Elucidating the Mechanisms Underlying Fetal Alcohol Spectrum Disorders and Therapeutics: The Effects of Postnatal Choline Supplementation and Exercise Intervention on Cellular Proliferation in the Neurogenic Niche of the Hippocampus

by

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We acknowledge and respect the Lək̓ʷəŋən (Songhees and Esquimalt) Peoples on whose territory the university stands, and the Lək̓ʷəŋən and W̱SÁNEĆ Peoples whose historical relationships with the land continue to this day.

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Abstract

Alcohol exposure to a developing fetus can produce a wide variety of deleterious and transient effects; these effects present as a collection of symptoms and in clinical populations, are referred to as Fetal Alcohol Spectrum Disorders (FASDs). With prevalence rates of around 7 in 1000 cases, globally, FASDs are among the leading causes of neurodevelopmental damage. The amount and timing of alcohol use are factors that play into the severity and type of damage seen in patients with an FASD. Symptom types can range from craniofacial abnormalities to central nervous system (CNS) dysfunction. The third trimester of fetal development contains a growth spurt for the hippocampus, a region of the brain responsible for learning and memory processes and during this time, it is particularly susceptible to teratogenic insult. Additionally, the hippocampus contains one of the few neurogenic niches in the peri-adolescent brain, within the subgranular zone (SGZ) of the dentate gyrus (DG). As a result, it is expected that alcohol exposure during this period will disrupt neurogenic processes, but whether this effect is transient remains unclear. In recent years, professionals have moved towards novel approaches for the treatment of FASDs involving choline and aerobic exercise as effective treatment options; research is ongoing. Choline is an essential nutrient that plays a vital role in many neural and bodily processes including cell membrane synthesis, methylation, and neurotransmission. Aerobic exercise involves sustained, rhythmic physical activity and improves cardiovascular endurance through an increase in heart rate and oxygen flow to muscles. Previous work has demonstrated choline as an effective treatment to counter the aberrant effects of prenatal alcohol exposure (PAE) on the developing brain, particularly in hippocampal-dependent behaviours and similarly, it has been reported that aerobic exercise can enhance neurogenesis in the hippocampus and counteract deficits caused by PAE. To determine the effects of each of these treatments on the first stage of neurogenesis, cellular proliferation, a postnatal binge model of ethanol exposure with postnatal choline supplementation and juvenile chronic moderately-paced forced running was employed, and animals were sacrificed during the peri-adolescent period (PD36). Corresponding tissue was subject to immunohistological staining against bromodeoxyuridine (BrdU) and Ki67, and cell-positive densities for each stain were determined. Results showed no significant variations in the densities of proliferating cells of any treatment (ethanol exposure or choline supplementation) except forced running, which showed significant increases in Ki67 cell densities only. Similarly, two-way combinations of treatments and all three treatments showed no significant differences. Although this study provides insight into the relationship between hippocampal neurogenesis and ethanol, choline, and aerobic exercise, it is limited by methodological constraints which may influence the generalizability of the findings.

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List of Abbreviations

FASDs	Fetal Alcohol Spectrum Disorders	**RNA**	Ribonucleic acid
FAS	Fetal Alcohol Syndrome	**GCL**	Granule cell layer
CNS		**MFP**	Mossy Fiber pathway
pFAS	partial Fetal Alcohol Syndrome	**MPP**	Medial Perforant pathway
ARND	Alcohol-Related Neurodevelopmental Disorder	**LPP**	Lateral Perforant pathway
ARBD	Alcohol-Related Birth Defects	**ED**	Embryonic Day
ND-PAE	Neurobehavioral Disorders Associated with Prenatal Alcohol Exposure	**PND**	Postnatal Day
APA	American Psychiatric Association	**PAE**	Prenatal ethanol exposure
WHO	World Health Organization	**PBS**	Phosphate buffered saline
CDC	Center for Disease Control	**PFA**	Paraformaldehyde
US	United States	**BrdU**	Bromodeoxyuridine
ADH	Alcohol dehydrogenase	**NA**	Numerical aperture
ALDH	Acetaldehyde dehydrogenase	**RI**	Refractive Index
MEOS	Microsomal ethanol-oxidising system	**ANOVA**	Analysis of variance
ROS	Reactive oxygen species		
BBB	Blood brain barrier		
BAC	Blood alcohol concentration		
CBT	Cognitive behavioural therapy		
ADHD	Attention deficit/hyperactivity disorder		
DNA	Deoxyribonucleic acid		
CA	Cornu Ammonis		
DG	Dentate Gyrus		
EC	Entorhinal Cortex		
NSC	Neural Stem Cell		
NPC	Neural Progenitor Cell		
SGZ	Subgranular Zone		
GABA	Gamma-aminobutyric acid		
G1	Gap 1		
S	Synthesis		
G2	Gap 2		
M	Mitosis		
dsDNA	Double stranded DNA		
ssDNA	Single stranded DNA		

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Dedication

I'd like to dedicate this thesis work to my grandmother – Nanny. You always encouraged me in every academic pursuit, and you have stayed with me throughout every step of my journey. This goes out to you.

Chapter 1: Introduction

1.1 Fetal Alcohol Spectrum Disorders

1.1.1 Overview and Background

Fetal Alcohol Spectrum Disorders (FASDs) is an umbrella term used to describe a variety of symptoms that arise in individuals who were exposed to alcohol during fetal development. The term FASDs is used to represent a few disorders that are separated by differing diagnostic criteria. The most extreme is Fetal Alcohol Syndrome (FAS), which is characterized as severe damage to the Central Nervous System (CNS) as well as minor facial feature and growth problems. Partial FAS (pFAS) encompasses deficits of the same nature, but to a lesser degree. Other diagnoses include Alcohol-Related Neurodevelopmental Disorder (ARND), presenting cognitive deficits and Alcohol-Related Birth Defects (ARBD), presenting heart, kidney, bones, and hearing complications. Lastly, Neurobehavioral Disorders Associated with Prenatal Alcohol Exposure (ND-PAE) encompasses the deficits in thinking and memory, behavioral problems, and trouble with day-to-day living. Diagnosis of ND-PAE requires the mother of the child to have consumed more than the minimal level of alcohol before the child's birth, defined by the American Psychiatric Association (APA) as more than 13 alcoholic drinks per month of pregnancy (CDC, 2024e).

For centuries, alcohol consumption was widespread and often unregulated. Scientific inquiry into the effects of alcohol on physical and mental health began to gain momentum in the late 19th century, but it wasn't until the mid-20th century that comprehensive research into the teratogenic effects of alcohol became widely known and disseminated to the public. In 1968, a French publication, (Lemoine et al., 1968), described observed deficits in the offspring

of alcoholic mothers. If pregnancies reached full term without resulting in miscarriage or stillbirth, offspring were identified based on four key domains – very peculiar face, considerable growth delay, high frequency malformations and psycho-motor retardations. Later, these symptoms were similarly described by (Jones et al., 1973) and (Jones & Smith, 1973), leading to the initial recognition of the risks associated with alcohol use during pregnancy by medical professionals. By 1977, financial support for biomedical research of FASDs and public health efforts were initiated (Lange et al., 2017).

1.1.2 Epidemiology

FASDs are among the leading causes of preventable neurodevelopmental damage (NIAAA, 2023). It is well-established that ethyl alcohol, the primary component found in all alcoholic beverages, can easily cross the placental barrier (Popova et al., 2023). Alcohol use during pregnancy is a significant risk factor for adverse outcomes such as spontaneous abortions and stillbirth. In cases where the fetus is carried to term, alcohol exposure can result in premature birth, low birthweight, and the onset of lifelong cognitive, behavioural, physical, and emotional deficiencies (Popova et al., 2017). Global estimates from the World Health Organization (WHO) report that approximately 1 in 13 women who drink alcohol during a pregnancy will give birth to a child with an FASD (Lange et al., 2017).

Several risk factors for maternal alcohol use in pregnancy have been identified, such as alcohol use disorder, mental health disorders (including depression), a history of physical and/or sexual abuse, substance abuse by the mother's partner (Esper & Furtado, 2014), higher gravidity and parity (Mulat et al., 2022), and delayed pregnancy recognition (Popova et al.,

2023). Unfortunately, the risk for alcohol consumption during pregnancy is higher in low socioeconomic communities that are associated with inadequate access to prenatal healthcare, lack of education on the harmful effects of alcohol use during pregnancy and higher consumption rates driven by stress and poverty (Skagerström et al., 2011).

Few estimates for the prevalence of FASDs are available, since the disorder can be challenging to diagnose, and many cases go unrecognized. Nonetheless, recent reports have suggested that the disorders persist in 7.7 cases per 1000 individuals in the global population (Lange et al., 2017). Prevalence rates tend to be higher in developed countries with robust diagnostic frameworks and awareness programs and lower in developing countries where cases are underreported. Studies cited by the Center for Disease Control (CDC) have identified ranges of 1-5% of children in the US (United States) and some areas of the Western Europe (CDC, 2024; May et al., 2009, 2014, 2018). The government of Canada cited reports of prevalence rates of 0.1% of youth living in private dwellings, and 1.2% of youth in Indigenous communities (Palmeter et al., 2021). The highest reported prevalence rates include South Africa (11.1%), Croatia (5.3%) and Ireland (4.8%) where drinking rates are high (Popova et al., 2017). The lowest reports are in countries with high abstinence from alcohol such as Saudi Arabia, Qatar, and the United Arab Emirates, with 0 reported cases (Popova et al., 2017). Of note, 76 countries have an FASD prevalence rate of over 1%, a rate that is like the prevalence of autism spectrum disorders (1.1-2.5%) (Popova et al., 2023).

FASDs incur high economic burdens, globally. The applicable costs of supporting an individual with an FASD include medical care like hospital admissions, health professional services, and medication; educational services like home schooling, special schooling and

residential programs; social services like respite care, foster care and legal aid; productivity losses, and other out-of-pocket costs (Popova et al., 2011, 2015, 2016). The lifetime cost of supporting an individual with an FASD in North America is estimated to cost over 1 000 000 CAD dollars (average age at death: 71.6 years of age) (Popova et al., 2011).

1.1.3 Pathology

The damage associated with alcohol to the developing fetus is profuse and persistent. Ethyl alcohol crosses the placental barrier at the time of use and creates systemic (i.e. organ damage and epigenetic changes) and neurodevelopmental (i.e. brain structure anomalies, neuron death and neurochemical imbalances) alterations. The damage induced can be attributed to the amount, period(s), and frequency of alcohol consumed.

Alcohol is rapidly absorbed from the stomach into the bloodstream, occurring within 5 to 10 minutes following consumption (Cederbaum, 2012). Once in the bloodstream, ethyl alcohol circulates throughout the body before reaching the liver, where it is primarily metabolized through an oxidative pathway by an enzyme called alcohol dehydrogenase (ADH) to form a compound called acetaldehyde. This compound is highly toxic and reactive and causes damage at the cellular and genomic level. As such, it is rapidly metabolized by acetaldehyde dehydrogenase (ALDH) to form acetic acid, a less toxic compound. Acetic acid is converted to carbon dioxide and water and are easily dispensed from the body. Under circumstances of high alcohol use or chronic consumption, another system known as the microsomal ethanol oxidizing system (MEOS) becomes activated to enable the use of the enzyme CYP2E1 to also metabolize alcohol. However, metabolism using the MEOS pathway

produces reactive oxygen species (ROS) as by-products of the reaction, that will ultimately lead to oxidative stress and tissue damage (Cederbaum, 2012; Koop, 2006). It is important to note that metabolism rates and the abundance of enzymes involved vary across individuals based on genetic factors, epigenetic changes, nutrition, and other factors (Cederbaum, 2012). ADH and MEOS pathways contribute to the majority of ethanol metabolism (around 90%) and most of ethanol elimination, but ethanol can also be removed from the body without being metabolized first. Excretion through breath, urine, saliva, tears, and sweat can eliminate ethanol from the body, but this makes less than 10% of alcohol elimination (Cederbaum, 2012). Ethanol is a psychoactive compound, as it can cross the blood brain barrier (BBB) and enter the brain. Once there, ethanol causes a general depression of the CNS by impacting neurotransmitter systems in the prefrontal cortex, cerebellum, hippocampus, amygdala, and brainstem (Koop, 2006). The enzymes in the ADH and MEOS pathways are present in certain structures of the brain as well, and ethanol and its metabolites will eventually be eliminated.

Measurement of the amount of alcohol present in a person's bloodstream can be defined as their blood alcohol concentration (BAC). Absorption rates vary based on several factors like body weight and composition, food intake, rate of consumption and type of alcohol use, but the time taken to reach peak BACs occur at around 30 minutes to 2 hours following alcohol use (Mitchell et al., 2014). BACs are typically used to assess a person's level of intoxication and depend on factors like body weight and food intake. Estimates in women with lean body weights of 120 – 180 pounds, suggest that consumption of 1 standard drink (a 12-ounce bottle of beer at 5% alcohol OR a 5-ounce glass of wine at 12% alcohol OR a 1.5-ounce shot of hard liquor at 40% alcohol OR any drink containing 13.5 g of pure alcohol) will induce a

BAC of around 0.03% – 0.04% (Hope Gillette, 2023). This is considered a low BAC and can be characterized by mild impairment (Kristina Ackermann, 2024). A moderate BAC of around 0.04% – 0.08% BAC can be achieved through 1.5 – 3.5 standard drinks (Hope Gillette, 2023) and is characterized by lower alertness and impaired judgement (Kristina Ackermann, 2024). High BACs of around 0.08% – 0.2% are the result of 2 – 8 standard drinks (Hope Gillette, 2023) and ranges from reduced muscle coordination and slurred speech to loss of balance and muscle control (Kristina Ackermann, 2024). The consumption of 5-10 standard drinks creates very high BAC levels, exceeding 0.2% (up to 0.3%) (Hope Gillette, 2023) and is characterized by confusion, drowsiness, and vomiting. Anything above a BAC of 0.3% will likely result in alcohol poisoning (Kristina Ackermann, 2024).

The amount of maternal alcohol use during pregnancy plays a massive role in determining the severity of symptoms present in the offspring. Fetal blood alcohol concentrations take only 1 to 2 hours to reach levels nearly equivalent to maternal levels following consumption (Burd et al., 2012) and enzymes in the ADH and MEOS pathways are also found in the placenta and fetus, but at much lower quantities. Consequently, when ethanol crosses the placental barrier, its abnormal presence persists in the fetus, with low quantities of enzymes responsible for its metabolism, disrupting development. At this point, ethanol accumulates in the amniotic fluid before crossing the placental barrier again for maternal metabolism and elimination (Burd et al., 2012). Higher doses of alcohol are associated with more severe effects on the developing fetus and produces more profound outcomes (Burd et al., 2012).

The period of exposure plays a massive role in determining the type of symptoms present in the offspring. The stages of pregnancy can be measured through fetal weeks, starting at conception, and continuing until birth, and are denoted as three trimesters (Figure 1). During differing stages of pregnancy, certain structures are particularly susceptible to insult. The first trimester of human pregnancy occurs from conception to 12 weeks and marks a critical period for the development of the heart, arms, legs, eyes, ears, teeth, palette, lungs, stomach, external genitalia, and the central nervous system (NIH, 2023). Damage during the first trimester of development is associated with facial dysmorphologies, heart defects, abnormal development of the corpus collosum (a region of the brain responsible for communication between the two hemispheres) and deficits to the cerebellum (a region of the brain involved in motor control and balance) (C. Coles, 1994). The second trimester, 13 to 27 weeks, induces growth of developed structures and organs as well as continuation of CNS development (NIH, 2023). During this trimester, the brain undergoes a growth spurt, and neurons begin migrating to their appropriate locations in the brain. Damage during this period of pregnancy is associated with growth defects and neurological deficits in neuron placement and network formation. During the third trimester of development, 28-40 weeks, structures in the CNS, organs and systems continue to develop (NIH, 2023). Damage during this period of pregnancy is also associated with growth defects and neurological deficits, as well as the risk of pregnancy complications like preterm delivery (OTIS, 2023). Of note, the hippocampus, a structure responsible for learning and memory, undergoes a growth spurt, during which one of its regions, the dentate gyrus, undergoes extensive structural maturation. This is a crucial period of development that involves synaptic development, dendritic arborization, and the formation of new neurons, increasing the

density of neural networks in the hippocampus (Hur et al., 2022). Not only has teratogenic exposure during this period has been linked to disruptions in emotional and cognitive processing (Thomas et al., 2007), but also learning and memory deficits (Guerri et al., 2009) and neurobehavioral dysfunction (Roediger et al., 2021).

The frequency of maternal alcohol use during pregnancy plays a massive role in determining the severity and type of symptoms in the offspring. Continuous consumption, or consistent exposure of alcohol to the fetus will cause cumulative damage over time. As such, the frequency of alcohol use during pregnancy will affect the severity of damage to the offspring, with a high frequency of alcohol use increasing the severity. In addition, fetal development occurs across the entire period of pregnancy, with highly conserved stages of development, and continuous exposure across varying phases of development will increase the amount and types of damage that persist in the offspring. Chronic alcohol exposure will increase the severity of outcomes presented, even at lower doses, although the impact may be more subtle (Burd et al., 2012).

Overall, the pathology of FASDs is difficult to characterize precisely, due to the numerous factors that can affect its onset. The severity and type of damage will be dependent on the dose, period(s) and frequency of alcohol use, as well as individual susceptibility (Hope Gillette, 2023). It is important to remember that there is no safe amount and no safe time to consume alcohol during pregnancy (CDC, 2024c).

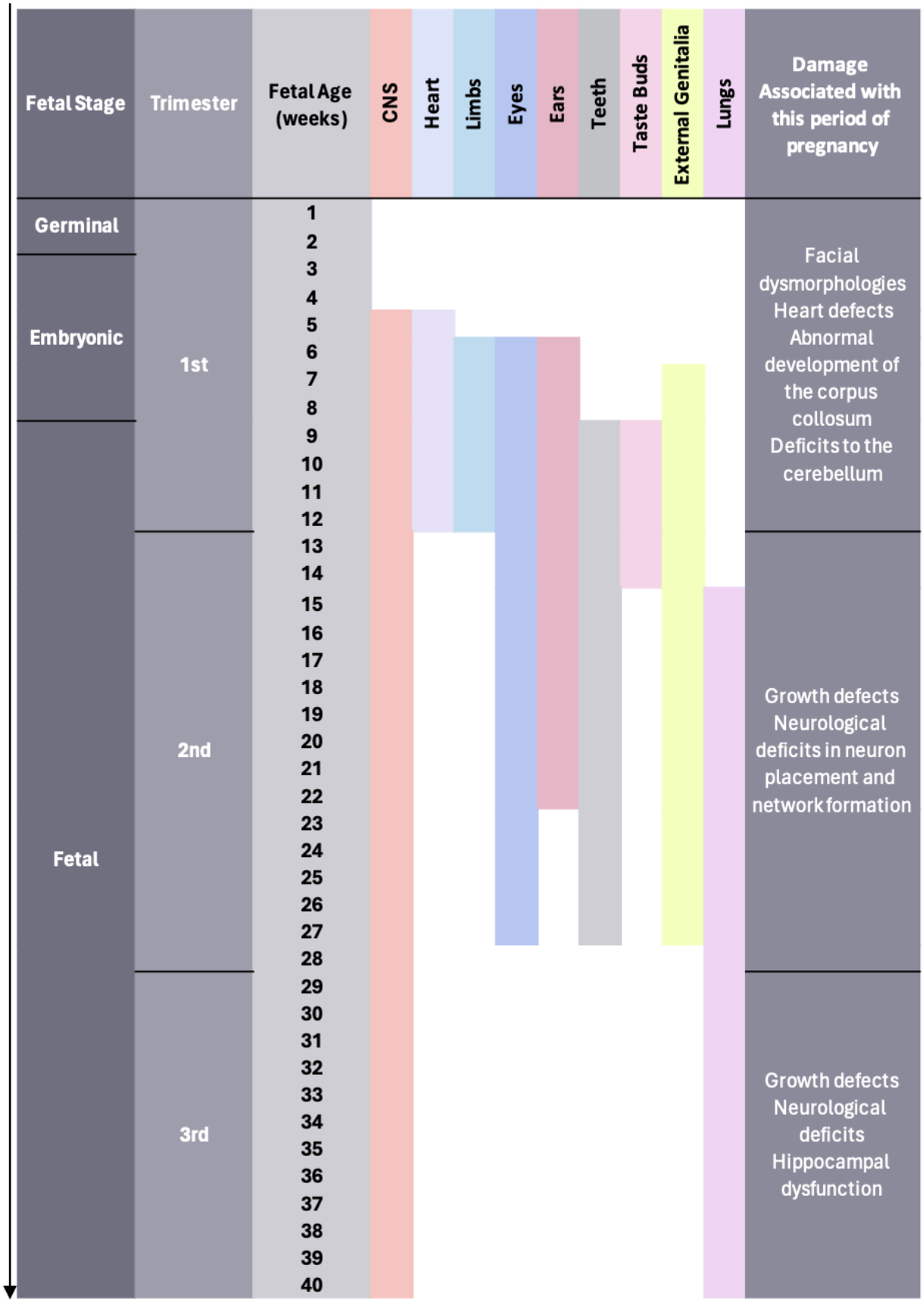
Fetal Stage
Trimester
Fetal Age (weeks)
CNS
Heart
Limbs
Eyes
Ears
Teeth
Taste Buds
External Genitalia
Lungs
Damage Associated with this period of pregnancy
Germinal
Embryonic
Fetal
1st
2nd
3rd
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Facial dysmorphologies
Heart defects
Abnormal development of the corpus collosum
Deficits to the cerebellum
Growth defects
Neurological deficits in neuron placement and network formation
Growth defects
Neurological deficits
Hippocampal dysfunction

Figure 1: Stages of fetal development across trimesters of human pregnancy. Highlighting the developmental periods of multiple organs and systems, indicating periods by which they are vulnerable to teratogenic insult. Damage associated with each trimester of pregnancy is listed on the right. Adapted from(Bleyl & Schoenwolf, 2010) and (OTIS, 2023).

1.1.4 Primary and Secondary Outcomes

The variability of maternal alcohol consumption across the entire period of pregnancy can lead to differences in the presence, onset, and severity of symptoms among patients. However, individuals with FASDs will exhibit symptoms from the following collection of traits: (1) CNS deficits like hyperactivity, learning and memory impairments and reduced executive functioning capacity (2) physical growth abnormalities like delayed growth and small head size and (3) abnormal facial features, such as a smooth philtrum, thin upper lip, and small palpebral fissures (CDC, 2024e). These congenital anomalies can lead to secondary conditions that affect early life development including mental health issues, behavioural problems, academic difficulties, and substance use (CDC, 2024d). Their symptoms will also persist into and throughout adulthood (NIAAA, 2023) where they are at a greater risk for encountering issues with the legal system, difficulties holding job(s), unemployment, substance use and homelessness (CDC, 2024d). It is important to note that the risk for these outcomes is lessened if individuals are treated with appropriate interventions (CDC, 2024d).

1.2 Interventions for Fetal Alcohol Spectrum Disorders

1.2.1 Protective Factors

Individuals with FASD or FAS-related symptoms are ideally diagnosed at around 5-6 years old, since as these individuals age, any untreated symptoms tend to worsen, making an early diagnosis and immediate intervention highly beneficial. Several other protective factors can reduce FASD symptomology, such as the presence of a nurturing and stable home environment and participation in specialized service and education programs (CDC, 2024a).

1.2.2 Therapeutic Interventions

There is no known cure for FASDs, but studies have suggested that treatment and intervention strategies can help improve cognitive dysfunction to reduce the likelihood of secondary outcomes of affected individuals (Andreu-Fernández et al., 2024). These usually take an integrated approach, and can involve multiple disciplines such as psychiatry, neurology, and education services. Therapeutic interventions, including Cognitive Behavioural Therapy (CBT), for individuals with FASDs and their families, are always incorporated into treatment plans to aid in the adherence of their assigned interventions and improve quality of life. In an ideal world, treatment and intervention strategies are designed to target the symptomology present in the affected individual and are tailored to their specific needs (Andreu-Fernández et al., 2024; Kalberg & Buckley, 2007; Petrenko, 2015).

1.2.3 Pharmacological Interventions

Symptomology surrounding damage to the CNS has often required a pharmacological approach (CDC, 2024a). Individuals with FASDs often present comorbid disorders such as anxiety, depression, and attention deficit hyperactivity disorder (adhd). Even if the persistence of their symptoms does not reach the severity or threshold for diagnosis of these comorbidities, pharmacological treatments of these disorders are still typically seen as the appropriate approach. For example, mood stabilizers, drugs that are used to treat difficulty with emotional regulation, a symptom that is often seen in individuals with an FASD, can provide symptom relief (Ozsarfati & Koren, 2015). Pharmacological interventions are used to target specific disorders and have been effective in doing so, but FASD patients may require multiple medications to treat a multitude of symptoms, which increases the risk of drug interactions and adverse effects (CDC, 2024b). Additionally, medications may exhibit limited effectiveness in individuals with FASD due to the presence of widespread damage, and present timely and costly efforts in finding the correct one(s) (CDC, 2024b).

1.2.4 Nutritional and Lifestyle Interventions

In recent years, professionals have moved towards novel approaches involving natural and non-invasive interventions as effective and more affordable treatment options, particularly to improve cognitive outcomes. The first intervention is choline supplementation. Choline is an essential nutrient that aids in the development of the brain and has been shown to reduce cognitive deficits (Derbyshire & Obeid, 2020). It is found in many foods including liver, egg yolks, fish, and broccoli. There is a daily dietary intake requirement of choline to achieve

adequate levels, at around 425 mg for women above the age of 19, and 450 mg – 550 mg for pregnant or lactating women (NIH, 2022). Choline supplementation is currently being assessed in clinical trials where it is administered at either the prenatal (administered during the gestational period) or postnatal (administered following birth) period (Derbyshire & Obeid, 2020). Both periods of supplementation offer benefits to the fetus/child (Ernst et al., 2022a). Prenatal choline supplementation will provide additional support to the developing fetus via acetylcholine synthesis, cell membrane composition and deoxyribonucleic acid (DNA)/histone methylation, and postnatal choline supplementation will continue this support after birth (NIH, 2022).

The second intervention is aerobic exercise, encompassing any form of cardiovascular conditioning that induces a moderate level of exertion over a sustained period. Physical activity in general is highly recommended by health professionals, with adults requiring at least 150 minutes of moderate-intense physical activity per week (CDC, 2023). This recommendation applies to pregnant women as well, with an emphasis on safety and comfort (CDC, 2023). However, aerobic exercise in particular aids in brain development and function by increasing blood flow, promoting the growth of new neurons, strengthening neural connections, and improving cognitive performance. In pregnant women, it can lead to improved placental function (Hopkins & Cutfield, 2011), healthier birth weights (Kramer & McDonald, 2009; Piercy et al., 2018), improved cardiovascular health (ACOG, 2020), and lower risk of chronic diseases (Piercy et al., 2018), all while improving maternal health outcomes (ACOG, 2020; Hopkins & Cutfield, 2011; Kramer & McDonald, 2009; Piercy et al., 2018). Following birth, children should begin mild forms of physical activity at as early as 1 year of age, through

walking, running, jumping, and climbing around their environments, to encourage healthy growth and development (Sommer et al., 2021). By the age of 6, children are recommended to partake in a minimum of 60 minutes of aerobic physical activity per day, to improve physical, cognitive, and mental health outcomes (Okely et al., 2021). These interventions provide a holistic approach to wellness and can target specific areas of cognitive function for which pharmacological interventions currently do not. Seeing as these are novel approaches, research is ongoing.

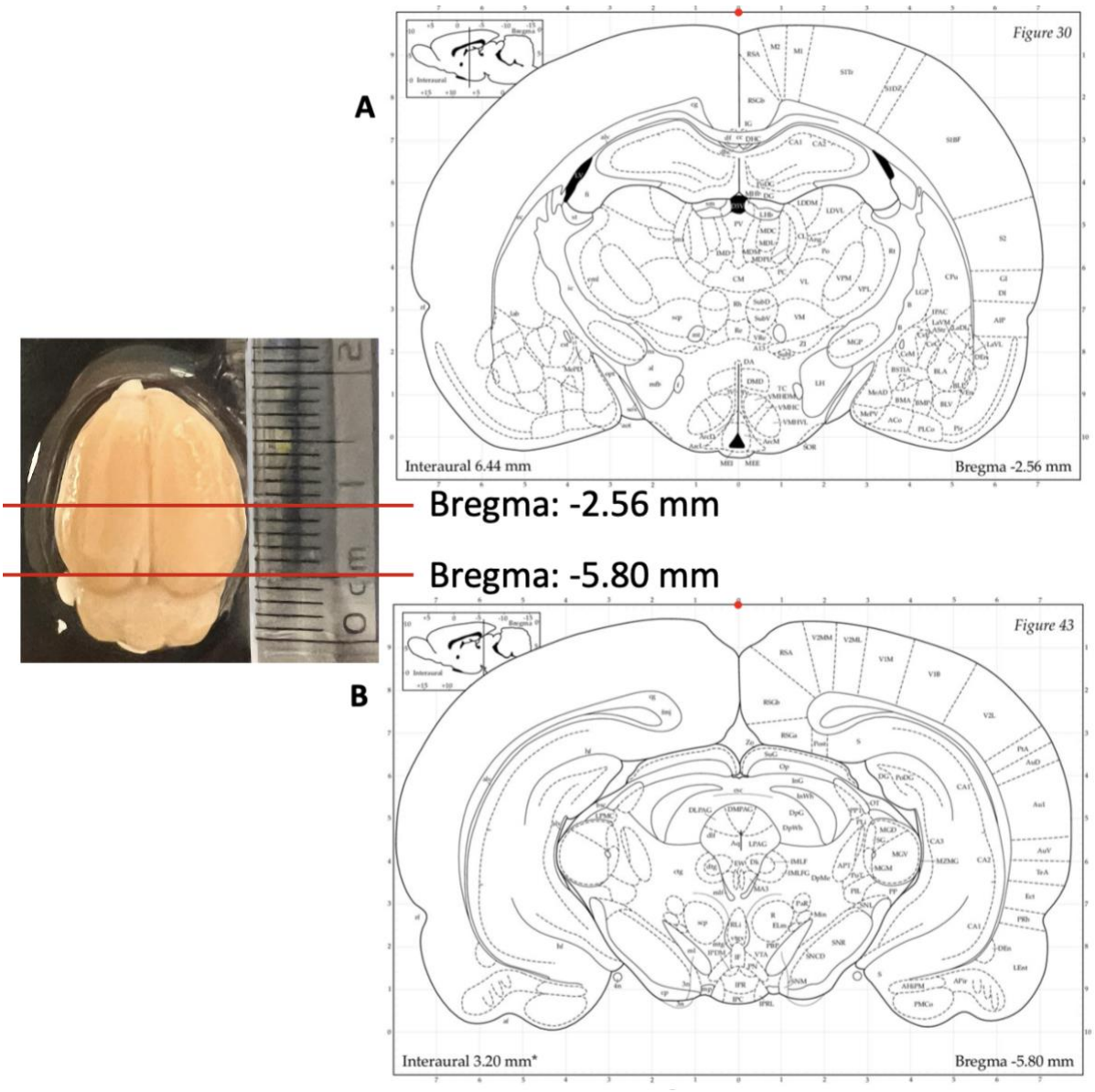

Figure 2: Dorsal view of a fixed 36 day old rodent brain. Red lines indiciate Bregma coordinates of interest moving coronally through the brain along the anterior to posterior axis with (A) the start of the hippocampus and the beginning of slice collection of intereest and (B) the end of the hippocampus and the end of slice collection of interest. *Images of Bregma positions taken from the Paxinos and Watson Rat Brain Atlas.*

1.3 The Hippocampus

1.3.1 Overview

The hippocampus is a structure in the brain located in the medial temporal lobe, just below the surface of the cortex. This structure plays a central role in learning and memory processes including the formation, organization, and retrieval of information, as well as spatial navigation and emotions (Chauhan et al., 2021). The hippocampus can be divided along the anterior-posterior axis (segmented from the front of the brain to the back of the brain), denoted dorsal to ventral hippocampus. When moving along this axis, the hippocampus exists towards the posterior end and is oriented such that the dorsal hippocampus is further anterior than the ventral hippocampus. The hippocampus is present in slices from Bregma -2.56 to -5.80 (Figure 2).

The hippocampal formation is a comprehensive structure that encompasses the Cornu Ammonis areas (CA1, CA2, CA3 and CA4), the dentate gyrus (DG) and the subiculum (Chauhan et al., 2021) (Figure 3). The CA1, situated next to the subiculum, plays crucial roles in outputting information to other parts of the brain, particularly the Entorhinal Cortex (EC). The CA2, a smaller and less studied region, sits between the CA1 and CA3, while the CA3, sits next to the CA2 and is connected to the DG. The CA4 is located deep within the hippocampal formation and is sometimes considered to be part of the DG.

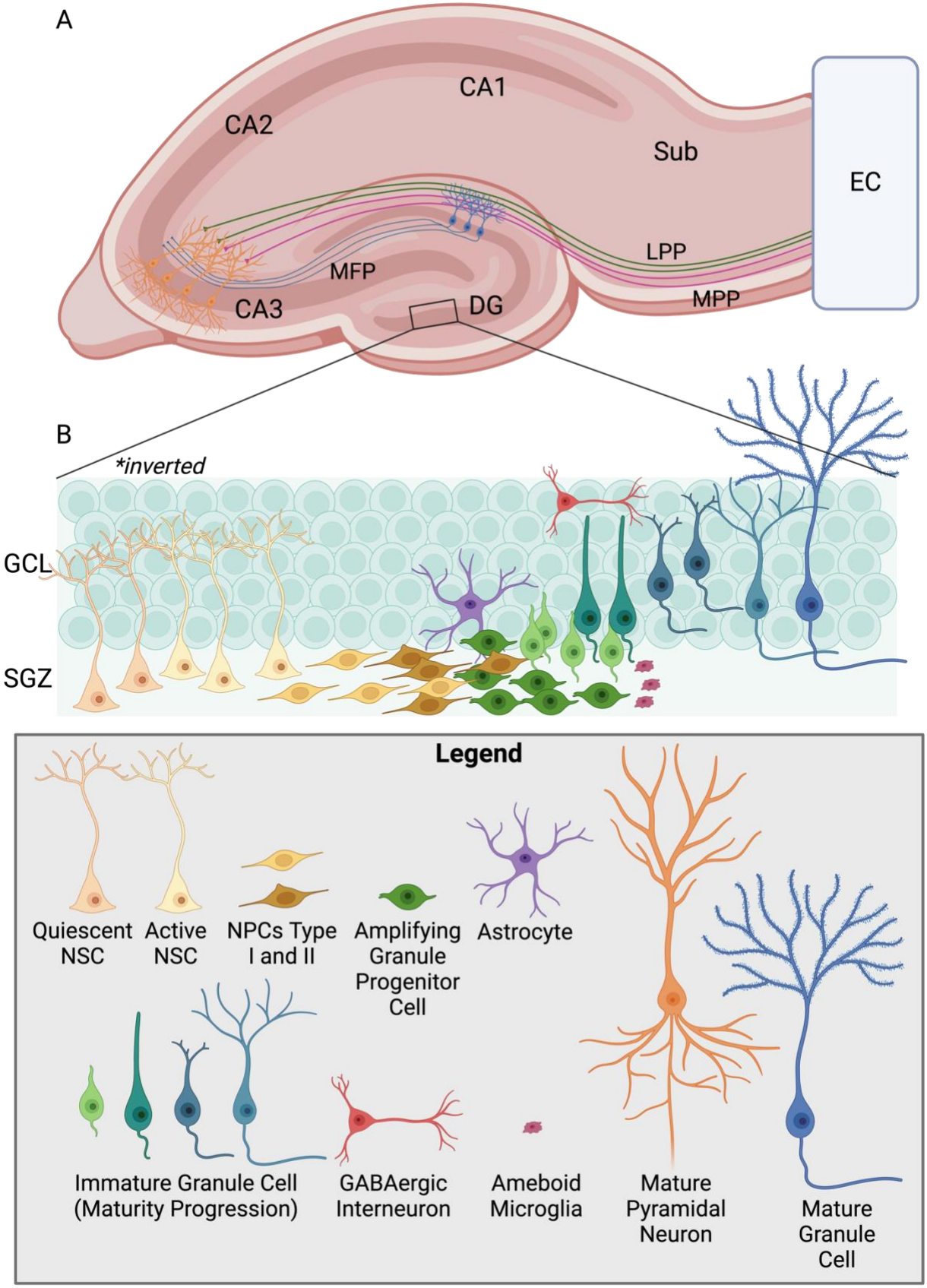
A
CA1
CA2
Sub
EC
LPP
MFP
MPP
CA3
DG
B
*inverted
GCL
SGZ
Legend
Quiescent NSC
Active NSC
NPCs Type I and II
Amplifying Granule Progenitor Cell
Astrocyte
Immature Granule Cell (Maturity Progression)
GABAergic Interneuron
Ameboid Microglia
Mature Pyramidal Neuron
Mature Granule Cell

Figure 3: Illustration of the hippocampus and circuitry in the DG. MFP is mossy fiber pathway. LPP is lateral perforant pathway. MPP is medial perforant pathway (A) Simplified illustration of neurogenesis in the GCL and SGZ of the DG (B) Legend for cell types is displayed below. *This figure was generated in Biorender.*

1.3.2 Hippocampal Neurogenesis and Cellular Proliferation

Neurogenesis is the process by which new neurons are formed and incorporated into neural circuits. Neurogenesis begins during early embryonic development and continues into adulthood (Toda et al., 2019). Like other cellular mechanisms, these processes occur in response to their extracellular environment, that provide specialized support and signalling. Neurogenesis has the capacity to take place in what is known as the "neurogenic niche" due to its containment of neural stem cells (NSCs) and neural progenitor cells (NPCs), a specialized extracellular matrix and vascular niche, extracellular signalling pathways that target neurogenic processes and its neuroinflammatory environment (Vicidomini et al., 2020). There are only a few neurogenic niches in the adult brain, one of which is the subgranular zone (SGZ) within the DG of the hippocampus. Newly formed neurons arising from the SGZ are excitatory DG granule cells that are involved in the formation of new memories and spatial navigation. The process of neurogenesis will typically occur through the following stages: cellular (1) proliferation, (2) migration, (3) differentiation, (4) integration, (5) maturation and (6) survival (Vicidomini et al., 2020).

The first stage of neurogenesis is cellular proliferation, which refers to the process by which specific cells, that have the capacity to proliferate, increase in their abundance through division via the cell cycle. The cell cycle is made up of four phases: (1) Gap 1 (G1) (2) Synthesis

(S) (3) Gap 2 (G2) and (4) Mitosis (M) (Frade & Ovejero-Benito, 2015). During the G1 phase, cells prepare for genomic replication through the synthesis of proteins that aid in deoxyribonucleic acid (DNA) replication. DNA is composed of two polynucleotide strands that consist of covalently bonded nucleotides – a deoxyribose sugar molecule and a nitrogen-containing base (adenine, guanine, thymine and cytosine). The two polynucleotide chains are bound together by hydrogen bonds that link complementary base pairs (adenine with thymine and guanine with cytosine). During the S phase, DNA replication occurs. First, double stranded DNA (dsDNA) is unwound into two strands of single stranded DNA (ssDNA) that serve as templates for the synthesis of new DNA. Short fragments of ribonucleic acid (RNA) called primers bind to each ssDNA template before the enzyme DNA polymerase adds the remaining complementary base pairs. At this point, another enzyme, exonuclease, strips away the primers and complementary base pairs are substituted by DNA polymerase. Finally, DNA ligase seals two identical copies of dsDNA, and S phase is complete. During the G2 phase, the proteins that are responsible for cellular division are synthesized. Lastly, during mitosis cellular division occurs, where the two copies of dsDNA are separated into new nuclei, producing two identical copies of the original cell (Frade & Ovejero-Benito, 2015).

Cell types that are capable of cellular proliferation in the neurogenic niche of the hippocampus are NSCs and NPCs (Overall et al., 2016). NSCs can exist in a quiescent or inactive state but can also be activated for either self-renewal or asymmetric division to form an identical NSC and a progenitor cell with a more specified state. NSC's have the capacity to differentiate into neurons, astrocytes, and oligodendrocytes. NPCs are highly proliferative progenitor cells that are derived from NSCs and gradually differentiate into immature neurons.

Once generated, they migrate to their appropriate position in the granule cell layer (GCL), where they might continue to proliferate and mature before full integration into neural circuits. Of note, many of these generated immature neurons will not survive and will undergo cell death (Overall et al., 2016).

Neurogenesis plays a crucial role in the maintenance and development of DG circuits. The DG is one of the only regions of the brain that contains a neurogenic niche, and its implications are largely essential to the functionality of the DG in its role of learning and memory formation.

1.3.3 The Dentate Gyrus (DG)

The DG is a critical part of the hippocampal formation (Amaral et al., 2018; Amaral & Lavenex, 2007; Aniol et al., 2022). The cells in the DG originate from proliferating neuroepithelial cells in the medial pallium at around embryonic day (ED) 13 to 14. The newly formed and differentiated granule cell progenitors (from the proliferating neuroepithelial cells) migrate along radial glial cells, from the ventricular zone to the dentate neuroepithelium. This region will eventually become the DG. During the early second trimester, the granule cell progenitors continue to proliferate and migrate until they form the distinct densely packed layer, known as the granule cell layer. Gradually, granule cells begin to mature and form synaptic connections with other neurons. By the late second trimester, granule cells have begun to extend their dendrites and axons to form connections with neurons in other hippocampal regions. Synaptic refinement of neural circuits, completion of the formation of DG layers and development of granule cells occurs during the third trimester of development. This

period is largely important as it lays the foundation for the function of the DG following birth and into adulthood. During this period, the DG is extremely susceptible to teratogenic insult, and disruptions in development could lead to aberrant neurogenesis and neural circuit function (Aniol et al., 2022). Neurogenesis continues after birth and as previously described, is essential for learning and memory functions in the DG.

The DG serves as the primary input region to the hippocampus. Information is processed in the DG before being passed along to the CA3 via the mossy fiber pathway (MPP) (Figure 3) (Amaral et al., 2018). The DG is composed of three primary layers: (1) the molecular layer, containing dendrites of granule cells and axons of other hippocampal neurons (2) the granule cell layer, containing densely packed granule cells and (3) the polymorphic layer (hilus), containing interneurons and axons of granule cells, also known as mossy fibers (Amaral & Lavenex, 2007). The adolescent DG is comprised of key cell types that contribute to learning and memory processes, like (1) granule cells, excitatory neurons that receive input from the EC and project to the CA3 via the mossy fiber pathway (2) neural stem cells and neural progenitor cells, proliferating cells in the SGZ (3) GABAergic interneurons, inhibitory neurons that regulate the excitatory activity of granule cells (4) mossy cells, excitatory neurons that project back to the DG (5) astrocytes, glial cells that provide structural support and maintain homeostasis (6) microglia, the resident immune cells of the brain that are involved in synaptic pruning, neuroprotection and inflammatory responses (Amaral & Lavenex, 2007). Two major pathways in the DG are the medial and lateral perforant pathway (MPP and LPP), that projects from the EC to the DG and the MPP, that projects from the DG to the CA3. In the dorsal hippocampus, the MPP and LPP connect from the medial EC and transmit detailed spatial and sensory

information to the DG, whereas in the ventral hippocampus, they connect from the lateral EC and transmit emotional and contextual information to the DG. In contrast, the mossy fiber pathway remains structurally consistent along the dorsal-ventral hippocampal axis, but the dorsal pathway is involved in episodic memory encoding, while the ventral pathway is implicated more in emotional memory (Amaral & Lavenex, 2007). Disruptions to neurogenesis in DG could disrupt these processes.

1.4 Preclinical Research and FASDs

1.4.1 Animal Models of FASDs

Animal models can be used to study FASDs through the provision of ethanol during fetal development to replicate the effects that are seen in clinical populations and perform experimental testing. Various animals have been used to model FASDs, including drosophila, zebrafish, rodents, and non-human primates (Patten et al., 2014a). Rodent models are the most seen in preclinical research, likely due to their display of maternal-infant characteristics that closely resemble humans as well as shorter generation periods (Wilson & Cudd, 2011). The utilization of an animal model allows for precise experimental design that involves three main factors: period and length of exposure, dose of ethanol and exposure mechanism.

The stages of human pregnancy can be separated into three trimesters, each of which can be defined by their containment of unique developmental markers and stages. Like humans, rats have well-defined periods of development including early gestation, mid-gestation, late gestation, and the postnatal period. By comparing the neurodevelopmental stages in both species, researchers can study the brain by creating animal models that mimic

exposure and treatment during those periods (Figure 4). The first trimester of human pregnancy, conception to 12 weeks, is equivalent to early and mid-gestation in rats, fertilization to ED 10. The second trimester, 13 to 27 weeks, is equivalent to late gestation to birth in rats, ED10 to ED22 (birth). The third trimester of development, 27-40 weeks, is equivalent to the initial postnatal period, postnatal day (PND) 0 to 10 in rats (Zeiss, 2021).

Typically, blood alcohol concentrations are dosed in varying amounts depending on the nature of the study. As an example, binge levels of ethanol exposure require high doses of around 5-6g/kg, producing BAC's of around 200-300 mg/dL, or 0.2-0.3%. These levels of intoxication are potentially dangerous and are estimated to be equivalent to approximately 5 to 9 standard drinks.

Several methods for delivery of ethanol to rat dams or pups can be used for the study of PAE. These include 1) artificial rearing 2) lactation exposure 3) vapour inhalation exposure and 4) intragastric intubation. For artificial rearing, pups are separated from their mothers and housed in cups floating in a temperature-controlled tank (Nagy, 2002). They are automatically reared on a controlled diet through feeding tubes; this method is designed to mimic the conditions within the womb and has been used in multiple studies (Girard et al., 2003; West et al., 1984). Lactation exposure involves having the mother consume a controlled diet, and having the pups receive their diets from lactation exposure. This method leaves pups unbothered, but limits precision with dosing. While many studies that use this method do so with the intention of assessing the effects of breastfeeding, they are often done so during the early postnatal period (the third trimester equivalent) (Perez et al., 2023). Vapour inhalation is not as commonly used, as it does not mimic the route of intake in human beings. However, it elicits a

quick increase in BAC without the handling and stress involved in other procedures (Pal & Alkana, 1997; Patten et al., 2014b). Here, intragastric intubation was used; this involves the oral insertion of a feeding tube, for the delivery of liquid diet directly into the stomach of the pup. This method is beneficial because it allows for the precise, controlled, and consistent delivery of substances, by bypassing gastric digestion and first-pass metabolism.

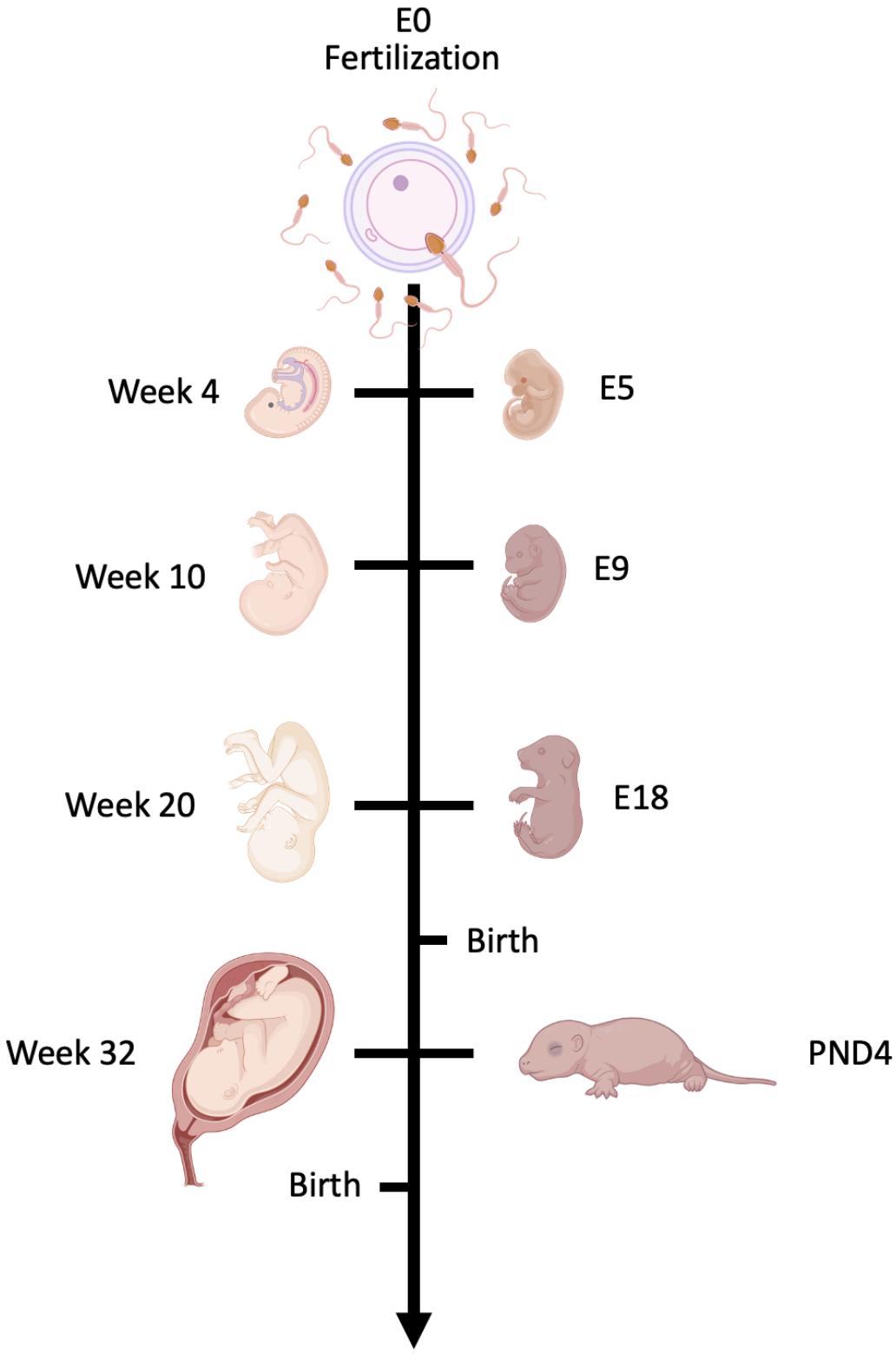

Figure 4: Comparison of human and rat embryonic/fetal development based on neurodevelopmental similarities. Human age in weeks is based on the fetal age. *Figure generated using Biorender.*

1.5 Hippocampal-Dependent Symptomology

1.5.1 Learning and Memory Deficits

Children with an FASD often display a variety of learning and memory-related deficits because of widespread neurodevelopmental damage to structures like the prefrontal cortex, corpus callosum, cerebellum, thalamus, temporal lobes, and hippocampus (Mattson et al., 2019). In general, this damage has been shown to manifest as slower learning, impaired recall, and impaired discrimination (Mattson et al., 2019), but at a more specific level, prenatal alcohol exposure (PAE) impairs many types of learning and memory including episodic memory (Du Plooy et al., 2016), spatial memory (Green et al., 2009; Kaemingk & Halverson, 2000; Uecker & Nadel, 1998), contextual memory (Fuglestad et al., 2022), the encoding and consolidation of new information (C. D. Coles et al., 2011), working memory (C. D. Coles et al., 2010; Rasmussen, 2005; Wheeler et al., 2012)and context-dependent emotional memory (Rasmussen, 2005). These types of learning and memory deficits are all hippocampal-dependent, the focus of this work. Previous work has observed the effects of interventions on learning and memory deficits relating to the hippocampus. This will be discussed in the upcoming sections.

1.5.2 Choline Supplementation

Choline supplementation has been examined prenatally and postnatally in clinical studies, assessing its effects in children with a history of prenatal alcohol exposure (Ernst et al., 2022b). Clinical studies assessing choline supplementation during the prenatal period have reported promising results in their effects on hippocampal-dependent symptomology. (Jacobson et al., 2018) reported that children who received prenatal choline supplementation

showed better recognition memory at 12 months of age. Additionally, (Warton et al., 2021) reported that children who received prenatal choline supplementation showed better recognition memory than the placebo group and that this finding correlated with significantly larger volumes of certain brain structures, including the left and right hippocampus. Clinical studies assessing choline supplementation during the postnatal period, have reported more contrasting findings in that choline supplementation improves working and spatial memory. (Wozniak et al., 2020) reported that younger children (aged 2-3 years) who received choline showed improved sequential memory and in a 4-year follow-up study, reported that children, now 6.5-9 years old, showed improvements in visual spatial skills, working memory and verbal memory. In contrast, (Nguyen et al., 2016) reported there were no effects of choline reported on memory. Altogether, these clinical findings suggest that choline supplementation is a hopeful treatment for memory-related deficits in patients with FASD.

Similarly, several preclinical studies have been conducted assessing the effects of choline supplementation before, during and after ethanol exposure. Postnatal choline supplementation has been shown to attenuate ethanol-induced deficits on working memory and learning, in prenatal models of ethanol exposure. Thomas and colleagues performed a study examining postnatal choline supplementation from PD2-21 in rats following moderate 1st and 2nd trimester prenatal ethanol exposure, and its effects on visuospatial discrimination abilities in rats. All choline-treated animals displayed improved task memory, with the largest improvements seen in ethanol-exposed animals that received choline treatment (Thomas et al., 2000). Additionally, Waddell & Mooney examined postnatal choline supplementation from PD16-30 in the offspring of rats that were exposed to ethanol during a portion of the second

trimester equivalent period of development (ED16-20). Rats were subject to a T-maze working memory task in young adulthood, following training in adolescence. Rats that received choline treatment showed improved working memory on both control animals, as well as ethanol-exposed animals (Waddell & Mooney, 2017).

Postnatal choline supplementation has also been shown to attenuate ethanol-induced deficits in spatial learning, spatial working memory, and spatial navigation, following ethanol exposure during the third trimester equivalent period of pregnancy in rats (PND4-9). Ryan and colleagues conducted a preclinical study assessing the effects of postnatal choline supplementation on rats following ethanol insult during the third trimester equivalent of pregnancy. Rats were administered choline chloride on PND11-20, PND21-30 or PND11-30, before being subject to the spatial learning task Morris Water Maze on PND45. Ethanol exposure produced significant impairments in behaviour during the probe trial, assessing memory retention of visual cues and spatial navigation, and these deficits were mitigated by choline treatment, regardless of the period of administration (Ryan et al., 2008). Similarly, in 2016, Schneider and Thomas conducted another postnatal choline supplementation study on ethanol-exposed rats during the third trimester of development. Choline was administered from PND40-60, and rats were subject to a series of behavioural tasks assessing activity levels, spatial learning, and spatial working memory. Ethanol-induced deficits were seen in all behavioural tasks, and choline supplementation attenuated the negative effects in spatial learning and spatial working memory but not in activity levels (Schneider & Thomas, 2016). As such, there is evidence to support that choline supplementation can attenuate the hippocampal-dependent behavioural deficits seen following PAE.

1.5.3 Aerobic Exercise

Using physical activity as an intervention can reduce the executive function deficits seen in individuals with neurodevelopmental disability (Sung et al., 2022). For example, two pilot studies conducted by (Anderson-Hanley et al., 2011), reported significant improvements in working memory in children with autism following aerobic exercise intervention. Additionally, previous work has shown that aerobic exercise can attenuate some of the behavioural and neuropsychological deficits seen in children with a history of PAE. (Keiver et al., 2016) published a study in which the effects of a physical intervention denoted FAST club was provided as an after-school activity to children with histories of PAE. The purpose of FAST club was to provide physical activity to children, with the goal of improving executive functioning. Improvements in executive functioning, seen by the Children's Colour Trail Test, measuring attention, divided attention, and speed of mental processing, were seen immediately following intervention, and sustained for 3 months.

PAE has been reported to induce deficits in reference and working memory but have been shown to be attenuated by exercise. (Christie et al., 2005) conducted a preclinical study in rats, in which ethanol was maternally exposed during the first two trimesters of pregnancy. Offspring were housed individually and given access to a running wheel at PD54. At PD60, rats were tested using a spatial learning and spatial working memory task. Deficits were seen in ethanol-exposed offspring, but these impairments were mitigated if given access to a running wheel. (Redila et al., 2006) conducted a similar study in which ethanol was administered during the first two trimesters of development, and adult offspring were individually housed and given access to a running wheel. Animals were sacrificed at either PND58 or PND86 and neurogenic

cell populations were assessed. It was reported that populations of proliferating cells in the neurogenic niche of the hippocampus were reduced in ethanol-exposed offspring, but that these reductions were rescued in offspring that were given access to a running wheel. Additionally, ethanol-exposed offspring showed reductions in the abundance of surviving cells, but these impairments were also rescued when given access to a running wheel.

1.6 Project Aims

The purpose of this work is to elucidate the mechanisms underlying the positive effects seen from postnatal choline supplementation and aerobic exercise on animals exposed to ethanol during the postnatal period of rodent development. More specifically, the goal is to determine if adolescent neurogenesis is impacted by postnatal ethanol exposure, and whether this impact is attenuated by choline supplementation and aerobic activity. This was done using an animal model of binge postnatal ethanol exposure, postnatal choline supplementation and chronic moderately-paced forced running. At PD36, animals were sacrificed and various cell populations in the dentate gyrus of the hippocampus were examined using immunohistochemistry. There are four main aims. First, are the densities of proliferating cells in the dorsal and ventral subgranular zone and granule cell layer decreased by postnatal ethanol exposure? Are they increased by either postnatal choline supplementation and/or forced running? Next, are the densities of proliferating cells in the dorsal and ventral subgranular zone and granule cell layer impacted by a combination of postnatal ethanol exposure, postnatal choline supplementation or forced running? Do all three treatments have a differential effect?

Chapter 2: Materials & Methods

2.1 Animal Generation

2.1.1 Subjects

The procedures used to generate animals for this work were approved by the San Diego State University (SDSU) Institutional Animal Care and Use Committee (IACUC) and are in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* (Animal Use Protocol #17-04-004T).

A total of 118 Sprague Dawley rats (59 male and 59 female) were derived using procedures that have been described previously (Thomas et al., 2007). Male and female breeders were paired, and after pregnancy was confirmed, pregnant females were given ad libitum access to regular rat chow (LabDiet 5001; Richmond, IN) and water. On the morning after birth, litters were culled to 10 pups (5 males and 5 females, if possible). No more than 1 pup per sex per litter were assigned to any treatment group, to help control for litter effects.

2.1.2 Animal Model & Treatment Groups

For a schematic presentation of the treatment paradigm used, see Figure 5. Pups from each litter were randomly assigned, in series, to either an experimental condition or control group. Treatments included ethanol administration, choline treatment and exposure to forced running. The precise parameters are listed in Table 1.

(1) Ethanol administration. From PND4-9, pups received either vehicle (**sham**) or ethanol (2.625g/kg) in vehicle **(ethanol)**, via intragastric intubation (Kelly & Lawrence, 2008a). This procedure involved the oral insertion of a feeding tube, for the delivery of liquid diet directly

into the stomach of the pup. The vehicle used was a nutritionally balanced milk diet, and was administered twice daily, 2 hours apart.

(2) Choline Treatment. From PND10-30, sham and ethanol pups received either vehicle (**saline**) or choline (100mg/kg) in vehicle (**choline**), via subcutaneous injections. The vehicle used was saline solution.

(3) Forced Running. From PND26-35, sham-saline, sham-choline, ethanol-saline and ethanol-choline animals were exposed to either a locked running wheel (**no forced running**) or an automated running wheel (**forced running**). Animals were acclimatized to the apparatus from PND22-25.

The animal model used produced 8 treatment groups: sham-saline-no forced running (control), ethanol-saline-no forced running (ethanol), sham-choline-no forced running (choline), sham-saline-forced running (forced running), sham-choline-forced running (choline forced running), ethanol-choline-no forced running (ethanol choline), ethanol-saline-forced running (ethanol forced running), and ethanol-choline-forced running (ethanol choline forced running).

2.1.3 Blood Alcohol Concentrations

Blood alcohol concentrations were collected from each ethanol-exposed pup at PND6, 90 minutes after ethanol intubation. Blood was collected via tail clip and plasma was separated using the Analox Alcohol Analyzer (Model AM1; Analox, Instruments, Lunenberg, ME, USA). Blood was collected from sham-intubated pups as well, but not analyzed.

2.1.4 Body Weight

All pups were weighed daily from the first day of treatment (PND4) to the day of sacrifice (PND36). Body weight gained was assessed across varying timepoints, to determine variations in body growth during each treatment procedure. Weight gained was calculated as the difference between animal weights on the first and last days of interest.

2.1.5 Bromodeoxyuridine (BrdU) Injections

On PND36, bilateral intraperitoneal injections of bromodeoxyuridine (BrdU) injections were administered to all animals, 12 hours apart. Each dose was 200mg/kg, reported to be the saturating dose for BrdU, for a complete daily dose of 400mg/kg (Taupin, 2007).

2.1.6 Transcardial Perfusions

Within 24 hours of the final BrdU injection, animals were weighed, and then given a lethal dose of pentobarbital (10% pentobarbital in sterile saline, 3 mL/kg, i.p.), followed by transcardial perfusion with 1x phosphate buffered saline (PBS) to clear blood vessels, then 4% paraformaldehyde solution (PFA) to fix tissue. Once perfused, the brains were collected, weighed, and stored in 4% PFA overnight to post-fix, and then immersed in 30% sucrose solution for cryopreservation.

Table 1: Animal Treatment and Brain Collection Parameters

AGE (POSTNATAL DAY, PD)	TREATMENT/ MILESTONE	DOSAGE/ CONCENTRATION IN SOLUTION	ADMINISTRATION TECHNIQUE	CONTROLS
0	Birth	--	--	--
4 – 9	Ethanol Exposure	Dose: 5.25g/kg EtOH 11.9% v/v EtOH in milk	Gastric Intubation 2 feedings/day, 2h apart	Milk Diet (same method of administration & volume as EtOH)
10 – 30	Choline Treatment	Dose: 100mg/kg 2.15% of 70% Choline Solution in saline solution	Subcutaneous Injections Administered at 0.0067 x body weight (g)	Saline Solution (same method of administration & volume as choline)
22 – 25	Forced-Wheel Running Acclimation	--	2m/min for 30 mins (morning and evening sessions) Speed increased 2m/min each day (final speed of 8m/min)	Locked wheel for 30 mins (morning and evening sessions)
26 – 35	Forced-Wheel Running	--	10m/min for 30 mins (morning and evening sessions, 4h apart)	Locked wheel for 30 mins (morning and evening sessions, 4h apart)
35	Bromodeoxyuridine Injections	Concentration: 10mg/ml Dose: 200mg/kg per injection (400mg/kg daily)	Bilateral Intraperitoneal Injection	--
36	Euthanasia and Tissue Collection (within 24 hours of BrdU Injections)	1x Phosphate Buffered Saline Solution (to flush system) and 4% Paraformaldehyde (to fix tissue)	Intracardial Perfusions Brain, Heart & Liver Tissue Collected	--

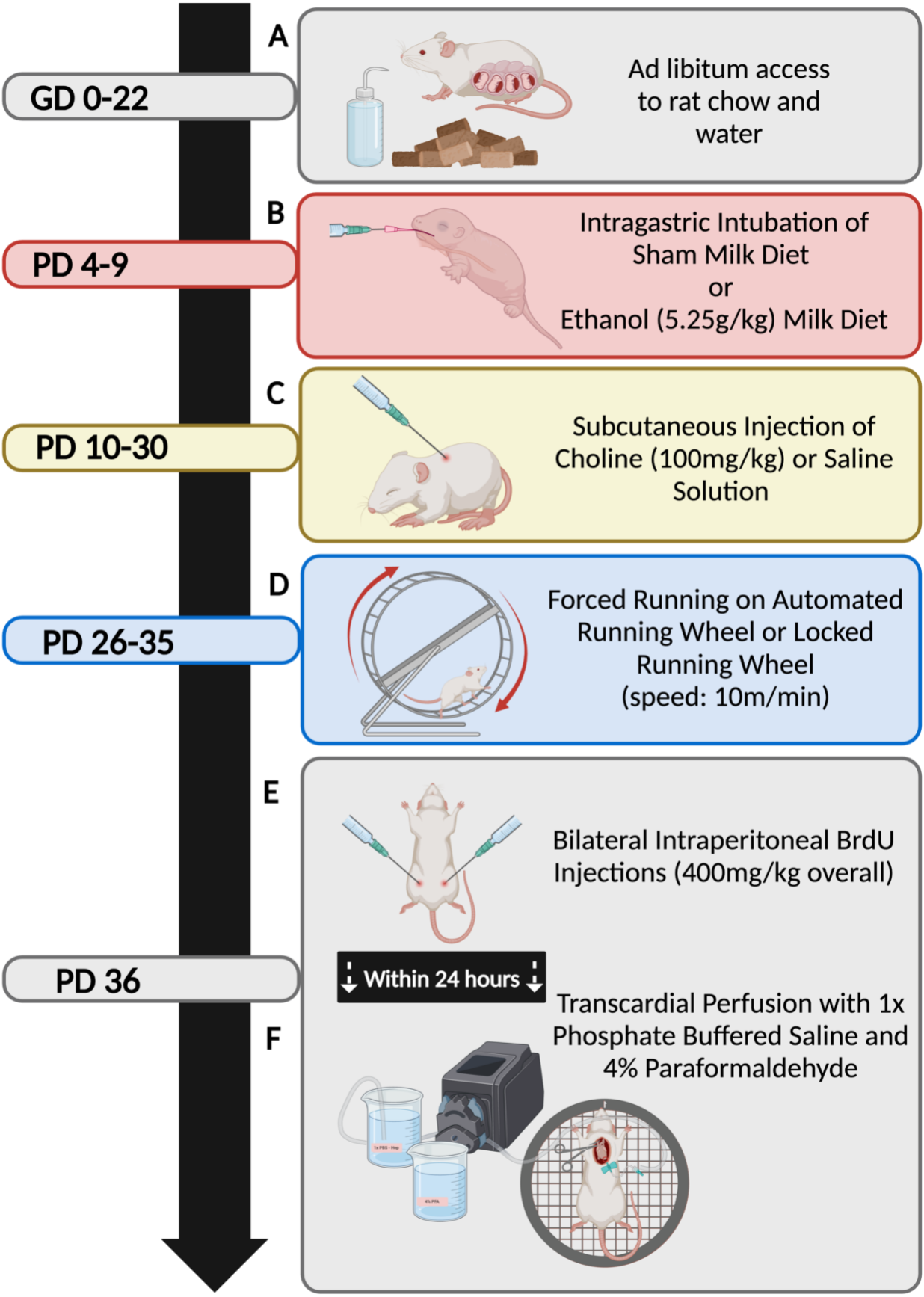
A
GD 0-22
Ad libitum access to rat chow and water
B
PD 4-9
Intragastric Intubation of Sham Milk Diet or Ethanol (5.25g/kg) Milk Diet
C
PD 10-30
Subcutaneous Injection of Choline (100mg/kg) or Saline Solution
D
PD 26-35
Forced Running on Automated Running Wheel or Locked Running Wheel (speed: 10m/min)
E
Bilateral Intraperitoneal BrdU Injections (400mg/kg overall)
PD 36
Within 24 hours
F
Transcardial Perfusion with 1x Phosphate Buffered Saline and 4% Paraformaldehyde

Figure 5: Schematic presentation of animal treatments and brain collection (A) From GD (gestational day) 0-22, pregnant dams were given ad libitum access to rat chow and water (B) From PD (postnatal day) 4-9, offspring underwent intragastric intubation of sham milk diet (SHAM) or ethanol milk diet (EtOH) (C) From PD10-30, offspring were given subcutaneous injections of saline or choline (CHOLINE) solution (D) From PD 26-35, offspring were exposed to forced running on a locked running wheel or automated running wheel (FORCED RUNNING) (E) On PD36, offspring were given 2 bilateral intraperitoneal Bromodeoxyuridine (BrdU) injections then sacrificed via intracardial perfusion within 24 hr. *This figure was generated using Biorender.*

2.2 General Tissue Processing

Brains were shipped to the University of Victoria for processing and further experimental testing. Brains were sectioned into 50μm slices using a vibratome (Leica VT1000; Germany) (Figure 6). Sections were cut with the speed set to 1mm/sec, and the amplitude of the blade vibration set to 1.2mm. These settings allowed for undamaged sections to be reliably obtained. Sections were collected in series, in PBS and 0.01% sodium azide, using a 96-well cell culture plate (Avantor VWR Tissue Culture Plate). Sections were stored at 4 °C until use for immunohistochemical staining.

Slices were selected in a 1 in 12 series, to ensure that for each series there were at least three dorsal hippocampal sections (Bregma -2.56 to -3.80) and three ventral hippocampal sections (Bregma -4.80 to -5.80) (Figure 7). Free-floating dorsal and ventral hippocampal-containing slices were removed from storing solution and given three 1x PBS washes for 10 minutes at room temperature, to remove sodium azide.

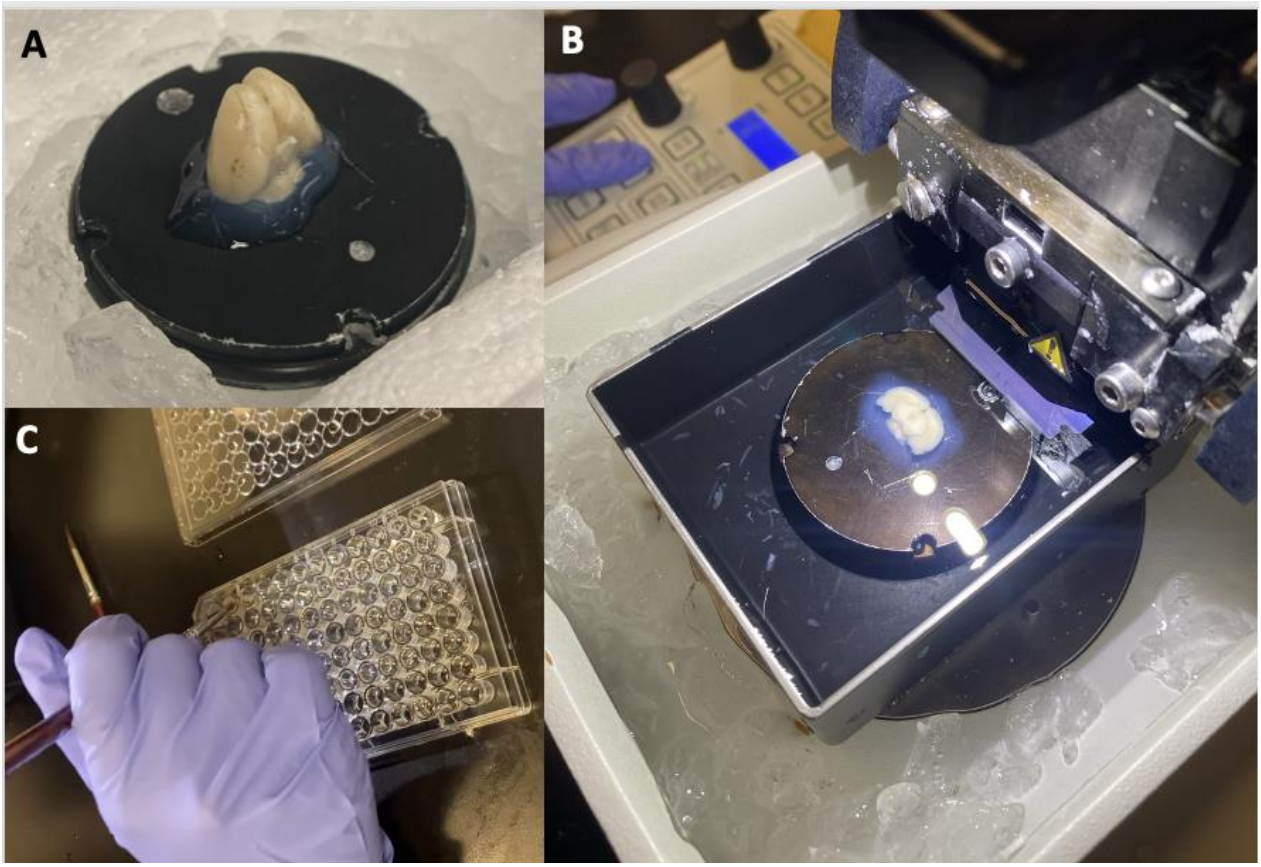

Figure 6: Slices were coronally sectioned into 50 μm slices. PD36 rat brains were sliced such that the cerebellum was removed and the brain could sit on a flat on its posterior end, and then mounted onto the vibratome stage using Krazy glue and 2% Agarose-PBS (A) Brains were sectioned using a Leica Vibratome (B) Slices were collected in a 96-well plate (C)

2.3 Imumunohistochemistry

2.3.1 Bromodeoxyuridine (BrdU)

Slices were incubated in 0.6% hydrogen peroxide for 30 minutes at room temperature, to block endogenous peroxidases, followed by incubation in a blocking solution of 3% normal goat serum – 0.5% Triton X – 1x PBS (blocking solution) for 1 hour at room temperature, to block endogenous binding sites. They were then placed into primary antibody of 1:2000 mouse anti-BrdU (Millipore Sigma MAB3424) diluted in blocking solution, for a 16-hour incubation at 4

°C. Subsequent antibody steps, diluted in blocking solution, consisted of secondary (1:500 goat anti-mouse IgG (H+L) biotinylated) for 2 hours at room temperature and avidin biotin complex (VECTPK4000 ABC-Peroxidase kit). A 3,3' Diaminobenzidine-nickel substrate (VECTSK4100 DAB HRP Substrate kit) reaction was used for chemiluminescent detection. Slices were mounted in 1x PBS and arranged in a dorsal to ventral fashion, with a maximum of 6 slices per slide (Figure 7). They were then placed in dehydration solution (50%, 70%, 100% ethanol, Citrisolv for 5 minutes at room temperature) 24 hours later. Slides were coverslipped (RI: 1.518-1.521) immediately following Citrisolv dehydration, using Permount solution.

2.3.2 Ki67

Slices were incubated with 10mM Sodium Citrate (pH 6.0) at 80 °C for 20 minutes before being cooled to room temperature for around 10 minutes. Slices were washed in 1x PBS 3 times, for 5 minutes before being incubated in a blocking solution of 5% normal goat serum-0.25% Triton-X 100-1x PBS for 2 hours at room temperature. They were then placed into primary antibody of 1:1000 rabbit anti-Ki67 (Abcam, Ab15580), diluted in blocking solution, for a 24-hour incubation at 4 °C. Next, slices were washed 3 times for 10 minutes in PBS and were then incubated for 2 hours at room temperature in secondary antibody (1:500 goat anti-mouse IgG (H+L) biotinylated), diluted in blocking solution. Following 3 more 10-minute wash steps, slices were incubated in avidin biotin complex (VECTPK4000 ABC-Peroxidase kit) diluted in PBS-Triton X. Subsequent 10-minute wash steps were done before a final incubation in 3,3' Diaminobenzidine-nickel substrate (VECTSK4100 DAB HRP Substrate kit). Slices were mounted in 1x PBS and arranged in a dorsal to ventral fashion, with a maximum of 6 slices per slide

(Figure 7). Slides were coverslipped (RI: 1.518-1.521) immediately following Citrisolv dehydration, using Permount solution.

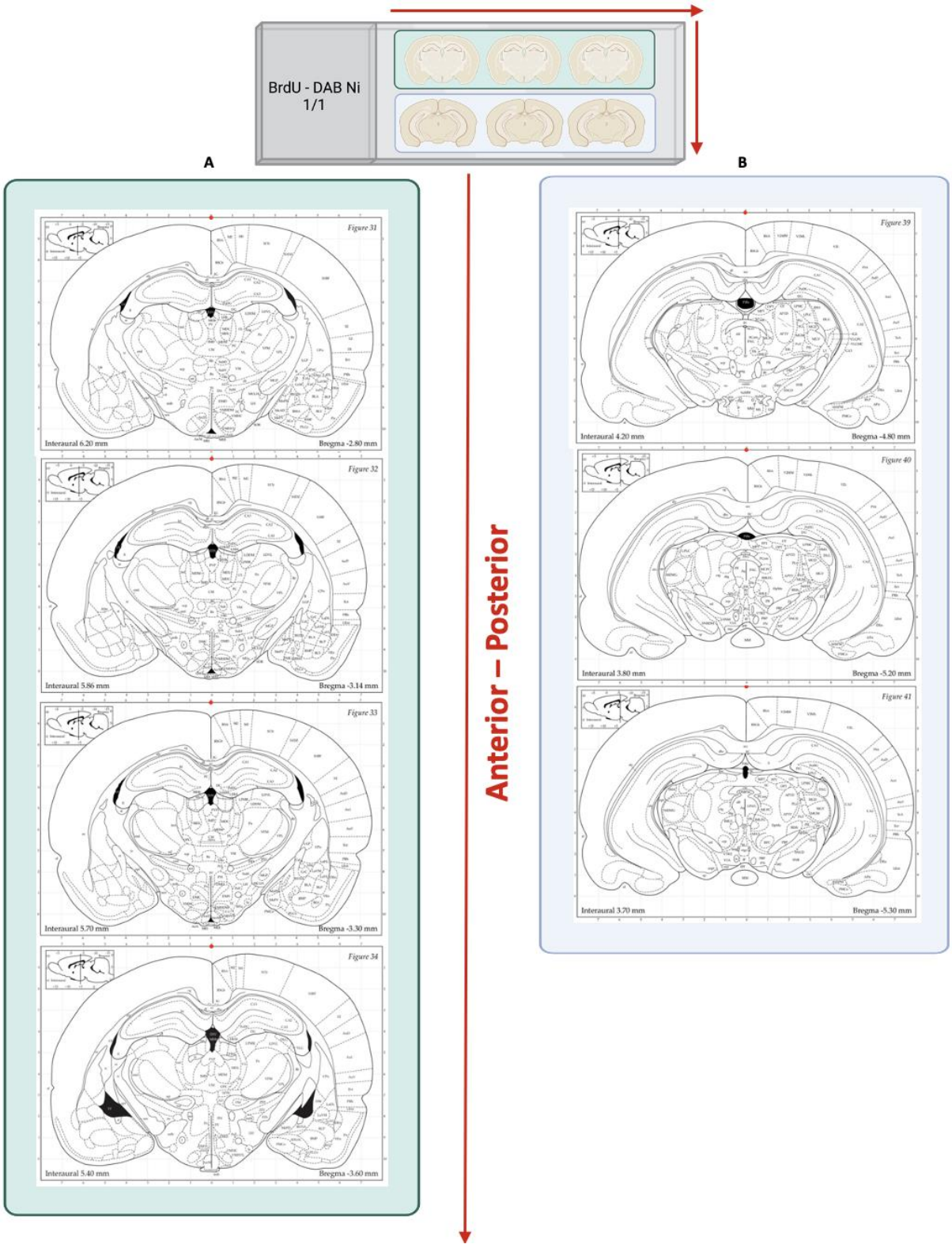

Figure 7: Mounting parameters following Immunohistological staining and schematic representation of (A) dorsal and (B) ventral slices, ordered along the anterior-posterior axis.

This figure was partially generated using Biorender. *Images of Bregma positions taken from the Paxinos and Watson Rat Brain Atlas.*

2.4 Microscopy

All sections were examined on an Olympus BX51 brightfield microscope (MBF Bioscience LLC, Williston, VT USA), equipped with Stereo Investigator Software 2022.2.3 (MBF Bioscience LLC, Williston, VT USA). For each animal, there were 1-2 slides, with the first slide containing 4-6 slices, and the second slide containing 0-2 slices, for a total of 6 slices across the hippocampus. Sections were analyzed from top left to bottom right, to maintain the dorsal to ventral order across subjects. A 10x objective (0.25 numerical aperture (NA)) was used to locate regions of interest within each section.

2.4.1 Profile Counts

BrdU and Ki67 cell numbers were obtained from the subgranular zone and granule cell layer of each slice using profile counting to assess cell proliferation. Cells were counted at 40x (0.75 NA), and clusters of cells were identified. A 100x (1.3 NA) objective was then used to resolve and count the identified clusters of cells. Cells were counted manually and tracked using a tally counter, then recorded on an Excel spreadsheet.

2.4.2 Area Measurements

Area measurements of the granule cell layer and subgranular zone of each slice were conducted in Stereoinvestigator, using the trace and contour measurement functions (Figure 8 and 9). Traces around the granule cell layer and subgranular zone were manually drawn using a

40x objective (0.75 NA), and the area of each trace, represented in microns squared, was automatically measured and displayed, using scaling information provided by Stereoinvestigator.

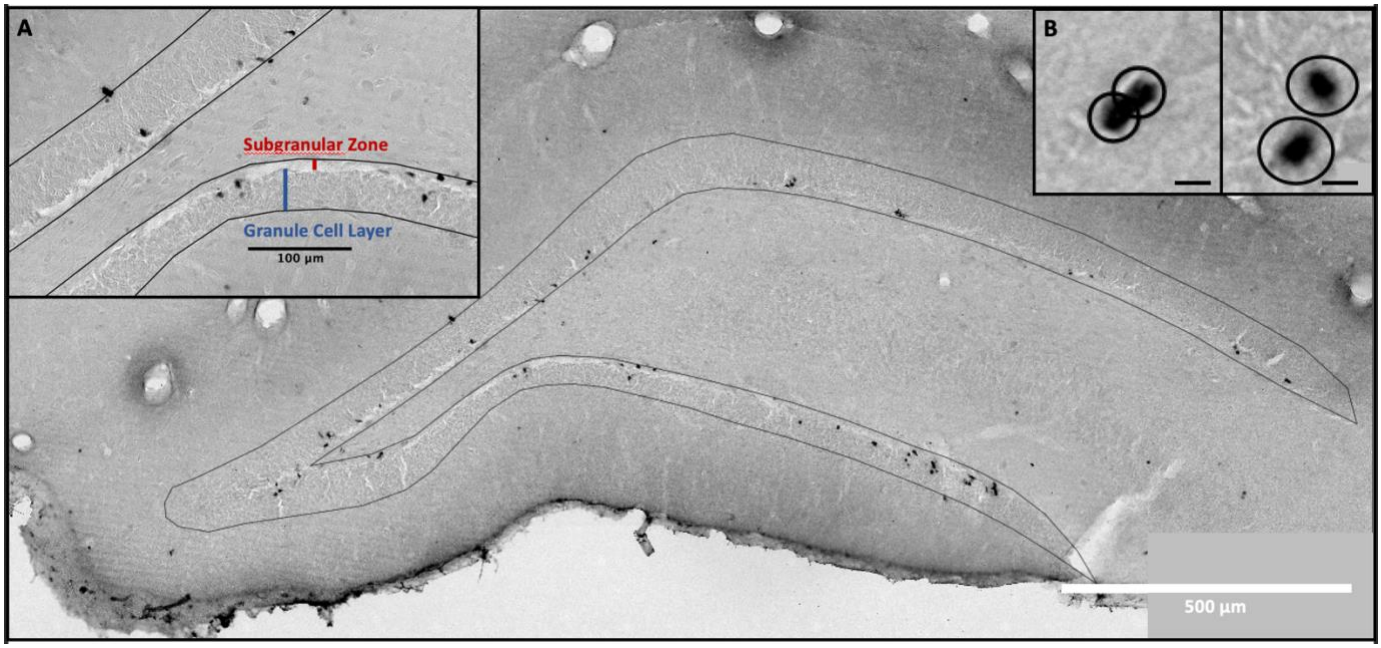

Figure 8: BrdU cells were counted in the granule cell layer and subgranular zone (A) of the dentate gyrus at 40x magnification or 100x magnification for clusters of cells that were difficult to differentiate at 40x magnification. Snapshot of BrdU positive cells at varying phases of proliferation (100x magnification) (B). Scale bars for insert 10 µm.

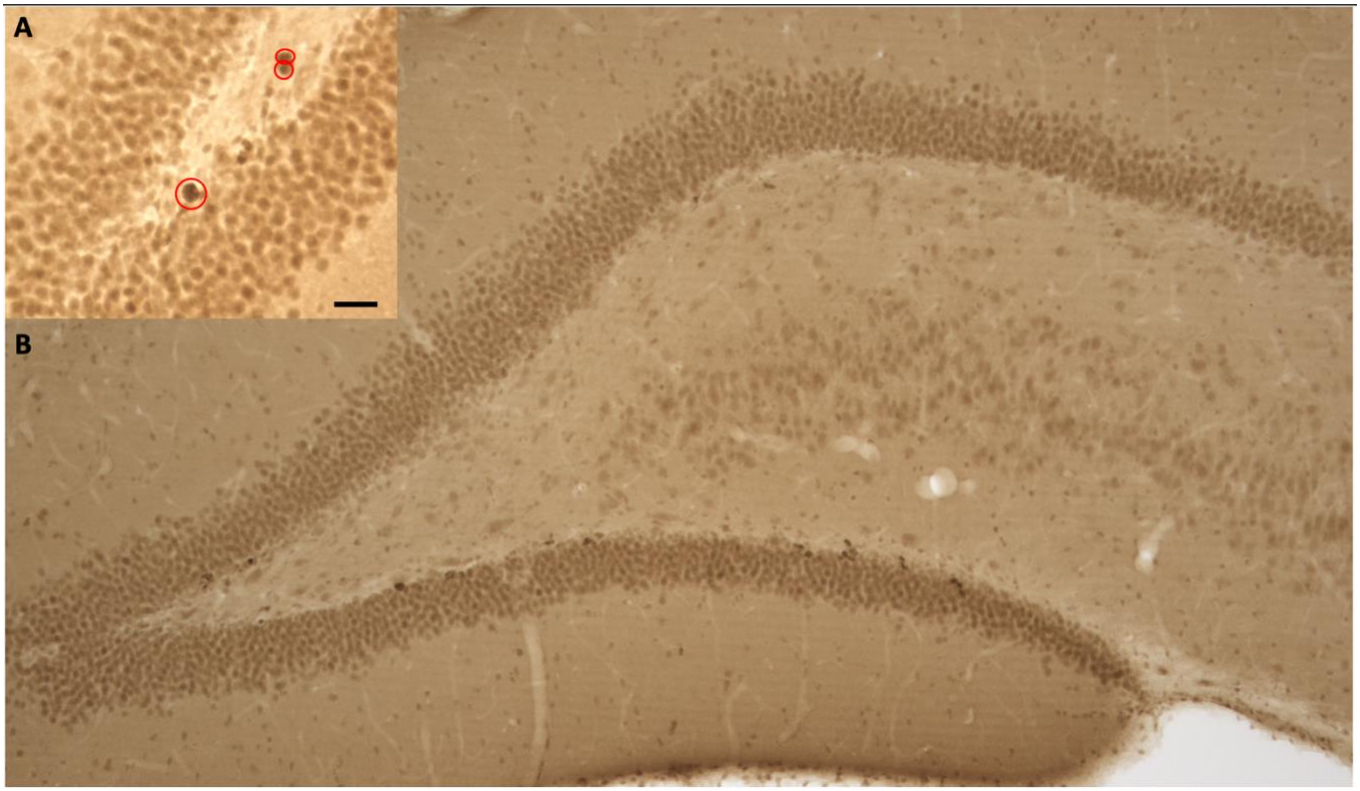

Figure 9: Ki67 positive cell counts at 40x magnification (A). Area Measurements were acquired from the granule cell layer and subgranular zone (10x magnification). Scale bars for insert 40 μm.

2.5 Analysis

Cell densities were calculated as the number of counted cells per region of interest (mm^2) and represented as a fraction of the area of the region of interest (mm^2).

Table 2: Cell density Calculation

$$BrdU\ Cell\ Densities\left(\frac{Count}{Area}(mm^2)\right) = \frac{Particle\ Count}{Area\ Measurements\ (\mu m^2)} \times 1000000$$

Profile counts and area measurements for each slice were calculated as a density value. For initial analysis, density values were averaged to produce two individual datapoints encompassing slices that fell within the dorsal criteria and slices that fell within the ventral criteria. Further statistical testing involved averaging density values from all slices to produce a singular datapoint encompassing all slices within a brain. Each datapoint represents the average density of cells across all documented slices in a brain.

2.5.1 Statistical Analysis

All statistical analyses for two-way analysis of variance (ANOVAs) and three-way ANOVAs were conducted with JASP Team (2024) JASP (Version 0.18.3) [Computer software]. A Shapiro Wilks test was used to reveal if each dataset was normally distributed for all columns of data and Levine's Test for Equality of Variances was used to assess homogeneity of variance. If assumptions of normality were not met, a reciprocal transformation was applied to the dataset,

and are indicated in results. Statistical significance was established *a priori* as $p < .05$. Acquisition of effect sizes for additional statistical testing were also collected using JASP.

Further statistical testing using *post-hoc* power analyses were conducted using G*Power3, to compute the achieved power for each statistical test.

2.5.2 Graph Generation

All graphical figures were created using GraphPad Prism version 10.0.0 for Windows (Boston, Massachusetts, USA) and G*Power3 (Faul, Erdfelder, Lang, & Buchner, 2007). The software was used to generate custom line graphs with error bars, two-way interleaved scatterplots with bars, and three-way interleaved bar graphs superimposed with scatterplots. Each datapoint is the mean density value for each animal across slices (slice n=6). All error bars ± SEM.

Chapter 3: Results

3.1 Postnatal choline supplementation increases body growth in female rats.

All animals were generated in the Thomas laboratory at San Diego State University. Body growth of rats in each treatment group (Control, Ethanol, Choline, Exercise, Ethanol Choline, Ethanol Exercise, Choline Exercise and Ethanol Choline Exercise), were measured throughout postnatal development. Daily measures of body weight, represented as an average per treatment group, are presented in Appendix 1. Two-way ANOVAs were conducted to compare male and female weight gains between specific timepoints, assessing the potential effects of procedures on body growth (Figure 3.1). First, the impact of postnatal ethanol exposure was assessed in pups from PND4-9 (See Table 3.1 for mean values). There were no significant differences in body weight gained (Sex: $F_{1,64} = 2.122$, $p = .150$; Ethanol: $F_{1,64} = 1.257$, $p = .266$; Sex x Ethanol: $F_{1,64} = 0.031$, $p = .860$). Next, body growth during postnatal choline supplementation was assessed from PND10-30 (See Table 3.2 for mean values). No significant variation was seen in sex ($F_{1,64} = 3.055$, $p = .085$) or sex and choline interactions ($F_{1,64} = 0.930$, $p = .399$). However, a main effect of choline was seen ($F_{1,64} = 6.710$, $p = .012$), and a simple main effects test revealed significance between the group means of female animals ($p = .013$) but not males ($p = .261$), indicating that female animals were driving the main effect. Lastly, body growth during forced running was evaluated from PND26-35 (See Table 3.3 for mean values). There were no significant differences in body weight gained (Sex: $F_{1,64} = 3.076$, $p = .084$; Forced Running: $F_{1,64} = 0.575$, $p = .451$; Sex x Forced Running: $F_{1,64} = 0.146$, $p = .704$).

Table 3: Average Weight Gained (g) in male and female rats from PND 4 to 9.

Treatment Group	Male		Female	
	Weight Gained ± SEM	# of animals	Weight Gained ± SEM	# of animals
Control	11.88 ± 0.41	17	10.95 ± 0.47	18
Ethanol	11.14 ± 0.73	16	10.41 ± 0.61	17

Table 4: Average Weight Gained (g) in male and female rats from PND 10 to 30.

Treatment Group	Male		Female	
	Weight Gained ± SEM	# of animals	Weight Gained ± SEM	# of animals
Control	81.41 ± 3.94	8	74.09 ± 3.39	9
Ethanol	84.01 ± 4.88	8	75.36 ± 6.59	8
Choline	86.43 ± 4.40	8	88.93 ± 4.37	9
Ethanol Choline	88.50 ± 3.40	9	81.48 ± 2.52	9

Table 5: Average Weight Gained (g) in male and female rats from PND 26 to 35.

Treatment Group	Male		Female	
	Weight Gained ± SEM	# of animals	Weight Gained ± SEM	# of animals
Control	61.82 ± 4.95	5	60.08 ± 3.67	5

Ethanol	65.84 ± 7.50	5	59.83 ± 8.16	4
Choline	66.90 ± 7.03	5	77.13 ± 7.10	4
Ethanol Choline	69.10 ± 2.65	5	48.45 ± 7.09	4
Exercise	70.40 ± 2.72	3	50.95 ± 5.95	4
Ethanol Exercise	79.07 ± 3.58	3	55.50 ± 4.96	4
Choline Exercise	50.53 ± 3.01	3	57.08 ± 7.80	5
Ethanol Choline Exercise	60.10 ± 8.79	4	64.60 ± 3.91	5

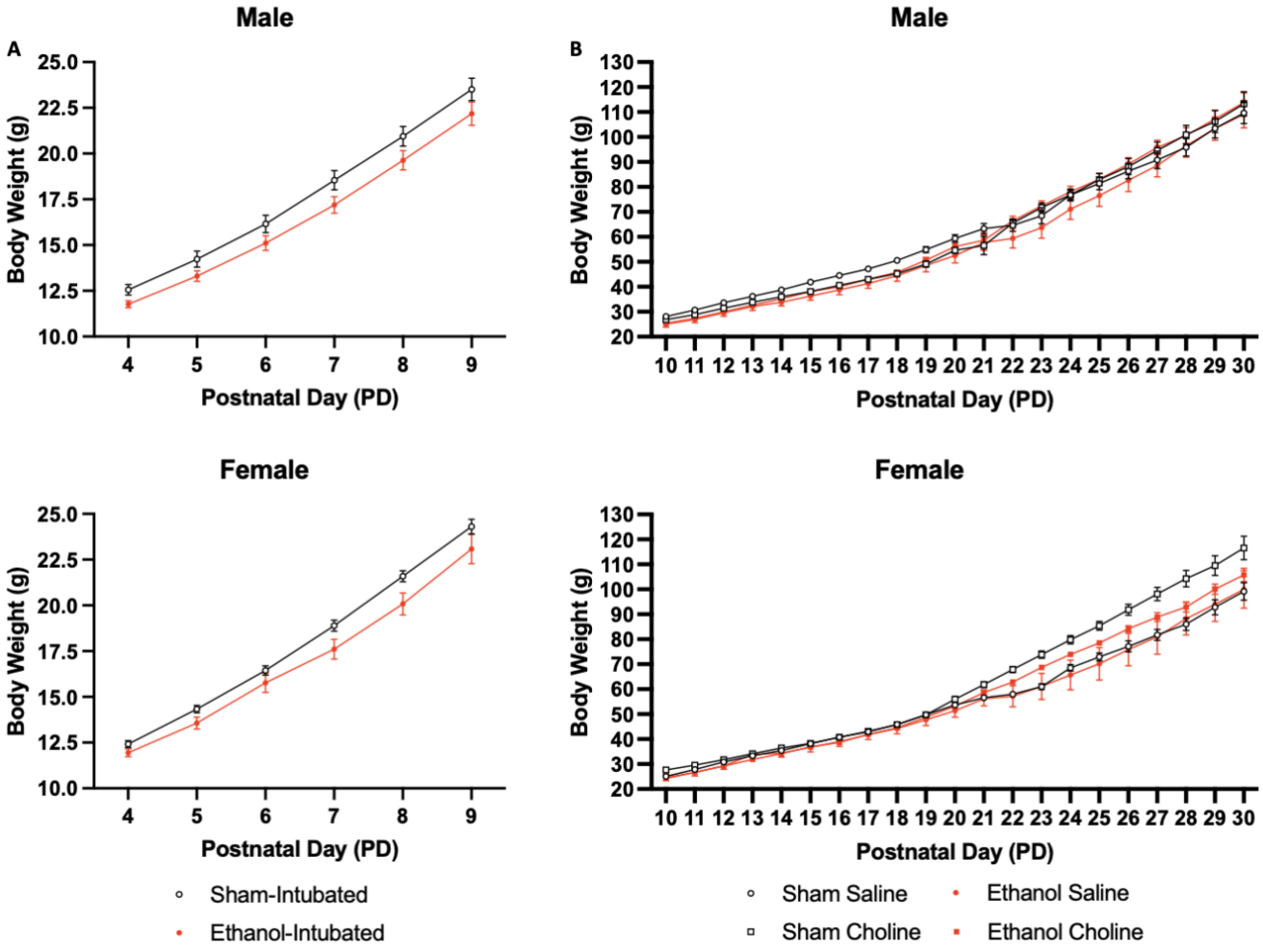

Figure 10: Male and Female Body Weights (g) across postnatal development. (A) Sham and Ethanol-Intubated groups from PND4-9 (B) Sham Saline, Ethanol Saline, Sham Choline and Ethanol Choline groups from PND 10-30. All error bars ± SEM.

3.2 Main effect of sex seen in body growth within treatment groups across the entire developmental period.

The average weight gained (g) across postnatal developmental periods of interest in each treatment group are displayed in Table 3.4. Variations in body weight gained across the entire postnatal period of development was next assessed using a two-way analysis of variance between sexes and across all treatment groups: control, ethanol, choline, exercise, choline exercise, ethanol choline, ethanol exercise, ethanol choline exercise (See Table 3.3 for means). A main effect of sex was seen ($F_{1,52}$ = 5.360, p = .025), with a simple main effects test revealing significant differences in ethanol choline (p = .030), ethanol exercise (p = .005) and exercise (p = .028) groups. There was no variation seen across treatment groups ($F_{7,52}$ = 1.204, p = .317), without an interaction with sex ($F_{7,52}$ = 2.417, p = .032). Post hoc testing for sex and treatment revealed no significance, likely due to small sample sizes in certain groups.

Table 6: Average Weight Gained (g) in each treatment group across postnatal development.

Treatment Group	Males		Females	
	Weight Gained ± SEM	# of animals	Weight Gained ± SEM	# of animals
Control	134.86 ± 8.37	5	127.96 ± 5.51	5
Ethanol	130.80 ± 6.43	5	127.85 ± 14.75	4
Choline	143.28 ± 11.37	5	158.08 ± 9.91	4
Ethanol Choline	149.46 ± 4.68	5	120.80 ± 7.4	4
Exercise	146.73 ± 5.27	3	113.63 ± 8.25	4
Ethanol Exercise	158.23 ± 11.43	3	114.88 ± 15.27	4
Choline Exercise	124.20 ± 6.96	3	133.14 ± 8.98	5
Ethanol Choline Exercise	134.08 ± 11.97	4	137.86 ± 5.15	5

3.3 Postnatal choline supplementation and forced running increase female body weight to levels comparable to males.

Immediately prior to sacrifice, body weights were measured, and subsequently analyzed to determine variation in demographic information between treatment groups and sexes. A two-way analysis of variance was run on body weights at PND36 and revealed a significant main

effect of sex ($F_{1,52}$ = 50.583, p < .001). The body weights of male animals were significantly higher in control (p = .028), ethanol (p = .002), ethanol choline (p < .001), ethanol exercise (p = .008) and exercise (p = .003), or trending towards significance in choline (p = .080) and ethanol choline exercise (p = .056), than females. Interestingly, the group with the largest p-value was choline exercise (p = .273). There wasn't significant variation across treatment groups ($F_{7,52}$ = 1.836, p = .096), or between sex and treatment ($F_{7,52}$ = 0.829, p = .567). Immediately following animal perfusions, brains were extracted and weighed. Data violated the homogeneity of variance assumption, and as such, Kruskal Wallis tests were run to compare brain weights between sexes (H_1 = 3.508, p = .061) and treatment groups (H_7 = 9.163, p = 241), with no significance seen.

Table 7: Mean Body Weights of Male and Female animals at PD36.

Treatment Group	Male		Female	
	Body Weight ± SEM	**# of animals**	**Body Weight ± SEM**	**# of animals**
Control	175.32 ± 8.73	5	148.75 ± 4.71	5
Ethanol	177.77 ± 3.87	5	141.38 ± 6.41	4
Choline	177.50 ± 6.97	5	156.48 ± 6.62	4
Exercise	163.05 ± 16.29	5	127.40 ± 7.41	4
Ethanol Choline	184.82 ± 5.21	3	142.30 ± 5.98	4
Ethanol Exercise	165.70 ± 7.36	3	133.36 ± 6.85	4
Choline Exercise	159.84 ± 12.07	3	148.02 ± 4.17	5
Ethanol Choline Exercise	161.45 ± 4.14	4	138.40 ± 13.42	5

3.4 Blood Alcohol Concentrations (BACs) did not differ across ethanol-exposed groups.

Blood alcohol concentrations were evaluated at PND6 in all ethanol-exposed conditions. A two-way analysis of variance revealed no significant differences in male ($F_{3,14} = 0.628$, $p = .605$) and female ($F_{3,17} = 0.444$, $p = .724$) animals. The average peak BAC of each ethanol-exposed treatment group are listed in Table 3.2.

Table 8: Peak BAC (mg/dL) of Male and Female animals in ethanol treatment groups.

Treatment Group	**Male**		**Female**	
	Peak BAC ± SEM	**# of animals**	**Peak BAC ± SEM**	**# of animals**
Ethanol	285.11 ± 0.05	5	270.33 ± 0.05	4
Ethanol Choline	291.47 ± 0.04	3	268.18 ± 0.05	4
Ethanol Exercise	254.68 ± 0.09	3	245.98 ± 0.02	5
Ethanol Choline Exercise	257.62 ± 0.06	4	268.18 ± 0.06	5

3.5 Preliminary Cell Density Analysis Reveals No Differences Across Anterior-Posterior Axis

BrdU, an exogenous thymidine analogue, was administered to animals within a 24-hour window before sacrifice to label newly synthesized DNA in dividing cells within 24 hours. After tissue processing, immunohistochemical staining against BrdU was used to label cells in the S phase of the cell cycle, during the proliferative stage of neurogenesis. In addition to BrdU, immunohistochemical staining against Ki67 was used to assess cell proliferation, for its expression in the nucleus during all stages of the cell cycle, capturing a larger window of proliferating cells (Kee et al., 2002). Statistical testing for this dataset was conducted following the same procedures as the BrdU dataset.

The functionality of the hippocampus has been shown to change along its dorsal-ventral axis, due to differences in its connectivity (Fanselow & Dong, 2010; A. R. Lee et al., 2017). Based on these anatomical variations, the dorsal and ventral hippocampus may respond differently to the treatments administered in this study. As such, profile counts and area measurements of dorsal and ventral slices, as well as density values for each, were recorded and calculated separately. First, to assess whether variation between the densities of proliferating cells in dorsal and ventral slices exists in the current dataset, a two-way analysis of variance was run using stereotaxic divisions (i.e. dorsal or ventral) and all treatment groups as factors. All data was normalized using a reciprocal transformation. A main effect of stereotaxic division ($F_{1,73} = 6.757$, $p = .011$) but not treatment ($F_{7,73} = 0.4163$, $p = .889$) was found in BrdU densities, as well no significant interactions ($F_{7,73} = 0.1985$, $p = .985$) between the two factors. A general increase in BrdU densities was observed in the dorsal condition of all groups, consistent with literature stating that the rate of neurogenesis is higher in the dorsal

hippocampus(Huckleberry et al., 2018). No main effects or interactions (Stereotaxic Divisions: $F_{1,80} = 0.444$, $p = .507$; Treatment: $F_{7,80} = 1.680$, $p = .126$; Interactions: $F_{7,80} = 0.045$, $p = .999$) were seen in Ki67 densities. Consequently, dorsal and ventral datasets were combined and analyzed together for all further statistical testing.

Table 9: Mean BrdU Cell Densities Between Dorsal & Ventral Conditions Across Treatments

Treatment Group	**Dorsal**		**Ventral**	
	Average Density ± SEM	**# of animals**	**Average Density ± SEM**	**# of animals**
Control	229.8 ± 30.8	7	204.4 ± 24.8	7
Ethanol	300.8 ± 47.9	8	245.1 ± 39.5	7
Choline	311.8 ± 48.5	5	251.4 ± 65.8	4
Exercise	323.4 ± 53.6	4	240.3 ± 21.6	4
Choline Exercise	253.6 ± 47.2	7	216.2 ± 49.9	5
Ethanol Choline	317.3 ± 83.4	4	236.6 ± 56.6	5
Ethanol Exercise	330.9 ± 61.8	5	296 ± 61.3	6
Ethanol Choline Exercise	283.8 ± 39.9	9	251.3 ± 33.6	8

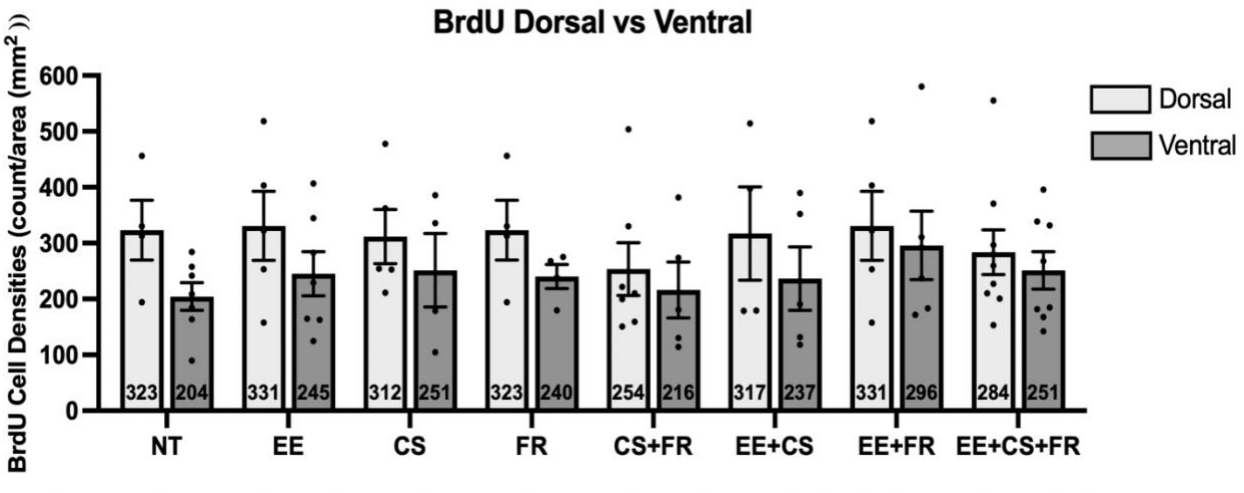

Figure 11: BrdU Cell Densities in dorsal vs. ventral hippocampal slices, across treatments. Each datapoint is the mean density value for each animal across slices (slice n=6). NT is no treatment, EE is ethanol exposure, CS is choline supplementation, FR is forced running. All error bars ± SEM. * $p < .05$, ** $p < .01$, *** $p < .001$, ns = not significant.

Table 10: Mean Ki67 Cell Densities Between Dorsal & Ventral Conditions Across Treatments

Treatment Group	Dorsal		Ventral	
	Average Density ± SEM	**# of animals**	**Average Density ± SEM**	**# of animals**
Control	740.1 ± 64.2	7	777.6 ± 66.9	7
Ethanol	713 ± 57.9	8	745.2 ± 45.2	8
Choline	684.8 ± 75.9	6	691.7 ± 69.2	6
Exercise	767.9 ± 147.4	6	819.4 ± 119.4	6
Choline Exercise	755 ± 83.3	6	802.2 ± 41	6
Ethanol Choline	665.4 ± 80	6	732.1 ± 70.1	6
Ethanol Exercise	948.1 ± 152	4	955.5 ± 171.1	4
Ethanol Choline Exercise	870.6 ± 97.6	5	856.1 ± 77	5

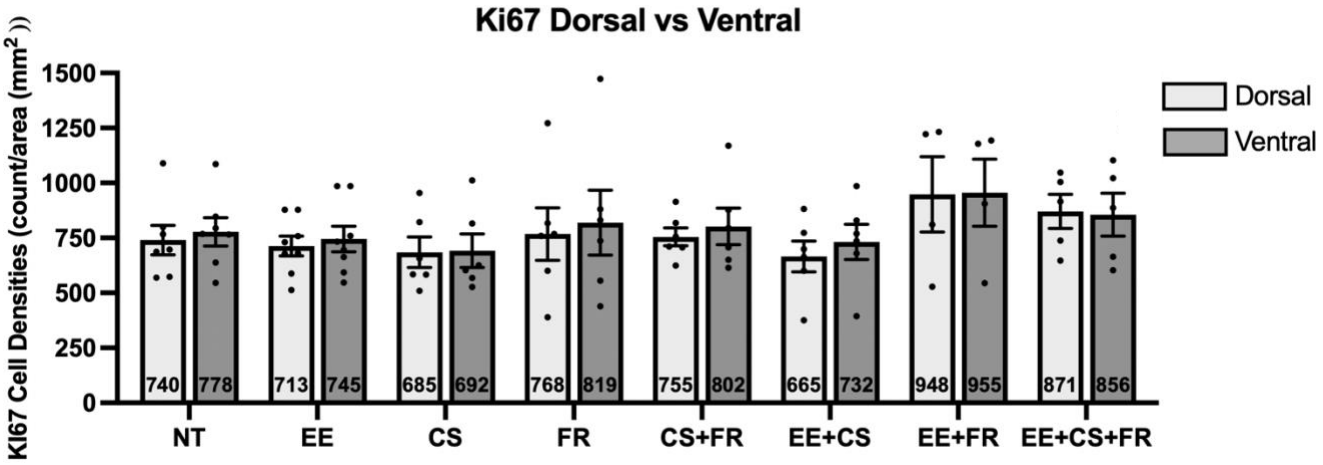

Figure 12: Ki67 Cell Densities in dorsal vs. ventral hippocampal slices, across treatments. Each datapoint is the mean density value for each animal across slices (slice n=6). NT is no treatment, EE is ethanol exposure, CS is choline supplementation, FR is forced running. All error bars ± SEM. * $p < .05$, ** $p < .01$, *** $p < .001$, ns = not significant.

3.6: No effect of treatments on the densities of proliferating cells in the SGZ and GCL (combined) of the entire hippocampus

Next, to determine if there were any effects of ethanol exposure, choline supplementation and forced running, a three-way ANOVA was run on BrdU and Ki67 datasets. No significant main effects were found in BrdU cell densities (Ethanol: $F_{1,43} = 1.315$, $p = .258$; Choline: $F_{1,43} = 0.219$, $p = .6424$; Forced Running: $F_{1,43} = 0.1381$, $p = .712$). Similarly, ethanol exposure ($F_{1,39} = 0.6170$, $p = .437$) and choline supplementation ($F_{1,39} = 0.5011$, $p = .483$) yielded no significant main effects on Ki67 cell densities, but forced running showed a significant main effect ($F_{1,39} = 4.777$, $p = .035$, $n^2_p = 0.116$). There were no significant interactions found in BrdU densities (Choline vs. Exercise: $F_{1,43} = 2.350$, $p = .133$; Ethanol vs. Choline: $F_{1,43} = 0.3274$, $p = .570$; Ethanol vs. Exercise: $F_{1,43} = 0.0165$, $p = .899$; Ethanol vs. Choline vs. Exercise: $F_{1,43} = 0.1003$, $p = .753$) or Ki67 densities (Choline vs. Exercise: $F_{1,39} = 0.0163$, $p = .899$; Ethanol vs. Choline: $F_{1,39} = 0.001$, $p = .987$; Ethanol vs. Exercise: $F_{1,39} = 1.382$, $p = .247$; Ethanol vs. Choline vs. Exercise: $F_{1,39} = 0.3316$, $p = .568$).

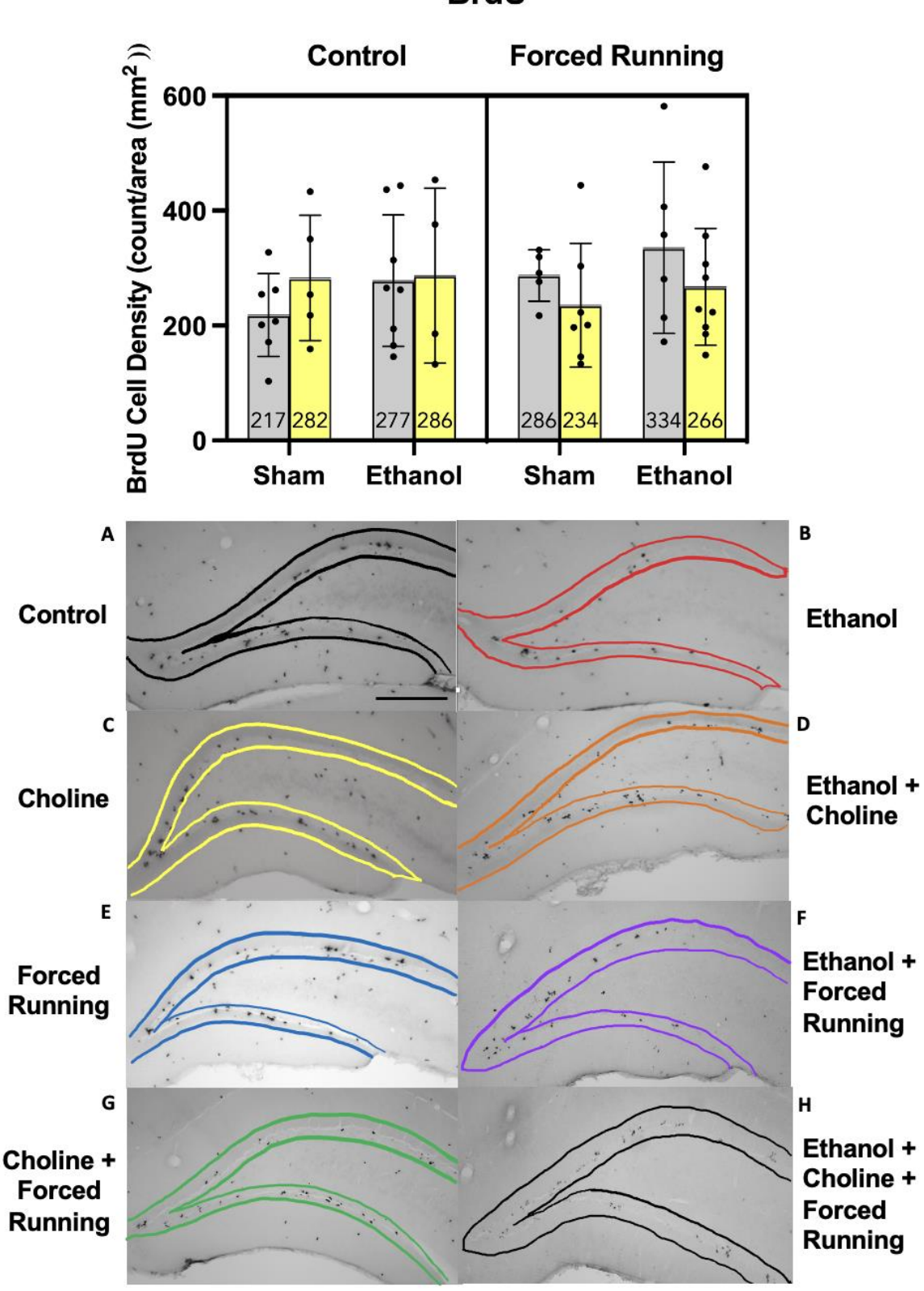
BrdU
Control
Forced Running
BrdU Cell Density (count/area (mm2))
600
400
200
0
217
282
277
286
286
234
334
266
Sham
Ethanol
Sham
Ethanol
A
Control
B
Ethanol
C
Choline
D
Ethanol + Choline
E
Forced Running
F
Ethanol + Forced Running
G
Choline + Forced Running
H
Ethanol + Choline + Forced Running

Figure 13: BrdU cell densities (count/area (mm^2)) across treatment groups. Each datapoint is the mean density value for each animal across slices (slice n=6). All error bars ± SEM. * $p < .05$, ** $p < .01$, *** $p < .001$. Representative Images of BrdU cell densities in the GCL and SGZ (combined) for control (A) ethanol (B) choline (C) ethanol and choline (D) forced running (E) ethanol and forced running (F) choline and forced running (G) ethanol and choline and forced running (H). Scale bars for insert 500 μm.

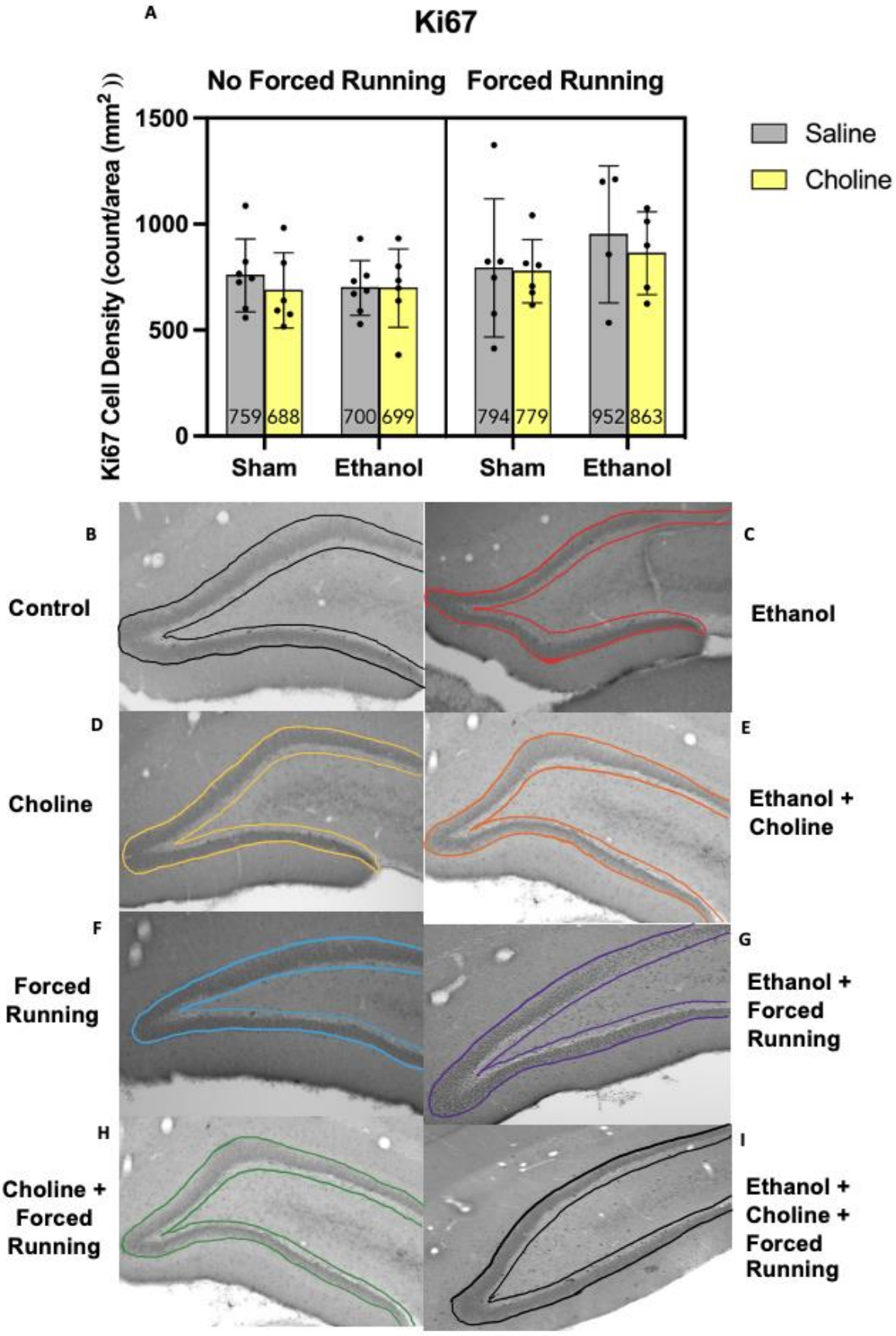
A
Ki67
No Forced Running
Forced Running
Ki67 Cell Density (count/area (mm2))
1500
1000
500
0
759
688
700
699
794
779
952
863
Sham
Ethanol
Sham
Ethanol
Saline
Choline
B
Control
C
Ethanol
D
Choline
E
Ethanol +
Choline
F
Forced
Running
G
Ethanol +
Forced
Running
H
Choline +
Forced
Running
I
Ethanol +
Choline +
Forced
Running

Figure 14: Ki67 cell densities (count/area (mm^2)) across treatment groups. Each datapoint is the mean density value for each animal across slices (slice n=6). All error bars ± SEM. * $p < .05$, ** $p < .01$, *** $p < .001$. Representative Images of BrdU cell densities in the GCL and SGZ (combined) for control (A) ethanol (B) choline (C) ethanol and choline (D) forced running (E) ethanol and forced running (F) choline and forced running (G) ethanol and choline and forced running (H). Scale bars for insert 500 μm.

Chapter 4: Discussion

4.1 Summary of Major Findings

Immunohistological staining was used in this work to assess variations in the densities of proliferating cells in the dentate gyrus of the hippocampus. Cellular proliferation of dorsal and ventral hippocampal slices showed no significant impact across treatment conditions – ethanol exposure, choline supplementation and forced running. Ethanol exposure and choline supplementation showed no significant main effects, but forced running did in the Ki67 dataset only. Choline supplementation and forced running failed to show a significant interaction with one another, and individually, each showed no significant interactions with ethanol exposure. All three combined treatments showed no significant interactions.

4.2 Demographic Data Reveals Validation in the Usage of the Animal Model

While the combination of treatments used in this animal model is unique, each component is well-established and not novel in its usage. Postnatal ethanol exposure from PD4-9, via intragastric intubation, is used to study the third trimester of neurodevelopment and has been used in previous work in rats. Studies that have assessed group differences in body weights following intragastric intubation have reported no significant differences in growth between control and ethanol-intubated pups, but rather, decreases in growth following intragastric intubation in general (Kelly & Lawrence, 2008b; Kelly & Tran, 1997; Light et al., 1998; Tran et al., 2000). This effect is likely due to the stress and handling involved in the administration of the procedure. The results reported in this work support these findings, in both sexes. Blood alcohol content was assessed at PD6 in all ethanol-exposed groups, revealing concentrations of around 200-300mg/dL. These concentrations, of around 0.2-0.3% blood

alcohol content, are considered dangerously high levels of intoxication, and are seen in studies assessing the effects of binge alcohol exposure. Postnatal choline supplementation during PD10-30, has been used in various preclinical rodent studies, with varying reports on body growth (Ryan et al., 2008; Schneider & Thomas, 2016; Thomas et al., 2004). Here, choline-supplemented females showed significantly more growth than control animals, with weight gains comparable to male animals. Forced running is a form of physical activity that has the potential to decrease body weight, however the effects vary based on the intensity, timing, and frequency of activity; for example, a moderate intensity workout would burn fewer calories than a high intensity workout (Leasure & Jones, 2008). Here a moderately-paced 9-day aerobic exercise regimen was used, and as expected, no significant variation was seen in weight gained in either sex.

4.3 Experimental Treatments Do Not Have Differential Effects on Cellular Proliferation Across the Dorsal-Ventral Axis

The functional role of neural circuitry within the hippocampus shifts along the dorsal-ventral axis, based on its connectivity to different regions of the brain. The dorsal portion, which can be found with lower Bregma coordinates along the anterior-posterior axis (Figure 7), is involved in spatial learning and memory (S. L. Lee et al., 2019). In contrast, the ventral portion, found with higher Bregma coordinates along the anterior-posterior axis, is involved in stress and emotion (Fanselow & Dong, 2010). In addition to the rate of neurogenesis being different (Huckleberry et al., 2018), due to the variance in neural connections, cellular proliferation in each region can be differentially impacted by the conditions imposed on the

hippocampus. For example, previous work has shown that corticosteroid-induced anxiety and neurogenesis interact differently along the dorsal-ventral axis of the hippocampus, with a higher sensitivity in the ventral region (Shi et al., 2023). In contrast, environmental enrichment has been shown to promote cellular proliferation via BrdU immunohistochemistry in the dorsal but not ventral hippocampus (Tanti et al., 2012). In the context of ethanol exposure, choline supplementation or forced running, cellular proliferation along the dorsal-ventral axis of the hippocampus has not been reported. Here, in both datasets, there were no significant interactions seen between dorsal/ventral and treatments, suggesting that impacts to cellular proliferation at the dorsal/ventral level, were equivalent across all treatments. This is surprising considering the age of the tissue being examined – there is a higher level of neural plasticity due to ongoing maturation during adolescence, which would likely lead to a greater sensitivity to external stimuli and higher variability between conditions. This could be the result of transient treatment effects that will be further discussed in upcoming sections.

4.4 Postnatal Binge Ethanol Exposure Does Not Produce Long-Lasting Effects on Cellular Proliferation

Ethanol, commonly known as alcohol, is widely used for recreation, stress relief, mood enhancement and as a coping mechanism; however, ethanol is a psychoactive substance and its presence in the brain is abnormal. As such, it has the capability to create widespread disruptions to neural and cellular processes throughout the brain, and in, of interest, to the neurogenic niche of the hippocampus. Some studies assessing cellular proliferation during the peri-adolescent to adolescent period, have reported reductions in neonatal ethanol-exposed

groups (Hamilton et al., 2011), while others have reported no changes (Gil-Mohapel et al., 2014; Hamilton et al., 2016; Helfer et al., 2009a). Despite its temporal implications, the effects of binge postnatal ethanol exposure on hippocampal neurogenesis have been minimally studied. During the temporal window of PD4-9, developmentally equivalent to the third trimester of human pregnancy, the rodent hippocampus undergoes extensive development; a series of dramatic changes including neurogenesis, involving cell proliferation, migration, differentiation, and maturation as well as synaptogenesis and synaptic integration occur (Aniol et al., 2022). Ethanol can cross the blood brain barrier where infiltration to the developing hippocampus can occur. Previous work has shown dramatic reductions in cellular proliferation immediately following postnatal binge ethanol insult (Nixon & Crews, 2002; Vetreno & Crews, 2015). However, Klintsova and colleagues demonstrated that postnatal ethanol exposure produced no variation from controls in the number of BrdU and Ki67 positive cells at PD50 (Klintsova et al., 2007). Similarly, the results shown here show no significant changes in the densities of BrdU and Ki67 cells from controls at PD36, following postnatal ethanol exposure. These results provide evidence to support the notion that the effects seen following postnatal binge ethanol exposure on the abundance of proliferating cells in the hippocampal dentate gyrus are transient.

4.5 Choline Supplementation Has No Effect Cellular Proliferation during the Peri-Adolescent Period

Choline is an essential nutrient that possesses the capability to pass the blood brain barrier. An adequate intake of the nutrient choline is vital as it plays an essential role in various

physiological processes. This includes the synthesis of phosphatidylcholine, a major constituent of cell membranes, and acetylcholine, a neurotransmitter involved in memory storage and muscle control, as well as its participation as a methyl group donor (Derbyshire & Obeid, 2020). Through its role in these processes, choline availability has also been shown to influence components of neurogenesis including neuronal proliferation, differentiation, and migration (Derbyshire & Obeid, 2020). Previous work has demonstrated that choline supplementation increases cellular proliferation through its involvement in methylation. (Zeisel, 2004, 2011), and is essential for membrane synthesis (Zeisel, 2011). Choline supplementation has been shown to have positive effects on neurogenesis. These effects have been observed in models of Down Syndrome, and choline-supplemented normalization of neurogenesis was shown to be correlated with improvements in spatial cognition (Velazquez et al., 2013). Of note, prenatal choline supplementation has been shown to increase BrdU cell densities in the dentate gyrus of adult rats (Glenn et al., 2007). Rodent models assessing postnatal choline supplementation and hippocampal-dependent behaviours have shown that choline improves spatial learning and working memory during the peri-adolescent period (Ryan et al., 2008; Thomas et al., 2000) and this result has been mirrored in clinical work (Wozniak et al., 2020). These dorsal hippocampal-dependent improvements in behaviour could be the result of increases in neurogenesis within the dentate gyrus, yet postnatal choline supplementation and neurogenesis during the peri-adolescent period have not been assessed. Here, choline supplementation produced no main effect in the abundance of BrdU and Ki67-positive cells in the dentate gyrus, providing evidence that postnatal choline supplementation does not change the density of proliferating cells at this time point, 6 days after administration. Previous work has shown that postnatal choline

supplementation can attenuate the behavioural deficits seen following perinatal ethanol exposure (Ryan et al., 2008; Schneider & Thomas, 2016; Thomas et al., 2000, 2010). Here, there were no significant interactions found between ethanol exposure and choline supplementation in either BrdU or Ki67 datasets. This indicates that the effects of choline supplementation on the density of proliferating cells are consistent between sham and ethanol conditions. This could be explained by the lack of effect to cell proliferation densities seen following ethanol exposure.

4.6 Aerobic Running Increases Ki67 Cell Densities during the Peri-Adolescent Period

Running has been shown to positively influence cellular proliferation in the hippocampus (Boehme et al., 2011; Helfer et al., 2009b; Van Praag et al., 1999). Mechanistically, it has been linked to the production of neurotrophic factors that promote processes that regulate hippocampal function-like neurogenesis, as well as the modulation of stress via alterations in receptor levels (Stranahan et al., 2008). It is well known that certain molecular and cellular changes associated with neurogenesis start to occur within hours following running and can continue for up to several days. Cellular proliferation of neural stem cells typically occurs within days and can take place for up to several weeks (Van Praag et al., 1999). In this study, the time window assessed for cellular proliferation was within 48 hours of the final forced running session. As expected, results showed that forced running had a significant main effect on Ki67 cell densities, with observed increases in the cell densities of the forced running condition, but surprisingly, it did not on BrdU cell densities. The statistical significance found in the Ki67 dataset was accompanied by a low effect size, suggesting that the

practical impact of forced running in this dataset is low. This indicates that there was an increase in the number of dividing cells (shown by Ki67), but not new cells entering the cell cycle (shown by BrdU). Aerobic exercise as an intervention has been shown in clinical and preclinical rodent models to prevent and reduce FASD-like neuropathology in the hippocampus (Christie et al., 2005; Helfer et al., 2009a; Keiver et al., 2016). Here, there were no significant interactions seen between ethanol exposure and forced running, suggesting that forced running impacts cellular proliferation in sham-intubated animals and ethanol-intubated animals comparably. Like the non-significant interaction between ethanol and choline, this could be explained by the lack of effect following ethanol exposure.

4.7 Combined Effects of Treatments Yield No Substantial Outcomes on Cellular Proliferation

The two interventions assessed in this study, choline, and aerobic running, are commonly used to improve overall physical health and cognitive function. Individually, both choline and aerobic running have been shown in the literature to enhance neurogenesis through the various mechanisms discussed earlier. As such, it is possible that if utilized together, choline supplementation and aerobic running might produce a synergistic effect in enhancing neurogenesis. However, the results show that there were no significant interactions between choline supplementation and forced running in either BrdU or Ki67 datasets. Similarly, in both datasets, no interactions between all three treatments (ethanol exposure, choline supplementation and forced running) were seen, suggesting that there was no impact of their combined effects.

4.8 Limitations and Future Directions

While the results of this study contribute to our understanding of cellular proliferation in relation to each treatment, the work has some limitations that may impact the generalizability of the findings. To assess the statistical sensitivity of each test, post-hoc power analyses were conducted on BrdU (Ethanol: 5.05%; Choline: 5.07%; Ethanol by Choline: 5.05%; Forced Running: 5.14%; Ethanol by Forced Running: 5.02%; Choline by Forced Running: 5.74%; Ethanol by Choline by Forced Running: 5.21%) and Ki67 (Ethanol: 5.28%; Choline: 5.12%; Ethanol by Choline: 5.01%; Forced Running: 11.18%; Ethanol by Forced Running: 6.07%; Choline by Forced Running: 5.00%; Ethanol by Choline by Forced Running: 5.17%) datasets. The experimental design lacked sufficient power, likely due to a combination of a small sample size and high variability. The tissue used in this experiment was adolescent, a period in which the hippocampus is still undergoing structural and functional maturation, which could lead to higher variability. Additionally, the animals generated for this study were done so during the COVID-19 pandemic. During this time, animal procedures were altered, and staffing was reduced. Moreover, the timing of routine animal care procedures varied throughout the study, due to animals being bred at different intervals. This further resulted in variability in the timing of tissue handling, processing and the execution of technical procedures. Data quantification was performed manually, although consistent and validated, leaving room for human error. Lastly, fluctuations in hormonal levels including corticosterone and estrogen were not assessed in this study. Corticosterone, the hormone associated with stress, has been shown to inhibit cellular proliferation and certain procedures used in this model likely induced stress at the time of handling (Borsini et al., 2023). It would be interesting to determine whether elevated stress

could have counteracted the positive effects of procedures (ex. following subcutaneous injections of choline). Corticosterone could have been taken through blood, urine feces. In contrast, estrogen has been shown to have neuroprotective effects and promote cellular proliferation of NPCs in the hippocampus (Bustamante-Barrientos et al., 2021). Estrogen levels fluctuate in males and females (although less pronounced in males) and could have contributed to some of the variability seen in the datasets. Estrogen levels could have been assessed through blood, urine or feces.

With respect to the techniques used in this work, every method offers its benefits and limitations. BrdU, while being the standard for studying neurogenesis, involves stressful handling procedures to label in vivo, and lengthens the cell cycle, altering cell cycle dynamics (Taupin, 2007). Additionally, it involves harsh denaturation protocols while immunostaining that could degrade surrounding proteins and limit the capacity for co-labelling. This is a huge consideration for future studies, since BrdU is a marker for only newly synthesized DNA and does not differentiate cell repair from cell division, as well as the cell types that are undergoing DNA synthesis (Cameron, 2006). Similarly, Ki67 immunostaining highlights cells in the active phases of the cell cycle (G1, S, G2 and M) but does not differentiate cell types that are in these processes (Di Rosa et al., 2021). Lastly, a systematic stereological approach to the quantification of BrdU and Ki67 positive cells could have been used to increase the reproducibility of the work (Zhao & van Praag, 2020).

To provide further insight into the potential mechanisms governing the negative symptoms observed following postnatal ethanol exposure, and the attenuation of these symptoms following postnatal choline supplementation and aerobic physical activity, the

remaining components of neurogenesis – all of which have been linked to the improvement of spatial and working memory – could be investigated. Follow up immunohistochemistry studies assessing the cellular density of the biomarker doublecortin (DCX), a marker for immature neurons and glial fibrillary acidic protein (GFAP), an astrocytic marker, and ionized binding adaptor molecule 1 (IBA1), a marker for microglia, could elucidate the types of proliferating cells seen following BrdU staining. This would determine which types of cells are being generated in each condition. An additional follow up immunohistochemistry study assessing the survival of proliferating cells into adulthood could provide more insight into neurogenic processes following treatments. This could be achieved by aging animals into adulthood (around 4 weeks following BrdU injections at the same time point), before sacrifice and assessment of tissue. Quantifying BrdU positive cells, in this tissue, will provide insight into the number of cells that have survived following proliferation at around PD36 (when BrdU entered the system).

Chapter 5: Conclusion

The goal of this research was to determine whether a binge model of postnatal ethanol exposure could induce transient decreases in cellular proliferation in the SGZ and GCL of the DG, using Ki67 and BrdU immunostaining. No significant changes were seen. Additional treatments, postnatal choline supplementation and juvenile chronic aerobic (forced) running were employed to assess whether increases to cellular proliferation and/or attenuation of decreases following ethanol exposure would occur. Forced running produced a significant increase in Ki67 densities, but not BrdU. No changes were seen following choline supplementation, and no synergistic effects were observed following choline supplementation and forced running. Similarly, no significant changes were seen following ethanol exposure and choline supplementation, ethanol exposure and forced running and all three treatments (ethanol exposure, choline supplementation and forced running). However, constraints to the design of this experiment were seen, leading to high variability and small sample sizes of the data analysed. Altogether, this work provided valuable insights into the potential trends seen in the densities of proliferating cells in the SGZ and GCL of the DG, following postnatal binge ethanol exposure, postnatal choline supplementation and juvenile aerobic running, providing a foundation for future research. Future studies should increase the sample sizes used for these statistical tests to ensure that the tests are powered. Additional studies should assess the types of cells that are proliferating, as well as the survival of proliferating cells into adulthood, to continue to provide a further understanding into the mechanisms governing the negative behavioural effects following ethanol exposure and positive behavioural effects following choline supplementation and aerobic running, seen during the peri-adolescent period.

Chapter 6: Appendix

6.1: Demographic Data

Table 6.1: Body Weights (g) of male and female offspring across postnatal development. Body Weight at ethanol intubation (PD4-9). Body Weight at Saline/Choline injection (PD10-30). Body weight at running wheel (PD26-35).

	Sham Saline No Exercise		**Sham Saline Exercise**		**Sham Choline No Exercise**		**Sham Choline Exercise**		**Ethanol Saline No Exercise**		**Ethanol Saline Exercise**		**Ethanol Choline No Exercise**		**Ethanol Choline Exercise**	
Sex	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
n	8	9	7	6	8	9	8	7	8	10	5	5	9	8	6	6
PD 4	11.625 ± 1.93	12.477 ± 1.09	12.114 ± 2.62	12.916 ± 0.97	11.662 ± 2.05	11.788 ± 1.33	12.35 ± 1.50	12.728 ± 1.28	12.112 ± 1.57	13.21 ± 0.50	12.24 ± 1.96	13.66 ± 1.06	12.244 ± 1.00	13.275 ± 1.05	12.15 ± 1.29	12.7 ± 1.74
PD 5	14.212 ± 2.40	14.911 ± 1.02	14.928 ± 3.23	15.666 ± 1.04	14.125 ± 2.49	14.422 ± 1.49	14.9 ± 2.19	15.228 ± 1.92	13.325 ± 1.75	14.34 ± 0.80	13 ± 2.50	14.64 ± 1.46	13.188 ± 1.13	14.637 ± 1.21	13.2 ± 1.91	13.75 ± 1.83
PD 6	16.187 ± 2.83	17.144 ± 1.00	16.742 ± 3.93	17.983 ± 1.12	16.425 ± 3.09	16.577 ± 1.76	17.2 ± 2.98	17.428 ± 2.34	15.125 ± 2.01	16.94 ± 0.64	14.88 ± 2.79	16.62 ± 1.47	14.866 ± 1.92	16.75 ± 1.27	14.75 ± 1.97	15.6 ± 2.24
PD 7	18.737 ± 2.87	19.766 ± 2.07	20.157 ± 4.46	20.733 ± 1.74	18.662 ± 3.38	18.855 ± 1.86	20.112 ± 3.93	20.514 ± 2.68	17.325 ± 2.19	19.56 ± 0.99	17 ± 3.30	19.08 ± 2.12	17.2 ± 1.94	19.412 ± 1.50	16.866 ± 2.55	17.766 ± 2.61
PD 8	21.137 ± 2.84	22.333 ± 1.05	21.957 ± 5.26	23.583 ± 1.55	21.3 ± 3.72	21.533 ± 2.06	22.15 ± 3.99	22.457 ± 3.28	19.562 ± 2.30	21.16 ± 0.96	19.36 ± 3.43	21.82 ± 2.01	19.1 ± 2.08	21.5 ± 1.80	19.166 ± 2.31	20.65 ± 3.05
PD 9	23.562 ± 4.03	25.022 ± 1.10	25.757 ± 5.55	26.533 ± 1.74	24.187 ± 4.22	24.911 ± 2.68	25.612 ± 4.41	25.242 ± 3.84	21.75 ± 2.75	24.81 ± 1.34	21.84 ± 3.97	24.26 ± 1.58	21.522 ± 2.33	24.625 ± 2.29	21.816 ± 2.33	57.616 ± 85.0
PD 10	27.15 ± 3.65	28.555 ± 1.70	28.414 ± 6.65	29.8 ± 1.88	27.15 ± 5.22	27.777 ± 2.80	28.712 ± 5.45	29.071 ± 4.41	24.662 ± 3.01	27.47 ± 1.31	24.38 ± 4.15	27.3 ± 2.47	24.488 ± 2.21	27.275 ± 2.80	24.383 ± 2.84	25.816 ± 3.82
PD 11	29.062 ± 3.74	30.577 ± 2.10	30.171 ± 6.88	32.283 ± 1.95	29.275 ± 5.31	29.9 ± 3.10	31.275 ± 5.70	31.314 ± 4.86	27.087 ± 3.05	30.09 ± 1.23	26.52 ± 4.28	29.68 ± 2.29	27.155 ± 2.39	29.725 ± 3.36	26.4 ± 2.95	28.05 ± 4.07
PD 12	31.6 ± 3.50	33.177 ± 3.15	31.871 ± 6.75	35.033 ± 1.89	31.75 ± 6.14	32.566 ± 3.30	33.875 ± 5.84	33.685 ± 5.08	29.912 ± 3.11	33.28 ± 1.55	29.26 ± 4.53	32.44 ± 2.45	29.888 ± 2.53	32.6 ± 3.12	29.05 ± 3.03	31.066 ± 4.52
PD 13	34.2 ± 4.03	35.911 ± 4.30	33.885 ± 6.87	38 ± 2.06	34.5 ± 6.34	35.277 ± 3.10	36.562 ± 6.27	36.114 ± 2.64	32.725 ± 3.31	35.61 ± 1.60	31.94 ± 4.36	35.2 ± 2.80	32.511 ± 2.55	35.6 ± 3.35	32.883 ± 2.76	33.65 ± 4.58

PD 14	36.8 37 ± 3.88	37.9 11 ± 5.90	36.1 57 ± 7.24	40.3 83 ± 2.55	37.0 5 ± 6.35	37.7 55 ± 2.81	38.8 87 ± 6.63	38.7 28 ± 5.08	35.1 12 ± 3.08	38.2 9 ± 1.97	34.0 8 ± 4.69	37.2 8 ± 3.18	35.2 ± 3.01	38.0 ± 3.22	33.5 ± 2.92	36.0 5 ± 4.89
PD 15	38.9 75 ± 4.16	40.9 66 ± 6.97	37.8 28 ± 7.38	43.1 66 ± 2.80	39.6 ± 6.11	40.5 88 ± 2.97	41.3 62 ± 6.86	41.2 85 ± 5.14	37.6 5 ± 3.64	41.5 3 ± 1.93	36.5 2 ± 4.46	40.0 4 ± 3.87	37.5 77 ± 3.47	40.7 87 ± 3.83	35.5 5 ± 3.23	38.2 66 ± 5.20
PD 16	41.6 37 ± 4.36	43.5 77 ± 8.27	39.6 42 ± 7.40	45.7 66 ± 3.16	42.1 ± 7.03	42.8 77 ± 2.93	43.9 87 ± 7.49	44.2 71 ± 5.41	40.3 25 ± 4.14	44.1 3 ± 1.89	38.8 6 ± 5.38	42.3 4 ± 3.58	40.0 22 ± 4.00	43.5 ± 4.01	37.7 16 ± 3.49	40.5 66 ± 5.25
PD 17	44.0 5 ± 3.86	46.0 88 ± 9.40	42.0 85 ± 7.21	48.2 66 ± 3.28	44.2 37 ± 6.99	45.4 55 ± 3.08	46.7 87 ± 7.51	46.0 28 ± 5.76	42.1 5 ± 3.66	46.6 1 ± 1.58	41.6 6 ± 4.99	44.9 4 ± 4.50	42.0 66 ± 3.87	46.1 12 ± 4.31	40.3 66 ± 3.28	43.3 83 ± 5.59
PD 18	46.8 87 ± 4.55	49.2 33 ± 10.9	44.6 14 ± 8.21	52.0 16 ± 3.52	47 ± 7.99	48.4 11 ± 3.72	50.1 25 ± 8.14	50.4 28 ± 6.36	44.5 75 ± 4.38	50.5 6 ± 1.81	44.3 6 ± 5.65	48.1 4 ± 3.85	45.0 11 ± 4.47	49.3 75 ± 5.29	43.0 33 ± 3.89	46.2 5 ± 6.16
PD 19	50.3 5 ± 4.94	53.7 66 ± 12.2	47.6 14 ± 9.07	56.0 5 ± 3.43	51 ± 8.35	52.5 ± 4.54	54.4 12 ± 9.05	55 ± 7.32	48.5 87 ± 4.54	54.7 1 ± 2.11	48.4 ± 6.36	52.3 8 ± 3.59	48.2 33 ± 4.75	53.6 12 ± 5.84	47.0 66 ± 4.05	50.7 16 ± 6.62
PD 20	54.3 37 ± 5.63	58.4 22 ± 12.9	51.3 57 ± 10.0	60.6 5 ± 4.06	55.1 5 ± 8.96	57.3 55 ± 5.43	59.7 25 ± 10.1	59.7 57 ± 8.68	52.4 ± 5.33	59.6 7 ± 1.76	53.4 8 ± 6.86	57.9 2 ± 2.78	52.0 44 ± 4.67	58.4 5 ± 7.42	51.6 83 ± 3.37	55.3 5 ± 7.50
PD 21	59.8 87 ± 6.52	64.5 22 ± 13.1	56.0 28 ± 15.2	66.2 16 ± 4.36	56.8 25 ± 10.1	62.8 88 ± 5.08	65.2 37 ± 11.0	65.0 71 ± 9.41	57.4 25 ± 5.79	65.6 4 ± 2.31	58.6 ± 7.81	63.3 ± 3.61	56.7 77 ± 5.12	61.0 87 ± 6.51	56.5 5 ± 11.8	61.1 ± 7.23
PD 22	61.6 75 ± 8.46	67.3 66 ± 13.8	58.6 57 ± 13.1	69.7 5 ± 6.24	63.3 25 ± 11.1	66.2 ± 7.36	68.5 75 ± 10.8	66.9 28 ± 11.6	59.4 62 ± 7.33	69.5 3 ± 5.29	61.0 6 ± 9.41	65.8 8 ± 5.87	59.9 66 ± 5.80	68.5 87 ± 8.19	57.6 ± 5.58	63.9 33 ± 7.78
PD 23	66.9 62 ± 8.51	74.5 33 ± 14.9	63.2 14 ± 13.8	75.0 83 ± 7.41	68.4 87 ± 11.7	71.7 66 ± 9.43	73.6 37 ± 11.2	70.9 42 ± 14.7	63.8 37 ± 7.90	74.9 3 ± 6.78	65.5 6 ± 10.3	69.5 6 ± 7.60	65.1 44 ± 6.16	73.8 ± 8.98	61.4 33 ± 7.74	68.2 66 ± 8.03
PD 24	72.5 12 ± 8.74	81.0 44 ± 15.2	68.2 ± 14.2	81.3 33 ± 5.54	74.1 62 ± 11.8	78.6 22 ± 10.8	79.5 ± 11.2	77.8 14 ± 16.2	69.7 25 ± 8.35	80.8 3 ± 4.29	69.9 8 ± 11.5	77.8 4 ± 6.19	70.0 22 ± 6.88	81.0 37 ± 7.96	65.7 16 ± 6.38	75 ± 6.42
PD 25	77.1 25 ± 8.47	86.5 55 ± 14.7	71.7 57 ± 14.4	86.4 83 ± 6.41	79.0 12 ± 11.6	83.2 66 ± 10.9	84.8 37 ± 11.6	84.1 28 ± 17.8	75.1 75 ± 8.74	87.0 6 ± 5.51	74.3 6 ± 11.4	83.4 ± 6.84	74.4 77 ± 7.48	87.3 87 ± 8.24	68.9 5 ± 5.14	80.0 16 ± 6.33
PD 26	81.2 75 ± 10.3	93.6 33 ± 15.8	75.1 85 ± 15.4	92.4 5 ± 7.20	84.0 37 ± 13.4	89.7 ± 9.97	87.6 75 ± 13.9	88.1 42 ± 18.7	79.4 5 ± 10.4	93.0 8 ± 7.49	79.2 ± 12.5	87.5 ± 8.28	78.7 44 ± 8.60	93.7 37 ± 8.17	74.9 83 ± 4.71	85.6 5 ± 6.64
PD 27	86.7 12 ± 11.9	101. 17 ± 16.0	78.6 ± 16.5	98.3 83 ± 7.83	90.5 87 ± 15.0	96.8 77 ± 11.4	92.5 75 ± 12.9	93.4 42 ± 20.1	85.2 75 ± 10.8	100. 87 ± 8.44	82.8 8 ± 12.8	92.5 4 ± 9.53	84.7 33 ± 9.53	101. 66 ± 7.31	80.0 66 ± 6.34	89.9 16 ± 7.01

PD 28	92.6 37 ± 11.1	108. 21 ± 15.9	83.4 28 ± 15.9	102. 23 ± 8.77	96.0 5 ± 14.2	104. 2 ± 9.87	95.8 37 ± 14.0	100. 17 ± 19.2	91.1 62 ± 11.0	108. 63 ± 11.5	87.4 2 ± 12.5	98.6 4 ± 10.2	90.3 33 ± 10.2	108. 52 ± 7.24	85.9 16 ± 7.20	96.1 66 ± 6.34
PD 29	97.5 5 ± 12.0	116. 43 ± 16.5	88.1 14 ± 16.5	110. 28 ± 8.45	101. 51 ± 15.3	111. 98 ± 9.25	101. 26 ± 14.7	106. 41 ± 20.6	95.7 37 ± 11.7	116. 41 ± 11.7	93.0 2 ± 13.7	106. 66 ± 12.2	95.7 ± 10.2	115. 73 ± 8.30	91.8 ± 8.38	103. 71 ± 5.93
PD 30	103. 82 ± 13.4	123. 93 ± 16.0	92.2 28 ± 17.0	116. 66 ± 8.17	108. 07 ± 15.7	119. 62 ± 8.81	106. 3 ± 14.7	112. 77 ± 21.4	103. 02 ± 14.0	124. 31 ± 13.9	97.3 4 ± 14.6	113 ± 14.7	102. 87 ± 11.7	124. 67 ± 7.68	96.7 83 ± 9.02	109. 46 ± 6.26
PD 31	106. 3 ± 14.8	121. 54 ± 16.4	98.2 8 ± 13.6	124. 8 ± 9.23	121. 1 ± 11.1	129. 2 ± 10.0	113. 26 ± 8.19	123. 24 ± 21.4	108. 75 ± 21.2	125. 3 ± 16.9	100. 85 ± 12.2	119. 05 ± 17.1	108. 05 ± 11.4	129. 44 ± 3.67	98.9 4 ± 10.5	119. 22 ± 5.43
PD 32	115. 18 ± 11.7	138. 2 ± 15.7	101. 08 ± 13.6	131. 64 ± 7.07	128. 45 ± 12.9	137. 8 ± 10.4	120. 5 ± 8.85	131. 14 ± 22.3	113. 3 ± 15.7	132. 88 ± 20.4	106. 5 ± 13.3	127. 57 ± 21.2	111. 85 ± 11.7	137. 6 ± 4.37	103. 9 ± 12.4	126. 48 ± 4.64
PD 33	121. 6 ± 12.9	146. 52 ± 16.3	108. 24 ± 15.2	140. 9 ± 8.75	135. 35 ± 11.9	146. 02 ± 11.6	128. 96 ± 10.4	140. 34 ± 24.9	122. 67 ± 16.3	143. 76 ± 22.2	111. 32 ± 13.5	136. 45 ± 22.9	120. 7 ± 13.4	147. 36 ± 5.19	110. 98 ± 12.7	135. 9 ± 4.71
PD 34	128. 58 ± 13.2	155. 2 ± 17.1	114. 12 ± 15.9	149. 84 ± 10.0	141. 95 ± 12.2	156. 14 ± 11.5	139. 05 ± 8.98	148. 72 ± 25.3	129. 15 ± 18.8	153. 22 ± 23.1	116. 85 ± 13.9	145. 25 ± 23.9	127. 8 ± 13.8	157. 46 ± 6.14	118 ± 13.9	142. 78 ± 5.21
PD 35	135. 38 ± 13.5	167. 92 ± 15.5	120. 02 ± 14.5	157. 88 ± 8.78	150. 17 ± 14.1	166. 42 ± 12.4	142. 1 ± 7.40	156. 58 ± 26.2	134. 85 ± 18.9	162. 66 ± 25.0	122. 87 ± 15.1	154. 85 ± 24.7	132. 15 ± 15.3	166 ± 5.42	122. 16 ± 15.0	152. 28 ± 6.26

6.2 BrdU Cell Counts and Area Measurements

Table 6.2: Average BrdU cell counts and sample sizes (n) in dorsal, ventral and combined slices across treatments

Treatment Group	Dorsal		Ventral		Combined	
	Cell Count	n	Cell Count	n	Cell Count	n
Control	51.98	7	56.00	7	53.99	7
Ethanol	57.86	9	58.70	8	58.26	9
Choline	70.57	6	77.89	5	73.90	6
Exercise	73.13	4	71.43	3	72.40	4
Choline Exercise	59.45	7	60.46	5	59.87	7
Ethanol Choline	73.03	4	59.28	5	65.39	5
Ethanol Exercise	83.36	5	91.37	6	87.72	6
Ethanol Choline Exercise	54.01	8	58.67	7	56.18	8

Table 6.3: Average BrdU area measurements and sample sizes (n) in dorsal, ventral and combined slices across treatments

Treatment Group	Dorsal		Ventral		Combined	
	Area Measurement (μm^2)	n	Area Measurement (μm^2)	n	Area Measurement (μm^2)	n
Control	226116.89	7	269756.65	7	247936.77	7
Ethanol	205163.42	9	261177.94	8	231523.19	9

Choline	243967.53	6	337477.01	5	286471.84	6
Exercise	228695.63	4	278234.98	3	249926.78	4
Choline Exercise	239528.41	7	287732.32	5	259613.37	7
Ethanol Choline	220767.79	4	248217.91	5	236017.86	5
Ethanol Exercise	202538.03	5	264641.94	6	236412.89	6
Ethanol Choline Exercise	219775.70	8	259325.46	7	238232.26	8

6.3 Ki67 Cell Counts and Area Measurements

Table 6.4: Average Ki67 cell counts and sample sizes (n) in dorsal, ventral and combined slices across treatments

Treatment Group	Dorsal		Ventral		Combined	
	Cell Count	n	Cell Count	n	Cell Count	n
Control	144.357143	7	124.430952	7	134.394048	7
Ethanol	135.37619	7	163.297619	7	149.336905	7
Choline	174.1125	6	152.980556	6	171.395139	6
Exercise	141.816667	6	168.677778	6	147.398611	6
Choline Exercise	175.577778	6	170.047222	6	172.8125	6
Ethanol Choline	135.055556	6	123.652778	6	129.354167	6
Ethanol Exercise	221.033333	5	204.183333	5	212.608333	5
Ethanol Choline Exercise	117.916667	5	134.19	5	126.053333	5

Table 6.5: Average Ki67 area measurements and sample sizes (n) in dorsal, ventral and combined slices across treatments

Treatment Group	Dorsal		Ventral		Combined	
	Area Measurement (μm^2)	n	Area Measurement (μm^2)	n	Area Measurement (μm^2)	n
Control	211829.636	7	231306.335	7	221567.986	7
Ethanol	191015.766	7	216858.73	7	203937.248	7

Choline	229742.536	6	246318.772	6	238030.654	6
Exercise	201776.024	6	203303.578	6	202539.801	6
Choline Exercise	215918.112	6	224100.88	6	220009.496	6
Ethanol Choline	203665.441	6	235489.989	6	219577.715	6
Ethanol Exercise	205564.076	5	203314.892	5	204439.484	5
Ethanol Choline Exercise	198388.098	5	237548.409	5	217968.254	5

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