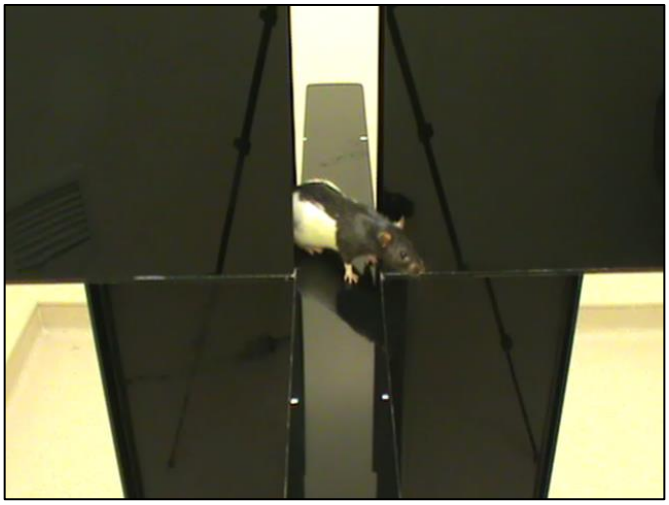

Figure 4.3: Elevated plus maze, used to assess anxiety-like behavior in adolescent (P36) and adult (P90) offspring.

4.2.4.6 Whishaw Tray Reaching

Offspring were tested in the Whishaw tray reaching task from P92 – 114. The testing chambers were boxes that measured 20cm wide, 27cm deep, and 19.5cm high. The front of the cage was made up of 3mm metal bars that were spaced 10mm apart; the three other walls and ceiling of the boxes were solid, clear Plexiglas®. The floor of the chamber was wire mesh and lifted 5cm off the table such that any food that was dropped could not be retrieved. Immediately outside the front of the cage sat a food tray, 7mm deep and 5cm wide, that contained food pieces (Prostock® poultry feed) that were approximately 5mm long (Figure 4.4). Animals were food restricted to 90% of their initial weight to increase motivation to reach. For the first week of the task, rats were habituated to the testing chambers for 30min each day, during which time the food tray was empty. Training began with 1hr sessions each day for three days, which was then reduced to 30min for four days, then 10min for seven days. During training, a research

assistant was present in the room to encourage the rats to reach through the bars with their forepaws while discouraging use of their tongue to reach the food, and to refill the food tray as it was emptied. All animals successfully reached within the first two days of training but overtraining ensured that all animals had ample time to reach peak accuracy. On the final day of the task, rats were filmed for 5min while in the testing chambers. Videos were double scored by two assistants blind to the treatment groups for the number of reach attempts with each forepaw and the number of attempts that led to the animal successfully consuming the food piece ("hits"). Reach success was calculated as the number of hits divided by the number of attempts.

Figure 4.4: Whishaw tray reaching chamber, a measure of fine motor skill.

4.2.4.7 Morris Water Task

Animals were tested in a place version of the Morris water task described by Morris (1984) from P115 – 119. The pool had a diameter of 1.5m, white interior walls, and was filled with water (24°C) that was tinted white using non-toxic tempera paint to a

depth of 35cm. Submerged 5cm beneath the surface of water was a platform constructed of clear Plexiglas® with a surface measuring 12cm x 12cm. The pool was conceptually divided into four quadrants (NE, SE, SW, NW), with the platform located in the center of the NW quadrant. Positioned above the pool was a camera connected to HVS Image tracking software. The pool itself contained no distinguishing features, but the walls of the testing room each contained unique visual cues. Animals completed four training trials each day for five days, with each trial starting from one of the cardinal directions; the order of the starting locations was pseudorandomized such that the same order was not repeated. For each trial, the animal was placed in the pool close to and facing the wall of the pool at the appropriate location, then released. Animals were allowed up to 60s to locate the hidden platform, after which time an experimenter would gently direct them to the platform with their hand. Regardless of whether the animal found the platform on its own, it would be given 5s to visually scan the room prior to being placed in its holding cage until the next trial (~5-10min). Following the four training trials on the fifth day of training, the platform was removed from the pool and each animal was placed back in the pool for one 30s probe trial. For analysis of the training data, the latency (s) and distance (m) were averaged across the four trials each day. For the probe trial, we analyzed distance (m), percent of time in the target quadrant (NW), percent of time in the opposite quadrant (SE), and percent of time in the adjacent quadrants (average of NE and SW).

4.2.5 Neuroanatomy

4.2.5.1 Euthanasia and Perfusion

A subset of the offspring was randomly selected across litters to be euthanized at weaning (P21, $n = 33$), in adolescence (P45, $n = 26$) and in adulthood (P120, $n = 34$).

Remaining animals were used in a different experiment and will not be discussed here. Dams were euthanized following a second round of breeding (data not shown), at approximately 10 months of age. Animals were administered an overdose of pentobarbital sodium (i.p.; 300mg/kg) and in the absence of hindlimb reflexes, intracardially perfused with 50 – 200mL of 0.9% saline, depending on age and body weight. Animals were then decapitated, and their brains were removed, weighed, and placed in Golgi-Cox solution in dark (light-blocking) bottles for two weeks. The Golgi-Cox solution was replaced with 30% sucrose for at least two weeks prior to the brains being sectioned on a vibratome at 200μm. Every section of brain tissue was placed on 2% gelatin-coated slides, then stained as per Gibb & Kolb (1998).

4.2.5.2 Spine Density

Basilar dendritic spine density for P21 and P120 offspring and dams was estimated for pyramidal cells in the orbitofrontal cortex (OFC; layer III AID), medial PFC (layer V Cg3), parietal cortex (layer III Par1), and hippocampus (layer III CA1; P120 only; Figure 4.5). Dendrites were traced at 1250x using a camera lucida if they satisfied the following criteria: 1) at least 30μm long; 2) terminal; 3) at least third branch order; 4) seemingly complete (untruncated); and 5) unobstructed by blood vessels, cells, or staining artefacts. Preferably five but no less than three dendrites per area were drawn for each hemisphere to ensure an accurate representation of the spine density for each area; the densities for the 3 – 5 dendrites per hemisphere per area were averaged and this average was the unit of analysis. Dendrites from the brain of one male and one female per litter were drawn to control for potential litter effects. Drawings were analyzed by an assistant blind to the treatment groups using the Cell Counter plugin for ImageJ

(https://imagej.nih.gov/ij/plugins/cell-counter.html) and density was reported as the number of spines per 10μm of dendrite.

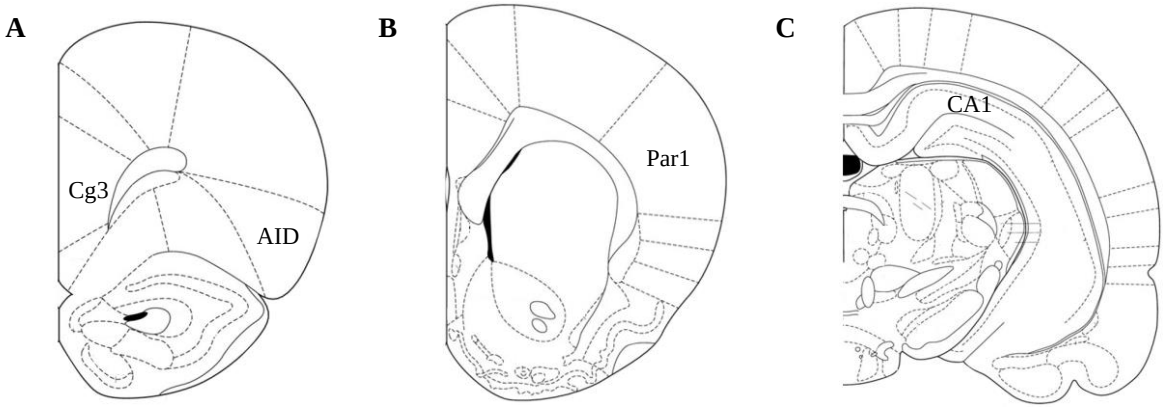

Figure 4.5: Representation of the areas sampled for dendritic spine density. (A) Dendrites were traced in Cg3 and AID from Bregma 4.2 – 2.7mm (Bregma 3.7mm is shown) (B) Dendrites were traced in Par1 from Bregma 2.2 – -0.4mm (Bregma 1.7mm is shown) (C) Dendrites were traced in CA1 from Bregma -3.6 – -5.8mm (Bregma -4.8mm is shown).

4.2.6 Statistical Analysis

All statistical analysis was completed using IBM SPSS 27. As the dam is the experimental unit, behavioral data for males and females within a litter was averaged to yield one male value and one female value per litter to control for litter effects. Data was similarly averaged for offspring body and brain weight. For spine density, we controlled for potential litter effects by randomly selecting and drawing only one male and one female per litter. Data was analyzed using one-way, two-way, or mixed analysis of variance (ANOVA) as appropriate. For mixed-ANOVA, the Huynh-Feldt correction was applied to control for departures from sphericity when necessary. Whishaw tray reaching

was analyzed with analysis of covariance (ANCOVA) to adjust for the influence of the number of reach attempts on success rate. The Bonferroni correction was used to control for multiple comparisons. The level of alpha was set at $p = .05$.

4.3 Results

4.3.1 Maternal Ethanol Consumption

Mixed-ANOVA revealed a significant Week x Condition interaction for the daily volume of water consumed, $F(7, 56) = 12.132$, $p < .001$, $\eta_P^2 = .603$; beginning in week 2 of ethanol dosing, alcohol dams consumed significantly less water on a daily basis than control dams (all p's < .001 after Bonferroni correction applied), but there was no group difference during baseline water assessment, $F(1, 8) = 0.614$, $p = 1.000$, $\eta_P^2 = .071$, or week 1 of dosing, $F(1, 8) = 0.109$, $p = 1.000$, $\eta_P^2 = .013$ (data not shown). Furthermore, one-way ANOVA revealed that the percent change in body weight between baseline and week 7 of dosing was greater in control dams ($M = 13.8\%$, $SE = 0.67$) than in alcohol dams ($M = 8.3\%$, $SE = 0.71$), $F(1, 12) = 31.785$, $p < .001$, $\eta_P^2 = .726$. The average weekly intake of ethanol in g/kg of body weight is depicted in Figure 4.6. The overall average across all seven weeks was 11.9 g/kg.

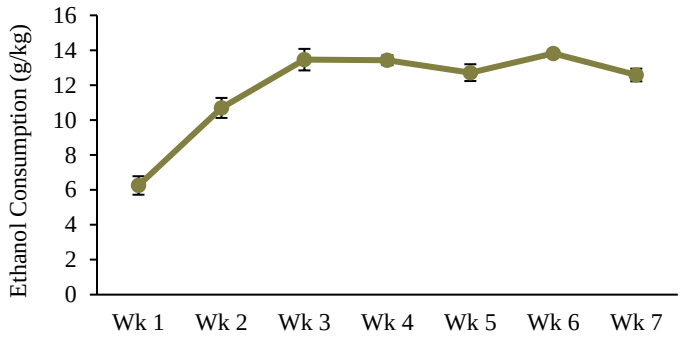

Figure 4.6: Ethanol consumption (g/kg body weight) across the seven weeks of dosing (weekly mean ± SE). Ethanol concentration was 2.5% - 10% during week 1, 10% to 17.5% during week 2, and 20% from week 3 through 7.

4.3.2 Litter Characteristics

One-way ANOVA revealed no significant difference between the litter size of control dams (M = 14.4, SE = 0.748) and alcohol dams (M = 13.6, SE = 0.748), $F(1, 8)$ = 0.571, p = .471, η_P^2 = .067. Furthermore, there was no significant difference for the percent male offspring per litter, $F(1, 8)$ = 0.440, p = .526, η_P^2 = .052 (for control dams M = 51.3%, SE = 8.0; for alcohol dams M = 44.8%, SE = 5.8%).

4.3.3 Behavior

4.3.3.1 Maternal Care

Mixed ANOVA revealed no significant main effects of pup age or Age x Condition interactions for any of the maternal behaviors (p's > .05); therefore, data was averaged across the three ages for further analysis. Further, due to heterogeneity of variance, Welch's F-statistic is reported. There was no significant main effect of maternal condition on the overall percent of time spent nursing pups, $F_W(1, 6.8)$ = 2.224, p = .181, η_P^2 = .218, percent of time spent licking and grooming pups, $F_W(1, 5.3)$ = 0.000, p = .998,

η_P^2 = .000, or the percent of time spent in non-contact with pups, F_W(1, 5.4) = 2.677, p = .158, η_P^2 = .251 (Figure 4.7).

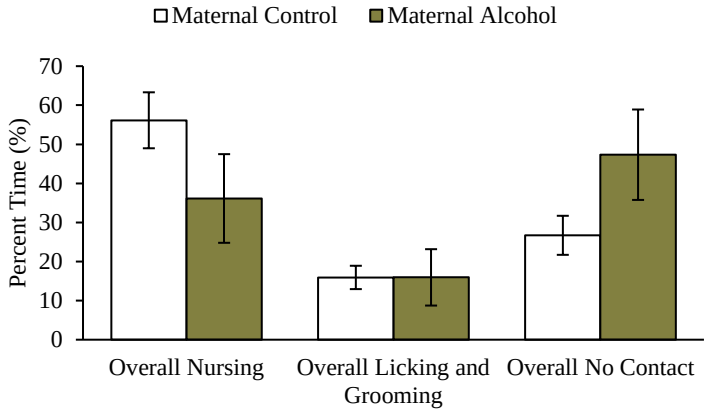

Figure 4.7: Maternal care provided by dams in the control and alcohol conditions (mean ± *SE*). There were no significant differences in the overall time spent nursing, licking, and grooming, or not touching pups.

4.3.3.2 Negative Geotaxis

Mixed-ANOVA revealed a significant Age x Sex interaction for the time spent rotated above the horizontal plane in negative geotaxis, F(1, 16) = 5.344, p = .034, η_P^2 = .250; males spent significantly more time rotated upright between P9 and P10, F(1, 16) = 18.167, p = .001, η_P^2 = .532, but females did not improve with age, F(1, 16) = 0.986, p = .336, η_P^2 = .058 (Figure 4.8A). The same interaction was observed for the number of falls from the platform, F(1, 16) = 6.212, p = .024, η_P^2 = .280; males fell significantly fewer times on P10 compared to P9, F(1, 16) = 24.360, p < .001, η_P^2 = .604, but females did not improve with age, F(1, 16) = 1.990, p = .177, η_P^2 = .111 (Figure 4.8B). There was no significant main effect of condition for either time upright, F(1, 16) = 0.373, p = .550, η_P^2

= .023, or number of falls, $F(1, 16) = 0.030$, $p = .865$, $\eta_P^2 = .002$, nor were there any significant interactions with condition for either measure (p's > .05).

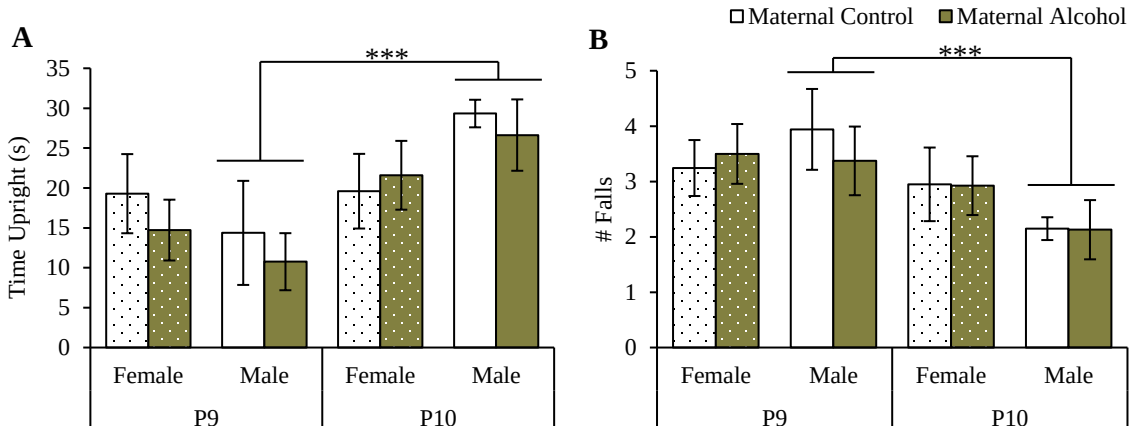

Figure 4.8: Negative geotaxis outcomes (mean ± SE). There were significant Sex x Age interactions for both the time spent rotated upright (A) and the number of falls from the platform (B) such that males improved with age, but females did not. *** $p \leq .001$

4.3.3.3 Open Field

The Huynh-Feldt value of epsilon was used to correct for departures from sphericity (ε = .978) for the number of novel squares entered across pup age. A 5x2x2 mixed ANOVA with age as a within-subject factor and maternal condition and pup sex as between-subject factors revealed a significant Age x Condition interaction for the number of novel squares, $F(3.911, 62.570) = 2.574$, $p = .047$, $\eta_P^2 = .139$. Bonferroni *post hoc* tests comparing each day to the subsequent day indicated that pups born to control dams reached their peak level of exploration sooner than pups born to alcohol treated dams (Figure 4.9A). Pups born to control dams entered significantly more unique squares between P12 and P13, $t(9) = -3.271$, $p = .048$, but showed no further improvement

between P13 and P15, $t(9) = 0.047$, $p = 1.000$. Pups born to alcohol treated dams did not show a significant increase in the number of novel squares until between P13 and P15, $t(9) = 3.289$, $p = .046$. No other day-by-day comparisons were significant for either group (p's > .05). When comparing the effect of maternal condition within each pup age, there was a nearly significant difference between control and alcohol pups on P15, $t(9) = 2.091$, $p = .053$, such that alcohol pups entered marginally more novel squares than control pups. There was no significant main effect of sex, $F(1, 16) = 0.001$, $p = .971$, $\eta_P^2 = .000$, nor any interactions with sex (p's > .05) for the number of novel squares. Therefore, data are graphically represented collapsed across sex for simplicity.

For the total number of squares, there was no deviation from sphericity ($\varepsilon = 1.000$). A 5x2x2 mixed ANOVA revealed only a significant main effect of pup age, $F(4, 64) = 11.365$, $p < .001$, $\eta_P^2 = .415$ (Figure 4.9B). Bonferroni post hoc tests revealed that pups entered significantly more total squares between P12 and P13, $t(19) = 4.860$, $p = .002$, but there were no other significant differences between consecutive days (p's > .05). There were no main effects of maternal condition, $F(1, 16) = 0.007$, $p = .934$, $\eta_P^2 = .000$, or pup sex, $F(1, 16) = 0.170$, $p = .686$, $\eta_P^2 = .011$, nor were there any significant interactions (p's > .05).

We also analyzed what percent of novel and total squares occurred within the inner portion of the open field as an early indicator of anxiety-like behavior. We only did this for P15 data as this is the age of eye opening in developing pups. For the percent inner novel squares, two-way ANOVA revealed no main effect of maternal condition, $F(1, 16) = 0.933$, $p = .348$, $\eta_P^2 = .055$, or pup sex, $F(1, 16) = 0.066$, $p = .800$, $\eta_P^2 = .004$, nor a Condition x Sex interaction, $F(1, 16) = 0.103$, $p = .752$, $\eta_P^2 = .006$. Likewise, for the

percent inner total squares, there was no main effect of condition, $F(1, 16) = 0.809$, $p = .382$, $\eta_P^2 = .048$, sex, $F(1, 16) = 0.000$, $p = .985$, $\eta_P^2 = .000$, or an interaction, $F(1, 16) = 0.285$, $p = .601$, $\eta_P^2 = .017$ (Figure 4.9C).

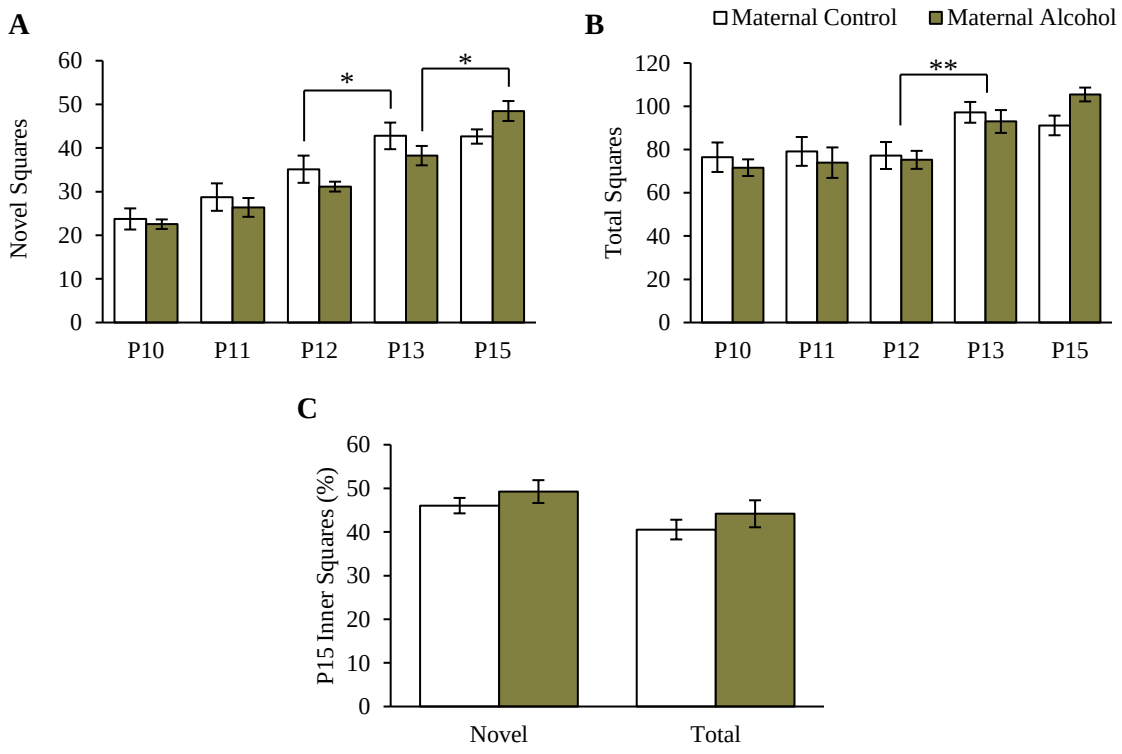

Figure 4.9: Open field results (mean ± *SE*) based on pup age and maternal condition. Data are presented collapsed across sex due to an absence of sex differences. (A) For the number of novel squares, control pups reached their maximum number of squares on P13, whereas alcohol pups did so on P15. (B) For the total number of squares, all pups experienced a jump in the number of total squares between P12 and P13 regardless of maternal condition. (C) There were no differences in the proportion of novel or total squares that were in the inner part of the open field. P = postnatal day. * $p \leq .05$; ** $p \leq .01$.

4.3.3.4 Activity Box

On P35, two-way ANOVA revealed no significant main effects or interactions for distance travelled, movement time, percent of time in the center of the open field, or number of rears. On P90, two-way ANOVA revealed significant main effects of sex for distance travelled (female > male), movement time (female > male), percent time in the center (male > female), and number of rears (female > male; Table 4.1; Figure 4.10A-D).

Table 4.1: Summary of statistical findings for activity box

	Condition			Sex			Condition x Sex		
	F	p	η_P^2	F	p	η_P^2	F	p	η_P^2
P35									
Distance (cm)	0.074	.790	.005	0.831	.375	.049	0.041	.842	.003
Movement (s)	0.034	.857	.002	1.154	.299	.067	0.036	.853	.002
Center (%)	2.117	.165	.117	4.077	.061	.203	1.637	.219	.093
Rears	1.509	.237	.086	0.456	.509	.028	0.575	.459	.035
P90									
Distance (cm)	0.135	.719	.008	**18.686**	**.001**	**.539**	1.702	.210	.096
Movement (s)	0.086	.773	.005	**11.187**	**.004**	**.411**	1.707	.210	.096
Center (%)	3.577	.077	.183	**9.699**	**.007**	**.377**	0.008	.928	.001
Rears	3.243	.091	.169	**5.143**	**.038**	**.243**	0.427	.523	.026

Model $df = 1$, Error $df = 16$
Boldface = $p \leq .05$

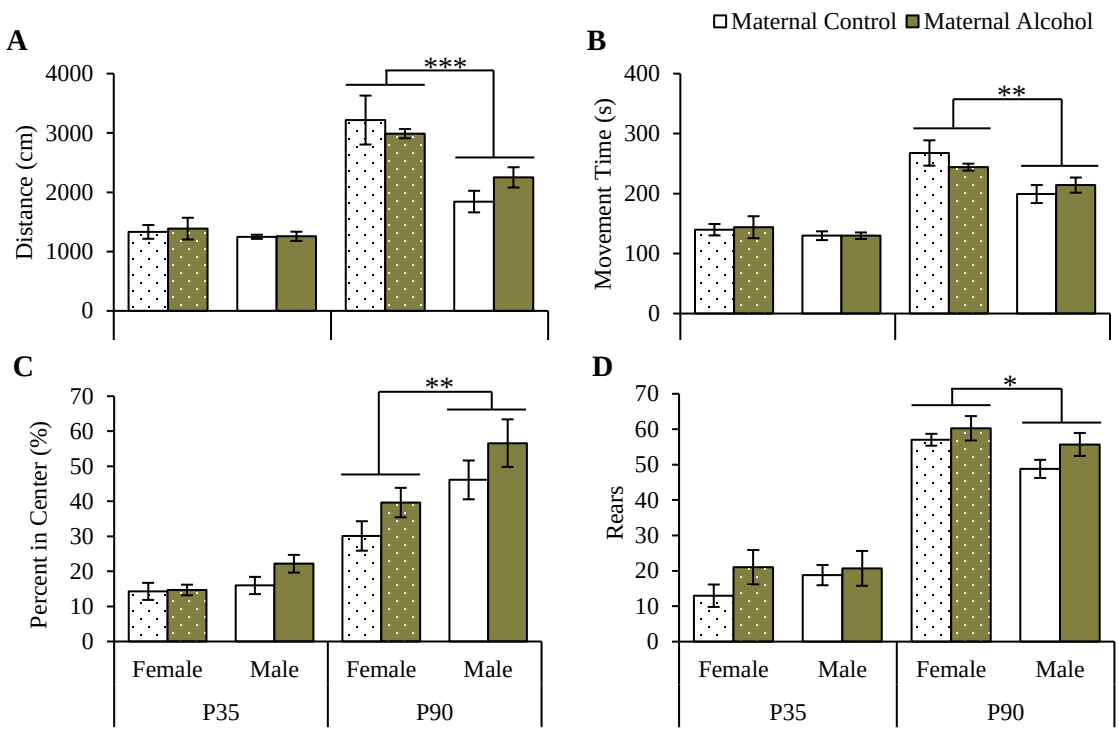

Figure 4.10: Activity box results for adolescent (P35) and adult (P90) offspring (mean ± *SE*). There were significant differences between adult male and female offspring for distance travelled (A), time spent moving (B), percent of time spent in the center of the open field (C), and number of rears (D). P = postnatal day.* $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$

4.3.3.5 Elevated Plus Maze

On P36, two-way ANOVA revealed significant main effects of condition for time spent in the open arms (maternal alcohol > maternal control) and closed arms (maternal control > maternal alcohol), and for the total number of arm entries (maternal alcohol > maternal control). There was no significant effect of condition for the time spent in the ends of the open arms on P36, nor were there any significant main effects of sex or

Condition x Sex interactions. On P91, two-way ANOVA revealed no significant main effects or interactions for any of the measures (Table 4.2; Figure 4.11A-D).

Table 4.2: Summary of statistical findings for elevated plus maze.

	Condition			Sex			Condition x Sex		
	F	p	η_P^2	F	p	η_P^2	F	p	η_P^2
P36									
Open (s)	**5.762**	**.029**	**.265**	1.139	.302	.066	0.230	.638	.014
End Open (s)	1.151	.299	.067	0.800	.384	.048	0.017	.898	.001
Closed (s)	**6.090**	**.025**	**.276**	0.381	.546	.023	0.597	.451	.036
Entries	**10.163**	**.006**	**.388**	0.554	.469	.033	0.034	.857	.002
P91									
Open (s)	0.912	.354	.054	0.574	.460	.035	0.311	.585	.019
End Open (s)	0.554	.467	.033	0.098	.759	.006	0.478	.499	.029
Closed (s)	0.869	.365	.051	0.059	.811	.004	0.098	.758	.006
Entries	0.238	.632	.015	4.059	.061	.202	0.605	.448	.036

Model $df = 1$; Error $df = 16$
Boldface = $p \leq .05$

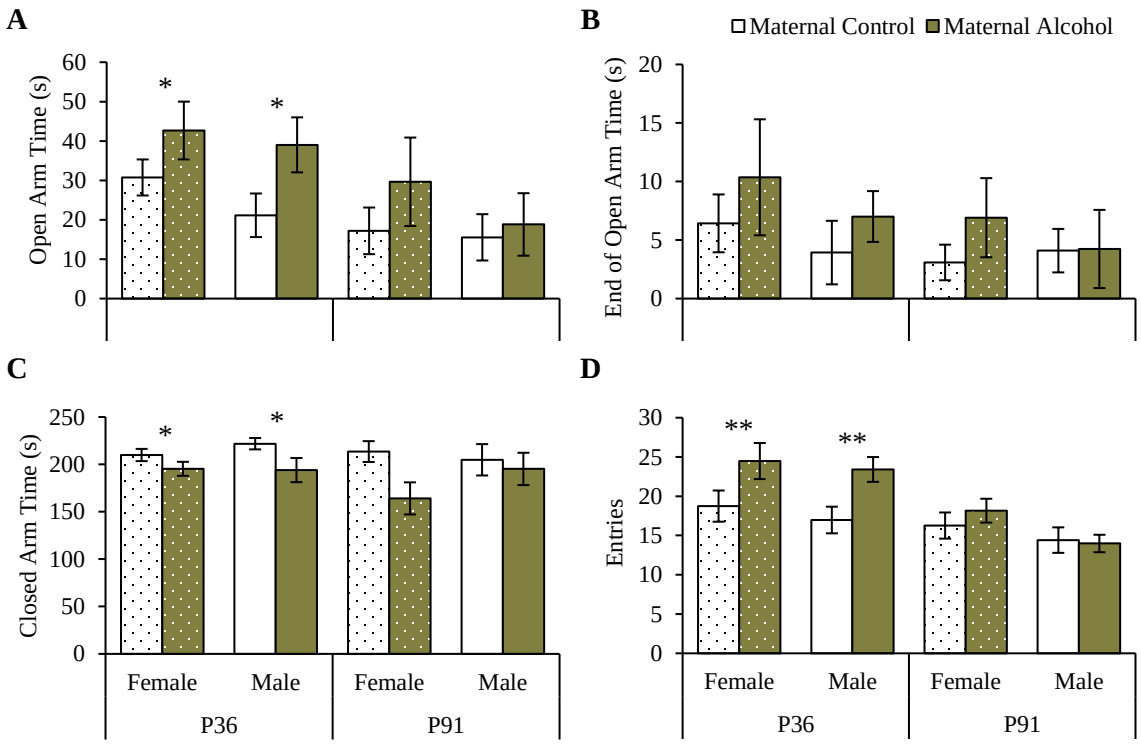

Figure 4.11: Elevated plus maze results for adolescent (P36) and adult (P91) offspring (mean ± *SE*). In adolescence, there were significant effects of maternal alcohol for time spent in the open arms (A), time spent in the closed arms (C) and the total number of arm entries (D). There were no significant effects for the time spent in the end of the open arms (B). P = postnatal day. * $p \leq .05$, ** $p \leq .01$

4.3.3.6 Whishaw Tray Reaching

Two-way ANOVA revealed no significant main effect of condition, $F(1, 16) = 0.178$, $p = .679$, $\eta_P^2 = .011$, main effect of sex, $F(1, 16) = 2.292$, $p = .150$, $\eta_P^2 = .125$, or a Condition x Sex interaction, $F(1, 16) = 0.637$, $p = .637$, $\eta_P^2 = .014$, for the total number of reach attempts (Figure 4.12A). Two-way ANCOVA revealed that the number of reach attempts was not a significant covariate for reach success, $F(1, 15) = 2.177$, $p = .161$, η_P^2

= .127, so two-way ANOVA was used instead. There was no significant main effect of condition, $F(1, 16) = 0.178$, $p = .679$, $\eta_P^2 = .011$, or sex, $F(1, 16) = 0.948$, $p = .345$, $\eta_P^2 = .056$, nor a Condition x Sex interaction, $F(1, 16) = 0.280$, $p = ,604$, $\eta_P^2 = .017$, for reach success (Figure 4.12B).

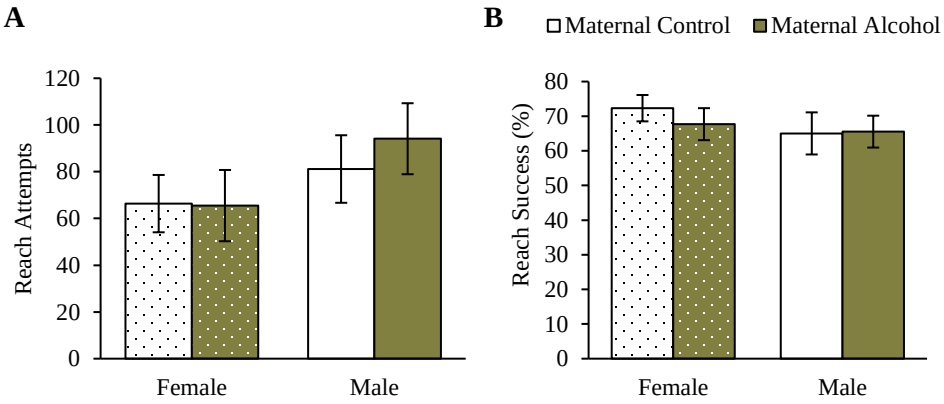

Figure 4.12: Whishaw tray reaching outcomes (mean ± *SE*). There were no effects on the number of reach attempts (A) or the reach accuracy (B).

4.3.3.7 Morris Water Task

Three-way mixed ANOVA revealed a significant Condition x Sex interaction for the average latency to locate the hidden platform across the five days of training, $F(1, 16) = 4.884$, $p = .042$, $\eta_P^2 = .234$. Female offspring of alcohol-exposed dams had a lower overall latency than females born to control dams, $F(1, 16) = 7.238$, $p = .016$, $\eta_P^2 = .311$, but there was no effect of maternal alcohol among male offspring, $F(1, 16) = 0.189$, $p = .669$, $\eta_P^2 = .012$. There was also a main effect of training, with all animals locating the platform more quickly as training progressed, $F(4, 64) = 92.552$, $p < .001$, $\eta_P^2 = .853$. Animals' latency significantly improved between days 1 and 2, $F(1, 16) = 98.577$, $p <$

.001, η_P^2 = .860, days 2 and 3, $F(1, 16) = 8.972$, $p = .036$, η_P^2 = .359, and days 4 and 5, $F(1, 16) = 10.026$, $p = .024$, η_P^2 = .385, but not between days 3 and 4, $F(1, 16) = 1.049$, $p = .963$, η_P^2 = .062 (Bonferroni-corrected). There were no significant interactions with training (p's > .05; Figure 4.13A).

Similar effects were observed for swim distance during training. There was a significant Condition x Sex interaction for the average distance across all days of training, $F(1, 16) = 8.032$, $p = .012$, η_P^2 = .334; maternal alcohol significantly decreased average swim distance in female offspring, $F(1, 16) = 7.598$, $p = .014$, η_P^2 = .322, but not in males, $F(1, 16) = 1.566$, $p = .229$, η_P^2 = .089. There was also a main effect of training, $F(4, 64) = 127.2$, $p < .001$, η_P^2 = .888. Distance significantly decreased between days 1 and 2, $F(1, 16) = 109.7$, $p < .001$, η_P^2 = .873, days 2 and 3, $F(1, 16) = 22.601$, $p < .001$, η_P^2 = .585, and days 4 and 5, $F(1, 16) = 10.600$, $p = .020$, η_P^2 = .399, but not between days 3 and 4, $F(1, 16) = 0.349$, $p = 1.000$, η_P^2 = .021 (Bonferroni-corrected). There were no significant interactions with training (p's > .05; Figure 4.13B).

There were no significant main effects or interactions for any of the probe trial measures (Table 4.3; Figure 4.13C-D).

Table 4.3: Summary of statistical findings for Morris water task probe trial.

	Condition			Sex			Condition x Sex		
	F	p	η_P^2	F	p	η_P^2	F	p	η_P^2
Distance (m)	0.073	.790	.005	0.155	.699	.010	0.196	.664	.012
Target (%)	0.371	.551	.023	0.003	.958	.000	0.003	.955	.000
Adjacent (%)	0.009	.928	.001	0.076	.786	.005	0.256	.620	.016
Opposite (%)	0.974	.338	.057	0.294	.595	.018	0.421	.526	.026

Model df = 1, Error df = 16

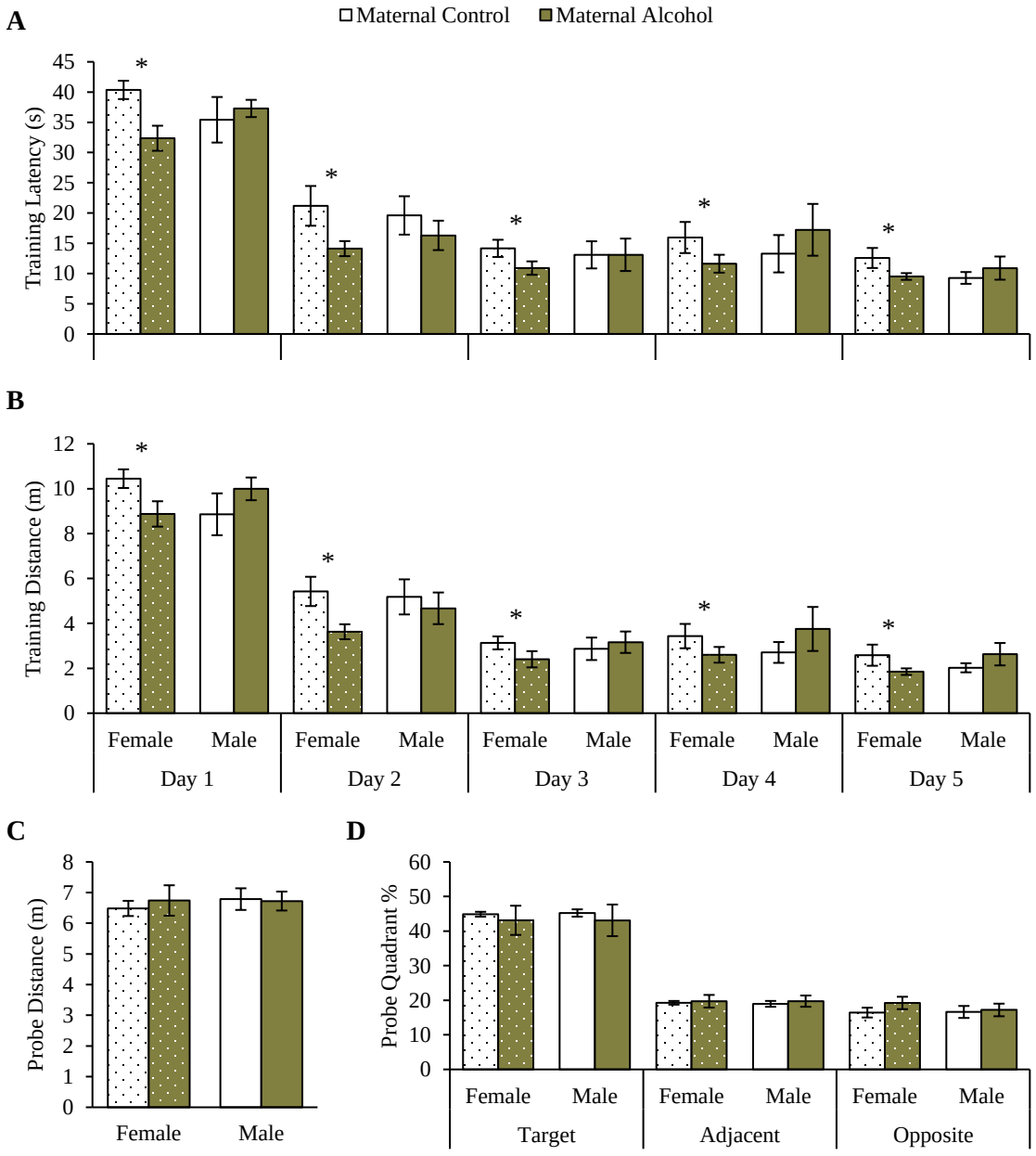

Figure 4.13: Morris water task performance (mean ± *SE*). Offspring latency (A) and swim distance (B) across the five days of training. There were significant Condition x Sex interactions in which alcohol-treated females located the platform significantly faster and with less distance than control females. During the probe trial, there were no effects for

the swim distance (C) or the percent of time spent in any quadrant of the pool (D). * $p \leq .05$.

4.3.4 Offspring Weight

4.3.4.1 Body Weight

Body weight was analyzed separated by sex due to the invariability of a sex difference; therefore, one-way ANOVA was performed for each sex. The sample size is small for P45 (n = 9 for female, n = 8 for male) because not every litter was represented at this time point. On P21, there was no effect of condition for either female, $F(1, 8) = 0.195$, $p = .671$, $\eta_P^2 = .024$, or male body weight, $F(1, 8) = 0.052$, $p = .826$, $\eta_P^2 = .006$. On P45, there was again no effect of condition for either female, $F(1, 7) = 0.046$, $p = .836$, $\eta_P^2 = .007$, or male body weight, $F(1, 6) = 0.143$, $p = .718$, $\eta_P^2 = .023$. And finally, on P120 there was no effect of condition of female, $F(1, 8) = 0.126$, $p = .732$, $\eta_P^2 = .015$, or male body weight, $F(1, 8) = 0.000$, $p = .983$, $\eta_P^2 = .000$, body weight (Figure 4.14A).

4.3.4.2 Brain Weight

Brain weight was also analyzed separated by sex, using one-way ANOVA for each sex. There was no effect of condition on the brain weight of P21 female, $F(1, 8) = 3.812$, $p = .087$, $\eta_P^2 = .323$, or male offspring, $F(1, 8) = 0.000$, $p = .996$, $\eta_P^2 = .000$. On P45, maternal alcohol had no impact on the brain weight of female offspring, $F(1, 7) = 0.458$, $p = .520$, $\eta_P^2 = .061$, but males born to alcohol exposed dams had significantly larger brains than males born to control dams, $F(1, 6) = 8.970$, $p = .024$, $\eta_P^2 = .599$. And on P120, females born to alcohol exposed dams had significantly larger brains than

females born to control dams, $F(1, 8) = 7.083$, $p = .029$, $\eta_P^2 = .470$, but there was no effect in male offspring, $F(1, 8) = 3.066$, $p = .118$, $\eta_P^2 = .277$ (Figure 4.14B).

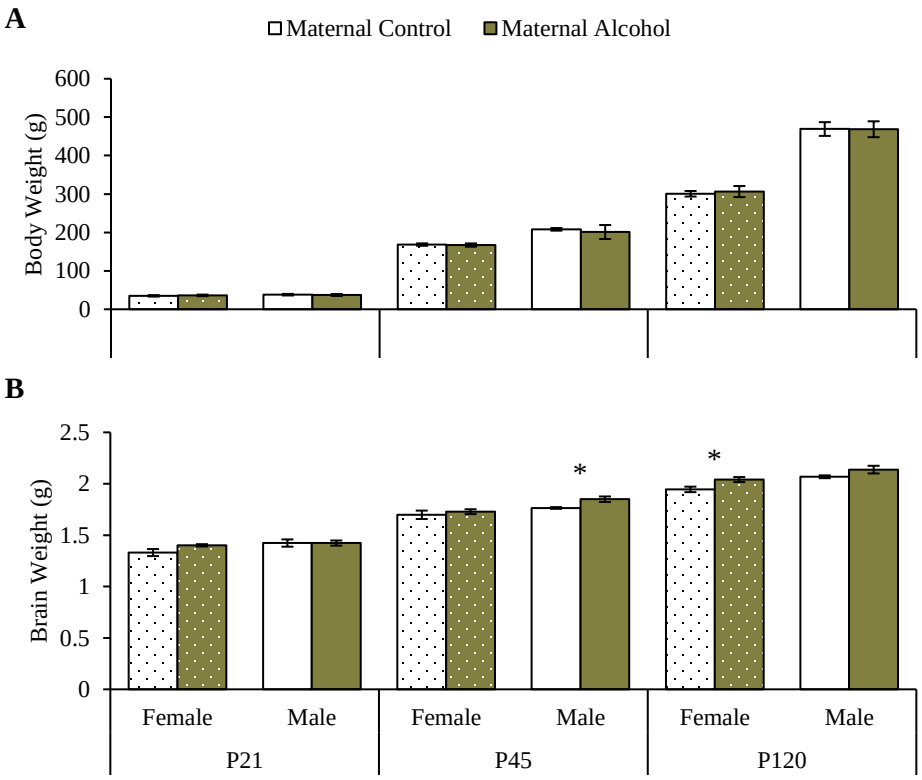

Figure 4.14: Offspring body (A) and brain (B) weight throughout development. There were no significant effects for body weight. For brain weight, adolescent (P45) alcohol-treated males had larger brain than control males, and adult (P120) alcohol-treated females had larger brains than control females. P = postnatal day. * $p \leq .05$

4.3.5 Spine Density

4.3.5.1 P21

There were no significant effects of hemisphere, so left and right values were averaged for further analysis. Two-way ANOVA revealed no significant main effects or

interactions for basilar dendritic spine density in AID, Cg3, or Par1 of P21 offspring (Table 4.4; Figure 4.15).

Table 4.4: Summary of statistical findings for offspring dendritic spine density.

	Condition			Sex			Condition x Sex		
	F	p	η_P^2	F	p	η_P^2	F	p	η_P^2
P21									
Basilar AID	0.960	.342	.057	2.590	.127	.139	0.108	.746	.007
Basilar Cg3	0.002	.967	.000	0.092	.765	.006	0.116	.737	.007
Basilar Par1	0.404	.534	.025	0.135	.718	.008	0.001	.972	.000
P120									
Basilar AID	0.844	.372	.050	0.026	.874	.002	0.576	.459	.035
Basilar Cg3	**22.866**	**<.001**	**.588**	**7.770**	**.013**	**.327**	**12.911**	**.002**	**.447**
Basilar Par1	1.704	.210	.096	0.802	.384	.048	0.615	.444	.037
Basilar CA1	0.647	.433	.039	0.000	.985	.000	**7.002**	**.018**	**.304**

Model $df = 1$, Error $df = 16$
Boldface = $p \leq .05$

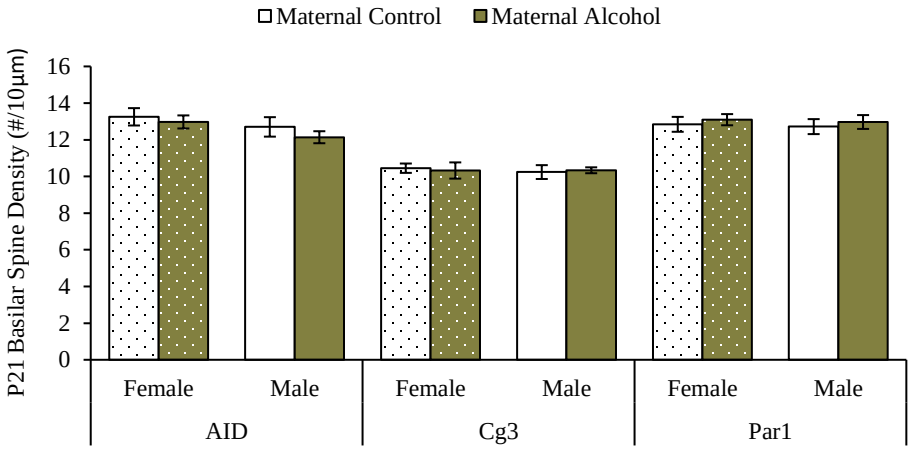

Figure 4.15: Weanling offspring (P21) basilar spine density (mean ± *SE*). There were no significant differences.

4.3.5.2 P120

There were no significant main effects or interactions with hemisphere, so left and right values were averaged for further analysis. Two-way ANOVA revealed a significant Condition x Sex interaction for both Cg3 and CA1 (Table 4.4). In Cg3, female offspring of alcohol-exposed dams had significantly greater basilar spine density than females from control dams, $F(1, 16) = 35.070$, $p < .001$, $\eta_P^2 = .687$, but there was no effect of condition for male offspring, $F(1, 16) = 0.706$, $p = .413$, $\eta_P^2 = .042$. In CA1, there was no effect for female offspring, $F(1, 16) = 1.696$, $p = .211$, $\eta_P^2 = .096$, but male offspring showed a maternal alcohol-induced reduction in basilar spine density, $F(1, 16) = 5.953$, $p = .027$, $\eta_P^2 = .271$ (Figure 4.16).

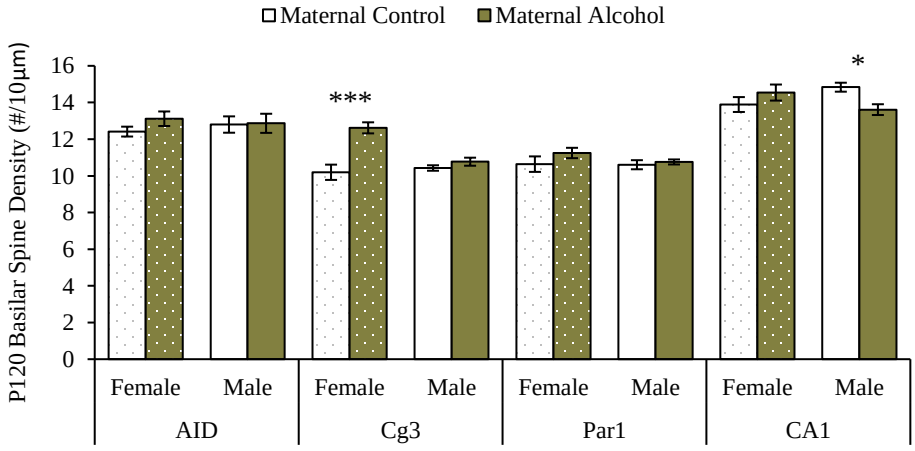

Figure 4.16: Adult offspring (P120) basilar spine density (mean ± *SE*). In medial PFC (Cg3), female alcohol offspring had significantly greater spine density than control females. In hippocampus (CA1), male alcohol offspring had significantly reduced spine density compared to control males. * $p \leq .05$, *** $p \leq .001$

4.3.5.3 Dams

There were no significant effects of hemisphere, so left and right values were averaged for further analysis. One-way ANOVA revealed no effect of alcohol exposure on dam basilar dendritic spine density in either area of the PFC, AID, $F(1, 8) = 1.151$, $p = .315$, $\eta_P^2 = .126$, or Cg3, $F(1, 8) = 0.717$, $p = .422$, $\eta_P^2 = .082$. However, dams exposed to preconception alcohol had significantly reduced spine density in area Par1, $F(1, 8) = 5.599$, $p = .046$, $\eta_P^2 = .412$ (Figure 4.17).

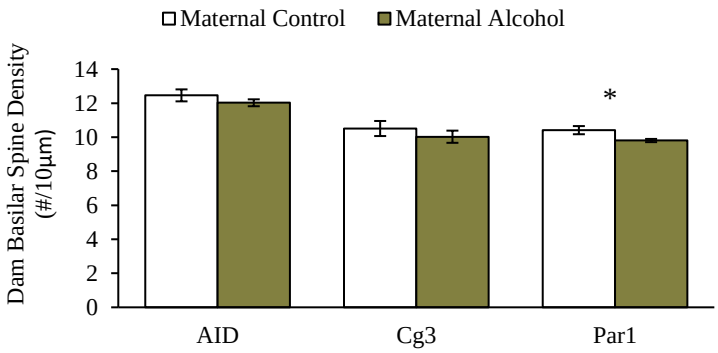

Figure 4.17: Dam basilar spine density (mean ± *SE*). There were no effects of alcohol in the PFC (AID or Cg3), but alcohol resulted in a decrease in spine density in the parietal cortex (Par1). * $p \leq .05$

4.4 Discussion

Here we show that chronic maternal alcohol exposure, mimicking alcoholism in humans, in the time immediately preceding but not overlapping pregnancy has long-term impacts on offspring behavior and brain in the absence of significant changes in maternal care. Specifically, we report: 1) a decrease in anxiety-like behavior in adolescent, but not adult, female and male offspring of alcohol-exposed dams; 2) an apparent improvement

in spatial learning among female offspring of alcohol-exposed dams; 3) a sex- and age-dependent increase in brain weight due to maternal alcohol; 4) and sex- and region-dependent changes in basilar dendritic spine density in adult offspring.

4.4.1 Maternal Preconception Alcoholism Does Not Significantly Alter Maternal Care

We did not find that maternal preconception alcohol had any negative affect on the quality of maternal care towards pups, especially when considering the time spent licking and grooming pups. Jabbar and colleagues (2016) similarly found no difference in maternal care when they exposed females to 3 weeks of 6.7% ethanol in drinking water two weeks prior to mating. Maternal care in rodents, especially the frequency of licking and grooming, has profound influences on the long-term developmental trajectory of offspring (Curley & Champagne, 2016). Therefore, any conclusions that could be drawn from the results of the current study are predicated on equal maternal care between treatment groups.

However, dams in the control and alcohol-treated groups were significantly different with respect to the volume of water consumed and their percent change in body weight between the start and finish of the alcohol administration. Chronic ethanol consumption is known to decrease food intake, water intake, and growth rate in rodents (Štrbák et al., 1998). To compensate for this, many ethanol-exposure paradigms for rodents use a pair-fed control group, in which the non-alcohol-treated animals receive a daily quantity of food that matches that consumed by the alcohol-treated animals (Harper et al., 2014; Jabbar et al., 2016; Tunc-Ozcan et al., 2016; Al-Yasari et al., 2021). The paradigm we used following Jamerson and colleagues (2004) did not use a pair-fed

control group, so the effects observed in the offspring are possibly due in part to the reduced food intake of the alcohol dams. Interestingly, others have reported that in many respects offspring of preconception control-fed (*ad libitum* access to food) and pair-fed animals do not significantly differ from one another, suggesting that the reduction in food intake is inconsequential for select measures (Harper et al., 2014; Jabbar et al., 2016; Tunc-Ozcan et al., 2016; Al-Yasari et al., 2021). Unfortunately, our data does not allow us to parse out the influences of ethanol and food restriction for the current experiment. Therefore, differences observed between the offspring of control and alcohol-treated dams may be due to preconception ethanol, preconception food restriction, or a combination of both.

4.4.2 Maternal Preconception Alcoholism Has Selective Impacts on Offspring Behavior

Of the six behavioral tests used to assess the offspring, three were impacted by maternal preconception alcohol. There was no effect of maternal alcohol on negative geotaxis performance, no changes in activity level in adolescence or adulthood, and no effects on fine motor control in Whishaw tray reaching.

In an assessment of open field exploration in developing pups, pups born to alcohol treated dams took longer to reach their maximum level of exploration than control pups; control pups reached their maximum number of novel squares on P13, but alcohol pups did not achieve this until P15. This suggests that although maternal alcohol did not affect the onset of locomotor behavior, it did impact the rate of development. This is the first study to examine the impacts of maternal alcohol on the emergence of locomotion in offspring. Other have examined locomotion around the time of weaning;

following prenatal (Brys, Pupe, & Bizarro, 2014) or preconception (Choi et al., 2012) exposure to alcohol, pups (P19 – 22) display hyperactivity compared to control pups. However, by this age, locomotor competency is already achieved. The current study did observe a nearly significant increase in exploration on P15, which may indicate the first signs of alcohol induced hyperactivity. However, we found no indication of hyperactivity in adolescent or adult offspring, aside from increased movement around the elevated plus maze.

In elevated plus maze, adolescent alcohol-treated offspring of both sexes displayed a significant decrease in the expression of anxiety-like behavior (spending more time in the open arms and less time in the closed arms) and increased movement around the maze (more arm entries) compared to adolescent control animals. There was no effect of maternal alcohol in adult offspring. Although elevated plus maze is not an assessment of risk-taking, a decrease in anxiety in potentially dangerous situations could be indicative of increased risk-taking tendencies. Adolescent male rats exposed to prenatal alcohol do exhibit increased impulsivity/risk-taking in a "transitional bridge" task in which rats must cross from one platform to another using a progressively narrower bridge, and also have increased levels of dopamine in the nucleus accumbens (Muñoz-Villegas, Rodríguez, Giordano, & Juárez, 2017). However, others have not found that maternal preconception alcohol decreases anxiety. Jabbar and colleagues (2016) report the opposite, with adult male offspring of ethanol treated dams spending less time in the open arms than controls and no effect in females. In this study, male and female offspring also presented with heightened physiological reactivity to stress. However, these offspring were tested in adulthood only, so it is unknown if this effect would be the same

in adolescent offspring. Brancato and colleagues (2018) exposed dams to ethanol during preconception + gestation + lactation using either a habitual or binge exposure schedule. They found that juvenile male offspring of habitual drinkers exhibited increased anxiety, whereas the male offspring of binge drinkers showed decreased anxiety. They also found that the offspring of binge-drinking dams had an elevated propensity to consume ethanol as adolescents. Prenatal alcohol exposure is typically associated with increased anxiety-like behavior, but one study reported that adult female offspring exhibited a decrease in anxiety assessed by open field and elevated plus maze, whereas males showed increased anxiety. These authors also found that females had decreased expression of glucocorticoid receptors in the hippocampus, suggesting dysregulation of the HPA axis (Lam et al., 2019). Therefore, it is evident that the effect of maternal preconception alcohol on anxiety-like behavior is dependent on the timing and dose of maternal exposure, as well as the age of the offspring at the time of assessment. Future work should focus on elucidating how preconception alcohol impacts risk-taking, given the relationship between prenatal alcohol and risky behaviors in rodents and humans (Rasmussen & Wyper, 2007).

In Morris water task, female offspring of alcohol-treated dams showed a spatial learning advantage; regardless of the day of training, females born to alcohol dams located the hidden platform significantly faster and with less distance than control females, but there was no difference among male offspring. Prolonged alcohol exposure typically results in impaired learning and memory, as well as hippocampal neurodegeneration and impaired long-term potentiation (LTP; Kutlu & Gould, 2016). Two studies to date have examined maternal preconception ethanol exposure and learning

and memory functioning. Second-generation offspring of dams prenatally exposed to ethanol display impaired contextual fear memory and altered hippocampal gene expression (Tunc-Ozcan et al., 2016). Offspring of dams exposed to intermittent ethanol during preconception, gestation, and lactation are slower to learn the location of the hidden platform in Morris water task and impaired at remembering the target quadrant during the probe trial (Brancato, Castelli, Lavanco, & Cannizzaro, 2020). Others have found that low to moderate levels of prenatal ethanol has no impact on learning or memory in either the Y-maze (Cullen, Burne, Lavidis, & Moritz, 2014) or the Morris water task (Dursun, Jakubowska-Doğru, & Uzbay, 2006; Cullen et al., 2014). Cullen and colleagues (2014) also report no changes in hippocampal cell number, size, or density. One study of direct ethanol exposure has shown that although high doses impair contextual fear conditioning, moderate doses have no effect, and low doses actually improve fear conditioning; these authors speculate that N-methyl-d-aspartate (NMDA) receptor activity in the hippocampus and amygdala may be responsible, such that low doses of ethanol potentiate NMDA activity (Gulick & Gould, 2007). Therefore, whereas alcohol is most commonly associated with impaired learning and memory and hippocampal dysfunction, some doses of ethanol can also facilitate hippocampus-dependent learning. More work is required to understand the mechanism underlying the learning advantage in the present study.

4.4.3 Maternal Preconception Alcoholism Altered Spine Density in Adult Offspring and Dams

We observed no differences in basilar spine density in P21 offspring born to control and alcohol-exposed dams. We did, however, find a drastic increase in medial

PFC (Cg3) spine density in P120 females, and a significant decrease in hippocampal (CA1) spine density in P120 males. This is the first study to examine dendritic spine density following maternal preconception alcohol. Others have reported that postnatal alcohol decreases spine density (Whitcher & Klintsova, 2008; De Giorgio & Granato, 2015; Hamilton, Criss, & Klintsova, 2015) or changes the proportion of mature to immature spines in medial PFC and hippocampus (Hamilton, Whitcher, & Klintsova, 2010; Risher et al., 2015).

An increase in spine density in the medial PFC in the current work could be due to an impairment in synaptic pruning. Pruning is an important process in synaptic plasticity that removes unnecessary connections as an animal matures (Segal, Korkotian, & Murphy, 2000). Increased spine density due to aberrant pruning is seen in neurodevelopmental conditions such as autism spectrum disorder (Hutsler & Zhang, 2010) and Fragile X syndrome (Comery et al., 1997). There are also normal processes of apoptosis that occur in the medial PFC as animals mature, which are more pronounced in females; female undergo a greater loss in neuron number in the medial PFC as they age than males (Markham, Morris, & Juraska, 2007; Willing & Juraska, 2015). It would be valuable to investigate if this apoptosis is affected by maternal preconception alcohol, such that alcohol-exposed female offspring would have greater cell numbers than control females as they mature. One study investigated neuron number in the medial PFC following prenatal alcohol but only examined male offspring; they found a significant decrease in prenatally exposed males (Mihalick et al., 2001). If a similar sex difference also exists for synaptic pruning, this may explain why the female offspring in the current

experiment showed increased spine density in the medial PFC due to maternal alcohol, but the males did not.

Hippocampal spine density and learning and memory performance are highly dependent on gonadal hormones. In female rats, hippocampal spine density fluctuates with the five-day estrous cycle; spine density is the greatest when the circulating levels of estradiol and progesterone are the highest (Woolley & McEwen, 1993). In orchiectomized males, administration of either estradiol or testosterone increases hippocampal spine density (CA1) and improves object recognition (Jacome et al., 2016). We observed a decrease in CA1 spine density in the male offspring of alcohol-treated dams, but no effect in females. It is possible that the estrous cycle of the female offspring overwhelmed any alcohol-induced changes in spine density that may have existed. For the males, a decrease in spine density is in line with what others have reported following alcohol exposure. Prenatal ethanol results in decreased spine density in CA1 and CA3 in P1 offspring, although the effect disappears by P10 (Jakubowska-Doğru, Elibol, Dursun, & Yürüker, 2017). Adolescent ethanol does not decrease spine density, but it does change the relative proportion of mature to immature spines in CA1 (Risher et al., 2015). Finally, in a mouse model of Wernicke-Korsakoff syndrome, which features a thiamine deficiency that is commonly associated with alcoholism, mice show impaired hippocampal long-term memory, hippocampal neuron loss, and decreased spine density in the dentate gyrus (Inaba et al., 2016).

We also found that following direct exposure to high doses of ethanol, the dams exhibited decreased spine density in the parietal cortex (Par1). Dams were euthanized nearly 6-months after the cessation of alcohol treatments; therefore, this decrease in spine

density represents a long-term consequence of alcohol abuse. Withdrawal from alcohol can induce changes in spine density that are unique to the changes observed during alcohol use in the nucleus accumbens (Peterson, McCool, & Hamilton, 2015) and OFC (McGuier et al., 2015). Both of these studies used a withdrawal period of only seven days. One study examined the effects of long-term alcohol withdrawal on neuronal architecture in the hippocampal formation. Although they did not observe withdrawal-induced changes in spine density, neurons were smaller following 6-months of alcohol withdrawal compared to during active alcohol exposure (Paula-Barbosa, Brandão, Madeira, & Cadete-Leite, 1993). Therefore, it is evident that chronic alcohol use continues to impact brain morphology long after cessation.

4.4.4 Limitations and Future Directions

As mentioned, the ethanol exposure paradigm we followed did not used a pair-fed control group to account for decreased food intake in the alcohol-treated animals. This impairs our ability to delineate the effects of preconception ethanol from those of preconception food restriction. However, alcohol abuse interferes with appetite regulation and is often associated with malnutrition (Kokavec, 2008), so the combination of ethanol intake with food restriction may provide a more naturalistic picture of the effects of maternal preconception alcoholism. The ethanol exposure paradigm we followed (Jamerson, Wulser, & Kimler, 2004) used male rats and reported that the addition of ethanol to the drinking water did not reduce water intake compared to baseline levels; this was not true for the alcohol-treated females in the current study, suggesting that the paradigm we followed is not ideal for female animals. Furthermore, female rats tend to consume greater volumes of ethanol when it is freely available; one study found that in a

two-bottle choice paradigm, female Long-Evans consumed 9g/kg of ethanol, whereas males consumed half this amount (Priddy et al., 2017). Indeed, in the current study, the females consumed on average 11.9g/kg, a drastically larger amount than is typically administered (between 3 and 6g/kg). Future work should consider such sex differences to ensure that females are consuming an ecologically valid quantity of alcohol.

4.4.5 Concluding Remarks

We have shown that the offspring of female rats subjected to chronic, high dose ethanol in the weeks preceding conception have altered locomotor development, decreased anxiety in adolescence, enhanced spatial learning in adulthood (females only), and changes in basilar dendritic spine density in adulthood. This work contributes to the relatively limited collection of studies that examine maternal preconception alcohol exposure and provides the first evidence as to how this experience impacts offspring brain structure.

References

Al-Yasari, A., Jabbar, S., Cabrera, M. A., Rousseau, B., & Sraker, D. K. (2021). Preconception alcohol exposure increases the susceptibility to diabetes in the offspring. *Endocrinology, 162*, 1 – 18. DOI:10.1210/endocr/bqaa188

Asimes, A., Kim, C. K., Cuarenta, A., Auger, A. P., & Pak, T. R. (2018). Binge drinking and intergenerational implication: Parental preconception alcohol impacts offspring development in rats. *Journal of the Endocrine Society, 2*, 672 – 686. DOI:10.1210/js.2018-00051

Asimes, A., Torcaso, A., Pinceti, E., Kim, C. K., Zeleznik-Le, N. J., & Pak, T. R. (2017). Adolescent binge-pattern alcohol exposure alters genome-wide DNA methylation patterns in the hypothalamus of alcohol-naïve male offspring. *Alcohol, 60*, 179 – 189. DOI;10.1016/j.alcohol.2016.10.010

Brancato, A, Castelli, V., Lavanco, G., & Cannizzaro, C. (2020). Environmental enrichment during adolescence mitigates cognitive deficits and alcohol vulnerability due to continuous and intermittent perinatal alcohol exposure in adult rats. *Frontiers in Behavioral Neuroscience, 14*, 583122. DOI:10.3389/fnbeh.2020.583122

Brancato, A., Castelli, V., Cavallaro, A., Lavanco, G., Plescia, F., & Cannizzaro, C. (2018). Pre-conceptional and peri-gestational maternal binge drinking produces inheritance of mood disturbances and alcohol vulnerability in adolescent offspring. *Frontiers in Psychiatry, 9*, 150. DOI:10.3389/fpsyt.2018.00150

Brys, I, Pupe, S., & Bizarro, L. (2014). Attention, locomotor activity and developmental milestones in rats prenatally exposed to ethanol. *International Journal of Developmental Neuroscience, 38*, 161 – 168. DOI:10.1016/j.ijdevneu.2014.08.007

Choi, I., Kim, P., Joo, S. H., Kim, M. K., Park, J. H., Kim, H. J., Ryu, J. H., Cheong, J. H., & Shin, C. Y. (2012). Effects of preconceptional ethanol consumption on ADHD-like symptoms in Sprague-Dawley rat offsprings. *Biomolecules and Therapeutics, 20*, 226 – 233. DOI:10.4062/biomolther.2012.20.2.226

Chudley, A. E., Conry, J., Cook, J. L., Loock, C., Rosales, T., & LeBlanc, N. (2005). Fetal alcohol spectrum disorder: Canadian guidelines for diagnosis. *Canadian Medical Association Journal, 172* (5 suppl), S1 – S21. DOI:10.1503/cmaj.1040302

Comery, T. A., Harris, J. B., Willems, P. J., Oostra, B. A., Irwin, S. A., Weiler, I. J., & Greenough, W. T. (1997). Abnormal dendritic spines in fragile X knockout mice: Maturation and pruning deficits. *Proceedings of the National Academy of Sciences, 94*, 5401 – 5404.

Cook, J. L., Green, C. R., Lilley, C. M., Anderson, S. M., Baldwin, M. E., Chudley, A. E., Conry, J. L., LeBlanc, N., Loock, C. A., Lutke, J., Mallon, B. F., McFarlane, A. A., Temple, V. K., Rosales, T., for the Canada Fetal Alcohol Spectrum Disorder Research Network. *Canadian Medical Association Journal, 188,* 191 – 197. DOI:10.1503/cmaj.141593

Cullen, C. L., Burne, T. H. J., Lavidis, N. A., & Moritz, K. M. (2014). Low dose prenatal alcohol exposure does not impair spatial learning and memory in two tests in adult and aged rats. *PLoS One, 9*, e101482. DOI:10.1371/journal.pone.0101482

Curley, J. P., & Champagne, F. A. (2016). Influence of maternal care on the developing brain: Mechanisms, temporal dynamics and sensitive periods. *Frontiers in Neuroendocrinology, 40,* 52 – 66. DOI:10.1016/j.yfrne.2015.11.001

De Giorgio, A., & Granato, A. (2015). Reduced density of dendritic spines in pyramidal neurons of rats exposed to alcohol during early postnatal life. *International Journal of Developmental Neuroscience, 41*, 74 – 79. DOI:10.1016/j.ijdevneu.2015.01.005

Dursun, I., Jakubowska-Doğru, E., & Uzbay, T. (2006). Effects of prenatal exposure to alcohol on activity, anxiety, motor coordination, and memory in young adult Wistar rats. *Pharmacology, Biochemistry and Behavior, 85*, 345 – 355. DOI:10.1016/j.pbb.2006.09.001

Easey, K. E., Dyer, M. L., Timpson, N. J., & Munafò, M. R. (2019). Prenatal alcohol exposure and offspring mental health: A systematic review. *Drug and Alcohol Dependence, 197*, 344 – 353. DOI:10.1016/j.drugalcdep.2019.01.007

Finegersh, A., Rompala, G. R., Martin, D. I. K., & Homanics, G. E. (2015). Drinking beyond a lifetime: New and emerging insights into paternal alcohol exposure on subsequent generations. *Alcohol, 49*, 461 – 470. DOI:10.1016/j.alcohol.2015.02.008

Gibb, R., & Kolb, B. (1998). A method of vibratome sectioning of Golgi-Cox stained whole rat brain. *Journal of Neuroscience Methods, 79*, 1 – 4. DOI:10.1016/S0165-0270(97)00163-5

Goldberg, L. R., & Gould, T. J. (2019). Multigenerational and transgenerational effects of paternal exposure to drugs of abuse on behavioral and neural function. *European Journal of Neuroscience, 50*, 2453 – 2466. DOI:10.1111/ejn.14060

Gulick, D., & Gould, T. J. (2007). Acute ethanol has biphasic effects on short- and long-term memory in both foreground and background contextual fear condition in C57BL/6 mice. *Alcoholism: Clinical and Experimental Research, 31*, 1528 – 1537. DOI:10.1111/j.1530-0277.2007.00458.x

Gupta, K. K., Gupta, V. K., & Shirasaka, T. (2016). An update on fetal alcohol syndrome – Pathogenesis, risks, and treatment. *Alcoholism: Clinical and Experimental Research, 40,* 1594 – 1602. DOI:10.1111/acer.13135

Hamilton, G. F., Criss, K. J., & Klintsova, A. Y. (2015). Voluntary exercise partially reverses neonatal alcohol-induced deficits in mPFC layer II/III dendritic morphology of male adolescent rats. *Synapse, 69,* 405 – 415. DOI:10.1002/syn.21827

Hamilton, G. F., Whitcher, L. T., & Klintsova, A. Y. (2010). Postnatal binge-like alcohol exposure decreases dendritic complexity while increasing the density of mature spines in the mPFC layer II/III pyramidal neurons. *Synapse, 64,* 127 – 135. DOI:10.1002/syn.20711

Harper, K. M., Tunc-Ozcan, E., Graf, E. N., & Redei, E. E. (2014). Intergenerational effects of prenatal ethanol on glucose tolerance and insulin response. *Physiological Genomics, 46,* 159 – 168. DOI:10.1152/physiolgenomics.00181.2013

Haycock, P. C. (2009). Fetal alcohol spectrum disorders: The epigenetic perspective. *Biology of Reproduction, 81,* 607 – 617. DOI:10.1095/biolreprod.108.074690

Hutsler, J. J., & Zhang, H. (2010). Increased dendritic spine densities on cortical projection neurons in autism spectrum disorder. *Brain Research, 1309,* 83 – 94. DOI:10.1016/j.brainres.2009.09.120

Inaba, H., Kishimoto, T., Oishi, S., Nagata, K., Hasegawa, S., Watanabe, T., & Kida, S. (2016). Vitamin B1-deficient mice show impairment of hippocampus-dependent memory formation and loss of hippocampal neurons and dendritic spines: Potential microendophenotypes of Wernicke-Korsakoff syndrome. *Bioscience, Biotechnology, and Biochemistry, 80,* 2425 – 2436. DOI:10.1080/09168451.2016.1224639

Jabbar, S., Chastain, L. G., Gangisetty, O., Cabrera, M. A., Sochacki, K., & Sarkar, D. K. (2016). Preconception alcohol increases offspring vulnerability to stress. *Neuropsychopharmacology, 41,* 2782 – 2793. DOI:10.1038/npp.2016.92

Jacome, L. F., Barateli, K., Buitrago, D., Lema, F., Frankfurt, M., & Luine, V. N. (2016). Gonadal hormones rapidly enhance spatial memory and increase hippocampal spine density in male rats. *Endocrinology, 157,* 1357 – 1362. DOI:10.1210/en.2015-1959

Jakubowska-Doğru, E., Elibol, B., Dursun, I., & Yürüker, S. (2017). Effects of binge-like ethanol exposure and maternal stress on postnatal morphological development of hippocampal neurons in rats. *International Journal of Developmental Neuroscience, 61,* 40 – 50. DOI:10.1016/j.ijdevneu.2017.06.002

Jamerson, P. A., Wulser, M. J., & Kimler, B. F. (2004). Neurobehavioral effects in rat pups whose sires were exposed to alcohol. *Developmental Brain Research, 149*, 103 – 111. DOI:10.1016/j.devbrainres.2003.12.010

Kokavec, A. (2008). Is decreased appetite for food a physiological consequence of alcohol consumption? *Appetite, 51*, 233 – 243. DOI:10.1016/j.appet.2008.03.011

Koren, G., Nulman, I., Chudley, A. E., & Loocke, C. (2003). Fetal alcohol spectrum disorder. *Canadian Medical Association Journal, 169*, 1181 – 1185.

Kutlu, M. G., & Gould, T. J. (2016). Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: Contributions to development and maintenance of addiction. *Learning and Memory, 23*, 515 – 533. DOI:10.1101/lm.042192.116

Lam, V. Y. Y., Raineki, C., Wang, L. Y., Chiu, M., Lee, G., Ellis, L., Yu, W., & Weinberg, J. (2019). Role of corticosterone in anxiety- and depressive-like behavior and HPA regulation following prenatal alcohol exposure. *Progress in Neuropharmacology, Biology, and Psychiatry, 90*, 1 – 15. DOI:10.1016/j.pnpbp.2018.10.008

Lee, Y. J., Kim, J. Y., Lee, D. Y., Park, K. J., Kim, G. H., Kim, J., E., Roh, G. S., Lim, J. Y., Koo, S., Lim, M. K., Park, H. Y., & Kim, W.-H. (2020). Alcohol consumption before pregnancy causes detrimental fetal development and maternal metabolic disorders. *Scientific Reports, 10*, 10054. DOI:10.1038/s41598-020-66971-1

Livy, D. J., Maier, S. E., & West, J. R. (2004). Long-term alcohol exposure prior to conception results in lower fetal body weights. *Birth Defects Research (Part B), 71*, 135 – 141. DOI:10.1002/bdrb.20007

Markham, J. A., Morris, J. R., & Juraska, J. M. (2007). Neuron number decreases in the rat ventral, but not dorsal, medial prefrontal cortex between adolescence and adulthood. *Neuroscience, 144*, 961 – 968. DOI:10.1016/j.neuroscience.2006.10.015

McGuier, N. S., Padula, A. E., Lopez, M. F., Woodward, J. J., & Mulholland, P. J. (2015). Withdrawal from chronic intermittent alcohol exposure increases dendritic spine density in the lateral orbitofrontal cortex of mice. *Alcohol, 49*, 21 – 27. DOI:10.1016/j.alcohol.2014.07.017

Mihalick, S. M., Crandall, J. E., Langlois, J. C., Krienke, J. D., & Dube, W. V. (2001). Prenatal ethanol exposure, generalized learning impairment, and medial prefrontal deficits in rats. *Neurotoxicology and Teratology, 23*, 453 – 462.

Morris, R. (1984). Developments of a water maze procedure for studying spatial learning in the rat. *Journal of Neuroscience Methods, 11*, 47-60. DOI:10.1016/0165-0270(84)90007-4

Motz, B. A., & Alberts, J. R. (2005). The validity and utility of geotaxis in young rodents. *Neurotoxicology and Teratology, 27*, 529 – 533. DOI:10.1016/j.ntt.2005.06.005

Muñoz-Villegas, P., Rodríguez, V., M., Giordano, M., & Juárez, J. (2017). Risk-taking, locomotor activity and dopamine levels in the nucleus accumbens and medial prefrontal cortex in male rats treated prenatally with alcohol. *Pharmacology, Biochemistry and Behavior, 153*, 88 – 86. DOI:10.1016/j.pbb.2016.12.011

Nieto, S. J., & Kosten, T. A. (2019). Who's your daddy? Behavioral and epigenetic consequences of paternal drug exposure. *International Journal of Developmental Neuroscience, 78*, 109 – 121. DOI:10.1016/j.ijdevneu.2019.07.002

Paula-Barbosa, M. M., Brandão, F., Madeira, M. D., & Cadete-Leite, A. (1993). Structural changes in the hippocampal formation after long-term alcohol consumption and withdrawal in the rat. *Addiction, 88*, 237 – 247. DOI:10.1111/j.1360-0443.1993.tb00807.x

Peterson, V. L., McCool, B. A., & Hamilton, D. A. (2015). Effects of ethanol exposure and withdrawal on dendritic morphology and spine density in the nucleus accumbens core and shell. *Brain Research, 1594*, 125 – 135. DOI:10.1016/j.brainres.2014.10.036

Priddy, B. M., Carmack, S. A., Thomas, L. C., Vendruscolo, J. C. M., Koob, G. F., & Vendruscolo, L. F. (2017). Sex, strain, and estrous cycle influence on alcohol drinking in rats. *Pharmacology, Biochemistry and Behavior, 152*, 61 – 67. DOI:10.1016/j.pbb.2016.08.001

Przybycien-Szymanska, M. M., Rao, Y., S., Prins, S. A., & Pak., T. R. (2014). Parental binge alcohol abuse alters F1 generation hypothalamic gene expression in the absence of direct fetal alcohol exposure. *PLoS ONE, 9*, e89320. DOI:10.1371/journal.pone.0089320

Rasmussen, C., & Wyper, K. (2007). Decision making, executive functioning, and risky behaviors in adolescents with prenatal alcohol exposure. *International Journal on Disability and Human Development, 6*, 405 – 416. DOI:10.1515/IJDHD.2007.6.4.405

Risher, M.-L., Fleming, R. L., Risher, W. C., Miller, K. M., Klein, R. C., Wills, T., Acheson, S. K., Moore, S. D., Wilson, W. A., Eroglu, C., & Swartzwelder, H. S. (2015). Adolescent intermittent alcohol exposure: Persistence of structural and functional hippocampal abnormalities into adulthood. *Alcoholism: Clinical and Experimental Research, 39*, 989 – 997. DOI:10.1111/acer.12725

Rompala, G. R., & Homanics, G. E. (2019). Intergenerational effects of alcohol: A review of paternal preconception ethanol exposure studies and epigenetic mechanisms in the male germline. *Alcoholism Clinical and Experimental Research, 43*, 1032 – 1045. DOI:10.1111/acer.14029

Segal, M., Korkotian, E., & Murphy, D. D. (2000). Dendritic spine formation and pruning: Common cellular mechanisms? *Trends in Neuroscience, 23*, 53 – 57.

Sokol, R. J., Delaney-Black, V., & Nordstrom, B. (2003). Fetal alcohol spectrum disorder. *JAMA, 260*, 2996 – 2999. DOI:10.1001/jama.290.22.2996

Statistics Canada. (2021). Table 13-10-0096-11 Heavy drinking by age group. https://doi.org/10.25318/1310009601-eng

Štrbák., V., Benický, J., Macho, L., Ježová, D., & Nikodémová, M. (1998). Four-week ethanol intake decreases food intake and body weight but does not affect plasma leptin, corticosterone, and insulin levels in pubertal rats. *Metabolism, 47*, 1269 – 1273. DOI:10.1016/s0026-0495(98)90335-3

Tunc-Ozcan, E., Harper, K. M., Graf, E. N., & Redei, E. E. (2016). Thyroxine administration prevents matrilineal intergenerational consequences of *in utero* ethanol exposure in rats. *Hormones and Behavior, 82*, 1 – 10. DOI:10.1016/j.yhbeh.2016.04.002

Vassoler, F. M., Byrnes, E. M., & Pierce, R. C. (2014). The impact of exposure to addictive drugs on future generations: Physiological and behavioral effects. *Neuropharmacology, 76*, 269 – 275. DOI:10.1016/j.neuropharm.2013.06.016

Whitcher, L. T., & Klintsova, A. Y. (2008). Postnatal binge-like alcohol exposure reduces spine density without affecting dendritic morphology in rat mPFC. *Synapse, 62*, 566 – 573. DOI:10.1002/syn.20532

Willing, J., & Juraska, J. M. (2015). The timing of neuronal loss across adolescence in the medial prefrontal cortex of male and female rats. *Neuroscience, 301*, 268 – 275. DOI:10.1016/j.neuroscience.2015.05.073

Woolley, C. S., & McEwen, B. S. (1993). Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *The Journal of Comparative Neurology, 336*, 293 – 306.

Yohn, N. L., Bartolomei, M. S., & Blendy, J. A. (2015). Multigenerational and transgenerational inheritance of drug exposure: The effects of alcohol, opiates, cocaine, marijuana, and nicotine. *Progress in Biophysics and Molecular Biology, 118*, 21 – 33. DOI:10.1016/j.pbiomolbio.2015.03.002

Zilles, K. (1985). *The Cortex of the Rat: A Stereotaxic Atlas*. Springer-Verlag: Berlin, Germany.

CHAPTER 5:

General Conclusion

Abstract

This chapter provides a brief overview of the major findings of the preceding chapters, discusses the importance of these findings with respect to how they contribute to the current literature, and proposes some promising avenues of investigation for future studies in this area.

This thesis has explored how the use of recreational drugs of abuse prior to conception impacts the long-term development of the next generation in terms of behavior and brain. Collectively, these studies have demonstrated that the effects of maternal preconception drug use are highly dependent on offspring sex and age. Furthermore, the consequences of exposure to moderate levels of nicotine appear to be more pronounced than those following heavy alcohol use, at least with respect to the chosen measures. However, such comparisons must be made with caution as methodological differences inhibit direct comparison.

5.1 Overview of Thesis

Chapter 1 reviewed what is currently known about the effects of nicotine and alcohol when exposure occurs during gestation and prior to conception. As was evident from this chapter, our understanding of maternal preconception drug exposure is severely lacking. Therefore, Chapters 2 through 4 described experiments that sought to identify how chronic use of nicotine or alcohol in the time immediately preceding conception impacts the next generation using a rodent model. Chapter 2 revealed that although chronic exposure to moderate levels of nicotine prior to conception does not impair maternal care, pre-weaning offspring exhibit delayed progression through developmental milestones. Furthermore, maternal enrichment induced hypoactivity in developing female offspring and increased basilar spine density in the orbitofrontal cortex (OFC). Interestingly, there was no interaction between maternal nicotine and enrichment for pre-weaning offspring; maternal nicotine and enrichment each exerted unique influences over pup development. Chapter 3 provided a long-term follow-up of these offspring and revealed that whereas nicotine reduced anxiety in adolescent females, enrichment

increased anxiety in adolescent males. In adult offspring, nicotine impaired the control of complex forelimb movement in adult females, and enrichment facilitated spatial learning in adult males, but only if the dam was not exposed to nicotine. Enrichment also decreased brain weight and basilar spine density in the parietal cortex of adult males born to dams not exposed to nicotine. Therefore, maternal preconception nicotine and enrichment continued to exert unique, sex-dependent effects on offspring development throughout life. Finally, Chapter 4 reported on the consequences of maternal preconception alcoholism. Adolescent offspring of both sexes displayed decreased anxiety, suggesting decreased inhibition towards novelty and potentially risky situations. Furthermore, adult female offspring had an apparent spatial learning advantage in the Morris water task. Maternal alcoholism also induced sex-specific changes in basilar spine density, increasing density in the medial PFC of adult females but decreasing density in the hippocampus of adult males.

5.2 Importance of Findings

These experiments have considerably added to our knowledge regarding the effects of maternal preconception exposure to nicotine and alcohol. Previously, the majority of studies in this area have focused on paternal exposure, with very few experiments exploring exclusively preconception use by the mother (see Chapter 1). Therefore, the experiments presented in this thesis are valuable in that they build upon a very limited body of research. Furthermore, our results contribute some novel findings. First and foremost, these studies provide the very first evidence as to how maternal preconception nicotine and alcohol impact the offspring brain. Our analysis of dendritic spine density is the first attempt to explore how these maternal experiences impact brain

structure and allow us to make inferences regarding changes to synaptic plasticity. Secondly, we are the first to demonstrate that maternal preconception nicotine, and to a lesser extent alcohol, impacts the emergence of behavior in young rat offspring, specifically the rotating reflex and the emergence of locomotion. We also confirm the finding of one other study that maternal preconception nicotine does not impact maternal care and are the first to our knowledge to demonstrate that this is also true for alcohol. Thirdly, we are the first to show that maternal preconception nicotine impairs the control of complex forelimb movement in adult females, and we provide further support of the link between preconception nicotine and alcohol and the expression of anxiety-like behavior. Lastly, we showed that although preconception nicotine and enrichment exert several unique influences over offspring development, the two experiences do interact such that in certain cases (i.e. spatial learning and brain weight), nicotine blocks the effect of enrichment, likely by inhibiting plasticity. Additionally, as a general strength of the current studies that distinguishes these works from that of others, both experiments employed a lifespan approach in which offspring were examined throughout life and examined both sexes, which as we can see was essential to understanding the impact of maternal preconception nicotine/enrichment and alcohol.

5.3 Future Directions

These compiled works have significantly contributed to the body of literature on maternal preconception drug exposure. However, there remains many unanswered questions. First, although we have demonstrated that maternal exposure to nicotine and alcohol impacts dendritic spine density, it is important to also explore how these experiences alter other aspects of neuronal structure, such as size and complexity. Also,

more technologically advanced techniques, such as two-photon imaging, could be used to examine the density, shape, and turnover of dendritic spines in real time. Furthermore, it would be informative to investigate other indicators of synaptic plasticity, including the expression of various receptors, to gain a more complete picture of how and to what extent the mechanisms of synaptic plasticity are affected. As it is well established that prenatal nicotine and alcohol impact neurotransmission, especially acetylcholine, dopamine, and serotonin, future work should also investigate how preconception exposure impacts these systems. Epigenetic studies should focus of the expression of nicotinic acetylcholine receptors (nAChRs), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) as a starting point. Conversely, exploratory studies of the whole genome could identify potential behavioral or molecular alterations that may otherwise go undetected.

Here we reported on the effects for the first generation of offspring. Future work should investigate if these changes in behavior and brain propagate for multiple generations, despite repeated exposure. It would also be valuable to investigate how the timing of maternal exposure moderates the effects. For example, rather than exposing the dams to the drug in adulthood immediately prior to conception, future studies could expose adolescent females followed by a period of sobriety prior to mating. Additionally, our results indicate that the effects of maternal preconception drug use are often quite subtle. It is possible that we may have observed additional behavioral aberrations if we had chosen more sensitive behavioral assessments. Indeed, many of the tests we used were initially developed using brain lesioned animals that exhibit much larger deficits. Therefore, future work in this area should use variations of these tests that are more

capable of detecting slight deficits in functioning. And of course, complementary studies that use the same exposure paradigm but with the sires are necessary if we want to directly compare the relative effects of maternal and paternal preconception drug exposure. And lastly, the goal of this thesis was to investigate how the abuse of legal, socially accepted drugs prior to conception impacts the next generation. We have presented on nicotine and alcohol, but the effects of cannabis and caffeine should also be explored.