Postnatal Choline Supplementation Ameliorates Synaptic Plasticity Deficits Following Prenatal Ethanol Exposure in a Sex-Specific Manner

By

Erin Lisalotte Gräfe

B.Sc., McGill University, 2017

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Division of Medical Sciences (Neuroscience)

Postnatal Choline Supplementation Ameliorates Synaptic Plasticity Deficits Following Prenatal Ethanol Exposure in a Sex-Specific Manner

By

Erin Lisalotte Gräfe

B.Sc., McGill University, 2017

Supervisory Committee

Dr. Brian R. Christie, Supervisor

Division of Medical Sciences

Dr. Raad Nashmi, Departmental Member

Department of Biology

Dr. Tamara Bodnar, Outside Member

University of British Columbia

Abstract

Background: Fetal Alcohol Spectrum Disorder (FASD) is one of the leading causes of neurodevelopmental impairment. FASD is the diagnostic term to encompass the range of physical, cognitive, or emotional impairments due to prenatal ethanol exposure (PNEE). One of the most notable consequences of PNEE are deficits in hippocampal synaptic plasticity, and consequently, learning and memory impairments. Currently, there is no treatment for FASD. However, there are promising data to demonstrate that the essential nutrient choline may improve outcomes following developmental ethanol exposure. Questions remain as to how postnatal choline supplementation improves hippocampal outcomes at a synaptic level, whether these occur in a sex-dependent manner, and if any changes will persist into adulthood.

Methods: This dissertation employed a first-two trimester moderate model of PNEE (Gestational day 1-22). Offspring were supplemented with choline chloride (100 mg/kg/day) from postnatal day (PND) 10-30, then tested either immediately following treatment (PND 31-36) or in adulthood (PND 60-90). In juvenile offspring bidirectional plasticity was evaluated, as well as behavioural changes using the Radial Arm Maze. In adulthood, saturating and subthreshold long-term potentiation (LTP) was evaluated, as well as alterations in GluN2B functionality.

Results: PNEE reduced the magnitude of LTP in male juvenile offspring, but not in females. Choline treatment increased LTP in both male and female PNEE offspring. However, choline treatment insignificantly decreased the amount of long-term depression (LTD) in male offspring, regardless of prenatal environment. Improvements in PNEE male LTP did not translate to behavioural improvements in the Radial Arm Maze, either in the working or reference memory performance. Female juvenile offspring did not learn the task over the course of the five trials and this lack of learning was not due to differences in search strategy. In adulthood, there were no

evident changes in LTP with PNEE or with choline treatment in either sex. Despite the lack of deficit or improvement, choline treatment altered the LTP threshold, such that lower frequency stimulating protocols still resulted in LTP in choline treated offspring. While it is not clear why this change in LTP threshold occurred, it could be due to alterations in GluN2B functionality; GluN2B antagonism increased field excitatory postsynaptic potential (fEPSP) size in control offspring, but not in saline treated PNEE adults.

Conclusion & Significance: This dissertation demonstrated that postnatal choline supplementation ameliorated the deficits in LTP in PNEE males and further increased LTP in PNEE females. However, these changes in synaptic plasticity did not persist in adulthood when using a saturating conditioning stimulus. There may still be alterations in hippocampal LTP threshold after treatment cessation, but the exact locus of action remains to be uncovered. These data further support choline as a treatment for PNEE, but also suggest that extended choline treatment may produce more long-lasting changes.

Table of Contents

List of Figures

Introductory Figures

Methodological Figures

Results

**Note: the introductory, methods, and conclusion figures were generated using biorender.com*

List of Tables

Acknowledgements

There are many people I would like to acknowledge for their role and influence in my graduate studies. First and foremost, I would like to thank Dr. Brian Christie. You like to mention at times the role of serendipity in research and I feel like this also came into play for how I found myself in the Christie Laboratory – having to interview from the University of Otago library, the place of your own PhD studies because I needed the free Wi-Fi while living abroad. Whether that was the reason you took the chance on me as a student I'll never know, but I will always appreciate that fortunate coincidence. I truly appreciate your mentorship throughout my time in the DMS; the patience while I was finding my footing in the first couple of years, to the confidence you've demonstrated in my abilities by the end of my degree. I have grown so much, both personally and professionally, and I am grateful to have found my way in an environment that allowed me to make mistakes and allowed me to learn from them.

I would also like to wholeheartedly thank my many past laboratory mentors. Dr. Cristina Pinar – thank you for being a strong feminist force in the department. You showed me how to stand up for what I believed in and kindled my passion for advocating for women in science. Dr. Juan Triviño Paredes – thank you for always being kind and supportive of my work. Your words of encouragement always meant so much when I was doubting myself. To Dr. Luis Bettio – thank you for answering all my electrophysiology questions. I appreciate all of the heartfelt and reminiscent conversations about laboratory life and beyond. To Dr. Jon Thacker – I'm not sure if you belong in this list, because you will never quite be a 'past' mentor, as I will probably still be asking you for mentorship advice after I graduate. Thank you for sending me jobs that are spot on for what I've said I want in a career and for all of our discussions on important issues in STEM. And finally, to Dr. Christine Fontaine. You are one of the most driven, kind, and exceptional scientists that I have ever met, and I am beyond grateful to have been working on the FASD project with you. Your mentorship and friendship was perhaps the most impactful influence in my graduate life.

Next I'd like to acknowledge the Christie lab mates who kept me sane as I figured out how to wrap up my studies. Hannah, thank you for adopting me as your introvert friend and enriching my life in so many ways. I am beyond excited to see how you absolutely take the world by storm. Taylor, you are such a positive and empathetic influence. You inspire me in so many ways and I

am grateful to have gotten to know you better over the last few years. Irene, you are one of the most capable and natural scientists that I have ever seen enter into the MSc program. Thank you for being my friend through the highs and lows. Allyson, my fantastic mentee. I'm glad that we got to bond over the struggles that are *in vitro* electrophysiology. There are few people that know exactly what that is like. To the rest of the Christie laboratory members, thank you for all the support and kindness over the years.

A shout out to all the Neuroscience colleagues who made this journey special. Especially to Jenessa and Alejandra, my NGSA team. I'm sorry that I mostly just showed up to quote the handbook and make some stubborn/sarcastic statements in our important meetings. You guys were both such shining lights in those COVID years. And to Carla, Penny, Essie, Katie, Laura, and all the people who were for advice or to just to unwind over a beer – thank you.

Finally, I want to thank my family for always being interested in my research (even if it didn't always make sense) and being so incredibly supportive. You guys always made me believe I could do anything if I set my mind to it.

Last but certainly not least, thank you to my partner Alex. There have been difficult times in this journey, but you have always been there to pick me up off the floor (both figuratively and literally). You know me better than anyone else, you love me unconditionally, and your belief in my abilities carried me through when I lost sight of them myself. I can't wait to build a life with you. I love you to the moon and back.

Dedication

This dissertation is dedicated to my Nannie, Ms. Kathryn Eaton.

You have loved, supported, and cheered me on at every step throughout my PhD.

I hope this dissertation makes you proud.

CHAPTER 1: INTRODUCTION

1.1 Fetal Alcohol Spectrum Disorder (FASD)

Fetal Alcohol Spectrum Disorder (FASD) is the clinical term to encompass the range of cognitive, social, and physical consequences when exposed to alcohol in the womb. The term '*fetal alcohol syndrome*' (FAS) was first characterized in the 1970s by American doctors Jones and Smith (Jones and Smith, 1973; Brown *et al.*, 2019) and in the decades to follow the term FASD was issued to include the less severe partial FAS (pFAS), alcohol-related birth defects (ARBD), and alcohol related neurodevelopmental disorder (ARND) (Manning and Eugene Hoyme, 2007; Chudley, 2018). These diagnostic terms are characterized based on the continuum of physical and cognitive symptoms, including facial abnormalities (short palpebral fissures, thin upper vermilion border, and smooth philtrum), growth retardation, cognitive disabilities, and often require confirmation of alcohol consumption during pregnancy (Manning and Eugene Hoyme, 2007).

The psychosocial factors surrounding alcohol consumption during pregnancy and FASD are complex. It has been well established that alcohol is teratogenic to the developing brain, yet alcohol consumption during pregnancy has remained at approximately 10% for several decades (Denny *et al.*, 2019). While it might appear straightforward to place the burden of responsibility on the pregnant individual, many factors contribute to the steady prevalence of FASD, including domestic abuse, violence, and inadequacies in birth control or sex education to prevent unwanted pregnancies (Skagerström, Chang and Nilsen, 2011; Walker *et al.*, 2011). Despite there being evidence for alcohol exposure in around 1 in 10 pregnancies, current estimates of FASD in North America range from 1-5% (May *et al.*, 2009; Popova *et al.*, 2017, 2019; Flannigan, Unsworth and Harding, 2018), highlighting the nonlinear relationship between alcohol consumption and FASD diagnosis. There are several factors that may make some pregnancies, and not others, vulnerable

to prenatal ethanol exposure, including the pattern of consumption (i.e. binge versus continuous), developmental timing, the medical history of the pregnant individual, their metabolism/nutrition, and genetic profile (May and Gossage, 2011). Take for instance the pattern of consumption. Seminal work by Bonthius and West (1990) exposed rodent offspring from postnatal day (PND) 4-10 with the alcohol in three unique patterns: two doses of ethanol (4.5 g/kg/d) two hours apart, four doses of ethanol (4.5 g/kg/d) over eight hours, or 12 exposures (6.6 g/kg/d) over the course of 24 hours. As expected, peak blood alcohol content was highest when the alcohol was provided in a higher concentration and decreased in a dose-dependent manner. Similarly, brain weight decreased as a measure of blood alcohol content and thus pattern of exposure, demonstrating that *how* alcohol is consumed is important when considering teratogenic outcomes (Bonthius and West, 1990). There are also social factors to be taken into consideration with the estimated prevalence of FASD, namely the high levels of stigma and shame associated with FASD that could prevent an individual from admitting to consuming alcohol (May and Gossage, 2011), the financial cost of receiving a professional diagnosis (Popova *et al.*, 2013), and lack of education or discomfort in assigning a FASD diagnosis from medical professionals (Petrenko *et al.*, 2014; Howlett *et al.*, 2019). Both the biological and social factors make it difficult to achieve a true estimate of the impact of prenatal alcohol exposure.

Individuals with FASD experience a myriad of lifelong cognitive impairments that can include issues with attention, learning and memory, problem solving, and decreased general intelligence (Mattson, Bernes and Doyle, 2019). These neurodevelopmental consequences often lead to secondary dysfunction. The majority of individuals with FASD have comorbid mental health disorders (Pei *et al.*, 2011), are ten times more likely to have attention deficit hyperactivity disorder (ADHD) (Weyrauch *et al.*, 2017), disproportionately enter foster care (Tenenbaum *et al.*, 2020),

and face more difficulties in the legal system (Fast and Conry, 2009). Alcohol use during pregnancy should not be considered completely preventable, as evidenced by the consistent consumption rates and the complex social influences surrounding the decision to consume alcohol during pregnancy. As even low amounts of ethanol exposure can lead to lifelong social and cognitive difficulties, it is of increased importance to investigate treatments for FASD.

1.1.1 Ethanol, Metabolism & the Placenta

Ethanol has a simple structure that allows it to diffuse into all the cells of the body and have pervasive effects. The ethanol molecule is polar due to the hydroxyl (-OH) group, yet lipophilic due to the short carbon chain (C_2H_5). This unique combination of structural properties is the reason ethanol can pass through lipid membranes, including through the blood brain barrier and placenta

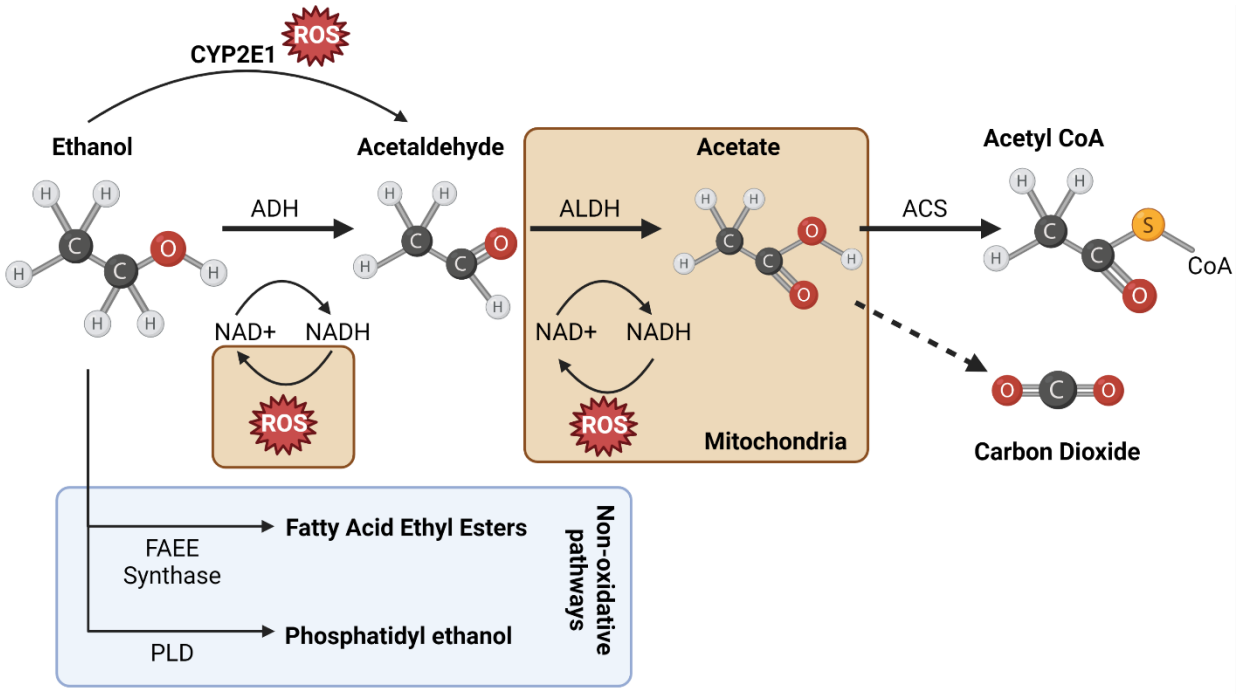


Figure 1 Ethanol Metabolism. Ethanol is metabolized through either (1) an oxidative pathway into acetaldehyde, acetate and finally acetyl-CoA or carbon dioxide; or (2) a non-oxidative pathway, generating FAEE and phosphatidyl ethanol. ROS (Reactive oxygen species), ADH (alcohol dehydrogenase), CYP2E1 (cytochrome P450 2E1), NAD (nicotinamide adenine dinucleotide), ALDH (aldehyde dehydrogenase), ACS (Acetyl CoA synthase), FAEE (fatty acid ethyl ester), PLD (phospholipase D).

(Little and VanBeveren, 1996), without requiring a specific receptor or other form of transporter. Therefore, when alcohol is consumed during pregnancy, the fetus quickly reaches similar blood alcohol concentrations as the maternal blood (Idänpään-Heikkilä *et al.*, 1972; Espinet and Argilés, 1984).

Ethanol is metabolized through three main pathways (**Figure 1**) (Zakhari, 2006). The major ethanol metabolism pathway involves the oxidation of ethanol into acetaldehyde by the enzyme alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD+). During high levels of alcohol consumption, however, ethanol is metabolized by the cytochrome P450 2E1 enzyme (CYP2E1) into acetaldehyde. Acetaldehyde is then further oxidized to acetate by acetaldehyde dehydrogenase (ALDH) and finally into acetyl-CoA through acetyl-CoA synthetase (ACS) or into carbon dioxide. Ethanol can also be broken down through non-oxidative pathways using fatty acid ethyl ester (FAEE) synthase to generate FAEEs or through phospholipase D to generate phosphatidyl ethanol (Zakhari, 2006; Cederbaum, 2012). The generation of FAEEs is translationally relevant, as it can be used as a biomarker of fetal alcohol exposure, such as in the meconium, following birth (Ostrea *et al.*, 2006). The molecular damage caused by ethanol does not occur through the ethanol molecule alone, but also through the products generated as it is metabolized. Ethanol metabolic pathways generate reactive oxygen species (ROS), either directly in the CYP2E1 pathway or indirectly through the regeneration of NAD+, and acetaldehyde has been shown to cause similar morphological impairments to ethanol (Shabtai *et al.*, 2018).

During prenatal development the fetus does not yet fully possess the enzymes required to metabolize alcohol. The fetal liver is thought to function at 5-10% of the adult capacity (Pikkarainen and RäIhä, 1967; Pikkarainen, 1971); ADH is at approximately a quarter of the adult capacity at birth and only reaches full functionality in the postnatal period (Räihä, Koskinen and

Pikkarainen, 1967; Zorzano and Herrera, 1989) and CYP2E1 begins to be present in the second trimester but is not near adult levels until several months after birth (Johnsrud *et al.*, 2003). Furthermore, while the placenta does contain ethanol metabolizing enzymes, the clearance rate of the placenta is relatively low (Burd, Blair and Dropps, 2012). This results in amniotic accumulation of ethanol (Guerri and Sanchis, 1985), prolonging the exposure of ethanol to the fetus even after alcohol is no longer detected in the maternal blood (Tranmer, 1985). Therefore, the fetus is reliant on the metabolic clearance of the pregnant individual.

1.1.2 Animal Models of FASD

Many animal models of FASD have been developed to further understand the associated neurodevelopmental changes. These paradigms include the liquid diet model, gavage, vapor exposure, and injection. Each model has its own advantages and translational barriers. A commonly used model, and the model used in this dissertation, is the liquid diet model (Lieber and DeCarli, 1982). This model administers an ethanol-containing diet throughout gestation and results in moderate blood alcohol concentrations (BAC; 80-180 mg/dl). A potential limitation of this design is that there may be nutritional deficiencies due to the change in diet (Patten, Fontaine and Christie, 2014). The change to a liquid diet is often controlled for by adding in a pair fed diet control which is fed an equivalent number of liquid calories per body weight of a non-ethanol containing diet. However, it has been recognized that the pair fed diet likely induces an additional stress due to the food restriction and produces a phenotype unique to that of PNEE (Weinberg *et al.*, 2008). Additionally, nutritional deficits are a consequence of chronic alcohol consumption, as alcohol interferes with either the availability or absorption of many key nutrients including vitamin A, zinc, DHA, folic acid, and choline (Young *et al.*, 2014). A second commonly used model is the gavage ethanol administration. This technique involves the insertion of a thin, flexible tube into

the stomach of newborn pups and results in relatively a high BAC (up to approximately 400 mg/dl), due to the lack of fetal alcohol enzymes discussed above. However, this technique is stressful and may involve the pup being reared artificially, with the pup-in-a-cup method (Patten, Fontaine and Christie, 2014). Finally, researchers have implemented vapor exposure and injection models. These techniques allow for precise BACs and controlled timing, however do not model translational methods of ethanol consumption (Patten, Fontaine and Christie, 2014). There is no model of FASD that will encompass all aspects of the human condition; each model provides additional and valuable insight into the mechanisms behind developmental ethanol exposure.

1.1.3 Effects of Ethanol are Impacted by Stage of Brain Development

The specific deficits associated with perinatal ethanol exposure are dependent on the timing of ethanol exposure in relation to the stage of brain development. Human and rats follow similar developmental trajectories, albeit with markedly different durations (Rice and Barone, 2000). The beginning of nervous system development occurs at neurulation, in which the ectoderm invaginates to form the neural tube and neural crest. This process occurs around GD 10.5–11 in rodents and within the first gestational month in humans (Rice and Barone, 2000). As the neural crest cells are heavily involved in the formation of the craniofacial bones (Dubey and Saint-Jeannet, 2017), alcohol exposure immediately preceding (i.e. GD 7-9) can result in the facial abnormalities characteristic of FAS (Sulik, 2005). Subsequently, the neural tube begins differentiating and expanding into the prosencephalon, mesencephalon, and rhombencephalon; these areas will become the forebrain, midbrain, and hindbrain, respectively (Stiles and Jernigan, 2010).

The developmental timing of the hippocampus confers specific vulnerability to ethanol exposure. The *cornu ammonus* (CA) region begins to form during GD 15-20 in rats and weeks 7-12 in humans, while the dentate gyrus (DG) forms from GD 19 into early postnatal development,

or weeks 12 to 40 in humans. These timelines are followed by a rapid growth period from postnatal day (PND) 1-10 (Rice and Barone, 2000). These dates are of particular importance when considering the behavioural and functional consequences of developmental ethanol exposure on the hippocampus. Ethanol exposure only during the second trimester-equivalent period reduced DG long-term potentiation (LTP) (Helfer, White and Christie, 2012), which was hypothesized to be due to an insult to the granule cell progenitors. Deficits in synaptic plasticity due to third trimester equivalent exposure are more variable (Bellinger *et al.*, 1999; Izumi *et al.*, 2005; Puglia and Valenzuela, 2010; Helfer, White and Christie, 2012; Anna R. Patten, Brocardo, *et al.*, 2013) and will be discussed at length in section *'1.2.9 Changes in Synaptic Plasticity with PNEE'*. Similarly, timing of developmental ethanol exposure correlates with behavioural changes (Reviewed in Schneider, Moore and Adkins, 2011). When offspring were exposed in either the prenatal period (GD 8-20) or in the third trimester equivalent period (PND 4-9) and tested on a spatial discrimination task, juvenile male offspring were impaired but only if they were exposed during neonatal development (O'Leary-Moore *et al.*, 2006). Conversely, ethanol exposure during either the first or second trimester resulted in increased locomotive and anxiety-like behaviours, which were not evident with third trimester equivalent exposure (Mantha, Kleiber and Singh, 2013). These parameters are critical when considering the heterogeneity of preclinical PNEE data and when considering the efficacy of any treatments to human populations with FASD.

1.1.4 Developmental Ethanol Exposure Alters the Brain

When ethanol reaches the brain, it can quickly pass through lipid membranes and alter a myriad of processes within the neuron (**Figure 2**). Ethanol increases the generation of reactive oxygen species (ROS), alters the functioning of many receptors or channels, causes cell death, disrupts the

methylation signature, and impacts immune and supportive cells (Reviewed in (Fontaine *et al.*, 2016)).

Part of the etiology of prenatal ethanol exposure involves the formation of ROS (Brocardo, Gil-Mohapel and Christie, 2011). ROS are unstable and reactive molecules containing an oxygen and an unpaired electron, such as hydrogen peroxide (H_2O_2). ROS are naturally occurring in the cell, such as in the mitochondrial electron transport chain, but result in oxidative stress when the levels of ROS are at an imbalance of the antioxidant capacities of the cell. The unstable nature of ROS results in broad damage to all molecules, including DNA, RNA, lipids, and proteins (Auten and Davis, 2009). As ethanol metabolism leads to the generation of ROS (see *1.1.1 Ethanol, Metabolism & the Placenta*), a marked oxidative imbalance is characteristic of prenatal ethanol exposure (PNEE) (Henderson *et al.*, 1995; Brocardo *et al.*, 2012). Indeed, many treatment options have arose from the oxidative stress model of FASD,

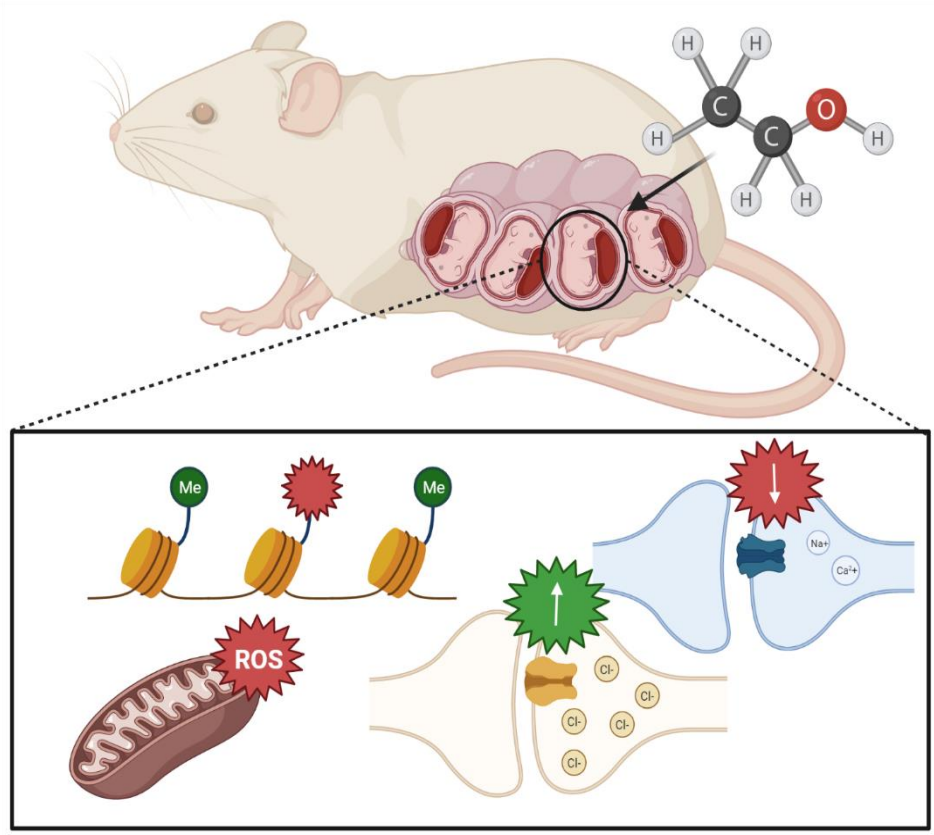


Figure 2 PNEE disrupts a myriad of processes in the brain. Ethanol can readily cross through the placenta where it disrupts neural development. These impairments include altering the methylation signature in the epigenome, generating reactive oxygen species (ROS), as well increasing GABA receptor activity (yellow neuron) an decreasing NMDA receptor function (blue).

including supplementing omega-3 fatty acids (Anna R. Patten, Sickmann, *et al.*, 2013; Patten, Brocardo and Christie, 2013), vitamin E (Marino, Aksenov and Kelly, 2004; Shirpoor *et al.*, 2009), resveratrol (Kumar *et al.*, 2011), and epigallocatechin-3-gallate (Almeida-Toledano *et al.*, 2021).

Ethanol can also modulate the functioning of many receptors and channels which can contribute to long-lasting alterations in synaptic plasticity (Fontaine *et al.*, 2016). Perhaps the most notable example of the modulatory role of ethanol is on the $GABA_A$ receptor. Ethanol can directly bind to the $GABA_A$ receptor (reviewed in Förstera *et al.*, 2016), with specific sensitivity to the δ-subunit (Wallner, Hanchar and Olsen, 2003). Ethanol has been considered a GABAmimetic, as binding can stimulate $GABA_A$ receptors, as well as increase channel open time and frequency (Suzdak *et al.*, 1986; Tatebayashi, Motomura and Narahashi, 1998), GABA release is excitatory during gestational development and into the first postnatal week in rodents (Valeeva, Valiullina and Khazipov, 2013). The depolarizing actions of the $GABA_A$ receptor occurs until there is an upregulation of the KCC2 transporter (the potassium chloride cotransporter 2), known as the 'GABA switch' (Rivera *et al.*, 1999). Until this developmental timepoint there is a relatively increased chloride ion (Cl^-) concentration in the intracellular space, therefore Cl^- will exit the cell down its electrochemical gradient causing depolarization (Valeeva, Valiullina and Khazipov, 2013). While ethanol-induced activity of $GABA_A$ receptors during gestational development could lead to excitotoxicity, this is unlikely as ethanol also inhibits NMDA receptors (Lovinger, White and Weight, 1990; Wright, Peoples and Weight, 1996). Ethanol acts in a non-competitive manner to decrease NMDA receptor activity, specifically the frequency and duration of channel opening (Wright, Peoples and Weight, 1996). Ethanol can also modulate the activity of nicotinic acetylcholine (nACh) receptors, although the directionality is dependent on the subunits present (Aistrup, Marszalec and Narahashi, 1999). For instance, α7 nACRs, which are α-bungarotoxin

sensitive, are inhibited by ethanol (Yu *et al.*, 1996; Aistrup, Marszalec and Narahashi, 1999; Oz *et al.*, 2005) while α-bungarotoxin insensitive nACh receptors (likely α4β2-containing receptors) were potentiated by ethanol exposure (Aistrup, Marszalec and Narahashi, 1999). Additionally, while ethanol is known to inhibit voltage-gated calcium channels in neuronal culture (Walter and Messing, 1999), during the embryonic period ethanol potentiates L-type voltage-gated calcium channels which impedes neuronal migration (Lee, Yeh and Yeh, 2021). Finally, ethanol can potentiate serotonin (5HT-3) receptors (Yu *et al.*, 1996), which may contribute to anxiety-like behaviours later into adulthood in ethanol-exposed offspring (Oubraim *et al.*, 2022).

Prenatal ethanol exposure can also lead to cell loss, including in the hippocampus (Reviewed in Gil-Mohapel *et al.*, 2010), cerebellum (Chen, Berryhill and West, 2001; Klintsova *et al.*, 2002) and prefrontal cortex (Mihalick *et al.*, 2001). This cell loss is partly due to the increase in oxidative stress (Ramachandran *et al.*, 2001) but also due to the combined stimulation of $GABA_A$ receptors and inhibition of NMDA receptors (Ikonomidou *et al.*, 2000). While ethanol-induced neurodegeneration is widespread, there are specific spatial and temporal windows of vulnerability. For instance, the thalamus and hypothalamus are more sensitive to ethanol exposure around the end of the gestational period, damage in the hippocampus peaks around PND 3, and cortical neurodegeneration peaks around PND 7 (Ikonomidou *et al.*, 2000). Despite cell death being evident in all developmental ethanol exposure, the third trimester-equivalent brain growth spurt is regarded as a period of particular vulnerability (Ikonomidou *et al.*, 2000; Olney *et al.*, 2000). This neurodegeneration is not thought to occur due to excitotoxicity, but the lack of synaptic input to developing neurons signals acts as an apoptotic cue (Olney *et al.*, 2002). Interestingly, the CA1 subregion of the hippocampus is often more affected by ethanol-induced cell loss than the DG (Barnes and Walker, 1981; Miller, 1995; Tran and Kelly, 2003). This may be due to the capacity

for neurogenesis in the DG. A recent systematic review by Reid et al. (2020) supports this hypothesis, as it did not find convincing evidence for a negative effect of perinatal ethanol exposure on neurogenesis (Reid *et al.*, 2020).

Prenatal ethanol exposure also has the potential to disrupt the methylation signature of the epigenome (Zeisel, 2011; Mandal *et al.*, 2017). Ethanol ingestion can interfere with many components of the 1-carbon metabolism cycle, which generates methyl groups. Ethanol impairs folate uptake in the digestive tract (Hamid and Kaur, 2009), directly inhibits 1-carbon metabolism enzymes (Kenyon, Nicolaou and Gibbons, 1998), and reduces glutathione content (Patten, Brocardo and Christie, 2013). While there are many ways in which ethanol disrupts methylation, the data examining disruptions in the epigenetic signature in PNEE have many inconsistencies that make overall trends difficult to assess (Bestry *et al.*, 2022). A more broad examination of ethanol-induced alterations point to a decrease in methylation (Garro *et al.*, 1991), but regional and gene-specific examinations detail the complexity of PNEE. For instance, Otero and colleagues unexpectedly found that postnatal ethanol exposure induced a *hyper*methylation in the hippocampus and prefrontal cortex (Otero *et al.*, 2012). Examining changes at the epigenetic level demonstrated that embryos bathed in an ethanol-containing medium had an approximately equivalent number of genes hypomethylated and hypermethylated (Liu *et al.*, 2009; Alberry and Singh, 2020). The consequences of high-level analysis of complex methyl alterations are difficult to discern, therefore it may be more useful to focus on individual genes of importance, such as the *insulin-like growth factor 2* (*Igf2*) gene which is involved in the proliferation and survival in neuronal development (D'Ercole *et al.*, 1996). In this instance, binge ethanol exposure resulted in hypomethylation of the *Igf2* gene and a subsequent reduction in mRNA transcript expression (Downing *et al.*, 2011).

The alterations in the methylation signature are so profound that several groups have examined this area of study to develop a potential biomarker for FASD. An advantage of epigenetics to traditional biomarkers is that they can be utilized long past when FAEEs are found in nails, hair, and meconium (Portales-Casamar *et al.*, 2016; Lussier *et al.*, 2018; Cobben *et al.*, 2019). A model by Lussier et al. (2018) analyzing the epigenetic signature was able to predict FASD diagnosis with a sensitivity of 0.877 and a specificity of 0.944. Additionally, this model was almost completely successful in distinguishing between children with FASD and those with autism spectrum disorder (Lussier *et al.*, 2018), which would have great translational applications as these neurodevelopmental disorders have many overlapping symptoms. While these studies show great promise in the eventual use of epigenetics as a biomarker for FASD, there are still limitations. For instance, Cobben et al. (2019) similarly identified loci distinct in FASD, but very few overlapped with previous reports (Cobben *et al.*, 2019). Lastly, a separate study analysing methylation in maternal alcohol consumption, although not necessarily resulting in FASD, did not find a correlation between groups (Sharp *et al.*, 2018). These studies once again highlight the stochastic nature of FASD and the complex influence of environment, maternal factors, timing, and dose on establishing a global biomarker.

Finally, it is important to also consider the effects on glial cells within the brain, which are likewise impacted by developmental ethanol exposure (Wilhelm and Guizzetti, 2016). Microglia are mesodermal-derived, formed in the yolk sac, and migrate into the developing brain early in gestational development (Tay *et al.*, 2017). These critical immune cells in the brain replicate infrequently (Réu *et al.*, 2017), and therefore an early life insult may have long-lasting effects on neuronal immune function. While total number of microglia are not reduced following developmental ethanol exposure, more subtle properties are impacted (Komada *et al.*, 2017). These

include an increase in pro-inflammatory cytokine release, a shift towards a more activated state, and an exaggerated response to a secondary immune challenge (Drew *et al.*, 2015; Terasaki and Schwarz, 2016; Komada *et al.*, 2017). Astrocytes also have a critical role in the brain, they form part of the tripartite synapse, process neurotransmitter, maintain the blood brain barrier, and contribute to energy homeostasis (Perea, Navarrete and Araque, 2009; Sofroniew and Vinters, 2010). PNEE alters astrocyte reactivity, as evidenced by a regionally-specific increase (Goodlett *et al.*, 1993; Topper, Baculis and Valenzuela, 2015) or decrease (Vallés *et al.*, 1997; Li *et al.*, 2021) in glial fibrillary associated protein (GFAP). However, an increase in astrogliosis could be a protective measures against the ethanol insult (Watts *et al.*, 2005).

The acute molecular consequences of developmental ethanol exposure culminate with long-term effects at an anatomical level. PNEE causes a temporary reduction in spine density in adolescent development, but not in adulthood (Reyes *et al.*, 1983; Pentney, Cotter and Abel, 1984; Lopez-Tejero *et al.*, 1986; Galofré *et al.*, 1987; Berman *et al.*, 1996), and decreases dendritic complexity (Whitcher and Klintsova, 2008; Hamilton, Whitcher and Klintsova, 2010; Rice *et al.*, 2012). The combination of the consequences of developmental ethanol exposure converges on severe functional deficits, which are discussed in more detail below.

1.1.5 Sex Differences with Prenatal Ethanol Exposure & FASD

Perhaps one of the most intriguing factors surrounding developmental disorders is that of biological sex (Summarized in **Figure 3**). Many neurodevelopmental disorders, including autism and ADHD, appear to predominantly affect male offspring (Arnett *et al.*, 2015; Loomes, Hull and Mandy, 2017). Sex differences are also apparent in the case of FASD (Thanh *et al.*, 2014). For instance, one study demonstrated that girls are more likely to be exposed to high levels of binge alcohol and be classified as having FAS, despite lower overall incidence of FASD (May *et al.*,

2017). This could be that the historical FASD diagnostic criteria for less severe presentations were centered around male experiences; perhaps girls with low ethanol exposure are missed and thus do not receive the support they need. A more recent investigation further delved into potential sex differences with FASD. Flannigan and colleagues examined over 2,500 records of prenatal alcohol exposure from the Canadian national FASD database and determined there was indeed an equivalent rate in each diagnostic criteria for male and female children, but the physical, cognitive, and social outcomes were significantly different with biological sex: male children had more cognitive impairments, higher rates of ADHD, and had more problems in school and experienced incarceration; female children with prenatal alcohol exposure had an increased incidence of endocrine problem and mood disorders, and experienced more incidences of trauma, abuse, and problems with custody (Flannigan *et al.*, 2023). These works demonstrate that the relationship

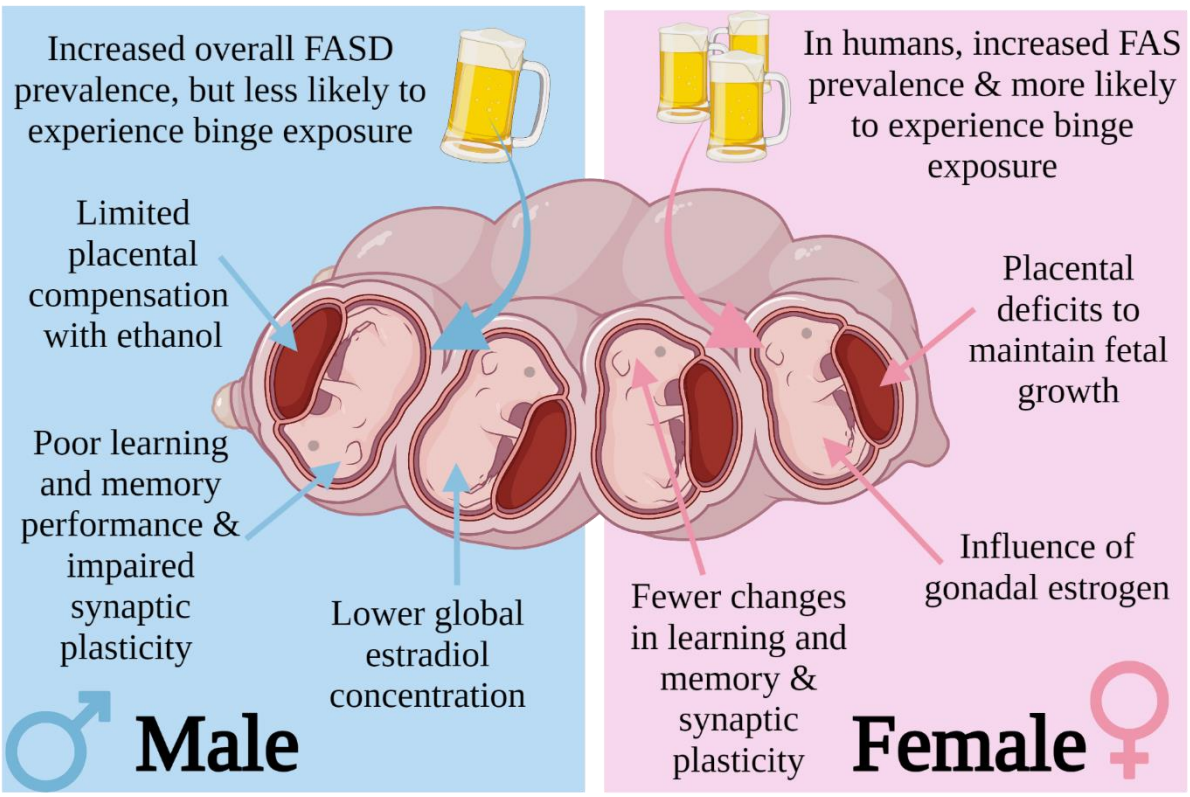


Figure 3 Sex Differences in Gestational Ethanol Exposure. Both biological and physiological factors contribute to sex differences following prenatal ethanol exposure. These include rates of ethanol exposure in humans, the reaction of the placenta, outcomes in neurological function, and hormonal interactions.

between sex-specific vulnerabilities to prenatal ethanol exposure and social outcomes are complex and constantly evolving.

Evidence in rodents also suggests that there is a sex-specific vulnerability to ethanol exposure. Indeed, only male mice exposed to ethanol during the third trimester-equivalent period exhibited deficits in hippocampal-dependent spatial memory tasks (Ieraci and Herrera, 2020). These deficits in spatial memory may not persist, as aged ethanol-exposed males and females (8-18 months old) did not have any impairments in the Y maze or water maze as compared to non-exposed animals (Cullen *et al.*, 2014). Many anxiety-like behaviours, however, persist into adulthood, but whether PNEE animals display decreased or increased behaviours, as well as sex-specific effect, varies with methodological design (Weinberg *et al.*, 2008). For instance, Ieraci & Herrera (2020) found decreased anxiety-like behaviours in both male and female offspring, while Cullen and colleagues (2013) determined PNEE was anxiogenic (Cullen *et al.*, 2013; Ieraci and Herrera, 2020).

The reason for the differential effects in male and female offspring exposed to ethanol remains unclear. One hypothesis that could account for differences in outcomes following PNEE is that the male and female placenta responds in a sex-dependent manner. The placenta is an organ that develops from trophoblasts within the zygote to provide oxygen and nutrients, therefore the placenta is either male or female and is unique to each fetus. Work from Kwan and colleagues (2020) found that the male and female placenta react differently to developmental ethanol exposure; the male placenta did not alter with PNEE, while the female placenta compensated to maintain vital organ growth (Kwan *et al.*, 2020). In additional to a placental origin, sex differences following PNEE could also be due to an interaction with gonadal hormones. Estrogen is considered in many cases neuroprotective, such as in cases of ischemic stroke or neurodegenerative disorders

(reviewed in Brann *et al.*, 2007). Indeed, estrogen has an intimate role in modulating synaptic plasticity (reviewed in Sheppard, Choleris and Galea, 2019). Few studies have examined naturally circulating hormones, but available results indicate estrogen increases spine density in a regional-specific manner (Woolley *et al.*, 1990), modulate long-term potentiation (Warren *et al.*, 1995; Bi *et al.*, 2001) and influence neurogenesis (Tanapat *et al.*, 1999). While PNEE has been shown to slightly delay puberty, as evidenced by vaginal opening, PNEE does not cause long-term changes in estrus cycling nor did it change basal levels of estradiol (Lan *et al.*, 2009). Therefore, the sex differences between males and females exposed to ethanol gestationally could stem from an altered placental response, as well as the neuroprotective effect of estrogens later in postnatal development.

1.2 Synaptic Plasticity

This dissertation will examine the effects of PNEE and choline supplementation on synaptic plasticity in the dentate gyrus during adolescence and adulthood. Therefore, the foundational principles around synaptic plasticity will be introduced and then discussed in the context of the ethanol-exposed brain.

1.2.1 Glutamatergic Transmission

Glutamate is the main excitatory neurotransmitter in the brain and plays a critical role in neuroplasticity at excitatory synapses. Glutamate can be formed through many intermediately components of the Kreb's cycle, as well as from glutamine. The enzyme phosphate-activated glutaminase converts glutamine to glutamate via a deamidation reaction in both glutamatergic neurons and astrocytes. Glutamate is packaged into vesicles by the vesicular glutamate transporters (VGLUTs), where it is then released into the synaptic cleft (Andersen *et al.*, 2021). Glutamate activates a variety of receptors, including AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, NMDA (N-methyl-D-aspartate) receptors, as well as kainite

receptors and perisynaptic metabotropic receptors (mGluRs) (Scheefhals and MacGillavry, 2018). The unique chemical structure of each of these receptors lends to their specific role in synaptic transmission and will be discussed below. After being released, extracellular glutamate can be taken back into either the presynaptic neuron or into astrocytes via the glutamate transporter-1 (GLT-1). Astrocytes are the only cells in the brain to contain the enzyme glutamine synthase, which converts glutamate into glutamine. Glutamine can then be transported back into glutamatergic neurons to continue the glutamate-glutamine cycle (Andersen *et al.*, 2021). A discussion on the influence of PNEE on glutamatergic transmission is found below and summarized in **Figure 4**.

The AMPA receptor is one of the main glutamatergic receptors involved in synaptic plasticity. As reviewed in Chater & Goda (2014), the AMPA receptor is a tetramer, containing four subunits (GluA1-4) which form a "dimer of dimers". Activation of AMPA receptors with glutamate release initiates the influx of Na^+ and efflux of K^+, resulting in depolarization of the neuron (Chater and Goda, 2014). Generally, AMPA receptors do not flux Ca^{2+} due to RNA editing processes on the GluA2 subunit which result in the pore lining being more positively charged. However, a class of AMPA receptors which have not undergone editing, or GluA2-lacking AMPA receptors, are calcium-permeable (CP-). These role of these CP-AMPA receptors in adulthood is still being uncovered, however they are likely involved early in synapse development (Stubblefield and Benke, 2010; Park *et al.*, 2018). AMPA receptors also interact with a series of proteins, such as Glutamate receptor-interacting proteins (GRIPs), protein kinase interacting with C kinase (PICK1), postsynaptic density 95 (PSD95) and stargazin. These proteins intricately regulate the trafficking, removal, and stabilization of AMPA receptors at the synapse (Bissen, Foss and Acker-Palmer, 2019).

As reviewed in Paoletti and colleagues (2013), the NMDA receptor is also critical for many forms of synaptic plasticity. The NMDA receptor is unique as it is a 'coincidence detector'; it requires both postsynaptic depolarization and glutamate release in order to activate. This is due to a magnesium ion (Mg^{2+}) resting within the pore of the NMDA receptor, which is removed by postsynaptic depolarization following AMPA receptor activation. The NMDA receptor also requires glycine or D-serine to be released as co-agonist and is permeable to Ca^{2+}. The NMDA receptor consists of four subunits from three classes of subunits: GluN1-3. All NMDA receptors contain two obligatory GluN1 subunits, which binds glycine/D-serine, and two GluN2 or GluN3 subunits which bind glutamate. The two elective subunits dictate the functional properties of the receptor, including channel conductance, efficacy of the Mg^{2+} block, localization, and binding properties of agonists or antagonists. GluN2A-containing NMDA receptors have a higher open probability and a faster decay time than GluN2B-containing NMDA receptors. It has been speculated that due to these distinct functional properties GluN2A and GluN2B subunits participate differentially in plasticity, specifically that the former is predominantly involved in LTP while the latter is involved in LTD. However, this relationship has been debated and likely is more complex (Paoletti, Bellone and Zhou, 2013). There is also a developmental regulation of NMDA receptor expression. The GluN2B receptor is the dominant form of GluN2 subunits during brain development and expression decreases into adulthood. Expression of GluN2A follows the inverse pattern, with low expression into the first postnatal week and dominant hippocampal expression in adulthood (Monyer *et al.*, 1994). The localization of NMDA receptors has also been thought to regulate its functionality, with synaptic NMDA receptors promoting survival while extrasynaptic NMDA receptors initiate an apoptotic cascade. The regulation of synaptic or extra-

synaptic localization is dependent on protein interactions, including PSD-95 and synapse-associated protein 102 (SAP-102) (Gladding and Raymond, 2011).

Finally, glutamate can activate mGluRs and kainite receptors. mGluRs are G-protein coupled receptors (GPCR) and their function is dependent on the type of associated G-protein. There are three classes of mGluRs: class 1 (mGluR1 and 5) are G_q-coupled and are involved in excitatory transmission, and class 2 (mGluR2 and 3) and class 3 (mGluR4 and 6-8) are G_{io}-coupled and are involved in inhibitory transmission. Class 1 mGluRs are typically found in the perisynaptic zone, while class 2 and 3 mGluRs are found presynaptically; both function to modulate synaptic activity (Bodzęta, Scheefhals and MacGillavry, 2021). Indeed, mGluRs are involved in some forms of LTD (Fontaine *et al.*, 2020). Notably, mGluRs are also found on glial cells, where they contribute to a neuroprotective role (Spampinato *et al.*, 2018). Kainate receptors are ionotropic glutamate receptors that largely found on the mossy fibers projecting to CA3 pyramidal cells. Kainate receptors are found both pre- and post- synaptically and regulate many aspects of neurotransmission (Vissel *et al.*, 2001; Carta *et al.*, 2014).

1.2.2 PNEE alters glutamatergic signalling.

As glutamatergic signalling is one of the most critical and metabolically demanding components of cognition, it is unsurprising that it is negatively affected by PNEE at many levels. In a zebrafish model of PNEE, increasing ethanol exposure resulted in reduced activity of both Na^+/K^+ ATPase and glutamine synthase, decreased glutamate uptake, and decreased glutamate binding (Baggio *et al.*, 2017, 2020). Interestingly, ethanol exposure did not alter VGLUT2 expression nor mitochondrial function in ethanol-exposed zebrafish (Baggio *et al.*, 2020), but rodent models of FASD demonstrated reductions in hippocampal VGLUT2 (Zhang *et al.*, 2015). Therefore, the model utilized must be carefully considered when translating results between animal

models and clinical research. Additionally, a separate study in rodents found an increase in glutamine synthetase expression, but only in female offspring (Sickmann *et al.*, 2014).

There is also evidence that PNEE alters glutamatergic receptor expression. However, results in the hippocampus, and especially in the DG, are mixed. In one study, low to moderate maternal ethanol exposure (60-90 mg/dl) decreased overall synaptic AMPA receptors (Staples, Porch and Savage, 2014) and GluN2B expression, but increased GluN1 and GluN3A expression (Brady *et al.*, 2013). A separate study of moderate PNEE (~140 mg/dl) found no change in expression of GluN1, GluN2A, or GluN2B (Sickmann *et al.*, 2014), while another found a decrease in GluN1, GluN2A and GluN2B with moderate ethanol exposure (4 g/kg/d; estimated BAC 120-180 mg/dl) (Lu *et al.*, 2018). With postnatal ethanol exposure, hippocampal GluN2B expression may be unaffected (Nixon *et al.*, 2002, 2004) or altered (Hughes *et al.*, 1998; Ieraci and Herrera, 2020); similarly, GluN2A expression can be altered (Nixon *et al.*, 2004) or unchanged (Nixon *et al.*, 2002; Ieraci and Herrera, 2020). Conversely, in the cortex there were often no change in the expression of AMPA receptors, most NMDAR subunits, mGluRs, or kainate receptors (Dettmer *et al.*, 2003; Nixon *et al.*, 2004; Bird *et al.*, 2015). However, some studies report cortical changes in GluN2B expression, such as increased specifically in the agranular insular cortex (Bird *et al.*, 2015) or decreased expression in guinea pig offspring (Dettmer *et al.*, 2003). A lack of change in *total* expression does not necessarily equate to no change in *local* expression, as the transport of NMDA receptors may also be impacted by developmental ethanol exposure (Hughes, Wilson and Leslie, 2001). As GluN2B receptor expression is dominant in development (Monyer *et al.*, 1994) and the switch to GluN2A receptors requires synaptic activity (Barria and Malinow, 2002), it can be theorized that PNEE delays this developmental switch by depressing synaptic activity.

Alterations in glutamatergic receptors also occur at a functional level, but differing experimental parameters and regional vulnerability can make determining trends difficult. For instance, the basolateral amygdala and dorsomedial striatum are areas that have increased spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs, respectively) (Baculis, Diaz and Valenzuela, 2015; Cheng *et al.*, 2018). Conversely, in the hippocampus the CA1 region has decreased excitation (Vaglenova *et al.*, 2008; Wijayawardhane *et al.*, 2008), while no overall changes are evident in the CA3 (Krawczyk *et al.*, 2016) nor in the DG (Kajimoto *et al.*, 2016). Similarly, the prefrontal cortex can also be highlighted as an area of increased vulnerability

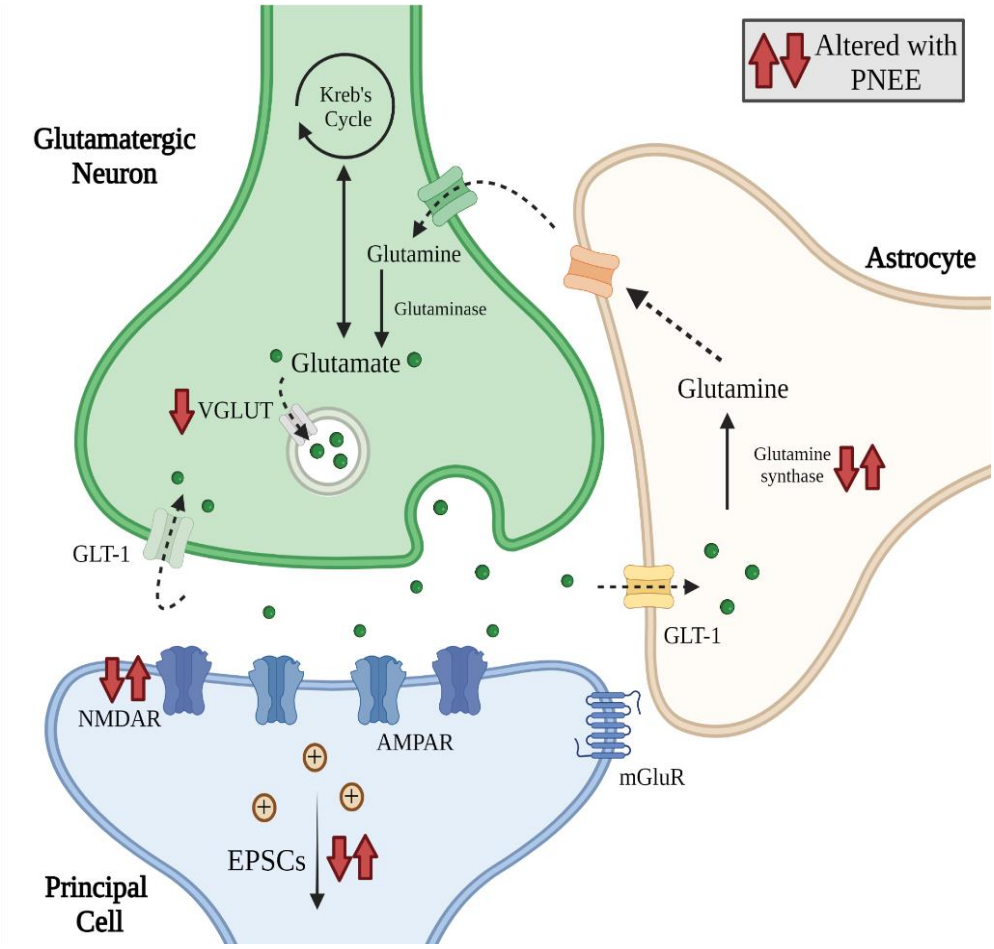


Figure 4 Glutamatergic Signaling Alterations Following PNEE. PNEE reduces VGLUT expression, and alterations in glutamine synthase, NMDA receptors, and excitatory postsynaptic currents (EPSCs).

(Skorput *et al.*, 2015; Skorput and Yeh, 2016). The discrepancy in regional EPSC outcomes may closely model behavioural changes characteristics of FASD, such as decreased learning, memory, and executive functioning (Mattson, Crocker and Nguyen, 2011), in addition to increased anxiety and hyperactivity (Nanson and Hiscock, 1990; Barr *et al.*, 2006).

1.2.3. Inhibitory Transmission

The main inhibitory neurotransmitter in the brain is GABA (γ-aminobutyric acid) which acts on ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors to dampen neuronal activity. GABA is synthesized from glutamate by the enzymes glutamic acid decarboxylase (GAD) 65 and GAD67 (Deidda, Bozarth and Cancedda, 2014). While in some areas of the brain, such as the cerebellum (Greif *et al.*, 1991) and barrel cortex (Kiser, Cooper and Mower, 1998), expression of GAD65 lags behind that of GAD67, this trend does not appear in the hippocampus (Dupuy and Houser, 1996). GAD67 is critical in early development, as GAD67 double knockout is lethal, while GAD65 double knockout mice can survive into adulthood (Asada *et al.*, 1996, 1997). The functional roles of GAD67 and GAD65 differs, although the distinct role for these enzymes is still being uncovered. Generally, GAD67 is thought to maintain basal formation of GABA while GAD65 increases synthesis GABA in an activity-dependent manner (Asada *et al.*, 1997; Deidda, Bozarth and Cancedda, 2014; Jiang *et al.*, 2022), however others have demonstrated a decrease in basal GABA content with GAD65 knockout models (Stork *et al.*, 2000). Following synthesis, GABA is packaged into vesicles by vesicular GABA transporter (VGAT), released into the synaptic cleft to activate GABA receptors, then transported back into neurons or astrocytes via GABA transporters (GATs). GABA can then be degraded into succinic semialdehyde by GABA-transaminase (Deidda, Bozarth and Cancedda, 2014). A discussion on the influence of PNEE on GABAergic transmission is found below and summarized in **Figure 5**.

GABA release into the synaptic cleft can activate two main classes of GABA receptors, both which function to decrease the activity of the postsynaptic neuron. As reviewed by Ghit and colleagues (2021), the $GABA_A$ receptor is a heteropentamer, typically formed with two α, two β, and one γ subunits. However, there is substantial diversity in subunit classes as well as isoforms due to alternative splicing leading to many possible receptor compositions (Ghit *et al.*, 2021). Of note, δ-containing $GABA_A$ receptors are typically found extrasynaptically and are involved in tonic inhibition. This class of $GABA_A$ receptors are important, as they are particularly sensitive to

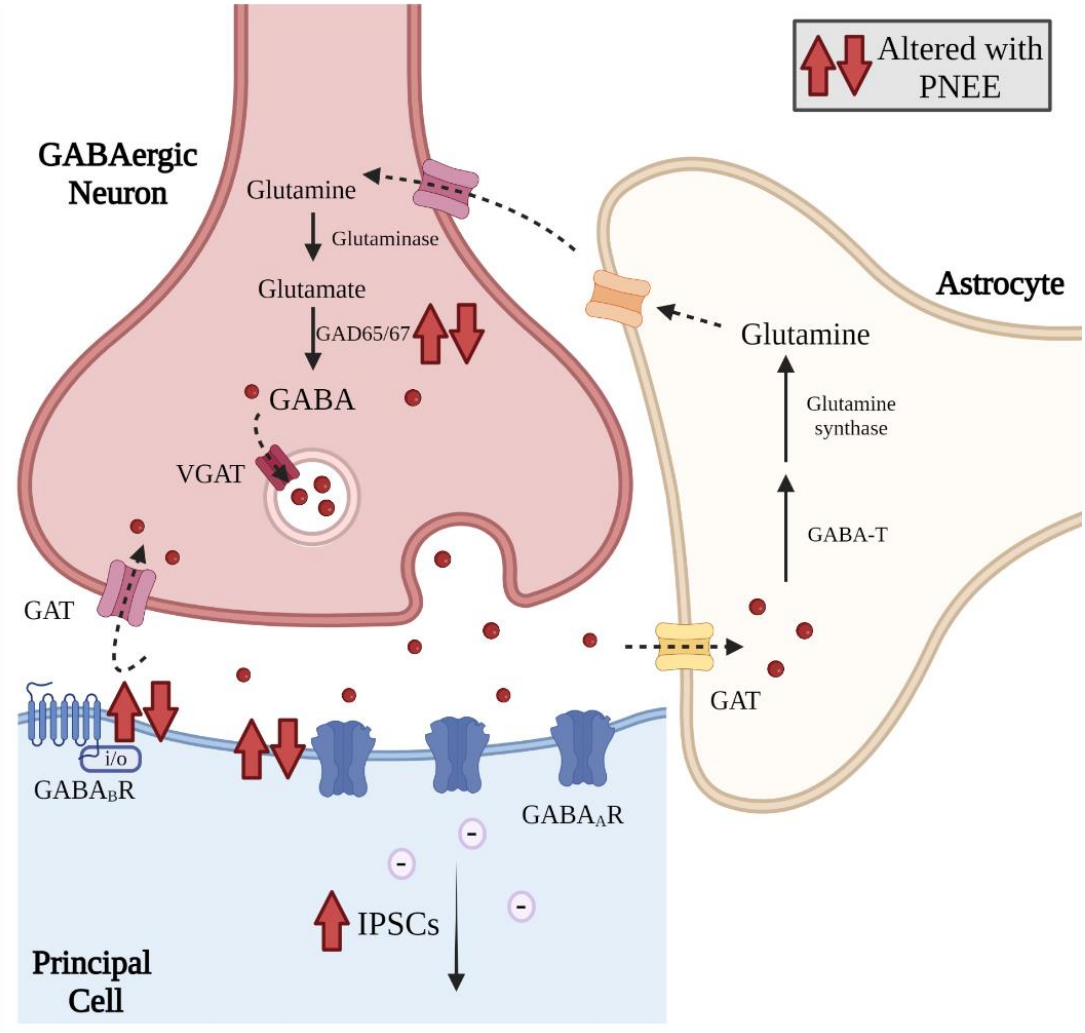


Figure 5 PNEE alters GABAergic transmission. PNEE alters GAD65/67 and both $GABA_A$ and $GABA_B$ receptor expression. Consistently, PNEE increases inhibitory postsynaptic currents (IPSCs).

ethanol (Wallner, Hanchar and Olsen, 2003). Upon GABA binding at the α-β junction, the $GABA_A$ receptor opens and allows selective passage of Cl^-, hyperpolarizing the neuron in the adult brain (Ghit *et al.*, 2021). $GABA_B$ receptors are metabotropic and coupled to the $Gi_{/o}$ protein. $GABA_B$ receptors are found both pre- and post- synaptically, where they function to reduced neurotransmitter release and hyperpolarize the neuron by inhibiting voltage-gated calcium channels and inhibiting adenyl cyclase (Heaney and Kinney, 2016).

1.2.4. PNEE alters GABAergic signalling.

PNEE has been demonstrated to alter the function of GABAergic transmission into adulthood. The number of GABAergic neurons is altered in a regional-specific manner. PNEE increased the number of GABAergic neurons in CA3 and DG (Lu *et al.*, 2018), as well as calretinin positive neurons in the female orbitofrontal cortex (Kenton *et al.*, 2020). In the cerebellum, however, PNEE decreased GABAergic interneurons density (Nirgudkar *et al.*, 2016). PNEE decreased GAD65 expression in the hypothalamus and increased GAD67 expression in the hippocampus (Lu *et al.*, 2018). Additionally, PNEE decreased expression of the α5, but not α1 or β2/3, $GABA_A$ receptor subunit in the embryonic and neonatal brain, however $GABA_A$ subunit expression was increased as compared to control offspring in adulthood (Iqbal *et al.*, 2004; Toso *et al.*, 2006). With $GABA_B$ receptors there is similarly a developmentally regulated, but complex, change in receptor expression with PNEE (Li *et al.*, 2005; Lee *et al.*, 2008). In terms of functionality, if inhibitory postsynaptic currents (IPSCs) are demonstrated to be altered with PNEE, consistently IPSC amplitude and frequency are increased (Skorput and Yeh, 2015; Skorput *et al.*, 2015; Montgomery *et al.*, 2018; Delatour, Yeh and Yeh, 2019, 2020; Kenton *et al.*, 2020).

1.2.5 Excitatory/Inhibitory Imbalance following PNEE.

The excitatory/inhibitory (E/I) balance is critical and disruptions in this delicate equilibrium may underlie the pathophysiology of neurodevelopmental disorders, including autism, schizophrenia (Gao and Penzes, 2015), and FASD. While generally studies show an increase in inhibition and variable alterations in excitation, few studies examine both excitation and inhibition within the same study. Despite the paucity of data some conclusions can be drawn and, like many results in PNEE models, the results are regionally specific. Layer V of the prefrontal cortex and the basal forebrain have been demonstrated to have an shift towards inhibition with low (BAC 20-80 mg/dl) and moderate (264 mg/dl) PNEE models, respectively (Skorput and Yeh, 2015; Skorput *et al.*, 2015). This shift in the E/I balance could underlie deficits in attentional processes in PNEE offspring. However, the basolateral amygdala, somatosensory cortex and CA3 region of the hippocampus demonstrate a shift towards excitation (Baculis, Diaz and Valenzuela, 2015; Krawczyk *et al.*, 2016; Delatour, Yeh and Yeh, 2019). The shift towards excitation could contribute to anxiety-like phenotypes, hyperactivity, and learning impairments. It should be noted that not all studies find an E/I imbalance following PNEE, specifically in layer V/VI of the somatosensory cortex in juveniles (Delatour, Yeh and Yeh, 2020) and in the DG of adult PNEE offspring (Kajimoto *et al.*, 2016). Despite the variability in E/I outcomes, exploring this imbalance will be an interesting area to examine in future studies.

1.2.6 Cholinergic Transmission

Another aspect of neurotransmission that is important to consider in the context of FASD is the modulatory role of the neurotransmitter acetylcholine. Cholinergic neurons are found in the brainstem, cortex, as well in subcortical areas, including the medial septum and the diagonal band of Broca (MS/DB). It is the MS/DB that projects to the hippocampus (Li *et al.*, 2017).

Acetylcholine (ACh) is produced in cholinergic neurons from acetyl CoA and choline with the enzyme choline acetyltransferase (ChAT). ACh is then packaged into vesicles through vesicular ACh transporter (VAChT) and released into the synapse. Unlike glutamatergic and GABAergic signaling, ACh is not taken back into astrocytes or neurons, but is metabolized with acetylcholinesterase into acetate and choline. Choline can then be transported back into the neuron through the Na^+/Choline transporter (Purves *et al.*, 2001).

There are two main types of ACh receptors: the ionotropic nicotinic ACh (nACh) receptor, and the metabotropic ACh receptor (mAChR). The nACh receptors are in the same family as $GABA_A$ receptors; nACh receptors are composed of five subunits (combinations of α and β subunits) which create a centralized pore permeable to Na^+, K^+, and Ca^{2+}. The subunit composition also dictates sensitivity to ethanol; α7 nACh receptors are inhibited with ethanol while α2 and α4-containing nACh receptors can be potentiated with acute ethanol (Yu *et al.*, 1996; Aistrup, Marszalec and Narahashi, 1999; Hendrickson, Guildford and Tapper, 2013). mACh receptors are GPCRs that can be further subdivided into excitatory (M1/M5) and inhibitory (M2/4) which are G_q or $G_{i/o}$ coupled, respectively. nACh and mACh receptors can be found pre- and post-synaptically and therefore can modulate diverse aspects of neurotransmission and synaptic plasticity (Picciotto, Higley and Mineur, 2012).

1.2.7 PNEE alters cholinergic transmission.

The cholinergic system may be particularly vulnerable to gestational ethanol exposure and thus is interesting in terms of the efficacy of choline supplementation as a treatment (**Figure 6**). Historically, PNEE has decreased the concentration of ACh in both fetal and neonatal brains, altered ChAT activity, but did not alter ChAT+ neuron density in the medial septum (Rawat, 1977; Swanson *et al.*, 1995, 1996). More recent work has found cell loss in the medial septum with

postnatal exposure, as a single binge ethanol exposure on PND 7 (estimated BAC 350-450 mg/dl) resulted in a 42% sex-independent decrease in hippocampal-projecting ChAT positive neurons, but not cholinergic neurons in the brainstem (Smiley *et al.*, 2021). Similarly, cortical-projecting ChAT positive neurons in the nucleus basalis of Meynert were reduced with postnatal ethanol exposure (Milbocker and Klintsova, 2020). This reduction is not always seen, however, as a similar exposure (PND 2-10; BAC 318 mg/dl) did not result in any change in ChAT positive cells in the medial septum, nor in choline acetyltransferase, α7 nACh receptors, or VAChT. Interestingly, ACh efflux was reduced in ethanol intubated offspring (Perkins, Fadel and Kelly, 2015). Within the hippocampus, PNEE increased the combined M1 and M2 expression (Carneiro *et al.*, 2005). When examining specific receptor types, ethanol exposure from PND 4-9 (BAC ~330 mg/dl) decreased expression of M1 receptors and increased M2/4 expression, significantly altering the M1/M2 balance (Monk, Leslie and Thomas, 2012). M1 functionality may also be altered with developmental ethanol exposure, as PNEE offspring have

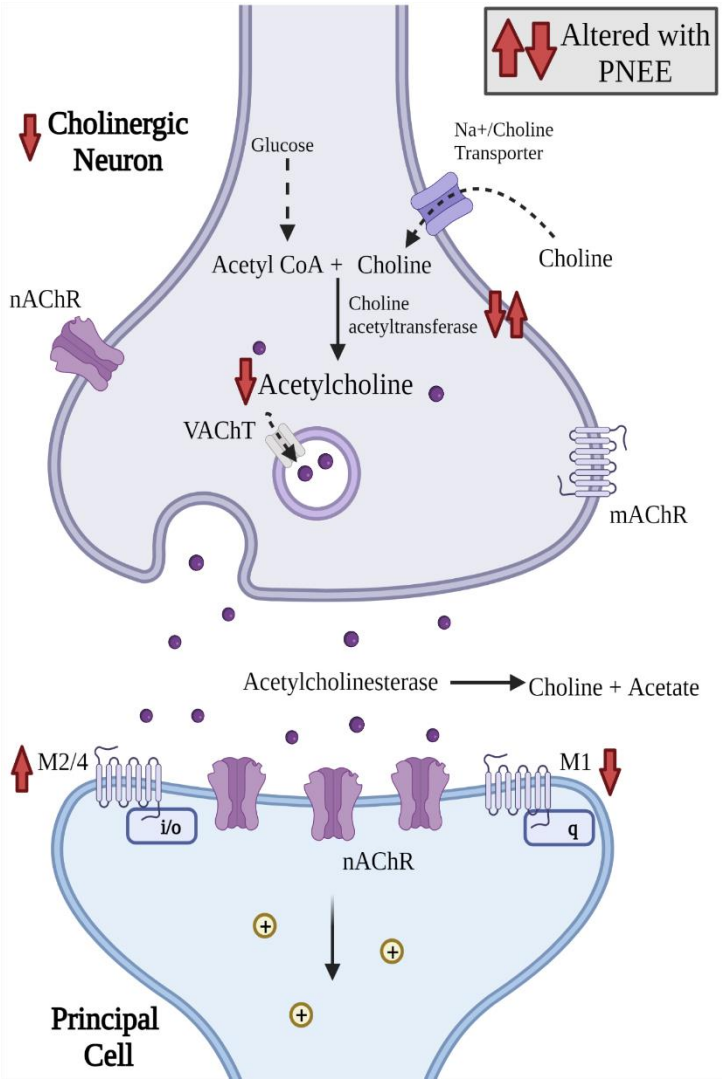


Figure 6 Cholinergic transmission is altered with PNEE. Developmental ethanol exposure decreases cholinergic neuron density, acetylcholine production, alters choline acetyltransferase and the M1:M2/4 ratio.

an exaggerated acute depression following M1 activation as compared to control offspring (Grafe *et al.*, 2021).

1.2.5 The Hippocampal Formation

This dissertation examines changes in synaptic plasticity within the DG of the hippocampus. As the hippocampal formation is foundational and already described in numerous places in the literature, only a brief overview will feature here (Andersen, 2007).

Hippocampal inputs originate in layer II and III of the entorhinal cortex (EC; **Figure 7**), which project to the DG/CA3 and CA1/subiculum, respectively. The fibers projecting to the DG are classified according to their location in the EC, as the more medially located fibers form the Medial Perforant Pathway (MPP) and terminate in the middle third of the molecular layer and the more laterally located fibers form the Lateral Perforant Pathway (LPP) which terminate in the outer third of the molecular layer. The MPP and LPP, despite their proximal location, have different functional properties. The MPP is more likely to potentiate while the LPP tends to depress following stimulation (Collitti-Klausnitzer *et al.*, 2021), the LPP more reliably demonstrates paired pulse facilitation as compared to the MPP (Petersen *et al.*, 2013), and the MPP is involved in allocentric representations while the LPP is involved in egocentric representations of spatial environments (Wang *et al.*, 2018). The inner third of the molecular layer receives input from GABAergic interneurons, contralateral hippocampal cells, and septal cholinergic projections. The principal cell of the DG, the granule cell, reside in the granule cell layer. The final layer of the DG is the polymorphic layer, which contains many types of interneurons as well as mossy cells. Mossy cells are the only excitatory non-principal cell in the hippocampus and provides feedback to both ipsilateral and contralateral granule cells. Interestingly, mossy cells are particularly vulnerable to

injury (Scharfman, 2016). The sparse firing of granule cells, as well as the feedback from mossy cells, is thought to contribute to the DG role in spatial pattern separation (Yassa and Stark, 2011).

The DG granule cells then project to the CA3 pyramidal cells via mossy fibers. The CA3 and CA1 regions are comprised of the *stratum oriens*, where the basal dendrites are located, the *strata radiatum, lacunosum,* and *moleculare* which house the apical dendrites of the pyramidal cells.

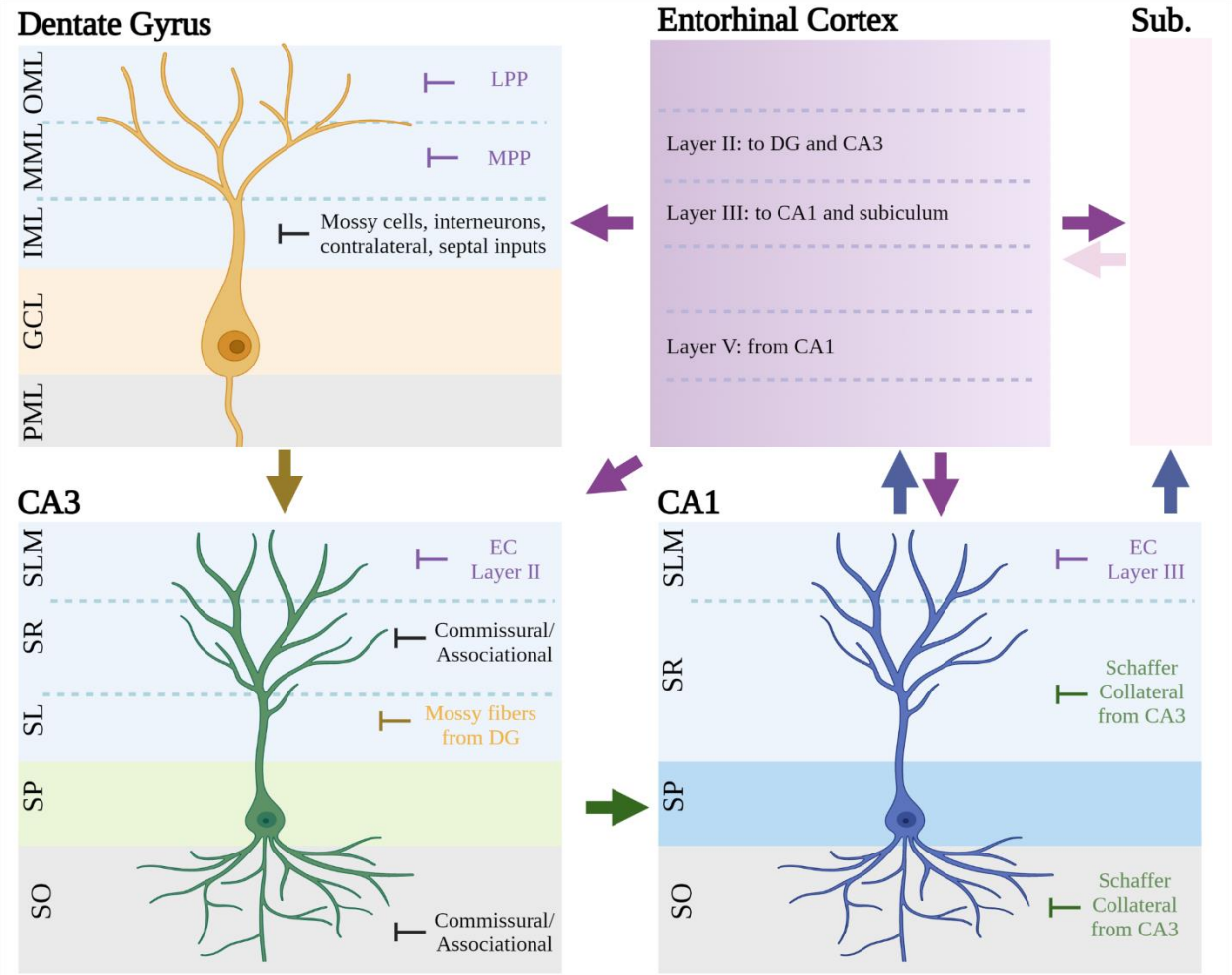


Figure 7 Simplified Hippocampal Diagram. The molecular layer of dentate gyrus (DG) is divided into the inner, middle, and outer layers (IML, MML, OML). The OML and MML receive inputs from the lateral and medial perforant pathway, respectively (purple arrow). The IML receives inputs from external regions, mossy cells, and interneurons. The DG projects to the stratum lucidum (SL) of the CA3 via mossy fibers (yellow arrow). The CA3 also receives inputs from largely external regions in the stratum radiatum (SR) and the stratum oriens (SO), and the entorhinal cortex (EC) in the stratum lacunosum-moleculare (SLM). The CA3 projects to CA1 via Schaffer Collateral fibers (green arrow) into the SO and SR layers. CA1 neurons also receive inputs from Layer 11 EC into the SLM. CA1 projects to the subiculum (Sub.) and Layer V of the EC.

CA3 neurons also receive strong input from associational and commissural connections. The CA3 pyramidal cells project to the CA1 pyramidal cells via Schaffer Collaterals. Finally, the CA1 fibers project to the subiculum, as well as back to layer V/VI of the EC.

1.2.6. Long-Term Potentiation (LTP)

The concept of 'memory' has fascinated scientist for centuries. While many theories were generated, it was the discovery of long-term potentiation (LTP) that finally began to answer the question of, *how do we remember*? This historic discovery was made by Bliss and Lømo in the early 1970s. In this seminal work, Bliss and Lømo found that repeated stimulation would result in the potentiation of excitatory postsynaptic potentials (EPSP) in the DG of anesthetized rabbits that could persist for hours (Bliss and Lømo, 1973). Since the 1970s, the understanding of LTP and synaptic plasticity has grown exponentially to our current understanding today.

The classical process of LTP (**Figure 8**) has been reviewed extensively (Lisman, Yasuda and Raghavachari, 2012; Nicoll and Roche, 2013; Herring and Nicoll, 2016; Bugra Baltaci *et al.*, 2019). Glutamate release activates AMPA receptors which begin to influx Na^+. The resulting depolarization of the neurons expels the Mg^{2+} blockade in NMDA receptors to allow Na+ and, critically, Ca^{2+}, to influx through the NMDA receptor pore. Ca^{2+} forms a complex with calmodulin (Ca^{2+}/CaM) which binds to the regulatory domain of the kinase CaMKII. Binding of Ca^{2+}/CaM triggers autophosphorylation of CaMKII on threonine 286/287 which allows the kinase to remain in a constitutively active state. CaMKII can then bind to the NMDA receptor and phosphorylate both accessory proteins and serine 831 on the GluR1 subunit of AMPA receptors. PKA (protein kinase A) can also phosphorylate serine 845, which is a necessary component for LTP. These phosphorylation events result in an increased conductance through the AMPA receptor. Additionally, structural changes are evident in early-LTP as the synapse enlarges to accommodate

an increase in AMPA receptor insertion through exocytosis and lateral diffusion. While early-LTP is dominated by kinase activity, the maintenance of late-LTP is caused by the change in translation

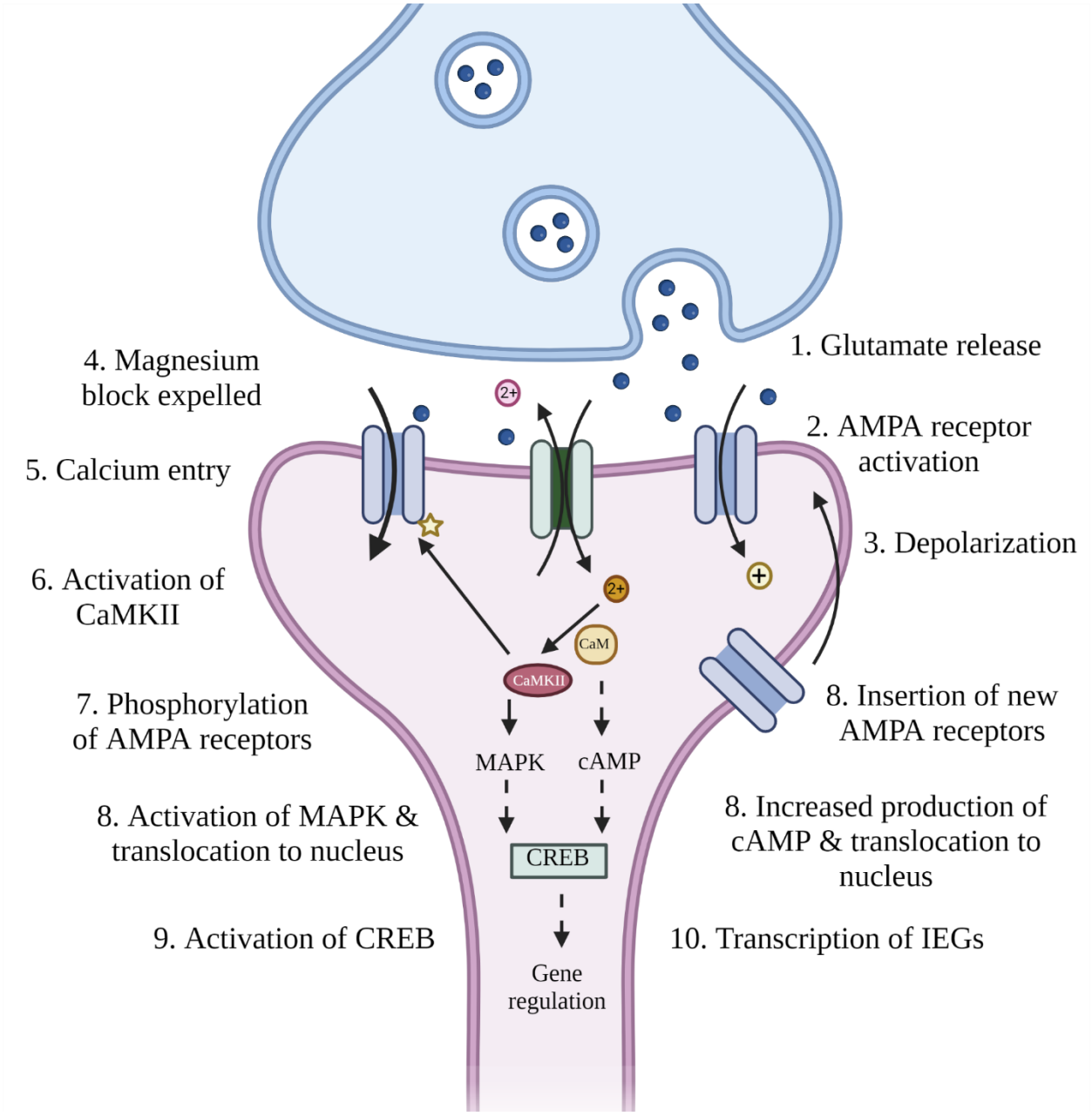


Figure 8 Simplified Long Term Potentiation (LTP). Glutamate release activates AMPA receptors which causes a depolarization of the postsynaptic neuron. Sufficient depolarization expels the magnesium block in the NMDA receptors which then influxes calcium. An increase in intracellular calcium will activate the kinase CaMKII, which will initiate downstream cascades. Final increase in synaptic strength is due to the insertion of AMPA receptors, an increase in conductance due to phosphorylation, and a change in protein synthesis.

of new proteins. This can occur through several mechanisms. Ca^{2+}/CaM can activate adenyl cyclase which increase production of cyclic AMP (cAMP). cAMP then activates PKA. Additionally, CaMKII can also activate MAPK (mitogen-activated protein kinase). Both PKA and MAPK can translocate to the nucleus to stimulate transcription regulatory proteins such as CREB (cAMP response element-binding protein) through phosphorylation. CREB acts as a transcription factor for intermediate early genes, such as Zif268 (Zinc finger-containing transcription factor 268) and Arc (activity-regulated cytoskeletal).

1.2.7. Long-Term Depression

Following the old adage, what goes up must go down, an increase in synaptic strength must also have an ability to weaken synaptic strength. The process of decreasing the efficacy of synaptic strength is known as long-term depression (LTD; **Figure 9**). LTD plays an important role in hippocampal spatial memory by fine tuning synaptic circuits to prevent memory generalization and flexibility in memory reversal tasks (Stacho and Manahan-Vaughan, 2022).

LTD can be induced through many mechanisms, mainly through the activation of the NMDA receptor or mGluRs (Collingridge *et al.*, 2010). NMDA receptor-dependent LTD involves many of the same components as LTP: glutamate activates AMPA receptors which depolarizes the postsynaptic neuron and expels the Mg^{2+} block, which then begins to influx Ca^{2+}. However, the intracellular Ca^{2+} concentration remain low and thus activates the more sensitive phosphatase calcineurin. Calcineurin then activates inhibitor-1, which subsequently activates protein phosphatase 1 (PP1), ultimately dephosphorylating serine 845 on the GluA1 subunit of the AMPA receptor. Low levels of calcium can also activate other proteins, including hippocalcin and protein interacting with C kinase 1 (PICK1), to induce endocytosis of AMPA receptors from the synapse. In addition to changes in protein synthesis, it is these mechanisms which function to weaken

synaptic strength. mGluR-dependent LTD functions in a similar, yet distinct, mechanism. mGluRs activate phosphate lipase C (PLC) which hydrolyzes PIP2 into DAG and IP3. Both IP3, which stimulates Ca^{2+} from intracellular stores, and DAG can lead to the activation of protein kinase C (PKC), ultimately phosphorylating serine 880 on the GluA2 subunit of the AMPA receptor. This phosphorylation events leads to the removal of AMPA receptors from the synapse.

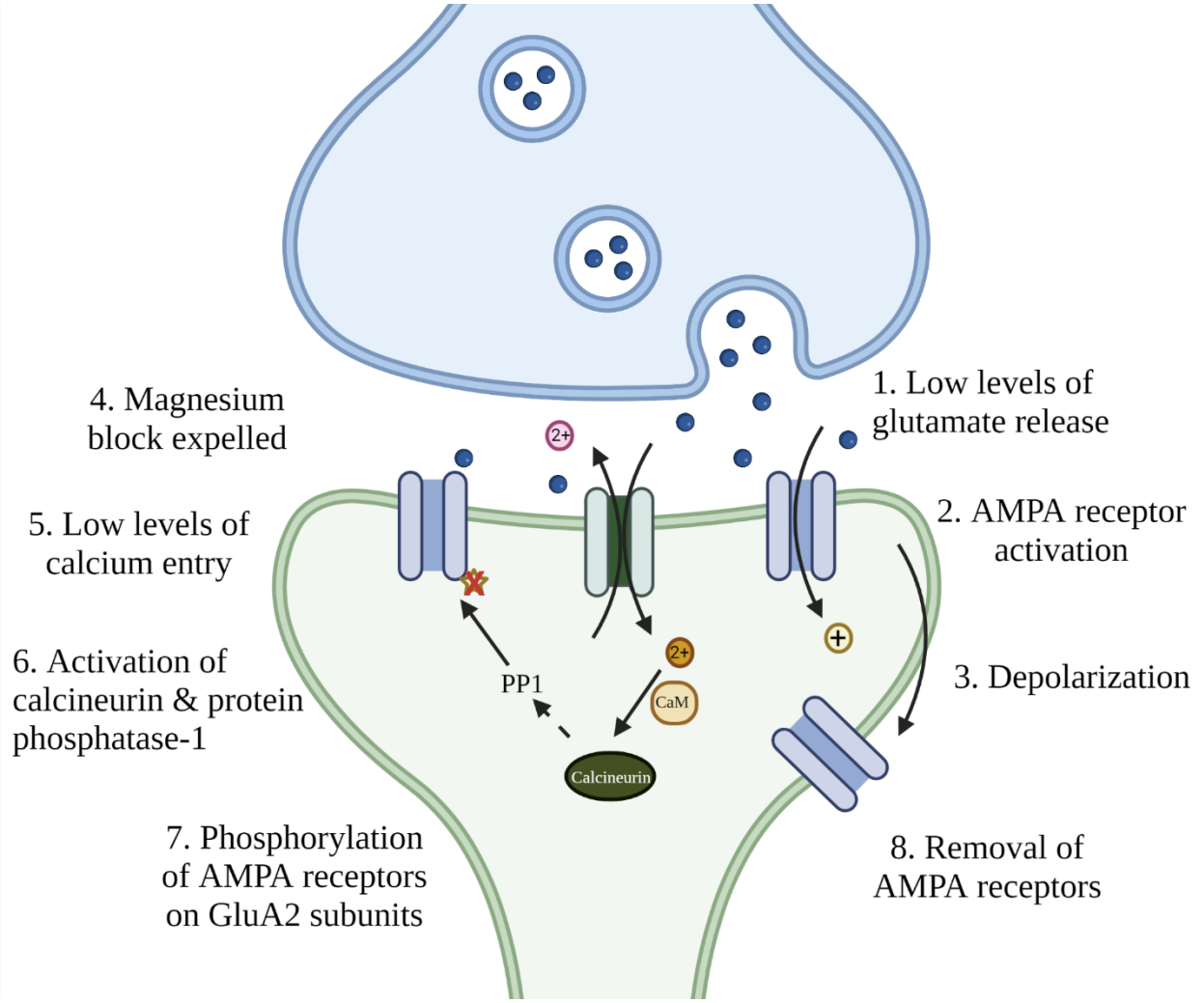


Figure 9 Simplified Long Term Depression (LTD). LTD begins in a similar fashion as LTP, with glutamate release which activates AMPA receptors and causes depolarization of the postsynaptic neuron. However, in this instance there are lower levels of glutamate release which results in a smaller increase in intracellular calcium concentration. Low intracellular calcium will activate phosphatases, which will remove phosphorylated sites. The final result is a removal of AMPA receptors at the synapse and reduction in overall synaptic strength.

It should be noted that several other forms of LTD are possible, including M1-dependent LTD (Auerbach and Segal, 1996; Grafe *et al.*, 2021) and presynaptic endocannabinoid LTD (Peñasco *et al.*, 2019; Fontaine *et al.*, 2020). However, as these are not explored directly in the current dissertation they will not be further discussed.

1.2.8 Sex-differences in Synaptic Plasticity

While the mechanisms of synaptic plasticity are fairly well conserved in males and females, there are differences to note between the sexes. In many instances adult males have increased LTP as compared to age-matched females (Summarized in **Table 1**) (Maren, De Oca and Fanselow, 1994; Maren, 1995; Yang *et al.*, 2004; Monfort *et al.*, 2015; Safari *et al.*, 2021; Le *et al.*, 2022). However, this is not always true when the estrous cycle is considered. In the female rat, the estrous cycle occurs over 4 or 5 days and involves four distinct phases. (1) Proestrus is the stage in which estradiol levels peak and occurs for approximately 14 hours. (2) Estrus lasts for 24-48 hours and is characterized with high progesterone levels. (3) Metestrus is the shortest phase, lasting 6-8 hours and is sometimes omitted due to the relatively short duration. (4) Diestrus is the longest phase of the estrus cycle and occurs for 48-72 hours (Cora, Kooistra and Travlos, 2015). Estrogen has the potential to impart a large impact on plasticity. In the canonical pathway estradiol binds to estrogen receptors (ERα or ERβ) which then dimerize, translocate to the nucleus, and bind to estrogen response elements to modulate transcription. Estrogens can also act through a separate and more rapid pathway which involves the activation of difference signalling cascades, such as through adenyl cyclase or PLC (Bean, Ianov and Foster, 2014). There are many studies examining exogenous estradiol on learning and memory, however these data are complicated by the timing, dose, and duration of treatment (Sheppard, Choleris and Galea, 2019). Generally, low or high doses of estrogen impair learning and memory processes, while doses falling in the middle of a

normalized curve improve outcomes (Bean, Ianov and Foster, 2014; Sheppard, Choleris and Galea, 2019). While there are few studies examining the effects of endogenous hormones on synaptic plasticity, there is some evidence demonstrating elevated CA1 and CA3 LTP in adult females during proestrus which is either equivalent or surpasses male LTP (Warren *et al.*, 1995; Bi *et al.*, 2001; Harte-Hargrove *et al.*, 2015). Additionally, cyclic changes are evident in CA1 spine density, however there were no changes in spine density in CA3 or the DG (Woolley *et al.*, 1990). Notably, there is a distinct paucity of data examining estrous-dependent changes in LTP in the DG and little evidence to conclusively say the natural fluctuations in hormones confer increased variability in naturally cycling females (Prendergast, Onishi and Zucker, 2014). Differences in synaptic plasticity are evident before puberty and thus there is a strong potential for age- and sex-dependent factors to influence hippocampal outcomes. A recent study by Le and colleagues (2022) found that female adolescent rats had greater LTP than male adolescent rats. However, into adulthood this outcome was reversed and adult female rats had reduced LTP as compared to adult males (Le *et al.*, 2022). While there is a paucity of evidence examining sex-dependent LTD, females generally have increased LTD as compared to males in both the juvenile (Titterness and Christie, 2008) and adult timepoints (Dursun *et al.*, 2018).

These sex dependent outcomes in plasticity may originate from differences in receptor composition. Le and colleagues (2022) found an age-dependent increase in the α5-GABA receptor subunit in females only, which, when antagonized, increased LTP in adult females (Le *et al.*, 2022). However, others have demonstrated that the increased LTP in males was related to increased GluR1 insertion and serine 845 phosphorylation as compared to females (Monfort *et al.*, 2015). Finally, another study concluded the sex difference in plasticity involved the NMDA receptor. Although males and females were not directly compared, Monfort & Felipo (2007) demonstrated

that age (2 vs 8 month old) decreased the amount of LTP in males but not in females, which was thought to be associated with a lower density of GluN1 and GluNN2 receptor subunits in aged males as compared to young males (Monfort and Felipo, 2007). Therefore, while the mechanism of sex-differences is not completely elucidated, it is clear that males generally have increased LTP as compared to females which may originate at the level of the receptor and may be influenced by gonadal hormone levels.

Table 1 Summary of sex differences in LTP. LTP is presented as a percent change from baseline (0%). PND: postnatal day; P or pro: proestrus; E: estrous; D: diestrus.

Region	**Age** (PND)	**Male LTP**	**Female LTP**	**Male vs Female**	**Ref.**
CA1	90-100	20-75%	P: 25-75% E: -10-50% D: -20-25%	M = F_{pro}	(Warren *et al.*, 1995)
	60-90	125%	59%	M > F	(Monfort *et al.*, 2015)
	Adult	49%	-1%	M > F	(Yang *et al.*, 2004)
	21-28	4%	29%	F > M	(Le *et al.*, 2022)
	56-70	35%	8%	M > F	
CA3	60-120	9%	P: 40% E: 17% D: 11-15%	M = F_{pooled} F_{pro} > M	(Harte-Hargrove *et al.*, 2015)
DG	15-20	0%	0%	M = F	(Maren, De Oca and Fanselow, 1994)
	32-35	20%	5-10%	M > F	
	55-60	25%	5%	M > F	
	55-60	40%	20%	M > F	(Maren, 1995)
	60	60%	40%	M > F	(Safari *et al.*, 2021)
	60-120	30%	20%	M = F	(Dursun *et al.*, 2018)

1.2.9 Changes in Synaptic Plasticity with PNEE

PNEE results in synaptic plasticity changes which are sex-dependent (summarized in **Table 2**). In male offspring, PNEE consistently decreases magnitudes of LTP (Sutherland, McDonald and Savage, 1997; Puglia and Valenzuela, 2010; Varaschin *et al.*, 2010; Titterness and Christie, 2012; An, Yang and Zhang, 2013; Anna R. Patten, Sickmann, *et al.*, 2013; Helfer, White and Christie, 2014; Kervern *et al.*, 2015; Subbanna and Basavarajappa, 2022) and often increases the amount of LTD (An and Zhang, 2013; An, Yang and Zhang, 2013; Kervern *et al.*, 2015; Silvestre de Ferron *et al.*, 2017; Subbanna and Basavarajappa, 2022), but not always (Titterness and Christie, 2008; Fontaine *et al.*, 2019). This may indicate an age-dependent influence on LTD, as the reported decrease or no change in LTD occurred in adolescent animals. In females, however, PNEE often results in an increase in LTP (Titterness and Christie, 2012; An and Zhang, 2013, 2015) or does not change LTP (Anna R. Patten, Sickmann, *et al.*, 2013; Patten, Brocardo and Christie, 2013; Helfer, White and Christie, 2014; Sickmann *et al.*, 2014). Few studies have examined the effects of PNEE specifically on female LTD, however, females had less depotentiation (An and Zhang, 2015) or no change in LTD (Titterness and Christie, 2008; Fontaine *et al.*, 2019) when compared to control offspring. These data indicate an impairment in bidirectional plasticity in ethanol exposed male offspring, but not always in female offspring.

Table 2 Summary of hippocampal synaptic plasticity changes following PNEE. GD: gestational day; IP: intraperitoneal; LD: liquid diet; W: ethanol in water; IT: intragastric intubation; M: males; F: females; PND: postnatal day; TBS: theta burst stimulation; HFS: high frequency stimulation; † = study conducted in guinea pigs; Depot: depotentiation experiment; T; number of trains

Region	Ethanol paradigm (BAC)	Sex	Age (PND)	LTP	LTD	Ref.
CA1	GD 8 & 12 (IP; 300 ± mg/dL)	M&F	~ 90	↓ (TBS)	↑ (1 Hz)	(Subbanna and Basavarajappa, 2022)

	GD -7 – 22 (W; ~258 mg/dl)	F	36+	↑ (HFS)	↓ Depot.(1 Hz)	(An and Zhang, 2015)
	GD -7 – 22 (W; ~258 mg/dl)	M	36+	↓ M (HFS)	↑ Depot.(1 Hz)	(An, Yang and Zhang, 2013)
	GD -7 – 22 (W; ~258 mg/dl)	M, F	36+	↓ M;↑ F (HFS)	↑ Depot. M; ↓ F (1 Hz)	(An and Zhang, 2013)
	GD 5- PND 7 (W; 62 mg/dl)	Unclear	17-30	= / ↓ (HFS)	-	(De La Fuente-Ortega *et al.*, 2019)
	GD 2-67 † (IT; 416 mg/dl)	M&F	~ 40-80	↓ (HFS)	-	(Richardson *et al.*, 2002)
	GD 2-67 † (IT; 416 mg/dl)	M&F	~40-80	= (HFS)	-	(Byrnes *et al.*, 2004)
	GD -28 – lactation (W; ~100 mg/dl)	M	~50	↓ (HFS)	↑ (1 Hz; 600 pulses)	(Kervern *et al.*, 2015)
	GD -28 – lactation (W; ~100 mg/dl)	M	45-55	-	↑ (1 Hz; 600 pulses)	(Silvestre de Ferron *et al.*, 2017)
	GD 1-21 (LD; 192 mg/dl)	M, F	30-35	-	= M, = F (3 Hz)	(Titterness and Christie, 2008)
	PND 2-9 (V; 124 or 340 mg/dl)	M&F	7-9	= (124) ↓(340; HFS)	-	(Puglia and Valenzuela, 2010)
DG	GD 1-21 (LD; 83 mg/dl)	M	120-150	↓ (HFS)	-	(Sutherland, McDonald and Savage, 1997)
	GD 1-21 (LD; (87 mg/dL)	M, F	30-35	↓ M ; ↑ F (TBS)	-	(Titterness and Christie, 2012)
	GD 1-21 (LD; 145 mg/dl)	M, F	50-70	↓ M; = F (TBS)	-	(Helfer, White and Christie, 2014)
	GD 1-21 (LD; 146 mg/dl)	M, F	55-70	↓ M; = F (TBS)	-	(Sickmann *et al.*, 2014)
	GD 1-21 (LD)	M, F	21-28	↓ M; ↓ F (HFS)	↓ M; = F (1 Hz)	(Fontaine *et al.*, 2019)
	GD 1-21 (LD; 101 mg/dl)	M, F	55-70	= M; = F (TBS)	-	(A R Patten *et al.*, 2013)
	GD 1-21 (LD; 135 mg/dl)	M,F	55-65	↓ M; = F	-	(Anna R. Patten,

						Sickmann, *et al.*, 2013)
	GD -5 – 22 (W; 69 mg/dl)	M&F	60-300	↓ (TBS) = (HFS)	-	(Brady *et al.*, 2013)
	GD 1-22 (W; 84 mg/dl)	M	105-140	=(HFS 10T) ↓ (HFS 3T)	-	(Varaschin *et al.*, 2010)

1.3 Choline as a Treatment for FASD

Despite the relatively high prevalence of FASD globally, there is no specific treatment for individuals who were exposed to alcohol in the womb. Current therapeutic options for individuals with FASD, as listed by the Centre for Disease Control and Prevention (CDC), involve symptom management, such as medications to reduce hyperactivity, anxiety, and depression, and behavioural therapies (CDC, 2022). However, none of these options address the underlying pathophysiology of developmental ethanol exposure.

1.3.1 Choline: an Essential Nutrient

Adequate choline intake is fundamental throughout the lifespan and consequently was designated an essential nutrient by the Food and Nutrition Board of the National Academies of Medicine in 1998 (**Table 3**) (Leermakers *et al.*, 2015; Wallace *et al.*, 2018). Despite the importance of choline, many individuals do not meet these adequate intakes (AI) (Wallace and Fulgoni, 2017). There are several factors that contribute to the individual required amount of dietary choline, including sex, age, and genetic profile. For instance, a single nucleotide polymorphism in a the promoter region of a gene involved in *de novo* choline synthesis, *phosphatidylethanolamine N-methyltransferase* (*PEMT*), can confer individual vulnerability to organ dysfunction during periods of choline deficiency (Costa *et al.*, 2006). There is also an increased need of choline during fetal growth and the immediate postnatal period for optimal brain development (Zeisel and da Costa, 2009; Derbyshire and Obeid, 2020). During these times the recommended AI increases

from 425 mg/day for non-pregnant individuals to 450 mg/day in pregnancy and 550 mg/day while lactating (Institute of Medicine Food and Nutrition Board, 1998). The need for choline early in perinatal development is emphasized by the six to seven-fold increase in choline concentration in the fetus and neonates (Zeisel and Wurtman, 1981; Ozarda Ilcol, Uncu and Ulus, 2002) which facilitates cell division and tissue growth (Albright *et al.*, 1999; Craciunescu *et al.*, 2003; Wang *et al.*, 2016), neural tube closure (Shaw *et al.*, 2009; Imbard, Benoist and Blom, 2013), as well as enhanced neurogenesis and hippocampal function later in life (Mellott *et al.*, 2004; Glenn *et al.*, 2007; Meck *et al.*, 2008). There also exists a correlative relationship between maternal choline intake and cognitive outcomes. When choline intake in pregnant women was increased to 930 mg/day during the third trimester, supplemented children had faster information processing speeds 13 months after birth (Caudill *et al.*, 2018). Finally, increased maternal choline intake has been related to improved outcomes following second trimester infections (Freedman *et al.*, 2019) which could be beneficial for long-term mental health (Bergdolt and Dunaevsky, 2019). These studies highlight the essential role of choline during early development and have motivated experts in the field to advocate for improved education and policies surrounding choline supplementation during critical time windows (Caudill *et al.*, 2020; Wallace *et al.*, 2020).

Information regarding choline in adolescence and young adulthood is lacking. The Institute of Medicine set the AI at 400 mg/day and 550 mg/day for female and male youth (14-18 years old), respectively. Once again, actual choline intake is estimated to be much lower than the recommended AI. One study of European diets determined young males were consuming 309-373 mg/day and young females ranged from 244-335 mg/day (Vennemann *et al.*, 2015). Similarly, a study of American choline consumption found that youth (14-18) were the least likely age group to be consuming adequate choline (Wallace and Fulgoni, 2017). As there is significant growth

during puberty, it is likely choline plays an important role in adolescent development. The paucity of data at this timepoint poses a significant gap in our understanding of choline requirements throughout puberty.

Sufficient choline intake remains of importance into and throughout adulthood. A systematic review conducted by Leermakers and colleagues (2015) examined 50 studies which explored choline in the diet, blood levels of choline, or choline supplementation. These data determined potential benefits of elevated choline for cognitive health and insulin sensitivity, however inconsistencies made it difficult to determine whether benefits exist in cardiovascular health, body composition, or lipid levels (Leermakers *et al.*, 2015). Additionally, recent evidence supports adequate choline intake in improving cognitive performance as elevated phosphatidylcholine levels are correlated with reduced dementia risk (Ylilauri *et al.*, 2019). Evidence in mouse models of Alzheimer's disease have shown improvements in spatial memory tasks and a decrease in amyloid-β plaque following choline supplementation from 2.5 to 10 months of age (Velazquez *et al.*, 2019). While choline plays a vital role during pregnancy and in the maintenance of long-term cognitive health, gaps remain in the understanding of the importance of choline in adolescence, healthy adulthood, and in the normal aging processes, emphasizing the need for longitudinal studies.

Despite the many benefits of sufficient choline intake, there are some potential harms from excessive choline intake, which is above the tolerable upper limit of 3.5g in adults. These side effects include a fishy body odor, nausea, liver toxicity, and hypotension (Institute of Medicine Food and Nutrition Board, 1998). Choline is also involved in the formation of Trimethylamine-N-Oxide (TMAO) (Zhu *et al.*, 2017). High levels of TMAO due to increase choline intake can lead to cardiovascular disease, including formation of atherosclerotic plaques (Wang *et al.*, 2011).

Therefore, while there are many benefits of choline supplementation, especially for those are consuming inadequate amounts, it should be evaluated on a personalized basis to limit consequences for individuals who may be at risk for cardiovascular disease.

Table 3 Adequate Intakes (AI) of choline across the lifespan by sex.

Age	**AI for Males** (mg/kg)	**AI for Females** (mg/kg)
0-1 year old	125 - 150	125 - 150
1-8 years old	200 - 250	200 - 250
9-18 years old	375 - 550	375 - 400
19 + years old	550	425
Pregnancy (any age)	-	450
Lactation (any age)	-	550

1.3.2 The Role of Choline during Development

Adequate choline is essential throughout perinatal development (Derbyshire and Obeid, 2020). Low maternal choline consumption is thought to be related to failed neural tube closure (NTC), as choline and folate have similar roles in the 1-carbon metabolism cycle (Shaw *et al.*, 2009). However, others have not found an association between choline intake and NTC and instead found a relationship between single nucleotide polymorphisms in *PEMT* and NTC (Mills *et al.*, 2014). In terms of cognitive outcomes, supplementing twice the recommended choline during the third trimester improved visual processing speeds in infants (Caudill *et al.*, 2018). The benefits of choline during prenatal development may only occur when it is in excess of the set AI, as there was no correlation between estimated choline consumption during the second trimester and measures of cognitive development at 3-5 years of age (Irvine *et al.*, 2023). In rodents, adequate choline is necessary for proper cell proliferation and preventing apoptosis within the hippocampus and cortex (Craciunescu *et al.*, 2003; Wang *et al.*, 2016). These changes in hippocampal anatomy

may underlie long-term changes in spatial memory and synaptic plasticity (Pyapali *et al.*, 1998; Meck *et al.*, 2008).

1.3.3 From Gut to Brain: Choline Transport in the Body

Due to its chemical structure ($C_5H_{14}NO$), choline cannot pass through membranes by simple diffusion and thus uses a variety of transporters to access target tissues. There are three main classes of choline transporters: the high-affinity choline transporter (CHT1), the choline transporter-like proteins (CLT1-5), and the organic cation transporter (OCT1-2). These transporters vary in their localization and affinity for choline. The main choline transporters in the adult brain are CHT1, CLT1, and CLT2 (Reviewed in Inazu, 2019) . In development there are significant elevations of choline concentrations in newly born rodents (Zeisel and Wurtman, 1981) that are maintained via the placental CTL-1 and CLT-2 (Baumgartner *et al.*, 2015) with minimal action of OCTs (Kekuda *et al.*, 1998). There also exists a relationship between maternal choline intake and transport to the fetus. Transcript levels of *Ctl1* and *Oct3* were increased with maternal choline supplementation in a dose-dependent and time-dependent manner (Kwan *et al.*, 2017). Permeability of the blood brain barrier has also been shown to be altered in new born rabbits and during the first week postnatally to allow for increased choline transport (Cornford, Braun and Oldendorf, 1982). However, increased maternal choline intake does not necessarily lead to a prolonged increase in the concentration of choline in fetal brain tissue. Kwan and colleagues (2017) supplemented choline to either two or four times the adequate levels in the diet of pregnant Swiss non-albino mice and found that, while male fetal tissue at GD 15.5 demonstrated a dose-dependent increase in choline, this was no longer evident at GD 18.5. Choline was also converted to acetylcholine, phosphocholine and betaine in a sex-dependent manner (Kwan *et al.*, 2017).

Into adulthood the blood brain barrier permeability of choline is decreased as compared to neonates (Cornford, Braun and Oldendorf, 1982) however choline can still cross via CLT-1 and CLT-2 (Inazu, 2019). The blood brain barrier has an efficient and saturable mechanism of choline transport with a maximal transport capacity of 3 nmol/min/g (Allen and Smith, 2001). The high affinity of this process possesses the potential for choline supplementation from the diet to impact choline concentrations within the brain into adulthood.

1.3.4 Potential Mechanisms of Choline as a Treatment

One of the intriguing possibilities of choline as a treatment for FASD rests in the multitude of processes choline is involved in (**Figure 10**), as this mirrors the myriad of disruptions caused

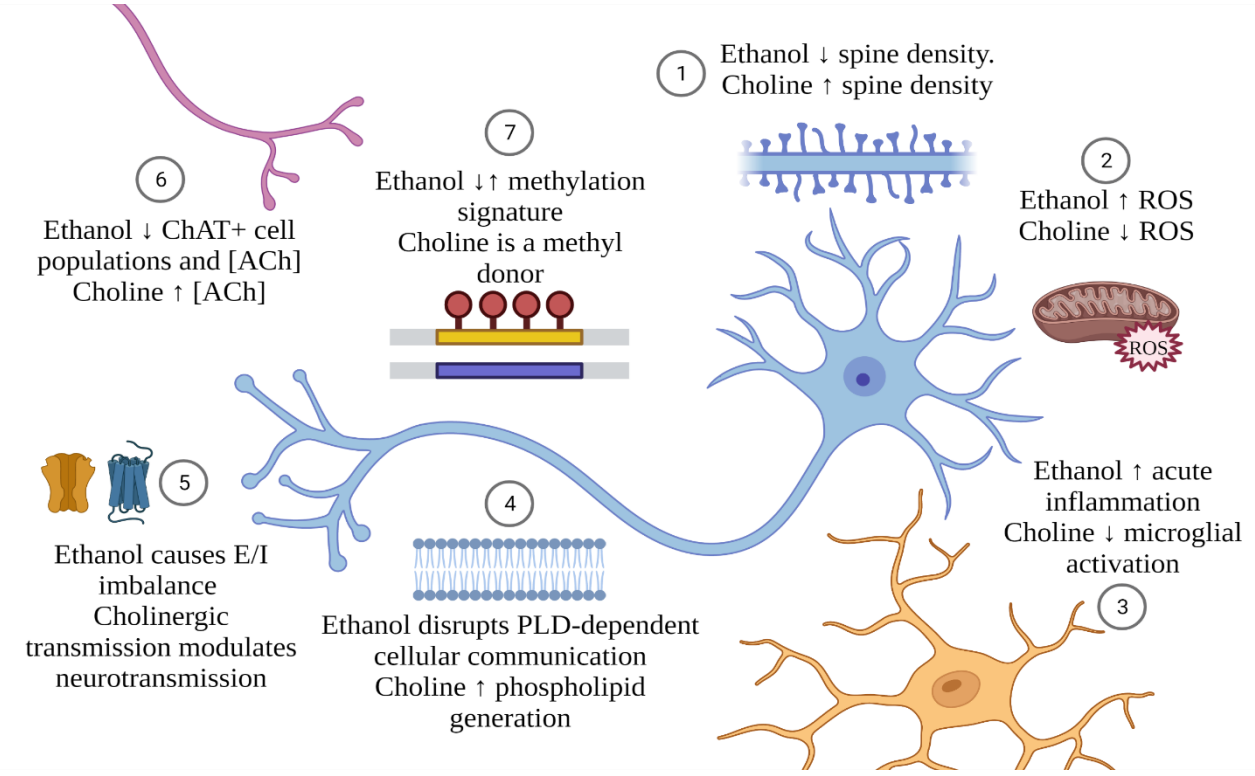


Figure 10 Mechanism of choline as a treatment. Choline and ethanol interact in many similar pathways which is one rationale for choline as a treatment for FASD. These events can be grouped as synaptic changes (receptor functioning, cholinergic transmission), anatomical changes (spine density, phospholipid membrane), intracellular alterations (epigenetic signature and mitochondrial ROS generation), and immune response.

by prenatal ethanol exposure. The four most relevant functions of choline are (1) formation of the phospholipid phosphatidylcholine, (2) epigenetic signature alterations through methyl donation in the 1-carbon metabolism cycle, (3) acetylcholine formation, and (4) anti-inflammatory effects.

Choline is required for the formation of phosphatidylcholine, which comprises approximately 40-50% of cell membranes (Zeisel *et al.*, 1991; Zeisel and da Costa, 2009). Meck and colleagues (2008) supplemented choline through distinct time windows including embryonic, early postnatal, juvenile and into adulthood. Only when choline was supplemented during GD 12-17 or PND 16-30 did animals exhibit an increase in spine density in CA1 and the DG at adulthood, as well as an improvement in performance on the radial arm maze (Meck *et al.*, 2008). The authors suggested these periods correlate with depletion of maternal stores of choline (Zeisel *et al.*, 1995) and a deficiency in the diet during weaning, respectively, as well as critical times for brain development, and thus restricting the window when additional nutrients could be beneficial. To the best of our knowledge, there has not been an investigation into the effects of postnatal choline supplementation on dendritic spine density or dendritic complexity with PNEE. However, Goeke and colleagues (2019) did determine that choline supplementation (100 mg/kg/day) provided simultaneously to ethanol exposure (5 g/kg/day; BAC 60-65 mM) from PND 4-9 did not ameliorate increases in apical complexity. Choline supplemented in controls did decrease many basal dendritic parameters (Goeke *et al.*, 2018). Whether similar results will also appear when choline is supplemented after the ethanol exposure has ceased is yet to be seen.

Choline can be acetylated to form the neurotransmitter acetylcholine in cholinergic neurons, but also in non-neuronal tissues, including the placenta (Bhuiyan, Murad and Fant, 2006) and lymphocytes (Cox *et al.*, 2019). Increased choline consumption may not necessarily lead to increased levels of acetylcholine in the brain. Although there are some reports to indicate an

accumulation of ACh following supplementation (Cohen and Wurtman, 1976), increased choline availability may more likely influence ACh synthesis after heavy acetylcholine activity (Zeisel, 1994). Additionally, while lifelong choline supplementation may not increase the total number of ChAT positive neurons in the MSDB (Wang *et al.*, 2019), others have reported a lasting change in the morphology of MSDB neurons with perinatal choline supplementation (Williams *et al.*, 1998; Kelley *et al.*, 2014). Interestingly, there are many ways in which the body and brain adapt to deficient levels of choline to maintain proper functioning of the cholinergic system. For instance, during embryonic development and lactation the maternal stores of choline are depleted to ensure adequate choline levels for the offspring (Zeisel, 2006). If a completely choline deficient diet is fed to gestating rats, while ACh levels may be lower, there is a remarkable level of plasticity through adjusting ACh recycling to maintain ACh signalling (Cermak *et al.*, 1998). These studies indicate the importance of choline for normal developing brain and long-term brain health. Maternal choline supplementation can also decrease the threshold, not the absolutely magnitude, of LTP in the CA1 of adult offspring (Pyapali *et al.*, 1998). In the context of age-related disease, a murine model of Alzheimer's disease demonstrated that lifelong choline supplementation (2-11 months) increased the density of ChAT positive neurons. Although the density did not resume back to control levels, it did correlate with improved performance on hippocampal and amygdala-dependent tasks (Wang *et al.*, 2019). Maternal choline supplementation is also being actively explored in mouse models of Down Syndrome, where it was found to increase the number of basal forebrain cholinergic neurons in (Ash *et al.*, 2014; Kelley *et al.*, 2014).

One of the metabolites of choline, betaine, actively participates in the methylation of DNA. Betaine can donate one of its three methyl groups to homocysteine to form methionine in a reaction catalyzed by betaine homocysteine methyltransferase (BHMT). Methionine can further be

converted into s-adenosylmethionine (SAM), the most prominent methyl donor in the body (Obeid, 2013). Choline and methyl-donor availability in diet can alter gene expression. For example, Wolff and colleagues (1998) fed pregnant a/a dams diets with varying methyl-donor concentrations and determined the degree of epigenetic modulation in offspring through the degree of eumelanic mottling in their coat, which would occur by differing epigenetic-controlled expressions of the *agouti* gene. Dams fed methyl-donor enriched diets had more offspring with brown coats (Wolff *et al.*, 1998), showing that the nutritional environment during development can influence gene expression. It is likely that the window of greatest efficacy for choline to restore effects on methylation patterning may occur during gestation (Zeisel, 2011) and therefore may pose a critical difference between the mechanism of prenatal choline supplementation and postnatal choline supplementation.

As PNEE results in changes to methylation patterns and a metabolite of choline, betaine, is a methyl donor, it is hypothesized that choline supplementation will restore a normal epigenetic signature. Paradoxically, a study with alcohol exposure from PND 2-10 found hypermethylation induced by PNEE that was reverted by choline supplementation from PND 2-20 (100 mg/kg/day) in the prefrontal cortex and hippocampus (Otero *et al.*, 2012). While the reason for this unexpected result is still not clear, it provides a foundation that postnatal choline supplementation can mitigate adverse effects of PNEE and provides motivation for more targeted investigations at specific genes of interest. For example, gestational choline supplementation (642 mg/L) once again reduced hypermethylation of POMC genes caused by moderate PNEE (Bekdash, Zhang and Sarkar, 2013). However, as Akison and colleagues (2018) noted, choline itself has an effect on several mRNA levels of histone-modifying enzymes, including reducing G9a levels in supplemented PNEE animals and pair-fed controls, reducing Setdb1 and Dnmt1 in choline supplemented PNEE

animals, increasing Dnmt3 above control levels in all choline supplemented animals and decreasing MeCP2 in pair-fed choline-supplemented animals (Bekdash, Zhang and Sarkar, 2013). Therefore, in future work it will be crucial to study the effects of choline supplementation at the level of single gene or transcript level, as well as the effects of choline itself, which may be exerting an effect separate from its actions mitigating PNEE-induced deficits.

Choline has been studied as an anti-inflammatory mechanism in many models of neurodegeneration (Pavlov and Tracey, 2005; Maurer and Williams, 2017). This is in large part to the fact that microglia, as well as astrocytes, express α7 nicotinic ACh receptors (Suzuki *et al.*, 2006; Shen and Yakel, 2012; Egea *et al.*, 2015) as well as σ1 receptors (Jia *et al.*, 2018). Subsequent activation of α7 nicotinic ACh receptors alters cytokine production to induce an anti-inflammatory effect (Parada *et al.*, 2013; Egea *et al.*, 2015). Cholinergic action on glial cells within the hippocampus likely contributes to neuronal firing (Maurer and Williams, 2017), and thus the actions of glia within the brain would be misrepresented if its functionality was limited to only inflammatory action. Additionally, there likely exists a relationship between TLR4 (Toll like receptor 4) and choline in microglia and macrophages, as TLR4-activated macrophages increase the amount of choline taken into the cell to metabolize into phosphatidylcholine for proper mitochondrial function (Sanchez-Lopez *et al.*, 2019). Therefore, there is exists a complex relationship between choline and glia cells in the balance between acting on ACh receptors to mediate cytokine production and uptake to maintain microglia function. While there are no studies examining postnatal choline supplementation on microglia in models of PNEE to date, choline supplementation has been studied within the context of two other disease models: traumatic brain injury (TBI) and Alzheimer's disease. In the former study, a choline-rich diet (increased choline component from 0.2% to 2%) was supplied for two weeks prior to a controlled cortical impact and

within the recovery period. Animals supplemented choline had slightly improved scores on the Morris Water Maze, and perhaps more importantly, had reduced inflammation, as characterized by decreased microglial activation and attenuated the decreased α7 nicotinic ACh receptor binding seen in the non-supplemented impacted group (Guseva *et al.*, 2008). In the latter study, a choline supplemented diet (increased choline volume from 1.1 g/kg to 5.0 g/kg) from 2.5 to 10 months in a mouse model of Alzheimer's disease reduced microglial activation and expression of microglial α7 nicotinic ACh receptors (Velazquez *et al.*, 2019).

1.3.5. Choline Supplementation in Clinical Trials

Clinical trials have begun examining the potential of choline as a treatment for FASD. There are two time periods in which this treatment is being explored: gestational choline supplementation and postnatal choline supplementation. Both periods target the brain at distinct periods of development and also differ in the social context of treatment feasibility. For instance, within the context of FASD, there is a high stigma surrounding alcohol use during pregnancy and this may prevent individuals from seeking medical information and some doctors from offering treatment (Howlett *et al.*, 2019). It is not uncommon for many children to not be diagnosed with FASD until they begin falling behind, academically or socially, in school (Alberta Medical Association, 2003). Thus, treatment options for both maternal and postnatal time periods are necessary.

There have been several studies examining maternal choline supplementation which have provided promising results. Firstly, there are clear benefits of maternal choline supplementation in children not exposed to ethanol. For instance, third trimester choline supplementation (930 mg/day) improved saccade reaction time within the first year of life, a developmental test for memory performance and visual processing, as compared to the control choline intake group (480 mg/day) (Caudill *et al.*, 2018). At a seven year follow up, choline supplemented children continued to

outperform on memory and visual attentional tasks (Bahnfleth *et al.*, 2019, 2022). As such, health care experts in the United Kingdom feel that the evidence that choline is critical to fetal development is overwhelming and have recommended to the NHS to include choline in prenatal supplements, equivalent to that of folic acid (Caudill *et al.*, 2020).

Results in ethanol-exposed offspring are similarly as promising. This work largely stems from studies conducted in Cape Town, South Africa. South Africa has the highest known rates of FASD globally with an estimated prevalence between 13-20% (May *et al.*, 2013). Furthermore, women with children who have FASD often have a dietary profile that is deficient in many key nutrients, including choline (May *et al.*, 2014). Thus, this population of women is one in which choline supplementation may be exceptionally beneficial. Studies by the Jacobson & Jacobson laboratory have demonstrated that maternal choline supplementation (2 g/day until parturition) resulted in improved eyeblink conditioning, faster body weight and head circumference growth, improved visual memory, as well as an increase in volume of six brain region (Jacobson *et al.*, 2018; Warton *et al.*, 2021). Studies on maternal choline supplementation have also been conducted in the Ukraine, a country which has an estimated FASD prevalence four times that of the global average (Lange *et al.*, 2017). While choline was not supplemented alone in the study by Coles and colleagues (2015), prenatal vitamins and minerals improved scores on the Bayley Scales of Infant Development in alcohol-exposed offspring. However, the addition of choline (750 mg/day) had no further benefit. Of note, there was a trend to negative scores on motor testing at 6 months in the choline supplemented group that should be considered in future studies (Coles *et al.*, 2015). In a separate study from the same laboratory, the addition of choline to prenatal vitamins improved a visual-based cognitive task while vitamins alone had no significant benefit (Kable *et al.*, 2015). These clinical data provide evidence that choline supplementation during brain development may

be particularly beneficial for improving cognitive outcomes in both the exposed and non-exposed brain.

Since not all individuals who consume alcohol during pregnancy will be able to supplement choline during the gestational period, there is an essential need for treatments which are effective during childhood. Wozniak and colleagues supplemented choline (500 mg/day for nine months) in young children with FASD (2.5 to 5 years old) and examined the effects on cognitive outcomes immediately following the treatment, as well as at a four and seven year follow up. Younger choline supplemented children (2.5 – 4 years old) had an improved performance on some cognitive measures, in such as in the delayed memory task, but there were no changes in scores of global cognition (Wozniak *et al.*, 2015). At the four-year follow up, some improvements were still evident, including in non-verbal IQ, working memory, and in one memory task. However, there was no change in other forms of IQ, executive functioning, most memory tasks, or behavioural functioning (Wozniak *et al.*, 2020). At the seven year follow up there were no longer benefits in working memory but choline treatment did result in long-lasting changes in visuo-motor performance and improved white matter ultrastructure (Gimbel *et al.*, 2022). The age in which choline is supplemented appears to be critical, as a trial with older children with FASD (6-10 years old), found that choline supplementation (625 mg/day for six weeks) resulted in no improvements in memory, attention, or executive functioning tasks (Nguyen *et al.*, 2016). There are a couple of caveats and limitations to highlight with these findings. For one, Nguyen and colleagues supplemented choline for a shorter period, six weeks versus nine months, which may contribute to the lack of significant findings. Furthermore, neither study analyzed the data by biological sex. As discussed earlier, preclinical work has demonstrated that male offspring are more vulnerable to

developmental ethanol exposure, therefore stratifying data by sex may be important, especially as puberty is reached in this cohort.

1.3.6. Choline Supplementation in Preclinical Trials

The promising results in clinical trials is further supported by preclinical research, which will also help to elucidate the mechanism behind choline supplementation in the ethanol-exposed brain. Behavioural tests have examined the effects of choline supplementation on learning and memory, executive functioning, anxiety, and in motor functioning (Reviewed in Akison *et al.*, 2018). Choline supplementation at doses of 10, 50, and 100 mg/kg all improved performance in the Morris Water Maze in ethanol exposed females. Interestingly, in this study males were not impaired with postnatal ethanol exposure (PND 4-9) and had no further benefit with choline (Thomas *et al.*, 2007). Choline-treated animals also had a shorter path length in the Morris Water Maze when choline was supplemented from PND 10-30 or from PND 10-20. Interestingly, choline from PND 21-30 or from PND 40-60 had no benefits on spatial memory (Ryan, Williams and Thomas, 2008; Schneider and Thomas, 2016). Choline supplementation also improved other cognitive factors in ethanol exposed offspring, including working memory (Waddell and Mooney, 2017), anxiety-like behaviour (Monk, Leslie and Thomas, 2012), and motor development (Bearer *et al.*, 2015). Choline supplementation has been demonstrated to ameliorate changes in the methylation signature (Otero *et al.*, 2012) and modulate both the neural and peripheral response to immune challenge (Baker *et al.*, 2022). While these data indicate that postnatal choline supplementation may be beneficial to hippocampal functioning when provided during adolescent brain development, it is unknown as to the changes at a synaptic level, nor the mechanism of action.

1.4 Project Aims

This dissertation aims to understand how postnatal choline supplementation alters synaptic plasticity in the PNEE brain. Throughout all aims both male and female offspring will be utilized to further explore sex-dependent effects in PNEE and choline efficacy. This dissertation can be further stratified into three questions:

1. Can postnatal choline supplementation improve long-term potentiation and long-term depression in the juvenile PNEE dentate gyrus?
2. Do any changes in juvenile hippocampal plasticity result in parallel changes in learning and memory performance?
3. Do the improvements seen in juvenile hippocampal synaptic following the treatment persist into adulthood?

CHAPTER 2: METHODS

2.1 Animal Generation

All procedures were approved by the UVic Animal Care Committee. A summary of animal generation can be seen in **Figure 11**. Sprague Dawley adult males and nulliparous females were housed together overnight. The following morning pregnancy was determined through the detection of sperm in a vaginal lavage. The day of sperm detection was marked as Gestational Day (GD) 1. Females were singly housed and randomly assigned to either control or ethanol conditions. Control dams remained on the rat chow (PicoLab Rodent Diet 5053 or Lab Diet Rodent 5001, Lab Diets, St. Louis, MO) and had *ad libitum* access to both chow and water. Ethanol dams had their food removed and were provided an ethanol-containing liquid diet (35.5% ethanol-derived calories; Dyets Inc, Bethlehem, PA) in addition to *ad libitum* access to water. Ethanol dams were weaned onto the liquid diet, beginning with 1/3 ethanol diet and 2/3 a pair-fed liquid diet on GD 1, 2/3 ethanol diet and 1/3 pair-fed liquid diet on GD 2, and finally 3/3 ethanol diet on GD 3. The amount of diet provided and the amount remaining after 24 hours was weighed daily. Diet consumption was analyzed by subtracting the amount of provided diet by the amount of remaining diet. Dams were weighed on GD 1, 8, 15 and 21. Ethanol dams were transferred back to the control rat chow on GD 21 and remained on this diet for the remainder of their lives. All dams typically gave birth in the evening of GD 22. Offspring were sexed and assessed on PND 2 for signs of health, including clear milk lines, pink colour, and movement. Any pups that did not meet these health requirements were culled on PND 2. Offspring and dams were weighed on postnatal day (PND) 2, 4, and daily throughout the injection period. On PND 22, offspring were weaned into sex-matched, like-condition cages.

Offspring were pseudo-randomly assigned to either a saline control or choline injected group on PND 10, such that there were representative saline and choline pups from all litters, when possible. Pups were weighed daily and received a subcutaneous injection of either sterilized saline (0.9% NaCl, volume matched to choline offspring) or choline chloride (100 mg/kg/day, dissolved in sterile saline) from PND 10 until PND 30. Two adult females were only injected with saline from PND 10-21 but were included as there was no change in magnitudes of LTP with the shortened saline exposure into adulthood (30.2 ± 17.4% vs 38.7 ± 12.7%, p = 0.703). During the injection period (between 2-5 pm PST), eye opening was assessed in later litters as a developmental marker. Eye opening criteria was set such that it was an all-or-nothing event requiring both eyes to be fully open.

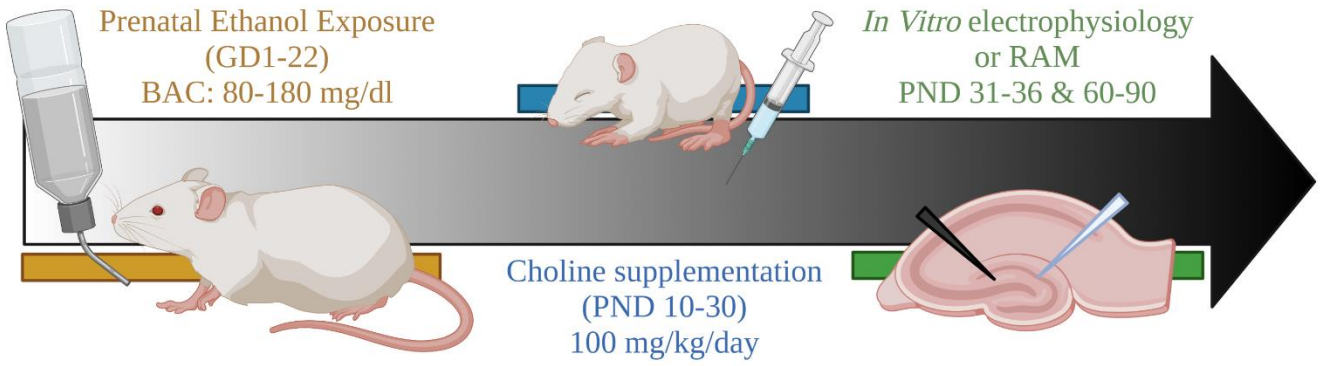


Figure 11 Summary of animal generation. Pregnant dams begin liquid ethanol diet procedure following sperm detection and throughout the entirety of gestation (gestational day; GD1-22). Beginning on postnatal day (PND) 10 until PND 30, offspring are randomly assigned to saline or choline (100 mg/kg/day) treatment. Offspring are analyzed for behaviour or *in vitro* electrophysiology from PND 31-36 or PND 60-90.

2.2 Hippocampal Slice Preparation

Offspring were examined in adolescence, between PND 31-36, and in early adulthood, PND 60-90. Animals were deeply anesthetized with isoflurane until toe pinch and tail pinch reflexes were no longer evident. They were then quickly euthanized with decapitation and the brain was carefully extracted. The extracted brain was placed into ice cold (4°C), oxygenated (95% O_2,

5% CO_2) artificial cerebral spinal fluid (aCSF; 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$ and 10 mM Dextrose) to remove any additional debris. The brain was then placed on filter paper dampened with aCSF on an inverted petri dish. The cerebellum and prefrontal cortex were removed and the brain was hemisected. Each hemisphere was placed onto its medial surface. A portion of the dorsal surface was the brain was removed at approximately a 30° angle to provide a flat surface and optimal orientation of fibers. Each hemisphere was rotated 90° onto the newly cut dorsal surface of the brain, the white matter was then removed, and each hemisphere was fixed to a vibratome chuck with VetBond™. After a brief drying period, the chuck was secured in the Vibratome filled with ice cold, oxygenated aCSF. Each hemisphere was sliced into 400 µm sections. Slices containing the hippocampus were transferred to a prepared holding container, containing warmed (32°C), oxygenated aCSF. Slices were allowed to recover for one hour before electrophysiology experiments.

2.3 In Vitro Electrophysiology

An upright microscope (Olympus BX5OWI) was used to place the concentric bipolar (FHC, Bowdoinham, ME) and glass micropipette recording electrodes in the medial perforant pathway of the dentate gyrus (**Figure 12**). Electrodes were situated such that a minimum field excitatory postsynaptic potential (fEPSP) of 0.7 mV was generated. The maximum fEPSP was determined by increasing the magnitude of the provided current until the fEPSP stopped increasing in size. Slices used in LTP experiments were set at 50% of the maximum fEPSP amplitude and slices used in LTD experiments were set at 70% of the maximum fEPSP amplitude. A stable preconditioning baseline was recorded by delivering a 0.12 ms pulse of current at a frequency of 0.067 Hz for 20 minutes either in the presence of PTX (100 µM) or in aCSF for LTP and LTD experiments, respectively. Following the preconditioning recording, a paired pulse test and input/output curve

were run. The paired pulse test consisted of two pulses delivered 50 ms apart repeated six times. The average slope of the second pulse was divided by the average slope of the first pulse to determine the paired pulse ratio. The input/output curve consisted of ten pulses of increasing pulse width from 0.3 µs to 300 µs. The amplitude of the fiber volley and the fEPSP were analyzed for each pulse.

A conditioning stimulus was then applied to the tissue that corresponded to the either LTP (high frequency stimulation (HFS), 50 pulses @ 100 Hz, repeated 4x with a 30 second intertrain interval) or LTD (low frequency stimulation (LFS), 900 pulses @ 1 Hz). Slices were included in the data set if the amount of potentiation 50-60 minutes following the HFS exceeded 10%, or else were

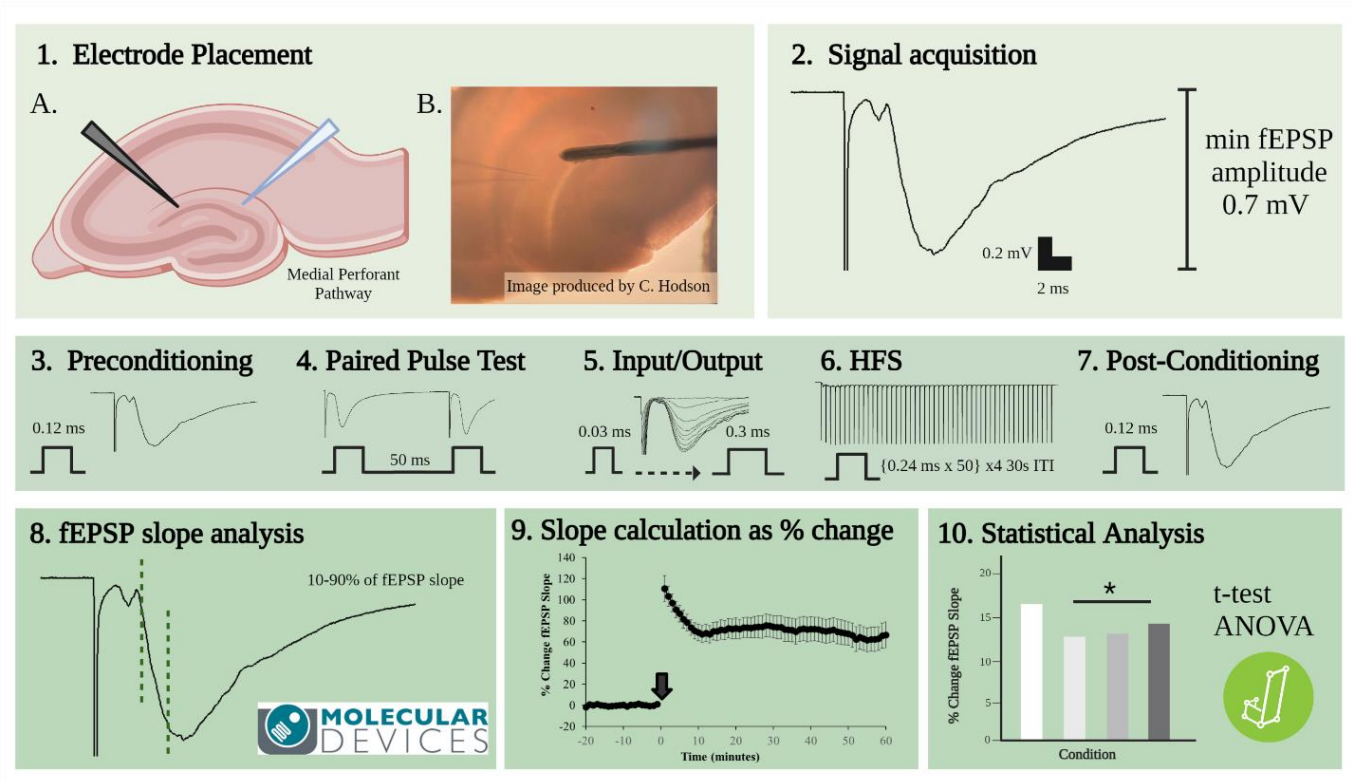


Figure 12 Summary of electrophysiology procedure. 1A. Schematic of electrodes are placed in the medial perforant pathway (MPP) of the dentate gyrus (DG). 1B. Photograph of electrode placement in a hippocampal slice. 2. Representative fEPSP representing signal acquisition. 3-7. Protocol steps, including preconditioning recording, paired pulse test, input/output curve, high frequency stimulation (HFS), and pos-conditioning recording. 8. Representative cursor placement for fEPSP slope analysis. 9. Slope is presented as a percent change from preconditioning average. 10. Statistical analysis using JASP software.

considered a return to baseline and thus not potentiated. Following the conditioning stimulation, all slices were returned back to the 0.12 ms @ 0.067 Hz protocol on aCSF for an additional 60 minutes. The slope (between 10% - 90% of the rising phase of the fEPSP slope) of the fEPSP was analyzed using pClamp 11 software (Molecular Devices, San Jose, California) and binned to 1 minute intervals. The postconditioning recording was plotted as a percent change from the average preconditioning slope.

For the threshold experiments, all of the previously described experiments remained the same, however the frequency of the HFS conditioning stimulation was reduced to either 30, 50 or 70 Hz. All protocols continued to deliver 50 pulses four times with a thirty second intertrain interval.

For GluN2B isolation experiments, the original signal was collected in regular aCSF and set to 70% of the maximum. The aCSF was replaced with an aCSF containing low Mg^{2+} (0.1 µM), glycine, and NBQX (2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline;10 µM) for 12 minutes. After the signal was no longer visible due to the AMPA receptor antagonism, the stimulation intensity was increased to evoke the maximum fEPSP. After 12 minutes stimulating with a 0.12 ms pulse width at a frequency of 0.067 Hz, a paired pulse test was performed. The aCSF was replaced with an aCSF containing low Mg^{2+}, glycine, NBQX, and ifenprodil (10 µM) for 12 minutes on the 0.12 ms pulse width at a frequency of 0.067 Hz protocol. Following drug application, an additional paired pulse test was performed. Finally, this paradigm was repeated in the presence of APV (2R)-amino-5-phosphonovaleric acid; 50 µM) to determine the resulting signal was NMDA receptor dependent.

2.4 Radial Arm Maze

A subset of animals were tested on their spatial memory using the 8-arm radial arm maze (RAM; Maze Engineers, Cambridge, MA) from PND 28-34 (**Figure 13**). All experiments occurred in the dark phase of the light cycle to increase animal activity. Animals were habituated from PND 28-30. Animals were brought into the procedure room in a darkened box and placed into the centre of the maze. Habituation consisted of 5 minutes over three days of exploration with treats (Froot Loops™, Kellogg's) randomly distributed throughout the maze. For all non-habituation trials, the food rewards were placed in a paper cup and spatial cues were visible above arms 3 and 7. Additional treats were placed outside of all arms to interfere with any scent cues and the maze was wiped down with Virkon™ disinfectant between trials. All videos were recorded with EthoVision XT 11.5 software (Noldus, Netherlands). Prior to the task the food was removed from offspring cages to increase motivation and was provided again immediately following task completion.

A working memory task was performed on PND 31. In the working memory task all 8 arms were baited with a food reward in paper cups. The animal was placed in the center of the maze and allowed to explore for 5 minutes. A successful completion of the task occurred once all arms were visited at least one time. Number of entries to complete the task, time required to enter all arms at least once, and total number of arms visited in the 5 minute allotted time were recorded. The distance the animal travelled during the five minutes was analyzed with EthoVision software after all animals had completed the task.

The reference memory task ran from PND 32 until PND 34. In this task, 4 pseudo-randomly chosen arms were baited for each animal. Some arm combinations were assigned to ensure that there was at least one animal in every condition performing the maze at each complexity level. The baited arms stayed consistent throughout the trials for each animal for the remainder of the

task. The animal was placed in the center of the maze and given 5 minutes or 3 minutes to explore for Trials 1 and 2-5, respectively. A successful task was determined to be when all 4 of the baited arms were visited at least once. On days in which two iterations of the task were performed, a minimum of 1 hour separated each trial. The number of entries until the task was completed, time to finish, and search strategy was recorded. Search strategies were defined as follows:

(1) Serial exploration: over 2/3 of entries consisted of turns in single direction (i.e., traveling from arm 1 to 2 to 4);

(2) Direct exploration: the animal travelled directly to the baited arms in less than 6 entries;

(3) Random exploration: the animal explored the arms with no discernable pattern (i.e., sporadic left and right turns or crossing the center of the maze to a non-baited arm).

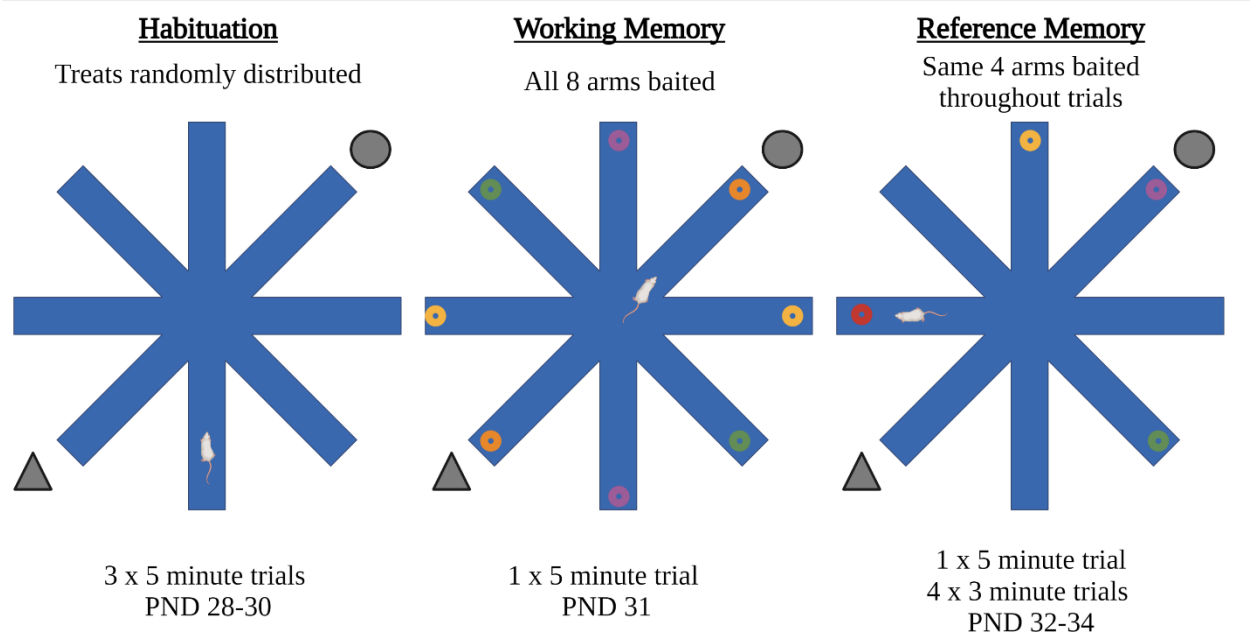


Figure 13 Radial arm maze procedure overview. The Radial Arm Maze (RAM) takes place from PND 28-34. Habituation occurs over 3 days where rewards are randomly distributed over the maze to encourage exploration and decrease anxiety. Spatial cues (circle and triangle) are placed over two arms. Working memory takes place on PND 31. In this task, all arms are baited and placed in Dixie cups. The reference memory task takes place over PND 32-34. In this task, four arms are pseudo-randomly selected to be baited and stay constant over the remainder of the trial.

If an animal failed to complete the task their score was penalized. 10 seconds of time was added for each arm not explored and their number of entries increased by 1/(# of arms completed). For example, if 3 out of 4 arms were explored in the 3 minutes of Trial 3 with 20 entries, the final score was logged as 190 seconds (180+10) and 27 entries (20 +20/3). The number of failed tasks was recorded and compared between conditions, but there was no difference in the prevalence of failed trials between groups. Occasionally no exploration would occur. In these instances, the data were removed from analysis. Finally, data were segregated into difficulty of task. As the arms were randomly baited, the task could either have 0 arms of separation (i.e., baited arms 3, 4, 5, and 6), up to 3 arms of separation on the shortest path (i.e., baited arms 2, 4, 6, and 8).

2.5 Statistical Analysis

Statistical analysis was performed with JASP (ANOVAs) or Excel (Student t-tests and chi squared tests). Statistical significance was established *a priori* as $p < 0.05$.

Litter characteristics and initial deficits in LTP between control and PNEE offspring were compared using a student's t-test. Normality was examined with a Shapiro-Wilkes test and homogeneity of variance was assessed with Levene's Test. Effect sizes were represented as *Cohen's d* (*d*). For electrophysiology experiments and the working memory data, a 2-way ANOVA was used to analyze the data, using prenatal environment (Control, PNEE) and postnatal treatment (Saline, Choline) as factors. Data were analyzed for male and female offspring separately. Normality was examined with a Shapiro-Wilkes test and homogeneity of variance was assessed with Levene's Test. If assumptions were not met a transformation was applied and is noted in the respective results section. In rare instances where transformation of the data did not meet requirements, a non-parametric Kruskal-Wallis test was used. Effect sizes were reported as partial eta squared (η_p^2). When a positive interaction was found, a Tukey *post hoc* analysis was utilized.

A Repeated-Measures ANOVA was used to analyze input/output curves, weight gain, and reference memory performance. For input/output tests the assumption of sphericity was not met and therefore a Greenhouse-Gessier correction was applied. Effect sizes were reported as partial eta squared (η_p^2). When a positive interaction was found, Bonferroni correction for multiple comparisons was utilized as a *post hoc* analysis. A chi squared test or Fisher's exact test (if any values were less than 5) were performed in Excel to determine patterns in discrete data, such as in the case of eye opening, number of slices demonstrating depression, and search strategy. The chi squared value was calculated using the following formula: $x^2 = \sum \frac{(O-E)^2}{E}$

CHAPTER 3: RESULTS

Some of the following work has been published in different formats and has been adapted for this dissertation.

Juvenile LTP data: Grafe, E. L. Wade, M.M.M. Hodson, C.E., Thomas, J.D., Christie, B.R. (2022) 'Postnatal Choline Supplementation Rescues Deficits in Synaptic Plasticity Following Prenatal Ethanol Exposure', *Nutrients*, 14(10). doi: 10.3390/NU14102004.

	Design	Animal Generation	Data Collection	Data Analysis	Manuscript Preparation
Grafe, E.L.	Yes	Yes	Yes	Yes	Yes
Wade, M.M.M.	-	Yes	-	-	-
Hodson, C.E.	-	Yes	Yes	-	-
Thomas, J.D.	-	-	-	-	Yes
Christie, B.R.	Yes	-	-	-	Yes

3.1 Prenatal Ethanol Consumption Does Not Alter Maternal or Litter Characteristics

Control and ethanol dams were weighed throughout gestation. While the average dam weight at the beginning (GD1) and end of gestation (GD22) were similar between groups (**Table 4**; $p > 0.05$), dams fed the ethanol-containing diet had a significantly overall lower percent weight gain than control dams ($p = 0.018$, Cohen's $d = 0.130$, Student's two tailed t-test). On average, PNEE dams consumed a daily average of 64.9 ± 2.2 g of diet, resulting in a consumption of 12.1 ± 0.2 g/kg of ethanol per day. Dams from both conditions had similar gestational lengths of 22 days ($p > 0.05$) and the ethanol diet throughout gestation also did not change the number of pups born ($p > 0.05$), nor the ratio of male to female offspring ($p > 0.05$). Offspring in the ethanol-exposed offspring did not weigh differently than control offspring at PND 2 nor at PND 4 ($p > 0.05$).

Dams	**Control Diet** (n=15)		**PNEE Diet** (n=15)		**p value**
Weight at GD1 (grams)	259.3 ± 10.0		273.9 ± 11.0		0.349
Weight at GD21 (grams)	388.8 ± 10.2		374.5 ± 17.0		0.479
Weight Gain (% from baseline)	151.2 ± 3.9%		139.2 ± 2.6%		0.018*
Gestational Length (days)	21.8 ± 0.2		22.0 ± 0.0		0.343
# Pups born	10.7 ± 0.6		9.7 ± 0.6		0.289
Male:Female ratio	1.0 ± 0.2		1.0 ± 0.1		0.954
Offspring	**Male** (n=19)	**Female** (n=19)	**Male** (n=22)	**Female** (n=23)	**p value**
Pup weight PND 2 (grams)	7.54 ± 0.22	7.29 ± 0.23	7.13 ± 0.23	6.73 ± 0.21	M: 0.184 F: 0.108
Pup weight PND 4 (grams)	9.76 ± 0.38	9.14 ± 0.27	9.64 ± 0.37	8.97 ± 0.37	M: 0.327 F: 0.356

Table 4. Maternal characterics during and follow pregnancy. * indicates significance $p < 0.05$. PND: postnatal day. Offspring n = number of litters, as pup weight is averaged across all males and females.

3.2 PNEE Delayed Development in Exposed Offspring

All offspring were weighed daily throughout the injection period from PND 10 until PND 30. There was a significant effect of PNEE on weight gained in males (**Figure 14A**; $F(1,29) = 6.70$, $p = 0.015$, $\eta_p^2 = 0.188$), but there was no main effect of choline supplementation ($F(1,29) = 0.59$, $p = 0.449$, $\eta_p^2 = 0.020$). There was a significant interaction between prenatal condition and postnatal day ($F(2.64,76.55) = 7.39$, $p < 0.001$, $\eta_p^2 = 0.203$). *Post hoc* analysis determined that PNEE males weighed less than controls on PND 26 ($p < 0.1$), PND 27 ($p < 0.05$) and PND 28-30 ($p < 0.01$). Similarly in females (**Figure 14B**), there was a significant main effect of prenatal exposure ($F(1,36) = 9.02$, $p = 0.005$, $\eta_p^2 = 0.200$), but no effect of choline treatment on weight gain

(F(1,36) = 0.82, p = 0.371, η_p^2 = 0.022). There was a significant interaction of prenatal environment and day (F(2.19,78.86) = 7.44, p < 0.001, η_p^2 = 0.171). *Post hoc* analysis determined PNEE

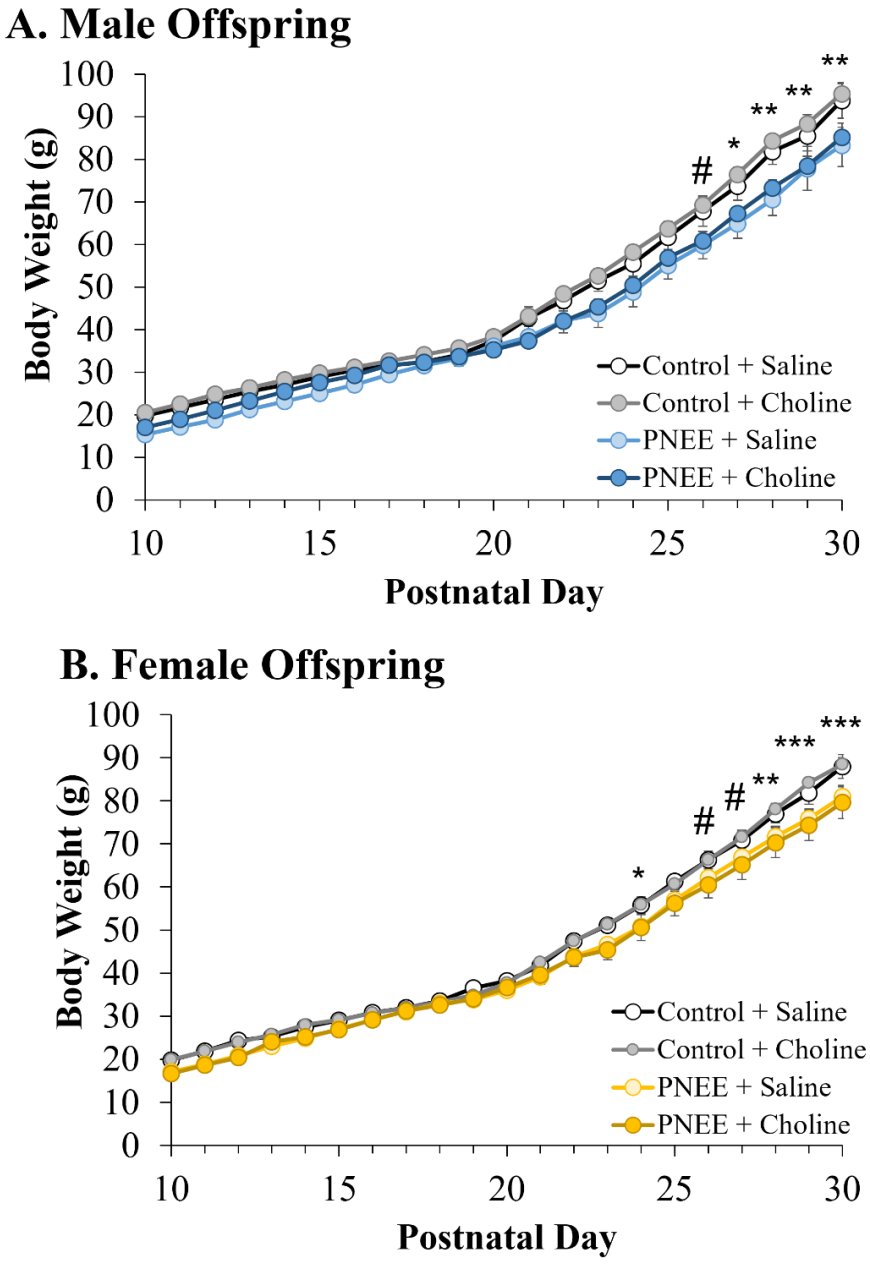


Figure 14 PNEE decreased weight gain in offspring. PNEE decreased weight gain in both male (A) and female (B) offspring. N=7-13 animals for males and N=9-14 animals for females. # p <0.1, * p < 0.05, ** p < 0.01, and *** p < 0.001.

offspring weighed less than control offspring on PND 24 ($p = 0.040$), PND 26 to 27 ($p < 0.1$), PND 28 ($p = 0.01$) and PND 29-30 ($p < 0.001$).

Eye opening was recorded as a measurement of neuronal development. The *a priori* criteria required both eyes to be fully open and, as no sex differences were evident, male and female offspring were combined for this measurement. Due to the discrete nature of the data and the low N in some groups (N less than 5), the data were also collapsed by postnatal treatment in order to perform a Fisher exact test. While this limits the ability to determine an interaction between ethanol exposure and choline treatment, there was no evidence of an effect choline treatment. PNEE delayed eye opening on PND 16 as compared to control offspring (**Figure 15**; $p < 0.001$).

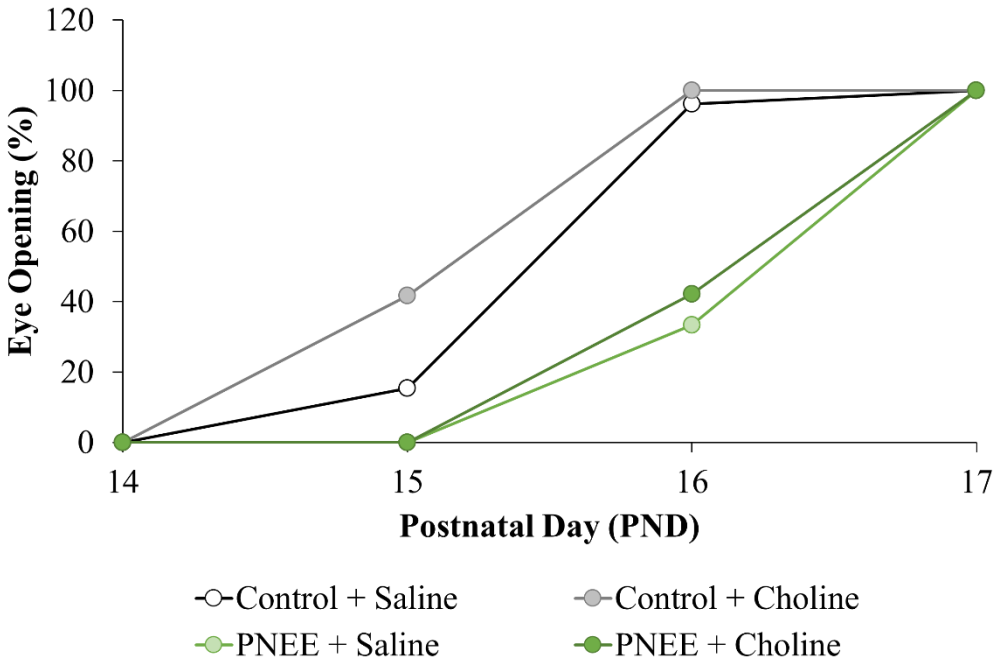


Figure 15 Eye opening is delayed in PNEE offspring. Eye opening was assessed as a binary event. The percent of pups with their eyes open is reported by discrete postnatal day. Male and female pups were combined. N = 18-27 per group.

Aim 1. Can postnatal choline supplementation improve long-term potentiation and long-term depression in the juvenile PNEE dentate gyrus?

3.3 PNEE Increased Basal Excitability in Juvenile Females

Initial analysis of basal synaptic properties between conditions was conducted, including paired pulse ratios (PPR) and input/output curves (I/O), to determine changes in presynaptic and postsynaptic properties, respectively. On average, PPR showed slight paired pulse facilitation in all groups. In juvenile male offspring (**Figure 16A**) there was no effect of prenatal exposure ($F(1,59) = 0.09$, $p = 0.768$, $\eta_p^2 = 0.001$), postnatal treatment ($F(1,59) = 0.61$, $p = 0.440$, $\eta_p^2 = 0.010$),

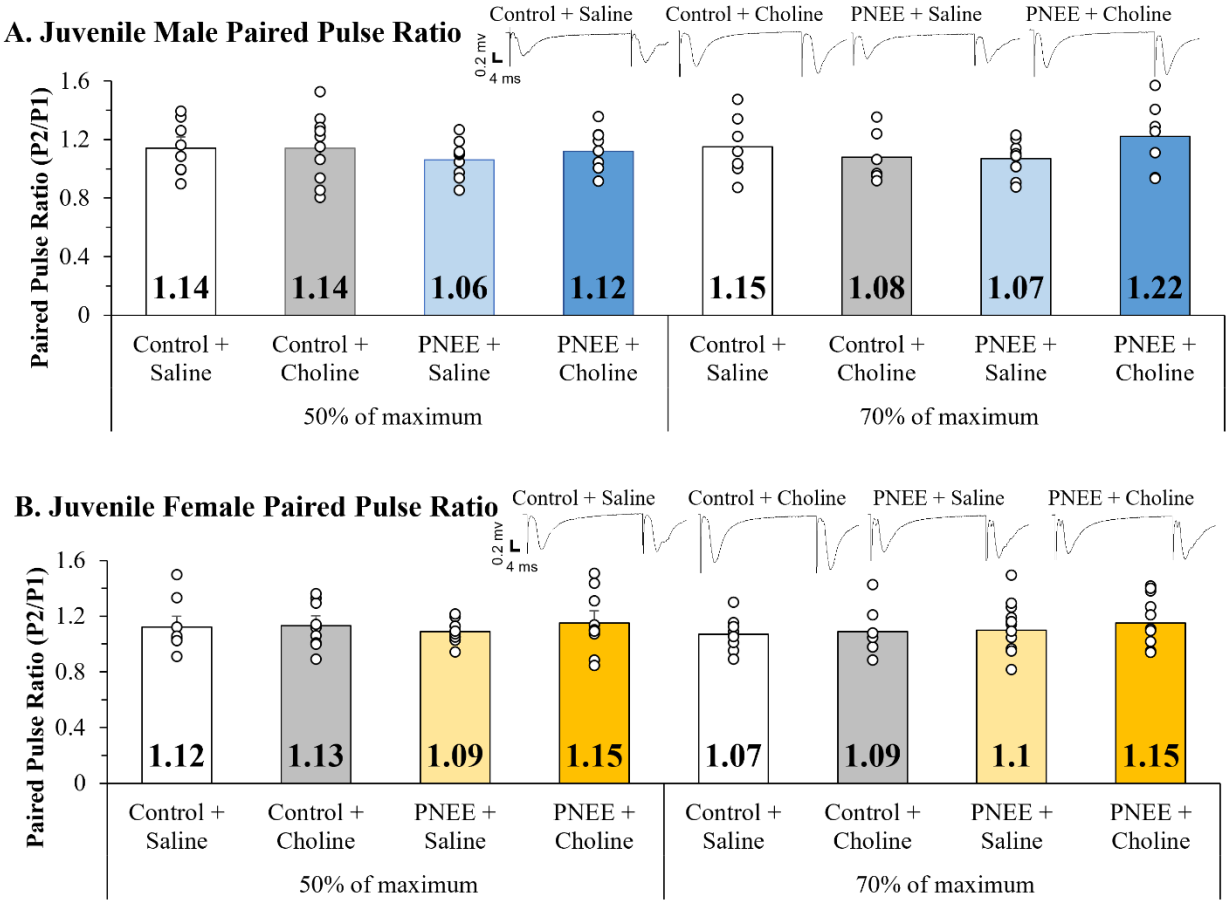


Figure 16 Juvenile paired pulse ratios did not differ between conditions. Paired pulse ratios for males (A) and females (B) demonstrate equivalent amounts of facilitation in all experimental conditions. Representative traces are located above graphs. Each point is the PPR per slice and the bars represent the average PPR. All error bars are ± SEM.

or experimental condition (i.e. set to 50 or 70% of the maximum; $F(1,59) = 0.06$, $p = 0.805$, $\eta_p^2 = 0.001$) on the PPR. Similarly in female offspring (**Figure 16B**) there was no effect of prenatal exposure ($F(1,66) = 0.70$, $p = 0.404$, $\eta_p^2 = 0.011$), postnatal treatment ($F(1,66) = 0.60$, $p = 0.441$, $\eta_p^2 = 0.009$), or experimental condition ($F(1,66) = 0.02$, $p = 0.668$, $\eta_p^2 = 0.003$) on the PPR.

I/O tests, however, uncovered sex-specific effects of PNEE and postnatal choline supplementation. In all conditions, there was a significant overall effect of pulse width on fEPSP

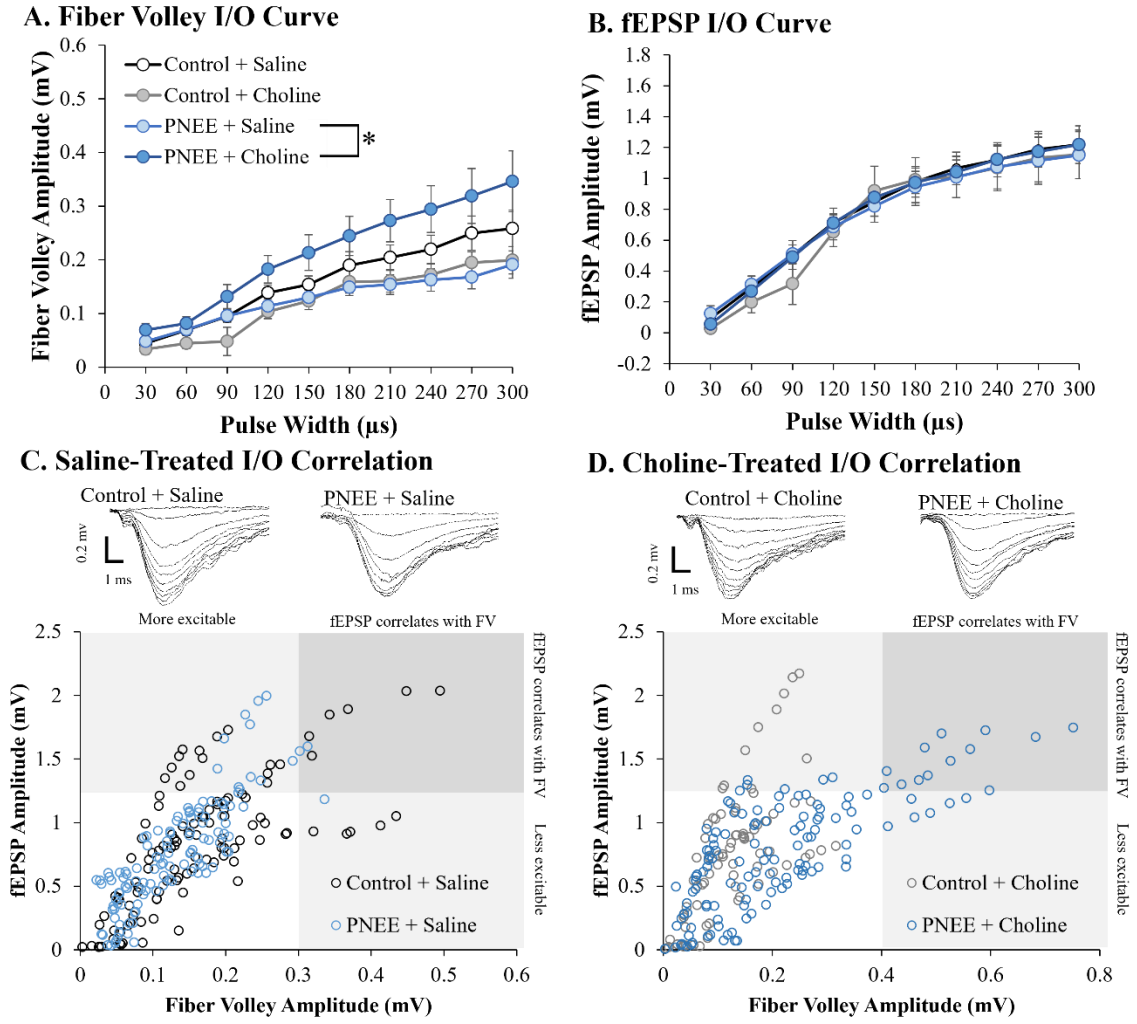


Figure 17 Increase in FV amplitude in PNEE males. Analysis of fiber volley (A) and fEPSP amplitude (B) across increasing pulse widths. Each point is averaged across slices and error bars are ± SEM. Representative traces for each condition are visible above (C) and (D). The relationship between fiber volley and fEPSP amplitudes for saline-treated (C) and choline-treated juvenile male offspring (D). Each point represents a single waveform, therefore each slice is represented by ten points (1 per pulse width). * $p < 0.05$.

amplitude ($p < 0.001$). In the male LTP condition (fEPSP set to 50% of the maximum and in the presence of PTX), there was a significant between-subject interaction with prenatal exposure and postnatal treatment in fiber volley amplitude (**Figure 17A**; $F(1,39) = 6.20$, $p = 0.017$, $\eta_p^2 = 0.137$). Follow up Tukey *post hoc* analysis determined this effect was between choline-treated and saline-treated PNEE males ($p = 0.039$).When considering pulse width as a factor, there was again a significant interaction between prenatal environment and postnatal treatment ($F(1.43,55.61) = 5.33$, $p = 0.015$, $\eta_p^2 = 0.120$). The Bonferroni correction for multiple comparisons, however only indicated a significant different between saline-treated and choline-treated PNEE offspring at the 300 µs pulse width ($p = 0.037$). There was no effect of either prenatal condition nor postnatal treatment on the fEPSP amplitude (**Figure 17B**; Prenatal: $F(1,42) = 0.07$, $p = 0.791$, $\eta_p^2 = 0.002$; Postnatal: $F(1,42) = 0.86$, $p = 0.360$, $\eta_p^2=0.020$). This relationship can be seen when the FV is directly compared to the fEPSP amplitude (**Figure 17C & D**) – choline-treated PNEE males overall have a wider range of FV amplitudes which is not seen to the same extent in other treatment conditions and does not increase excitability.

In the male LTD condition (fEPSP set to 70% of the maximum without PTX), there was no effect of prenatal diet nor postnatal choline treatment on fiber volley (**Figure 18A**; Prenatal: $F(1,31) = 0.534$, $p = 0.471$, $\eta_p^2 = 0.017$; Postnatal: $F(1,31) = 0.237$, $p = 0.629$, $\eta_p^2 = 0.008$) or fEPSP amplitude (**Figure 18B**; Prenatal: $F(1,32) = 0.069$, $p = 0.794$, $\eta_p^2=0.002$; Postnatal: $F(1,32)=2.680$, $p = 0.111$, $\eta_p^2=0.077$). While it may appear that choline-treated increased fEPSP amplitude, following the Greenhouse-Geisser correction there was no effect of treatment with increasing pulse width ($F(1.20,38.43) = 3.60$, $p = 0.058$, $\eta_p^2 = 0.101$). The relationship between fiber volley and fEPSP is represented in (**Figure 18C & D**).

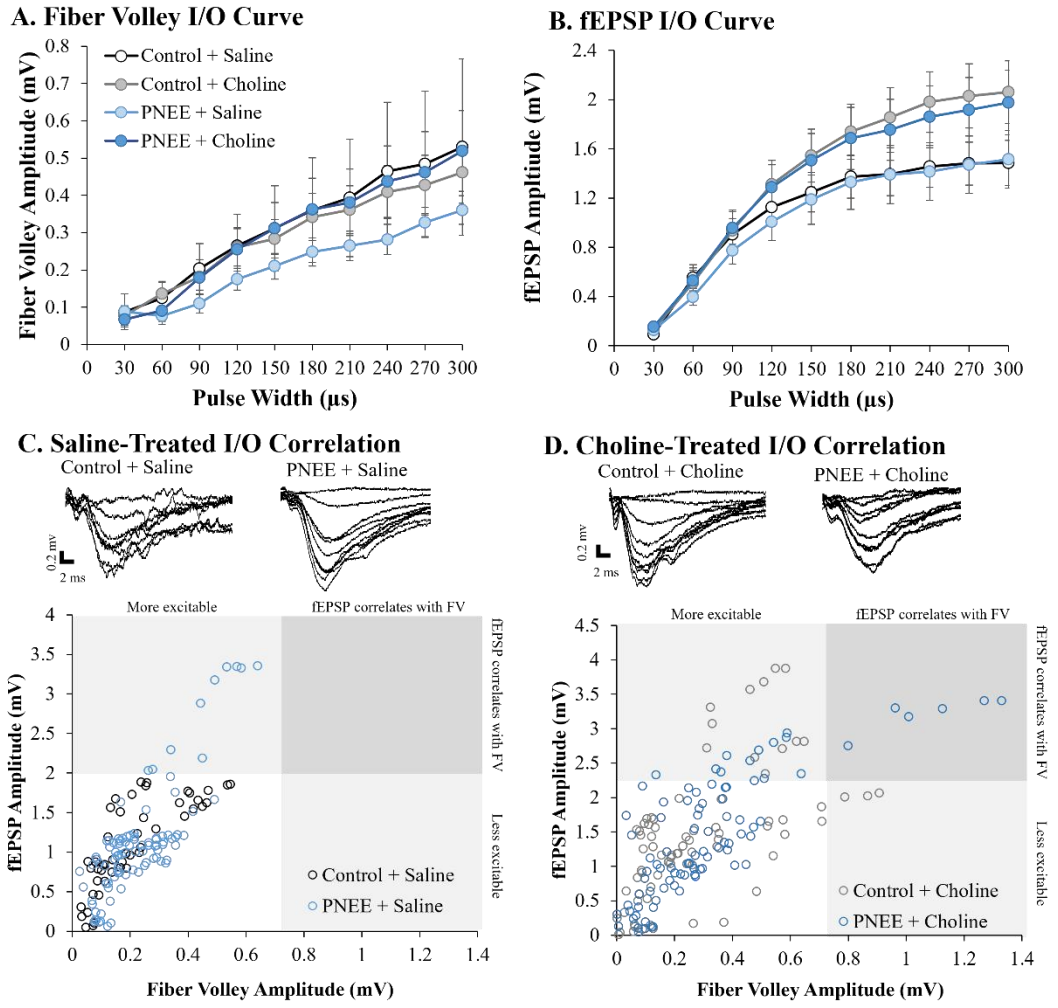


Figure 18 No change in basal excitability when set to 70% of maximum. Analysis of fiber volley (A) and fEPSP amplitude (B) across increasing pulse widths. Each point is averaged across slices and error bars are ± SEM. Representative traces for each condition are visible above (C) and (D). The relationship between fiber volley and fEPSP amplitudes for saline-treated (C) and choline-treated juvenile male offspring (D). Each point represents a single waveform; therefore each slice is represented by ten points (1 per pulse width).

In female offspring a different picture emerges. Within LTP conditions, there was not a significant effect of prenatal condition or choline treatment on fiber volley amplitude (**Figure 19A**; Prenatal: $F(1, 40) = 3.08$, $p = 0.087$, $\eta_p^2 = 0.007$; Choline: $F(1, 40) = 0.43$, $p = 0.518$, $\eta_p^2 = 0.001$). When examining fEPSP amplitude across pulse width (**Figure 19B**), there was a significant interaction between prenatal exposure and choline treatment ($F(1.44, 60.32) = 6.21$, $p = 0.008$, $\eta_p^2 = 0.130$). *Post hoc* analysis determined that saline-treated PNEE females were significantly

different than choline-treated PNEE females at 240 µs (p=0.044), 270 µs (p=0.025), and 300 µs (p=0.020). The relationship between fiber volley and fEPSP amplitude is evident in **Figure 19C** and **19D**, as saline-treated PNEE females generally had increased fEPSP amplitude for the corresponding fiber volley. In the LTD condition (i.e. fEPSP set to 70% of maximum and no $GABA_A$ receptor antagonism), there was no change in fiber volley amplitude with either prenatal

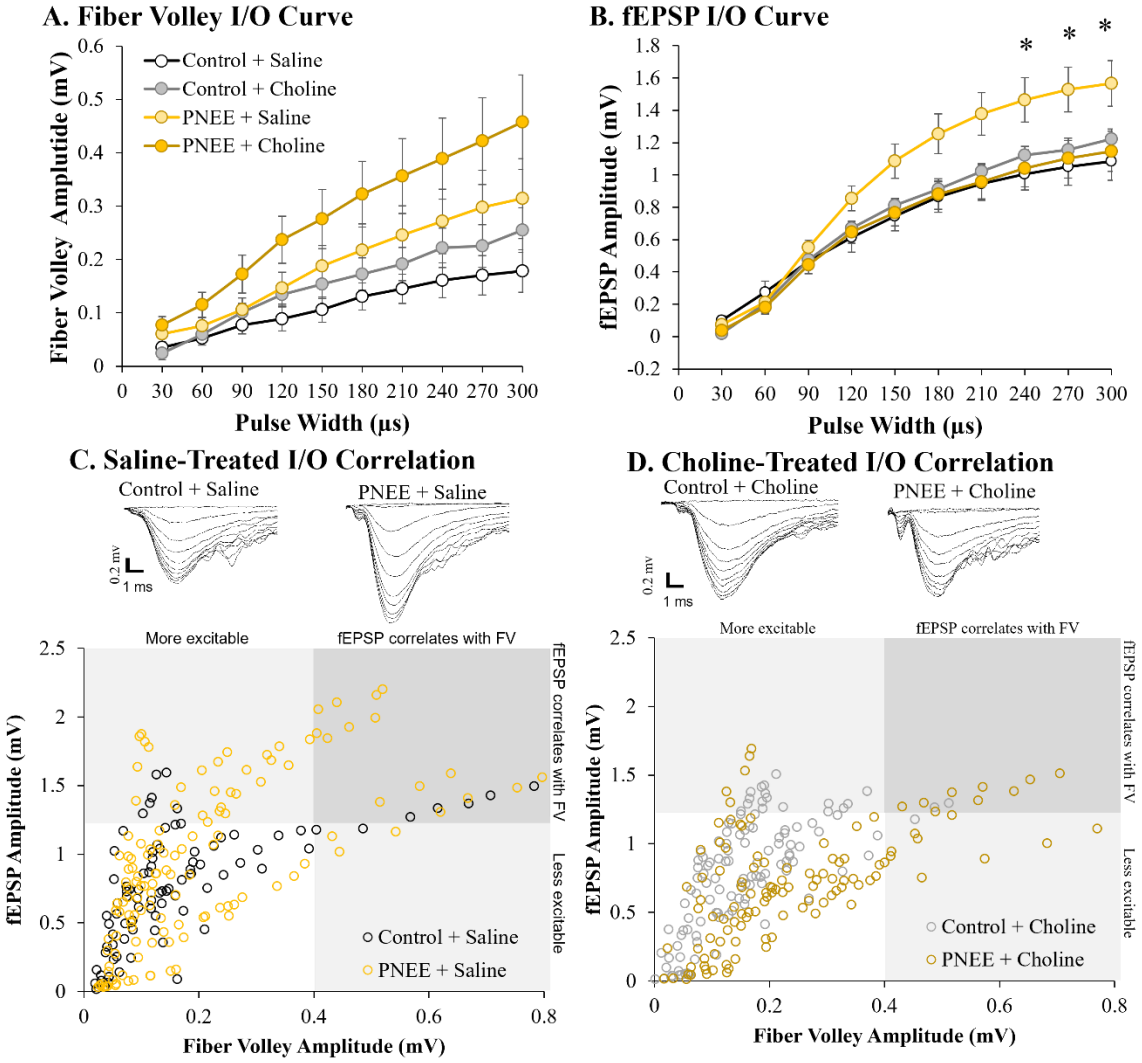


Figure 19 Increased excitability in saline treated PNEE offspring. Analysis of fiber volley (A) and fEPSP amplitude (B) across increasing pulse widths. Each point is averaged across slices and error bars are ± SEM. Representative traces for each condition are visible above (C) and (D). The relationship between fiber volley and fEPSP amplitudes for saline-treated (C) and choline-treated juvenile female offspring (D). Each point represents a single waveform; therefore each slice is represented by ten points (1 per pulse width). * $p < 0.05$.

condition (**Figure 20A.** $F(1,35) = 0.03$, $p = 0.856$, $\eta_p^2 = 0.00$) or choline treatment ($F(1,35) = 5.15$, $p = 0.478$, $\eta_p^2 = 0.014$). Despite a similar trend of saline-treated PNEE offspring having an increased fEPSP amplitude at higher pulse widths, there was no change in fEPSP amplitude across conditions (**Figure 20B.** Prenatal: $F(1,39) = 3.35$, $p = 0.075$, $\eta_p^2 = 0.079$; Choline: $F(1,39) = 2.56$, $p = 0.117$, $\eta_p^2 = 0.062$). The lack of clear relationship between fiber volley and fEPSP amplitude is displayed in **Figure 20C** and **20D.**

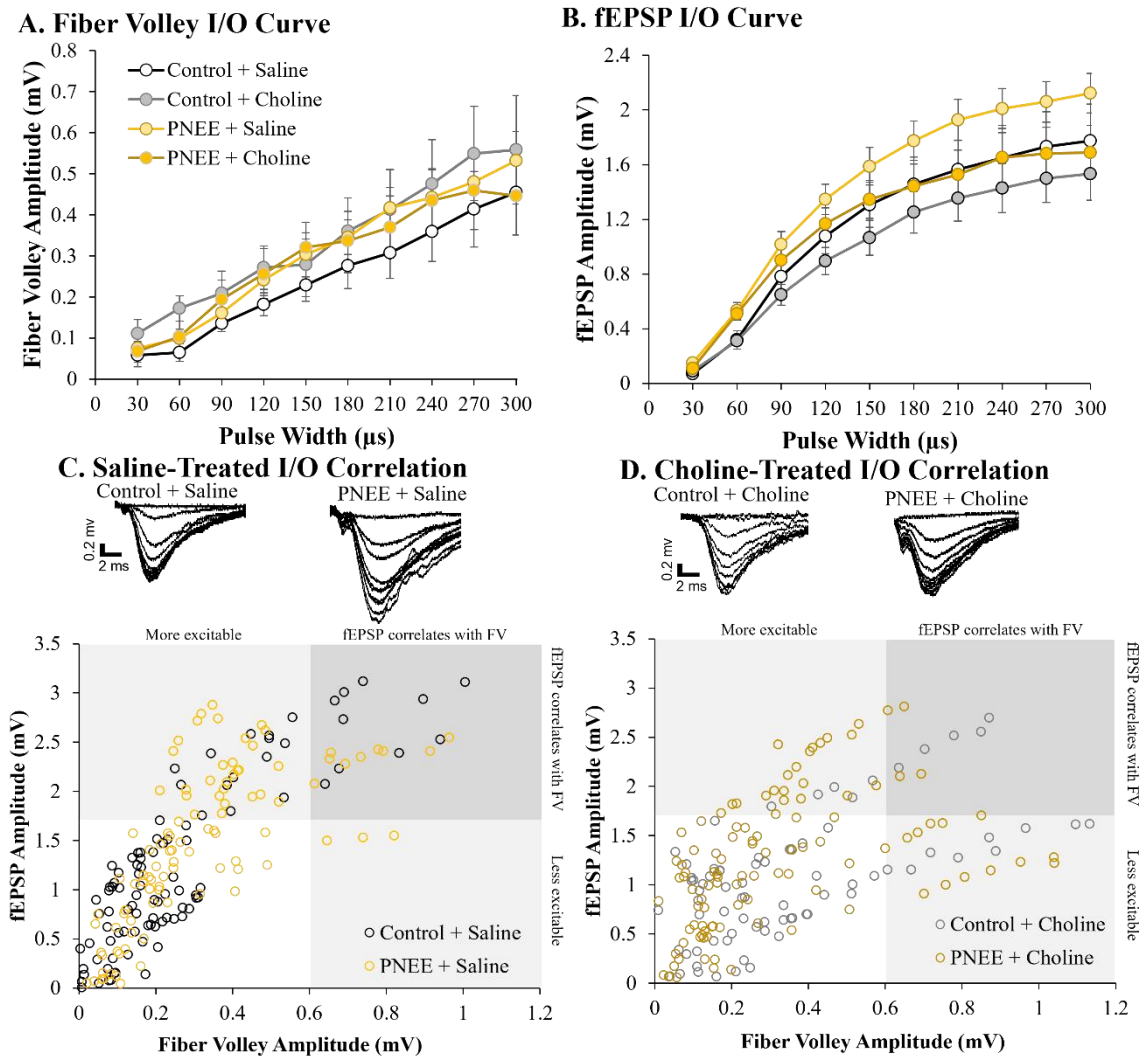


Figure 20 No change in excitability in female offspring at 70% of maximum. Analysis of fiber volley (A) and fEPSP amplitude (B) across increasing pulse widths. Each point is averaged across slices and error bars are ± SEM. Representative traces for each condition are visible above (C) and (D). The relationship between fiber volley and fEPSP amplitudes for saline-treated (C) and choline-treated juvenile female offspring (D). Each point represents a single waveform; therefore each slice is represented by ten points (1 per pulse width).

3.4 Choline Restored LTP in Juvenile PNEE Male, But Not Female, Offspring

To determine whether postnatal choline supplementation could improve synaptic plasticity, long-term potentiation (LTP) was analyzed in juvenile offspring following the cessation of the choline treatment (PND 31-36). A summary of average LTP across all conditions, as well as slice, animal and litter numbers are found in **Table 5**. In juvenile male offspring (**Figure 21A**), the

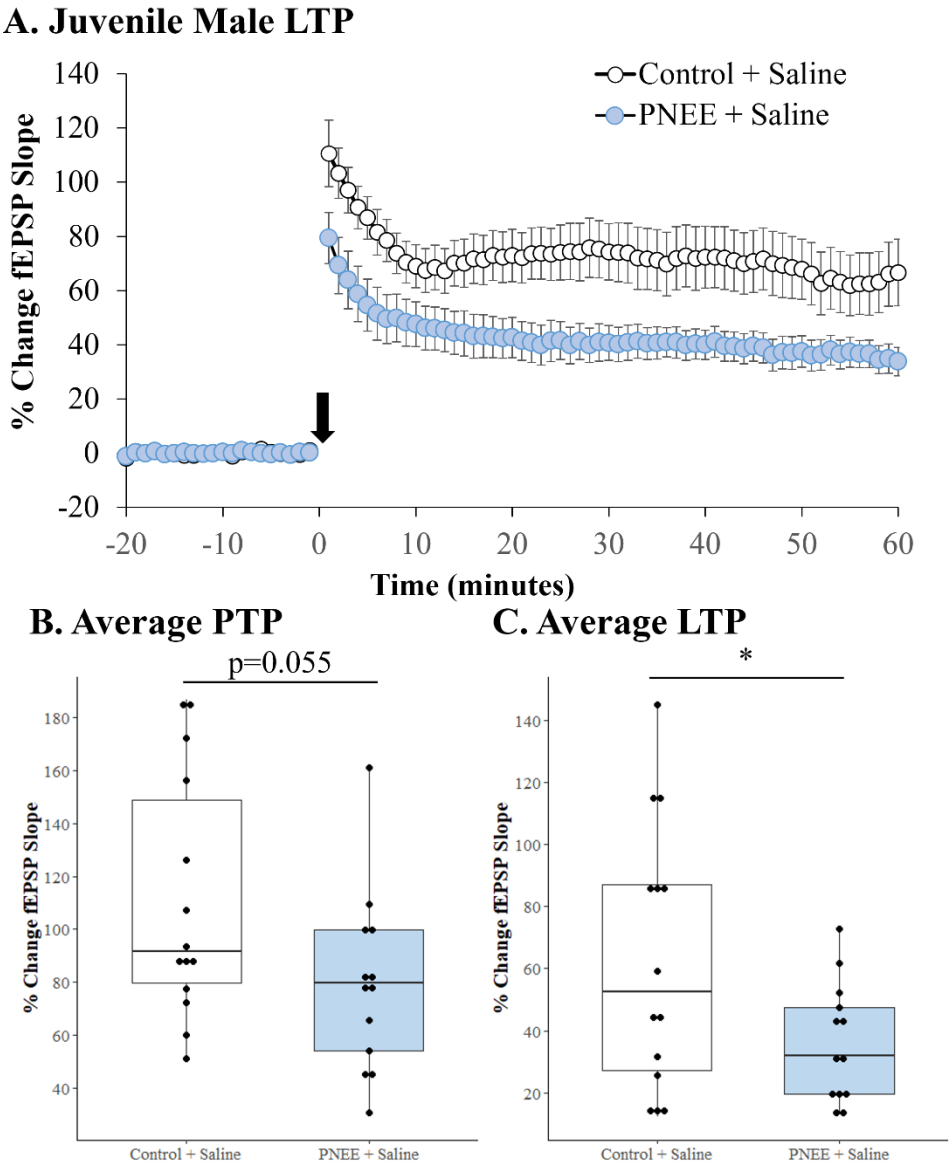


Figure 21 PNEE decreases LTP in male offspring. A. Average change in fEPSP slope binned to one-minute intervals. B. Average PTP and C. LTP. Each point represents an individual slice. Black arrow indicates HFS. *p<0.05. All error bars are ± SEM.

magnitude of post-tetanic potentiation (PTP; **Figure 21B**) was decreased with PNEE, albeit non-significantly (p = 0.055, Cohen's *d* = 0.77; Student's two-tailed t-test). However, there was a significant decrease in the magnitude of LTP with PNEE in male offspring (**Figure 21C**; p = 0.047, Cohen's *d* = 0.50).

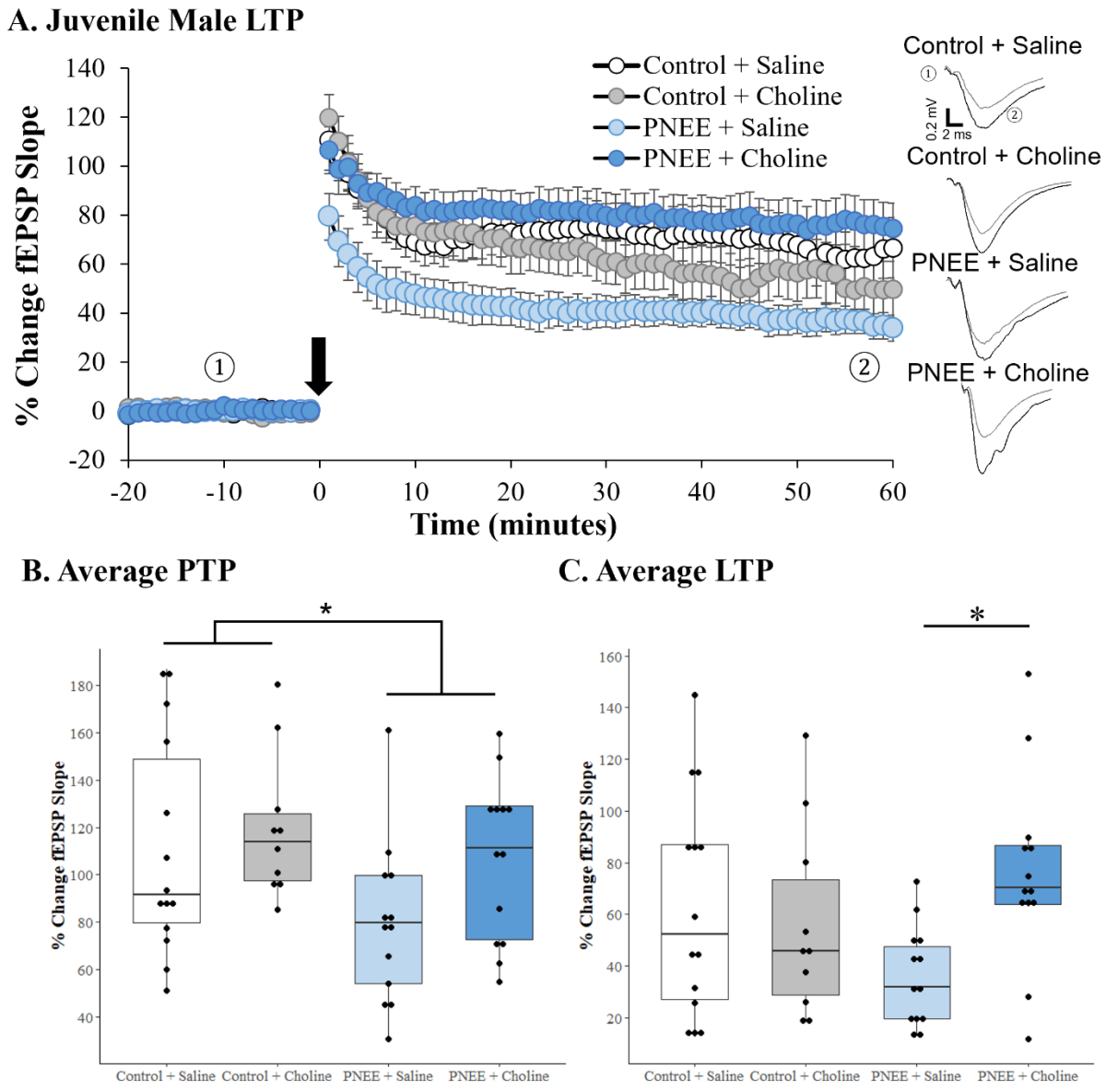


Figure 22 Choline treatment ameliorated deficits in LTP in PNEE male offspring. A. Average change in fEPSP slope binned to one minute intervals. The black arrow indicates HFS. Representative traces are featured to the right with the pre-conditioning record ① in grey and post-conditioning recording ② in black. B. Average PTP and C. LTP. Each point represents an individual slice. *p<0.05. All error bars are ± SEM.

Choline treatment was next examined to determine if it could ameliorate deficits due to PNEE (**Figure 22A**). PNEE decreased PTP as compared to control animals (**Figure 22B**; $F(1, 46) = 4.35$, $p = 0.043$, $\eta_p^2 = 0.09$); choline treatment did not alter magnitudes of PTP ($F(1, 46) = 2.90$, $p = 0.096$, $\eta_p^2 = 0.06$). However, there was a significant interaction between PNEE and choline treatment in the magnitude of LTP (**Figure 22C**; $F(1, 46) = 4.17$, $p = 0.047$, $\eta_p^2 = 0.11$). Further post-hoc analysis determined there was a significant increase in LTP in choline-treated PNEE male

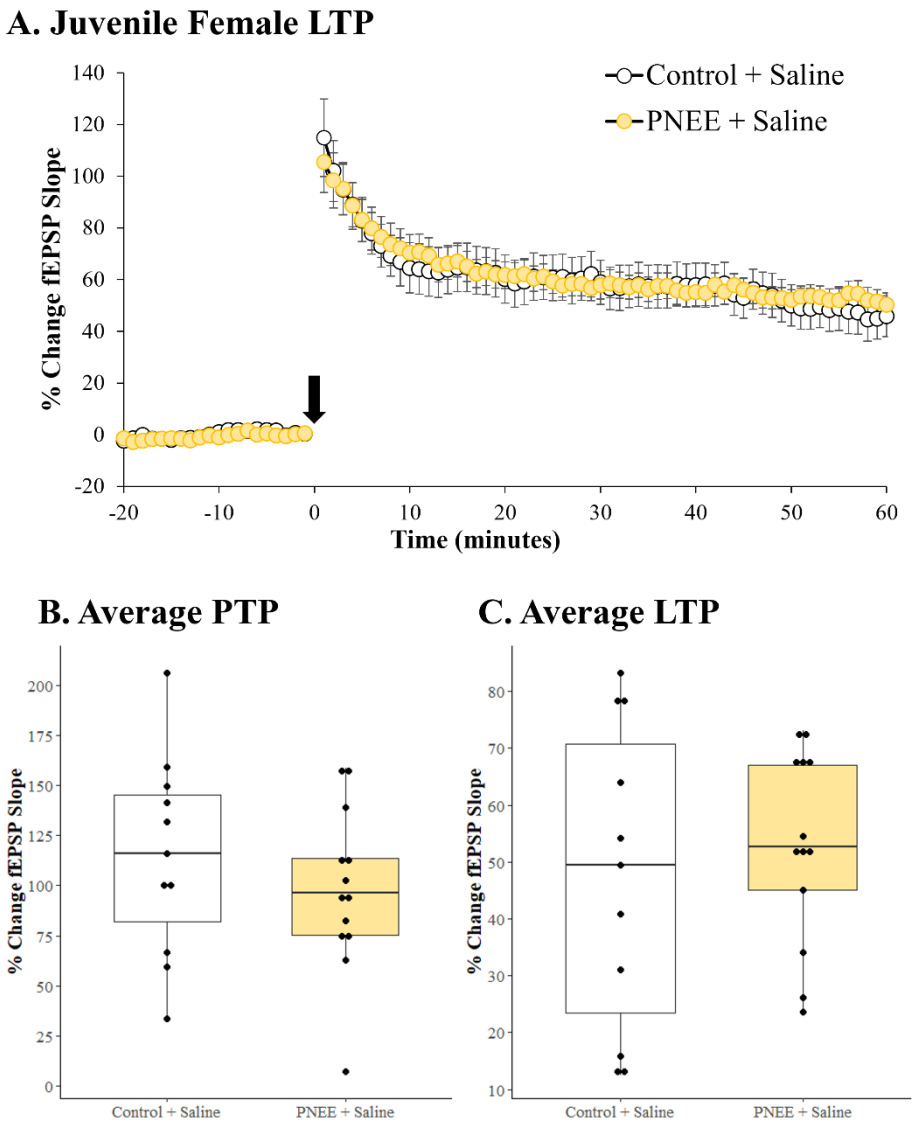


Figure 23 No Change in LTP with PNEE in female offspring. A. Average change in fEPSP slope binned to one minute intervals. B. Average PTP and C. LTP. Each point represents an individual slice. All error bars are ± SEM.

offspring as compared to saline-treated PNEE males (p = 0.039).

Female PNEE offspring were also examined for effects of PNEE (**Figure 23A**). Unlike in male offspring, there was no decrease in the degree of PTP in due to PNEE in female offspring (**Figure 23B**; p = 0.376, Cohen's *d*= 0.53, two-tailed t-test). Additionally, there was no difference in overall magnitudes of LTP in PNEE offspring (**Figure 23C**; p = 0.577, Cohen's *d*= 0.25, two-

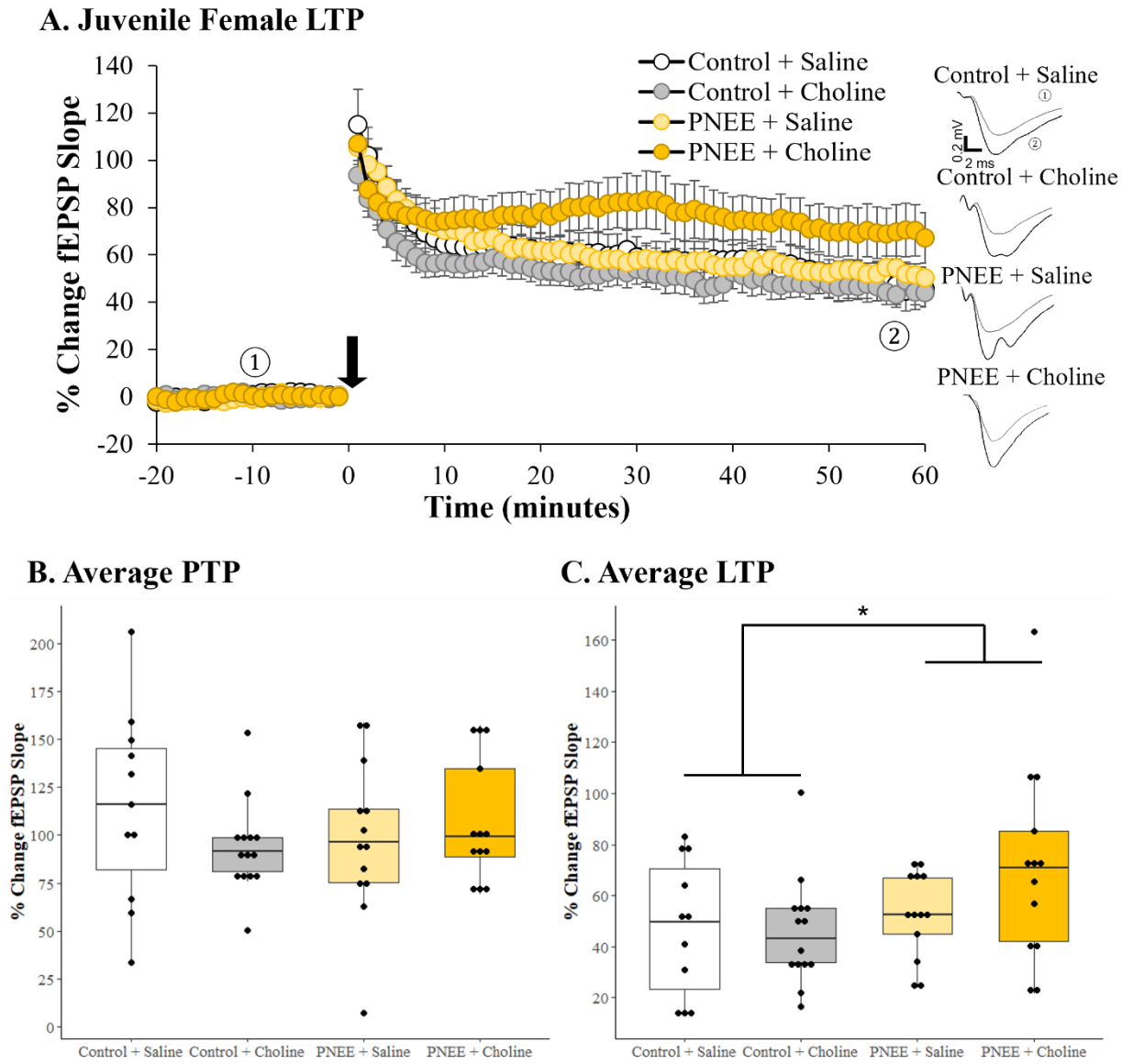


Figure 24 Choline supplementation increased LTP in female PNEE offspring. A. Average change in fEPSP slope binned to one minute intervals. The black arrow indicates HFS. Representative traces are featured to the right with the pre-conditioning record ① in grey and post-conditioning recording ② in black. B. Average PTP and C. LTP. Each point represents an individual slice. *p<0.05. All error bars are ± SEM.

tailed t-test).

Finally, LTP was evaluated in female PNEE offspring treated with choline (**Figure 24A**). There was no main effect of prenatal condition (**Figure 24B.** $F(1, 46) = 0.02$, $p = 0.888$, $\eta_p^2 = 0.00$) nor postnatal treatment ($F(1, 46) = 0.34$, $p = 0.557$, $\eta_p^2 = 0.010$) on PTP. However, there was a main effect on prenatal condition on LTP, such that that PNEE increased LTP in female offspring (**Figure 24C.** $F(1, 46) = 4.20$, $p = 0.046$, $\eta_p^2 = 0.080$). While choline-treated female PNEE offspring increased LTP as compared to saline-treated PNEE females, there was no effect of choline treatment ($F(1, 46) = 0.87$, $p = 0.356$, $\eta_p^2 = 0.050$).

Table 5 Juvenile LTP Summary for male and female offspring. LTP: long-term potentiation, SEM: standard error of the mean, s: slice number, a: animal number, l: litter number.

	Male Offspring		**Female Offspring**	
	LTP ± SEM	**N (s, a, l)**	**LTP ± SEM**	**N (s, a, l)**
Control + Saline	63.9 ± 11.5%	14s, 5a, 4l	47.4 ± 8.2%	11s, 6a, 5l
Control + Choline	52.7 ± 11.7%	10s, 4a, 3l	45.9 ± 5.8%	14s, 6a, 3l
PNEE + Saline	36.0 ± 5.4 %	13s, 4a, 4l	52.9 ± 4.6 %	13s, 4a, 4l
PNEE + Choline	76.0 ± 10.1%	13s, 6a, 5l	69.5 ± 10.9%	13s, 5a, 4l

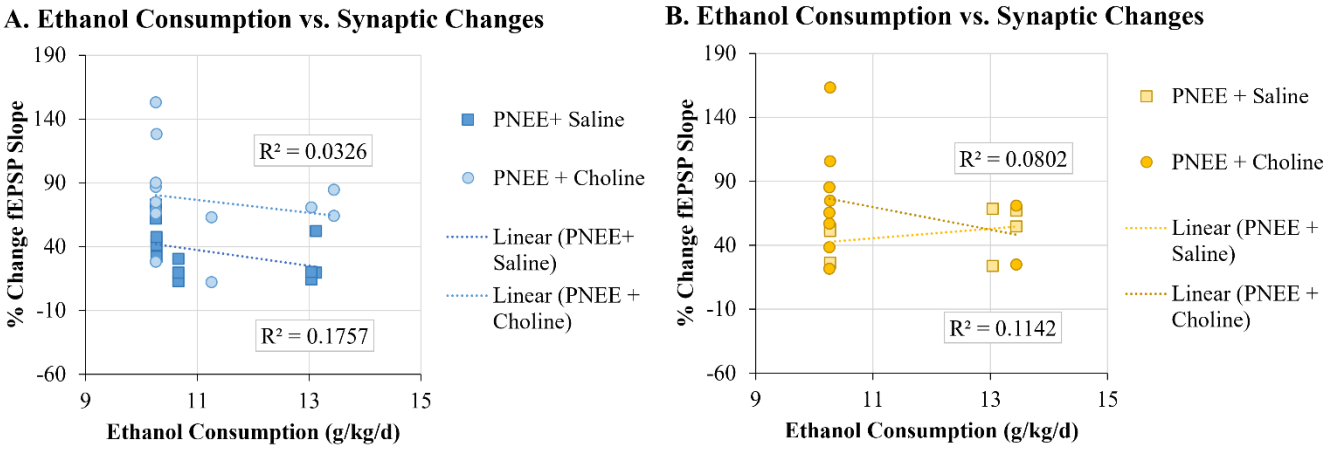


Figure 25 No correlation evident between ethanol consumption and synaptic plasticity outcomes. Correlative outcomes for (A) male and (b) female offspring across average daily ethanol consumption by maternal weight. Each point represents an individual slice.

It was unclear whether the amount of ethanol consumption during gestation could impact synaptic plasticity later in development. Therefore, a correlative analysis was performed comparing the amount of LTP with average daily consumption of their respective dam. However, no correlation was evident for males (**Figure 25A**) or females (**Figure 25B**) between maternal ethanol consumption and the magnitude of LTP.

3.5 Choline Supplementation Did Not Increase the Magnitude of Long-Term Depression

The increase in LTP in PNEE male offspring initiated two hypotheses regarding the mechanism of choline supplementation as a treatment for ethanol-dependent deficits in hippocampal synaptic plasticity. The first possibility was that choline supplementation was improving hippocampal synaptic plasticity, i.e., both LTP and long-term depression (LTD), by potentially increasing the overall plastic nature or health of the tissue. The second possibility was that choline supplementation was biasing the hippocampal network towards excitation. These two hypotheses were tested by analyzing the effects of choline supplementation on the magnitude of LTD.

In male offspring, there was no effect of prenatal condition or choline treatment on short-term depression (**Figure 26A & 26B**; Prenatal: $F(1,34)=0.62$, $p = 0.436$, $\eta_p^2 = 0.018$; Treatment: $F(1,34)=0.15$, $p=0.703$, $\eta_p^2= 0.004$). LTD was also not reduced by PNEE (**Figure 26C;** $F(1,39) = 0.019$, $p = 0.892$, $\eta_p^2=0.000$), but LTD did not occur in both choline-treated groups. While the main effect of choline treatment was not significant ($F(1,39) = 3.97$, $p = 0.053$, $\eta_p^2=0.092$), it is evident that postnatal choline supplementation did not improve overall bidirectional synaptic plasticity. This is further evidenced by examining the cumulative probability (**Figure 27**). Within both conditions, control (**Figure 27A**) and PNEE (**Figure 27B**), choline treatment shifted the cumulative probability towards the right, towards more excitation. Additionally, examining the

percentage of slices that had any depression (i.e., percent change fEPSP less than 0%), it is clear that the majority of slices in the saline-treated groups depressed (72.7%), while ~60% of choline-

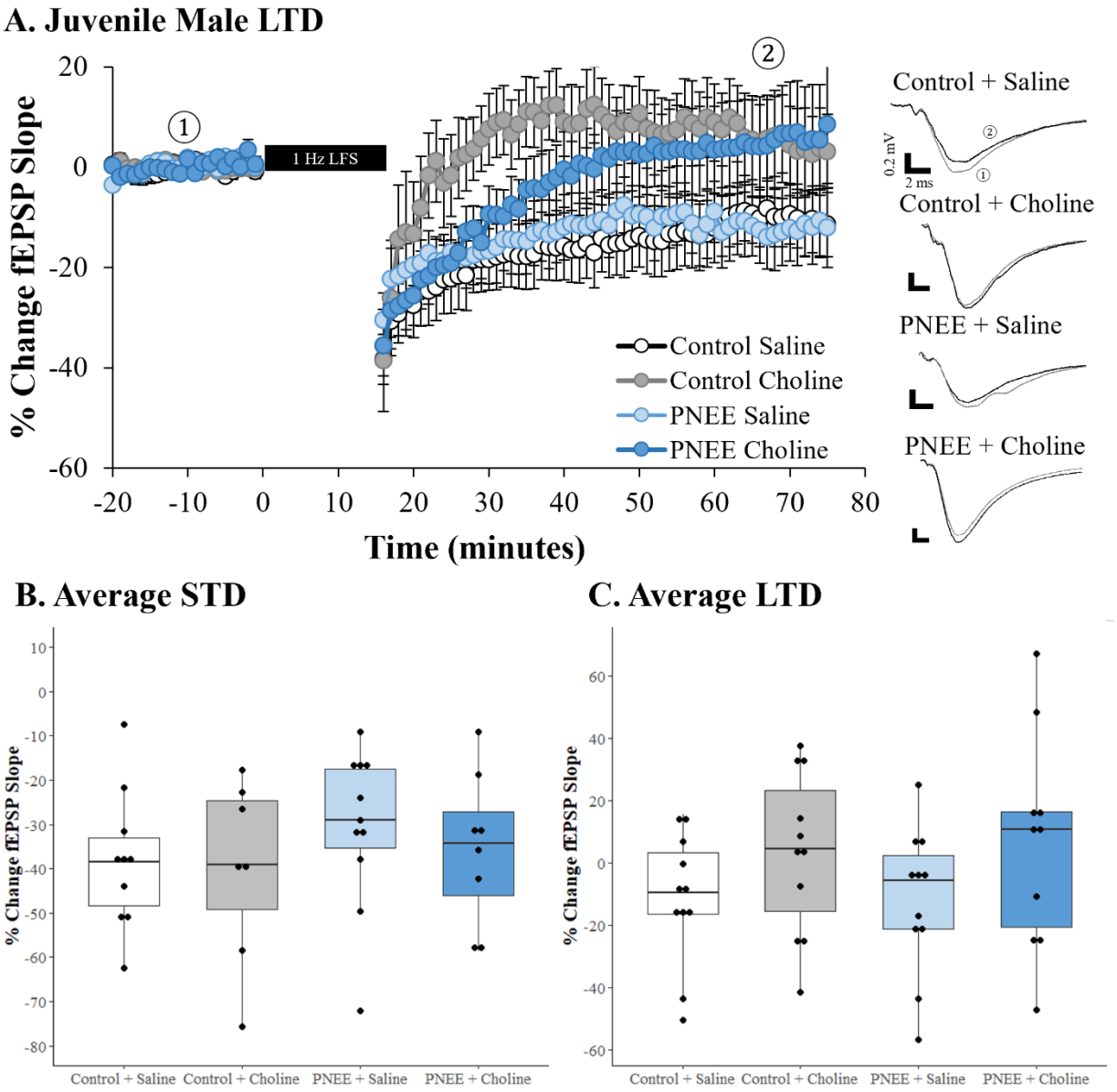


Figure 26 Choline treatment decreased LTD in juvenile males. A. Summary change in fEPSP slope for juvenile males, including in the preconditioning period (-20 to 0 minutes) and post-conditioning (15 to 60 minutes). The black bar represents the LFS. Representative traces are to the right for pre-conditioning ① and post-conditioning ②. Average short-term depression (B) and long-term depression (C) are represented by bars. Individual slices are represented by a point. All error bars are ± SEM.

treated slices did not. Collapsing the data by choline or saline treatment and analyzing the categorical variables number of slices depressed vs potentiated, there is a significant effect of treatment $X^2(1, N=43) = 3.92$, $p = 0.048$ using chi-square analysis.

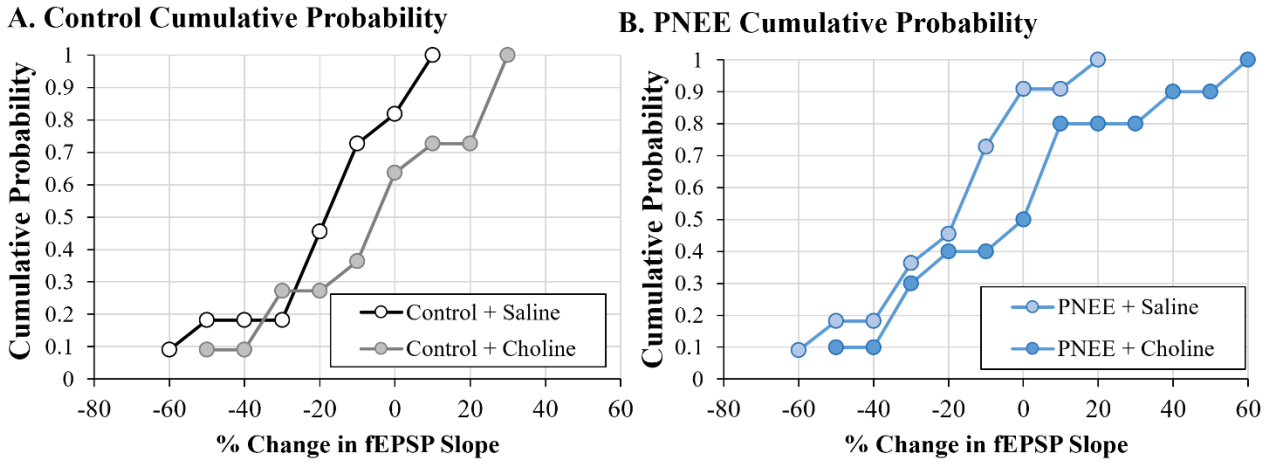


Figure 27 Cumulative probability shifted to the right with choline treatment. Probability that the magnitude of LTD would fall into each 10% bin for control (A) and PNEE (B) offspring.

In female offspring (**Figure 28**) there was a significant increase in the degree of short-term depression due to ethanol exposure (**Figure 28A**, $F(1,44)=4.83$, $p=0.033$, $\eta_p^2=0.099$). However, there was no effect of either prenatal diet ($F(1,44)=0.26$, $p=0.614$, $\eta_p^2=0.006$) or choline treatment on the degree of LTD (**Figure 28C**; $F(1,44)=0.049$, $p=0.825$, $\eta_p^2=0.001$). Interestingly, none of the female conditions had a significant amount of depression after one hour of post-conditioning recordings, as all groups had on average depression less than 10% LTD. There was no shift in the cumulative probabilities for female control (**Figure 29A.**) and PNEE offspring (**Figure 29B.**). When collapsing across treatment condition, the ratio of the number of successful to unsuccessful slices was similar between conditions ($p > 0.05$). Data of slice, animal, and litter number are summarized in **Table 6**.

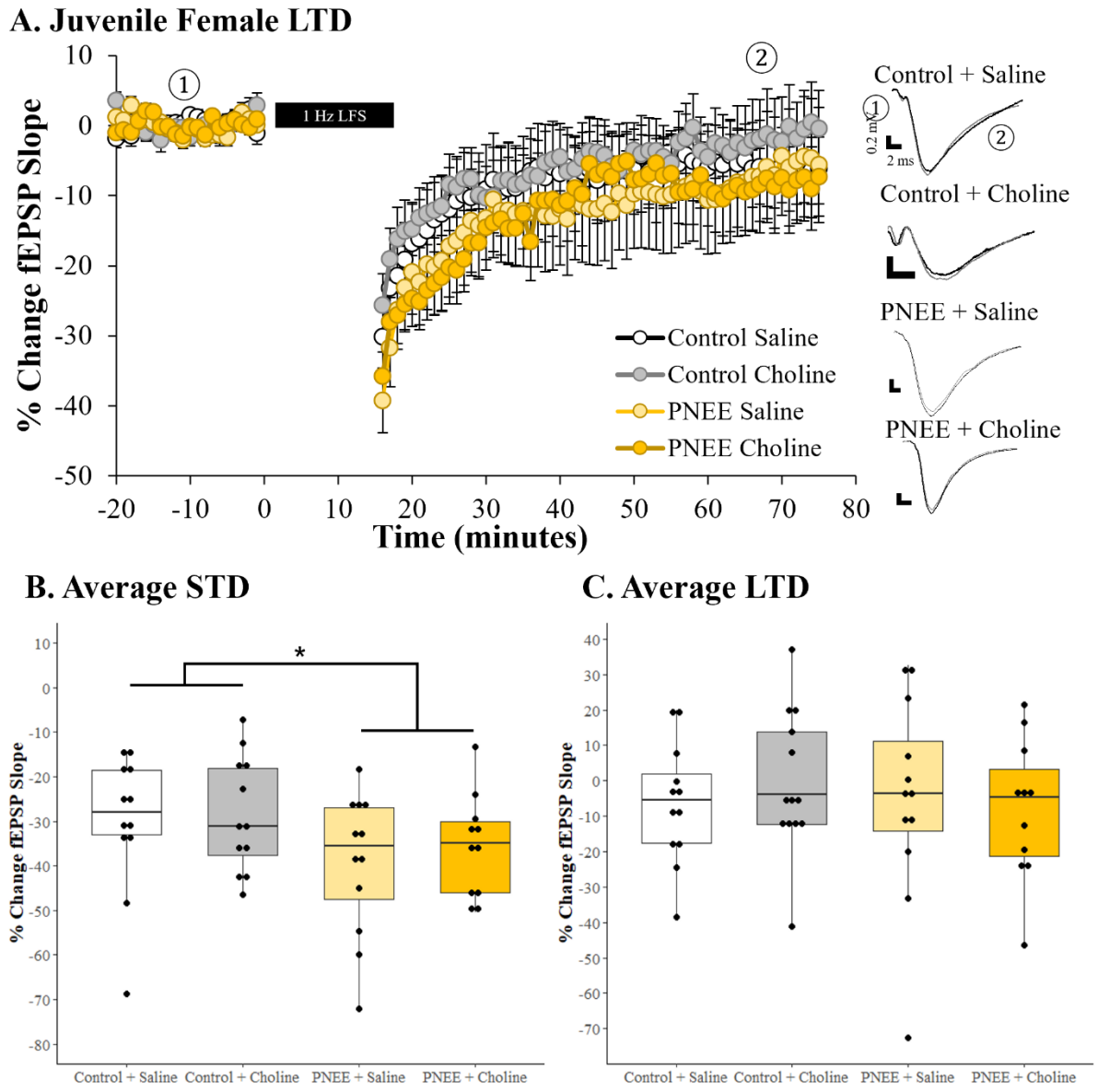


Figure 28 Increase in short-term depression with PNEE. A. Summary change in fEPSP slope for juvenile males, including in the preconditioning period (-20 to 0 minutes) and post-conditioning (15 to 60 minutes). The black bar represents the LFS. Representative traces are to the right for pre-conditioning ① and post-conditioning ②. Average short-term depression (B) and long-term depression (C) are represented by bars. Individual slices are represented by a point. All error bars are ± SEM. *p<0.05.

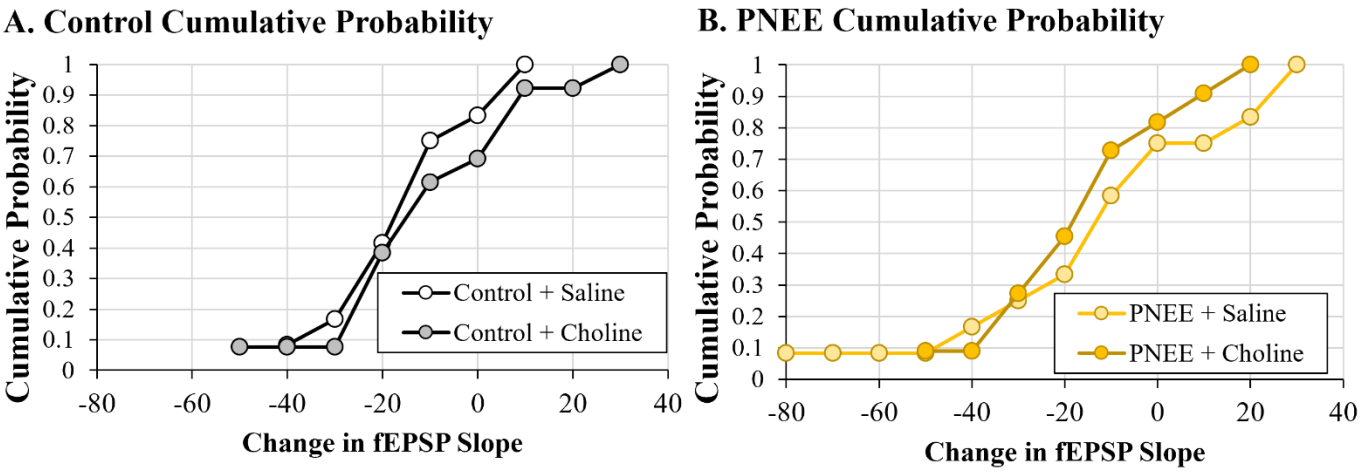


Figure 29 No change in cumulative probability in female offspring. Probability that the magnitude of LTD would fall into each 10% bin for control (A) and PNEE (B) offspring.

Table 6 Summary data from LTD experiments. LTD: long-term depression, SEM: standard error of the mean, s: slice number, a: animal number, l: litter number. The final column is the percentage of slices that depression in the last 10 minutes of post-conditioning recording.

	Male Offspring		
	LTD ± SEM	**N (s, a, l)**	**% depressed slices**
Control + Saline	-11.0 ± 6.3%	11s, 4a, 4l	72.7%
Control + Choline	+3.3 ± 7.9%	11s, 3a, 3l	36.4%
PNEE + Saline	-11.8 ± 7.1 %	11s, 3a, 2l	72.7%
PNEE + Choline	+6.3 ± 11.0%	10s, 4a, 3l	40.0%
	Female Offspring		
	LTD ± SEM	**N (s, a, l)**	**% depressed slices**
Control + Saline	-6.3 ± 5.0%	12s, 3a, 3l	75.0%
Control + Choline	-0.5 ± 5.5%	13s, 4a, 4l	61.5%
PNEE + Saline	-5.1 ± 8.4 %	12s, 5a, 3l	58.3%
PNEE + Choline	-8.1 ± 6.0%	11s, 3a, 3l	72.7%

Finally, the amount of maternal ethanol consumption was correlated with amount of LTD in the PNEE groups. There was no correlation between ethanol consumption and LTD in saline-treated PNEE males, however there was a moderate correlation in choline-treated PNEE males

(**Figure 30A.** R^2 = 0.673). This correlation indicated that offspring which had the potential to be more exposed to ethanol had greater LTD magnitudes following treatment. In females (**Figure 30B**), there was a weak correlation between saline-treated PNEE females and ethanol consumption (R^2=0.323), such that increased exposure resulted in poorer LTD. There was no correlation in choline treated PNEE females.

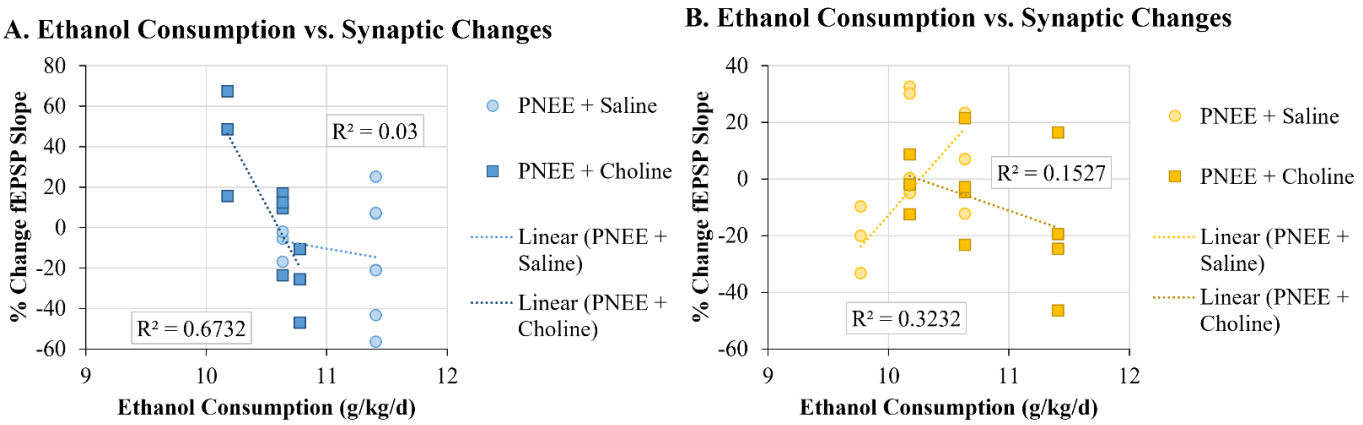


Figure 30 No correlation evident between ethanol consumption and LTD outcomes. Correlative outcomes for (A) male and (b) female offspring across average daily ethanol consumption by maternal weight. Each point represents an individual slice.

Aim 2: Do any changes in juvenile hippocampal plasticity result in parallel changes in learning and memory performance?

3.6 Choline supplementation improved working memory in control females

To determine whether alterations in hippocampal synaptic plasticity resulted in behavioural improvements, offspring performed the Radial Arm Maze (RAM). The first phase of the RAM involved a working memory task. In male offspring, choline-treated offspring explored the arms

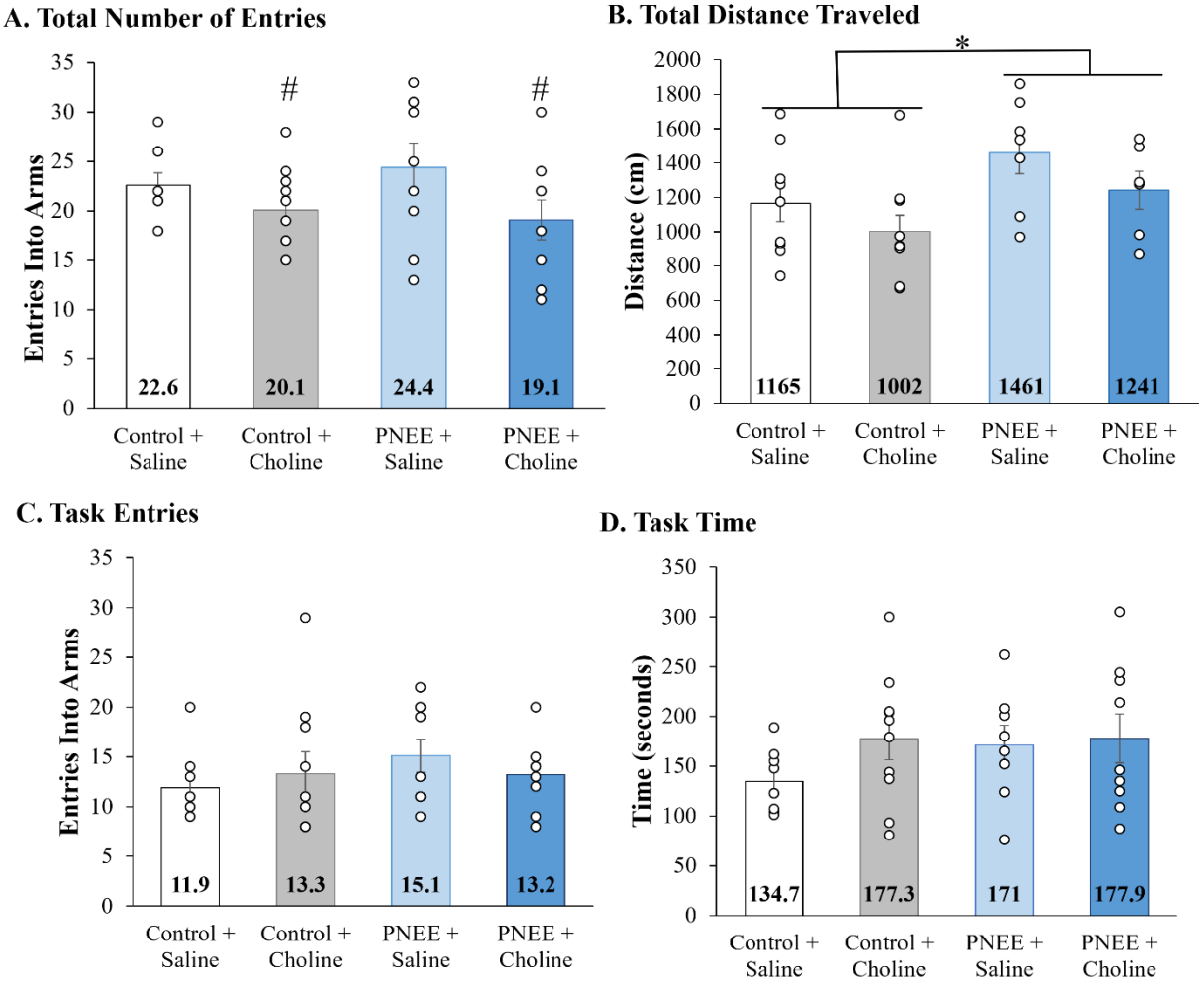


Figure 31 Working Memory Performance was Equivalent Across Groups. Activity across the entire trial (5 minutes) was analyzed as number of arm entries (A) and total distance traveled (B). Task performance was assessed as number of entries (C) and time until all 8 arms were explored (D). # Main effect of choline treatment $p < 0.05$. * Main effect of PNEE $p < 0.05$. Each point is an individual animal. All error bars are ± SEM.

of the maze less than saline-treated offspring (**Figure 31A**; $F(1,33) = 4.591$, $p = 0.040$, $\eta_p^2 = 0.122$), however PNEE males traveled a longer distance in the maze than control males (**Figure 31B;** $F(1,28) = 5.93$, $p = 0.022$, $\eta_p^2 = 0.175$). Distance traveled was not altered with choline-treatment ($F(1,28) = 3.03$, $p = 0.093$, $\eta_p^2 = 0.098$). When considering task performance, all groups completed the task equally well when considering the number of arm entries (**Figure 31C**; Prenatal: $F(1,33) = 2.159$, $p = 0.151$, $\eta_p^2 = 0.061$; Postnatal: $F(1,33) = 0.263$, $p = 0.611$, $\eta_p^2 = 0.008$), as well as the time to complete the task (**Figure 31D**; Prenatal: $F(1,33) = 0.865$, $p = 0.359$, $\eta_p^2 = 0.026$; Postnatal: $F(1,33) = 1.556$, $p = 0.221$, $\eta_p^2 = 0.046$). Of note, working memory task entries in male offspring was skewed (Shapiro-Wilkes test $p<0.05$) and therefore reciprocal transformed data were analyzed.

In females there was no effect of prenatal exposure (**Figure 32A;** $F(1,35)=0.02$, $p=0.878$, $\eta_p^2=0.00$) nor postnatal treatment ($F(1,35)=0.42$, $p=0.519$, $\eta_p^2=0.012$) on total exploration of the maze. Similarly, there was no difference in the distance travelled between groups (**Figure 32B;** Prenatal: $F(1,33)=0.92$, $p=0.343$, $\eta_p^2=0.027$; Postnatal: $F(1,33)=0.16$, $p=0.692$, $\eta_p^2=0.005$). As many female offspring completed the working memory task in approximately 8 entries by using a serial strategy, the task completion data were heavily skewed and transformations were applied. However, the transformed entry data were still not normally distributed (Shapiro-Wilkes $p=0.049$), therefore a Kruskal-Wallis test was utilized. All female offspring completed the task in a similar number of entries (**Figure 32C;** Prenatal: $H(1)=2.46$, $p=0.117$, Postnatal: $H(1)=0.61$, $p=0.435$). Finally, while there was no main effect of prenatal environment on the time to complete the working memory task (**Figure 32D;** $F(1,35)=1.86$, $p=0.182$, $\eta_p^2=0.050$) nor postnatal treatment ($F(1,35)=0.11$, $p=0.742$, $\eta_p^2=0.003$), there was a significant interaction between the two (PNEE *

Choline: F(1,35)=0.5.68, p=0.023, η_p^2=0.140). *Post-hoc* analysis determined this difference was between choline-treated control offspring and choline-treated PNEE offspring (p=0.052).

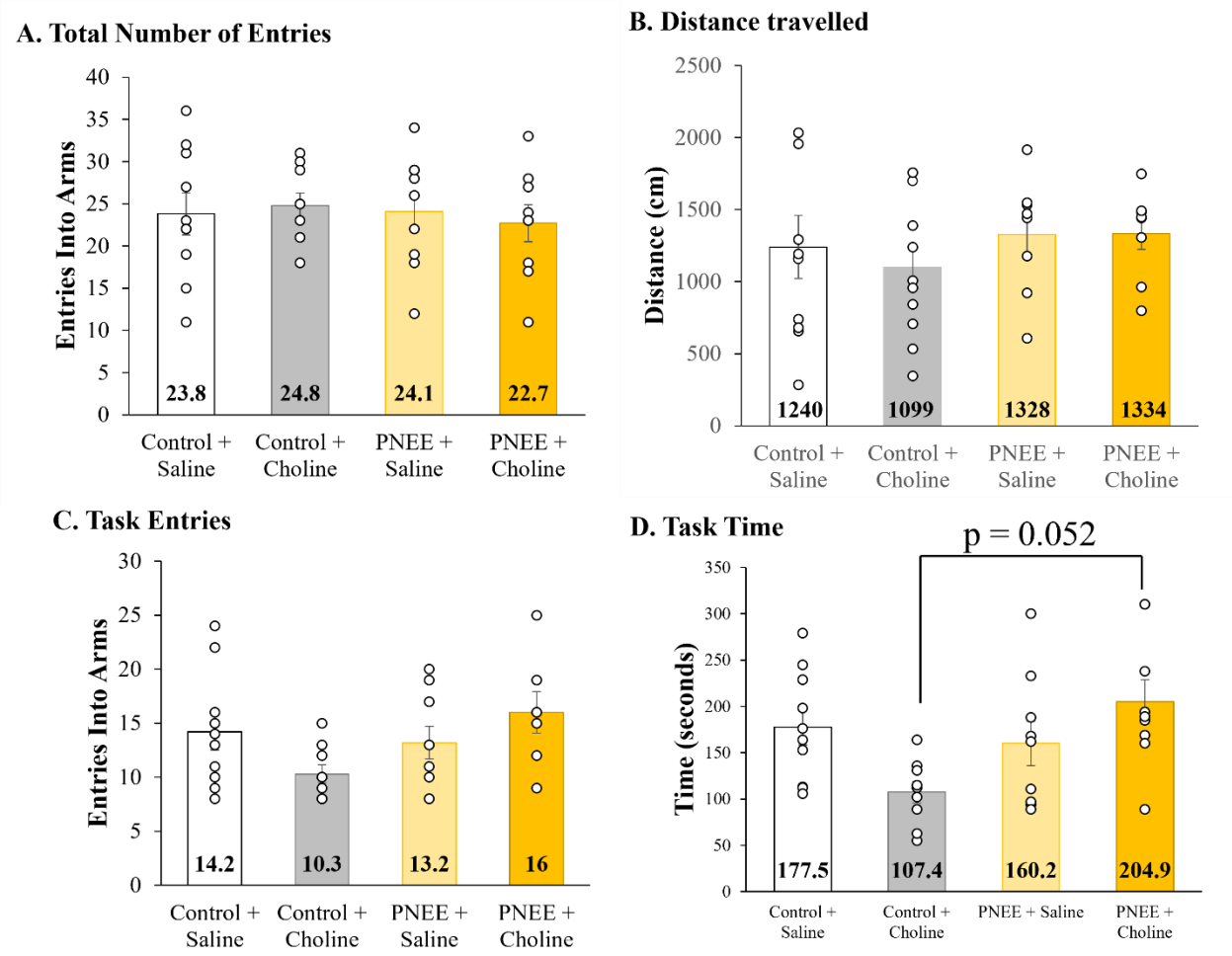


Figure 32. Choline-treated PNEE females performed working memory task slower. Activity across the entire trial (5 minutes) was analyzed as number of arm entries (A) and total distance traveled (B). Task performance was assessed as number of entries (C) and time until all 8 arms were explored (D). Each point is an individual animal. All error bars are ± SEM.

3.7 No effect of PNEE or choline treatment on reference memory performance

Subsequently, offspring performed the reference memory RAM task over three days. In male offspring there was a significant effect of trial for both the number of entries (**Figure 33A;** F(2.9, 85.1) = 2.86, p = 0.043, η_p^2 = 0.090) and time to complete the task (**Figure 33B;** F(2.6, 76.3) = 4.03, p = 0.013, η_p^2 = 0.122). *Post hoc* analysis determined that performance was the most

poor on the first trial ($p<0.05$), indicating a degree of learning after the initial day. However, there

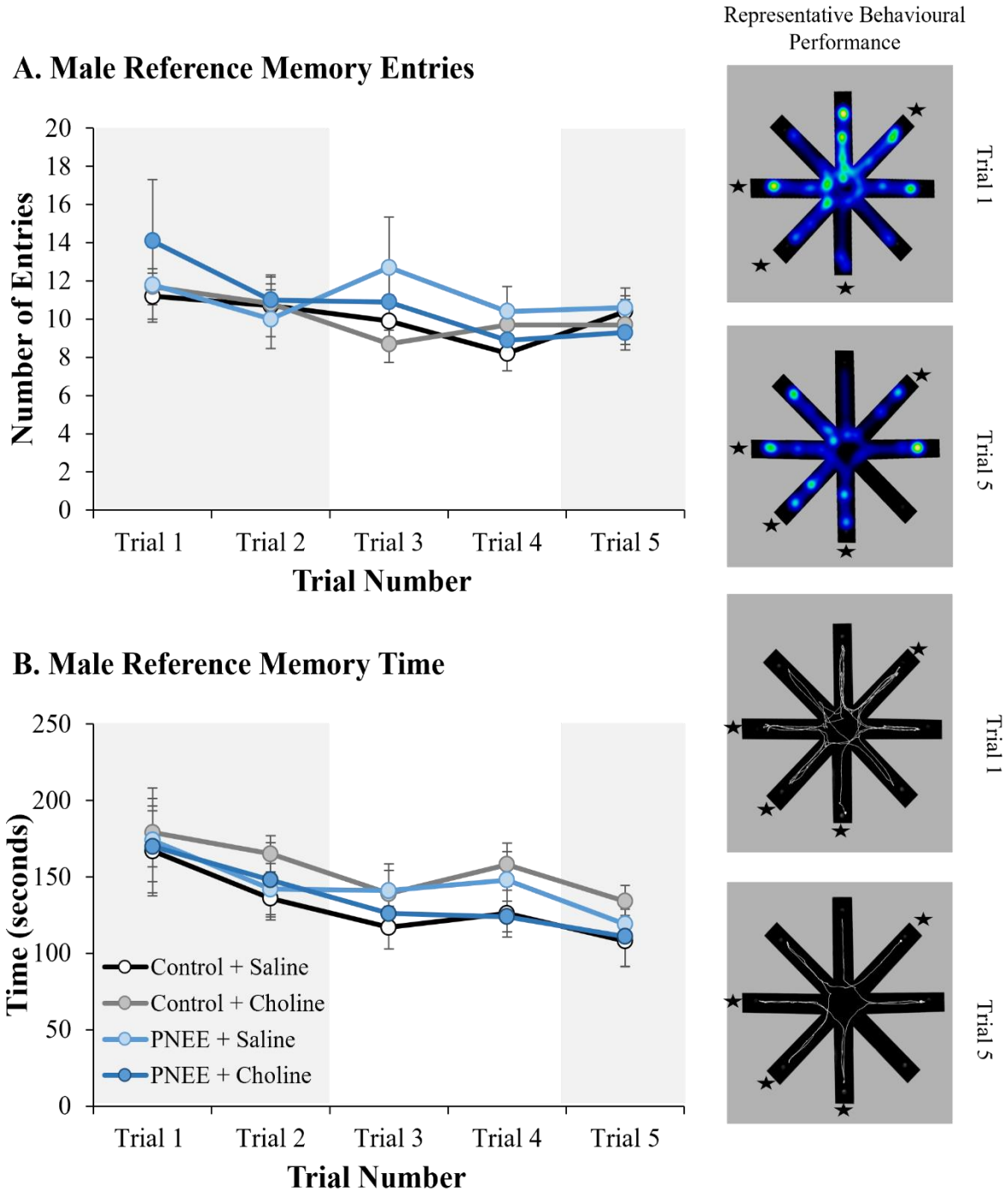


Figure 33 No effect of PNEE or choline treatment on reference memory performance in males. Reference memory performance was assessed by number of entries (A) and time (B) to complete the task over the course of five trials. The grey and white bars represent different days. Performance was averaged across trials and error bars are ± SEM. A representative performance from a saline-treated control male is located to the right, with a heat map (top two figures) and path trace (bottom two figures) for trial 1 and trial 5. Stars indicate the baited arms.

was no effect of prenatal condition (Entries: $F(2.9, 85.1) = 0.26$, $p = 0.848$, $\eta_p^2 = 0.009$; Time: $F(2.6, 76.3) = 0.10$, $p = 0.944$, $\eta_p^2 = 0.003$) nor choline treatment (Entries: $F(2.9, 85.1) = 0.50$, $p = 0.677$, $\eta_p^2 = 0.017$; Time: $F(2.6, 76.3) = 0.20$, $p = 0.874$, $\eta_p^2 = 0.007$) on task performance.

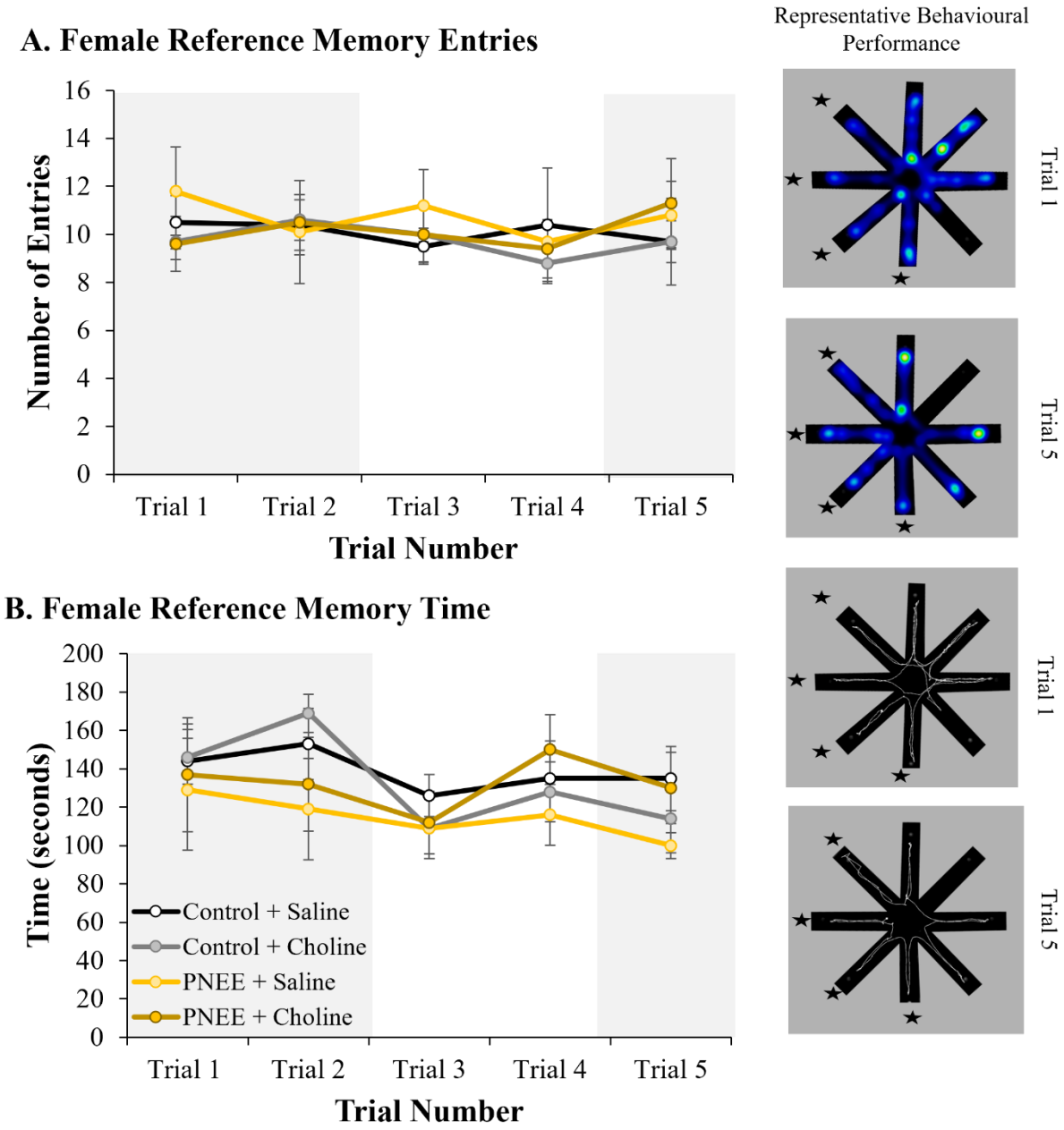


Figure 34. No improvement in female reference memory. Reference memory performance was assessed by number of entries (A) and time (B) to complete the task over the course of five trials. The grey and white bars represent different days. Performance was averaged across trials and error bars are ± SEM. A representative performance from a saline-treated control female is located to the right, with a heat map (top two figures) and path trace (bottom two figures) for trial 1 and trial 5. Stars indicate the baited arms.

In female offspring there was no effect of trial number on the number of entries to finish the task (**Figure 34A;** $F(3.24, 110.40)=0.26$, $p=0.870$, $\eta_p^2 = 0.008$), but there was a significant effect of trial when the time to complete the task was analyzed (**Figure 34B;** $F(3.40, 115.74)=4.35$, $p=0.004$, $\eta_p^2 = 0.113$). Time to complete trial 2 was significantly longer than on trial 3 ($p=0.003$) and trial 5 ($p=0.042$), indicating these offspring were completing the task quickly but still making the same number of errors. There was no overall effect of prenatal condition (Entries: $F(1,34)=0.46$, $p=0.505$, $\eta_p^2 = 0.013$, Time: $F(1,34)=0.60$, $p=0.674$, $\eta_p^2 = 0.005$) nor postnatal treatment (Entries: $F(1,34)=0.05$, $p=0.825$, $\eta_p^2 = 0.001$, Time: $F(1,34)=0.45$, $p=0.446$, $\eta_p^2 = 0.017$) on reference memory performance in female offspring. Data were also analyzed without any penalty applied for failed trials (**Supplementary Figure 2**), but this did not alter any outcomes.

3.8 Relationships between task performance, complexity, and search strategies

One advantage of the RAM task, and a factor that was considered when choosing a spatial memory task, is that the difficulty level of the task can be altered to further probe learning and memory differences. In this task the baited arms were pseudo-randomized and stratified by complexity in terms of the degree of separation (i.e., no separation of arms = complexity 0, while maximum separation = complexity 3). It was hypothesized that there would be a correlation between performance and complexity, such that increasing complexity would translate to a poorer task performance. However, in male offspring there was no relationship between task performance and time (**Figure 35A**) or number of entries (**Figure 35B**). In females, while there was no correlation between performance and time (**Figure 35C**), there was a weak correlation in saline-treated PNEE females with the number of entries and complexity (**Figure 35D**), such that saline-treated PNEE females performed better with increasing maze complexity. Therefore, the female data were separated into low complexity (0-1 separation of baited arms) and high complexity (2-3

separation of baited arms) trials for additional *post hoc* analysis. Since dividing the data in this way decreased the N, assumptions for use of ANOVA were violated and the nonparametric Kruskal-Wallis test was used. Low complexity trials in female offspring demonstrated a potential trend of PNEE (**Figure 36A**; H(1) = 3.25, p = 0.072), with female PNEE offspring potentially performing more poorly in these trials. There was no difference in female offspring performance in high complexity trials (**Figure 36B**; H(1) = 0.01, p = 0.922).

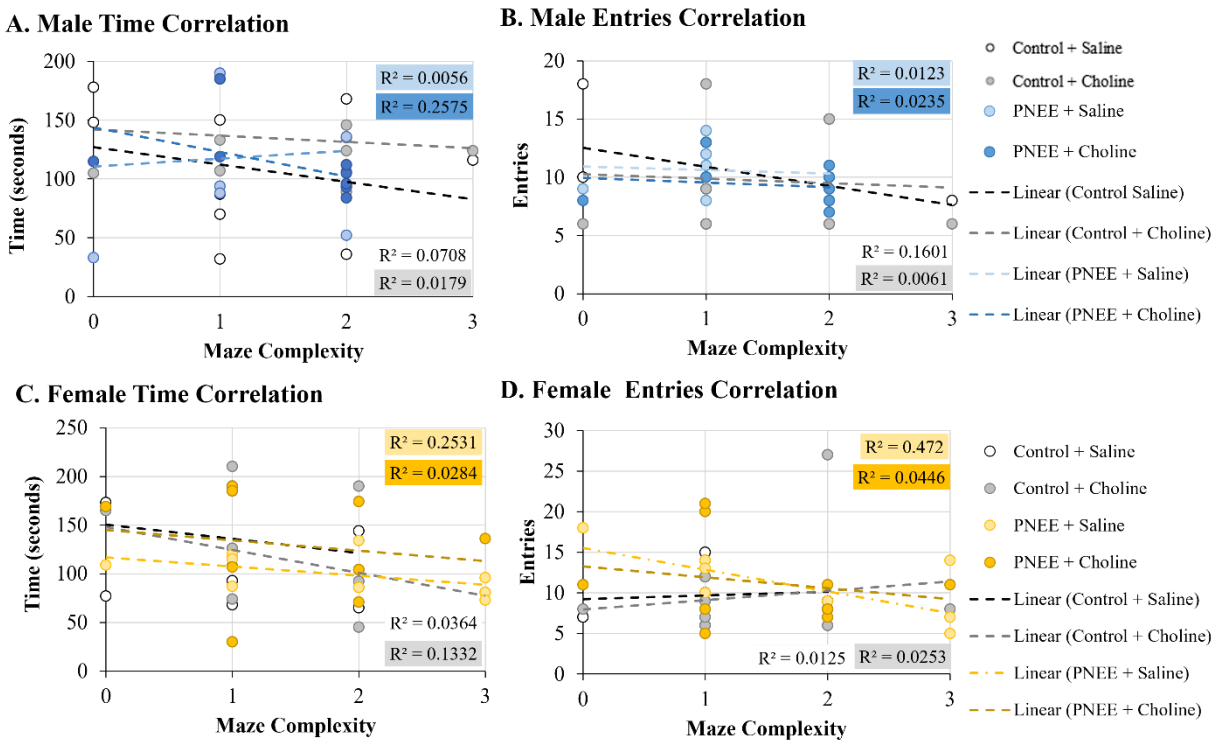


Figure 35 Limited correlation between task complexity and performance. Maze complexity was binned by the minimum degree of separation between baited arms. In males, there was no relationship between maze complexity and time (A) or entries (B). In female offspring, there was no correlation with time (C), but PNEE females had a weak correlation with number of entries and complexity (D).

It was also hypothesized that the lack of learning in female offspring was due to the employment of an alternative learning strategy as compared to male offspring. Three search strategies were used to characterize each trial: serial, random, or direct (summarized in **Figure 37A**). As search strategy varied within animal across the trials, the data were recorded by trial rather than collapsing by animal. There was no difference in the search strategy used within male (**Figure 37B.** X^2 (6, N = 180) = 4.12, p > 0.05) or female offspring (**Figure 37C.** X^2 (6, N = 196) = 2.68, p > 0.05). While more males employed the direct strategy and more females utilized the serial strategy, there was no significant sex difference in strategies used (**Figure 37D.** X^2 (2, N = 376) = 4.84, p > 0.05).

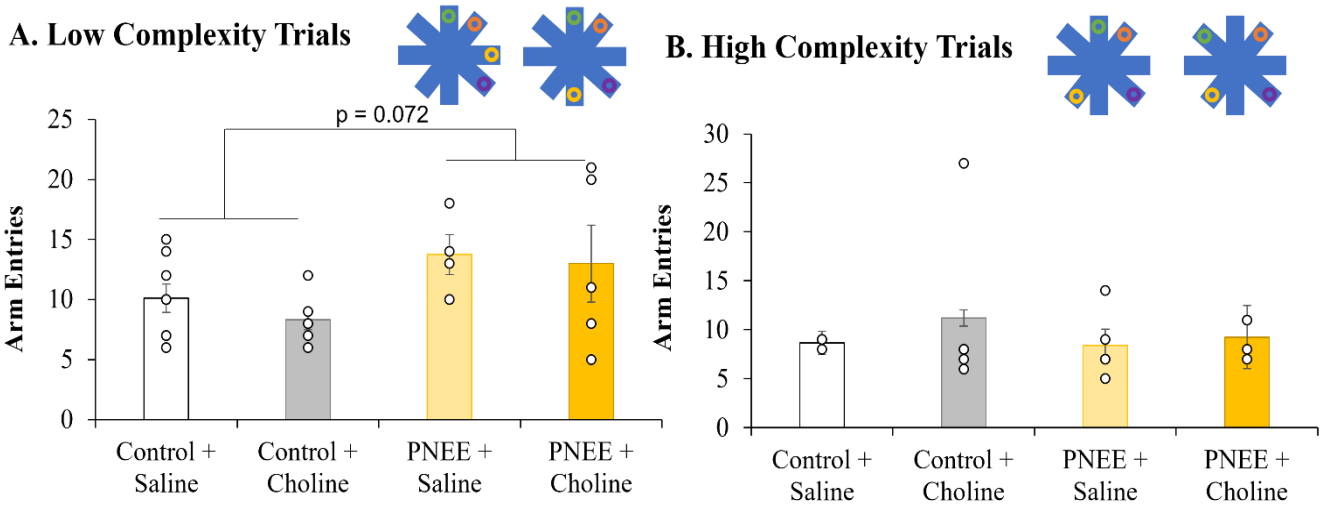


Figure 36 Potential relationship with female performance and maze complexity. Female task performance was separated by low (A) and high complexity trials (B).

Finally, rates of trial failure and success were compared across condition. There was once again no significant difference in males (*data not shown*; X^2 (3, N = 180) = 4.06, p > 0.05) or in females (X^2 (3, N = 196) = 5.49, p > 0.05).

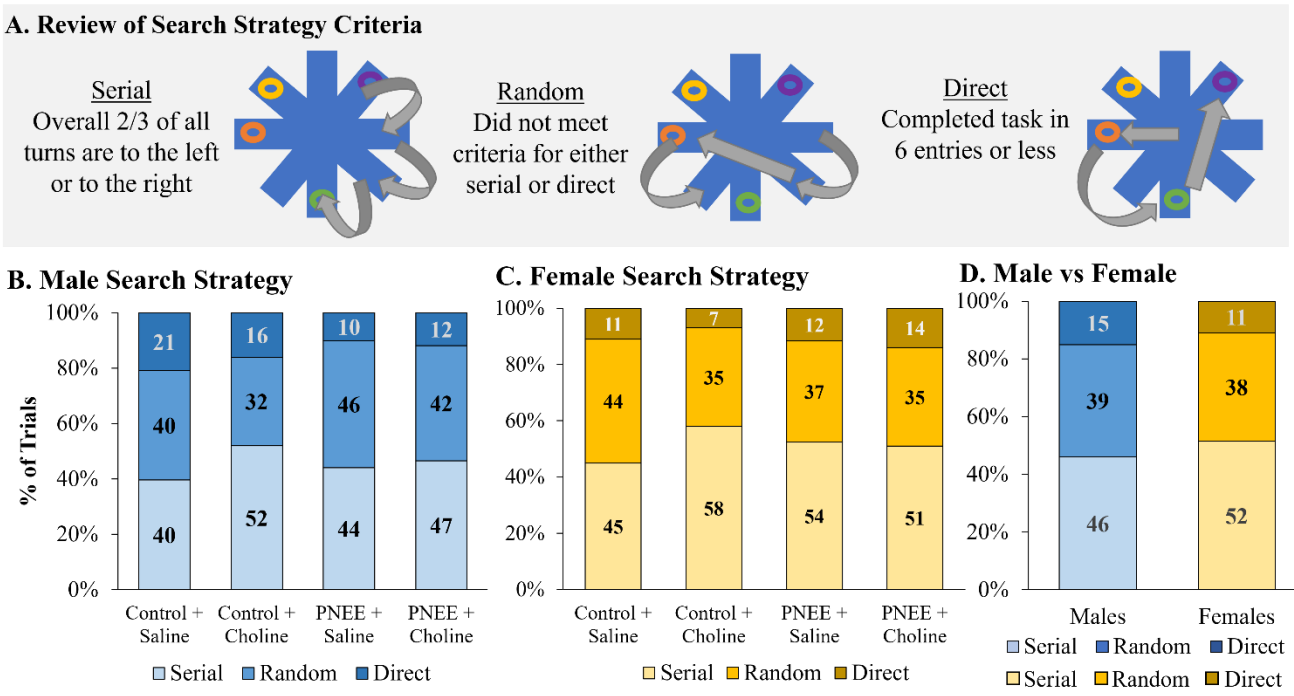


Figure 37 Search strategy utilized to complete the RAM was similar across conditions. A. Summary of search strategies. The percent of all trials that utilized each search strategy in male (B.) and female (C.) offspring, as well as a comparison between male and female search strategy.

Aim 3: Do the improvements seen in juvenile hippocampal synaptic following choline treatment persist into adulthood?

3.9 PNEE alters basal excitability into adulthood.

The final aim of this project was to determine if changes in synaptic plasticity would persist following treatment cessation. The first measure examined was basal excitability, as there were marked sex and treatment dependent effects in juvenile offspring. Therefore, input/output tests were performed in adulthood to determine if changes in basal excitability persisted. In male adult

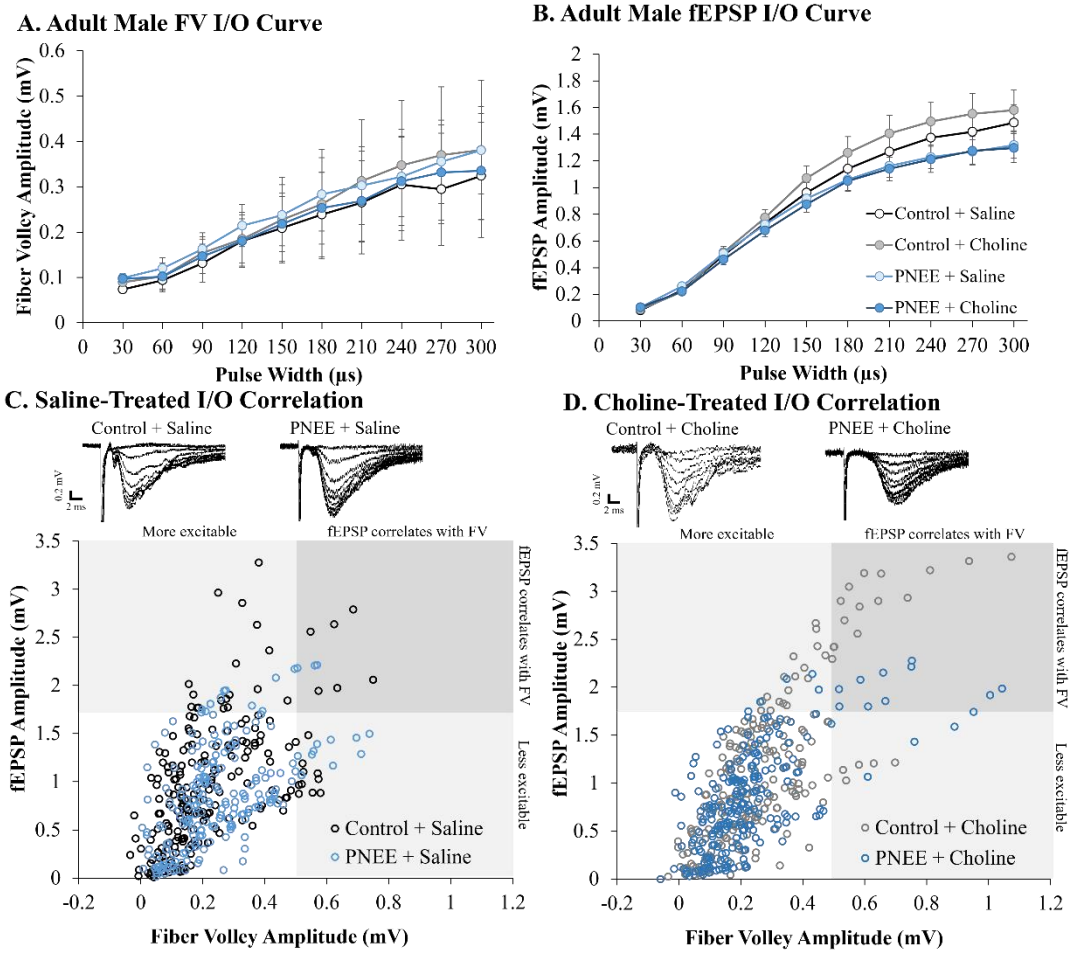


Figure 38 PNEE does not change excitability in adult male offspring. Input/output curve for male offspring did not demonstrate significant changes in either fiber volley (A) or fEPSP (B). The relationship between fiber volley and fEPSP are represented for saline-treated (C) and choline-treated adult males (D). Each point represents a single waveform fEPSP at its corresponding fiber volley, such that each slice is represented with ten points (1 per pulse width). Error bars are ± SEM.

offspring, there were no changes in either fiber volley (**Figure 38A**) or fEPSP amplitude (**Figure 38B**) with increasing pulse width with PNEE (FV: $F(1.18, 155.75) = 0.32$, $p = 0.323$, $\eta_p^2 = 0.004$; fEPSP: $F(1.18, 101.61) = 3.16$, $p = 0.072$, $\eta_p^2 = 0.035$) or choline treatment (FV: $F(1.18, 155.75) = 0.55$, $p = 0.564$, $\eta_p^2 = 0.006$; fEPSP: $F(1.18, 101.61) = 0.37$, $p = 0.578$, $\eta_p^2 = 0.004$). The relationship between fiber volley and fEPSP is represented for saline-treated (**Figure 38C**) and choline-treated male offspring (**Figure 38D**).

In adult females, however, both fiber volley (**Figure 39A**) and fEPSP amplitude (**Figure 39B**) were increased in PNEE females with increasing pulse width (Main effect of PNEE. FV: $F(1.71, 121.63) = 17.79$, $p < 0.001$, $\eta_p^2 = 0.200$; fEPSP: $F(1.21, 93.13) = 8.87$, $p = 0.002$, $\eta_p^2 = 0.103$). *Post hoc* analysis determined this was occurred with fEPSP amplitude at the pulse widths 240 µs ($p = 0.097$), 270 µs ($p= 0.047$) and 300 µs ($p = 0.041$). Similarly, PNEE fiber volley was increased at 150 µs ($p = 0.055$), 180 µs ($p = 0.036$), and 210-300 µs ($p < 0.001$). There was no effect of choline treatment on either measure (FV: $F(11.71, 121.63) = 0.80$, $p = 0.436$, $\eta_p^2 = 0.011$; fEPSP: $F(1.21, 93.13) = 0.07$, $p = 0.832$, $\eta_p^2 = 0.00$). The relationship between fiber volley and fEPSP is represented for saline-treated (**Figure 39C**) and choline-treated female adults (**Figure 39D**). These correlations demonstrate that, even though fEPSP is increased with PNEE, this largely appears to be due to an increase in fiber volley input and is not necessarily indicative of increased basal excitability.

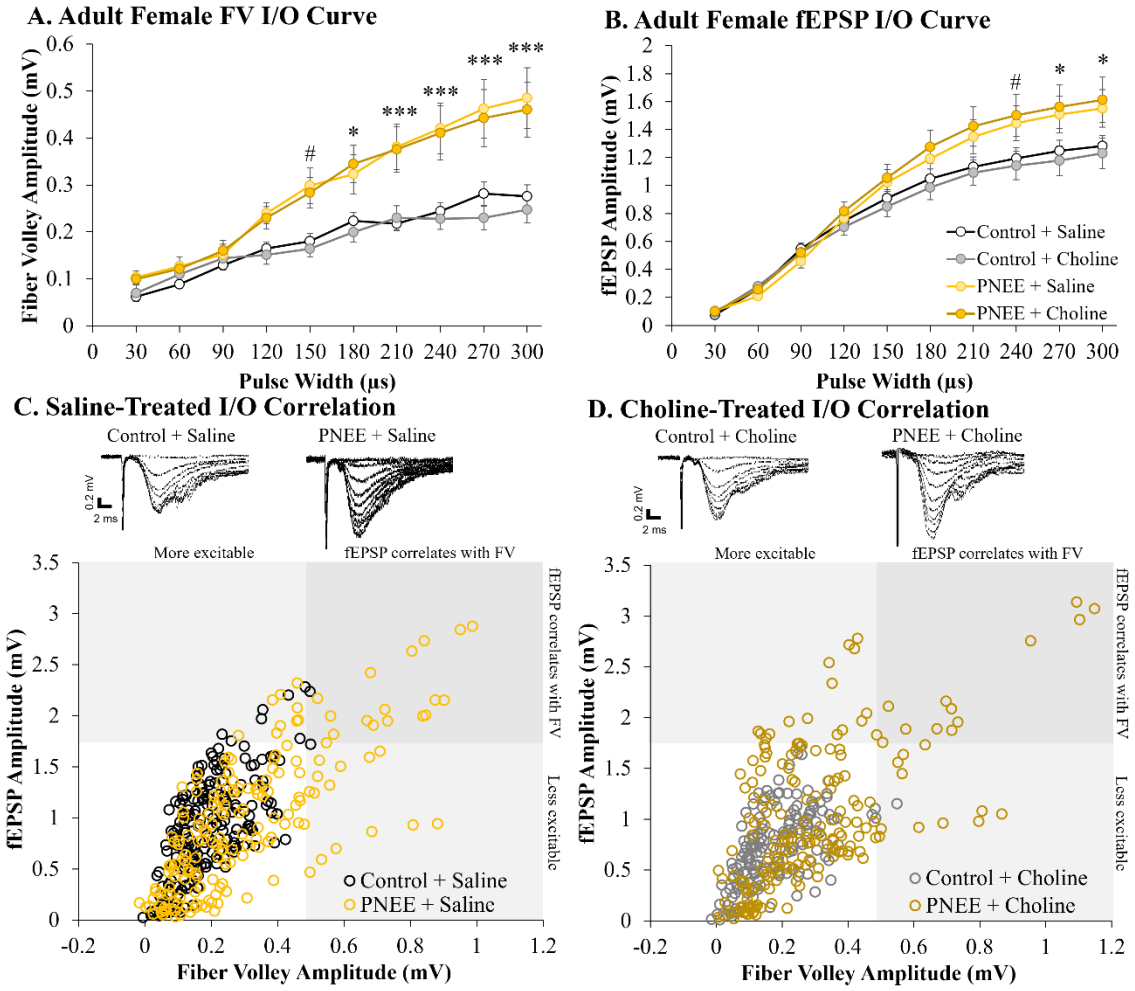


Figure 39 PNEE increases excitability in adult female offspring. Input/output curve for female offspring demonstrates a significant increase in fiber volley (A) and fEPSP (B) amplitude with PNEE. The relationship between fiber volley and fEPSP are represented for saline-treated (C) and choline-treated adult females (D). Each point represents a single waveform fEPSP at its corresponding fiber volley, such that each slice is represented with ten points (1 per pulse width). Error bars are ± SEM. # $p < 0.1$, * $p < 0.05$, and *** $p < 0.001$.

3.10 Benefits of choline supplementation do not persist into adulthood.

While there were fewer changes in basal excitability into adulthood, it was next to determine if changes in synaptic plasticity would persist into adulthood. Following the injection period, offspring were allowed to age up into adulthood (PND 60-90) and synaptic plasticity was examined (**Figure 40A & Figure 41A**). Of note, male LTP and female PTP data were not normally distributed (Shapiro-Wilk's test $p < 0.05$) and therefore analysis was performed on transformed

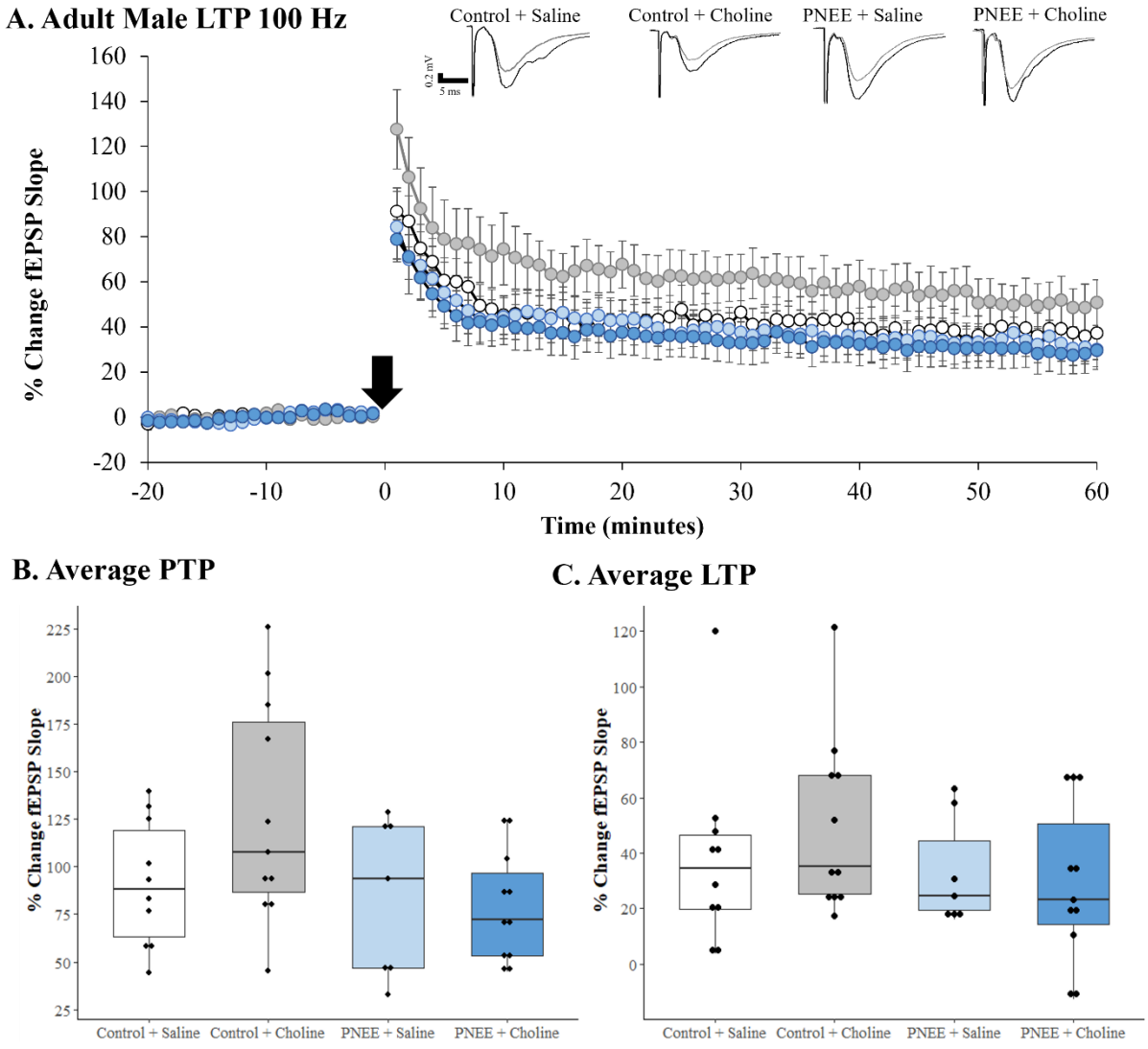


Figure 40. No long-term changes in adult male LTP. (A) Average change in fEPSP slope. The black arrow represents the HFS. Each point is the average change in fEPSP slope binned per minute. Error bars are ± SEM. (B) No change in the average PTP (Average change in fEPSP slope one minute following HFS) or (C) LTP (average change in fEPSP slope 50-60 minutes following HFS

data. Additionally, since there was not an equal number of slices demonstrating less than 10% LTP across conditions, all slices were included in this analysis. In adult males offspring there was no effect of PNEE (**Figure 40C** $F(1,35)=1.794$, $p=0.189$, $\eta_p^2=0.049$) nor choline treatment ($F(1,35)=0.038$, $p=0.846$, $\eta_p^2=0.001$) in the magnitude of LTP. However, control adult males had

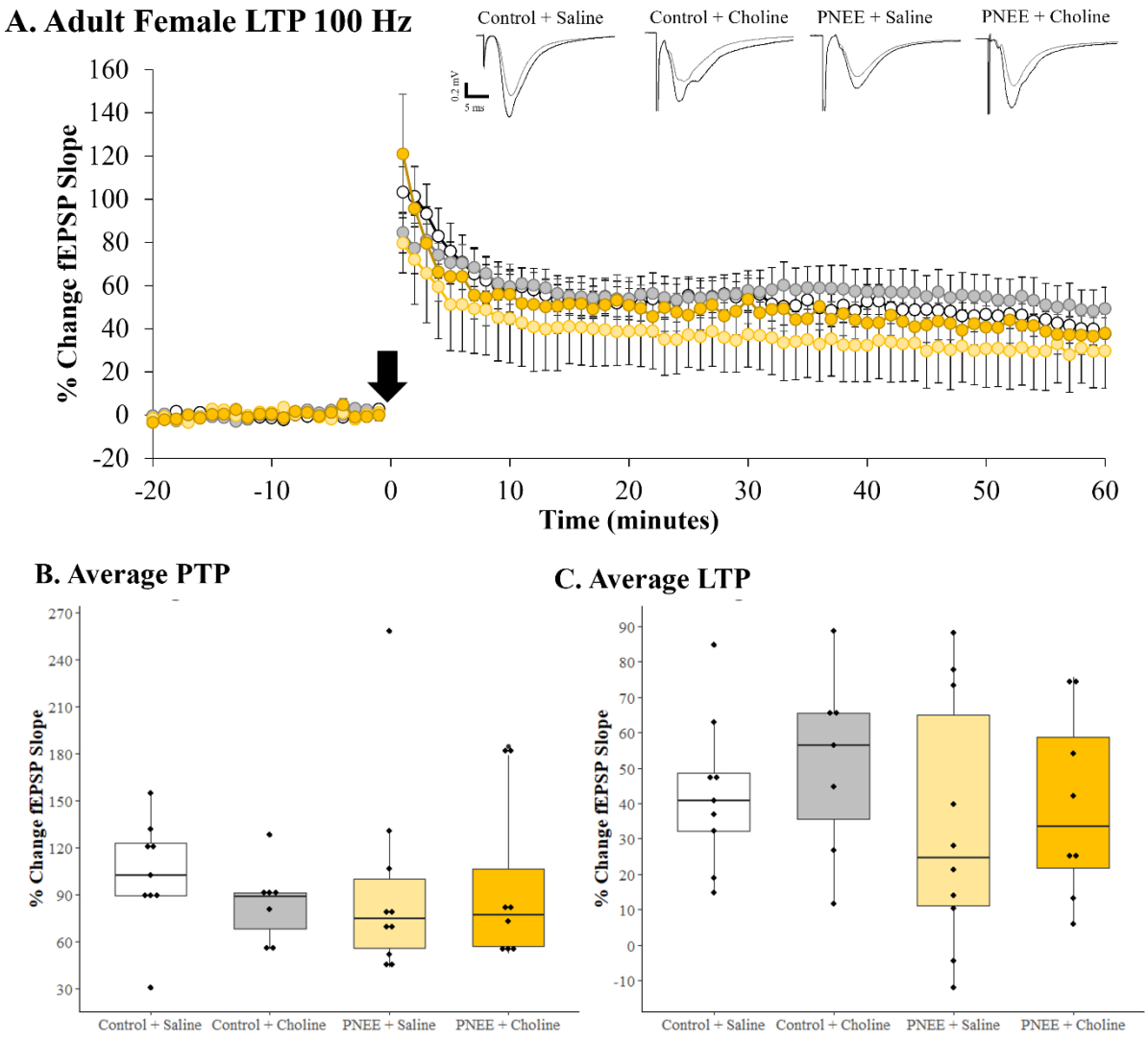


Figure 41. No changes in adult female LTP with PNEE or choline treatment. (A) Average change in fEPSP slope. The black arrow represents the HFS. Each point is the average change in fEPSP slope binned per minute. Error bars are ± SEM. (B) No change in the average PTP (Average change in fEPSP slope one minute following HFS) or (C) LTP (average change in fEPSP slope 50-60 minutes following HFS).

a non-significant increase in the magnitude of PTP, which was largely due to an increase in choline-treated controls (**Figure 40B** $F(1,35)=3.961$, $p=0.054$, $\eta_p^2=0.102$).

Similarly in adult female offspring (**Figure 41A**), there was no significant effect of PNEE (**Figure 40B.** $F(1,30)=0.171$, $p=0.682$, $\eta_p^2=0.006$) nor choline treatment ($F(1,30)=0.112$, $p=0.740$, $\eta_p^2=0.004$) on PTP. Additionally, neither PNEE or choline treatment altered LTP in adult females (**Figure 41C.** Prenatal: $F(1,30) = 1.193$, $p = 0.283$, $\eta_p^2 = 0.038$; Choline: $F(1,30) = 0.519$, $p = 0.477$, $\eta_p^2 = 0.017$).

Table 7 Adult Saturating LTP Summary. LTP: long-term potentiation, SEM: standard error of the mean, s: slice number, a: animal number, l: litter number.

	Male Offspring		**Female Offspring**	
	LTP ± SEM	**N (s, a, l)**	**LTP ± SEM**	**N (s, a, l)**
Control + Saline	38.3 ± 10.7%	10s, 3a, 3l	42.8 ± 7.3%	9s, 4a, 3l
Control + Choline	49.4 ± 9.7%	11s, 5a, 4l	51.3 ± 9.9 %	7s, 3a, 2l
PNEE + Saline	33.1 ± 7.4%	7s, 4a,3l	32.6 ± 11.2 %	8s, 4a, 4l
PNEE + Choline	29.3 ± 8.7%	11s, 6a, 5l	39.2 ± 9.5%	13s, 5a, 4l

3.11Alterations in LTP threshold evident in adulthood

Despite the lack of changes in LTP evident with either PNEE or choline treatment into adulthood, it was hypothesized that more subtle changes in LTP threshold induction might be present. Therefore, the frequency of the HFS was decreased to 70, 50, and 30 Hz to determine the LTP induction threshold. The other properties of the conditioning stimulus, including the number of pulses, the number of trains, and the intertrain interval, were kept consistent. As it was the intention that some slices would not potentiate, there was also no minimum potentiation requirement for inclusion. However, slices were considered outliers and thus excluded if they were more than twice the standard deviation from the average. With this criteria, two male slices were

removed (one PNEE + Saline at 50 Hz and one PNEE + Choline at 70 Hz) and one female slice (Control + Choline at 50 Hz).

In adult male offspring, there were no differences in LTP or PTP at 70 Hz between control and PNEE offspring (**Figure 42A-C**. LTP: F(1,33)=0.412, p=0.526, η_p^2=0.012; PTP: F(1,34) =

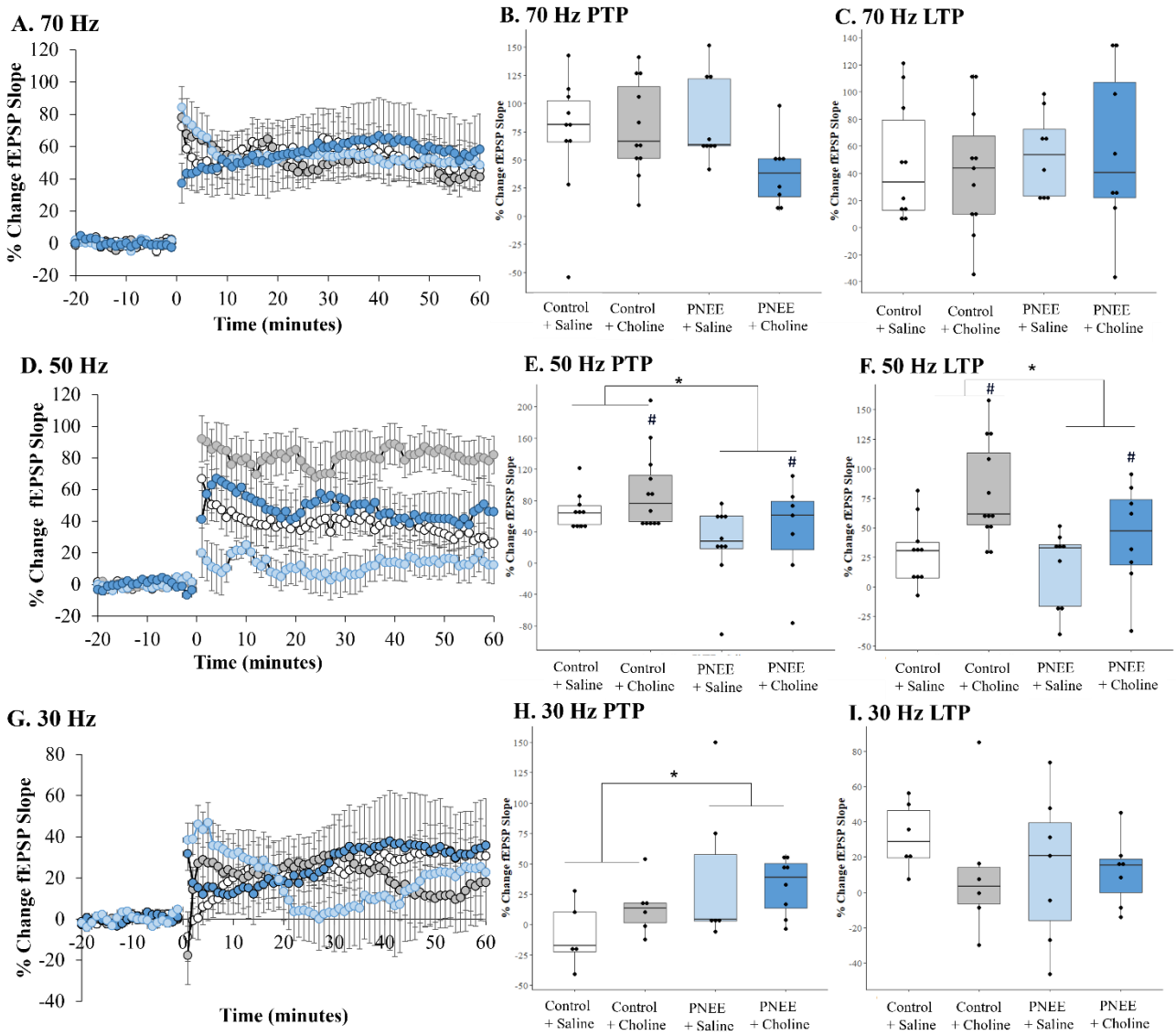


Figure 42 Postnatal choline supplementation alters LTP threshold. Male LTP threshold was analyzed using a conditioning stimulus at 70 Hz (A), 50 Hz (D), and 30 Hz (G). Each point is the average percent change in fEPSP slope binned to a 1 minute interval. Error bars are ± SEM. PTP (B, E, F) was calculated as the average change of fEPSP slope from baseline during the first minute. LTP (C, F, I) was calculated as the averaged percent change of fEPSP slope from baseline during the last ten minutes. Each bar is the average PTP or LTP and the points represent individual slices* $p < 0.05$. # $p < 0.05$ main effect of choline treatment.

0.949, p=0.337, η_p^2=0.027) or with choline treatment (LTP: F(1,33)=0.010, p=0.0920, η_p^2=0.00; PTP: F(1,34) = 2.035, p = 0.163, η_p^2=0.056). While it appeared that there may be an interaction between prenatal condition and choline treatment with PTP, this interaction was not significant (F(1,34) = 3.349, p = 0.076, η_p^2=0.090). In saline-treated controls, 70 Hz and 100 Hz protocols produced the same amount of LTP and therefore was not subthreshold (*Student's t-test*, p=0.591). Using a 50 Hz stimulation protocol (**Figure 42D-F**), differences between conditions emerged. Control adult males had significantly more LTP than PNEE males (F(1,35)=4.426, p=0.043, η_p^2=0.112). Additionally, choline treatment significantly increased the amount of LTP (F(1,35)=9.925, p=0.003, η_p^2=0.221). There was no interaction between prenatal and postnatal conditions (F(1,35) = 0.87, p = 0.868, η_p^2=0.024). Similarly, PTP was increased with prenatal (F(1,31) = 4.28, p = 0.047, η_p^2=0.121) and postnatal conditions (F(1,31) = 6.64, p = 0.015, η_p^2=0.176). However, in saline-treated control animals there was no significant difference in the amount of LTP between 50 Hz and 100 Hz (Student's t-test, p=0.537). Finally, at the 30 Hz conditioning stimulus there was only a significant effect of PNEE on PTP (F(1,21) = 6.18, p = 0.021, η_p^2=0.227), however there was not a significant effect of prenatal ethanol exposure **Figure 42G-I**. Prenatal: F(1,22)=0.504, p=0.485, η_p^2=0.022), nor choline treatment on LTP (F(1,22)=0.742, p=0.398, η_p^2=0.033). Interestingly, in saline-treated control males there was no difference in the magnitude of LTP at 30 Hz or 100 Hz (Student's t-test, p=0.619), suggesting this protocol may not be subthreshold in adult males. Therefore, as an additional pilot experiment a 10 Hz HFS was run in adult males and this protocol produced no LTP in saline-treated control males (**Supplementary Figure 3**).

	70 Hz		50 Hz		30 Hz	
	LTP	**N (s, a, l)**	**LTP**	**N (s, a, l)**	**LTP**	**N (s, a, l)**
Control + Saline	47.9 ± 13.9%	10s, 5a, 3l	29.7 ± 8.8%	10s, 6a, 4l	31.7 ± 7.7%	6s, 2a, 1l
Control+ Choline	42.2 ± 14.1%	11s, 5a, 4l	78.9 ± 12.2%	12s, 5a, 4l	11.6 ± 16.1%	6s, 2a, 1l
PNEE + Saline	53.7 ± 10.4%	9s, 5a, 4l	15.6 ± 10.8%	9s, 6a, 4l	13.6 ± 16.0%	6s, 3a, 2l
PNEE + Choline	56.3 ± 21.6%	8s, 4a, 4l	42.3 ± 15.5%	7s, 4a, 4l	11.9 ± 7.4%	8s, 4a, 2l

Table 8 Summary of subthreshold LTP in adult male offspring. LTP: long-term potentiation, SEM: standard error of the mean, s: slice number, a: animal number, l: litter number.

In females, delivering an HFS at 70 Hz (**Figure 43A-C**) did not result in significant changes in LTP or PTP due to prenatal condition (LTP: $F(1,30) = 0.00$, $p=0.984$, $\eta_p^2=0.00$; PTP: $F(1,28) = 0.53$, $p = 0.473$, $\eta_p^2=0.019$) nor with choline treatment (LTP: $F(1,30) = 3.62$, $p=0.067$, $\eta_p^2=0.108$; PTP: $F(1,28) = 0.56$, $p = 0.462$, $\eta_p^2=0.019$). When the conditioning stimulus frequency was decreased to 50 Hz, there was a significant interaction between prenatal exposure and postnatal treatment (**Figure 43D-F.** $F(1,27) = 6.50$, $p=0.017$, $\eta_p^2=0.194$). Further Tukey *post hoc* analysis, however, did not find any significant differences between conditions ($p > 0.05$). There was also no effect on PTP (Prenatal: $F(1,28) = 0.03$, $p = 0.872$, $\eta_p^2 = 0.00$; Postnatal: $F(1,28) = 0.12$, $p = 0.735$, $\eta_p^2 = 0.004$). Similar to male offspring, control females had similar levels of LTP at 100 Hz and 70 Hz (Student's t-test, $p=0.208$), as well as 50 Hz (Student's t-test, $p=0.708$), demonstrating that neither protocol was subthreshold. Finally, adult female offspring were stimulated with a conditioning stimulus at 30 Hz (**Figure 43G-I**). In control females, this protocol produced significantly less LTP in controls than the HFS at 100 Hz (Student's t-test, $p=0.004$). While there was no effect of prenatal exposure ($F(1,17)=0.158$, $p=0.696$, $\eta_p^2=0.009$), choline treatment significantly increased the magnitude of LTP at the subthreshold stimulation ($F(1,17) = 4.67$, $p =$

0.045, $\eta_p^2 = 0.216$). There was no change in magnitudes of PTP (Prenatal: F(1,17) = 1.14, p = 0.300, $\eta_p^2 = 0.063$; Postnatal: F(1,17) = 0.03, p = 0.871, $\eta_p^2 = 0.002$). These data are summarized in **Figure 44.**

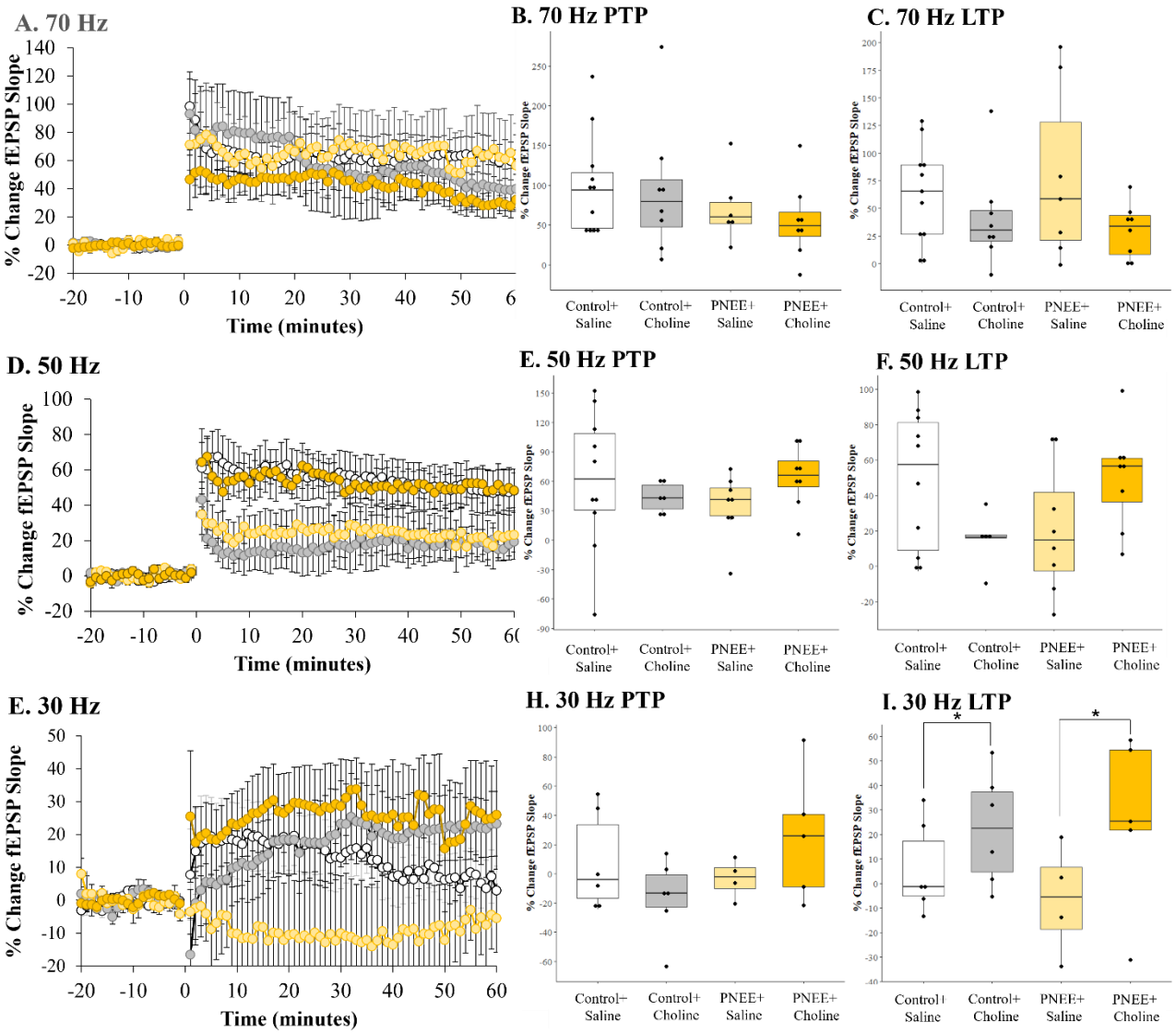


Figure 43 Choline supplementation alters LTP threshold in female adults. Female LTP threshold was analyzed using a conditioning stimulus at 70 Hz (A), 50 Hz (D), and 30 Hz (G). Each point is the average percent change in fEPSP slope binned to a 1-minute interval. Error bars are ± SEM.PTP (B, E, F) was calculated as the average change of fEPSP slope from baseline during the first minute. LTP (C, F, I) was calculated as the averaged percent change of fEPSP slope from baseline during the last ten minutes. Each bar is the average PTP or LTP and the points represent individual slices. * $p < 0.05$ main effect of choline treatment. (Figure on previous page).

Table 9 Summary of subthreshold LTP in adult female offspring. LTP: long-term potentiation, SEM: standard error of the mean, s: slice number, a: animal number, l: litter number.

	70 Hz		50 Hz		30 Hz	
	LTP	**N (s, a, l)**	**LTP**	**N (s, a, l)**	**LTP**	**N (s, a, l)**
Control + Saline	62.7 ± 13.3%	11s, 6a, 4l	48.3± 12.4%	10s, 6a, 4l	5.9 ± 5.9%	6s, 2a, 2l
Control+ Choline	41.0 ± 15.5%	8s, 5a, 3l	17.9 ± 6.5%	6s, 3a, 3l	22.3 ± 9.4%	6s, 3a, 2l
PNEE + Saline	79.0 ± 29.7%	6s, 5a, 3l	20.8 ± 12.8%	8s, 4a, 3l	-6.5 ± 11.3%	4s, 2a, 2l
PNEE + Choline	29.8 ± 8.6%	8s, 6a, 3l	50.3 ± 10.1%	8s, 4a, 3l	25.8 ± 16.1%	5s, 3a, 2l

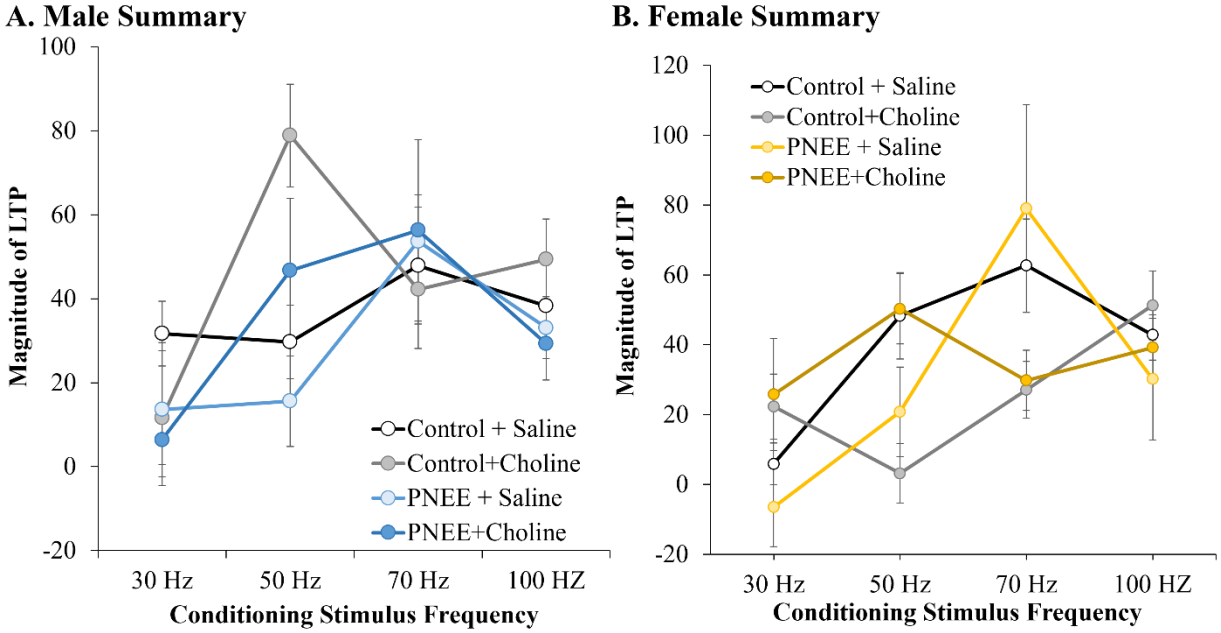


Figure 44 Summary of LTP threshold experiments. A summary of the average (A) male and (B) female LTP with varying conditioning stimulus frequency. All points are the average across slices and error bars are ± SEM.

3.12 GluN2B functionality is altered with PNEE.

The change in LTP threshold with choline supplementation could be due to alterations in receptor activity and therefore the GluN2B contribution to the fEPSP was assessed. In this experiment, the fEPSP was analyzed in a low Mg^{2+} aCSF with the AMPA receptor antagonist and compared with the fEPSP after the addition of the GluN2B-specific antagonist ifenprodil. Baseline

properties were compared between fEPSP waveforms with the AMPA receptor antagonist NBQX and fEPSP waveforms with NBQX and the GluN2B antagonist ifenprodil. The onset latency (time from fiber volley peak to fEPSP initiation) was not altered with GluN2B antagonism (**Figure 45A.** $F(1,24) = 0.50$, $p = 0.484$, $\eta_p^2 = 0.021$), nor with prenatal condition ($F(1,24) = 0.85$, $p = 0.366$, $\eta_p^2 = 0.034$) or postnatal treatment ($F(1,24) = 2.84$, $p = 0.105$, $\eta_p^2 = 0.106$). Upon measuring the peak latency (fEPSP initiation to peak fEPSP), there was a significant main effect of drug, such that ifenprodil increased peak latency (**Figure 44B.** $F(1,24) = 29.16$, $p < 0.001$, $\eta_p^2 = 0.549$). There was

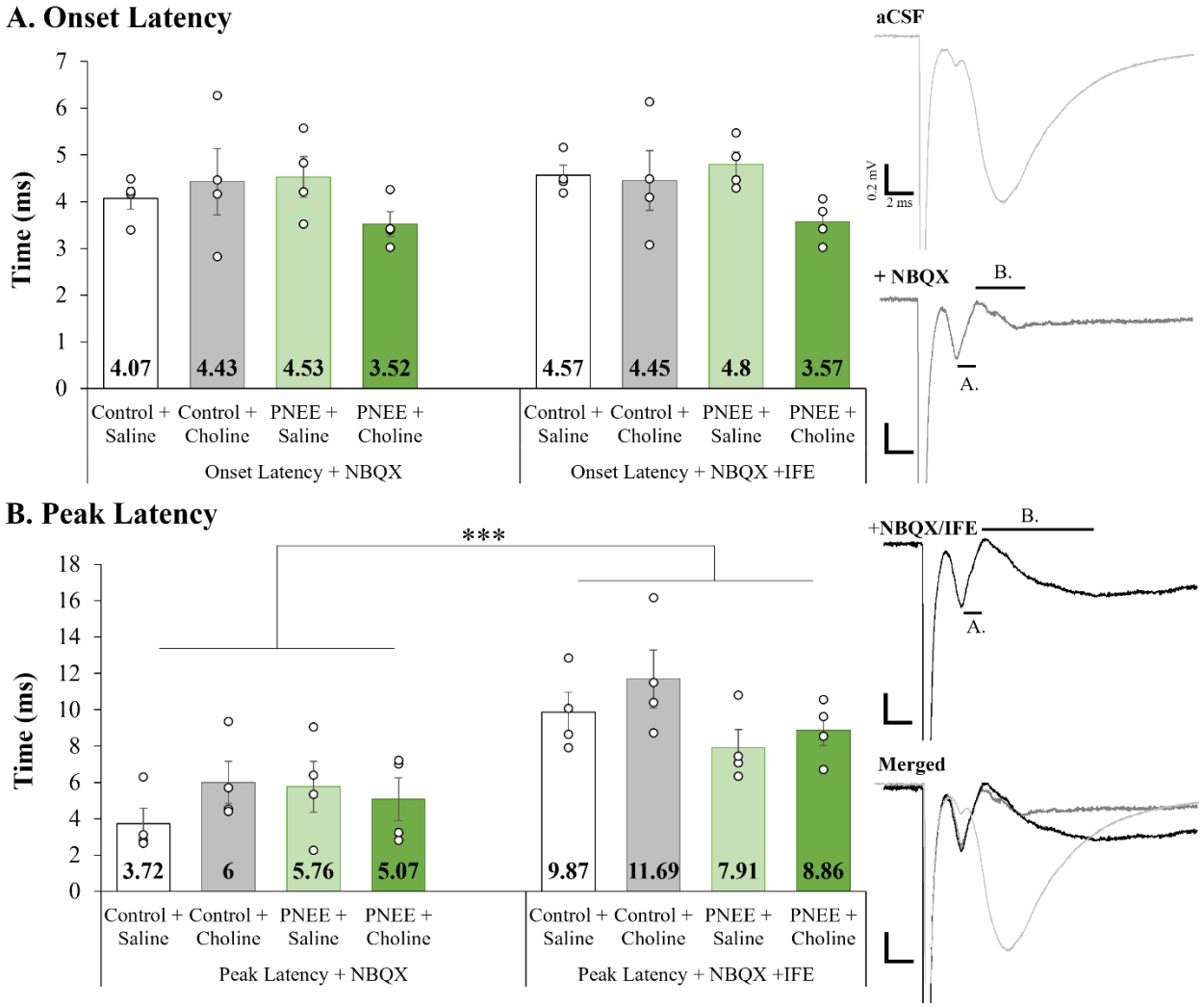


Figure 45 GluN2B antagonism increased peak latency. A. Onset latency and B. peak latency in the presence of the AMPA receptor antagonist NBQX (left) and NBQX with GluN2B antagonist IFE (right). Each bar is the average latency and slices are represented by individual points. n = 4 per group (2 male and 2 female slices). Error bars are ± SEM. *** $p < 0.001$. Representative traces are featured to the right.

no main effect of PNEE ($F(1,24) = 1.25$, $p = 0.276$, $\eta_p^2 = 0.049$) nor choline supplementation ($F(1,24) = 1.76$, $p = 0.198$, $\eta_p^2 = 0.068$). While the interaction between prenatal treatment and ifenprodil drug application was not significant ($F(1,24) = 3.21$, $p = 0.086$, $\eta_p^2 = 0.118$), this could be an interesting relationship to explore in future experiments.

A paired pulse ratio was assessed in the presence of NBQX and then again in NBQX and ifenprodil. There was no change in the PPR with ifenprodil (**Figure 45** $F(1,24) = 0.11$, $p = 0.739$, $\eta_p^2 = 0.005$). Similarly, there was no main effect of PNEE ($F(1,24) = 1.84$, $p = 0.188$, $\eta_p^2 = 0.071$) or choline supplementation ($F(1,24) = 1.01$, $p = 0.325$, $\eta_p^2 = 0.040$).

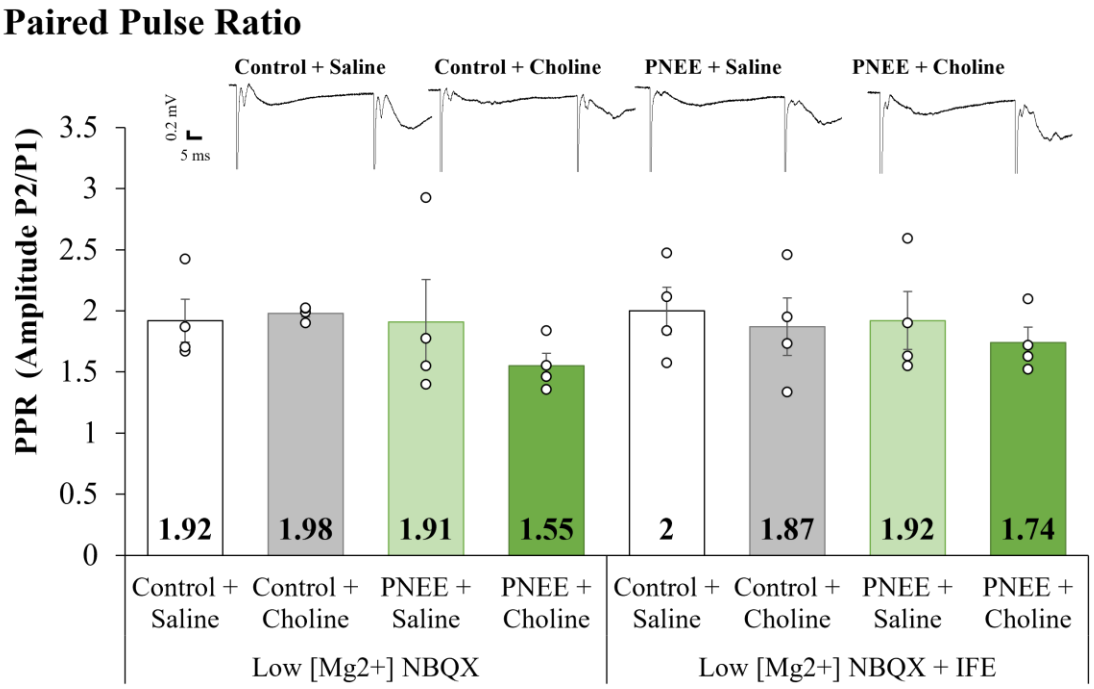


Figure 46 Paired pulse ratio did not change with GluN2B antagonism. PPR was calculated as the amplitude of pulse 2 divided by pulse 1. Each bar is the average PPR per group and slices are represented by individual points. n = 4 slices (2 male and 2 female slices). Error bars are ± SEM.

Finally, the area under the first pulse in the paired pulse test was analyzed to determine the degree of potentiation or depression with GluN2B antagonism (**Figure 47**). fEPSP area was significantly increased with ifenprodil as compared to fEPSP area without GluN2B antagonism ($F(1,24) = 27.35$, $p < 0.001$, $\eta_p^2 = 0.533$). Furthermore, fEPSP potentiation was significantly reduced with PNEE ($F(1,24) = 9.69$, $p = 0.005$, $\eta_p^2 = 0.288$) but not significantly ameliorated by choline treatment ($F(1,24) = 1.55$, $p = 0.225$, $\eta_p^2 = 0.061$).When the degree of potentiation was examined (**Figure 47B**), there was a significant interaction between prenatal environment and postnatal treatment ($F(1,12) = 6.26$, $p = 0.028$, $\eta_p^2 = 0.343$), such that saline treated PNEE offspring had less of an increase in fEPSP area than saline-treated controls (p=0.024), and an insignificant decrease from choline-treated controls (p=0.071) or choline-treated PNEE offspring (p=0.055).

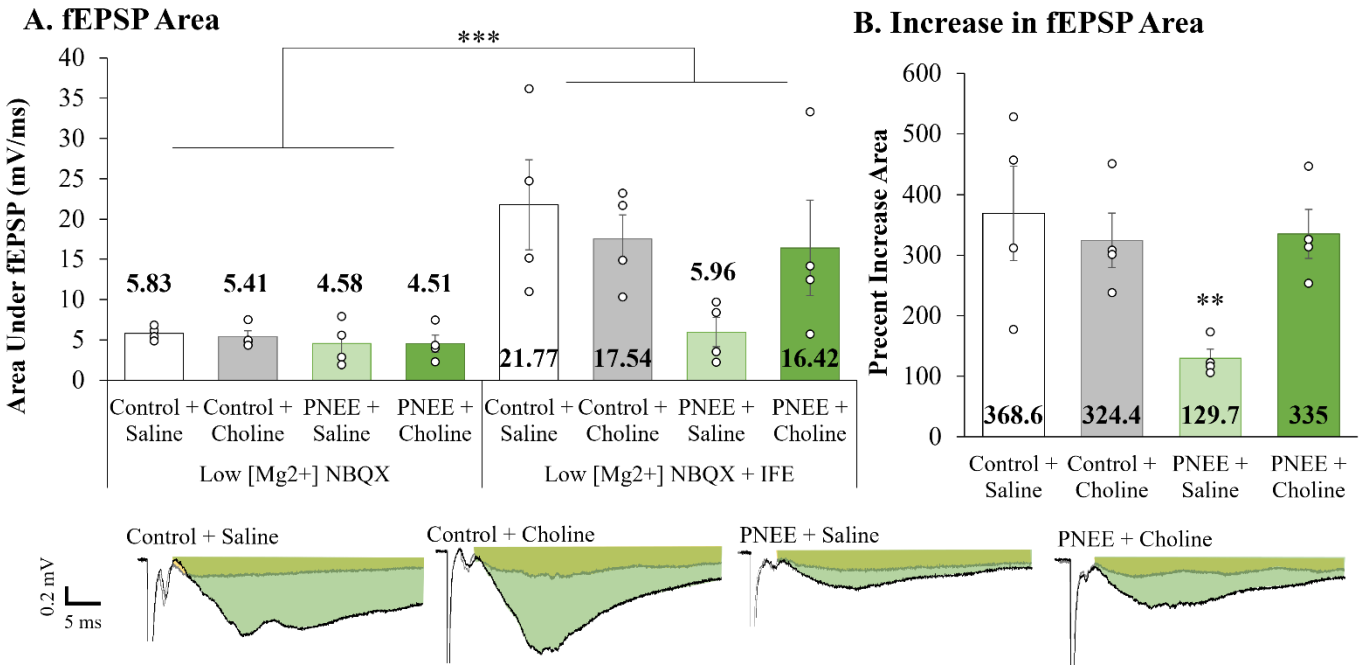


Figure 47 PNEE reduced fEPSP potentiation following GluN2B antagonism. A. fEPSP area during NBQX (left) or NBQX + ifenprodil (right) application. B. The percent change in fEPSP area after ifenprodil application. Each point is an individual slice (n = 4 slices; 2 male and 2 female slices). Representative traces are displayed for all treatment conditions on the bottom, such that the larger black waveform represents the fEPSP with ifenprodil application. All error bars are ± SEM. ** $p < 0.01$ and *** $p < 0.001$.

Finally, it was queried whether the resulting fEPSP following NBQX application in the low Mg2+ aCSF was indeed due NMDA receptor activation. Therefore, the following experiment was performed in which DL-APV (D-2-amino-5-phosphonovalerate) was washed over the slice either following the ifenprodil application (**Figure 48A**) or immediately after the NBQX application (**Figure 48B)**. Of note, the following data presented was performed in males only and the control was a cage control rather than a saline-injected offspring. In all groups there was an increase in fEPSP area with ifenprodil and a reduction with DL-APV. There were two interesting outcomes in this pilot experiment. The first was that saline treated PNEE males had a more blunted effect on fEPSP area with either drug application. Choline-treated PNEE slices mimicked either the control or PNEE waveform. The second outcome was that DL-APV did not completely reduce the fEPSP waveform, despite causing a great reduction.

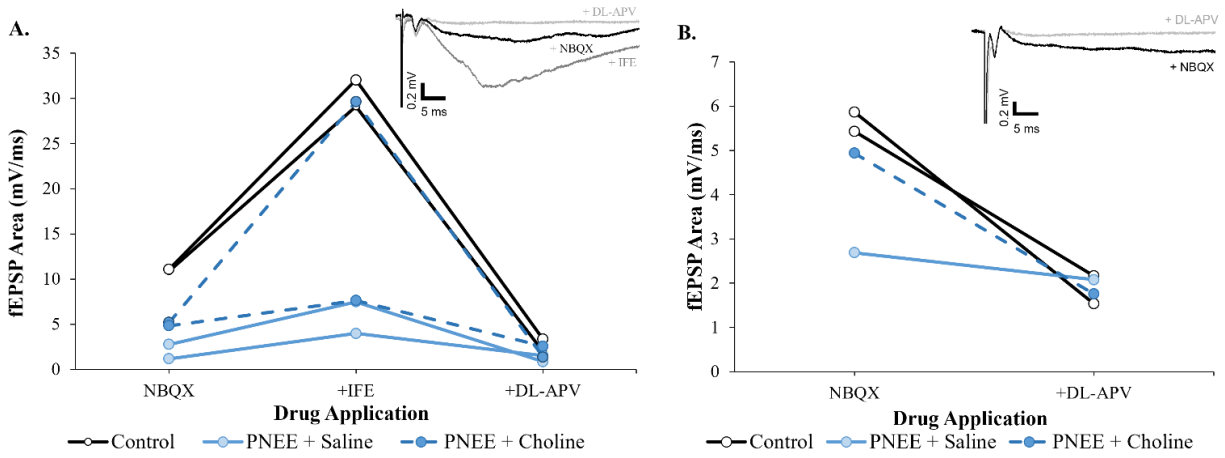


Figure 48 Resulting fEPSP area after DL-APV application. A. fEPSP area in low Mg^{2+} aCSF with NBQX (10 µM), ifenprodil (10 µM), and NBQX, and finally DL-APV (50 µM), ifenprodil, and NBQX. Each point represents a single slice (n_{slice} = 2 per condition). A representative trace from a control male is located in the top right inset. B. fEPSP area in low in low Mg^{2+} aCSF with NBQX or NBQX and DL-APV. A representative trace from a choline treated PNEE male is located in the top right inset.

CHAPTER 4: DISCUSSION

4.1 Summary of Major Findings

The data within this dissertation support the conclusion that PNEE delays neurodevelopmental processes and therefore creates a therapeutic window in which choline supplementation can be beneficial (**Figure 49**). This conclusion is evidenced by several key findings. The first is that PNEE delays eye opening and weight gain, demonstrating early signs of developmental delay. LTP is decreased in PNEE males and not in females. This deficit in synaptic plasticity is recovered into adulthood as PNEE animals 'catch up' to control offspring. LTP is increased in male and female PNEE juvenile offspring with choline treatment. However, control animals do not consistently have benefits in behavioural or physiological outcomes, which could

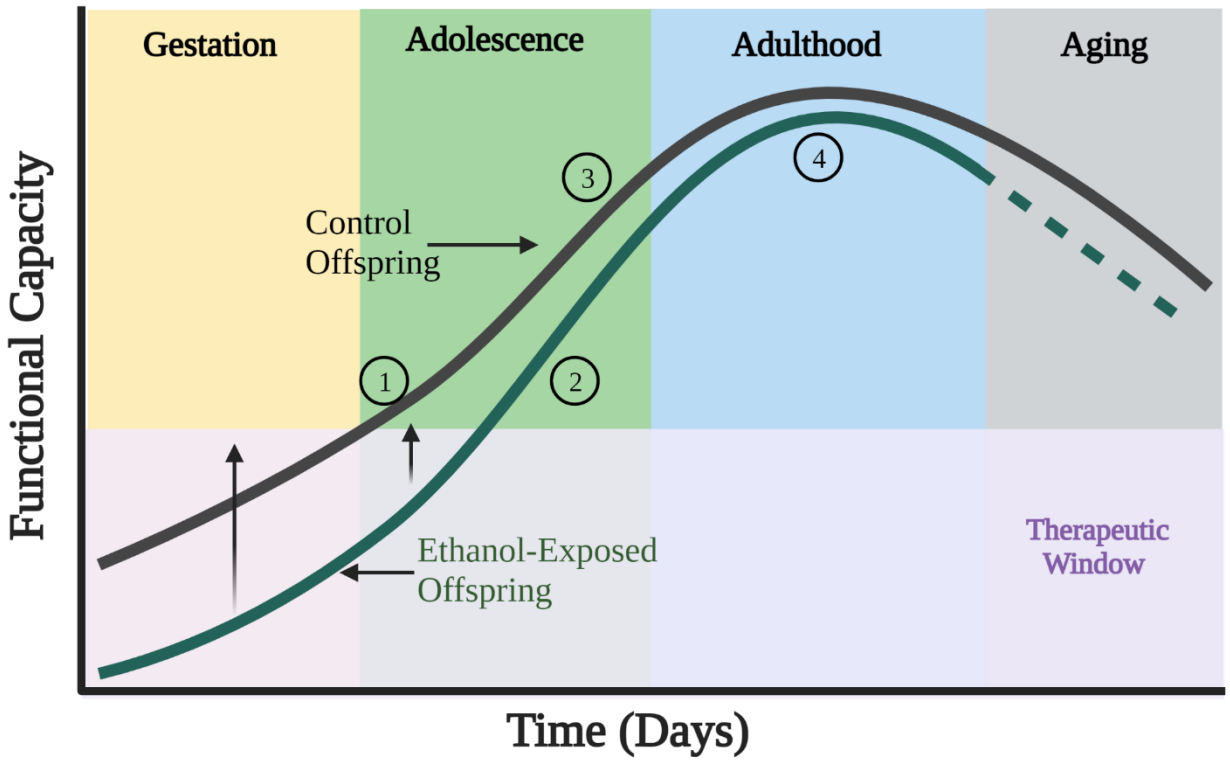


Figure 49 PNEE delays cognitive development which creates a therapeutic window for choline treatment. ① PNEE delays eye opening and ② weight gain as compared to control offspring. In adolescence, LTP is decreased in male ③ but not female offspring. However, in adulthood PNEE offspring "catch up" to controls ④.

indicate that the therapeutic window for hippocampal improvements in control animals has closed. Choline-treated control female offspring did demonstrate improvements in working memory performance, highlighting potential regional difference in the effects of choline treatment.

4.2 PNEE delays markers of neurodevelopment

The ethanol exposure paradigm used in this study is a well established, moderate ethanol-exposure paradigm (Weinberg, 1985). Previous work has determined that this paradigm produces blood alcohol content been 80-180 mg/dl (Christie *et al.*, 2005; Uban *et al.*, 2010; Anna R. Patten, Sickmann, *et al.*, 2013); in humans this range is equivalent to the legal intoxication level to being classified as 'impaired'. Blood alcohol content was not assessed in this study as the stress of the blood collection during pregnancy had a negative impact on maternal and offspring outcomes. However, due to the significant historical foundation of this paradigm and consistent maternal consumption of the diet, there is confidence the offspring in this study were exposed to a moderate amount of prenatal ethanol resulting in deficits. Dams on the ethanol diet gained less weight as compared to control dams (**Table 4**). A significant effect of the ethanol liquid diet on dam weight gain is commonly reported (Lan *et al.*, 2009; Sliwowska *et al.*, 2010; Uban *et al.*, 2010; Titterness and Christie, 2012), but this reduction is not always significant (A R Patten *et al.*, 2013; Anna R. Patten, Sickmann, *et al.*, 2013). Despite the decrease in maternal weight, PNEE did not result in significant changes in pup number, male to female ratio, or pup weight at PND 2 and 4. A decrease in weight in PNEE offspring was evident following weaning in both male and female offspring (**Figure 14**). Previous data has described different patterns in offspring weight gain using the first-two trimester ethanol exposure paradigm. Some report a reduction in weight gain specifically in males and only from PND 2-4 (Fontaine *et al.*, 2019), a reduction in both sexes from PND 2 -22 (Lan *et al.*, 2009; Anna R. Patten, Sickmann, *et al.*, 2013), a reduction in males beginning on PND

14 (Sliwowska *et al.*, 2010), and a reduction in females at PND 1 and PND 22, but not time points in between (Uban *et al.*, 2010). Therefore, while there is no specific date range in which PNEE offspring consistently weigh less than controls, PNEE generally results in slower weight gain at some point during postnatal development.

This moderate exposure paradigm also resulted in markers of developmental delay. Delayed eye opening has been utilized as a potential marker in other models of neurodevelopmental disorders, including in an autism-like model (Ruhela *et al.*, 2019) and prenatal exposure to methamphetamine (Rüedi-Bettschen and Platt, 2017). These data support that, despite the relatively moderate ethanol exposure paradigm, there were significant neural impacts. It also important to note that choline supplementation did not alter eye opening in either PNEE or control offspring. This is likely due to the short duration of choline treatment at the time of opening (eye opening occurred around PND 15 or 16 and choline treatment began at PND 10) and thus suggests sustained, rather than acute, choline treatment is required for positive effects in PNEE offspring.

4.3 PNEE and choline alters basal synaptic transmission in a sex-specific manner.

Both PNEE and choline treatment had a significant effect on basal transmission that were sex specific. In juvenile males choline-treatment with PNEE increased FV amplitude with increasing pulse width (**Figure 17A**). The FV represents the deflection of current across the presynaptic fibers (Mikulec *et al.*, 1998) and an increase in FV amplitude could suggest that more presynaptic fibers were recruited in the choline PNEE males. Interestingly, however, chronic choline treatment did not increase postsynaptic responsiveness with increasing pulse width (**Figure 17B**). This relationship is unlikely to be a measure of poor slice health due to the significant increase in LTP in choline treated PNEE males (**Figure 21C**). As there is the premise that choline is necessary for neuronal health, this conclusion is not supported. The picture is

perhaps clarified when the fiber volley is used as the input to fEPSP amplitude rather than pulse width (**Figure 17C & 17D**). The re-organization of data this way demonstrates that choline-treated PNEE males have decreased synaptic efficacy, as choline-treated PNEE males have a decreased fEPSP amplitude for the corresponding fiber volley.

These patterns in male neuronal excitability are no longer evident when the technical parameters are set for long-term depression experiments, meaning that the stimulation is set to a higher magnitude to elicit a response 70% of the maximum and is not conducted in the presence of a $GABA_A$ receptor antagonist. In these experiments, there was no significant difference in fiber volley (**Figure 18A**) or fEPSP amplitude, although there appeared to be a trend in an increase in fEPSP amplitude with choline treatment (**Figure 18B**). However, when considering the contribution of fiber volley to fEPSP in male offspring, it appears that PNEE or choline may increase synaptic efficacy. This poses a limitation of the excitability analysis. There was an unequal representation for corresponding fiber volley and therefore it was not possible to run a statistical analysis using FV as the input. Future experiments could use fiber volley amplitude instead of pulse width as the dependent variable to explore this relationship further.

In female offspring, saline-treated PNEE offspring had a significant increase in fEPSP amplitude (**Figure 19B**) that was still evident when considering fiber volley amplitude as the input mechanism (**Figure 19C & 19D**). This trend was present in the long-term depression conditions, despite not being significant, signifying that this is not necessarily due to a change in inhibition due to the presence of picrotoxin. The increase in excitability in PNEE females has not been reported previously, but could explain the lack of deficit in PNEE females at this age as demonstrated in this study and in others (Titterness and Christie, 2012). Interestingly, no changes in baseline excitability were previously reported in PNEE females from PND 21-28 and these

offspring had decreased LTP (Fontaine *et al.*, 2019). Therefore, there may be an age-dependent increase in excitability in PNEE females that could correspond with recovered LTP.

4.4 Sex-dependent changes in juvenile synaptic plasticity

There were sex-specific effects of synaptic plasticity following PNEE and choline treatment. Male PNEE offspring demonstrated deficits in LTP (**Figure 21C**) which was not seen in females (**Figure 23C**). Previous *in vivo* electrophysiology work from the Christie laboratory which examined LTP in PNEE offspring within the same juvenile period supports this finding (Titterness and Christie, 2012). The deficit in male PNEE offspring was ameliorated with postnatal choline treatment, such that magnitudes of LTP were equivalent to control males. These data are the first to demonstrate an improvement in LTP in PNEE male offspring with choline treatment. Control males treated with choline did not similarly benefit with postnatal choline treatment. Rodent studies examining behavioural outcomes of postnatal choline supplementation do not always exhibit further improvements in control offspring (Thomas *et al.*, 2000), however prenatal choline did improve performance on the Morris Water Maze (Thomas *et al.*, 2010). This evidence contributes to the conclusion that PNEE delays cognitive development and, as such, extends the therapeutic window for intervention (**Figure 49**). This window in PNEE offspring is not infinite, however, as choline supplementation from PND 40-60 in developmentally exposed offspring did not improve hippocampal-dependent spatial memory performance (Schneider and Thomas, 2016), nor did clinical trials of postnatal choline supplementation in older children with FASD (5-10 years old) display benefits in learning and memory tasks (Nguyen *et al.*, 2016). Nonetheless, the extended window of development and the unique neuronal environment of the ethanol-exposed brain provides a promising opportunity for intervention.

The mechanism behind improvements in LTP in choline treated PNEE offspring is currently unknown. As discussed previously, choline and ethanol parallel in the multitude of neuronal and developmental process that they can alter. While this makes choline a promising treatment for PNEE, this also generates many hypotheses as to its mechanism of action. Due to the limitations in the scope of a dissertation, only two overarching hypotheses were tested to narrow the possible mechanisms. The first option was that the improvement in LTP was due to an overall improvement in brain health, for instance due to phospholipid generation, reduction in ROS generation, or epigenetic changes. The second hypothesis was that the increase in LTP was a result of the fine balance of synaptic plasticity favouring excitatory action following choline treatment. The subsequent LTD experiment was performed, as an increase in LTD would support the former hypothesis while a decrease in bidirectional plasticity would suggest the latter.

In this study PNEE did not alter the magnitude of LTD in either male (**Figure 26**) or female offspring (**Figure 28**). Previous work has demonstrated decreased DG LTD in younger PNEE males (PND 21-28), but not in age-matched PNEE females (Fontaine *et al.*, 2019). The reason for the difference between the aforementioned study and the data in this dissertation could be that LTD mechanisms recover in the PNEE brain in the time between the two experimental groups. Indeed, an age-dependency of the magnitude of 1 Hz LTD has been shown in the non-exposed brain, such that LTD is greater in young animals (12-20 days old: 24 ± 3%) as compared to juvenile animals (31-40 days old: 11 ± 3%) (Kemp *et al.*, 2000). This aligns with work completed in the CA1 subregion of the hippocampus in which there were no deficits in LTD in juvenile (PND 30-35) PNEE males or females (Titterness and Christie, 2008). Furthermore, in this dissertation choline treatment in both control and PNEE male offspring caused a subtle decrease in the amount of LTD (**Figure 26**). While this was not significantly different than saline-treated males, it can be

concluded that choline treatment did not increase the amount of LTD and thus did not improve overall bidirectional synaptic plasticity. Furthermore, the cumulative probabilities demonstrate a shift rightwards, as well as a decreased percentage of slices that displayed any depression, indicating that choline treatment may be shifting the balance towards excitation in male offspring. In female offspring there were similarly no deficits due to PNEE nor any improvement with choline treatment (**Figure 28**). Despite the majority of slices showing a slight level of depression (i.e., a negative change from baseline fEPSP slope), the average magnitude of LTD was less than -10% for all conditions and thus can be considered a return to baseline. Choline treatment did not further decrease the amount of LTD in female offspring. While there is a paucity of data examining sex differences in LTD at the juvenile timepoint, previous work found that females may exhibit greater LTD than males, although males and females were not directly compared (Titterness and Christie, 2008). This study also explored the sex-specific effect of acute stress; LTD was increased in males following an acute stressor, but LTD was eliminated in stressed female offspring (Titterness and Christie, 2008). This finding could indicate that the lack of LTD in female offspring in this dissertation was due to the stress of repeated injections. However, a small pilot study of control female offspring that did not receive any injections also did not display a significant amount of LTD (**Supplemental Figure 1**).

The proposition that postnatal choline supplementation increases excitation in PNEE offspring should be further explored in future research. Two initial targets would be to examine a change in the cholinergic inputs to the hippocampus and changes in NMDA receptor functioning or composition. In regards to the former, third-trimester equivalent ethanol exposure decreased hippocampal-projecting cholinergic cells (ChAT+) in the medial septum/diagonal band of Broca by 42% (Smiley *et al.*, 2021), as well as cortically-projecting cholinergic neurons in the nucleus

basalis of Meynert in the basal forebrain (Milbocker and Klintsova, 2020). The timing of the postnatal choline treatment (PND 10-30) overlaps with the development of cholinergic neurons in the basal forebrain; ChAT and AChE functionality begins late in gestational development and continues to increase until around PND 30 (Thal *et al.*, 1992). Therefore, increased availability of choline may help to protect these vulnerable cholinergic neurons. Secondly, choline supplementation may influence the balance of excitation/inhibition in PNEE offspring. Future research should explore changes in NMDA receptor subunit composition, specifically in the developmental timeline of GluN2B and GluN2A, and alterations in excitatory and inhibitory currents in choline treated PNEE offspring.

4.5 Sex-specific outcomes in synaptic plasticity

In the LTP experiments there were sex-dependent effects of PNEE. Juvenile female offspring had an overall main effect of prenatal treatment, such that PNEE females had increased LTP as compared to control females (**Figure 24**). This sex-specific invulnerability to PNEE in the juvenile brain has been demonstrated previously in the literature (Titterness and Christie, 2012; An and Zhang, 2015). The juvenile time period used in this dissertation is around the time of sexual maturation in the female rat, which occurs around PND 32-34 and coincides with vaginal opening (Lewis *et al.*, 2002). Vaginal opening was monitored in these juvenile animals to limit the confounds of pubertal hormones. Therefore it is unlikely that influxes of estrogen may account for the lack of deficit in juvenile females in this study, despite a decrease seen previously at PND 21-28 (Fontaine *et al.*, 2019). Another explanation for these sex-differences originates much earlier; fundamental differences in the male and female placenta may account for sex-differences in the effects of PNEE. An important note, the placenta forms during the blastocyst phase of embryonic development, around GD 3.5-4.5 in rodents, and therefore the placenta is also of male or female

origin (Kalisch-Smith *et al.*, 2017; Woods, Perez-Garcia and Hemberger, 2018). The sexual dimorphism of the placenta is thought to convey unique susceptibility and protection to environmental conditions (Kalisch-Smith *et al.*, 2017). Indeed, in the context of PNEE the female placenta adapts differentially than the male placenta to preserve organ development (Kwan *et al.*, 2020). This early compensation in the female fetal environment could be the reason for lifelong benefits seen in many studies of PNEE.

4.6 Working Memory Performance Was Unchanged in Choline-Treated Animals

While changes in prefrontal cortex physiology were not examined in the synaptic plasticity experiments, working memory was analyzed as part of the training paradigm for the reference memory task. In male offspring there were no alterations in working memory performance with either PNEE or choline treatment (**Figure 31C & D**). It is perhaps not surprising that choline-treatment did not improve prefrontal-dependent behaviours, as previous work has found that choline treatment later in adolescent development improved working memory. Specifically choline treatment from PND 40-60 rescued ethanol-induced impairments in working memory, but not hippocampal-dependent memory (Schneider and Thomas, 2016), suggesting that the prefrontal cortex is can be modulated by choline supplementation later in the postnatal period.

There were significant effects of PNEE and choline treatment on hyperactivity and anxiety-like behaviours. PNEE males were hyperactive as compared control offspring (**Figure 31B**), which was not significantly ameliorated with choline treatment. Furthermore, choline-treated male animals explored fewer arms of the maze over the course of the entire trial (**Figure 31A**). There could be an increased stress response in choline treated males, either from a direct alteration of choline on the HPA (hypothalamus – pituitary – adrenal) axis or a heightened stress reaction to the injection protocol. The increase in anxiety-like behaviour may have prevented beneficial effects

of the treatment to be evident. Interestingly, the inverse was true for female offspring. There were no changes in anxiety-like behaviours in female offspring, as the total number of arm entries and distance travelled during the working memory task was equivalent between groups (**Figure 32A & B**). This sex difference has been noted in the literature previously, as male PNEE offspring are more likely to have a hyperactive HPA axis response to stressors than female PNEE offspring (Weinberg *et al.*, 2008). However, choline-treatment impaired working memory performance in female PNEE offspring. While there was a trend, but not a significant difference, in the total number of arm entries to complete the task (**Figure 32C**), choline-treated PNEE females required more time to complete the working memory task as compared to choline-treated female controls (**Figure 32D**). Importantly, choline-treated females did not differ from saline-treated offspring, showing a distinct response to choline treatment as a factor of prenatal environment rather than ameliorating or creating deficits in performance. The reason for this differential response to choline treatment is as of yet unknown. As previously mentioned, PFC-dependent behaviours were improved with choline treatment from PND 40-60 in PNEE offspring (Schneider and Thomas, 2016), therefore improvements in working memory tasks in control offspring could be due to the timing of choline treatment utilized in this study (PND 10-30) overlaps with the fine tuning of prefrontal circuitry (Kolb *et al.*, 2012).

The performance of all animals in the working memory task were above random chance. A simulated Radial Arm Maze was generated using RStudio (Version 1.4.1106; see **Supplementary Text 1**) and run 10,000 times. In this model each 'arm' was selected with pseudo-randomization, meaning that choices were limited as to not re-enter the arm currently 'resided' in and with an increased probability to choose an arm immediately adjacent. This was intended to mimic natural rodent behaviours as closely as possible. The simulated task was completed on

average in approximately 19 arm entries. In the experimental trials, each group completed the task in 11-15 entries on average, indicating that there was indeed the use of working memory processes and was not due to random chance.

4.7 Parallels between changes in synaptic plasticity and reference memory

Following habituation and the working memory task, a reference memory task was performed over the course of three days. In this task the same four arms were baited for each animal across trials and therefore this task analyzed the ability to discriminate between similar spatial inputs. Male offspring improved in their performance of this task; the number of entries and time to complete the maze decreased over the trials (**Figure 33**). However, there were no deficits in reference memory performance due to PNEE or improvements with choline treatment. While it is difficult to make direct comparisons between electrophysiological data and behavioural performance due to the multifold of regions and circuitry that are involved in each process, some hypotheses can be made when examining both pieces of evidence together. In particular, male offspring treated with choline did not outperform saline treated offspring on the radial arm maze, despite the significant increase in LTP in these animals. The bidirectional aspect of hippocampal plasticity may be critical in this aspect, as choline treatment decreased the magnitude of LTD. This could suggest that the fine tuning of synapses was altered and the ability to update the neural network from the previous working memory task, a task in which all arms contained a reward, was impaired. Previous work using a Delayed Non-Matching to Place (DNMP) version of the radial arm maze found that decreasing the amount of separation between the baited and sample arms uncovered differences between control animals and those with decreased adult neurogenesis. Differences in performance did not occur in these animal with the “easier” version of the task

where there was a large degree of separation (Clelland *et al.*, 2009). Interestingly, the complexity of the task had no bearing on task performance in male offspring (**Figure 35**).

In females there was no significant effect of trial in terms of the number of entries required to complete the task, but the task was completed in a shorter amount of time. This indicates that while female offspring may have learned that some arms were baited, they did not improve in spatial memory specific task performance over time. Sex differences in spatial memory have long been reported (Safari *et al.*, 2021) and therefore this finding is not surprising. It was supposed that this lack of learning was due to an altered search strategy utilization, specifically, that female offspring adopted a serial search strategy. This would prevent the direct formation of spatial memory, as it would be simpler to learn "to always turn left" and still receive all rewards. Despite this hypothesis, there were no sex difference in the search strategies used to complete the task over the course of all five trials (**Figure 37**). The search strategies utilized in this dissertation were generated based on the patterns of task completion witnessed. Perhaps sex differences would emerge with more strict criteria for serial exploration. Unlike in males, in PNEE female offspring there was a weak correlation with maze complexity and performance, but not in the direction predicted. Female PNEE offspring performed more poorly when the baited arms were closer together, and thus more separated from the non-baited arms (**Figure 35 & Figure 36**). It is not clear why a worse performance occurred with a lower amount of separation between baited arms but could be due to overlapping inputs of the closely adjacent arms. Unfortunately, due to the lower number of animals in each group resulting from dividing the data by complexity, this finding was not powered to determine if this trend is significant but could be followed up in future work.

Similar to the working memory task, a simulated RAM reference memory task was created with the same previous parameters but with the addition of only four distinct arms randomly

chosen for each 'animal' to be the correct arms. After 10,000 simulated trials, on average this task could be randomly completed in approximately 14-15 entries (**Supplementary Text 2**), demonstrating that, while improvements were not seen in all groups, task performance was better than random chance.

4.8 Deficits due to PNEE and benefits of choline did not persist into adulthood.

One of the major advantages of preclinical models of FASD is that long-term changes due to treatment can be explored. Therefore, benefits of postnatal choline supplementation in PNEE offspring were assessed into adulthood. Unexpectedly, there were no effects of PNEE on LTP magnitudes in animals from PND 60 to 90 (**Figure 40 & Figure 41**). Previous work has often indicated that ethanol-exposed male offspring have deficits in LTP that persist into adulthood (Sutherland, McDonald and Savage, 1997; Anna R. Patten, Sickmann, *et al.*, 2013; Sickmann *et al.*, 2014) while adult females show no deficits (A R Patten *et al.*, 2013; Anna R. Patten, Sickmann, *et al.*, 2013; Sickmann *et al.*, 2014) or even an increase in LTP with PNEE (Anna R. Patten, Gil-Mohapel, *et al.*, 2013). There are few reported instances in which PNEE did not cause long-term changes in adult male LTP, although these instances include some important caveats. For instance, previous work using the same paradigm from this study found no significance in male PNEE LTP, however, the analysis included treatment with N-Acetyl-Cysteine which may have occluded deficits that could have been evident with a direct comparison (A R Patten *et al.*, 2013). Additionally, another study which did not see deficits in LTP used only a single trimester ethanol exposure (Helfer, White and Christie, 2012; Anna R. Patten, Gil-Mohapel, *et al.*, 2013).

Alterations in synaptic plasticity due to postnatal choline supplementation were not evident in adulthood. It is likely that nutritional treatment should be continued throughout the lifespan in order for benefits to persist. Previous nutritional interventions performed in the Christie laboratory

have utilized omega-3 supplementation and in this paradigm the treatment spanned the entirety of the postnatal period. Benefits due to the prolonged treatment in adult male LTP were observed (Anna R. Patten, Sickmann, *et al.*, 2013). Similarly, a model of lifelong choline supplementation in a mouse model of Alzheimer's Disease found a reduction in amyloid beta plaque and improved spatial memory performance at 10 months of age (Velazquez *et al.*, 2019). Future experiments should therefore explore the effects of continued supplementation with choline on synaptic plasticity in ethanol exposed offspring. It would be suggested that method of administration be revised from subcutaneous injections to a choline-supplemented diet. While this would reduce the precision of the amount of administered choline, it would eliminate the stress from repeated injections and reduce the need for continued experimenter handling of the offspring.

4.9 Subtle changes are evident in LTP threshold.

After determining that there was no effect on LTP with PNEE and choline treatment, it was theorized that potential effects may be at a more subtle level. Previous work in non-exposed offspring has indicated that maternal choline supplementation (GD 12-17) decreased the induction threshold for LTP into adulthood (Pyapali *et al.*, 1998). Furthermore, Meck and colleagues found that maternal choline supplementation (GD 12-17) or postnatal choline treatment (PND 16-30) improved spatial memory performance on the 12-arm radial maze in seven-month-old offspring. These choline-treated animals also had increased spine density in the CA1 and in the DG (Meck *et al.*, 2008). Gestational and postnatal development are both demanding developmental times in terms of maternal supply of choline for offspring growth (Garner, Mar and Zeisel, 1995; Zeisel *et al.*, 1995), therefore choline treatment during these critical time windows may compensate for the nutritional depletion of maternal stores, even in non-exposed offspring. The cumulative evidence from these previous works prompted the exploration of changes in LTP threshold. As it was not

clear from the literature as to what would be considered a subthreshold LTP protocol when utilizing a high frequency stimulation protocol, several stimulation paradigms were tested. There were four main parameters that could have been altered: the number of pulses, the number of trains, the intertrain interval, and the frequency of the pulses. It was decided to alter the frequency of the pulses, as there are many studies which can still exhibit LTP with fewer trains and longer intertrain intervals. Frequency was decreased in incremental steps while maintaining all other parameters consistent. While there were no differences between conditions at 70 Hz, choline-treated groups had increased LTP at 50 Hz for male offspring (**Figure 42**) and 30 Hz in female offspring (**Figure 43**). These data confirm that choline supplementation during critical windows can still convey long-term changes in synaptic plasticity, but the effects are do not occur at saturating levels of stimulation. While subthreshold induction may be less clear in terms of the translational benefit to individuals with FASD, subtle changes in synaptic plasticity may in fact have a large impact in real world functioning.

An additional consideration is the potential effects in the aged brain, as adequate choline supplementation is important for long-term cognition (Tabassum *et al.*, 2017). There is a paucity of data examining bidirectional plasticity in aged PNEE offspring and therefore it is unknown if deficits in synaptic plasticity re-emerge into aging. Unfortunately, this experimental question may also have translational limitations. A 2016 study analyzing life expectancy for people FAS in Alberta, Canada found that the life expectancy for individuals with FAS was 34 years old and the average age of death was 28 years old (±19 years standard deviation) (Thanh and Jonsson, 2016). While there may be interesting biological potential for choline supplementation into adulthood with prenatal ethanol exposure, research into FASD should ultimately be focused on problems that concern individuals with FASD. Therefore, before research resources are invested into aged

research, it is perhaps more critical to investigate leading causes of death in individuals with FAS, which include suicide, accidents, and drug or alcohol use (Thanh and Jonsson, 2016).

4.10 Potential Role of GluN2B in deficits following PNEE.

While the original aim of this dissertation was to determine changes in synaptic plasticity following PNEE and choline supplementation, and thus the efficacy of choline as a treatment, a final pilot experiment was conducted to begin to unravel a potential mechanism for future study. The relative contribution of GluN2A and GluN2B subunits has been suggested to alter the properties of a synapse, such that more GluN2B subunit expression will decrease the LTP threshold (Brigman *et al.*, 2010). However, this is not always seen (Fox *et al.*, 2006) and thus is likely a simplification of the complex picture of synaptic transmission. However, the properties of GluN2A and GluN2B containing NMDA receptors do in fact differ. GluN2A subunits have a higher open probability, faster magnesium unblocking, and faster activation and deactivation kinetics than GluN2B-containing NMDA receptors (Clarke, Glasgow and Johnson, 2013). In this dissertation, the GluN2B antagonist ifenprodil did not alter the onset latency (the time from FV peak to the beginning of the fEPSP) but did increase the peak latency (the time from the start of the fEPSP to the peak amplitude). Unlike previous reports, which found that GluN2B antagonism caused a depression of the fEPSP area (Brady *et al.*, 2013), ifenprodil increased the area of the fEPSP (**Figure 47**). This could be due to the localization of GluN2B-containing NMDA receptors in the extrasynaptic space where they provide a “brake” in instances of high amount of glutamate spillover (Vizi, Kisfali and Lorincz, 2013). In saline treated PNEE offspring the degree of fEPSP area growth was significantly decreased as compared to all other groups. While the number of slices were limited in this pilot experiment, and thus sexes were combined, this change in GluN2B potentiation could suggest a mechanism by which choline-treatment is acting in PNEE offspring

to decrease the threshold for LTP induction. Interestingly, there was no further increase in choline-treated control offspring and therefore the mechanism behind any change in LTP threshold induction in control offspring is still unknown. If future works continues this line of investigation, both sexes should be included in sufficient power to determine sex-dependent differences.

4.11 Future experiments & Limitations

This work provides many opportunities for future exploration that could further uncover the mechanism by which postnatal choline supplementation alters synaptic plasticity in the PNEE brain. It was suggested that choline may alter the excitatory/inhibitory balance, as evidenced by the increase in LTP and the decrease in LTD, therefore subsequent work could use whole cell electrophysiology to examine the changes in E/I balance at a cellular level. Changes in plasticity could also be due to the cholinergic system. Examination of key cholinergic proteins, ChAT positive neuron densities in the medial septum/diagonal band of Broca, or antagonism of cholinergic receptors during HFS could provide evidence for this hypothesis. Additionally, as benefits did not persist when examined at PND 60-90, choline could be supplemented into adulthood. This would also provide an interesting model to examine the effects of PNEE and choline supplementation into aging.

This work possesses several limitations that should be discussed. The first limitation is the nutritional profile of the ethanol-containing diet. The Dyets Inc. High Protein Ethanol Diet contains 0.53 g/L of choline bitartrate, which is 0.218 g/L of choline. The Lab Diet Rodent 5001 contains an estimated 2.25 g/kg choline chloride. Factoring in the relative difference in consumption on the two diets (60 g/day on the ethanol diet and an estimated 15 g/day on the control diet), PNEE dams are likely consuming less choline than control dams. Controlling for this nutritional difference is difficult, as the classically used pair fed controls produce alterations

distinct from ethanol exposed offspring (Titterness and Christie, 2008; Weinberg *et al.*, 2008). It is thought that the stress of food deprivation negatively impacts fetal development in a unique manner and should be considered a maternal-stress model. However, providing *ad libitum* access to the currently available non-ethanol containing liquid diet would not control for choline intake. Thus, a choline-deficient liquid diet could be formulated, or the ethanol diet could be further supplemented with choline during prenatal development. Conversely, maternal choline stores could be examined between control and ethanol dams to determine the level of choline depletion. It could be argued, however, that pregnant individuals who are consuming alcohol are more likely to be simultaneously consuming a diet that is deficient in key nutrients (May *et al.*, 2014), in addition to the fact that the majority of pregnant individuals (alcohol consumption not considered) do not meet the recommend choline adequate intakes in pregnancy or during lactation (Wallace and Fulgoni, 2017). Therefore, there are translational parallels between this model and the real world. Nonetheless, the factor of nutrition should be considered when interpreting these results and applying them to future clinical research.

4.12 Translational applications

The objective of this work was to further understand the mechanisms of postnatal choline supplementation such that it can be applied to clinical studies involving individuals with FASD. The work in this dissertation provides several points to be considered. The first is that sex dependent effects should continued to be explored in clinical studies of FASD, as much of this work described unique changes in synaptic plasticity and behavioural performance between male and female offspring. This may also necessitate that clinical definitions of FASD be re-examined as current definitions may neglect to include some girls with alcohol exposure who may express distinct symptoms. The second important consideration is that the length of choline

supplementation should be continued. Choline is an essential nutrient which has few negative side effects when consumed in excess (Institute of Medicine Food and Nutrition Board, 1998) and is vastly under consumed in the majority of the population (Wallace and Fulgoni, 2017). Therefore, lifelong choline supplementation poses a small but manageable risk. This also may influence policy surrounding supplementation of choline in prenatal or children's vitamins. The final point to consider when translating this work to clinical research is that, while effects of choline treatment may be more subtle, any positive improvement early in vulnerable times may in fact be cumulate to produce large improvements in quality of life. Not to mention that other critical areas of the brain, such as the prefrontal cortex or the amygdala, may similarly show some level of benefit that could be meaningful when considering the large incidence of mental health problems in individuals with FASD.

5. Conclusion

The objective of this dissertation was to determine the effects of postnatal choline supplementation on hippocampal synaptic plasticity following prenatal ethanol exposure. This objective was divided into three main aims. The first aim was to explore changes in bidirectional plasticity in the juvenile offspring immediately following treatment. In this population, male, but not female, PNEE offspring had a deficit in LTP, but postnatal choline supplementation increased LTP in both sexes. This did not seem to be due to an overall improvement in brain health as LTD was not increased in either sex or, in fact, appeared to be decreased with choline treatment. The second aim was to determine if changes in hippocampal synaptic plasticity translated to improvements in spatial learning and memory. Other than hyperactivity in male PNEE animals, there were no clear deficits in learning or memory with PNEE, nor did choline treatment further improve task performance. Finally, the third aim was to determine if the benefits evident

immediately following choline treatment persisted into adulthood. While the 100 Hz saturating protocol demonstrated no deficits with PNEE, there were benefits of choline supplementation using a subthreshold stimulation protocol. This indicates that deficits due to PNEE and benefits of nutritional supplementation may be more subtle but are likely still impactful when considering the translational aspect of this research. The cumulative data displayed in this research supports the hypothesis that prenatal ethanol exposure delays neurodevelopment, but this delay provides a window of opportunity for treatments such as choline supplementation to be effective in improving hippocampal-dependent outcomes.

References

Aistrup, G. L., Marszalec, W. and Narahashi, T. (1999) 'Ethanol modulation of nicotinic acetylcholine receptor currents in cultured cortical neurons', *Molecular Pharmacology*, 55(1), pp. 39–49. doi: 10.1124/mol.55.1.39.

Akison, L. K. *et al.* (2018) 'Effect of Choline Supplementation on Neurological, Cognitive, and Behavioral Outcomes in Offspring Arising from Alcohol Exposure During Development: A Quantitative Systematic Review of Clinical and Preclinical Studies', *Alcoholism: Clinical and Experimental Research*, 42(9), pp. 1591–1611. doi: 10.1111/ACER.13817.

Alberry, B. L. J. and Singh, S. M. (2020) 'Hippocampal DNA Methylation in a Mouse Model of Fetal Alcohol Spectrum Disorder That Includes Maternal Separation Stress Only Partially Explains Changes in Gene Expression', *Frontiers in Genetics*, 11, p. 70. doi: 10.3389/fgene.2020.00070.

Alberta Medical Association (2003) 'Diagnosis of fetal alcohol syndrome (FAS).', *The Canadian Child and Adolescent Psychiatry Review*, 12(3), pp. 81–86. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2582739/ (Accessed: 2 September 2020).

Albright, C. D. *et al.* (1999) 'Choline availability alters embryonic development of the hippocampus and septum in the rat', *Developmental Brain Research*, 113(1–2), pp. 13–20. doi: 10.1016/S0165-3806(98)00183-7.

Allen, D. D. and Smith, Q. R. (2001) 'Characterization of the blood-brain barrier choline transporter using the in situ rat brain perfusion technique', *Journal of Neurochemistry*, 76(4), pp. 1032–1041. doi: 10.1046/j.1471-4159.2001.00093.x.

Almeida-Toledano, L. *et al.* (2021) 'Epigallocatechin Gallate Ameliorates the Effects of Prenatal Alcohol Exposure in a Fetal Alcohol Spectrum Disorder-Like Mouse Model.', *International journal of molecular sciences*, 22(2). doi: 10.3390/ijms22020715.

An, L., Yang, Z. and Zhang, T. (2013) 'Imbalanced Synaptic Plasticity Induced Spatial Cognition Impairment in Male Offspring Rats Treated with Chronic Prenatal Ethanol Exposure', *Alcoholism: Clinical and Experimental Research*, 37(5), pp. 763–770. doi: 10.1111/ACER.12040.

An, L. and Zhang, T. (2013) 'Spatial cognition and sexually dimorphic synaptic plasticity balance impairment in rats with chronic prenatal ethanol exposure', *Behavioural Brain Research*, 256, pp. 564–574. Available at: https://pubmed.ncbi.nlm.nih.gov/24050890/ (Accessed: 10 September 2020).

An, L. and Zhang, T. (2015) 'Prenatal ethanol exposure impairs spatial cognition and synaptic plasticity in female rats', *Alcohol*, 49(6), pp. 581–588. doi: 10.1016/J.ALCOHOL.2015.05.004.

Andersen, J. V. *et al.* (2021) 'Glutamate metabolism and recycling at the excitatory synapse in health and neurodegeneration', *Neuropharmacology*, 196, p. 108719. doi: 10.1016/J.NEUROPHARM.2021.108719.

Andersen, P. (2007) *The Hippocampus Book*. New York ; Oxford University Press (Oxford Neuroscience Ser.).

Arnett, A. B. *et al.* (2015) 'Sex differences in ADHD symptom severity', *Journal of Child Psychology and Psychiatry and Allied Disciplines*, 56(6), pp. 632–639. doi: 10.1111/JCPP.12337.

Asada, H. *et al.* (1996) 'Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures', *Biochemical and Biophysical Research Communications*, 229(3), pp. 891–895. doi: 10.1006/bbrc.1996.1898.

Asada, H. *et al.* (1997) 'Cleft palate and decreased brain γ-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase', *Proceedings of the National Academy of Sciences of the United States of America*, 94(12), pp. 6496–6499. doi: 10.1073/PNAS.94.12.6496/ASSET/B121F67E-10D1-45D8-B3F2-F3C08C06433D/ASSETS/GRAPHIC/PQ1271132003.JPEG.

Ash, J. A. *et al.* (2014) 'Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice', *Neurobiology of Disease*, 70, pp. 32–42. doi: 10.1016/j.nbd.2014.06.001.

Auerbach, J. M. and Segal, M. (1996) 'Muscarinic receptors mediating depression and long-term potentiation in rat hippocampus', *Journal of Physiology*, 492(2), pp. 479–493. doi: 10.1113/jphysiol.1996.sp021323.

Auten, R. L. and Davis, J. M. (2009) 'The Role of Oxygen in Health and Disease-A Series of Reviews Oxygen Toxicity and Reactive Oxygen Species: The Devil Is in the Details', *Pediatr Res*, 66, pp. 121–127.

Baculis, B. C., Diaz, M. R. and Valenzuela, C. F. (2015) 'Third trimester-equivalent ethanol exposure increases anxiety-like behavior and glutamatergic transmission in the basolateral amygdala.', *Pharmacology, biochemistry, and behavior*, 137, pp. 78–85. doi: 10.1016/j.pbb.2015.08.009.

Baggio, S. *et al.* (2017) 'Embryonic alcohol exposure promotes long-term effects on cerebral glutamate transport of adult zebrafish', *Neuroscience Letters*, 636, pp. 265–269. doi: 10.1016/j.neulet.2016.11.016.

Baggio, S. *et al.* (2020) 'Fetal alcohol spectrum disorders model alters the functionality of glutamatergic neurotransmission in adult zebrafish', *NeuroToxicology*, 78, pp. 152–160. doi: 10.1016/J.NEURO.2020.03.003.

Bahnfleth, C. *et al.* (2019) 'Prenatal Choline Supplementation Improves Child Color-location Memory Task Performance at 7 Y of Age (FS05-01-19)', *Current Developments in Nutrition*, 3(Supplement_1). doi: 10.1093/CDN/NZZ048.FS05-01-19.

Bahnfleth, C. L. *et al.* (2022) 'Prenatal choline supplementation improves child sustained attention: A 7-year follow-up of a randomized controlled feeding trial', *The FASEB Journal*, 36(1). doi: 10.1096/FJ.202101217R.

Baker, J. A. *et al.* (2022) 'Choline Supplementation Modifies the Effects of Developmental Alcohol Exposure on Immune Responses in Adult Rats'. doi: 10.3390/nu14142868.

Barnes, D. E. and Walker, D. W. (1981) 'Prenatal ethanol exposure permanently reduces the

number of pyramidal neurons in rat hippocampus', *Developmental Brain Research*, 1(3), pp. 333–340. doi: 10.1016/0165-3806(81)90071-7.

Barr, H. M. *et al.* (2006) 'Binge drinking during pregnancy as a predictor of psychiatric disorders on the structured clinical interview for DSM-IV in young adult offspring', *American Journal of Psychiatry*, 163(6), pp. 1061–1065. doi: 10.1176/AJP.2006.163.6.1061/ASSET/IMAGES/LARGE/Q519T2.JPEG.

Barria, A. and Malinow, R. (2002) 'Subunit-specific NMDA receptor trafficking to synapses.', *Neuron*, 35(2), pp. 345–353. doi: 10.1016/s0896-6273(02)00776-6.

Baumgartner, H. K. *et al.* (2015) 'Characterization of choline transporters in the human placenta over gestation', *Placenta*, 36(12), pp. 1362–1369. doi: 10.1016/j.placenta.2015.10.001.

Bean, L. A., Ianov, L. and Foster, T. C. (2014) 'Estrogen receptors, the hippocampus, and memory', *Neuroscientist*, 20(5), pp. 534–545. doi: 10.1177/1073858413519865/FORMAT/EPUB.

Bearer, C. F. *et al.* (2015) 'Choline Ameliorates Deficits in Balance Caused by Acute Neonatal Ethanol Exposure.', *Cerebellum (London, England)*, 14(4), pp. 413–20. doi: 10.1007/s12311-015-0691-7.

Bekdash, R. A., Zhang, C. and Sarkar, D. K. (2013) 'Gestational Choline Supplementation Normalized Fetal Alcohol-Induced Alterations in Histone Modifications, DNA Methylation, and Proopiomelanocortin (POMC) Gene Expression in β-Endorphin-Producing POMC Neurons of the Hypothalamus', *Alcoholism: Clinical and Experimental Research*, 37(7), pp. 1133–1142. doi: 10.1111/acer.12082.

Bellinger, F. P. *et al.* (1999) 'Ethanol exposure during the third trimester equivalent results in long- lasting decreased synaptic efficacy but not plasticity in the CA1 region of the rat hippocampus', *Synapse*, 31(1), pp. 51–58.

Bergdolt, L. and Dunaevsky, A. (2019) 'Brain changes in a maternal immune activation model of neurodevelopmental brain disorders', *Progress in Neurobiology*. Elsevier Ltd, pp. 1–19. doi: 10.1016/j.pneurobio.2018.12.002.

Berman, R. F. *et al.* (1996) 'Prenatal alcohol exposure and the effects of environmental enrichment on hippocampal dendritic spine density', *Alcohol*, 13(2), pp. 209–216. doi: 10.1016/0741-8329(95)02049-7.

Bestry, M. *et al.* (2022) 'Association of prenatal alcohol exposure with offspring DNA methylation in mammals: a systematic review of the evidence', *Clinical Epigenetics*, 14(1), p. 12. doi: 10.1186/s13148-022-01231-9.

Bhuiyan, M. B., Murad, F. and Fant, M. E. (2006) 'The placental cholinergic system: Localization to the cytotrophoblast and modulation of nitric oxide', *Cell Communication and Signaling*, 4(1), pp. 1–7. doi: 10.1186/1478-811X-4-4/FIGURES/5.

Bi, R. *et al.* (2001) 'Cyclic changes in estradiol regulate synaptic plasticity through the MAP kinase pathway', *Proceedings of the National Academy of Sciences of the United States of America*, 98(23), pp. 13391–13395. doi: 10.1073/PNAS.241507698/ASSET/59D49561-C12C-4710-9242-F7FF34C85E24/ASSETS/GRAPHIC/PQ2415076004.JPEG.

Bird, C. W. *et al.* (2015) 'Moderate prenatal alcohol exposure enhances GluN2B containing NMDA receptor binding and ifenprodil sensitivity in rat agranular insular cortex.', *PloS one*. Edited by V. K. Yaragudri, 10(3), p. e0118721. doi: 10.1371/journal.pone.0118721.

Bissen, D., Foss, F. and Acker-Palmer, A. (2019) 'AMPA receptors and their minions: auxiliary proteins in AMPA receptor trafficking', *Cellular and Molecular Life Sciences 2019 76:11*, 76(11), pp. 2133–2169. doi: 10.1007/S00018-019-03068-7.

Bliss, T. V. P. and Lømo, T. (1973) 'Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path', *The Journal of Physiology*, 232(2), pp. 331–356. doi: 10.1113/jphysiol.1973.sp010273.

Bodzęta, A., Scheefhals, N. and MacGillavry, H. D. (2021) 'Membrane trafficking and positioning of mGluRs at presynaptic and postsynaptic sites of excitatory synapses', *Neuropharmacology*, 200, p. 108799. doi: 10.1016/J.NEUROPHARM.2021.108799.

Bonthius, D. J. and West, J. R. (1990) 'Alcohol-Induced Neuronal Loss in Developing Rats: Increased Brain Damage with Binge Exposure', *Alcoholism: Clinical and Experimental Research*, 14(1), pp. 107–118. doi: 10.1111/j.1530-0277.1990.tb00455.x.

Brady, M. L. *et al.* (2013) 'Moderate prenatal alcohol exposure reduces plasticity and alters NMDA receptor subunit composition in the dentate gyrus.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 33(3), pp. 1062–7. doi: 10.1523/JNEUROSCI.1217-12.2013.

Brann, D. W. *et al.* (2007) 'Neurotrophic and Neuroprotective Actions of Estrogen: Basic Mechanisms and Clinical Implications', *Steroids*, 72(5), p. 381. doi: 10.1016/J.STEROIDS.2007.02.003.

Brigman, J. L. *et al.* (2010) 'Loss of GluN2B-Containing NMDA Receptors in CA1 Hippocampus and Cortex Impairs Long-Term Depression, Reduces Dendritic Spine Density, and Disrupts Learning', *The Journal of Neuroscience*, 30(13), p. 4590. doi: 10.1523/JNEUROSCI.0640-10.2010.

Brocardo, P. S. *et al.* (2012) 'Anxiety- and depression-like behaviors are accompanied by an increase in oxidative stress in a rat model of fetal alcohol spectrum disorders: Protective effects of voluntary physical exercise', *Neuropharmacology*, 62(4). doi: 10.1016/j.neuropharm.2011.10.006.

Brocardo, P. S., Gil-Mohapel, J. and Christie, B. R. (2011) 'The role of oxidative stress in fetal alcohol spectrum disorders', *Brain Research Reviews*. Brain Res Rev, pp. 209–225. doi: 10.1016/j.brainresrev.2011.02.001.

Brown, J. M. *et al.* (2019) 'A Brief History of Awareness of the Link Between Alcohol and Fetal Alcohol Spectrum Disorder', *Canadian Journal of Psychiatry*, pp. 164–168. doi: 10.1177/0706743718777403.

Bugra Baltaci, S. *et al.* (2019) 'Molecular Mechanisms of Early and Late LTP', *Neurochemical Research*, 44, pp. 281–296. doi: 10.1007/s11064-018-2695-4.

Burd, L., Blair, J. and Dropps, K. (2012) 'Prenatal alcohol exposure, blood alcohol concentrations and alcohol elimination rates for the mother, fetus and newborn', *Journal of*

Perinatology 2012 32:9, 32(9), pp. 652–659. doi: 10.1038/jp.2012.57.

Byrnes, M. L. *et al.* (2004) 'Spatial acquisition in the Morris water maze and hippocampal long-term potentiation in the adult guinea pig following brain growth spurt-prenatal ethanol exposure', *Neurotoxicology and Teratology*, 26(4), pp. 543–551. doi: 10.1016/J.NTT.2004.04.005.

Carneiro, L. M. V. *et al.* (2005) 'Behavioral and neurochemical effects on rat offspring after prenatal exposure to ethanol', *Neurotoxicology and Teratology*, 27(4), pp. 585–592. doi: 10.1016/j.ntt.2005.06.006.

Carta, M. *et al.* (2014) 'Kainate receptors in the hippocampus', *The European journal of neuroscience*, 39(11), pp. 1835–1844.

Caudill, M. A. *et al.* (2018) 'Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: A randomized, double-blind, controlled feeding study', *FASEB Journal*, 32(4), pp. 2172–2180. doi: 10.1096/fj.201700692RR.

Caudill, M. A. *et al.* (2020) 'Building better babies: should choline supplementation be recommended for pregnant and lactating mothers? Literature overview and expert panel consensus ', *European Gynecology and Obstetrics*, 2(3), pp. 149–161. Available at: https://www.egojournal.eu/wp-content/uploads/journal/2020/2.3/149/149-161.pdf (Accessed: 26 January 2021).

CDC (no date) *FASDs: Treatments*. Available at: https://www.cdc.gov/ncbddd/fasd/treatments.html (Accessed: 20 February 2023).

Cederbaum, A. I. (2012) 'Alcohol Metabolism', *Clinics in liver disease*, 16(4), p. 667. doi: 10.1016/J.CLD.2012.08.002.

Cermak, J. M. *et al.* (1998) 'Prenatal availability of choline modifies development of the hippocampal cholinergic system', *The FASEB Journal*, 12(3), pp. 349–357. doi: 10.1096/fasebj.12.3.349.

Chater, T. E. and Goda, Y. (2014) 'The role of AMPA receptors in postsynaptic mechanisms of synaptic plasticity', *Frontiers in Cellular Neuroscience*, 8(NOV), p. 401. doi: 10.3389/FNCEL.2014.00401/BIBTEX.

Chen, W. J., Berryhill, E. C. and West, J. R. (2001) 'Zinc supplementation does not attenuate alcohol-induced cerebellar Purkinje cell loss during the brain growth spurt period.', *Alcoholism, clinical and experimental research*, 25(4), pp. 600–605.

Cheng, Y. *et al.* (2018) 'Prenatal Exposure to Alcohol Induces Functional and Structural Plasticity in Dopamine D1 Receptor-Expressing Neurons of the Dorsomedial Striatum.', *Alcoholism, clinical and experimental research*. doi: 10.1111/acer.13806.

Christie, B. R. *et al.* (2005) 'Voluntary exercise rescues deficits in spatial memory and long-term potentiation in prenatal ethanol-exposed male rats', *European Journal of Neuroscience*, 21(6), pp. 1719–1726. doi: 10.1111/j.1460-9568.2005.04004.x.

Chudley, A. E. (2018) 'Diagnosis of fetal alcohol spectrum disorder: Current practices and future considerations', *Biochemistry and Cell Biology*, pp. 231–236. doi: 10.1139/bcb-2017-0106.

Clarke, R. J., Glasgow, N. G. and Johnson, J. W. (2013) ‘Mechanistic and Structural Determinants of NMDA Receptor Voltage-Dependent Gating and Slow Mg2+ Unblock’, *The Journal of Neuroscience*, 33(9), p. 4140. doi: 10.1523/JNEUROSCI.3712-12.2013.

Clelland, C. D. *et al.* (2009) ‘A functional role for adult hippocampal neurogenesis in spatial pattern separation’, *Science*, 325(5937), pp. 210–213. doi: 10.1126/science.1173215.

Cobben, J. M. *et al.* (2019) ‘DNA methylation abundantly associates with fetal alcohol spectrum disorder and its subphenotypes’, *Epigenomics*. Future Medicine Ltd., pp. 767–785. doi: 10.2217/epi-2018-0221.

Cohen, E. L. and Wurtman, R. J. (1976) ‘Brain acetylcholine: Control by dietary choline’, *Science*, 191(4227), pp. 561–562. doi: 10.1126/science.1251187.

Coles, C. D. *et al.* (2015) ‘Dose and Timing of Prenatal Alcohol Exposure and Maternal Nutritional Supplements: Developmental Effects on 6-Month-Old Infants’, *Maternal and child health journal*, 19(12), pp. 2605–2614. doi: 10.1007/S10995-015-1779-X.

Collingridge, G. L. *et al.* (2010) ‘Long-term depression in the CNS’, *Nature Reviews Neuroscience*, 11(7), pp. 459–473. doi: 10.1038/nrn2867.

Collitti-Klausnitzer, J. *et al.* (2021) ‘Preferential frequency-dependent induction of synaptic depression by the lateral perforant path and of synaptic potentiation by the medial perforant path inputs to the dentate gyrus’, *Hippocampus*, 31(9), pp. 957–981. doi: 10.1002/HIPO.23338.

Cora, M. C., Kooistra, L. and Travlos, G. (2015) ‘Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears.’, *Toxicologic pathology*, 43(6), pp. 776–93. doi: 10.1177/0192623315570339.

Cornford, E. M., Braun, L. D. and Oldendorf, W. H. (1982) ‘Developmental modulations of blood-brain barrier permeability as an indicator of changing nutritional requirements in the brain’, *Pediatric Research*, 16(4), pp. 324–328. doi: 10.1203/00006450-198204000-00017.

Costa, K.-A. *et al.* (2006) ‘Common genetic polymorphisms affect the human requirement for the nutrient choline’, *The FASEB Journal*, 20(9), pp. 1336–1344. doi: 10.1096/fj.06-5734com.

Cox, M. A. *et al.* (2019) ‘Choline acetyltransferase-expressing T cells are required to control chronic viral infection’, *Science (New York, N.Y.)*, 363(6427), pp. 639–644. doi: 10.1126/SCIENCE.AAU9072.

Craciunescu, C. N. *et al.* (2003) ‘Choline Availability During Embryonic Development Alters Progenitor Cell Mitosis in Developing Mouse Hippocampus’, in *Journal of Nutrition*. American Institute of Nutrition, pp. 3614–3618. doi: 10.1093/jn/133.11.3614.

Cullen, C. L. *et al.* (2013) ‘Low dose prenatal ethanol exposure induces anxiety-like behaviour and alters dendritic morphology in the basolateral amygdala of rat offspring.’, *PloS one*. Edited by K. Hashimoto, 8(1), p. e54924. doi: 10.1371/journal.pone.0054924.

Cullen, C. L. *et al.* (2014) ‘Low Dose Prenatal Alcohol Exposure Does Not Impair Spatial Learning and Memory in Two Tests in Adult and Aged Rats’, *PLOS ONE*, 9(6). doi: 10.1371/journal.pone.0101482.

D'Ercole, A. J. *et al.* (1996) 'The role of the insulin-like growth factors in the central nervous system', *Molecular Neurobiology*, 13(3), pp. 227–255. doi: 10.1007/BF02740625.

Deidda, G., Bozarth, I. F. and Cancedda, L. (2014) 'Modulation of GABAergic transmission in development and neurodevelopmental disorders: Investigating physiology and pathology to gain therapeutic perspectives', *Frontiers in Cellular Neuroscience*, 8(MAY), p. 119. doi: 10.3389/FNCEL.2014.00119/BIBTEX.

Delatour, L. C., Yeh, P. W. L. and Yeh, H. H. (2020) 'Prenatal Exposure to Ethanol Alters Synaptic Activity in Layer V/VI Pyramidal Neurons of the Somatosensory Cortex.', *Cerebral cortex (New York, N.Y. : 1991)*, 30(3), pp. 1735–1751. doi: 10.1093/cercor/bhz199.

Delatour, L. C., Yeh, P. W. and Yeh, H. H. (2019) 'Ethanol Exposure In Utero Disrupts Radial Migration and Pyramidal Cell Development in the Somatosensory Cortex.', *Cerebral cortex (New York, N.Y. : 1991)*, 29(5), pp. 2125–2139. doi: 10.1093/cercor/bhy094.

Denny, C. H. *et al.* (2019) 'Consumption of Alcohol Beverages and Binge Drinking Among Pregnant Women Aged 18–44 Years — United States, 2015–2017', *MMWR. Morbidity and Mortality Weekly Report*, 68(16), pp. 365–368. doi: 10.15585/mmwr.mm6816a1.

Derbyshire, E. and Obeid, R. (2020) 'Choline, neurological development and brain function: A systematic review focusing on the first 1000 days', *Nutrients*. MDPI AG, pp. 1–32. doi: 10.3390/nu12061731.

Dettmer, T. S. *et al.* (2003) 'Chronic prenatal ethanol exposure alters ionotropic glutamate receptor subunit protein levels in the adult guinea pig cerebral cortex.', *Alcoholism, clinical and experimental research*, 27(4), pp. 677–681. doi: 10.1097/01.ALC.0000060521.32215.E9.

Downing, C. *et al.* (2011) 'Subtle decreases in DNA methylation and gene expression at the mouse Igf2 locus following prenatal alcohol exposure: Effects of a methyl-supplemented diet', *Alcohol*, 45(1), pp. 65–71. doi: 10.1016/j.alcohol.2010.07.006.

Drew, P. D. *et al.* (2015) 'Pioglitazone Blocks Ethanol Induction of Microglial Activation and Immune Responses in the Hippocampus, Cerebellum, and Cerebral Cortex in a Mouse Model of Fetal Alcohol Spectrum Disorders', *Alcoholism: Clinical and Experimental Research*, 39(3), pp. 445–454. doi: 10.1111/acer.12639.

Dubey, A. and Saint-Jeannet, J. P. (2017) 'Modeling Human Craniofacial Disorders in Xenopus', *Current Pathobiology Reports*, 5(1), pp. 79–92. doi: 10.1007/S40139-017-0128-8/FIGURES/2.

Dupuy, S. T. and Houser, C. R. (1996) 'Prominent Expression of Two Forms of Glutamate Decarboxylase in the Embryonic and Early Postnatal Rat Hippocampal Formation', *The Journal of Neuroscience*, 16(21), p. 6919. doi: 10.1523/JNEUROSCI.16-21-06919.1996.

Dursun, N. *et al.* (2018) 'Sex Differences in Hippocampal Long-Term Depression and the N-Methyl-DAspartate Receptor in Rats - Positive Correlation between LTD and Glun2b Subunit', *Journal of Neurology and Neuroscience*, 9(5), pp. 0–0. doi: 10.21767/2171-6625.1000267.

Egea, J. *et al.* (2015) 'Anti-inflammatory role of microglial alpha7 nAChRs and its role in neuroprotection', *Biochemical Pharmacology*. Elsevier Inc., pp. 463–472. doi: 10.1016/j.bcp.2015.07.032.

Espinet, C. and Argilés, J. M. (1984) ‘Ethanol and acetaldehyde concentrations in the rat foeto-maternal system after an acute ethanol administration given to the mother’, *Archives of Physiology and Biochemistry*, 92(5), pp. 339–344. doi: 10.3109/13813458409080609.

Fast, D. K. and Conry, J. (2009) ‘Fetal alcohol spectrum disorders and the criminal justice system’, *Developmental disabilities research reviews*, 15(3), pp. 250–257. doi: 10.1002/DDRR.66.

Flannigan, K. *et al.* (2023) ‘Sex-related differences among individuals assessed for fetal alcohol spectrum disorder in Canada’, *Alcohol Clin Exp Res*, 47, pp. 613–623. doi: 10.1111/acer.15017.

Flannigan, K., Unsworth, K. and Harding, K. (2018) ‘The Prevalence of Fetal Alcohol Spectrum Disorder’, *Canada FASD Research Network*, July, pp. 1–4.

Fontaine, C. J. *et al.* (2016) ‘Effects of pre-natal alcohol exposure on hippocampal synaptic plasticity: Sex, age and methodological considerations’, *Neuroscience and Biobehavioral Reviews*, 64, pp. 12–34. doi: 10.1016/j.neubiorev.2016.02.014.

Fontaine, C. J. *et al.* (2019) ‘Impaired Bidirectional Synaptic Plasticity in Juvenile Offspring Following Prenatal Ethanol Exposure’, *Alcoholism: Clinical and Experimental Research*, 43(10), p. acer.14170. doi: 10.1111/acer.14170.

Fontaine, C. J. *et al.* (2020) ‘Endocannabinoid receptors contribute significantly to multiple forms of long-term depression in the rat dentate gyrus’, *Learning & Memory*, 27(9), pp. 380–389. doi: 10.1101/lm.050666.119.

Förstera, B. *et al.* (2016) ‘Potentiation of gamma aminobutyric acid receptors (GABAAR) by ethanol: How are inhibitory receptors affected?’, *Frontiers in Cellular Neuroscience*, 10(MAY), p. 114. doi: 10.3389/FNCEL.2016.00114/BIBTEX.

Fox, C. J. *et al.* (2006) ‘Contribution of NR2A and NR2B NMDA subunits to bidirectional synaptic plasticity in the hippocampus in vivo’, *Hippocampus*, 16(11), pp. 907–915. doi: 10.1002/HIPO.20230.

Freedman, R. *et al.* (2019) ‘Higher Gestational Choline Levels in Maternal Infection Are Protective for Infant Brain Development’, *Journal of Pediatrics*, 208, pp. 198-206.e2. doi: 10.1016/j.jpeds.2018.12.010.

Galofré, E. *et al.* (1987) ‘Effects of prenatal ethanol exposure on dendritic spines of layer V pyramidal neurons in the somatosensory cortex of the rat’, *Journal of the Neurological Sciences*, 81(2–3), pp. 185–195. doi: 10.1016/0022-510X(87)90095-5.

Gao, R. and Penzes, P. (2015) ‘Common Mechanisms of Excitatory and Inhibitory Imbalance in Schizophrenia and Autism Spectrum Disorders’, *Current Molecular Medicine*, 15(2), pp. 146–167. doi: 10.2174/1566524015666150303003028.

Garner, S. C., Mar, M. H. and Zeisel, S. H. (1995) ‘Choline distribution and metabolism in pregnant rats and fetuses are influenced by the choline content of the maternal diet’, *Journal of Nutrition*, 125(11), pp. 2851–2858. doi: 10.1093/JN/125.11.2851.

Garro, A. J. *et al.* (1991) ‘Ethanol Consumption Inhibits Fetal DNA Methylation in Mice: Implications for the Fetal Alcohol Syndrome’, *Alcoholism: Clinical and Experimental Research*,

15(3), pp. 395–398. doi: 10.1111/j.1530-0277.1991.tb00536.x.

Ghit, A. *et al.* (2021) 'GABAA receptors: structure, function, pharmacology, and related disorders', *Journal of Genetic Engineering and Biotechnology 2021 19:1*, 19(1), pp. 1–15. doi: 10.1186/S43141-021-00224-0.

Gil-Mohapel, J. *et al.* (2010) 'Hippocampal cell loss and neurogenesis after fetal alcohol exposure: Insights from different rodent models', *Brain Research Reviews*. Brain Res Rev, pp. 283–303. doi: 10.1016/j.brainresrev.2010.04.011.

Gimbel, B. A. *et al.* (2022) 'Long-term follow-up of a randomized controlled trial of choline for neurodevelopment in fetal alcohol spectrum disorder: corpus callosum white matter microstructure and neurocognitive outcomes', *Journal of neurodevelopmental disorders*, 14(1). doi: 10.1186/S11689-022-09470-W.

Gladding, C. M. and Raymond, L. A. (2011) 'Mechanisms underlying NMDA receptor synaptic/extrasynaptic distribution and function.', *Molecular and cellular neurosciences*, 48(4), pp. 308–320. doi: 10.1016/j.mcn.2011.05.001.

Glenn, M. J. *et al.* (2007) 'Prenatal choline availability modulates hippocampal neurogenesis and neurogenic responses to enriching experiences in adult female rats', *European Journal of Neuroscience*, 25(8), pp. 2473–2482. doi: 10.1111/j.1460-9568.2007.05505.x.

Goeke, C. M. *et al.* (2018) 'Neonatal Ethanol and Choline Treatments Alter the Morphology of Developing Rat Hippocampal Pyramidal Neurons in Opposite Directions', *Neuroscience*, 374, pp. 13–24. doi: 10.1016/J.NEUROSCIENCE.2018.01.031.

Goodlett, C. R. *et al.* (1993) 'Transient cortical astrogliosis induced by alcohol exposure during the neonatal brain growth spurt in rats.', *Brain research. Developmental brain research*, 72(1), pp. 85–97. doi: 10.1016/0165-3806(93)90162-4.

Grafe, E. L. *et al.* (2021) 'Effects of prenatal ethanol exposure on choline-induced long-term depression in the hippocampus', *Journal of Neurophysiology*. doi: 10.1152/JN.00136.2021.

Greif, K. F. *et al.* (1991) 'Postnatal Expression of Glutamate Decarboxylases in Developing Rat Cerebellum*', *Neurochemical Research*, 16(3), pp. 235–242.

Guerri, C. and Sanchis, R. (1985) 'Acetaldehyde and alcohol levels in pregnant rats and their fetuses', *Alcohol*, 2(2), pp. 267–270. doi: 10.1016/0741-8329(85)90057-6.

Guseva, M. V. *et al.* (2008) 'Dietary choline supplementation improves behavioral, histological, and neurochemical outcomes in a rat model of traumatic brain injury', *Journal of Neurotrauma*, 25(8), pp. 975–983. doi: 10.1089/neu.2008.0516.

Hamid, A. and Kaur, J. (2009) 'Role of signaling pathways in the regulation of folate transport in ethanol-fed rats', *The Journal of Nutritional Biochemistry*, 20(4), pp. 291–297. doi: 10.1016/J.JNUTBIO.2008.03.004.

Hamilton, G. F., Whitcher, L. T. and Klintsova, A. Y. (2010) 'Postnatal binge-like alcohol exposure decreases dendritic complexity while increasing the density of mature spines in mPFC Layer II/III pyramidal neurons', *Synapse*, 64(2), pp. 127–135. doi: 10.1002/syn.20711.

Harte-Hargrove, L. C. *et al.* (2015) 'Opioid Receptor-Dependent Sex Differences in Synaptic Plasticity in the Hippocampal Mossy Fiber Pathway of the Adult Rat', *The Journal of Neuroscience*, 35(4), p. 1723. doi: 10.1523/JNEUROSCI.0820-14.2015.

Heaney, C. F. and Kinney, J. W. (2016) 'Role of GABAB receptors in learning and memory and neurological disorders', *Neuroscience & Biobehavioral Reviews*, 63, pp. 1–28. doi: 10.1016/J.NEUBIOREV.2016.01.007.

Helfer, J. L., White, E. R. and Christie, B. R. (2012) 'Enhanced Deficits in Long-Term Potentiation in the Adult Dentate Gyrus with 2nd Trimester Ethanol Consumption', *PLOS ONE*. Edited by A. Dunaevsky, 7(12), p. e51344. doi: 10.1371/journal.pone.0051344.

Helfer, J. L., White, E. R. and Christie, B. R. (2014) 'Prenatal Ethanol (EtOH) Exposure Alters the Sensitivity of the Adult Dentate Gyrus to Acute EtOH Exposure', *Alcoholism: Clinical and Experimental Research*, 38(1), pp. 135–143. doi: 10.1111/acer.12227.

Henderson, G. I. *et al.* (1995) 'In Utero Ethanol Exposure Elicits Oxidative Stress in the Rat Fetus', *Alcoholism: Clinical and Experimental Research*, 19(3), pp. 714–720. doi: 10.1111/J.1530-0277.1995.TB01572.X.

Hendrickson, L. M., Guildford, M. J. and Tapper, A. R. (2013) 'Neuronal nicotinic acetylcholine receptors: Common molecular substrates of nicotine and alcohol dependence', *Frontiers in Psychiatry*, 4(APR), p. 29. doi: 10.3389/FPSYT.2013.00029/BIBTEX.

Herring, B. E. and Nicoll, R. A. (2016) 'Long-Term Potentiation: From CaMKII to AMPA Receptor Trafficking', *Annual review of physiology*, 78, pp. 351–365. doi: 10.1146/ANNUREV-PHYSIOL-021014-071753.

Howlett, H. *et al.* (2019) 'A Survey of Health Care Professionals' Knowledge and Experience of Foetal Alcohol Spectrum Disorder and Alcohol Use in Pregnancy.', *Clinical medicine insights. Reproductive health*, 13, p. 1179558119838872. doi: 10.1177/1179558119838872.

Hughes, P. D. *et al.* (1998) 'Effect of prenatal ethanol exposure on the developmental profile of the NMDA receptor subunits in rat forebrain and hippocampus', *Alcoholism: Clinical and Experimental Research*, 22(6), pp. 1255–1261.

Hughes, P. D., Wilson, W. R. and Leslie, S. W. (2001) 'Effect of gestational ethanol exposure on the NMDA receptor complex in rat forebrain: from gene transcription to cell surface.', *Brain research. Developmental brain research*, 129(2), pp. 135–145. doi: 10.1016/s0165-3806(01)00192-4.

Idänpään-Heikkilä, J. *et al.* (1972) 'Elimination and metabolic effects of ethanol in mother, fetus, and newborn infant', *American Journal of Obstetrics and Gynecology*, 112(3), pp. 387–393. doi: 10.1016/0002-9378(72)90483-8.

Ieraci, A. and Herrera, D. G. (2020) 'Early Postnatal Ethanol Exposure in Mice Induces Sex-Dependent Memory Impairment and Reduction of Hippocampal NMDA-R2B Expression in Adulthood', *Neuroscience*, 427, pp. 105–115. doi: 10.1016/J.NEUROSCIENCE.2019.11.045.

Ikonomidou, C. *et al.* (2000) 'Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome', *Science*, 287(5455), pp. 1056–1060. doi: 10.1126/science.287.5455.1056.

Imbard, A., Benoist, J. F. and Blom, H. J. (2013) 'Neural tube defects, folic acid and methylation', *International Journal of Environmental Research and Public Health*. Multidisciplinary Digital Publishing Institute (MDPI), pp. 4352–4389. doi: 10.3390/ijerph10094352.

Inazu, M. (2019) 'Functional expression of choline transporters in the blood-brain barrier', *Nutrients*, 11(10). doi: 10.3390/nu11102265.

Institute of Medicine Food and Nutrition Board (1998) *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*, *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. National Academies Press. doi: 10.17226/6015.

Iqbal, U. *et al.* (2004) 'Chronic prenatal ethanol exposure alters hippocampal GABAA receptors and impairs spatial learning in the guinea pig', *Behavioural Brain Research*, 150(1–2), pp. 117–125. doi: 10.1016/S0166-4328(03)00246-8.

Irvine, N. *et al.* (2023) 'Associations between maternal folate status and choline intake during pregnancy and neurodevelopment at 3-4 years of age in the Alberta Pregnancy Outcomes and Nutrition (APrON) study.', *Journal of developmental origins of health and disease*, pp. 1–13. doi: 10.1017/S2040174423000041.

Izumi, Y. *et al.* (2005) 'A single day of ethanol exposure during development has persistent effects on bi-directional plasticity, N-methyl-d-aspartate receptor function and ethanol sensitivity', *Neuroscience*, 136(1), pp. 269–279. doi: 10.1016/J.NEUROSCIENCE.2005.07.015.

Jacobson, S. W. *et al.* (2018) 'Efficacy of Maternal Choline Supplementation During Pregnancy in Mitigating Adverse Effects of Prenatal Alcohol Exposure on Growth and Cognitive Function: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial', *Alcoholism: Clinical and Experimental Research*, 42(7), pp. 1327–1341. doi: 10.1111/acer.13769.

Jia, J. *et al.* (2018) 'Sigma-1 Receptor-Modulated Neuroinflammation in Neurological Diseases', *Frontiers in Cellular Neuroscience*. Frontiers Media S.A. doi: 10.3389/fncel.2018.00314.

Jiang, W. *et al.* (2022) 'Impact of GAD65 and/or GAD67 deficiency on perinatal development in rats.', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 36(2), p. e22123. doi: 10.1096/fj.202101389R.

Johnsrud, E. K. *et al.* (2003) 'Human hepatic CYP2E1 expression during development', *The Journal of pharmacology and experimental therapeutics*, 307(1), pp. 402–407. doi: 10.1124/JPET.102.053124.

Jones, K. L. and Smith, D. W. (1973) 'Recognition of the fetal alcohol syndrome in early infancy.', *Lancet (London, England)*, 302(7836), pp. 999–1001. doi: 10.1016/s0140-6736(73)91092-1.

Kable, J. A. *et al.* (2015) 'The impact of micronutrient supplementation in alcohol-exposed pregnancies on information processing skills in Ukrainian infants', *Alcohol*, 49(7), pp. 647–656. doi: 10.1016/J.ALCOHOL.2015.08.005.

Kajimoto, K. *et al.* (2016) 'Prenatal alcohol exposure alters synaptic activity of adult hippocampal dentate granule cells under conditions of enriched environment', *Hippocampus*,

26(8), pp. 1078–1087. doi: 10.1002/hipo.22588.

Kalisch-Smith, J. I. *et al.* (2017) ‘Review: Sexual dimorphism in the formation, function and adaptation of the placenta’, *Placenta*, 54, pp. 10–16. doi: 10.1016/J.PLACENTA.2016.12.008.

Kekuda, R. *et al.* (1998) *Cloning and Functional Characterization of a Potential-sensitive, Polyspecific Organic Cation Transporter (OCT3) Most Abundantly Expressed in Placenta*, Journal of Biological Chemistry*. doi: 10.1074/jbc.273.26.15971.

Kelley, C. M. *et al.* (2014) ‘Maternal choline supplementation differentially alters the basal forebrain cholinergic system of young-adult Ts65Dn and disomic mice’, *Journal of Comparative Neurology*, 522(6), pp. 1390–1410. doi: 10.1002/cne.23492.

Kemp, N. *et al.* (2000) ‘Different forms of LTD in the CA1 region of the hippocampus: role of age and stimulus protocol’, *The European journal of neuroscience*, 12(1), pp. 360–366. doi: 10.1046/J.1460-9568.2000.00903.X.

Kenton, J. A. *et al.* (2020) ‘Moderate prenatal alcohol exposure alters the number and function of GABAergic interneurons in the murine orbitofrontal cortex.’, *Alcohol (Fayetteville, N.Y.)*, 88, pp. 33–41. doi: 10.1016/j.alcohol.2020.06.001.

Kenyon, S. H., Nicolaou, A. and Gibbons, W. A. (1998) ‘The Effect of Ethanol and Its Metabolites Upon Methionine Synthase Activity In Vitro’, *Alcohol*, 15(4), pp. 305–309. doi: 10.1016/S0741-8329(97)00134-1.

Kervern, M. *et al.* (2015) ‘Aberrant NMDA-dependent LTD after perinatal ethanol exposure in young adult rat hippocampus’, *Hippocampus*, 25(8), pp. 912–923. doi: 10.1002/hipo.22414.

Kiser, P. J., Cooper, N. G. F. and Mower, G. D. (1998) ‘Expression of Two Forms of Glutamic Acid Decarboxylase (GAD67 and GAD65) During Postnatal Development of Rat Somatosensory Barrel Cortex Indexing terms: GABA; barrel field; cortical development’, *J. Comp. Neurol*, 402, pp. 62–74. doi: 10.1002/(SICI)1096-9861(19981207)402:1<62::AID-CNE5>3.0.CO;2-M.

Klintsova, A. Y. *et al.* (2002) ‘Therapeutic effects of complex motor training on motor performance deficits induced by neonatal binge-like alcohol exposure in rats:: II. A quantitative stereological study of synaptic plasticity in female rat cerebellum’, *Brain Research*, 937(1), pp. 83–93. doi: https://doi.org/10.1016/S0006-8993(02)02492-7.

Kolb, B. *et al.* (2012) ‘Experience and the developing prefrontal cortex’, *Proceedings of the National Academy of Sciences of the United States of America*, 109(Suppl 2), p. 17186. doi: 10.1073/PNAS.1121251109.

Komada, M. *et al.* (2017) ‘Mechanisms underlying neuro-inflammation and neurodevelopmental toxicity in the mouse neocortex following prenatal exposure to ethanol’, *Scientific Reports*, 7(1), pp. 1–12. doi: 10.1038/s41598-017-04289-1.

Krawczyk, M. *et al.* (2016) ‘Hippocampal hyperexcitability in fetal alcohol spectrum disorder: Pathological sharp waves and excitatory/inhibitory synaptic imbalance’, *Experimental Neurology*, 280, pp. 70–79. doi: 10.1016/J.EXPNEUROL.2016.03.013.

Kumar, A. *et al.* (2011) ‘Resveratrol restores Nrf2 level and prevents ethanol-induced toxic

effects in the cerebellum of a rodent model of fetal alcohol spectrum disorders.', *Molecular pharmacology*, 80(3), pp. 446–457. doi: 10.1124/mol.111.071126.

Kwan, S. T. (Cecilia) *et al.* (2017) 'Maternal Choline Supplementation Modulates Placental Nutrient Transport and Metabolism in Late Gestation of Mouse Pregnancy', *The Journal of Nutrition*, 147(11), p. jn256107. doi: 10.3945/jn.117.256107.

Kwan, S. T. C. *et al.* (2020) 'An interaction between fetal sex and placental weight and efficiency predicts intrauterine growth in response to maternal protein insufficiency and gestational exposure window in a mouse model of FASD', *Biology of Sex Differences*, 11(1), p. 40. doi: 10.1186/s13293-020-00320-9.

De La Fuente-Ortega, E. *et al.* (2019) 'Prenatal ethanol exposure misregulates genes involved in iron homeostasis promoting a maladaptation of iron dependent hippocampal synaptic transmission and plasticity', *Frontiers in pharmacology*, 10, p. 1312. doi: 10.3389/FPHAR.2019.01312.

Lan, N. *et al.* (2009) 'Effects of prenatal ethanol exposure on hypothalamic-pituitary-adrenal function across the estrous cycle', *Alcoholism: Clinical and Experimental Research*, 33(6), pp. 1075–1088. doi: 10.1111/j.1530-0277.2009.00929.x.

Lange, S. *et al.* (2017) 'Global Prevalence of Fetal Alcohol Spectrum Disorder Among Children and Youth: A Systematic Review and Meta-analysis.', *JAMA pediatrics*, 171(10), pp. 948–956. doi: 10.1001/jamapediatrics.2017.1919.

Le, A. A. *et al.* (2022) 'Prepubescent female rodents have enhanced hippocampal LTP and learning relative to males, reversing in adulthood as inhibition increases', *Nature Neuroscience*, 25(2), pp. 180–190. doi: 10.1038/s41593-021-01001-5.

Lee, H. Y. *et al.* (2008) 'In vivo and in vitro ethanol exposure in prenatal rat brain: GABA(B) receptor modulation on dopamine D(1) receptor and protein kinase A.', *Synapse (New York, N.Y.)*, 62(7), pp. 534–543. doi: 10.1002/syn.20522.

Lee, S. M., Yeh, P. W. L. and Yeh, H. H. (2021) 'L-Type Calcium Channels Contribute to Ethanol-Induced Aberrant Tangential Migration of Primordial Cortical GABAergic Interneurons in the Embryonic Medial Prefrontal Cortex.', *eNeuro*. doi: 10.1523/ENEURO.0359-21.2021.

Leermakers, E. T. M. *et al.* (2015) 'Effects of choline on health across the life course: A systematic review', *Nutrition Reviews*, 73(8), pp. 500–522. doi: 10.1093/nutrit/nuv010.

Lewis, E. M. *et al.* (2002) 'SEXUAL MATURATION DATA FOR CRL SPRAGUE-DAWLEY RATS: CRITERIA AND CONFOUNDING FACTORS', *http://dx.doi.org/10.1081/DCT-120014794*, 25(4), pp. 437–458. doi: 10.1081/DCT-120014794.

Li, S. P. *et al.* (2005) 'Ethanol modulates the expression of GABA(B) receptor mRNAs in the prenatal rat brain in an age and area dependent manner.', *Neuroscience*, 134(3), pp. 857–866. doi: 10.1016/j.neuroscience.2005.05.001.

Li, X. *et al.* (2017) 'Generation of a whole-brain atlas for the cholinergic system and mesoscopic projectome analysis of basal forebrain cholinergic neurons', *Proceedings of the National Academy of Sciences of the United States of America*, 115(2), pp. 415–420. doi: 10.1073/PNAS.1703601115/SUPPL_FILE/PNAS.1703601115.SM03.MP4.

Li, Y. *et al.* (2021) 'Prenatal ethanol exposure impairs the formation of radial glial fibers and promotes the transformation of GFAPδ-positive radial glial cells into astrocytes', *Molecular Medicine Reports*, 23(4). doi: 10.3892/MMR.2021.11913.

Lieber, C. S. and DeCarli, L. M. (1982) 'The Feeding of Alcohol in Liquid Diets: Two Decades of Applications and 1982 Update', *Alcoholism: Clinical and Experimental Research*, 6(4), pp. 523–531. doi: 10.1111/j.1530-0277.1982.tb05017.x.

Lisman, J., Yasuda, R. and Raghavachari, S. (2012) 'Mechanisms of CaMKII action in long-term potentiation', *Nature Reviews Neuroscience*. NIH Public Access, pp. 169–182. doi: 10.1038/nrn3192.

Little, B. B. and VanBeveren, T. T. (1996) 'Placental transfer of selected substances of abuse', *Seminars in Perinatology*, 20(2), pp. 147–153. doi: 10.1016/S0146-0005(96)80082-6.

Liu, Y. *et al.* (2009) 'Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation', *Epigenetics*, 4(7), pp. 500–511. doi: 10.4161/epi.4.7.9925.

Loomes, R., Hull, L. and Mandy, W. P. L. (2017) 'What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis', *Journal of the American Academy of Child and Adolescent Psychiatry*, 56(6), pp. 466–474. doi: 10.1016/J.JAAC.2017.03.013.

Lopez-Tejero, D. *et al.* (1986) 'Effects of prenatal ethanol exposure on physical growth, sensory reflex maturation and brain development in the rat', *Neuropathology and Applied Neurobiology*, 12(3), pp. 251–260. doi: 10.1111/j.1365-2990.1986.tb00138.x.

Lovinger, D. M., White, G. and Weight, F. F. (1990) 'NMDA receptor-mediated synaptic excitation selectively inhibited by ethanol in hippocampal slice from adult rat', *Journal of Neuroscience*, 10(4), pp. 1372–1379. doi: 10.1523/jneurosci.10-04-01372.1990.

Lu, J. *et al.* (2018) 'Programming for increased expression of hippocampal GAD67 mediated the hypersensitivity of the hypothalamic-pituitary-adrenal axis in male offspring rats with prenatal ethanol exposure', *Cell Death and Disease*, 9(6).

Lussier, A. A. *et al.* (2018) 'DNA methylation as a predictor of fetal alcohol spectrum disorder', *Clinical Epigenetics*, 10(1). doi: 10.1186/s13148-018-0439-6.

Mandal, C. *et al.* (2017) 'Gestational alcohol exposure altered DNA methylation status in the developing fetus', *International Journal of Molecular Sciences*. MDPI AG. doi: 10.3390/ijms18071386.

Manning, M. A. and Eugene Hoyme, H. (2007) 'Fetal alcohol spectrum disorders: A practical clinical approach to diagnosis', *Neuroscience & Biobehavioral Reviews*, 31(2), pp. 230–238. doi: 10.1016/J.NEUBIOREV.2006.06.016.

Mantha, K., Kleiber, M. and Singh, S. (2013) 'Neurodevelopmental Timing of Ethanol Exposure May Contribute to Observed Heterogeneity of Behavioral Deficits in a Mouse Model of Fetal Alcohol Spectrum Disorder (FASD)', *Journal of Behavioral and Brain Science*, 3, pp. 85–99. doi: 10.4236/jbbs.2013.31009.

Maren, S. (1995) 'Sexually dimorphic perforant path long-term potentiation (LTP) in urethane-

anesthetized rats.', *Neuroscience letters*, 196(3), pp. 177–180. doi: 10.1016/0304-3940(95)11869-x.

Maren, S., De Oca, B. and Fanselow, M. S. (1994) 'Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning', *Brain research*, 661(1–2), pp. 25–34. doi: 10.1016/0006-8993(94)91176-2.

Marino, M. D., Aksenov, M. Y. and Kelly, S. J. (2004) 'Vitamin E protects against alcohol-induced cell loss and oxidative stress in the neonatal rat hippocampus', *INTERNATIONAL JOURNAL OF DEVELOPMENTAL NEUROSCIENCE*, 22(5–6), pp. 363–377. doi: 10.1016/j.ijdevneu.2004.04.005.

Mattson, S. N., Bernes, G. A. and Doyle, L. R. (2019) 'Fetal Alcohol Spectrum Disorders: A Review of the Neurobehavioral Deficits Associated With Prenatal Alcohol Exposure', *Alcoholism: Clinical and Experimental Research*, pp. 1046–1062. doi: 10.1111/acer.14040.

Mattson, S. N., Crocker, N. and Nguyen, T. T. (2011) 'Fetal Alcohol Spectrum Disorders: Neuropsychological and Behavioral Features', *Neuropsychology review*, 21(2), p. 81. doi: 10.1007/S11065-011-9167-9.

Maurer, S. V. and Williams, C. L. (2017) 'The cholinergic system modulates memory and hippocampal plasticity via its interactions with non-neuronal cells', *Frontiers in Immunology*. Frontiers Media S.A., p. 1489. doi: 10.3389/fimmu.2017.01489.

May, P. A. *et al.* (2009) 'Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies', *Developmental Disabilities Research Reviews*, 15(3), pp. 176–192. doi: 10.1002/ddrr.68.

May, P. A. *et al.* (2013) 'Approaching the prevalence of the full spectrum of fetal alcohol spectrum disorders in a South African population-based study.', *Alcoholism, clinical and experimental research*, 37(5), pp. 818–830. doi: 10.1111/acer.12033.

May, P. A. *et al.* (2014) 'Dietary intake, nutrition, and fetal alcohol spectrum disorders in the Western Cape Province of South Africa.', *Reproductive toxicology (Elmsford, N.Y.)*, 46, pp. 31–39. doi: 10.1016/j.reprotox.2014.02.002.

May, P. A. *et al.* (2017) 'Who is most affected by prenatal alcohol exposure: Boys or girls?', *Drug and alcohol dependence*, 177, pp. 258–267. doi: 10.1016/J.DRUGALCDEP.2017.04.010.

May, P. A. and Gossage, J. P. (2011) 'Maternal risk factors for fetal alcohol spectrum disorders: Not as simple as it might seem', *Alcohol Research and Health*, pp. 15–26.

Meck, W. H. *et al.* (2008) 'Developmental periods of choline sensitivity provide an ontogenetic mechanism for regulating memory capacity and age-related dementia', *Frontiers in Integrative Neuroscience*, 1(MAY), p. 7. doi: 10.3389/neuro.07.007.2007.

Mellott, T. J. *et al.* (2004) 'Prenatal choline supplementation advances hippocampal development and enhances MAPK and CREB activation.', *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 18(3), pp. 545–547. doi: 10.1096/fj.03-0877fje.

Mihalick, S. M. *et al.* (2001) 'Prenatal ethanol exposure, generalized learning impairment, and

medial prefrontal cortical deficits in rats.', *Neurotoxicology and teratology*, 23(5), pp. 453–462. doi: 10.1016/s0892-0362(01)00168-4.

Mikulec, A. A. *et al.* (1998) 'Halothane depresses action potential conduction in hippocampal axons', *Brain Research*, 796(1–2), pp. 231–238. doi: 10.1016/S0006-8993(98)00348-5.

Milbocker, K. A. and Klintsova, A. Y. (2020) 'Examination of cortically projecting cholinergic neurons following exercise and environmental intervention in a rodent model of fetal alcohol spectrum disorders'. doi: 10.1002/bdr2.1839.

Miller, M. W. (1995) 'Generation of Neurons in the Rat Dentate Gyrus and Hippocampus: Effects of Prenatal and Postnatal Treatment with Ethanol', *Alcoholism: Clinical and Experimental Research*, 19(6), pp. 1500–1509. doi: 10.1111/j.1530-0277.1995.tb01014.x.

Mills, J. L. *et al.* (2014) 'Maternal choline concentrations during pregnancy and choline-related genetic variants as risk factors for neural tube defects', *The American Journal of Clinical Nutrition*, 100(4), p. 1069. doi: 10.3945/AJCN.113.079319.

Monfort, P. *et al.* (2015) 'Gender Differences in Spatial Learning, Synaptic Activity, and Long-Term Potentiation in the Hippocampus in Rats: Molecular Mechanisms', *ACS Chem. Neurosci*, 6, pp. 1420–1427. doi: 10.1021/acschemneuro.5b00096.

Monfort, P. and Felipo, V. (2007) 'Hippocampal long-term potentiation is reduced in mature compared to young male rats but not in female rats', *Neuroscience*, 146(2), pp. 504–508. doi: 10.1016/J.NEUROSCIENCE.2007.02.008.

Monk, B. R., Leslie, F. M. and Thomas, J. D. (2012) 'The effects of perinatal choline supplementation on hippocampal cholinergic development in rats exposed to alcohol during the brain growth spurt', *Hippocampus*, 22(8), pp. 1750–1757. doi: 10.1002/hipo.22009.

Montgomery, K. S. *et al.* (2018) 'Effects of ethanol and varenicline on female Sprague-Dawley rats in a third trimester model of fetal alcohol syndrome.', *Alcohol (Fayetteville, N.Y.)*, 71, pp. 75–87. doi: 10.1016/j.alcohol.2018.02.006.

Monyer, H. *et al.* (1994) 'Developmental and regional expression in the rat brain and functional properties of four NMDA receptors.', *Neuron*, 12(3), pp. 529–540. doi: 10.1016/0896-6273(94)90210-0.

Nanson, J. L. and Hiscock, M. (1990) 'Attention Deficits in Children Exposed to Alcohol Prenatally', *Alcoholism: Clinical and Experimental Research*, 14(5), pp. 656–661. doi: 10.1111/J.1530-0277.1990.TB01223.X/FORMAT/PDF.

Nguyen, T. T. *et al.* (2016) 'Randomized, double-blind, placebo-controlled clinical trial of choline supplementation in school-aged children with fetal alcohol spectrum disorders 1', *Am J Clin Nutr*, 104(6), pp. 1683–92. doi: 10.3945/ajcn.116.142075.

Nicoll, R. A. and Roche, K. W. (2013) 'Long-term potentiation: Peeling the onion', *Neuropharmacology*, 74, pp. 18–22. doi: 10.1016/J.NEUROPHARM.2013.02.010.

Nirgudkar, P. *et al.* (2016) 'Ethanol exposure during development reduces GABAergic/glycinergic neuron numbers and lobule volumes in the mouse cerebellar vermis.', *Neuroscience letters*, 632, pp. 86–91. doi: 10.1016/j.neulet.2016.08.039.

Nixon, K. *et al.* (2002) 'NMDA receptor subunit expression following early postnatal exposure to ethanol.', *Brain research. Developmental brain research*, 139(2), pp. 295–299. doi: 10.1016/s0165-3806(02)00515-1.

Nixon, K. *et al.* (2004) 'NMDA Receptor Subunit Expression after Combined Prenatal and Postnatal Exposure to Ethanol', *Alcoholism: Clinical and Experimental Research*, 28(1), pp. 105–112. doi: 10.1097/01.ALC.0000106311.88523.7B.

O'Leary-Moore, S. K. *et al.* (2006) 'Reversal learning after prenatal or early postnatal alcohol exposure in juvenile and adult rats', *Alcohol (Fayetteville, N.Y.)*, 38(2), pp. 99–110. doi: 10.1016/J.ALCOHOL.2006.05.005.

Obeid, R. (2013) 'The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway', *Nutrients*. MDPI AG, pp. 3481–3495. doi: 10.3390/nu5093481.

Olney, J. W. *et al.* (2000) 'Ethanol-induced apoptotic neurodegeneration in the developing brain.', *Apoptosis : an international journal on programmed cell death*, 5(6), pp. 515–521. doi: 10.1023/a:1009685428847.

Olney, J. W. *et al.* (2002) 'The enigma of fetal alcohol neurotoxicity', *Annals of Medicine*. Taylor & Francis, pp. 109–119. doi: 10.1080/07853890252953509.

Ostrea, E. M. *et al.* (2006) 'Fatty Acid Ethyl Esters in Meconium: Are They Biomarkers of Fetal Alcohol Exposure and Effect?', *Alcoholism, clinical and experimental research*, 30(7), p. 1152. doi: 10.1111/J.1530-0277.2006.00131.X.

Otero, N. K. H. *et al.* (2012) 'Choline Supplementation and DNA Methylation in the Hippocampus and Prefrontal Cortex of Rats Exposed to Alcohol During Development', *Alcoholism: Clinical and Experimental Research*, 36(10), pp. 1701–1709. doi: 10.1111/j.1530-0277.2012.01784.x.

Oubraim, S. *et al.* (2022) 'Prenatal ethanol exposure causes anxiety-like phenotype and alters synaptic nitric oxide and endocannabinoid signaling in dorsal raphe nucleus of adult male rats', *Translational Psychiatry 2022 12:1*, 12(1), pp. 1–12. doi: 10.1038/s41398-022-02210-7.

Oz, M. *et al.* (2005) 'Additive Effects of Endogenous Cannabinoid Anandamide and Ethanol on α7-Nicotinic Acetylcholine Receptor-Mediated Responses in Xenopus Oocytes', *Journal of Pharmacology and Experimental Therapeutics*, 313(3), pp. 1272–1280. doi: 10.1124/JPET.104.081315.

Ozarda Ilcol, Y., Uncu, G. and Ulus, I. H. (2002) 'Free and phospholipid-bound choline concentrations in serum during pregnancy, after delivery and in newborns', *Archives of Physiology and Biochemistry*, 110(5), pp. 393–399. doi: 10.1076/apab.110.5.393.11832.

Paoletti, P., Bellone, C. and Zhou, Q. (2013) 'NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease', *Nature Reviews Neuroscience*, 14(6), pp. 383–400. doi: 10.1038/nrn3504.

Parada, E. *et al.* (2013) 'The microglial α7-acetylcholine nicotinic receptor is a key element in promoting neuroprotection by inducing heme oxygenase-1 via nuclear factor erythroid-2-related factor 2', *Antioxidants and Redox Signaling*, 19(11), pp. 1135–1148. doi: 10.1089/ars.2012.4671.

Park, P. *et al.* (2018) ‘The Role of Calcium-Permeable AMPARs in Long-Term Potentiation at Principal Neurons in the Rodent Hippocampus’, *Frontiers in Synaptic Neuroscience*, 10, p. 42. doi: 10.3389/FNSYN.2018.00042/BIBTEX.

Patten, Anna R., Gil-Mohapel, J., *et al.* (2013) ‘Effects of Ethanol Exposure during Distinct Periods of Brain Development on Hippocampal Synaptic Plasticity’, *Brain sciences*, 3(3), pp. 1076–1094. doi: 10.3390/BRAINSCI3031076.

Patten, Anna R., Brocardo, P. S., *et al.* (2013) ‘Impairments in hippocampal synaptic plasticity following prenatal ethanol exposure are dependent on glutathione levels’, *Hippocampus*. 2013/09/03, 23(12), pp. 1463–1475. doi: 10.1002/HIPO.22199.

Patten, A R *et al.* (2013) ‘Impairments in hippocampal synaptic plasticity following prenatal ethanol exposure are dependent on glutathione levels’, *Hippocampus*. 2013/09/03. doi: 10.1002/hipo.22199.

Patten, Anna R., Sickmann, H. M., *et al.* (2013) ‘Omega-3 fatty acids can reverse the long-term deficits in hippocampal synaptic plasticity caused by prenatal ethanol exposure’, *Neuroscience Letters*, 551, pp. 7–11. doi: 10.1016/J.NEULET.2013.05.051.

Patten, A. R., Brocardo, P. S. and Christie, B. R. (2013) ‘Omega-3 supplementation can restore glutathione levels and prevent oxidative damage caused by prenatal ethanol exposure’, *Journal of Nutritional Biochemistry*, 24(5), pp. 760–769. doi: 10.1016/j.jnutbio.2012.04.003.

Patten, A. R., Fontaine, C. J. and Christie, B. R. (2014) ‘A Comparison of the Different Animal Models of Fetal Alcohol Spectrum Disorders and Their Use in Studying Complex Behaviors’, *Frontiers in Pediatrics*, 2, p. 93. doi: 10.3389/fped.2014.00093.

Pavlov, V. A. and Tracey, K. J. (2005) ‘The cholinergic anti-inflammatory pathway’, *Brain, Behavior, and Immunity*. Academic Press Inc., pp. 493–499. doi: 10.1016/j.bbi.2005.03.015.

Pei, J. *et al.* (2011) ‘Mental health issues in fetal alcohol spectrum disorder’, *Journal of Mental Health*, 20(5), pp. 473–483. doi: 10.3109/09638237.2011.577113.

Peñasco, S. *et al.* (2019) ‘Endocannabinoid long-term depression revealed at medial perforant path excitatory synapses in the dentate gyrus’, *Neuropharmacology*, 153, pp. 32–40. doi: 10.1016/j.neuropharm.2019.04.020.

Pentney, R. J., Cotter, J. R. and Abel, E. L. (1984) ‘Quantitative measures of mature neuronal morphology after in utero ethanol exposure’, *Neurobehavioral toxicology and teratology*, 6(1).

Perea, G., Navarrete, M. and Araque, A. (2009) ‘Tripartite synapses: astrocytes process and control synaptic information’, *Trends in Neurosciences*. Elsevier Current Trends, pp. 421–431. doi: 10.1016/j.tins.2009.05.001.

Perkins, A. E., Fadel, J. R. and Kelly, S. J. (2015) ‘The effects of postnatal alcohol exposure and galantamine on the context pre-exposure facilitation effect and acetylcholine efflux using invivo microdialysis’, *Alcohol*, 49(3), pp. 193–205. Available at: /pmc/articles/PMC4414821/?report=abstract (Accessed: 15 September 2020).

Petersen, R. P. *et al.* (2013) ‘Electrophysiological identification of medial and lateral perforant path inputs to the dentate gyrus’, *Neuroscience*, 252. doi: 10.1016/j.neuroscience.2013.07.063.

Petrenko, C. L. M. *et al.* (2014) ‘Prevention of secondary conditions in fetal alcohol spectrum disorders: Identification of systems-level barriers’, *Maternal and Child Health Journal*, 18(6), pp. 1496–1505. doi: 10.1007/s10995-013-1390-y.

Picciotto, M. R., Higley, M. J. and Mineur, Y. S. (2012) ‘Acetylcholine as a Neuromodulator: Cholinergic Signaling Shapes Nervous System Function and Behavior’, *Neuron*. NIH Public Access, pp. 116–129. doi: 10.1016/j.neuron.2012.08.036.

Pikkarainen, P. H. (1971) ‘Metabolism of ethanol and acetaldehyde in perfused human fetal liver’, *Life Sciences*, 10(23 PART 2), pp. 1359–1364. doi: 10.1016/0024-3205(71)90187-1.

Pikkarainen, P. H. and RäIhä, N. C. R. (1967) ‘Development of alcohol dehydrogenase activity in the human liver’, *Pediatric Research*, 1(3), pp. 165–168. doi: 10.1203/00006450-196705000-00001.

Popova, S. *et al.* (2013) ‘Cost of Fetal Alcohol Spectrum Disorder Diagnosis in Canada’, *PLoS ONE*, 8(4). doi: 10.1371/journal.pone.0060434.

Popova, S. *et al.* (2017) ‘Prevalence of alcohol consumption during pregnancy and Fetal Alcohol Spectrum Disorders among the general and Aboriginal populations in Canada and the United States’, *European Journal of Medical Genetics*, 60(1), pp. 32–48. doi: 10.1016/J.EJMG.2016.09.010.

Popova, S. *et al.* (2019) ‘Population-based prevalence of fetal alcohol spectrum disorder in Canada.’, *BMC public health*, 19(1), p. 845. doi: 10.1186/s12889-019-7213-3.

Portales-Casamar, E. *et al.* (2016) ‘DNA methylation signature of human fetal alcohol spectrum disorder’, *Epigenetics and Chromatin*, 9(1). doi: 10.1186/s13072-016-0074-4.

Prendergast, B. J., Onishi, K. G. and Zucker, I. (2014) ‘Female mice liberated for inclusion in neuroscience and biomedical research’, *Neuroscience & Biobehavioral Reviews*, 40, pp. 1–5. doi: https://doi.org/10.1016/j.neubiorev.2014.01.001.

Puglia, M. P. and Valenzuela, C. F. (2010) ‘Repeated third trimester-equivalent ethanol exposure inhibits long-term potentiation in the hippocampal CA1 region of neonatal rats’, *Alcohol*, 44(3), pp. 283–290. doi: 10.1016/J.ALCOHOL.2010.03.001.

Purves, D. *et al.* (2001) ‘Acetylcholine’, in *Neuroscience*. 2nd editio. Sunderland (MA): Sinauer Associates. Available at: https://www.ncbi.nlm.nih.gov/books/NBK11143/ (Accessed: 17 February 2023).

Pyapali, G. K. *et al.* (1998) ‘Prenatal dietary choline supplementation decreases the threshold for induction of long-term potentiation in young adult rats’, *Journal of Neurophysiology*, 79(4), pp. 1790–1796. doi: 10.1152/jn.1998.79.4.1790.

Räihä, N. C., Koskinen, M. and Pikkarainen, P. (1967) ‘Developmental changes in alcohol-dehydrogenase activity in rat and guinea-pig liver.’, *The Biochemical journal*, 103(3), pp. 623–626. doi: 10.1042/bj1030623.

Ramachandran, V. *et al.* (2001) ‘In utero ethanol exposure causes mitochondrial dysfunction, which can result in apoptotic cell death in fetal brain: a potential role for 4-hydroxynonenal.’, *Alcoholism, clinical and experimental research*, 25(6), pp. 862–871.

Rawat, A. K. (1977) ‘DEVELOPMENTAL CHANGES IN THE BRAIN LEVELS OF NEUROTRANSMITTERS AS INFLUENCED BY MATERNAL ETHANOL CONSUMPTION IN THE RAT’, *Journal of Neurochemistry*, 28(6), pp. 1175–1182. doi: 10.1111/j.1471-4159.1977.tb12306.x.

Reid, H. M. O. *et al.* (2020) ‘A Systematic Review of the Effects of Perinatal Alcohol Exposure and Perinatal Marijuana Exposure on Adult Neurogenesis in the Dentate Gyrus’, *Alcoholism: Clinical and Experimental Research*, 44(6), pp. 1164–1174. doi: 10.1111/acer.14332.

Réu, P. *et al.* (2017) ‘The Lifespan and Turnover of Microglia in the Human Brain’, *Cell Reports*, 20(4), pp. 779–784. doi: 10.1016/j.celrep.2017.07.004.

Reyes, E. *et al.* (1983) ‘Effects of maternal administration of alcohol on fetal brain development’, *Neurobehavioral Toxicology and Teratology*, 5(2), pp. 263–267. doi: 10.1016/0091-3057(81)90153-2.

Rice, D. and Barone, S. (2000) ‘Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models.’, *Environmental Health Perspectives*, 108(suppl 3), pp. 511–533. doi: 10.1289/ehp.00108s3511.

Rice, J. P. *et al.* (2012) ‘Effects of exposure to moderate levels of ethanol during prenatal brain development on dendritic length, branching, and spine density in the nucleus accumbens and dorsal striatum of adult rats’, *Alcohol*, 46(6), pp. 577–584. doi: 10.1016/j.alcohol.2011.11.008.

Richardson, D. P. *et al.* (2002) ‘Impaired acquisition in the water maze and hippocampal long-term potentiation after chronic prenatal ethanol exposure in the guinea-pig’, *European Journal of Neuroscience*, 16(8), pp. 1593–1598. doi: 10.1046/J.1460-9568.2002.02214.X.

Rivera, C. *et al.* (1999) ‘The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation’, *Nature*, 397(6716), pp. 251–255. doi: 10.1038/16697.

Rüedi-Bettschen, D. and Platt, D. M. (2017) ‘Detrimental effects of self-administered methamphetamine during pregnancy on offspring development in the rat’. doi: 10.1016/j.drugalcdep.2017.03.042.

Ruhela, R. K. *et al.* (2019) ‘Negative geotaxis: An early age behavioral hallmark to VPA rat model of autism’, *ANNALS OF NEUROSCIENCES VOLUME*, 26. doi: 10.5214/ans.0972.7531.260106.

Ryan, S. H., Williams, J. K. and Thomas, J. D. (2008) ‘Choline supplementation attenuates learning deficits associated with neonatal alcohol exposure in the rat: effects of varying the timing of choline administration.’, *Brain research*, 1237, pp. 91–100. doi: 10.1016/j.brainres.2008.08.048.

Safari, S. *et al.* (2021) ‘Sex differences in spatial learning and memory and hippocampal long-term potentiation at perforant pathway-dentate gyrus (PP-DG) synapses in Wistar rats’, *Behavioral and Brain Functions*, 17(1), pp. 1–11. doi: 10.1186/S12993-021-00184-Y/FIGURES/7.

Sanchez-Lopez, E. *et al.* (2019) ‘Choline Uptake and Metabolism Modulate Macrophage IL-1β and IL-18 Production’, *Cell Metabolism*, 29(6), pp. 1350-1362.e7. doi: 10.1016/j.cmet.2019.03.011.

Scharfman, H. E. (2016) ‘The enigmatic mossy cell of the dentate gyrus’, *Nature Reviews Neuroscience 2016 17:9*, 17(9), pp. 562–575. doi: 10.1038/nrn.2016.87.

Scheefhals, N. and MacGillavry, H. D. (2018) ‘Functional organization of postsynaptic glutamate receptors’, *Molecular and Cellular Neuroscience*, 91, pp. 82–94. doi: 10.1016/J.MCN.2018.05.002.

Schneider, M. L., Moore, C. F. and Adkins, M. M. (2011) ‘The Effects of Prenatal Alcohol Exposure on Behavior: Rodent and Primate Studies’, *Neuropsychology review*, 21(2), p. 186. doi: 10.1007/S11065-011-9168-8.

Schneider, R. D. and Thomas, J. D. (2016) ‘Adolescent Choline Supplementation Attenuates Working Memory Deficits in Rats Exposed to Alcohol During the Third Trimester Equivalent’, *Alcoholism: Clinical and Experimental Research*, 40(4), pp. 897–905. doi: 10.1111/acer.13021.

Shabtai, Y. *et al.* (2018) ‘Acetaldehyde inhibits retinoic acid biosynthesis to mediate alcohol teratogenicity’, *Scientific Reports 2017 8:1*, 8(1), pp. 1–14. doi: 10.1038/s41598-017-18719-7.

Sharp, G. C. *et al.* (2018) ‘Maternal alcohol consumption and offspring DNA methylation: Findings from six general population-based birth cohorts’, *Epigenomics*, 10(1), pp. 27–42. doi: 10.2217/epi-2017-0095.

Shaw, G. M. *et al.* (2009) ‘Choline and Risk of Neural Tube Defects in a Folate-fortified Population’, *Epidemiology*, 20(5), pp. 714–719. doi: 10.1097/EDE.0b013e3181ac9fe7.

Shen, J. X. and Yakel, J. L. (2012) ‘Functional α7 nicotinic ACh receptors on astrocytes in rat hippocampal CA1 slices’, *Journal of Molecular Neuroscience*, 48(1), pp. 14–21. doi: 10.1007/s12031-012-9719-3.

Sheppard, P. A. S., Choleris, E. and Galea, L. A. M. (2019) ‘Structural plasticity of the hippocampus in response to estrogens in female rodents’, *Molecular Brain*. BioMed Central Ltd., pp. 1–17. doi: 10.1186/s13041-019-0442-7.

Shirpoor, A. *et al.* (2009) ‘Vitamin E protects developing rat hippocampus and cerebellum against ethanol-induced oxidative stress and apoptosis’, *Food Chemistry*, 113(1), pp. 115–120. doi: 10.1016/J.FOODCHEM.2008.07.034.

Sickmann, H. M. *et al.* (2014) ‘Prenatal ethanol exposure has sex-specific effects on hippocampal long-term potentiation’, *Hippocampus*, 24(1), pp. 54–64. doi: 10.1002/HIPO.22203.

Silvestre de Ferron, B. *et al.* (2017) ‘Increase of KCC2 in hippocampal synaptic plasticity disturbances after perinatal ethanol exposure’, *Addiction Biology*, 22(6), pp. 1870–1882. doi: 10.1111/adb.12465.

Skagerström, J., Chang, G. and Nilsen, P. (2011) ‘Predictors of drinking during pregnancy: A systematic review’, *Journal of Women’s Health*. Mary Ann Liebert, Inc., pp. 901–913. doi: 10.1089/jwh.2010.2216.

Skorput, A. G. J. *et al.* (2015) ‘Persistent Interneuronopathy in the Prefrontal Cortex of Young Adult Offspring Exposed to Ethanol In Utero.’, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 35(31), pp. 10977–10988. doi:

10.1523/JNEUROSCI.1462-15.2015.

Skorput, A. G. J. and Yeh, H. H. (2015) ‘Effects of ethanol exposure in utero on Cajal-Retzius cells in the developing cortex.’, *Alcoholism, clinical and experimental research*, 39(5), pp. 853–862. doi: 10.1111/acer.12696.

Skorput, A. G. J. and Yeh, H. H. (2016) ‘Chronic Gestational Exposure to Ethanol Leads to Enduring Aberrances in Cortical Form and Function in the Medial Prefrontal Cortex.’, *Alcoholism, clinical and experimental research*, 40(7), pp. 1479–1488. doi: 10.1111/acer.13107.

Sliwowska, J. H. *et al.* (2010) ‘Stress-induced suppression of hippocampal neurogenesis in adult male rats is altered by prenatal ethanol exposure’, *Stress*, 13(4), pp. 302–314. doi: 10.3109/10253890903531582.

Smiley, J. F. *et al.* (2021) ‘Neonatal ethanol causes profound reduction of cholinergic cell number in the basal forebrain of adult animals.’, *Alcohol (Fayetteville, N.Y.)*, 97, pp. 1–11. doi: 10.1016/j.alcohol.2021.08.005.

Sofroniew, M. V. and Vinters, H. V. (2010) ‘Astrocytes: biology and pathology’, *Acta Neuropathologica*, 119(1), p. 7. doi: 10.1007/S00401-009-0619-8.

Spampinato, S. F. *et al.* (2018) ‘Metabotropic Glutamate Receptors in Glial Cells: A New Potential Target for Neuroprotection?’, *Frontiers in Molecular Neuroscience*, 11. doi: 10.3389/fnmol.2018.00414.

Stacho, M. and Manahan-Vaughan, D. (2022) ‘The Intriguing Contribution of Hippocampal Long-Term Depression to Spatial Learning and Long-Term Memory’, *Frontiers in Behavioral Neuroscience*, 16. doi: 10.3389/fnbeh.2022.806356.

Staples, M. C., Porch, M. W. and Savage, D. D. (2014) ‘Impact of combined prenatal ethanol and prenatal stress exposures on markers of activity-dependent synaptic plasticity in rat dentate gyrus’, *Alcohol*, 48(6), pp. 523–532. doi: 10.1016/J.ALCOHOL.2014.06.006.

Stiles, J. and Jernigan, T. L. (2010) ‘The Basics of Brain Development’, *Neuropsychology Review*, 20(4), p. 327. doi: 10.1007/S11065-010-9148-4.

Stork, O. *et al.* (2000) ‘Postnatal development of a GABA deficit and disturbance of neural functions in mice lacking GAD65’, *Brain Research*, 865(1), pp. 45–58. doi: 10.1016/S0006-8993(00)02206-X.

Stubblefield, E. A. and Benke, T. A. (2010) ‘Distinct AMPA-type glutamatergic synapses in developing rat CA1 hippocampus.’, *Journal of neurophysiology*, 104(4), pp. 1899–1912. doi: 10.1152/jn.00099.2010.

Subbanna, S. and Basavarajappa, B. S. (2022) ‘Binge-like Prenatal Ethanol Exposure Causes Impaired Cellular Differentiation in the Embryonic Forebrain and Synaptic and Behavioral Defects in Adult Mice’, *Brain sciences*, 12(6). doi: 10.3390/BRAINSCI12060793.

Sulik, K. K. (2005) ‘Genesis of alcohol-induced craniofacial dysmorphism’, in *Experimental Biology and Medicine*, pp. 366–375. doi: 10.1177/15353702-0323006-04.

Sutherland, R. J., McDonald, R. J. and Savage, D. D. (1997) ‘Prenatal exposure to moderate

levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring', *Hippocampus*, 7(2), pp. 232–238. doi: 10.1002/(SICI)1098-1063(1997)7:2<232::AID-HIPO9>3.0.CO;2-O.

Suzdak, P. D. *et al.* (1986) *Ethanol stimulates y-aminobutyric acid receptor-mediated chloride transport in rat brain synaptoneurosomes (isolated brain vesicles/y-aminobutyric acid receptor complex/chloride uptake/barbiturates)*, *Neurobiology*.

Suzuki, T. *et al.* (2006) 'Microglial α7 nicotinic acetylcholine receptors drive a phospholipase C/IP3 pathway and modulate the cell activation toward a neuroprotective role', *Journal of Neuroscience Research*, 83(8), pp. 1461–1470. doi: 10.1002/jnr.20850.

Swanson, D. J. *et al.* (1995) 'Chronic Prenatal Ethanol Exposure Alters the Normal Ontogeny of Choline Acetyltransferase Activity in the Rat Septohippocampal System', *Alcoholism: Clinical and Experimental Research*, 19(5), pp. 1252–1260. doi: 10.1111/j.1530-0277.1995.tb01608.x.

Swanson, D. J. *et al.* (1996) 'Influence of chronic prenatal ethanol on cholinergic neurons of the septohippocampal system', *The Journal of Comparative Neurology*, 364(1), pp. 104–112. doi: 10.1002/(SICI)1096-9861(19960101)364:1<104::AID-CNE9>3.0.CO;2-9.

Tabassum, S. *et al.* (2017) 'Chronic choline supplementation improves cognitive and motor performance via modulating oxidative and neurochemical status in rats', *Pharmacology, biochemistry, and behavior*, 159, pp. 90–99. doi: 10.1016/J.PBB.2017.05.011.

Tanapat, P. *et al.* (1999) 'Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19(14), pp. 5792–801. doi: 10.1523/JNEUROSCI.19-14-05792.1999.

Tatebayashi, H., Motomura, H. and Narahashi, T. (1998) 'Alcohol modulation of single GABA(A) receptor-channel kinetics', *Neuroreport*, 9(8), pp. 1769–1775. doi: 10.1097/00001756-199806010-00018.

Tay, T. L. *et al.* (2017) 'Microglia across the lifespan: from origin to function in brain development, plasticity and cognition', *The Journal of Physiology*, 595, pp. 1929–1945. doi: 10.1113/JP272134.

Tenenbaum, A. *et al.* (2020) 'Fetal alcohol spectrum disorder among pre-adopted and foster children', *BMC Pediatrics*, 20(1), pp. 1–6. doi: 10.1186/S12887-020-02164-Z/TABLES/2.

Terasaki, L. S. and Schwarz, J. M. (2016) 'Effects of Moderate Prenatal Alcohol Exposure during Early Gestation in Rats on Inflammation across the Maternal-Fetal-Immune Interface and Later-Life Immune Function in the Offspring', *Journal of Neuroimmune Pharmacology*, 11(4), pp. 680–692. doi: 10.1007/s11481-016-9691-8.

Thal, L. J. *et al.* (1992) 'Development of the basal forebrain cholinergic system: Phenotype expression prior to target innervation', *Neurobiology of Aging*, 13(1), pp. 67–72. doi: 10.1016/0197-4580(92)90011-L.

Thanh, N. X. *et al.* (2014) 'Incidence and prevalence of fetal alcohol spectrum disorder by sex and age group in Alberta, Canada', *Journal of Population Therapeutics and Clinical Pharmacology*, 21(3), pp. e395–e404.

Thanh, N. X. and Jonsson, E. (2016) 'LIFE EXPECTANCY OF PEOPLE WITH FETAL ALCOHOL SYNDROME', *Journal of Population Therapeutics and Clinical Pharmacology*, 23(1). Available at: https://jptcp.com/index.php/jptcp/article/view/240 (Accessed: 22 March 2023).

Thomas, J. D. *et al.* (2000) 'Neonatal choline supplementation ameliorates the effects of prenatal alcohol exposure on a discrimination learning task in rats', *Neurotoxicology and Teratology*, 22(5), pp. 703–711. doi: 10.1016/S0892-0362(00)00097-0.

Thomas, J. D. *et al.* (2007) 'Choline supplementation following third-trimester-equivalent alcohol exposure attenuates behavioral alterations in rats', *Behavioral Neuroscience*, 121(1), pp. 120–130. doi: 10.1037/0735-7044.121.1.120.

Thomas, J. D. *et al.* (2010) 'Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats.', *Birth defects research. Part A, Clinical and molecular teratology*, 88(10), pp. 827–37. doi: 10.1002/bdra.20713.

Titterness, A. K. and Christie, B. R. (2008) 'Long-term depression in vivo: Effects of sex, stress, diet, and prenatal ethanol exposure', *Hippocampus*, 18(5), pp. 481–491. doi: 10.1002/HIPO.20407.

Titterness, A. K. and Christie, B. R. (2012) 'Prenatal ethanol exposure enhances NMDAR-dependent long-term potentiation in the adolescent female dentate gyrus', *Hippocampus*, 22(1), pp. 69–81. doi: 10.1002/hipo.20849.

Topper, L. A., Baculis, B. C. and Valenzuela, C. F. (2015) 'Exposure of neonatal rats to alcohol has differential effects on neuroinflammation and neuronal survival in the cerebellum and hippocampus', *Journal of Neuroinflammation*, 12(1), p. 160. doi: 10.1186/s12974-015-0382-9.

Toso, L. *et al.* (2006) 'Prenatal alcohol exposure alters GABA(A)alpha(5) expression: A mechanism of alcohol-induced learning dysfunction', *AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY*, 195(2), pp. 522–527. doi: 10.1016/j.ajog.2006.01.098.

Tran, T. D. and Kelly, S. J. (2003) 'Critical periods for ethanol-induced cell loss in the hippocampal formation', *Neurotoxicology and Teratology*, 25(5), pp. 519–528. doi: 10.1016/S0892-0362(03)00074-6.

Tranmer, J. E. (1985) 'Disposition of Ethanol in Maternal Venous Blood and the Amniotic Fluid', *Journal of Obstetric, Gynecologic, & Neonatal Nursing*, 14(6), pp. 484–490. doi: 10.1111/J.1552-6909.1985.TB02100.X.

Uban, K. A. *et al.* (2010) 'Prenatal alcohol exposure reduces the proportion of newly produced neurons and glia in the dentate gyrus of the hippocampus in female rats', *Hormones and Behavior*, 58(5), pp. 835–843. doi: 10.1016/j.yhbeh.2010.08.007.

Vaglenova, J. *et al.* (2008) 'Aniracetam reversed learning and memory deficits following prenatal ethanol exposure by modulating functions of synaptic AMPA receptors', *NEUROPSYCHOPHARMACOLOGY*, 33(5), pp. 1071–1083. doi: 10.1038/sj.npp.1301496.

Valeeva, G., Valiullina, F. and Khazipov, R. (2013) 'Excitatory actions of GABA in the intact neonatal rodent hippocampus in vitro', *Frontiers in Cellular Neuroscience*, (FEB). doi: 10.3389/fncel.2013.00020.

Vallés, S. *et al.* (1997) 'Ethanol exposure affects glial fibrillary acidic protein gene expression and transcription during rat brain development.', *Journal of neurochemistry*, 69(6), pp. 2484–2493. doi: 10.1046/j.1471-4159.1997.69062484.x.

Varaschin, R. K. *et al.* (2010) 'Effects of the cognition-enhancing agent ABT-239 on fetal ethanol-induced deficits in dentate gyrus synaptic plasticity', *Journal of Pharmacology and Experimental Therapeutics*, 334(1), pp. 191–198. doi: 10.1124/JPET.109.165027.

Velazquez, R. *et al.* (2019) 'Lifelong choline supplementation ameliorates Alzheimer's disease pathology and associated cognitive deficits by attenuating microglia activation', *Aging Cell*, 18(6). doi: 10.1111/acel.13037.

Vennemann, F. B. C. *et al.* (2015) 'Dietary intake and food sources of choline in European populations', *British Journal of Nutrition*, 114(12), pp. 2046–2055. doi: 10.1017/S0007114515003700.

Vissel, B. *et al.* (2001) 'The role of RNA editing of kainate receptors in synaptic plasticity and seizures', *Neuron*, 29(1), pp. 217–227. doi: 10.1016/S0896-6273(01)00192-1.

Vizi, E. S., Kisfali, M. and Lorincz, T. (2013) 'Role of nonsynaptic GluN2B-containing NMDA receptors in excitotoxicity: Evidence that fluoxetine selectively inhibits these receptors and may have neuroprotective effects', *Brain Research Bulletin*, 93, pp. 32–38. doi: 10.1016/J.BRAINRESBULL.2012.10.005.

Waddell, J. and Mooney, S. (2017) 'Choline and Working Memory Training Improve Cognitive Deficits Caused by Prenatal Exposure to Ethanol', *Nutrients*, 9(10), p. 1080. doi: 10.3390/nu9101080.

Walker, M. J. *et al.* (2011) 'The epidemiology of alcohol utilization during pregnancy: an analysis of the Canadian Maternity Experiences Survey (MES)', *BMC pregnancy and childbirth*, 11. doi: 10.1186/1471-2393-11-52.

Wallace, T. C. *et al.* (2018) 'Choline', *Nutrition Today*, 53(6), pp. 240–253. doi: 10.1097/NT.0000000000000302.

Wallace, T. C. *et al.* (2020) 'Choline: The Neurocognitive Essential Nutrient of Interest to Obstetricians and Gynecologists', *Journal of Dietary Supplements*, 17(6), pp. 733–752. doi: 10.1080/19390211.2019.1639875.

Wallace, T. C. and Fulgoni, V. L. (2017) 'Usual choline intakes are associated with egg and protein food consumption in the United States', *Nutrients*, 9(8). doi: 10.3390/nu9080839.

Wallner, M., Hanchar, H. J. and Olsen, R. W. (2003) 'Ethanol enhances α4β3δ and α6β3δ γ-aminobutyric acid type A receptors at low concentrations known to affect humans', *Proceedings of the National Academy of Sciences of the United States of America*, 100(25), pp. 15218–15223. doi: 10.1073/pnas.2435171100.

Walter, H. J. and Messing, R. O. (1999) 'Regulation of neuronal voltage-gated calcium channels by ethanol', *Neurochemistry International*. Pergamon, pp. 95–101. doi: 10.1016/S0197-0186(99)00050-9.

Wang, C. *et al.* (2018) 'Egocentric Coding of External Items in the Lateral Entorhinal Cortex',

Science (New York, N.Y.), 362(6417), p. 945. doi: 10.1126/SCIENCE.AAU4940.

Wang, Y. *et al.* (2016) 'Maternal dietary intake of choline in mice regulates development of the cerebral cortex in the offspring', *The FASEB Journal • Research Communication*, 30(4), pp. 1566–78. doi: 10.1096/fj.15-282426.

Wang, Y. *et al.* (2019) 'Choline Supplementation Ameliorates Behavioral Deficits and Alzheimer's Disease-Like Pathology in Transgenic *APP/PS1* Mice', *Molecular Nutrition & Food Research*, 63(18), p. 1801407. doi: 10.1002/mnfr.201801407.

Wang, Z. *et al.* (2011) 'Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease', *Nature*. doi: 10.1038/nature09922.

Warren, S. G. *et al.* (1995) 'LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats', *Brain Research*, 703(1–2), pp. 26–30. doi: 10.1016/0006-8993(95)01059-9.

Warton, F. L. *et al.* (2021) 'Maternal choline supplementation mitigates alcohol exposure effects on neonatal brain volumes', *Alcoholism: Clinical and Experimental Research*, 45(9), pp. 1762–1774. doi: 10.1111/ACER.14672.

Watts, L. T. *et al.* (2005) 'Astrocytes protect neurons from ethanol-induced oxidative stress and apoptotic death.', *Journal of neuroscience research*, 80(5), pp. 655–666. doi: 10.1002/jnr.20502.

Weinberg, J. (1985) 'Effects of Ethanol and Maternal Nutritional Status on Fetal Development', *Alcoholism: Clinical and Experimental Research*, 9(1), pp. 49–55. doi: 10.1111/j.1530-0277.1985.tb05049.x.

Weinberg, J. *et al.* (2008) 'Prenatal alcohol exposure: Foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome', in *Journal of Neuroendocrinology*. J Neuroendocrinol, pp. 470–488. doi: 10.1111/j.1365-2826.2008.01669.x.

Weyrauch, D. *et al.* (2017) 'Comorbid Mental Disorders in Fetal Alcohol Spectrum Disorders: A Systematic Review', *Journal of Developmental & Behavioral Pediatrics*, 38(4), pp. 283–291. doi: 10.1097/DBP.0000000000000440.

Whitcher, L. T. and Klintsova, A. Y. (2008) 'Postnatal binge-like alcohol exposure reduces spine density without affecting dendritic morphology in rat mPFC', *Synapse*, 62(8), pp. 566–573. doi: 10.1002/syn.20532.

Wijayawardhane, N. *et al.* (2008) 'Ameliorating effects of preadolescent aniracetam treatment on prenatal ethanol-induced impairment in AMPA receptor activity', *Neurobiology of Disease*, 29(1), pp. 81–91. doi: 10.1016/J.NBD.2007.08.001.

Wilhelm, C. J. and Guizzetti, M. (2016) 'Fetal Alcohol Spectrum Disorders: An Overview from the Glia Perspective', *FRONTIERS IN INTEGRATIVE NEUROSCIENCE*, 9, p. 65. doi: 10.3389/fnint.2015.00065.

Williams, C. L. *et al.* (1998) 'Hypertrophy of basal forebrain neurons and enhanced visuospatial memory in perinatally choline-supplemented rats', *Brain Research*, 794(2), pp. 225–238. doi: 10.1016/S0006-8993(98)00229-7.

Wolff, G. L. *et al.* (1998) 'Maternal epigenetics and methyl supplements affect agouti gene

expression in A vy /a mice', *The FASEB Journal*, 12(11), pp. 949–957. doi: 10.1096/fasebj.12.11.949.

Woods, L., Perez-Garcia, V. and Hemberger, M. (2018) 'Regulation of Placental Development and Its Impact on Fetal Growth—New Insights From Mouse Models', *Frontiers in Endocrinology*, 9, p. 570. doi: 10.3389/FENDO.2018.00570/BIBTEX.

Woolley, C. S. *et al.* (1990) 'Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons', *Journal of Neuroscience*, 10(12), pp. 4035–4039. doi: 10.1523/jneurosci.10-12-04035.1990.

Wozniak, J. R. *et al.* (2015) 'Choline supplementation in children with fetal alcohol spectrum disorders: A randomized, double-blind, placebo-controlled trial', *American Journal of Clinical Nutrition*, 102(5), pp. 1113–1125. doi: 10.3945/ajcn.114.099168.

Wozniak, J. R. *et al.* (2020) 'Four-year follow-up of a randomized controlled trial of choline for neurodevelopment in fetal alcohol spectrum disorder.', *Journal of neurodevelopmental disorders*, 12(1), p. 9. doi: 10.1186/s11689-020-09312-7.

Wright, J. M., Peoples, R. W. and Weight, F. F. (1996) 'Single-channel and whole-cell analysis of ethanol inhibition of NMDA-activated currents in cultured mouse cortical and hippocampal neurons', *Brain Research*, 738(2), pp. 249–256. doi: 10.1016/S0006-8993(96)00780-9.

Yang, D. W. *et al.* (2004) 'Sexual dimorphism in the induction of LTP: Critical role of tetanizing stimulation', *Life Sciences*, 75(1), pp. 119–127. doi: 10.1016/j.lfs.2003.12.004.

Yassa, M. A. and Stark, C. E. L. (2011) 'Pattern separation in the hippocampus', *Trends in neurosciences*, 34(10), p. 515. doi: 10.1016/J.TINS.2011.06.006.

Ylilauri, M. P. T. *et al.* (2019) 'Associations of dietary choline intake with risk of incident dementia and with cognitive performance: the Kuopio Ischaemic Heart Disease Risk Factor Study', *The American Journal of Clinical Nutrition*, 110(6), pp. 1416–1423. doi: 10.1093/ajcn/nqz148.

Young, J. K. *et al.* (2014) 'Nutrition Implications for Fetal Alcohol Spectrum Disorder', *Advances in Nutrition*, 5(6), p. 675. doi: 10.3945/AN.113.004846.

Yu, D. *et al.* (1996) 'Ethanol inhibition of nicotinic acetylcholine type alpha 7 receptors involves the amino-terminal domain of the receptor.', *Molecular Pharmacology*, 50(4).

Zakhari, S. (2006) 'Overview: How is alcohol metabolized by the body?', *Alcohol Research and Health*. Alcohol Res Health, pp. 245–254. Available at: https://pubmed.ncbi.nlm.nih.gov/17718403/ (Accessed: 30 October 2020).

Zeisel, S. H. *et al.* (1991) 'Choline, an essential nutrient for humans', *The FASEB Journal*, 5(7), pp. 2093–2098. doi: 10.1096/fasebj.5.7.2010061.

Zeisel, S. H. *et al.* (1995) 'Pregnancy and lactation are associated with diminished concentrations of choline and its metabolites in rat liver', *Journal of Nutrition*, 125(12), pp. 3049–3054. doi: 10.1093/jn/125.12.3049.

Zeisel, S. H. (2006) 'Choline: Critical Role During Fetal Development and Dietary

Requirements in Adults', *Annual Review of Nutrition*, 26(1), pp. 229–250. doi: 10.1146/annurev.nutr.26.061505.111156.

Zeisel, S. H. (2011) 'What Choline Metabolism Can Tell Us About the Underlying Mechanisms of Fetal Alcohol Spectrum Disorders', *Molecular Neurobiology*, 44(2), pp. 185–191. doi: 10.1007/s12035-011-8165-5.

Zeisel, S. H. and da Costa, K.-A. (2009) 'Choline: an essential nutrient for public health.', *Nutrition reviews*, 67(11), pp. 615–23. doi: 10.1111/j.1753-4887.2009.00246.x.

Zeisel, S. H. and Wurtman, R. J. (1981) 'Developmental changes in rat blood choline concentration.', *The Biochemical journal*, 198(3), pp. 565–570. doi: 10.1042/bj1980565.

Zhang, C. R. *et al.* (2015) 'Prenatal ethanol exposure alters adult hippocampal VGLUT2 expression with concomitant changes in promoter DNA methylation, H3K4 trimethylation and miR-467b-5p levels', *Epigenetics & Chromatin*, 8(1), p. 40. doi: 10.1186/s13072-015-0032-6.

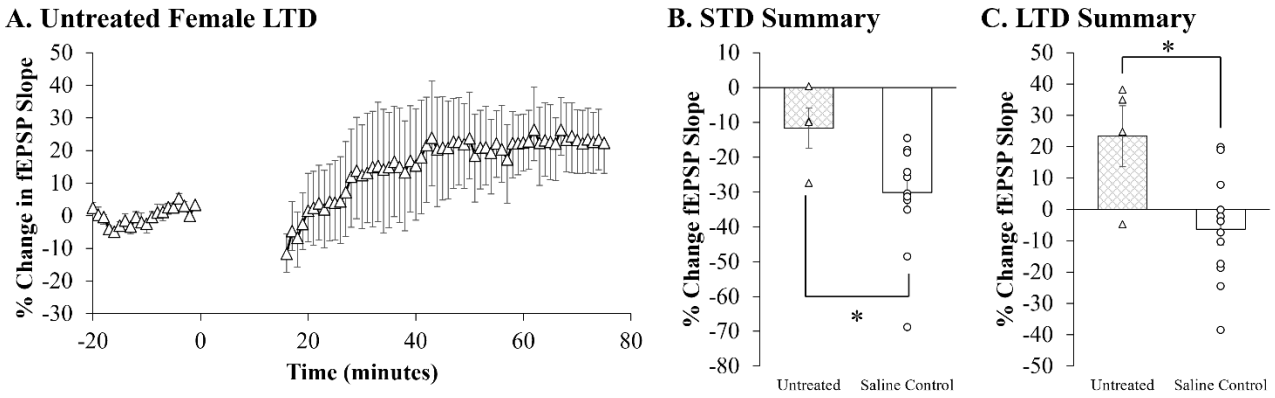
Zhu, W. *et al.* (2017) 'Gut Microbe-Generated TMAO from Dietary Choline Is Prothrombotic in Subjects', *Circulation*, 135(17), p. 1671. doi: 10.1161/CIRCULATIONAHA.116.025338.

Zorzano, A. and Herrera, E. (1989) 'Decreased in Vivo Rate of Ethanol Metabolism in the Suckling Rat', *Alcoholism: Clinical and Experimental Research*, 13(4), pp. 527–532. doi: 10.1111/j.1530-0277.1989.tb00372.x.

Supplementary Content

Supplementary Figure 1. Female control LTD without injections.

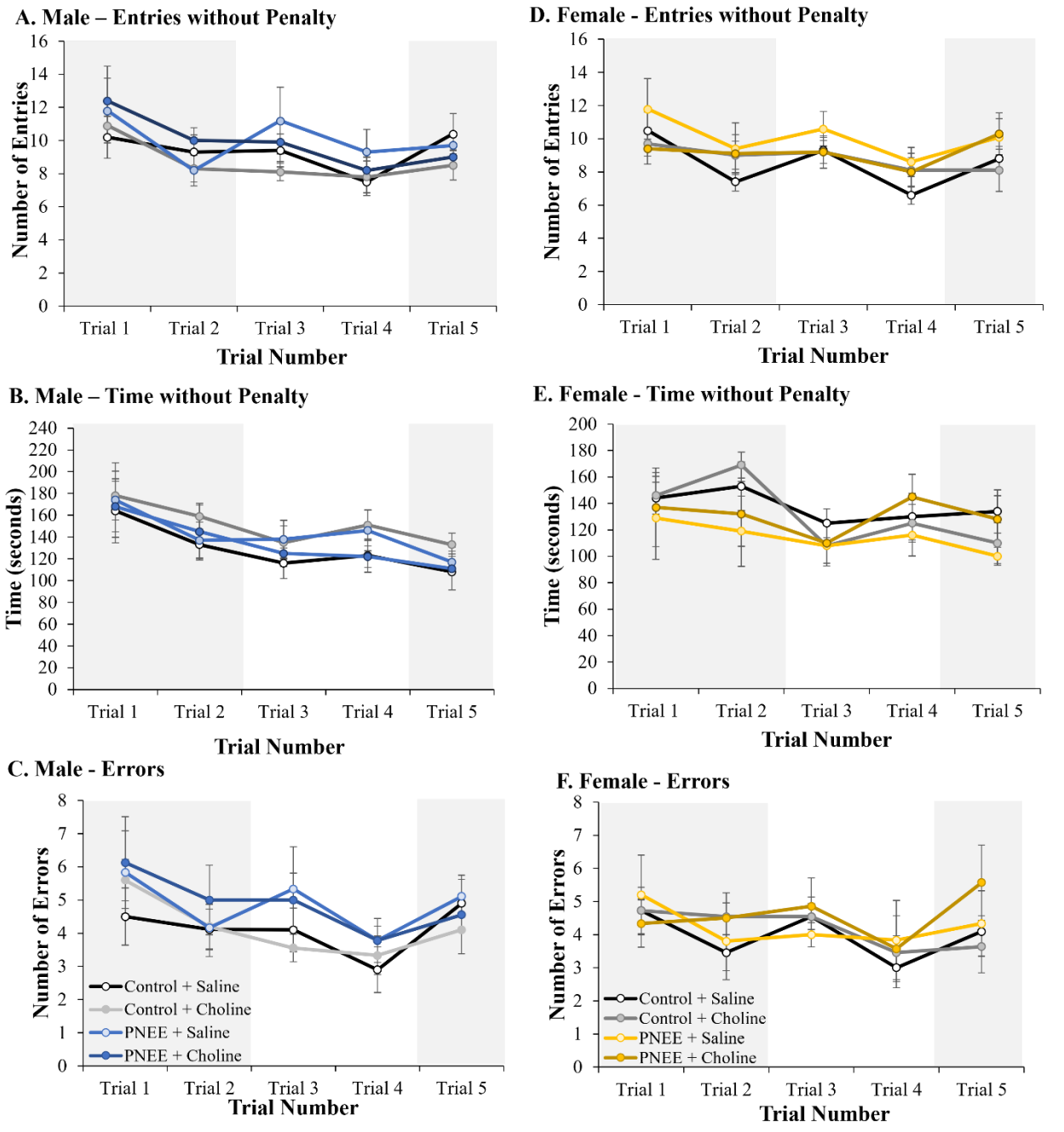
Saline injections did not decrease the amount of LTD in juvenile female offspring. Of note, slices were derived from four untreated female offspring (N=4, L=1) and therefore these data should be replicated in a separate litter to support these preliminary findings.



Supplementary Figure 1 Female LTD is not increased without stress. A. Summary change in fEPSP slope for untreated control female offspring. Average short-term depression (B) and long-term depression (C) are represented by bars. Individual slices (n=4) are represented by a point. All error bars are ± SEM. *p < 0.05, Student's two-tailed t-test.

Supplementary Figure 2. Reference Memory Performance without Penalization

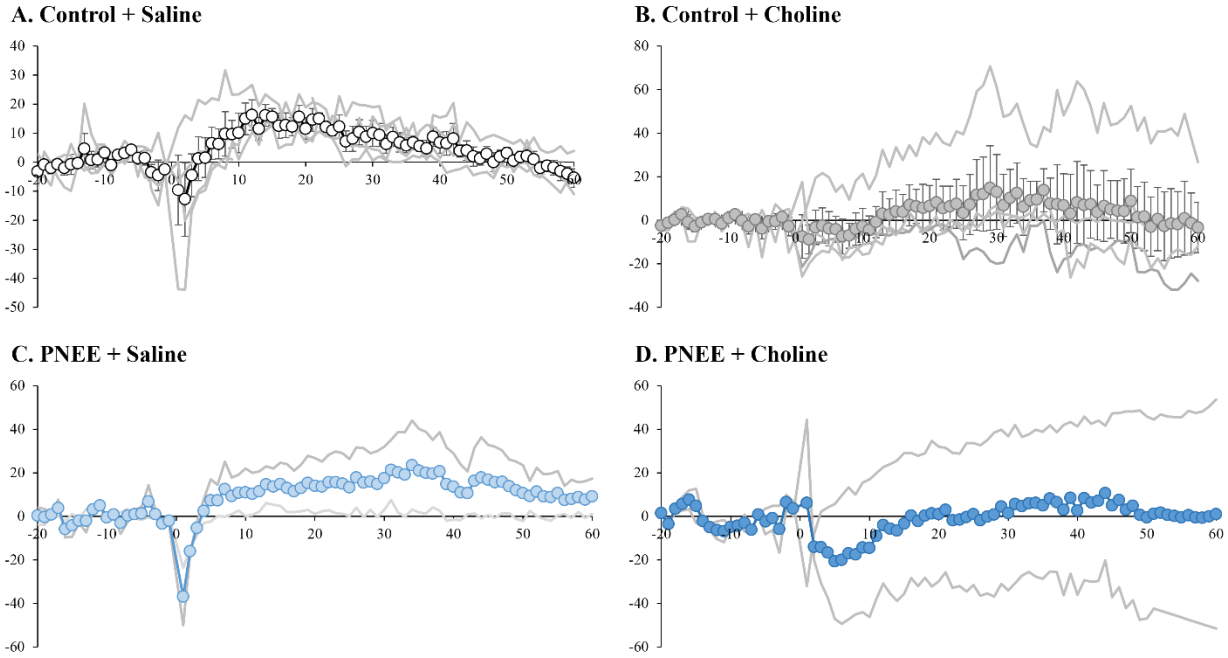
The reference memory task was additionally analyzed without a penalty applied for when the task was not completed. However, this did not change the overall outcomes seen in Aim 2. In male offspring, there was no change in number of entries (Sup Fig 2A. Prenatal: $F(2.82, 81.79) = 0.42$, $p = 0.730$, $\eta_p^2 = 0.014$; Treatment: $F(2.82, 81.79) = 0.54$, $p = 0.647$, $\eta_p^2 = 0.018$) and time to complete the task (Sup Fig 2B. Prenatal: $F(2.62, 75.97) = 0.07$, $p = 0.962$, $\eta_p^2 = 0.003$; Treatment: $F(2.62, 75.97) = 0.23$, $p = 0.854$, $\eta_p^2 = 0.008$). Similarly, in female offspring there were no change in term of number of entries (Sup Fig 2D. Prenatal: $F(4, 136) = 0.54$, $p = 0.707$, $\eta_p^2 = 0.016$; Treatment: $F(4, 136) = 1.13$, $p = 0.346$, $\eta_p^2 = 0.032$) or time to complete the task (Sup Fig 2E. Prenatal: $F(4, 136) = 0.45$, $p = 0.774$, $\eta_p^2 = 0.013$; Treatment: $F(4, 136) = 0.30$, $p = 0.876$, $\eta_p^2 = 0.009$). Finally, the number of errors was assessed as an additional measure to determine if PNEE or choline treatment altered reference memory performance. Errors were defined as entries into arms that were not a 'correct'/baited arm. In this measure, there was once again no differences in male offspring (Sup Fig 2C. Prenatal: $F(4, 108) = 0.26$, $p = 0.902$, $\eta_p^2 = 0.010$; Treatment: $F(4, 108) = 0.63$, $p = 0.645$, $\eta_p^2 = 0.023$) or female offspring (Sup Fig 2F. Prenatal: $F(4, 116) = 1.07$, $p = 0.375$, $\eta_p^2 = 0.036$; Treatment: $F(4, 116) = 0.86$, $p = 0.488$, $\eta_p^2 = 0.029$).



Supplementary Figure 2. Reference Memory Performance without Penalization. Reference memory task performance, including number of entries (A. Male, D. Female), time (B. Male, E. Female), and number of errors (C. Male, F. Female). Number of errors are classified as entries into arms that were not a 'correct' arm. Grey/white bars represent separate days. Error bars are ± SEM.

Supplementary Figure 3. Male subthreshold LTD (10 Hz)

As the 30 Hz HFS protocol still produced a level of potentiation in saline-treated control males, several slices were stimulated at 10 Hz to determine if this would be considered subthreshold. In control + saline adult males, there was no degree of potentiation. However, results were more variable in choline-treated offspring, but due to the low number of slices, it cannot be said yet if this is a meaningful increase in variability or a product of low power.



Supplementary Figure 3. Subthreshold LTD in control males occurs at 10 Hz. A. Saline-injected control males (n_{slices} = 4, $N_{animals}$ = 2). B. Choline-injected control males (n_{slices} = 4, $N_{animals}$ = 2). C. Saline-injected PNEE males (n_{slices} = 2, $N_{animals}$ = 1). D. Choline-injected PNEE males (n_{slices} = 2, $N_{animals}$ = 1). Each slice is represented by a grey line and the average is represented by circular points. Error bars for control graphs are ± SEM but are omitted in the PNEE graphs due to the low slice n.

Supplementary Text 1. *Working Memory Simulation*

```
final <- matrix(ncol=10000) #create matrix for all 10,000 trial outcomes
total_trials_run <- 0
repeat{
  total_trials_run <- total_trials_run + 1
  total <- 0 #count number of arms traveled
  current_matrix <- matrix(0, ncol=8) #create matrix to hold the current "animals" results
  p <- c(1,1,1,1,1,1,1,1) #set probability of all arms to 1

  repeat{
    current_arm <- sample(1:8, 1, prob=p) #select a random "arm" from 1-8
    p <- c(1,1,1,1,1,1,1,1) #reset probability to be equal
         p[current_arm]<- 0 #set current_arm probability to be 0 for next time
         #set adjacent arms to be twice as likely
         if(current_arm !=8){p[c(current_arm+1, current_arm-1)]<- 2}
         if(current_arm == 8){p[c(1,7)]<- 2}
         if(current_arm == 1){p[c(2,8)]<-2}

         #if animal hasn't explored that arm yet, put it in matrix
         if(current_matrix[1,current_arm] == 0){current_matrix[1,current_arm]<- current_arm}
         total <- total + 1

    #end trial When all 8 "arms" are explored end trial & record number of trials
    if (rowSums(current_matrix) == 36){final[1,total_trials_run] <- total
    break}}

if(total_trials_run == 10000){break}}
rowMeans(final) #calculate average of all the trials
```

Supplementary Text 2. *Reference Memory Simulation*

```
final <- matrix(ncol=10000) #create matrix for all trial outcomes
total_trials_run <- 0

repeat{
  total_trials_run <- total_trials_run + 1
  total <- 0
  correct_arms <- matrix(sample(1:8,4,replace=F)) #pick different 4 arms
  p <- c(1,1,1,1,1,1,1,1) #set probability

  repeat{
    current_arm <- sample (1:8, 1, prob=p) #select a random "arm" from 1-8
    p <- c(1,1,1,1,1,1,1,1) #reset probability

        #If correct arm is chosen, reset that value in the matrix to 0
        if(current_arm == correct_arms[1]){correct_arms[1] <- 0}
        if(current_arm == correct_arms[2]){correct_arms[2] <- 0}
        if(current_arm == correct_arms[3]){correct_arms[3] <- 0}
        if(current_arm == correct_arms[4]){correct_arms[4] <- 0}
        total <- total + 1

        p[current_arm]<- 0 #set current_arm probability to be 0 for next time, no re-entries
        #set adjacent arms to be twice as likely
        if(current_arm !=8){p[c(current_arm+1, current_arm-1)]<- 2}
        if(current_arm == 8){p[c(1,7)]<- 2}
        if(current_arm == 1){p[c(2,8)]<-2}

    #end trial when all correct_arms are chosen & record total number of trials
    if (colSums(correct_arms)== 0){final[1,total_trials_run] <- total
    break}}

    if(total_trials_run == 10000){break}}


rowMeans(final) #calculate average of all the trials
```