THE IMPACT OF NEONATAL ALCOHOL EXPOSURE AND VOLUNTARY EXERCISE ON CHOLINERGIC FIBER LENGTH AND CG2 VOLUME IN THE RAT MEDIAL PREFRONTAL CORTEX

by

Natalie Ginn

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Natalie Ginn

Approved: _____

Anna Klintsova, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Jeffrey Rosen, Ph.D.
Committee member from the Department of Psychological and Brain Sciences

Approved: _____

Jaclyn Schwarz, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____

Earl Lee II, Ph.D
Deputy Faculty Director, University Honors Program

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ABSTRACT

Prenatal alcohol exposure is known to cause chronic behavioral impairment and neurocognitive deficits diagnosed as Fetal Alcohol Spectrum Disorders (FASD). With a prevalence of one in every twenty children affected by prenatal alcohol exposure in the United States, FASD remains a serious public health concern. This study uses a rodent model of FASD to investigate the effects of third trimester-equivalent alcohol exposure and adolescent exercise intervention on the underexplored cortical-projecting pathway of the central cholinergic system, the nucleus basalis of Meynert (NBM)-cortical pathway. Recent literature has found that neonatal choline supplementation following prenatal alcohol exposure may ameliorate some alcohol-induced cognitive deficits, but the structural basis of these functional changes has not yet been investigated. Therefore, the current study examines cholinergic fiber length along the NBM-cortical cholinergic pathway and Cg2 volume of the medial prefrontal cortex (mPFC) in our experimental paradigm. Alcohol exposed (AE) male Long-Evans pups received ethanol in a binge-like manner (5.25 g/kg/day) twice a day during postnatal day (PD) 4-9. Control groups consisted of sham-intubated (SI) and suckle control (SC) rats. Pups were weaned on PD23 and socially housed in same-sex groups of three. Wheel running (WR) rats had 24-hour voluntary access to a running wheel from PD30-72. All animals were sacrificed on PD72 and forebrain tissue was collected and histochemically stained via a Hedreen and Bacon modified Karnovsky-Roots method to visualize acetylcholinesterase (AChE)-positive fibers in Cg2. Overall, there was no significant effect of postnatal treatment or behavioral

intervention on cholinergic fiber length or Cg2 volume in adulthood. No significant interactions were observed. These data indicate that cholinergic axons are innervating their mPFC target region in adulthood. Future investigation is necessary to elucidate cellular cholinergic mechanisms affected by neonatal alcohol exposure that may be contributing to the observed PFC-dependent cognitive deficits characteristic of FASD.

Chapter 1

INTRODUCTION

Fetal Alcohol Spectrum Disorders (FASD)

Prenatal alcohol exposure is the leading preventable cause of birth defects and developmental disorders in the United States (NIH, 2015). The effects of alcohol exposure *in utero* can persist into adulthood and may manifest as numerous atypical physical, behavioral and intellectual phenotypes classified under the broader diagnosis of Fetal Alcohol Spectrum Disorders (FASD). In the United States, ten to fifteen percent of women are estimated to consume alcohol during pregnancy (CDC, 2015; Popova et al., 2017), resulting in an estimated one out of every twenty school-aged children affected by FASD (CDC, 2015; May et al., 2014). With these numbers potentially higher than self-reported as well as the significant economic costs relating to fetal alcohol exposure, FASD remains a significant economic and public health concern (May et al., 2018; Popova et al., 2011).

The severity of symptoms experienced by FASD-affected individuals is often dependent on the quantity of alcohol consumed by a pregnant woman that the fetus is exposed to during pregnancy as well as the timing of alcohol exposure during fetal development (May et al., 2013). Three percent of women report binge drinking during pregnancy (defined as four or more drinks per episode) which increases the blood alcohol concentration (BAC) of both the mother and fetus and results in more severe neuroanatomical and cognitive teratogenic damage compared to chronic alcohol consumption (CDC, 2015; Maier & West, 2001). Additionally, alcohol exposure

during the first trimester results in the most severe diagnosis of FASD known as Fetal Alcohol Syndrome (FAS) which is characterized by facial dysmorphologies, growth deficits and central nervous system abnormalities.

However, the third trimester of pregnancy includes the brain growth spurt: a critical period of rapid neural development when the brain is particularly vulnerable to teratogenic effects (Dobbing & Sands, 2014). Alcohol exposure during this period can result in lifelong behavioral impairments and intellectual disabilities. Due to the lack of visible alcohol-related dysmorphologies, these symptoms are often overlooked or misdiagnosed as Attention-Deficit Hyperactivity Disorder and place children at a heightened risk to develop secondary disabilities due to lack of or improper early intervention (Bakhireva et al., 2018; Ira Chasnoff, 2015).

The severity of this public health concern is magnified by the estimated $2 million lifetime cost of care for one individual diagnosed with FAS (Lupton et al., 2004). This cost does not include individuals diagnosed with FASD, which is predicted to be five to ten times more prevalent than FAS (Sampson et al., 1997; Astley et al., 2002). Given the aforementioned risks to fetal development, the Centers for Disease Control and Prevention now recognizes no safe amount of alcohol consumption during pregnancy or when trying to become pregnant and recommends abstinence from alcohol during this time (CDC, 2018).

Rodent Model of FASD

The Klintsova lab in the Department of Psychological and Brain Sciences uses a previously developed well-established rodent model of FASD to investigate the neuroanatomical alterations and behavioral deficits resulting from a binge-like alcohol exposure during the third trimester. A rapid increase in brain development, axonal

growth and synaptogenesis occurs during the third trimester in human pregnancy (Dobbing & Sands, 1979) and first two postnatal weeks in rats (Bonthius & West, 1990). Previous literature has demonstrated that alcohol exposure during this third trimester-equivalent in rat pups alters the structure of the hippocampus and cerebellum long-term, impairing hippocampal-dependent behavioral tasks and motor ability observed in spatial memory and navigation tasks (Goodlett & Lundahl, 1996; Johnson & Goodlett, 2002). Dysfunction of prefrontal cortex (PFC) circuitry in both humans and rodents is also evident after alcohol exposure during the brain growth spurt, inducing executive functioning deficits such as memory impairments, attentional deficits and decision making symptomatic of FASD (Connor et al., 2000; Mattson et al., 2001; Driscoll et al., 1990). This study aims to expands on the investigation of neuroanatomical changes within the PFC after neonatal alcohol exposure.

Central Cholinergic System and Neonatal Alcohol Exposure

The central cholinergic system is a ubiquitous system of central nervous system neurons known to regulate higher-order cognitive functions such as learning, memory and attention. This neural system extends from the brainstem to the frontal cortex and innervates a multitude of brain regions through the release of its modulatory neurotransmitter acetylcholine (ACh). Specific cholinergic pathways, including the septohippocampal pathway, brainstem nuclei and nucleus basalis of Meynert (NBM)-cortical pathway innervate memory-related brain regions such as the hippocampus, thalamus and PFC, respectfully (Figure 1). As such, cholinergic modulation in these structures has been shown to be critical for learning and memory (Robinson-Drummer et al., 2017; Hamilton et al., 2001; Heroux et al., 2019).

The current study investigates the underexplored NBM-cortical cholinergic pathway and its cholinergic innervation of the frontal cortex. Prior research has primarily investigated the role of cholinergic septohippocampal pathway, projecting from the medial septum to the hippocampus, and hippocampal dysfunction resulting from alcohol exposure (Swanson et al., 1996; Moore et al., 1998). It is well documented that hippocampal-dependent cognitive tasks are disrupted in both FASD-affected individuals and in rodent models; disruption of cholinergic mechanisms may influence these deficits. However, executive functioning tasks regulated by the medial PFC (mPFC) are also impaired following neonatal alcohol exposure, yet this circuit remains understudied. Impaired PFC-dependent behaviors include deficits in inhibitory control, working memory, attention and mental flexibility (i.e. ability to plan, organize, or problem solve) (Connor et al., 2000; Nash et al., 2013; Wu et al., 2011). Additionally, rodent models of FASD show structural and molecular alterations within the PFC including decreased prefrontal dendritic complexity, spine density and altered early gene expression (Hamilton et al., 2010; Lawrence et al., 2012; Heroux et al., 2019). Alcohol-induced damage to the cholinergic system may influence these executive functioning deficits in patterns similar to those observed in the septohippocampal cholinergic pathway following neonatal alcohol exposure. Specifically, alcohol exposure may damage cholinergic neurons projecting along the NBM-cortical cholinergic pathway. The Basal Forebrain (BF), where the NBM nuclei is located, is the primary source of cholinergic innervation to the frontal cortex (Mesulam, 1990). Cholinergic dysfunction within this pathway would suggest impaired mPFC functioning and neurocognitive outcomes in affected individuals.

Loss or degeneration of cholinergic neurons within specific neural structures is characteristic of amnesic disorders such as Alzheimer's Disease (AD), dementia and Wernicke-Korskoff syndrome (Mizukawa et al., 1986). A dominant symptom of AD is progressive damage to and loss of cholinergic neurons within the NBM and BF correlated with cognitive decline as cholinergic innervation of the frontal cortex decreases (Davies & Maloney, 1976; Arendt et al., 1983). Wernicke-Korskoff syndrome, caused by thiamine deficiency after chronic alcohol abuse, is characterized by frontal lobe atrophy and symptoms similar to those of AD including persistent learning and memory deficits and cognitive dementia (Akhouri & Newton, 2019; Kopelman & Corn, 1988). These pathologies indicate that cholinergic neuronal decline is associated with impaired cognitive functioning and thus cholinergic damage is a probable component to the learning and memory deficits observed in FASD. However, cholinergic neuron morphology has been more thoroughly investigated in aging disorders rather than developmental disorders. As such, further investigation of cholinergic influence during development is necessary to better understand the mechanisms underlying life-long consequences of developmental disorders such as FASD.

Recent literature exploring the therapeutic effects of choline supplementation following developmental alcohol exposure supports our hypothesis that neonatal alcohol exposure damages cholinergic pathways and may contribute to impaired behavioral outcomes. In a study by Jacobson and lab (2018), prenatal choline supplementation via maternal administration during pregnancy correlates with rescued birth weight, head circumference and visual recognition memory in infants following heavy prenatal alcohol exposure. Wozniak and colleagues (2015) documented that

nine months of postnatal choline supplementation in young FASD-affected children showed improved hippocampal-dependent memory performance compared to their placebo group peers. These studies illustrate the rescuing effects of both prenatal and early postnatal choline supplementation on physical and cognitive deficits following alcohol exposure in-utero. Rodent literature has demonstrated similar findings, warranting further exploration of cholinergic mechanisms affected by developmental alcohol exposure yet partially ameliorated by choline administration. Schneider and Thomas (2016) and Ryan and colleagues (2008) are advancing this field of study with reports that early postnatal choline supplementation after alcohol exposure during the third trimester mitigates working and spatial memory deficits in adult rodents. These findings coincide with previous literature that hippocampal and PFC circuitry are impaired after neonatal alcohol exposure as well as support the novel idea that cholinergic innervation from the NBM to PFC may be damaged following alcohol exposure.

Cholinergic Fiber Length

The ability of choline supplementation to mitigate cognitive deficits following third trimester alcohol exposure indicates that ethanol may indeed target and alter the function of cholinergic neurons. While the literature supports PFC-circuitry damage and cholinergic dysfunction following neonatal alcohol exposure as well as the significant impact of the cholinergic system in learning and memory function, there remains a surprising gap in the literature investigating whether neonatal alcohol exposure affects the NBM-cortical cholinergic pathway.

We are one of the first research groups to investigate the effect of neonatal alcohol exposure on cholinergic neuron axon morphology within the mPFC. Past

literature provides evidence that impaired ACh transmission leads to impaired behavior, indicating that fiber integrity is critical for proper ACh signaling within cholinergic target regions (Schneider & Thomas, 2016; Robinson-Drummer et al., 2017; Hamilton et al., 2001). Furthermore, a decrease in the expression of choline acetyltransferase (ChAT), an enzyme implicated in the synthesis of ACh, and cholinergic markers in the cortex have also been documented in experiments investigating various periods of alcohol exposure on the cholinergic system, including third-trimester alcohol exposure, and forebrain target regions (Vetreno & Crews, 2018; Boutros et al., 2015; Robinson-Drummer et al., 2017). Alterations to fiber length may therefore exemplify a neuroanatomical change within the cholinergic NBM-cortical pathway after developmental alcohol exposure, contributing to impaired cellular cholinergic functions and behavioral learning and memory capacity in FASD.

The prelimbic mPFC, specifically the Cg2 sub-region as described in *The Rat Brain Atlas* by Paxinos and Watson (2014), was examined due to its implication in executive functioning tasks. Dysfunction of Cg2 results in ADHD-like symptoms such as hyperactivity and inattention, both of which are commonly observed in FASD patients (Passetti et al., 2002; Migliorini et al., 2015). A change in the cholinergic innervation to this region could result in symptoms associated with alcohol exposure during the third trimester of pregnancy. Additionally, frontal lobe size decreases after neonatal alcohol exposure, correlating with a decrease in white matter within the PFC (Wass et al., 2001; Nunez et al., 2011; Norman et al., 2009). This diminished volume of white matter indicates a loss of innervating axons within the PFC, supporting our hypothesis that Cg2 volume would decrease after third trimester alcohol exposure.

Voluntary Exercise as a Therapeutic Intervention in Adolescence

The therapeutic effects of exercise on physical health and cognitive performance have been well established in both human and rodent literature (Voss et al., 2013). Specifically, running is shown to improve neurotrophin levels such as brain-derived neurotrophic factor (BDNF) (Neeper et al., 1996), cellular proliferation and neurogenesis (Vivar et al., 2013), and synaptogenesis (Klintsova et al., 2002) observed in regions of the hippocampus and cerebellum and correlated with improved learning and memory. Voluntary exercise has also been shown to mitigate cognitive and behavioral deficits following neonatal alcohol exposure during the brain growth spurt (Thomas et al., 2011; Klintsova et al., 2013). These experiments elucidate the brain's plasticity throughout development and provide evidence in support of exercise interventions as a therapeutic treatment after FASD and similar amnesic conditions.

However, the effects of voluntary exercise on cholinergic system morphology after neonatal alcohol exposure remains underexplored. The majority of existing literature has investigated the effects of ethanol exposure and exercise on hippocampal-dependent behavioral tasks and the septohippocampal cholinergic pathway (Van Praag et al., 2005; Hall & Savage, 2016; Vetreno & Crews, 2018). Yet, because exercise has been shown to attenuate PFC morphological alterations and PFC-dependent behavioral impairment following alcohol exposure, exploration of the effect of voluntary exercise on the cholinergic NBM-cortical pathway could produce similar beneficial results (Hamilton et al., 2015; Connor et al., 2000).

Therefore, our experimental paradigm implemented a wheel-running exercise intervention following third-trimester alcohol exposure in rodents to observe the potential effects of voluntary exercise on cholinergic fiber length and Cg2 volume within mPFC (Figure 2). This intervention spans from adolescence to adulthood based

on the literature that long-term exercise increases hippocampal neurogenesis in adulthood as well as reverses hippocampal-dependent behavioral deficits following neonatal alcohol exposure (Helfter et al., 2009; Thomas et al., 2008). Voluntary exercise also has beneficial long-term effects on the brain by upregulating neurotrophin levels, specifically nerve growth factor (NGF) which regulates cholinergic maturation, signaling and survival (Hall et al., 2014; Hall & Savage, 2016; Connor et al., 2009). Cholinergic neurons are especially sensitive to increases in NGF concentration and are predicted to be receptive to the biomolecular changes induced by voluntary aerobic exercise. We therefore hypothesize that a long-term voluntary exercise intervention might alter cholinergic neuronal morphology and mitigate cognitive and behavioral impairments characteristic of mnemonic disorders. If found to ameliorate these symptoms in future studies, consistent aerobic exercise could be an effective non-invasive, low-cost and easily accessible therapeutic treatment for individuals affected by cholinergic system dysfunction.

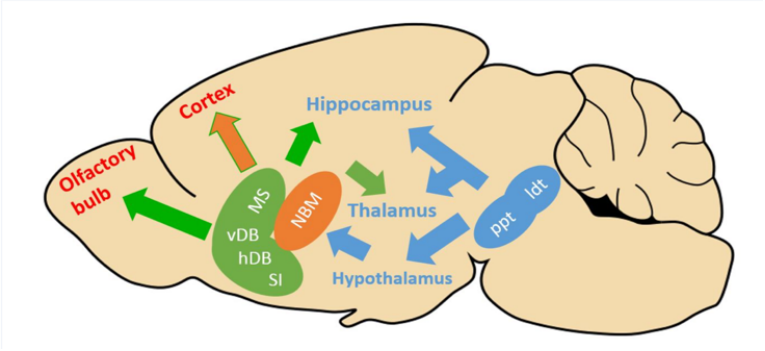

Figure 1 An illustration of the projections of the central cholinergic system and their target brain regions in a sagittal section of an adult rat brain. Pathways in green represent the septohippocampal cholinergic pathway. Pathways in blue represent projections from the brainstem nuclei. The pathway in orange represents the nucleus basalis of Meynert-cortical cholinergic pathway.

Chapter 2

METHODS

Experimental Subjects

Timed pregnant Long-Evans dams were obtained from Harlan Laboratories (Indianapolis, IN). Rats were maintained on a 12-hr light/dark cycle with light onset at 09:00 in a temperature-controlled colony room. On postnatal day (PD) 3, eleven litters were culled to eight pups each consisting of six males and two females when possible. On PD4, thirty-six male pups were randomly assigned to one of three treatment groups: alcohol-exposed (AE), sham intubated (SI), or suckle control (SC). All procedures were in accordance with the animal use protocol approved by the University of Delaware Institutional Animal Care and Use Committee. Pups underwent the alcohol exposure procedure from PD4-9 and afterwards were placed with their dams and left undisturbed until weaning on PD23. Animals were then socially housed in same-sex groups of three, counterbalanced for litter and neonatal condition. On PD30 during early adolescence, cages were assigned to one of two housing conditions: standard social housing (SH) or wheel running intervention (WR). Animals remained in these housing conditions until sacrifice on PD72 (Figure 2).

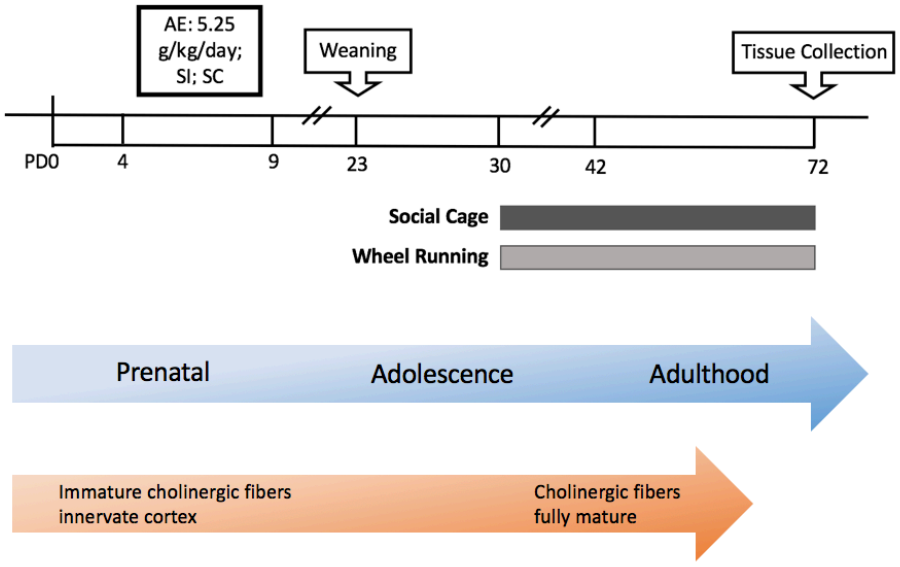

Figure 2 A timeline detailing a third trimester-equivalent binge-like alcohol exposure (AE) followed by adolescent behavioral intervention (voluntary wheel running, WR) in rats. The human-equivalent developmental periods correlating with the experimental design are highlighted in the first arrow below. The second arrow illustrates the development of cholinergic axons in humans and rodents in correlation with the experimental design.

Neonatal Alcohol Exposure and BAC Analysis

AE rats received ethanol (5.25 g/kg/day) twice a day at 09:00 and 11:00 on PD4-9 (Figure 2). Ethanol was delivered via intragastric intubation in 11.9% v/v in milk substitute isocaloric to the milk consumed by control pups. This postnatal alcohol exposure is comparable to acute binge-like drinking during the third trimester of human pregnancy (Dobbing & Sands, 1979). SI animals received intragastric intubation to control for stress of intubation but did not receive any liquid. SC animals were left undisturbed during this third trimester-equivalent besides daily weighing. On PD4, blood samples were collected 90 minutes after the second alcohol exposure from

both AE and SI pups via tail clip for BAC analysis. Blood plasma was analyzed for BAC using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA). The average BAC for AE animals was 338.458 mg/dL (± 30.874 SEM), which is comparable to a moderate to high binge-like alcohol exposure.

Voluntary Exercise Paradigm

On PD30, each cage of animals was assigned to one of two housing conditions spanning adolescence and adulthood (PD30-72): voluntary WR intervention or standard SH (Figure 2). All animals were caged in standard opaque cages (17 x 145 x 24 cm) in same-sex groups of three and balanced for postnatal treatment and litter when possible. WR rats had 24-hour voluntary access to an additional stainless-steel running wheel attached to the cage (Figure 3). Running distance per cage (calculated from the number of running wheel rotations) was recorded daily at 09:00. All animals were weighed approximately every 8 days and sacrificed on PD72.

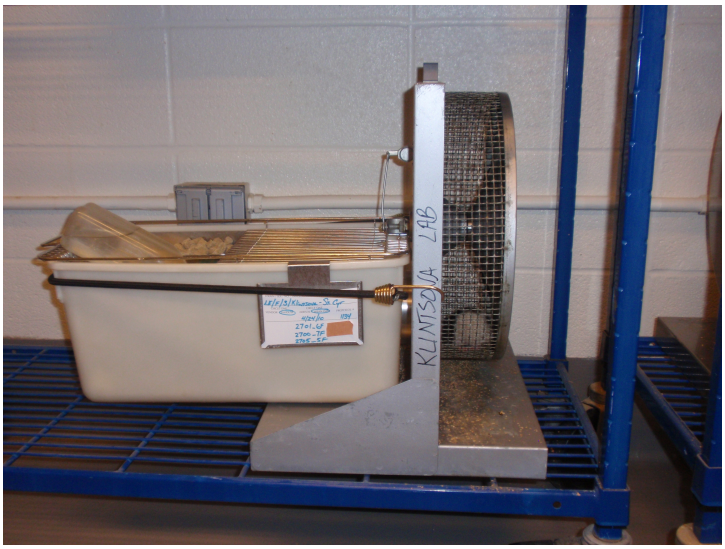

Figure 3 Image of standard housing cage with free access to a stainless-steel running wheel for wheel running (WR) intervention animals.

Tissue Collection

On PD 72, animals were deeply anesthetized via intraperitoneal injection of 2 mL/kg of ketamine/xylazine mixture (1.5 mL xylazine mixed with 10 mL of ketamine) (Gursky & Klintosva, 2017) and sacrificed via transcardial perfusion (0.1M phosphate buffered saline (PBS) solution with heparin followed by 4% paraformaldehyde, both at pH = 7.2). Brains were removed and stored in 4% paraformaldehyde in PBS at 4º C for 48 h. After two days, brains were transferred and stored in a solution of 30% sucrose added to 4% paraformaldehyde in PBS at 4º C. Forebrain tissue was collected and sectioned horizontally at 40 µm using a cryostat. Tissue sections were stored at -20º C in cryoprotectant.

Histochemistry: Visualization of Cholinergic Axons

Every eighth section of forebrain tissue was histochemically stained for acetylcholinesterase (AChE)-positive axons following a Hedreen and Bacon modified

Karnovsky-Roots method (Hedreen et al., 1985) (Figure 4, 5). Staining for the enzyme AChE, which densely surrounds cholinergic axons and is involved in the breakdown of ACh, allows for visualization of cholinergic fibers throughout the brain (Marrs & Bright, 1992; Hedreen et al., 1995; Anzalone et al., 2010). Tissue sections were singularly placed in bubble trays as free-floating sections and washed for ten minutes in 0.1M sodium acetate buffer (pH of 6.0). Sections were then submerged in an incubation medium consisting of 65% 0.1M sodium acetate buffer, 19% deionized water, 4% 0.1M sodium citrate, 10% 0.03M cupric sulfate, 2% 0.005M potassium ferricyanide and 25 mg of S-acetylthiocholine iodide (Acros Organics) for two hours. Control sections remained in 0.1M sodium acetate buffer only. Sections were then rinsed in a 0.1M sodium nitrate buffer solution for a total of five minutes before submersion in 4% ammonium sulfide solution for fifteen minutes. Another five-minute 0.1M sodium nitrate wash followed before sections were placed in 0.1% silver nitrate for fifteen minutes. A five-minute wash in 0.1M sodium nitrate wash followed by a ten-minute wash in 0.1M sodium acetate buffer solution concluded the staining procedure. Sections were immediately mounted onto slides and cover-slipped 48 hours later using DPX mounting media (Electron Microscope Series).

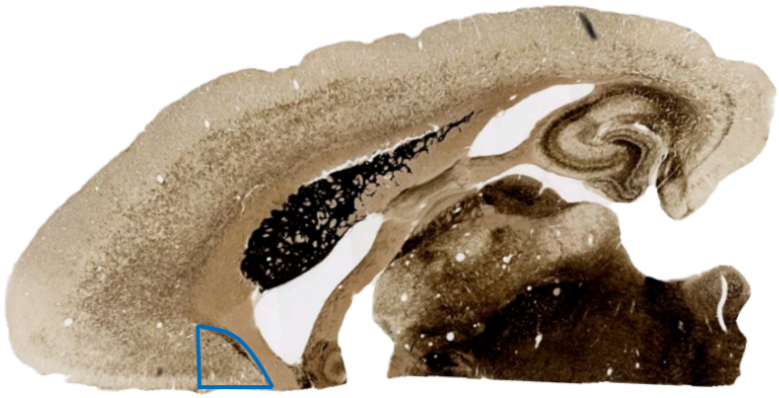

Figure 4 A 40 µm-thick horizontal tissue section of an adult rodent brain histochemically stained for AChE using a Hedreen and Bacon modified Karnovsky-Roots method. The Cg2 region of the medial PFC is outlined in blue.

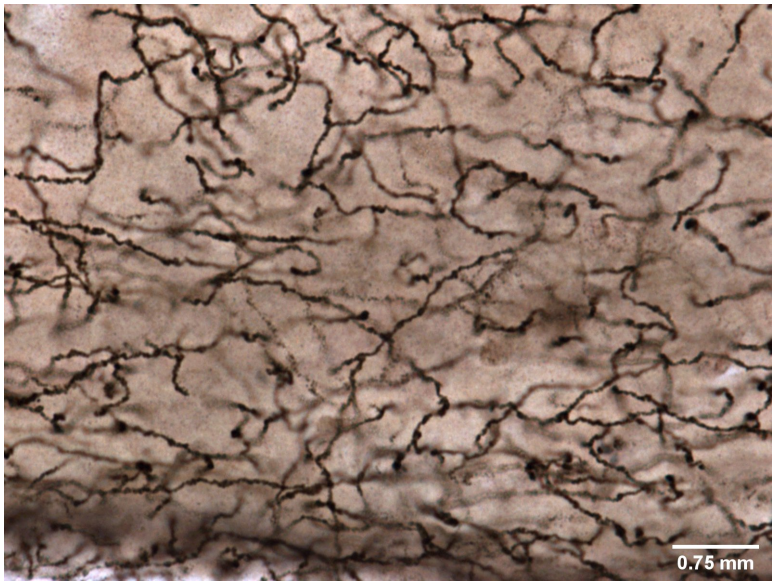

Figure 5 Visualization of histochemically stained AChE+ fibers in the Cg2 sub-region of the mPFC under 100x magnification in oil emersion.

Estimation of Fiber Length and Cg2 Volume

The Stereoinvestigator Spaceballs probe (MBF Bioscience software) was used for random, unbiased estimation of AChE-positive fiber length and structural volume at a $1/8^{th}$ sampling fraction within Cg2 (Figure 6). The region of interest was outlined under 40x magnification using a Zeiss Axioskop 2 plus microscope. Identification and tracing of Cg2 was performed by referencing the Paxinos and Watson Atlas (2014) and utilizing anatomical markers such as the corpus collosum, striatum and hippocampus to delineate the anterior, posterior, and dorsal boundaries of the desired region. After tracing, the Spaceballs probe was used under 100x magnification in oil emersion to estimate cholinergic fiber length within the Cg2 region (Figure 5). The probe estimates total fiber length as described in Smith and Guttman's publication of using spherical probes for length estimation (Smith & Guttman, 1953).

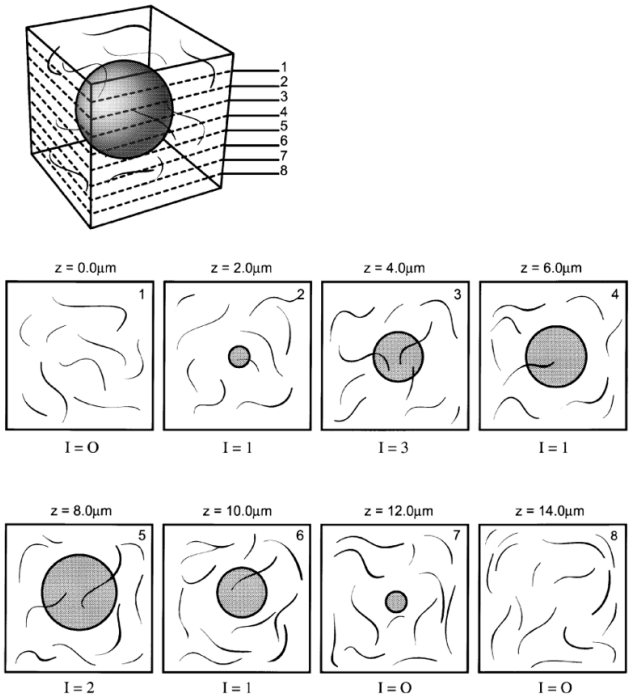

Figure 6 Visual representation of Spaceballs Probe adapted from Mouton et al. (2002). As the 3D spherical probe is focused through the depth of the tissue under the microscope, a 2D circular representation is presented to the researcher. Axon intersection points are marked along the circumference of the circle. Spaceballs software conducts algorithms explained by Smith and Guttman (1953) to estimate total fiber length throughout the depth of the tissue sample.

Chapter 3

RESULTS

Body Weights and Blood Alcohol Concentration (BAC)

Blood plasma was collected 90 minutes following the second alcohol exposure on PD4. Analysis of blood plasma shows that the BAC of AE animals in this experiment averaged 338.458 mg/dL (± 30.874 SEM). Additionally, a slight decrease in weight gain was observed in AE WR animals from PD30-72. However, this is an atypical finding that has not been historically found in our lab and may be the result of a cohort effect (Boschen et al., 2017).

Running History

WR animals ran an average running distance of 2.7 miles (± 1.2 SD) per 24 h for the first 12 days (PD31-42). The average running distance of WR animals for the last 30 days (PD43-72) was 8.5 miles (± 3.8 SD) per 24 h. This increase in running distance after the initial 12 days may be contributed to longer length of running stride as the animals aged or an increase in natural running endurance (Boschen et al., 2017). As animals were triple-housed in same-sex groups to avoid the negative outcomes of social isolation, the average running distance per individual rat could not be determined. However, previous work from our lab has demonstrated that running distance in WR animals does not significantly vary by neonatal treatment group (Helfer et al., 2009).

Analysis of Cholinergic Fiber length in mPFC

Cg2 Volume

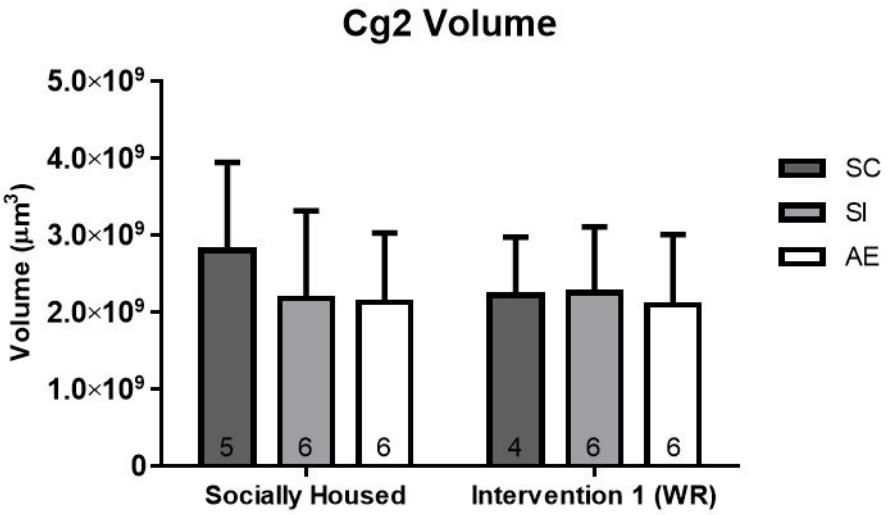

Figure 7 Cg2 volume in postnatal groups following adolescent intervention at PD72. SC=Suckle control, SI=Sham-intubated, AE=Alcohol exposed, ns=p>0.05

A two-way ANOVA was conducted to compare the effect of adolescent intervention and postnatal treatment on Cg2 structural volume in adulthood (n = 33). There was no significant difference between postnatal treatment groups on AChE-positive axonal length ($F(2, 27) = 0.59$, $p = 0.56$) nor a significant difference between WR/WR intervention groups on AChE-positive axonal length ($F(1, 27) = 0.38$, $p = 0.55$) No significant interaction was observed ($F(2, 27) = 0.44$, $p = 0.65$). Outliers that fell outside 1.5xIQR were excluded from analysis (n = 3).

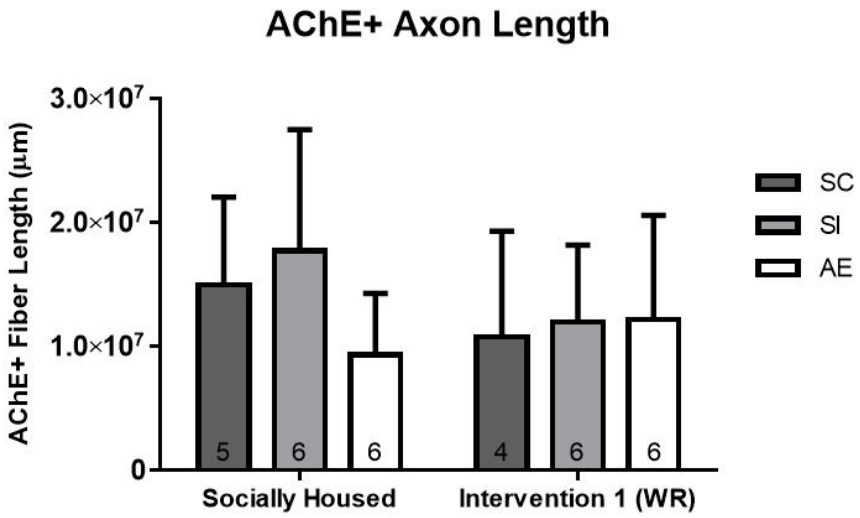

Figure 8 Estimated acetylcholinesterase-positive (AChE+) axonal length in Cg2 following adolescent intervention at PD72. SC=Suckle control, SI=Sham-intubated, AE=Alcohol exposed, ns=p>0.05.

A two-way ANOVA was conducted to compare the effect of adolescent intervention and postnatal treatment on AChE-positive axon length in adulthood (n = 33). There was no significant difference between postnatal treatment groups on AChE-positive axonal length ($F(2, 27) = 0.82$, $p = 0.45$) nor a significant difference between WRWR intervention groups on AChE-positive axonal length ($F(1, 27) = 0.87$, $p = 0.36$). No significant interaction was observed ($F(2, 27) = 1.06$, $p = 0.36$). Outliers that fell outside 1.5xIQR were excluded from analysis (n = 3).

Chapter 4

DISCUSSION

Summary of Results

This study expands upon current literature investigating the neuroanatomical changes underlying behavioral and cognitive deficits associated with FASD. The aim of the larger study, to which this experiment contributes, is to elucidate changes in cholinergic neuronal morphology within the understudied NBM-cortical cholinergic pathway following neonatal alcohol exposure and adolescent behavioral intervention. This experiment investigates the effect of third trimester alcohol exposure and a voluntary exercise intervention in adolescence on cholinergic fiber length in the mPFC target region. The data indicate that alcohol exposure during the third trimester-equivalent does not affect cholinergic axonal length in the Cg2 sub-region of the mPFC nor Cg2 volume as compared to control animals. Furthermore, voluntary exercise spanning from adolescence to adulthood did not affect cholinergic axonal length nor Cg2 volume in AE or control animals long-term. These results suggest that cortical-projecting cholinergic fibers are sufficiently present in the Cg2 target region in adulthood despite neonatal alcohol exposure and behavioral intervention. These findings generate a new platform of questions regarding the plasticity and resilience of the NBM-cortical pathway of the cholinergic system during development.

Postnatal Treatment Does Not Affect Cholinergic Fiber Length

The development of the cholinergic system occurs both pre- and postnatally in rodents, suggesting varied vulnerability to environmental exposures during different developmental periods. In rats, cholinergic BF neurons are generated between embryonic day (E) 11 and E16 and develop cholinergic properties by E14 (Hoffman &

Berger-Sweeney, 1998). However, afferents from these cholinergic neurons are the last modulatory afferents in the brain to mature, innervating the cerebral cortex and hippocampus only after birth and fully maturing by the second postnatal month (Hoffman & Berger-Sweeney, 1998). Additionally, ChAT activity and ACh synthesis, both of which are necessary for synapse formation and ACh transmission, peak during postnatal weeks 2 and 3 in rodents (Hoffman & Berger-Sweeney, 1998).

These developmental stages may suggest vulnerability to teratogens during the third trimester-equivalent in rodents, during which cholinergic projections and synapses within the cortex are maturing. In our experimental paradigm, rat pups are exposed to alcohol from PD4-9 and to voluntary exercise from PD30-72, paralleling the maturation of cholinergic fibers innervation in the PFC and reaching maturity by PD60 (Figure 2) (Hoffman & Berger-Sweeney, 1998). We hypothesized that developmental ethanol exposure would compromise cholinergic afferent integrity due to developmental vulnerability. However, our data as well as previous studies suggest remarkable resilience and plasticity of the cholinergic system after neonatal insult. In an experiment conducted by Hohmann and colleagues (1991), acute lesions to cholinergic BF neurons on PD1 in rodents showed an eighty-five percent depletion of immediate ChAT activity in the fronto-parietal neocortex but total rescue of cortical innervation and ChAT activity by the end of the first postnatal month. Similar results of reduced ChAT activity levels in the medial septum and hippocampus quickly followed by complete ChAT recovery in these regions were reported when examining the effects of chronic gestational alcohol exposure on the septohippocampal pathway in rodents (Swanson et al., 1995; Swanson et al., 1996). These studies suggest that neonatal insult to cholinergic innervation of target regions is transient and that the

plasticity of this system allows for full recovery by adolescence or adulthood in rodents. Future directions for our experiment may examine the effect of third trimester alcohol exposure on cortical cholinergic innervation within the first postnatal month of life to determine if neonatal cholinergic damage does occur but is ameliorated before adulthood.

Recent literature has expanded upon this area of interest by investigating the long-term effects of choline supplementation after neonatal alcohol exposure. These experiments by Ryan and colleagues (2008) and Schneider and Thomas (2018) have found that adolescent choline supplementation following third trimester-equivalent alcohol exposure in rats mitigates alcohol-induced working memory deficits. These findings suggest that neonatal alcohol does impair cholinergic functioning which may contribute to cognitive impairments characteristic in FASD. Since our findings showed no structural alterations to cholinergic morphology in the Cg2 following the same alcohol exposure paradigm, future studies should explore possible molecular cholinergic mechanisms affected by neonatal alcohol exposure which may induce these observed behavioral deficits.

These future directions investigating regulatory cholinergic mechanisms should explore ACh transmission within the NBM-cortical pathway following neonatal alcohol exposure. The decrease in ChAT activity observed in the previously mentioned studies after neonatal insult may reduce ACh production within the PFC (Swanson et al., 1996; Hohmann et al., 1991). This alteration in ACh transmission could underlie executive functioning impairments and cognitive deficit observed in FASD (Hall & Savage, 2016; Bloem et al., 2014; Robinson-Drummer et al., 2017). A decrease in BF ChAT levels may be further linked to a reactive increase in nerve

growth factor (NGF) gene expression after alcohol exposure, as has been reported in previous studies (Arendt et al., 1995; Boschen & Klintsova, 2016). NGF influences many aspects of cholinergic neuron functioning including modulating ChAT and AChE activity (Connor et al., 2009; Isaev et al., 2017), and thus abnormal changes in NGF concentration may disrupt ACh transmission in the frontal cortex. Future experiments are necessary to determine if alcohol exposure during the brain growth spurt does indeed affect molecular biosynthesis or neurotrophic levels within the cholinergic system, inducing neurocognitive deficits characteristic of FASD as the literature supports.

Additionally, NGF can upregulate muscarinic acetylcholine receptors (mAChR) which are correlated with attentional processing when located in the PFC and may be targeted by alcohol exposure (Bloem et al., 2014; Costa & Guizzetti, 1999; Robinson-Drummer et al., 2017). An increase in hippocampal mAChR density is observed into adulthood following third trimester or prenatal alcohol exposure (Nio et al., 1991; Kelly et al., 1998) and may indicate decreased concentrations of synaptic ACh (Rawat, 1997). If neonatal alcohol exposure reduces ACh levels but increases mAChR density in the PFC, we would expect to see fewer cholinergic connections within Cg2 rather than a change in cholinergic fiber length. However, based on the previously cited literature, these alcohol-induced changes may only cause transient neurocognitive alterations. This would corroborate our findings of insignificant cholinergic morphology changes within Cg2 in adulthood (Figure 7, 8).

Overall, this literature suggests that neonatal alcohol exposure may induce transient structural and molecular alterations to the cholinergic system, including cholinergic fiber length and innervation of the mPFC, but these changes may return to

normal by early adulthood, thus explaining why no significant changes in cholinergic fiber length were observed (Figure 8). Previous studies have shown that choline supplementation following third trimester alcohol exposure can ameliorate alcohol-induced cognitive deficits, indicating that neonatal alcohol exposure does indeed alter cholinergic function in the brain in adolescence (Ryan et al., 2008; Schneider & Thomas, 2018; McCann et al., 2005). Therefore, further investigation in this field of study is necessary to elucidate the mechanisms by which neonatal alcohol exposure effects the structure and function of the cholinergic system during development.

Adolescent Intervention Does Not Affect Cholinergic Fiber Length

A voluntary exercise intervention following third trimester alcohol exposure did not change cholinergic fiber integrity in either AE or control animals (Figure 8). Our hypothesis focused on changes in cholinergic morphology induced by chronic voluntary exercise because the behavioral intervention spans PD30-72, paralleling the development and maturation of cholinergic fiber innervation of the frontal cortex (Hoffman & Berger-Sweeney, 1998). Additionally, previous literature has demonstrated the therapeutic effects of exercise on cholinergic neurotransmission, suggesting that cholinergic neuronal damage in adolescence and young adulthood but can be rescued non-invasively. Work by Hall and Savage (2016) has contributed to these findings by utilizing a thiamine deficiency rat model of Wernicke-Korsakoff syndrome to examine the resulting deficits on cholinergic functioning within the septohippocampal pathway. Two weeks of voluntary exercise restored spatial working memory deficits observed in their model (Hall et al., 2014) as well as rescued hippocampal ACh efflux and the density of ChAT-positive cholinergic neurons within the medial septum/diagonal band (Hall & Savage, 2016). These findings demonstrate

the plasticity of the neural cholinergic system in response to exercise in adulthood. Perhaps investigating the molecular changes induced by voluntary exercise, specifically BF ChAT synthesis and PFC ACh transmission within the NBM-cortical pathway, will produce similar results and further our understanding of this underexplored pathway in our model.

Exercise may further modify cholinergic pathways by upregulating neurotrophin levels known to enhance cholinergic functioning. Specifically, increased levels of NGF have been found to ameliorate cognitive deficits observed after loss of cholinergic BF neurons (Fischer et al., 1987; Nagahara et al., 2009; Hall et al., 2016) as well as stimulate ACh release within hippocampal target regions when exogenously administered to cholinergic neurons within the medial septum and diagonal band (Huh et al., 2008; Knipper et al., 1994). Exercise is therefore a promising intervention to mitigate neurocognitive impairments resulting from cholinergic system dysfunction as it has been shown to upregulate NGF levels within the hippocampus and forebrain regions (Neeper et al., 1996; Chae & Kim, 2009). Hall and colleagues (2018) determined that NGF, not brain-derived neurotrophin factor (BDNF), following exercise recovered septohippocampal cholinergic neuron function and rescued spatial working memory deficit. The effects of long-term voluntary exercise on NGF levels and cholinergic fiber integrity following insult should be further investigated. If exercise is similarly found to upregulate NGF and modify ACh transmission within the NBM-cortical pathway, rescuing PFC-dependent behavioral deficits observed after third trimester alcohol exposure, a behavioral intervention would pose significant potential as a therapeutic intervention for FASD.

Limitations to the Study

This study only examines alcohol-induced changes on cholinergic morphology in male rodents. However, as the first experiment investigating potential structural alterations to an understudied cholinergic pathway following neonatal alcohol exposure and an adolescent behavioral intervention, this pilot study was designed as a foundation to direct future experiments. Studies by Hoffman and Berger-Sweeney suggest that sexual dimorphisms exist within the cholinergic system and its plasticity during development (1998). Thus, future studies should increase the sample size and include the examination of sex-specific effects of neonatal alcohol exposure and adolescent exercise intervention on cholinergic neuron morphology in mPFC. l

Additionally, while Cg2 is a relatively small mPFC sub-region to represent the PFC, this brain region holds significant relevance to our rodent model of FASD. Cg2 aids in the regulation of attention and hyperactivity (Hosseini et al., 2013), both of which are disrupted in FASD-affected individuals and indicate dysfunction in PFC circuitry. Further investigation of cholinergic innervation of the PFC should include additional regions of the cortex to explore potential patterns of cholinergic innervation across these sub-regions as well as to compromise a better understanding of overall cholinergic influence within the PFC.

Finally, future studies may use an AChE-specific stain or viral trace to more accurately investigate the effects of developmental ethanol exposure on cholinergic neuron morphology from the BF to the cortex. The Hedreen and Bacon modified Karnovsky-Roots method histochemically stains cholinoceptive fibers and is thus not AChE specific. However, this staining was sufficient to visualize cholinergic fibers in Cg2 and document significant changes in cholinergic fiber length as the NBM-cortical pathway is the primary source of cholinergic innervation to the PFC, and thus the

majority of fibers stained and analyzed are AChE-positive. This staining method is additionally supported by numerous past studies which have used a variation of this staining technique to successfully visualize and quantify AChE-positive fibers in the PFC (Hedreen et al., 1985; Struble et al., 1986; Anzalone et al., 2010).

Relevance to FASD

This study is the first to investigate how binge-like alcohol exposure during the third trimester and voluntary adolescent exercise intervention affects cholinergic morphology in an under-explored neural pathway. Our results indicate that cholinergic fiber morphology is not affected in adulthood by either postnatal treatment or behavioral intervention, suggesting that cortical-projecting cholinergic fibers are resilient to both negative and positive effects of these manipulations. This initial step toward elucidating the role of the cholinergic NBM-cortical pathway in FASD may direct future studies to investigate underlying molecular changes and neuroanatomical alterations earlier in life following alcohol exposure during the brain growth spurt. Advancing this field of study will enhance our understanding of the mechanisms underlying PFC-dependent behavioral deficits observed in FASD, as well as effective therapeutic interventions for FASD-affected individuals. Additionally, investigating individual cholinergic pathways will further our understanding of the central cholinergic system and its implementation in mnemonic disorders characterized by cholinergic system dysfunction.

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