FETAL ALCOHOL SPECTRUM DISORDERS: ALTERATION OF NEURONAL MORPHOLOGY BY ETHANOL, CHOLINE AND TISSUE PLASMINOGEN ACTIVATOR

By

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A DISSERTATION

Presented to the Department of Behavioral Neuroscience

and

Oregon Health & Science University

School of Medicine

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

May 2019

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADHD – Attention Deficit Hyperactivity Disorder

ARBD – Alcohol Related Birth Defects

ARND – Alcohol Related Neurodevelopmental Disorder

ARSB – Arylsulfatase B

BBB – Blood Brain Barrier

BDNF – Brain Derived Neurotrophic Factor

BECs – Blood Ethanol Concentrations

BSA – Bovine Serum Albumin

CA1 – Cornu Ammonis Area 1

CA3 – Cornu Ammonis Area 3

Chol – Choline

CNS – Central Nervous System

DMEM – Dulbecco's Modified Eagle Medium

DNMT – DNA Methyltransferase

DTI – Diffusion Tensor Imaging

ECM – Extracellular Matrix

EGFR – Epidermal Growth Factor Receptor

ERK 1/2 – Extracellular Signal-Regulated Kinases 1/2

EtOH – Ethanol

FAS – Fetal Alcohol Syndrome

FASD – Fetal Alcohol Spectrum Disorder

FBS – Fetal Bovine Serum

FDR – False Discovery Rate

GABA – Gamma-Aminobutyric Acid

GD – Gestational Day

LTP – Long Term Potentiation

mPFC – Medial Prefrontal Cortex

MRSI – Magnetic Resonance Spectroscopic Imaging

ND-PAE – Neurobehavioral Disorder Associated with Prenatal Alcohol Exposure

NGF – Nerve Growth Factor

NMDA – N-methyl-D-aspartate

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraxzolium bromide

PA – Phosphatidic Acid

PAE – Prenatal Alcohol Exposure

PAI-1 – Plasminogen Activator Inhibitor 1

PARP – Poly-ADP Ribose Polymerase

PC – Phosphatidylcholine

PD – Postnatal Day

PEMT – Phosphatidylethanolamine N-Methyltransferase

PEth – Phosphatidylethanol

pFAS – Partial Fetal Alcohol Syndrome

PKA – Protein Kinase A

PKC – Protein Kinase C

PLD – Phospholipase D

rtPA – Recombinant tPA

SAM – S-adenosylmethionine

sGAGs – Sulfated Glycosaminoglycans

siRNA – Small Interfering RNA

tPA – Tissue Plasminogen Activator

ACKNOWLEDGEMENTS

This document is the result of nearly 6 years of work; work which would not have been possible without a number of extraordinary people, whom I would like to take this opportunity to thank.

First, I would like to thank my mentor, Dr. Marina Guizzetti. From the very first day I wandered into her lab and refused to wander out, she has been amazingly supportive of everything, including both my successes and my mistakes. Without her guidance, I would never have gotten anywhere near as far as I did, as her patience and expertise brought me to where I am. I am a neuroscientist with experience, research and papers to my name because of her, and I will forever be indebted because of it.

Thank you to Dr. Deborah Finn, for being my lab-away-from-lab and chair of my Dissertation Committee. She constantly put up with me poking my head into her office at all hours of the day, and never once was anything less than receptive. Without her as an outside perspective, and a guide when Marina and I were still new at OHSU, I would have struggled with my work and my classes far more than I did. I would also like to thank the members of her lab, Michelle Nipper and Melinda Helms, who put up with me wandering in and (un)intentionally scaring them, as well as Jeremiah Jensen, who measured the blood ethanol concentrations.

I would also like to thank the other members of my Dissertation Committee: Dr. Andrey Ryabinin, Dr. Charles Allen, and Dr. Tamara Phillips. When I asked if they would take an enormous amount of effort and time out of their lives to work with me on my dissertation, none of them hesitated to say yes. They willingly assisted me with anything I needed help with throughout this process, and have provided invaluable scientific assistance with any snags I ran into along the way.

Also deserving of much thanks and praise are the members of the Guizzetti lab, past and present. Dr. Xiaolu Zhang, who gave me my first real introduction to bench work, never once giving up on me, even through my many, many mistakes. Joel Hashimoto, who introduced me to both the coding language of R and complicated statistics at the same time, and didn't laugh at my pain (much). Melissa

Roberts whose dedication toward freezing their hands off in the cryostat slicing brains made them invaluable to my research, as well as providing a source of companionship when I was at the microscope. Katie McNealy, my buddy in Golgi-Cox and the 'forever hungry' club. Lastly, Dr. Clare Wilhelm and Shelley Bloom who helped me with optimizing several sets of experimental methodology.

I also owe thanks to my fellow graduate students and postdocs in the Behavioral Neuroscience program. Specifically, Nadir Balba, Jordan Lueras, Elina Thomas, Sami Frederich, Erika Cuellar, John Mootz, Alex Stafford, and Antonia Savarese, all of whom never once minded my interruptions in their lives, and constant questions about whatever thoughts were passing through my brain at the time. I would also like to extend my thanks to Adam Munhall, of the Johnson lab, who assisted in osmolality measurements, and was a sounding board for many ideas during lunch.

I would also like to thank several people without whom I would never have set foot on this path. First, my undergraduate mentor at the State University of New York at Geneseo, Dr. Vincent Markowski. He was the first one who accepted me into a laboratory and gave me the foundations I needed to become a scientist. Tak Furuta and Clint Cross, my sensei in martial arts, who fostered my character development in ways I would never have imagined, giving me the support I needed to constantly challenge myself.

And lastly, I would like to thank my family. My mother, Corinne Goeke, and father, James Goeke, who have constantly supported me throughout all the trial and tribulations of my life, and my little sister, Janelle Goeke, who understood the world of a science graduate student far better than our computer engineer parents.

Thanks to all of you, as you were all integral to this process. Without even one of you giving me the endless support that you did, I would never have gotten where I am, and you all have my unending thanks for it.

OVERALL ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD), which results from gestational ethanol (a short-chain primary alcohol) exposure, is the most common form of preventable intellectual disability. Individuals with FASD have a variety of Central Nervous System dysfunctions, including impulsivity, difficulties with abstract thinking, problems with memory, and learning disabilities. FASD is highly prevalent throughout the world; in the United States alone, it is estimated that 24-48 in 1,000 individuals have FASD. While the mechanisms underlying the response of the developing brain to gestational ethanol exposure are not fully understood, a potential mechanism is tissue plasminogen activator (tPA), which is upregulated by ethanol, both *in vivo* and *in vitro.* There is also a potential treatment for FASD, choline, which has been shown to ameliorate some of the effects of prenatal ethanol exposure. Furthermore, some of the effects of ethanol on tPA, and the capacity for choline to be a treatment, may be mediated by astrocytes, which have been shown to influence neurons and neuronal morphology.

Therefore, the experiments in this dissertation examined the hypothesis that gestational ethanol exposure alters neuronal morphology, and that this alteration is, in part, due to astrocyte-mediated increases in tPA caused by ethanol. These experiments also examined the secondary hypothesis that astrocytes can mediate a rescue effect of choline on neuronal morphology alterations induced by ethanol. To test the main hypothesis, experiments examined *in vitro* astrocytic modulation of neurite outgrowth following treatment of astrocytes with ethanol, choline or tPA. Other experiments analyzed the effects of ethanol and choline *in vivo,* to examine alterations of hippocampal pyramidal CA1 neuronal morphology induced by these treatments (see Fig. 1.1 for mechanisms of choline and Fig. 1.2 for mechanisms of tPA).

Astrocytes were shown to mediate effects of ethanol, choline and tPA on neurite outgrowth. Specifically, neurons cultured *in vitro* with ethanol-treated astrocytes displayed reduced neurite outgrowth. However, astrocytes treated with choline alone did not display altered neurite outgrowth.

When neurons were cultured with astrocytes treated with ethanol and choline simultaneously, there was an astrocyte-mediated rescue effect on the ethanol-induced decrease in neurite outgrowth.

Astrocytes also mediated the effects of tPA, as decreasing levels of astrocytic *Plat,* the gene that encodes for tPA, via a small interfering (si) RNA, decreased neurite outgrowth. By contrast, neurons cultured with astrocytes exposed to a combination treatment of ethanol and siRNA *Plat* had neurite lengths similar to those of controls. Lastly, increasing astrocytic tPA protein levels also decreased neurite outgrowth in neurons cultured with siRNA *Plat* treated astrocytes. These experiments demonstrate that astrocytes are mediators of neurite outgrowth, and specifically mediate the effect of ethanol, alterations in the levels of tPA, and a rescue effect of choline *in vitro*.

Ethanol and choline also altered neuronal morphology *in vivo.* Neonatal rats, from postnatal day (PD) 4-PD9, were intubated with 5 g/kg/day ethanol (BECs between 59.96-64.27 mM), or injected with 100 mg/kg/day choline. Ethanol intubation resulted in increased parameters of hippocampal pyramidal neuron apical dendrite morphology, an effect which was seen more strongly in females. By contrast, choline decreased parameters of basilar dendrite morphology, an effect which was seen more strongly in males. When rats were intubated with ethanol from PD4-PD9, but choline injections were continued until PD30, there was a sex-specific effect of choline on apical dendrites; male rats given choline had decreased parameters of apical dendrite morphology compared to both male control rats and female rats given choline. These *in vivo* experiments demonstrate that ethanol and choline exposure during periods of development result in alterations of neuronal morphology, alterations which differ by sex and area of the cell examined.

In summary, the experiments of this dissertation indicate that gestational ethanol exposure can induce alterations in neuronal morphology. These alterations may be due, in part, to astrocyte-mediated, ethanol-induced increases in tPA. Furthermore, *in vitro,* astrocytes mediated a rescue effect of choline on ethanol-induced decrease in neurite outgrowth. However, *in vivo,* the effect of choline on

neuronal morphology was dependent on sex, age of the animal, and the area of the cell being examined. Overall, although these experiments were done in rats, and so many not translate to humans, these data support the hypothesis that increases in tPA seen following ethanol exposure are involved in how the brain responds to ethanol exposure, that astrocytes mediate some of the effects of ethanol exposure, and that choline may be a treatment for FASD, but may be dependent on a variety of factors, including the sex of the individual and the timing of the exposure.

Figure 1.1. Mechanisms of choline. Choline can be converted into betaine, which can in turn be converted into the universal methyl donor, thus influencing DNA methylation. Conversion of choline into betaine happens primarily in the liver and kidneys. Choline can also be converted into the neurotransmitter acetylcholine in cholinergic neurons. Lastly, choline can be converted into phosphatidylcholine (PC), a major membrane phospholipid. PC can be hydrolyzed by phospholipase D (PLD) to produce choline and phosphatidic acid (PA). PA is a second messenger that is involved in proliferation, cytoskeletal reorganization and vesicular trafficking. However, when ethanol is present, PC can instead be transphosphorylated by PLD into choline and phosphatidylethanol, preventing the downstream effects of PA.

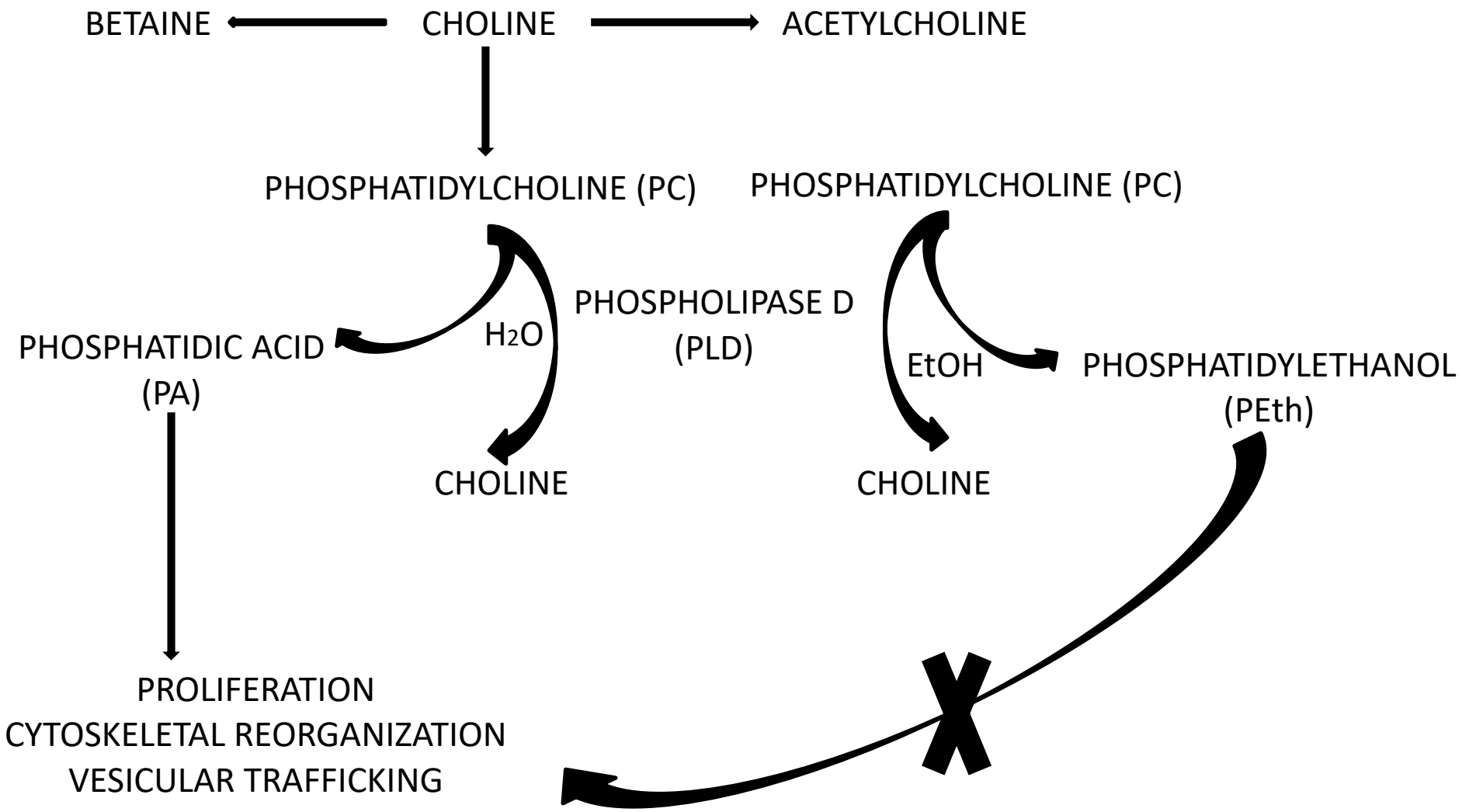
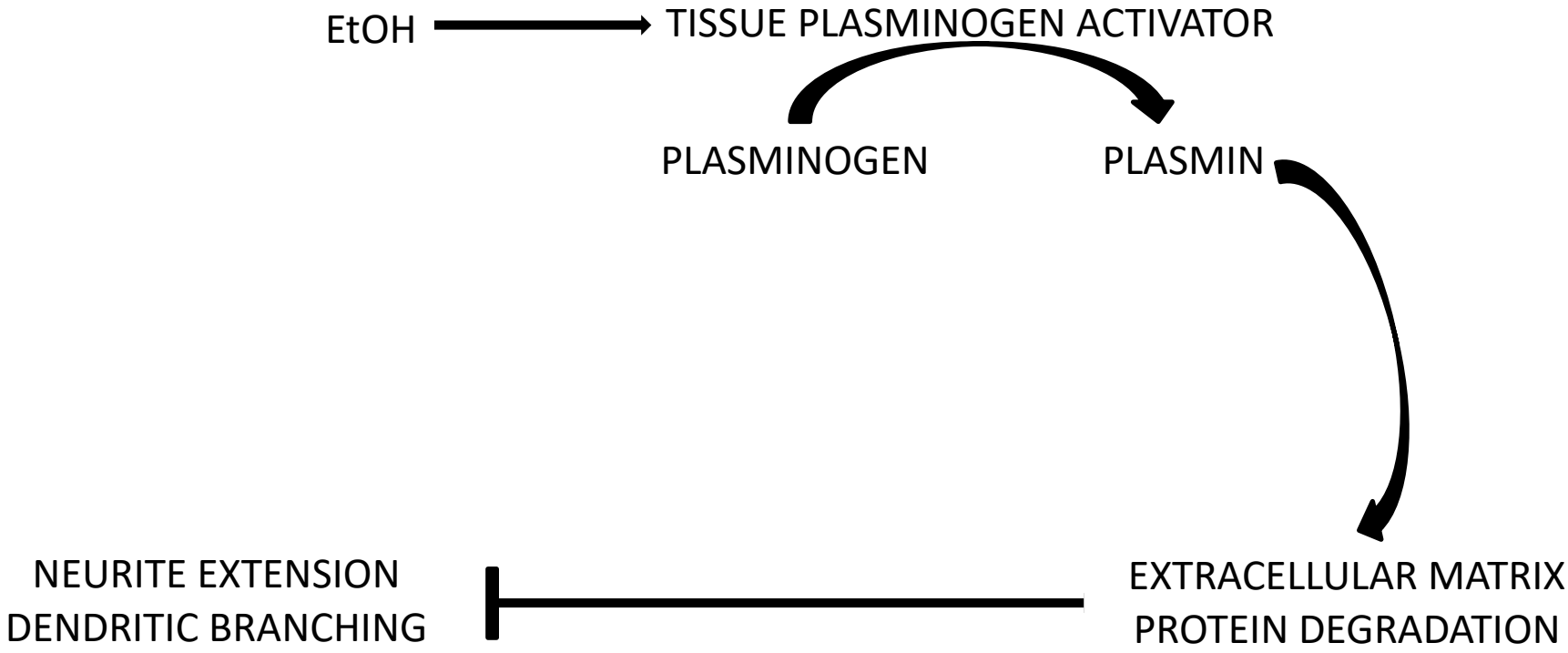

BETAINE
CHOLINE
ACETYLCHOLINE
PHOSPHATIDYLCHOLINE (PC)
PHOSPHATIDYLCHOLINE (PC)
PHOSPHATIDIC ACID
(PA)
H2O
PHOSPHOLIPASE D
(PLD)
EtOH
PHOSPHATIDYLETHANOL
(PEth)
CHOLINE
CHOLINE
PROLIFERATION
CYTOSKELETAL REORGANIZATION
VESICULAR TRAFFICKING

Figure 1.2. Mechanisms of tissue plasminogen activator. Ethanol increases tPA expression and release. tPA can synthesize the conversion of plasminogen into plasmin. Plasmin can then degrade extracellular matrix proteins, resulting in an inhibition of neurite outgrowth and dendritic branching.


EtOH
TISSUE PLASMINOGEN ACTIVATOR
PLASMINOGEN
PLASMIN
NEURITE EXTENSION
DENDRITIC BRANCHING
EXTRACELLULAR MATRIX
PROTEIN DEGRADATION

CHAPTER 1: GENERAL INTRODUCTION

Fetal Alcohol Spectrum Disorders (FASD)

Ethanol, a short-chain primary alcohol, can cross both the placenta and the blood-brain barrier (BBB), and therefore, drinking ethanol during pregnancy can directly affect the developing fetus (May et al., 2014; Medina, 2011). The effects of gestational ethanol exposure are termed Fetal Alcohol Spectrum Disorders (FASD) (Guizzetti, 2015; Popova et al., 2017; Riley et al., 2011). The capacity for ethanol to affect the developing fetus was first scientifically recognized in the 1970s (Jones & Smith, 1973), and was termed Fetal Alcohol Syndrome (FAS). FAS is characterized by facial dysmorphology, such as a smooth philtrum and a thin upper lip, and growth retardation, such as smaller than average height and lower than average body weight (Jones & Smith, 1973). FAS also presents with a wide range of Central Nervous System (CNS) dysfunctions, including attentional deficits, decreased intelligence, trouble with abstract thinking, language and vocabulary deficits, executive functioning impairment, and difficulties with memory (Aragon et al., 2008; Lebel et al., 2012; May et al., 2014; Riley et al., 2011). However, gestational ethanol exposure does not always result in the characteristic facial dysmorphology of FAS, even when CNS dysfunctions are present (Jones & Smith, 1973).

As a result, 'FASD' was created as an umbrella term to represent several different manifestations of gestational ethanol exposure. One, Alcohol Related Neurodevelopmental Disorder (ARND), requires confirmed gestational ethanol exposure, either a structural brain anomaly or evidence of cognitive/behavioral abnormalities (Riley et al., 2011), along with behavioral problems (Dejong et al., 2018). In ARND, none of these abnormalities or dysfunctions can be explained genetically or by family environment, and facial dysmorphology is not necessarily present (May et al., 2014). FASD also includes Alcohol Related Birth Defects (ARBD), characterized by organ malformations, and requiring confirmation of gestational ethanol exposure (Dejong et al., 2018; May et al., 2014). Along the FASD spectrum, FAS remains the most severe, associated with the most deleterious symptoms and greatest amounts of

gestational ethanol exposure (May et al., 2014). Lastly, the Diagnostic and Statistical Manual of Mental Disorders V (DSM V) includes 'Neurobehavioral Disorder Associated with Prenatal Alcohol Exposure' (ND-PAE) (Dejong et al., 2018). ND-PAE requires confirmed gestational ethanol exposure, as well as at least one symptom in each of three categories: CNS dysfunctions, behavioral problems, and trouble with daily life (Dejong et al., 2018; Hagan Jr. et al., 2016; Kable et al., 2016). It is therefore a recognized fact that gestational ethanol exposure can have deleterious, long-lasting effects on the developing fetus.

Unfortunately, drinking ethanol during pregnancy, and as a result FASD, is common across the world. A systematic review and meta-analysis found that many countries report very high estimated percentages of ethanol use during pregnancy, for example Ireland, where it was estimated that up to 60.4% of women consumed ethanol during gestation (Popova et al., 2017). In the United States alone, the estimate is that 24 – 48 in 1,000 live births are people with FASD. There are also some populations with much higher rates of FASD, such as a population in South Africa, where it is estimated that nearly 8% of 1,000 live births are individuals with FAS (Popova et al., 2017). Interestingly, there are a number of modifying factors that can change the rates of FASD independent of the rates of ethanol consumption during pregnancy. The aforementioned population in South Africa, for example, also has high rates of malnutrition, low socioeconomic status, and a long history of wine production, all factors which contribute to the high rates of FASD (Popova et al., 2017). While different populations have different prevalence of FASD, this meta-analysis clearly showed that drinking ethanol during pregnancy, and as a result FASD, is very common throughout the world (Popova et al., 2017). Due to this prevalence, a significant amount of research has been done on how gestational ethanol exposure affects the developing brain, both clinically and preclinically, and both *in vivo* and *in vitro*.

FASD in Humans: Imaging Studies

Some studies have examined FASD clinically, in human cohorts. In children with confirmed FASD, examined between the ages of 9.3 and 15.4 years, gestational ethanol exposure resulted in

decreased volume of the left hippocampus as compared to controls, but no difference in the right hippocampus (Willoughby et al., 2008). In the same study, only controls demonstrated the increase in left and right hippocampal volumes normally correlated with age. Children and adolescents with FASD also had decreased brain volume compared to matched controls (Lebel et al., 2012), which was associated with lower intelligence on a variety of assessments. Children with confirmed FASD and examined via resting state functional MRI analysis (Wozniak et al., 2013) showed a higher path length and lower global efficiency, indicating a network abnormality potentially representing cognitive deficits, which may not be correlated with facial dysmorphology.

When children with confirmed gestational ethanol exposure, both with and without Attention Deficit Hyperactivity Disorder (ADHD), were examined via proton magnetic resonance spectroscopic imaging (MRSI) and diffusion tensor imaging (DTI) (O'Neill et al., 2018), results indicated a white matter pathology that may be specific to FASD comorbidity with ADHD. In a longitudinal study, confirmed prenatal ethanol exposure resulted in cortical volume loss, due to a lack of cortical increase seen in control cohorts (Lebel et al., 2012). Lastly, children with confirmed FASD also routinely scored lower on a variety of intelligence tests (Aragon et al., 2008), although there were areas where children with FASD perform similarly to controls, such as the similarities and vocabulary subtests on a test of general intelligence, indicating that the effects of gestational ethanol exposure are not global. Therefore, FASD in humans is characterized by a number of brain abnormalities and altered connectivity, both of which underlie some of the cognitive and behavioral dysfunctions seen following gestational ethanol exposure.

Effects of Ethanol *In Vivo*

Other studies have examined the effects of ethanol in animal models. Some of these *in vivo* FASD models target specific periods of development, one of which is the brain growth spurt, a period of interest to FASD research. During the brain growth spurt, the brain undergoes a massive increase in size and weight, along with astrocyte and oligodendrocyte proliferation, synaptogenesis, and dendritic

arborization (Farhy-Tselnicker & Allen, 2018; Guerri, 1998; Semple et al., 2013). In humans, the brain growth spurt occurs during the third trimester of gestation, making it a target for fetal ethanol exposure (Dobbing & Sands, 1979; Semple et al., 2013). However, in many animals, including rodents commonly used in experimental models, the brain growth spurt occurs postnatally, allowing FASD animal models to target specific periods of development, including the brain growth spurt (Driscoll et al., 1990; Patten et al., 2014; Rice & Barone, 2000; Semple et al., 2013).

Some studies, however, target periods other than the brain growth spurt, representative of earlier time points in human gestation, and have examined the *in vivo* effect of ethanol on the normally occurring development during these periods. In one such study, Wistar rat pups born to dams fed 5% weight/volume ethanol throughout gestation (Blood Ethanol Concentrations (BECs): 22.5 ± 12 mM [dams], 23.3 ± 4 mM [fetuses], measured Gestational Day [GD] 12, 15, 21) had decreased proliferation of both neurons and astrocytes, an effect which is a potential reason for the reduced thickness of the cerebral cortex seen in FASD (Rubert et al., 2006). Wistar rat pups born to dams exposed to 6 g/kg/day ethanol (BECs of dams: 58.6 ± 9.7 mM, measured GD20) had smaller granule cell somas, as well as hippocampal pyramidal neurons with smaller dendritic parameters and a lower spine density (Jakubowska-Dogru et al., 2017). Long-Evans rats exposed to 5% ethanol in saccharin water throughout all gestation (dam BECs: 20 ± 1.3 mM, measured 3rd week of gestation), and examined in adulthood, demonstrated a reduced dendrite length and branching in the medium spiny neurons in the nucleus accumbens shell, with no differences in spine density (Rice et al., 2012). Therefore, even when ethanol is given at time points representative of early human gestation, ethanol still has a significant effect on the development of the rat brain.

In ICR mice, prenatal exposure to ethanol from GD6- GD18, to 25% weight/volume ethanol (dam BECs: 75.4 mM) decreased neural stem cell proliferation in the dorsal telencephalon, and also increased apoptosis, altered neuronal distribution and dopaminergic projections into the neocortex, changed

microglial activation state, and induced neocortical hypoplasia (Komada et al., 2017). Mouse pups (Ail4 tdTomato reporter) born to dams who voluntarily consumed ethanol, both before and during gestation (dam BECs: 23.8 mM), also had greater dorsomedial striatum medium spiny neuronal dendritic complexity, specifically in the neurons that expressed the D1 dopaminergic receptors (Cheng et al., 2018). C57BL/6J mice exposed to ethanol prenatally, starting at GD7 (BECs: 45.6 mM) and examined at postnatal day (PD) 56 had decreased development in barrel sizes in rows D and E, indicating that the impaired somatosensory capacity seen following ethanol exposure may be due to specific deficits in somatosensory barrel cortex development (Olateju et al., 2019). Therefore, the effects of ethanol on the developing brain are not species specific, as mice showed alterations following ethanol exposure, just as rats did.

Other studies have specifically targeted the brain growth spurt, equivalent to the third trimester of human gestation. In one such study, neonatal Long-Evans rats intubated with 5.25 g/kg/day ethanol (BECs: 85.4 ± 21 mM) during their brain growth spurt (PD4-PD9), and examined on PD10 had decreased microglial cell territory in the CA1 and dentate gyrus of the hippocampus, decreased microglial cell number, and increased levels of both pro- and anti-inflammatory cytokines (Boschen et al., 2016b). Long-Evans rats intragastrically intubated with ethanol between PD4-PD9 (BECs: 78.3 ± 3.6 mM, measured PD4), and euthanized on PD26-PD30, also demonstrated decreased overall basilar dendrite length of mPFC Layer II/III neurons, along with decreased intersections proximal to the soma (Hamilton et al., 2010). In another study using the same ethanol exposure paradigm as Hamilton et al (2010), (BECs: 79.3 ± 3.6 mM, measured PD4) and examining Long-Evans rats at the same age (Whitcher & Klintsova, 2008), ethanol exposure decreased spine density on mPFC Layer III pyramidal neuron apical dendrites, without changing the ratio of immature to mature spines. Neonatal ethanol exposure (PD4-PD9) in Long-Evans rats (BECs: 78 ± 3.6 mM, measured PD4) also increased expression of the gene coding for Brain Derived Neurotrophic Factor (BDNF), however, this was a transient effect that was not

seen later in the animal's life (Boschen et al., 2016a). Furthermore, even a short period of ethanol exposure can have deleterious effects, as Sprague-Dawley rats subcutaneously injected with ethanol only on PD7 (BECs: 47.6 mM), and euthanized a day later, had increased neurodegeneration due to cell death in the forebrain (Ikonomidou et al., 2000).

Adolescent mice (wildtype and adenylyl-cyclase 1/8 double knockout) exposed to ethanol neonatally (PD5-7), during their brain growth spurt (BECs: 59.4 mM) had smaller soma sizes in medium spiny neurons of the caudate putamen, as well as reduced dendritic complexity (Susick et al., 2014). Furthermore, subcutaneous injections of ethanol into neonatal C57BL/6J mice, from PD4-PD9 (BECs: 83.2 mM, measured PD9) resulted in a decreased capacity for experience-induced visual cortex plasticity, an effect which is not dependent on microglia (Wong et al., 2018). Adolescent ferrets exposed every other day from PD10-PD30 to ethanol (59.4 mM) that underwent monocular deprivation had reduced ocular dominance plasticity, an effect that was rescued by an injection of serum response factor, which has previously been shown to improve neuronal plasticity, especially in the area close to the site of the injection (Foxworthy & Medina, 2015). Therefore, ethanol exposure during the brain growth spurt, equivalent to the third trimester of human gestation, has pervasive impacts on the developing brain of rodents.

Overall, these data show that, in multiple animal models targeting different time points of human gestational equivalence, ethanol has a variety of deleterious effects, including alteration of dendritic morphology, changes in cell soma properties and altered projections between brain areas. As these alterations are wide-ranging and complex, they likely underlie some of the behavioral and CNS dysfunctions seen in individuals with FASD. Furthermore, ethanol had a deleterious effect at many different time periods of exposure, indicating that there is likely no safe time during development to consume ethanol.

Effects of Ethanol on Behavior in Rodents

Other research has examined the effects of neonatal and/or gestational ethanol exposure on behavior. In one study, Sprague-Dawley rats exposed to ethanol (36% of total calories in a liquid diet) throughout all of gestation, and tested on the Morris Water Maze in adolescence, showed specific deficits in spatial learning (Gianoulakis, 1990). These deficits included longer performance latencies, longer distances swam to the platform, and less concentrated searching behavior following platform removal. Mice pups (Ail4 tdTomato reporter mice) born to dams that voluntarily consumed ethanol throughout all of gestation, via a 2 bottle choice drinking procedure with free access to water and a 20% ethanol solution (BECs: 23.8 mM), also demonstrated increased hyperactive behavior and locomotor activity into adulthood (PD133), when compared to water drinking controls (Cheng et al., 2018). These data indicate that, even with ethanol exposure limited to gestation, behavioral deficits are present later in life.

Sprague-Dawley rats neonatally intubated with ethanol from PD4-PD9 (BECs: 63 mM) also demonstrated several deficits in the Morris Water Maze, especially at higher ethanol doses. These deficits included difficulties with acquisition of the task, and a less localized search pattern in the probe trial (Goodlett & Johnson, 1997). In another experiment, Wistar rats prenatally (all of gestation) and/or neonatally (until PD25) exposed to ethanol, via exposure of dams during both gestation and lactation (measured PD21 of lactation: 24.6 ± 13 mM [dams], 25.5 ± 4.3 mM [fetuses]), had deficits in spatial working and object recognition reference memory (Popovic et al., 2006). These deficits were most severe when the ethanol exposure was given neonatally and abruptly ended, via fostering with non-ethanol treated dams. Furthermore, ethanol exposure resulted in greater deficits in more complicated tasks of object discrimination and spatial learning, in comparison to the deficits seen in simpler tasks (Popovic et al., 2006).

Neonatal exposure to ethanol (PD4-PD9) via intragastric intubation (BECs: 90.7 ± 2.1 mM, measured PD4) also resulted in decreased novel object exploration in young adult Long-Evans rats;

however, this was only seen when there was a short delay between the exposure periods to the objects (MacIlvane et al., 2016). Long-Evans rats neonatally (PD4-PD9) intubated with either 4.00 g/kg or 5.25 g/kg ethanol (BECs: 4.00 g/kg – 81.4 ± 2.6 mM, 5.25 g/kg – 103.6 ± 3.1 mM, measured PD4) demonstrated impairments on a contextual fear conditioning paradigm at PD31 (Murawski et al., 2012). Long-Evans rats intragastrically intubated with ethanol from PD4 to PD9 (BECs: 29 ± 1.1 mM, measured PD4), and tested for a context pre-exposure facilitation effect, had decreased post-shock and retention-test freezing when compared to controls, as well as disrupted intermediate-early gene expression in the mPFC during context pre-exposure, an effect which was not seen in the hippocampus (Heroux et al., 2019). When neonatal C57BL/6 mice were subcutaneously injected with ethanol for either a single day (PD7) or three days (PD7-9) (BECs: PD7 – 112.2 mM), ethanol exposure induced mild difficulties in spatial learning during adolescence, and much greater deficits in adulthood (Wagner et al., 2014).

When astrocytes were examined *in vitro,* taken from ferrets intraperitoneally injected with 3.5 g/kg ethanol every other day between PD10 and PD30 (BECs: 59.4 mM), ethanol induced a long-lasting alteration of the astrocyte secretome (Trindade et al., 2016), including alterations in ECM proteins, such as laminin subunits, and Cadherin-1 and Cadherin-11 (Trindade et al., 2016). Lastly, *in vitro* cultures from fetal Wistar rats of dams fed 5% weight/volume ethanol throughout gestation (BECs: 22.5 ± 12 mM [dams], 23.3 ± 4 mM [fetuses]) had a decreased radial glia cell progenitor pool, as well as a downregulation of maintainers of radial glial progenitor states; Notch1 and Fibroblast Growth Factor Receptor 2 (Rubert et al., 2006). Overall, these data indicate that, not only does ethanol exposure change brain structure and function, of both individual cells and areas of the brain as a whole, but the changes induced by ethanol exposure in the brain also have distinct and, in some cases, long-lasting impacts on behavior.

Sex Differences in Rodent *In Vivo* Responses to Ethanol

Some studies examining FASD in rodent models have also demonstrated sex differences following neonatal exposure to ethanol. For example, when Long-Evans rats were intubated with ethanol from PD7-9 (BECs: 95.6 ± 3.8 mM, measured PD7) and tested via the Morris Water Maze at PD70, female animals did not have the deficits that male animals did in spatial learning and memory (Johnson & Goodlett, 2002). In contrast, in Sprague-Dawley rats exposed to ethanol from PD4-6, PD7-9 or PD4-9 (BECs: 54.7 mM – 64.5 mM, measured PD6 and PD9), both females and males performed worse on the Morris Water Maze in the PD4-9 ethanol exposure paradigm, when compared to controls of the same exposure timing (Goodlett & Peterson, 1995). However, males also had a worse performance following the PD7-9 only exposure, while females did not, indicating a possible sex-specific resistance to the effects of ethanol on spatial learning behavior. Sprague-Dawley rats of both sexes given ethanol from PD4 to PD10 via either a 7.5% v/v ethanol solution for 4 of 12 feedings or via a 2.5% v/v ethanol solution for all 12 feedings (BECs: 7.5% v/v ethanol – 73.2 ± 5.5 mM, 2.5% v/v ethanol – 14.6 ± 3.6 mM, measured PD6 and PD7), and tested on the Morris Water Maze at PD90, showed deficits in performance (Kelly et al., 1988). However, this impairment was specific to female rats, and specific to when ethanol was given as a 7.5% v/v solution for 4 of 12 total feedings. As there are sex-specific differences in rodent models of FASD, there may also be sex-specific differences in humans with FASD, although the mechanisms behind these potential differences are unclear.

Astrocytes in Brain Development

Research into FASD has also examined *in vitro* models of ethanol exposure. These models must account for non-neuronal cell types, such as glia, as there is increasing evidence that glia are heavily involved in the development and function of the brain (Allen & Eroglu, 2017; Allen & Lyons, 2018; Farhy-Tselnicker & Allen, 2018; Silver & Miller, 2004). For example, astrocytes, a kind of glia, are vital for neuronal growth, as neurons grow significantly more when cultured with astrocytes than when they are cultured alone (Giordano et al., 2009; Guizzetti et al., 2008), an effect which was more significant in

neonatal astrocytes than injured adult astrocytes (Geisert & Stewart, 1991). Furthermore, astrocytes do more than just increase the growth of cultured neurons, as culturing retinal ganglion cells with mixed glia, including astrocytes, resulted in a potentiated frequency and amplitude of spontaneous post-synaptic currents, as well as decreased amounts of transmission failures, resulting in more efficient and functional synapses (Pfrieger & Barres, 1997).

Astrocytes also provide support for neurons by expressing a number of extracellular matrix (ECM) proteins, including fibronectin and laminin, providing a framework for support of nearby cells. Astrocytes can thereby alter the neuronal environment and increase neurite outgrowth (Moore et al., 2009). Both ECM proteins and subsequent changes in neurite outgrowth are increased when neurons are cultured with astrocytes treated with the cholinergic agonist carbachol, indicating that astrocytes secrete factors of the ECM that induce neurite outgrowth (Guizzetti et al., 2008). Carbachol also increases the proliferation of both rat astrocytes and human astrocytoma cells (Guizzetti et al., 1996), which results in axonal elongation of neocortical pyramidal neurons, but not cerebellar granule neurons. Another study showed that immature, but not mature, astrocytes secrete the ECM proteins thrombospondins 1 and 2, which in turn increase synaptogenesis (Christopherson et al., 2005). Astrocytes are also responsible for the uptake of proBDNF from the synaptic space, regulating its availability and capacity to stimulate neuronal growth (Bergami et al., 2008). These data therefore describe the roles that astrocytes play in neurite outgrowth and synaptogenesis and, while astrocytes have more roles than those described here, these data demonstrate that astrocytes are involved in brain development and function, and can influence neurons and the neuronal environment.

Astrocytes in FASD

Research also shows that astrocytes are specifically involved in the brain's response to ethanol exposure (Guizzetti et al., 2014; Wilhelm & Guizzetti, 2016). For example, in human astrocytoma cells, *in vitro* exposure to 10-100 mM ethanol for 24h decreased astrocyte proliferation induced by carbachol

(Guizzetti et al., 2004). *In vitro* treatment of astrocytes for 24h with 25 mM, 50 mM or 75 mM ethanol also reduced neurite outgrowth in hippocampal pyramidal neurons cultured with these astrocytes (Zhang et al., 2014b). *In vitro* ethanol exposure (25-100 mM, 24h exposure) also decreased carbachol treated astrocyte-induced increase in neuritogenesis, specifically via inhibition of astrocytic release of the ECM proteins fibronectin and laminin, and plasminogen activator inhibitor-1 (PAI-1), a protein involved in the plasminogen activator system (Guizzetti et al., 2010). Another study, culturing neurons in astrocyte-conditioned media, showed that media taken from astrocytes exposed to 100 mM ethanol produced smaller amounts of neurotrophic factors, resulting in decreased neurite outgrowth (Kim & Druse, 1996). Lastly, 100 mM ethanol (24h exposure) treated *in vitro* astrocytes have altered signaling with neurons, increasing release of neuronal interferons, part of the innate immune signaling of the brain (Lawrimore et al., 2019).

In contrast, some studies have shown that astrocytes can protect against the effects of ethanol. One such study showed that astrocytes treated *in vitro* with 2.5 or 4.0 mg/mL ethanol for up to 24h had decreased cortical neuronal reactive oxygen species, and prevented neuronal apoptosis and glutathione reductions, indicating protection from oxidative stress by astrocytes (Watts et al., 2005). Furthermore, conditioned media from ethanol-treated (400 mg/dL, up to 6 days of exposure) cortical astrocytes prevented ethanol-induced decreases in neurite outgrowth, even when the neurons were continually exposed to ethanol throughout the culture period, an effect which is likely due to protective factors secreted by the astrocytes following ethanol exposure (Yanni et al., 2002). Astrocytes have therefore been shown to be involved in both normal brain development and the response of the brain to ethanol exposure, and are of interest to FASD research.

Effects of Ethanol *In Vitro*

Further *in vitro* research has analyzed the effects of ethanol on both astrocytes and neurons, to further the knowledge of the mechanisms behind the effects seen of ethanol exposure *in vivo.* Some *in*

vitro experiments have examined the effect of ethanol directly on hippocampal neurons. For example, ethanol exposure (200, 400, 600 mg/dL, 6 days of exposure) inhibits dendritic outgrowth of hippocampal pyramidal neurons, decreases both the total and mean dendritic length, induces a slightly higher number of dendrites per cell, and increases the number of processes less than 20 μm in length (Yanni & Lindsley, 2000; Yanni et al., 2002). A reduction in axonal outgrowth was also seen when rat hippocampal pyramidal neurons were exposed to astrocyte conditioned media containing ethanol (50-75 mM, 24h exposure) (VanDeMark et al., 2009b). In particular, this decreased axonal outgrowth was specifically due to ethanol-induced inhibition of protein kinase C (PKC) activity and extracellular signal-regulated kinases (ERK) 1/2 phosphorylation, indicating ethanol may affect specific signaling pathways (VanDeMark et al., 2009b).

However, another study that exposed neurons to ethanol (50, 100, 200, 400 mg/dL) via astrocyte conditioned media, rather than exposing neurons directly to ethanol, showed an increased overall rate of axon elongation following ethanol exposure, as a result of reduced retraction of axons during saltatory growth (Lindsley et al., 2003). The primary difference between this and the previous study, in which ethanol decreased axonal outgrowth, was the timing of ethanol exposure, as the first study added media with ethanol 30 minutes after plating the neurons (VanDeMark et al., 2009b), and the second study added ethanol 7 hours after plating the neurons (Lindsley et al., 2003). Thus, these data indicate that one of the factors influencing the effect ethanol has on neurons is the timing of administration.

Ethanol has also been shown to alter the function of carbachol. In control conditions, carbachol increases intracellular calcium. However, neurons directly exposed to ethanol (50 mM – 75 mM, 24h exposure) did not show this increase (VanDeMark et al., 2009b). In another experimental paradigm, in which fetal rat hippocampal pyramidal neurons were treated directly with 200-600 mg/dL ethanol for 6 days, after which the ethanol was removed and the neurons were examined at 14 days in culture,

ethanol decreased the overall dendritic arbor size (Lindsley & Clarke, 2004; Yanni & Lindsley, 2000), and increased cell death (Lindsley et al., 2002). Hippocampal neurons are therefore affected by ethanol exposure in a variety of ways, including alteration of neuronal morphology and neurite outgrowth.

The effects of ethanol *in vitro* are not limited to hippocampal neurons, as other neuronal populations, including cortical neurons, are also affected by ethanol exposure. For example, cortical neurons exposed to 50 mM ethanol for 24h *in vitro* had increased activity of Poly-ADP Ribose Polymerase (PARP), an enzyme involved in epigenetics, which in turn reduced mRNA expression of several downstream genes, including BDNF (Gavin et al., 2016). In rat embryonic cortical neurons ethanol altered biochemical signaling involved in dendrite growth, as ethanol initially activated Src Family Kinases, and Reelin-Dab 1 downstream. However, this activation was transient, as it was followed by a longer inactivation (Wang et al., 2019). This inactivation could potentially decrease filopodia speeds, resulting in limited neurite outgrowth. The effects of ethanol are therefore not limited to a specific population of neurons.

Studies have also examined the effects of ethanol on non-neuronal cell types, including astrocytes. Astrocytes treated *in vitro* with ethanol showed a decrease in the neurite length of hippocampal neurons cultured with these treated astrocytes (Zhang et al., 2014b). Ethanol also decreased activity of astrocytic Arylsulfatase-B (ARSB), an enzyme which degrades proteins inhibitory to neurite outgrowth, and increased the amount of sulfated glycosaminoglycans (sGAGs), proteins which inhibit to neurite outgrowth, indicating an ethanol treatment-induced alteration of the ECM, which could result in an environment that inhibits neurite outgrowth (Zhang et al., 2014b). Furthermore, simultaneously culturing astrocytes and fetal rat hippocampal pyramidal neurons, and treating these cells with 300 or 800 mg/dL ethanol shortly after culturing, increased the proportion of neurons with axons (Clamp & Lindsley, 1998; Lindsley et al., 2002). However, when 400 mg/dL ethanol was given at a later time points, ethanol exposure decreased the total dendrite length per cell, once again indicating

that the timing of ethanol exposure influences the effect ethanol has on neuronal morphology (Lindsley et al., 2002). Therefore, ethanol can significantly alter neuronal morphology *in vitro*.

Thus, there is ample evidence showing that ethanol can have effects both *in vitro* and *in vivo,* including altering dendritic morphology, neurite formation and growth, and gene expression. These data also show that the effect of ethanol on neurons can be modulated by astrocytes which are, themselves, affected by ethanol exposure. As there are substantial changes in neuronal morphology and growth following ethanol exposure, it is possible that some of the CNS dysfunctions seen following gestational ethanol exposure are due to neuronal alterations caused by the ethanol exposure.

FASD Treatments: Preclinical and Clinical

Voluntary Exercise

There are a number of potential treatments for FASD, one of which is voluntary exercise. In rat models, exercise and motor training reduced ethanol-induced working memory impairments, increased Long-Term Potentiation (LTP) induction in the dentate gyrus (ethanol exposure during gestation, BECs: 43.7 ± 11.9 mM) (Christie et al., 2005), reversed ethanol-induced decreases in cerebellar paramedian lobule volume, and increased the number of parallel fiber synapses per Purkinje cell (ethanol exposure from PD4-PD9, BECs: 53.4 ± 1.9 mM, measured on PD6) (Klintsova et al., 2002). Voluntary exercise in adult rats also improved motor performance deficits caused by neonatal (PD4-PD9) ethanol exposure (BECs: 62.6 ± 2.8 mM, measured PD6) (Klintsova et al., 1998). In adolescent rats, voluntary exercise reversed the neonatal (PD4-PD9) ethanol-induced decrease in the spine density and basilar dendrite length and complexity of mPFC Layer II/III neurons (BECs: 95.1 ± 2.9 mM, measured PD4) (Hamilton et al., 2015). Voluntary exercise and environmental complexity given during adolescence also reversed the dendritic simplification in rat immature dentate gyrus granule cells induced by neonatal (PD4-PD9) ethanol exposure (BECs: 78 ± 3.6 mM, measured PD4), and increased methylation of *Bdnf* exon I

(Boschen et al., 2016a). Thus, exercise has shown promise as a potential treatment for ethanol-related deficits in rodents, even when the exercise occurs after the ethanol exposure has finished.

Choline

Studies have also shown that maternal diet, including differing levels of essential nutrients during gestation, can modify the severity of FASD in rats (Idrus et al., 2016). As a result, there is an increasing amount of research examining dietary factors as a potential treatment for FASD, both preclinically and clinically. One such dietary factor is choline, a nutrient found in many foods, including liver and wheat germ (Jiang et al., 2014; Li & Vance, 2008; Niculescu & Zeisel, 2002; Zeisel & Niculescu, 2006). Choline, like ethanol, can cross the placenta and BBB, and thereby also impact the brain (Allen & Lockman, 2003; Cornford et al., 1978; Lockman et al., 2001; Mooradian, 1988; Radziejewska & Chmurzynska, 2019; Sawada et al., 1999; Shimon et al., 1988; Wecker & Trommer, 1984). Such impacts include greater recovery and neuronal plasticity following induced stroke in adult male rats (Hurtado et al., 2007), anti-inflammatory effects modulated by the nicotinic acetylcholine receptor (Parrish et al., 2008), larger somata, greater basilar dendrite arborization and reduced slow afterhyperpolarization in CA1 hippocampal pyramidal neurons (Li et al., 2004).

Of particular interest regarding choline research is the fact that, in both humans and rodents, there is an increased need for choline during pregnancy and lactation, although few human women reach the recommended levels of choline during these times (Jiang et al., 2014). Furthermore, there is evidence that inadequate choline in dams can increase the severity of gestational ethanol exposure (GD5-GD20, BECs: 56.8 ± 3.7 mM, dams, measured GD20) in rat pups born to these choline-deprived dams (Idrus et al., 2016). As choline supplementation has been shown to have beneficial effects, both in rodents and in humans, a lack of choline can compound the effects of ethanol, and there is an increased need for choline during gestation, research has examined the possibility of choline as a treatment for FASD.

Preclinical Choline Research

Some of this research has been on how choline can modify the molecular and biological changes induced by ethanol exposure. For example, neonatal rat cerebellar granule neurons exposed to 25mM ethanol for 24h *in vitro* had redistributed L1 cell adhesion molecules into lipid rafts, indicating an alteration in neurite outgrowth downstream, an effect which was partially prevented by 40 μm choline treatment (Tang et al., 2014). Choline also partially prevented the ethanol-induced decrease in rat cerebellar granule cell neurite length (Tang et al., 2014). Rats intragastrically intubated with ethanol from PD4-PD9 (BECs: 88.2 ± 3.637 mM, measured PD6), and treated with 100 mg/kg/day choline from PD4-PD21, demonstrated a reduction in the ethanol-induced microRNA variability in the hippocampus (Balaraman et al., 2017).

In rats given ethanol from PD2-PD10 (BECs: 84.5 ± 4.8 mM, measured PD6) and 100 mg/kg/day choline from PD2-PD20, ethanol altered epigenetics by inducing hypermethylation in the hippocampus and the PFC; choline, by contrast, reduced this hypermethylation (Otero et al., 2012). This study also showed that non-ethanol exposed rats treated with choline alone had increased methylation in the same brain regions, indicating choline has an effect even when ethanol is not present. Furthermore, male rats treated gestationally (GD7-GD21) with both 6.7% v/v ethanol and 642 mg/L choline (BECs: 79.4 ± 4 mM, dams), and examined in adulthood, had choline-induced normalization of ethanol-altered histone modifications and DNMT-induced methylation of the hypothalamic neuron proopiomelanocortin gene (Bekdash et al., 2013). Ethanol exposure in rats (5.25 mg/kg/day) from PD4-PD9 (BECs: 79.4 ± 4 mM, measured on PD6) also decreased the density of dorsal hippocampal M1 muscarinic receptors, and increased the density of M2/4 receptors, while choline exposure (100 mg/kg/day) from PD4-PD30 rescued the effect of ethanol on the M2/4 receptors (Monk et al., 2012). Choline has therefore been shown to rescue some of the biological and mechanistic alterations induced by ethanol exposure.

Behaviorally, choline treatment (PD2-PD21, 25 mg/mL in saline) in rats improved ethanol-induced deficits (35% of calories from a liquid diet of ethanol, exposure GD6-GD20) in performance on a visuospatial discrimination task, including decreasing the larger amount of acquisition errors seen in ethanol treated rats (Thomas et al., 2000). Even though choline also improved performance in control rats, the beneficial effect of choline was most strongly seen in rats given ethanol and choline in combination. These effects of choline were still seen at PD45, even when the choline exposure was limited to PD2 – PD21, indicating choline exposure can have long-lasting effects (Thomas et al., 2000). Rats given ethanol and choline gestationally (pups born to dams intubated with 6 g/kg/day ethanol and 250 mg/kg/day choline from gestational day 5-20, BECs: 82 mM, measured GD20) were more successful on a working memory variant of the Morris Water maze compared to rats treated with ethanol alone (Thomas et al., 2010). Choline (70% choline chloride solution) also reduced ethanol-induced hyperactivity in male rats given 6.6 g/kg/day ethanol from PD4-PD9 (BECs: 93 ± 6.182 mM, measured PD6) and choline from PD4-PD30 (Thomas et al., 2004a). In this same study, choline reduced the amount of perseverative errors on reversal learning tasks seen following ethanol exposure.

In another experimental paradigm, in which choline (10, 50, 100 mg/kg/day) was given to rats from PD10-PD30, after the 6 g/kg/day ethanol exposure had ended (PD4-PD9, BECs: 79.2 ± 4.4 mM, measured PD6), choline still decreased ethanol-induced hyperactivity and spatial learning deficits, showing that choline can ameliorate ethanol-induced deficits even when choline supplementation is given after ethanol exposure has ceased (Thomas et al., 2007). Rats exposed gestationally to 6 g/kg/day ethanol and 250 mg/kg/day choline simultaneously, from GD5-GD20 (BECs: 54.7 ± 3.7 mM, measured GD20), also had greater brain and body weights than animals given ethanol alone. These rats performed similarly to control animals on a variety of behavioral tasks (righting reflex, geotactic reflex, cliff avoidance, reflex suspension and hindlimb coordination), behaviors that normally have severe deficits following treatment with ethanol alone (Thomas et al., 2009). Choline has also been shown to provide a

rescue effect even in mice born to dams fed a diet lacking in choline. Pups born to dams fed a choline-deficient diet throughout gestation, and given 10 µL of 18.8 mg/mL choline from PD1-PD5, 6 g/kg ethanol on PD5, and choline from PD6-PD20, demonstrated a rescue effect of choline (BECs: 122.9 ± 13.1 mM, 2h post ethanol exposure, 94.4 ± 2.9 mM, 4hr post ethanol exposure) (Bearer et al., 2015).

Some studies have examined if choline's effectiveness in ameliorating the deficits and alterations in behavior and morphology seen following ethanol exposure is restricted to a specific point in development. One such study examined several different time frames of choline exposure, and found that the choline treatment was successful across multiple developmental periods. Specifically, choline rescued the effect of 5.25 g/kg/day ethanol given via intragastric intubation from PD4-PD9 (BECs: 76.1 ± 1.2 mM, measured on PD6) on the Morris Water Maze when 100 mg/kg/day choline was given at PD11-PD20, PD21-PD30, or PD11-PD30 (Ryan et al., 2008).

However, the rescue effects of choline are not global, as not all ethanol-related deficits and impairments are improved by choline treatment. For example, male rats given 6.6 g/kg/day ethanol from PD4-PD9 (BECs: 92.7 mM) and 0.1 mL of an 18.8 mg/mL choline solution from PD4-PD30 had ethanol-induced deficits on the parallel bar motor tasks, deficits which were not improved by choline treatment (Thomas et al., 2004b). Furthermore, 5.25 g/kg/day neonatal ethanol exposure (PD4-PD9) induced deficits in working memory and overactivity in the open field test. However, while 100 mg/kg/day choline treatment from PD40-PD60 did reduce the deficits in working memory, it did not change ethanol-induced alterations on the open field test (Schneider & Thomas, 2016). Lastly, postnatal choline treatment (PD4-PD20. 0.1 mL of an 18.8 mg/mL solution) did not ameliorate the impaired response habituation seen in rats treated with 5 g/kg/day ethanol neonatally (PD4-PD9) (Hunt et al., 2014). These data demonstrate that the effects of choline may be specific to certain deficits seen in preclinical models of FASD.

Clinical Trials of Choline

Preclinical research demonstrating choline's effects on ethanol-induced deficits has led to choline-related clinical trials in humans (Akison et al., 2018). These have found that, in children with FASD aged 2.5-5 years, 9 months of choline supplementation (500 mg) improved performance on a hippocampal dependent memory task (elicited imitation), an effect which was more significant in younger individuals (Wozniak et al., 2015). Choline, when given via supplement (750 mg) and in combination with multivitamins to ethanol-drinking pregnant women, also induced a greater change in heart rate over time in newborns tested on visual habituation trials, indicating increased success in differentiating old and novel objects (Kable et al., 2015). Furthermore, when pregnant women who were confirmed heavy drinkers were given 2 g/day choline supplements during pregnancy, choline increased eye blink conditioning in newborns; these newborns also had a greater preference for novel objects, indicating better visual and recognition memory (Jacobson et al., 2018). Despite this, not all results support choline's efficacy as a treatment for FASD, similar to the results of preclinical studies. For example, choline supplementation in children between 5 and 10 years of age with FASD did not change ethanol-induced cognitive dysfunctions (Nguyen et al., 2016).

However, overall, a combination of preclinical and clinical research supports the potential for choline to be a treatment for FASD, as many studies have shown that choline can provide a rescue effect that ameliorates some of the effects of gestational ethanol exposure.

Choline: Potential Mechanisms of Action

There are several potential ways through which choline could be exerting its influence (Fig. 1.1), as choline has multiple primary mechanisms of action (Zeisel & Niculescu, 2006). First, in cholinergic neurons, choline can be metabolized into the neurotransmitter acetylcholine, thereby replenishing the supply, as acetylcholine is enzymatically inactivated and needs to be resynthesized (Klein, 2005). Choline can also be converted, in the liver and kidneys, to betaine which, in turn, can be converted into

methionine, and then the universal methyl donor S-adenosylmethionine (SAM) (Jiang et al., 2014; Niculescu & Zeisel, 2002; Zeisel & Niculescu, 2006).

The third primary pathway is the conversion of choline into phosphatidylcholine (PC) (Li & Vance, 2008). PC is a major membrane phospholipid that provides membrane stabilization and integrity, assists in neurogenesis, synaptogenesis and cell proliferation, and increases a cell's capacity for plasticity (Jiang et al., 2014; Li & Vance, 2008; Radziejewska & Chmurzynska, 2019). PC can also be incorporated into the cell membrane (Li & Vance, 2008; Radziejewska & Chmurzynska, 2019). However, once in the cell membrane, PC can be hydrolyzed, via phospholipase D (PLD), into choline and phosphatidic acid (PA), an important second messenger (Guizzetti et al., 2014). PA has a variety of downstream actions, including cell proliferation, cytoskeletal reorganization, vesicular trafficking, neurite extension, dendritic branching and activation of protein kinase A (Zhu et al., 2016). Interestingly, ethanol is also a substrate for PLD, as it can be used in a transphosphorylation reaction that converts PC into choline and phosphatidylethanol (PEth) (Guizzetti et al., 2004). As this reaction produces PEth in place of PA, PA is no longer available to act downstream on any of the aforementioned processes, making this potentially a way through which ethanol exposure could be altering neuronal morphology and brain development. More research is needed to elucidate the specific mechanisms by which choline can affect the brain and alter the effect of ethanol.

Tissue Plasminogen Activator (tPA)

tPA in the Brain

Research regarding FASD must also examine how ethanol affects the developing brain. One potential way ethanol could be having its effects is through tissue plasminogen activator (tPA) (Chevilley et al., 2015; Melchor & Strickland, 2005), a serine protease that can have a variety of effects within the brain (Hebert et al., 2015; Lee et al., 2015; Lee et al., 2017; Samson & Medcalf, 2006; Wiera & Mozrzymas, 2015). For example, tPA assists in the movement of the growth cone, regulating the neurite

outgrowth of developing neurons (Garcia-Rocha et al., 1994). However, inhibition of tPA increased neurite outgrowth in cultures of neonatal rat superior cervical ganglia neurons, indicating a context-specific effect of tPA on the growth cone (Pittman et al., 1989). tPA has also been shown to be epigenetically regulated, as expression is increased by inhibition of histone deacetylation in human neurons and astrocytes (Olsson et al., 2016).

tPA also has a role in learning, as inhibition of tPA impaired late phase LTP in rat hippocampal slices, whereas exogenous tPA increased late phase LTP (Baranes et al., 1998). Furthermore, in fetal mouse cortical neuronal cultures, recombinant tPA downregulated NMDA receptor signaling via increased activation of epidermal growth factor receptor (EGFR) signaling (Bertrand et al., 2015). Through this mechanism, tPA mediated NMDA receptor-induced excitotoxicity and EGRF-induced anti-apoptotic effects. tPA has also been shown to prevent p35 degradation in the post-synaptic terminal, activating cyclin dependent kinase 5 and subsequently modulating the amount of AMPA receptors in the post-synaptic terminal, thus altering signaling (Diaz et al., 2019).

When examined *in vivo,* tPA can regulate neuronal migration, as $tPA^{-/-}$ mice had fewer granule cells migrating through the cerebellar molecular layer (Seeds et al., 1999). Also in the cerebellum, increased tPA decreased dendritic growth in Purkinje neurons, and impaired synaptogenesis between Purkinje neurons and parallel fibers (Li et al., 2013). $tPA^{-/-}$ mice in a different study (Imamura et al., 2010) had dysregulated osmotic homeostasis, indicating an increase in cerebrovascular permeability, including higher plasma osmolarity after water deprivation, and had less secretion of arginine-vasopressin from the neurohypophysis, which was reversed by addition of a recombinant tPA protein. Furthermore, intravenous administration of recombinant tPA to adult male rats increased anxiety-like behaviors three months later, the underlying pathogenesis of which was ERK1/2-GAD1-GABA cascade in the hippocampus (Dong et al., 2018).

Of particular interest is the conversion by tPA of plasminogen into plasmin (Fig. 1.2) (Salles & Strickland, 2002; Samson & Medcalf, 2006). Plasmin is a protease that can degrade several ECM proteins, including laminin and fibronectin (Guizzetti et al., 2010; Hebert et al., 2015), thus altering the neuronal environment and its capacity to support neurite outgrowth. Plasmin can also cleave the proteoglycan DSD-1/phosphacan, and prevent neurite extension in the supragranular/molecular border of the dentate gyrus (Wu et al., 2000). After excitotoxic injury in mice, there is a transient increase of tPA in the mossy fiber pathway of the hippocampus, followed by a decrease and subsequent increase in plasminogen levels, (Salles & Strickland, 2002). tPA's activation of plasmin in mice is also involved in the stress-induced decrease in NMDA receptors, as $tPA^{-/-}$ and $plasminogen^{-/-}$ mice did not have this decrease (Pawlak et al., 2005a). These data therefore indicate that tPA and plasmin are potentially involved in regulation of neuronal plasticity.

Interestingly, the amount of tPA present is likely a determinant in its action. For example, large amounts of tPA can have a neurotoxic effect, as a perfusion of recombinant tPA into the cortex of rats disrupted the BBB and caused lesions, the size of which increased in correlation to tPA concentration (Goto et al., 2007). By contrast, $tPA^{-/-}$ mice had decreased hippocampal neuronal degeneration following exposure to excitotoxins, and fewer seizures following pharmacological activation (Tsirka et al., 1995). Differing amounts of tPA are also involved in diseases, as a mouse model of Alzheimer's Disease had lower amounts of tPA in the cortex and subcortex while a mouse model of spinocerebellar ataxia type-1 had increased cerebellar tPA (Sashindranath et al., 2011). In another study describing the role of tPA in ischemic stroke, pigs given an exogenous variant of tPA that does not bind to NMDA receptors had reduced hippocampal neuronal necrosis in both the CA1 and CA3 (Armstead et al., 2017). Furthermore, expression of tPA in mice was increased following spinal cord injury, and $tPA^{-/-}$ mice had reduced neurite outgrowth and motor recovery (Bukhari et al., 2011).

tPA is also released by PLD1, which may be involved in temporal lobe epilepsy, as tPA is heavily concentrated at growth cones during excitatory events (Zhang et al., 2005). ERK1/2 activation by tPA also activated cAMP response element binding protein and activating transcription factor 3, resulting in tPA-induced protection from excitotoxin-induced neuronal death (Wu et al., 2013). tPA application also increased regeneration of axons and levels of Matrix Metalloproteinase 9-positive macrophages 7 days after sciatic nerve crush injury in mice (Zou et al., 2006). When examining sciatic nerve injury, tPA deficient mice had fewer migrating macrophages and delayed axonal regeneration (Ling et al., 2006). Mice overexpressing tPA also had better performance on spatial learning tasks than controls (Madani et al., 1999), and $tPA^{-/-}$ mice did not have stress-induced spine retraction in the medial amygdala (Bennur et al., 2007). Therefore, tPA can have a variety of impacts on the brain, an impact which depends on many factors, including the area of the brain and the amount of tPA.

tPA and Ethanol

tPA is involved in the response to ethanol exposure, as tPA protein and mRNA levels were upregulated by ethanol, both *in vivo* (5 g/kg/day via intragastric intubation) and *in vitro* (75 mM ethanol, 24h exposure) (Wilhelm et al., 2018). Due to this, tPA is of particular interest to research involving the effect of ethanol on the brain, including FASD research. For example, in a mouse model of gestational ethanol exposure (two subcutaneous injections of 2.5 g/kg ethanol on PD7), the neuronal loss seen following ethanol exposure was completely eliminated in $tPA^{-/-}$ mice, while $tPA^{+/-}$ mice had intermediate neuronal loss (Noel et al., 2011). In another experiment, 3 month old male $tPA^{-/-}$ mice (Skrzypiec et al., 2009) did not exhibit neurodegeneration following ethanol withdrawal (14 days of up to 10% ethanol liquid diet, ethanol withdrawal began day 15, BECs: wildtype – 43 ± 11 mM, $tPA^{-/-}$ - 42 ± 9 mM). Wildtype mice expressing tPA normally did show this neurodegeneration, an effect which was specifically due to the tPA-induced increase in plasmin, and resulting degradation of laminin. tPA expression and release were increased in astrocytes exposed *in vitro* to ethanol (25, 50, 75 mM, 24h

exposure), due to ethanol-induced decrease in DNA methylation (Zhang et al., 2014a). Overall, ethanol exposure, both *in vitro* and *in vivo*, has been shown to alter the brain. As tPA has also been shown to affect the brain, and is increased following ethanol exposure, tPA is likely involved in how ethanol induces these alterations (Zhang et al., 2014b).

tPA and Astrocytes

Astrocytes, which have been shown to be involved in FASD, are also potential regulators of tPA (Hebert et al., 2015). Specifically, tPA is involved in the cross-talk between astrocytes and neurons, potentially by functioning as a gliotransmitter (Casse et al., 2012). Astrocytes can endocytose neuron-released tPA through the low-density lipoprotein-related protein receptor, and exocytose tPA in a glutamate-controlled manner (Casse et al., 2012). The extracellular levels of plasmin near astrocyte cell surfaces can induce the astrocytes to also uptake both plasminogen and plasmin through actin-mediated endocytosis (Briens et al., 2017). *In vitro*, multipotent mesenchymal stromal cell application to astrocytes induced tPA release and reduced PAI-1, resulting in an astrocyte-mediated increase in neurite outgrowth (Xin et al., 2010). Astrocytes can also act as a surface for activation of plasmin by tPA by presenting binding molecules specific to tPA and plasminogen, resulting in downstream fibrinolysis and BDNF conversion (Briens et al., 2017). Furthermore, within astrocytes, plasminogen and plasmin are degraded by lysosomes, resulting in an overall control of the balance of plasmin levels by astrocytes (Briens et al., 2017). Lastly, astrocytes are the primary CNS cell type that express PAI-1, which can regulate tPA and PAI-1 itself upregulated in pathological conditions, such as stroke (Ko et al., 2015). It is worth noting that *in vitro* astrocytes express more tPA mRNA than *in vivo* whole brain homogenates, indicating that astrocytes may produce more tPA than other cell types of the brain, including neurons (Wilhelm et al., 2018).

Astrocytes are also likely involved in tPA's response to ethanol exposure. For example, in control astrocytes, DNMT usually methylates the promotor region of tPA, thereby preventing its

expression and release (Guizzetti, 2015). However, in astrocytes treated *in vitro* with 25 – 75 mM ethanol, there is an inhibition of DNMT, resulting in decreased methylation and increased tPA (Zhang et al., 2014a). An increase of tPA would then convert plasminogen into plasmin, and decrease neurite outgrowth via changes in ECM protein proteolysis levels (Wilhelm et al., 2018). Ethanol exposure also inhibited astrocytic PAI-1 (25-100 mM, 24h exposure) (Guizzetti et al., 2010), resulting in an increase in tPA levels (Wilhelm et al., 2018), and a subsequent alteration of neurite outgrowth caused by ethanol exposure, specifically as mediated by astrocytes (Guizzetti et al., 2014). Therefore, not only are astrocytes possibly involved in FASD, they likely also mediate the effect of ethanol-induced increases in tPA. This increase of tPA, in turn, is potentially the cause for some of the brain alterations and deficits seen in individuals with FASD.

Overall, these data have shown that gestational ethanol exposure has long-lasting, pervasive effects on the individual. Some of these effects may be due, in part, to astrocyte-mediated increases in tPA caused by ethanol exposure. Data also indicate that choline may be a treatment for FASD. This dissertation utilized these data to arrive at the hypothesis that astrocyte-mediated, ethanol-induced increases in tPA are involved in the alterations of neuronal morphology seen following ethanol exposure, as well as the hypothesis that choline can ameliorate the effects of ethanol, and may also be mediated by astrocytes. Experiments were performed *in vitro* to isolate the mediating effect of astrocytes, as well as *in vivo,* to examine neuronal morphology in a rodent model of FASD.

Goals of the Dissertation

The first set of experiments (chapter 2) tested astrocytic modulation of the effects of ethanol and choline on neurons via an *in vitro* astrocyte-neuron co-culture system (Roque et al., 2011; Zhang et al., 2014b), in order to specifically isolate how astrocytes mediated the effects of the treatments. Astrocytes mediated an ethanol-induced decrease in neurite outgrowth, but did not mediate an effect

of choline alone on neurite outgrowth. In contrast to this, astrocytes did mediate a rescue effect of choline on the ethanol-induced decrease in neurite length.

As choline did provide a rescue effect *in vitro* in the experiments of chapter 2*,* experiments in chapter 3 analyzed *in vivo* morphological changes following neonatal ethanol and choline exposure in a rat model of FASD. Rats were neonatally intubated with ethanol and/or subcutaneously injected with choline (Goodlett & Peterson, 1995; Goodlett & Johnson, 1997) during the rat equivalent of the human third trimester brain growth spurt (PD4-PD9) (Driscoll et al., 1990; Fontaine et al., 2016; Patten et al., 2014; Rice & Barone, 2000; Semple et al., 2013). In both female and male hippocampal pyramidal neurons, ethanol significantly increased parameters of apical dendrite morphology, while choline induced a decrease in some parameters of basilar dendrite morphology. This indicates that ethanol may be inducing a premature maturation or altering connectivity, and choline may be delaying or prolonging the maturation period, or altering the connectivity in an opposite manner.

As choline did not have any rescue effect on ethanol-induced changes in hippocampal neuronal morphology in PD9 rats, despite previous research showing such a rescue effect (Balaraman et al., 2017; Otero et al., 2012; Thomas et al., 2000; Thomas et al., 2004a; Thomas et al., 2007; Thomas et al., 2008; Thomas et al., 2009; Thomas et al., 2010), the next step was to determine if a longer period of choline exposure could have a rescue effect on ethanol-induced morphological changes. Thus, experiments in chapter 4 were similar to those in chapter 3, with ethanol exposure from PD4-PD9. However, choline exposure was from PD4-PD30. While there was only a small effect of ethanol, a decrease of female basilar terminal orders in choline exposed animals also given ethanol, there was a sex-specific effect of choline, as choline treatment decreased parameters of male apical dendrite morphology, in comparison to both male controls and female animals given choline.

As described earlier, tPA can regulate neurite outgrowth, and ethanol-induced changes in tPA may contribute to some brain alterations and deficits in individuals with FASD. The last set of

experiments (chapter 5) therefore examined astrocytic tPA's involvement in the effects of ethanol *in vitro*. These experiments utilized the same astrocyte-neuron co-culture system as in chapter 2, treating only the astrocytes, in order to isolate astrocyte-mediated effects on neuronal morphology. Decreasing levels of *Plat* in astrocytes decreased neurite outgrowth, as did exposure to ethanol alone. rtPA also decreased neurite outgrowth, replicating the effects of ethanol. However, decreased *Plat* levels in combination with ethanol brought the levels of neurite outgrowth back to the length of control neurites; no effects were mediated by astrocyte cell death.

These experiments tested the hypothesis that ethanol-induced increases in tPA may be responsible for some of the alterations in neuronal morphology seen in FASD, and that choline may be a treatment for FASD. These experiments showed that astrocytes mediated an effect of ethanol *in vitro,* resulting in decreased neurite outgrowth. Astrocytes also mediated, *in vitro,* a rescue effect of choline on the effect ethanol has on neurite outgrowth. Also *in vitro,* astrocytes mediated an effect of both decreased tPA mRNA and increased tPA protein levels on neurite outgrowth. *In vivo,* ethanol induced an increased complexity of apical dendrite arborization on hippocampal CA1 pyramidal neurons at PD9, while no longer having such a large effect at PD30. Choline, by contrast, delayed maturation and decreased basilar complexity at PD9, and resulted in a sex-specific alteration in apical dendrite morphology at PD30. In conclusion, these experiments show that astrocytes can mediate the effect of ethanol, that ethanol alters neuronal morphology; possibly through inducing increases in tPA, and that, under certain conditions, choline may rescue or prevent the effects of ethanol. However, more research is needed to determine the exact specificity of how and in what manner tPA is involved in the brain's response to ethanol, and the efficacy of choline as a treatment for FASD.

CHAPTER 2: EFFECTS OF ETHANOL- AND CHOLINE-TREATED ASTROCYTES ON HIPPOCAMPAL NEURON NEURITE OUTGROWTH *IN VITRO*

This chapter has been reformatted for inclusion in this dissertation from:

Goeke, C., M., Hashimoto, J., G., Guizzetti, M. Effects of ethanol- and choline- treated astrocytes on hippocampal neuron neurite outgrowth in vitro, submitted to 'Alcohol', 2019.

Support: This work was supported by the NIH/NIAAA F31AA024950-01A1 to CG, and by NIH/NIAAA R01AA021468, R01AA022948 and VA Merit Review Award I01BX001819 to MG.

ABSTRACT

Exposure to ethanol *in utero* can result in Fetal Alcohol Spectrum Disorders (FASD), which may cause long-lasting cognitive and behavioral abnormalities. Preclinical studies indicate that choline ameliorates the behavioral effects of developmental ethanol exposure in rodents, and clinical studies on the effectiveness of choline for the treatment of individuals with FASD are ongoing. However, little is known about the mechanisms behind the effects of choline. We have previously reported that astrocyte pre-treatment with 75 mM ethanol for 24h *in vitro* reduces neurite outgrowth in hippocampal neurons co-cultured with the pre-treated astrocytes. Our *in vitro* system allows us to isolate how astrocytes mediate the effects of ethanol and choline on neuronal development, as all treatments are carried out on the astrocytes and are removed prior to co-culture with neurons. In this study, we exposed astrocytes to ethanol and choline, alone and in combination, to test the hypothesis that choline can ameliorate the astrocyte-mediated effects of ethanol on neurite growth. We report that astrocyte exposure to 25 mM, 50 mM, and 75 mM ethanol decreases neurite outgrowth in co-cultured hippocampal pyramidal neurons, while astrocyte treatment with choline alone (10 mM, 50 mM, 100 mM) had no effect on neurite outgrowth. Astrocyte treatment with ethanol and choline (75 mM and 100 mM, respectively) in combination, however, prevented the effect of ethanol, leading to levels of neurite outgrowth similar to the control condition. Changes in neurite outgrowth were not modulated by changes in the osmolality of the medium in which neurons were cultured, or by reduced astrocyte viability. We conclude that choline prevents the inhibitory effect of ethanol-treated astrocytes on neurite outgrowth while not altering normal neuronal development. These results suggest a new, astrocyte-mediated mechanism by which choline ameliorates the effects of developmental ethanol exposure.

INTRODUCTION

In utero ethanol exposure can induce Fetal Alcohol Spectrum Disorders (FASD), which have been increasingly recognized as a major concern in the US, as well as world-wide (Popova et al., 2017). In the United States, FASD prevalence is estimated to be as high as 5% of the population (May et al., 2014; Riley et al., 2011), and is even higher in other communities across the word (Popova et al., 2017; Roozen et al., 2016). The most severe and long-lasting consequences of *in utero* ethanol exposure include cognitive and behavioral dysfunctions that have been associated with neuroanatomical abnormalities (Guizzetti et al., 2014; May et al., 2014; Riley et al., 2011).

Several preclinical studies suggest that choline supplementation may ameliorate the cognitive and behavioral effects of developmental ethanol exposure. For instance, in a rat FASD model equivalent to the third trimester of human gestation, choline supplementation improves ethanol-induced hyperactivity, decreases the number of reversal learning errors in a serial spatial discrimination task (Thomas et al., 2004a), and rescues ethanol-induced impairment of spatial memory in the Morris Water Maze (Ryan et al., 2008). However, choline does not rescue ethanol effects on motor coordination (Thomas et al., 2004b), although choline did ameliorate balance deficits caused by neonatal ethanol exposure in mice (Bearer et al., 2015). Furthermore, low dietary choline exacerbates the effects of developmental ethanol exposure in motor development tasks (Idrus et al., 2016). A comprehensive review of the ameliorating effects of choline in rodent models of FASD has been recently published (Akison et al., 2018). Based on this strong preclinical evidence, clinical trials assessing the effectiveness of choline in the treatment of children with FASD have been carried out, and more clinical trials are recommended before a conclusion on the effectiveness of choline treatments on FASD children can be reached (Akison et al., 2018).

Choline is an essential nutrient found in many foods, including liver, eggs and wheat germ (Zeisel & Niculescu, 2006). Like ethanol, it can pass through both the placenta and the blood brain barrier (BBB), and therefore has the ability to affect the development of the fetal brain (Allen & Lockman, 2003;

Cornford et al., 1978). There is an increased demand for choline during pregnancy, and it is essential for healthy fetal development (Radziejewska & Chmurzynska, 2019). Only a few studies have explored possible mechanisms by which choline may prevent the effects of ethanol during brain development. Choline supplementation decreases ethanol induced hypermethylation in the prefrontal cortex (Otero et al., 2012), and stabilizes ethanol induced micro-RNA variability in the hippocampus (Balaraman et al., 2017). Gestational choline supplementation normalizes the effects of ethanol on DNA methylation, histone acetylation, and the expression of the pro-opiomelanocortin gene in hypothalamic neurons (Bekdash et al., 2013). Choline also prevents the effects of ethanol on neurite outgrowth, cell adhesion molecule L1 signaling, and L1 distribution in lipid rafts in cerebellar neurons in culture (Tang et al., 2014). Finally, choline supplementation normalizes the upregulation of M2/4 muscarinic receptors induced by ethanol in the dorsal hippocampus (Monk et al., 2012).

Astrocytes play essential roles in all aspect of brain development, as they contribute to the development of the BBB and are highly involved in synapse formation, functional maturation, and pruning (Allen & Eroglu, 2017; Allen & Lyons, 2018; Farhy-Tselnicker & Allen, 2018). Our previous research has indicated that astrocytes also play an important role in neurite outgrowth and mediate the effects of ethanol on neuronal development (Guizzetti et al., 2008; Guizzetti et al., 2010; Zhang et al., 2014a). Specifically, we have reported that when neurons are cultured *in vitro* with astrocytes treated with ethanol, they display decreased neurite outgrowth (Zhang et al., 2014a).

In this study, we investigated the hypothesis that choline treatments may prevent the inhibition of pyramidal neuron neurite outgrowth induced by ethanol-treated astrocytes. In order to selectively investigate the effect of the treatments (choline and ethanol) on hippocampal neuron development, we treated primary cultures of astrocytes with ethanol and/or choline for 24h. At the end of the 24h we removed the treatments, replaced them with treatment-free medium, and added newly isolated hippocampal neurons on top of these astrocyte cultures for an additional 16h, using a method

previously described by us (Zhang et al., 2014a). Neurite outgrowth was quantified by morphometric analysis of β-III tubulin-immunolabeled neurons. We report that when neurons were cultured with ethanol-treated astrocytes, they displayed reduced neurite outgrowth in all ethanol concentrations tested (25 mM, 50 mM, 75 mM). Neurons cultured with astrocytes treated with choline (10 mM, 50 mM, 100 mM) or with 100 mM choline and 75 mM ethanol together were not significantly different from neurons incubated with control astrocytes. These results suggest that choline prevents the effects of ethanol on neuronal development via an astrocyte specific mechanism.

METHODS

Animals

Timed-pregnant Gestational Day (GD) 15 Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Upon arrival, animals were maintained at the VA Portland Health Care System, under a 12h light/dark cycle at 22 ± 1°C. Animals had *ad libitum* access to water and food (chow diet). All animal procedures were approved by the VA Portland Health Care System Institutional Animal Care and Use Committee, and followed US National Institutes of Health animal welfare guidelines. The animals were allowed a week to acclimate following arrival before tissue collection.

Cortical Astrocyte Primary Cultures

Cortical astrocyte cultures were prepared from GD21 fetuses, as previously described (Guizzetti et al., 1996; Zhang et al., 2014a). Each different experiment utilized a different cell preparation from a different litter, and astrocytes were collected from all viable fetuses. Fetuses were sexed, and female and male astrocytes were plated in separate flasks. Astrocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin (all from Gibco, Grand Island, NY), in a humidified incubator at 37°C under a 5% CO_2/95% air atmosphere for 10-16 days. Two flasks (one with female-only astrocytes and one with male-only astrocytes) were then trypsinized; cell suspensions derived from female and male astrocyte

flasks were combined and plated into 24-well plates containing circular glass coverslips ($1x10^5$ cells/well). Cultures prepared in this way are balanced, mixed-sex cultures. Astrocytes were cultured for three additional days and then switched to serum-free DMEM supplemented with 0.1% Bovine Serum Albumin (BSA, Sigma Aldrich, St. Louis, MO) and Penicillin/Streptomycin for 24h, followed by ethanol and/or choline treatments, prepared in DMEM/0.1% BSA medium, for an additional 24h. The medium containing treatments was then removed from astrocyte cultures and replaced with treatment-free DMEM/0.1% BSA.

Cortical Astrocyte-Hippocampal Neuronal Co-Cultures

Hippocampal neurons were prepared from GD21 fetuses as previously described (Guizzetti et al., 2008; Guizzetti et al., 2010; Zhang et al., 2014b). As with astrocytes, each different experiment was prepared using neurons from separate litters. For each preparation, the neurons from 5 female and 5 male pups were pooled and used in these cultures. Neurons were then plated of top of the astrocyte monolayer, at a concentration of $1x10^4$ neurons/coverslip, for 16h. Neurons were added 2h after the removal of treatments from the astrocyte cultures.

Ethanol and Choline Treatments

Astrocytes plated on glass coverslips placed in 24-well plates were incubated with 1 mL DMEM/0.1% BSA medium containing 25 mM, 50 mM, or 75 mM ethanol or control (ethanol-free) medium for 24h (Guizzetti et al., 2010; VanDeMark et al., 2009b; Zhang et al., 2014b). Ethanol treatments took place in sealed chambers with a dish of water containing the same ethanol concentration present in the cultures; each chamber contained astrocytes treated with a single ethanol concentration (Chen et al., 2013; Zhang et al., 2014b). A gas mixture of 5% CO_2/95% air was run through these chambers, after which the chambers were sealed and incubated at 37°C for 24h.

Choline treatments were carried out by incubating astrocyte cultures with 1 mL DMEM/0.1% BSA medium containing 10 mM, 50 mM or 100 mM choline chloride (Sigma Aldrich, St. Louis, MO) or

control (choline-free) medium for 24h (Parrish et al., 2008; Singh et al., 2017). In some experiments, astrocytes were incubated with 75 mM ethanol, 100 mM choline, 75 mM ethanol + 100 mM choline, or control (treatment-free) DMEM/0.1% BSA medium for 24h.

Ethanol Concentration Determination

Ethanol concentrations in the medium were determined at the beginning and the end of each treatment by head-space gas chromatography after mixing 20 µL of medium with 500 µL of a solution of 4 mM propanol internal standard in water, as previously described (Finn et al., 2007). There was no significant evaporation of ethanol during the exposure period.

Osmolality

Changes in osmolality have been shown to affect neurite outgrowth (Cubillan et al., 2012). It is also possible that the 100 mM choline chloride treatment could be affecting the osmolality of the medium even after choline is removed. Therefore, we analyzed the osmolality in control medium, medium containing 100 mM choline at the beginning and at the end of 24h treatments, and in treatment-free medium incubated on astrocytes for 2h following choline treatment removal (corresponding to the medium to which neurons are exposed). Osmolality was measured using a Vapro Pressure Osmometer 5520 (Wescor Inc., Logan, UT). For each condition, samples from three independent samples of media were tested; each sample was run three times and results are expressed as mmol/kg.

Cell Viability

Mixed-sex astrocytes exposed to choline and/or ethanol were tested for cell viability using the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetraxzolium bromide (MTT) assay (Sigma Aldrich, St. Louis, MO), as previously described (Gavin et al., 2016; VanDeMark et al., 2009a, 2009b). Cells were incubated with 500 µL DMEM/0.1% BSA containing 0.5 mg/mL MTT for 2h. The MTT-containing medium was then

removed from the astrocyte monolayer, 500 µL of DMSO was added, and the resulting solution was read on a spectrophotometer at 570 nm; data are expressed as percent of control.

Immunocytochemistry

Following astrocyte-neuron co-culture, cells were fixed via 4% paraformaldehyde in phosphate buffered saline, and immunostained with a β-III Tubulin antibody (1:150 dilution; catalog #: MAB1637, Millipore, Burlington, MA) followed by the Alexa Fluor 488 secondary antibody (1:300 dilution; catalog #: A11001, Thermo-Fisher Scientific, Waltham, MA). Coverslips were then mounted on microscope slides (Zhang et al., 2014b).

Microscopy

Neurons were imaged on a Leica DM500b microscope with a DFC36 FX camera, using the program Neurolucida (Version 11, MBF Bioscience, Williston, VT, USA). Neurons were selected for morphometric analysis by a researcher blind to the experimental treatments and traced from pictures taken with a 40x objective. Only cells that 1) had three or more neurites all longer than the cell body (a morphology indicating a pyramidal neuron, and not a bipolar neuron), 2) did not overlap with other neurons, 3) were fully on top of the underlying astrocytes, 4) were pyramidal neurons and 5) were intact were selected for analysis. Neurites were traced using the software Neurolucida, and analyzed by Neurolucida Explorer. For each cell, three measurements were taken: the length of the longest neurite, the average length of the minor neurites, and the number of neurites.

Statistics

Data were analyzed using a multi-level analysis in the statistical program R (Team & RC, 2017) with the package lme4 (Bates et al., 2015). Outliers were identified using a value equal to 1.5 the Inter-Quartile Range. We conducted nested multilevel analyses to account for multiple cells measured in the same coverslip and multiple coverslips within the same experiment (Aarts et al., 2014) (See Table 2.1 for a summary of the sample sizes). Benjamini-Hochberg tests were run to correct for multiple comparisons

and control for the false discovery rate (FDR) (Benjamini & Hochberg, 1995). ANOVA were run for non-neuronal morphological parameters, and Tukey's post-hoc test was run when appropriate. A post-hoc p-value of <0.05 and a FDR q-value of <0.05 were considered significant. Data are reported as mean ± S.E.M.

RESULTS

Ethanol exposure decreased neurite outgrowth

A multilevel analysis with corrections for multiple comparisons showed an effect of ethanol exposure on the longest neurite length ($q<0.001$, Table 2.2). Post-hoc analysis showed that ethanol exposure significantly decreased the longest neurite length at 50 mM and 75 mM ethanol (Control vs. 50 mM $q<0.001$, decrease of 18.92%, Control vs. 75 mM $q<0.001$, decrease of 14.63%; Fig. 2.1A). There was also a trend toward a decrease in 25 mM ethanol exposure (Control vs. 25 mM $q=0.069$, decrease of 14.3%). Furthermore, there was a trend toward main effect of ethanol on average minor neurite length ($q=0.063$), which post-hocs showed was due to a decrease in neurite length following 75 mM ethanol exposure ($q=0.025$, decrease of 8.7%, Fig. 2.1B, Table 2.2). Ethanol-treated astrocytes did not affect the number of neurites (Fig. 2.1C, Table 2.2).

Choline exposure did not change neurite outgrowth

Astrocytes were exposed to 10 mM, 50 mM, or 100 mM choline, and neurons were cultured on the treated astrocytes as described above. Choline did not affect the length of the longest or the minor neurites, nor the number of neurites, regardless of choline concentration (Fig. 2.2A, 2.2B, 2.2C, Table 2.3).

Choline prevents the effect of ethanol on neurite outgrowth

We found a significant interaction between ethanol and choline ($q<0.001$) and a significant main effect of ethanol ($q=0.01$) on the longest neurite length, but no effect of choline alone (Table 2.4). Ethanol and choline together resulted in longer neurite lengths than the ethanol treatment ($q<0.001$,

difference of 18.14%) and the choline treatment (q=0.011, difference of 11.47%), and ethanol treatment resulted in shorter neurite lengths than control treatments (decrease of 10.48%, q=0.02, Fig. 2.3A). There was no significant effect of choline on the average minor neurite length, but there was a trend toward an effect of ethanol (ethanol vs. control, decrease of 8.28%, q=0.064, Fig. 2.3B).

We also found a significant main effect of ethanol on the number of neurites (q=0.01), but no significant effect of choline or interaction (Fig. 2.3C; Table 2.4). Examining the main effect of ethanol showed that neurons cultured with ethanol and choline in combination had more neurites than the ethanol only treatment (q=0.008). Representative images of neurons in all four conditions can be seen in Figure 2.4.

Changes in neurite outgrowth following choline exposure were not due to changes in osmolality

As expected, 100 mM choline chloride significantly increased medium osmolality. Specifically, there was a main effect of choline treatment compared to control medium both before ($p<0.001$) and after ($p<0.001$) the 24h incubation (Fig 2.5) by Tukey's post-hoc [ANOVA: $F(3,8)=7387.574$, $p<0.001$]. There was a significant increase in the osmolality of the choline-containing medium incubated with astrocytes for 24h compared to the medium before incubation (see the two middle columns in Fig. 2.5; $p<0.01$The osmolality of the medium incubated with astrocytes for 2h following the 24h incubation with choline was not different from the osmolality of control medium, indicating that the effect of choline-treated astrocytes on neurite outgrowth is not due to changes in osmolality (Fig. 2.5, right column).

Changes in neurite outgrowth are not due to astrocyte cell death

Cell viability, assessed by the MTT assay, was not reduced by any of the ethanol (Fig. 2.6A) or choline (Fig. 2.6B) treatments. There was, however, a small but significant increase in cell viability induced by the highest concentration of ethanol (75 mM), however, this is likely not physiologically relevant [ANOVA: $F(3,32)=2.429$, $p=0.01$; control versus 75 mM ethanol, $p=0.026$ by the Dunnett's post-hoc test, Fig. 2.6A].

Table 2.1. Neuron morphometric analysis experimental sample sizes

Experiment Type	Treatment	Number of Experiments	Coverslips/ Condition	Cells/ Condition
EtOH Concentration - Response (Fig. 2.1)	Control	6	18	302
	25 mM EtOH		18	274
	50 mM EtOH		18	287
	75 mM EtOH		18	322
Choline Concentration - Response (Fig. 2.2)	Control	3	7	217
	10 mM Choline		8	206
	50 mM Choline		8	228
	100 mM Choline		7	176
EtOH+Choline Treatment (Fig. 2.3)	Control	3	9	256
	Choline		8	240
	EtOH		8	212
	EtOH+Choline		9	226

Table 2.2. Ethanol concentration-response multilevel analyses and FDRs

Parameter	χ^2	DF	p value	q value (FDR correction)
Longest Neurite Length	24.143	3	2.33E-05	7.00E-05
Average Minor Neurite Length	8.198	3	0.042	0.063
Average Number of Neurites	0.428	3	0.934	0.934

Table 2.3. Choline concentration-response multilevel analyses and FDRs

Parameter	χ^2	DF	p value	q value (FDR correction)
Longest Neurite Length	2.256	3	0.521	0.774
Average Minor Neurite Length	4.670	3	0.198	0.593
Average Number of Neurites	1.113	3	0.774	0.774

Figure 2.1. Effect of ethanol-treated astrocytes on neurite outgrowth of pyramidal neurons. Astrocytes plated on glass coverslips were treated for 24h in the presence or absence of ethanol (25, 50, or 75 mM); treatments were then washed out and replaced with fresh medium. 2h later, freshly isolated hippocampal neurons were plated on top of the astrocytes for an additional 16h, at the end of which neurons were fixed, labeled with a β-III tubulin antibody followed by a fluorescent secondary antibody, and morphometrically analyzed using the software Neurolucida. **A.** Length of the longest neurite. **B.** Average minor neurite length. **C.** Number of neurites/neuron. *q<0.05, ***q≤0.001 (after FDR corrections), +p<0.05 (before FDR corrections).

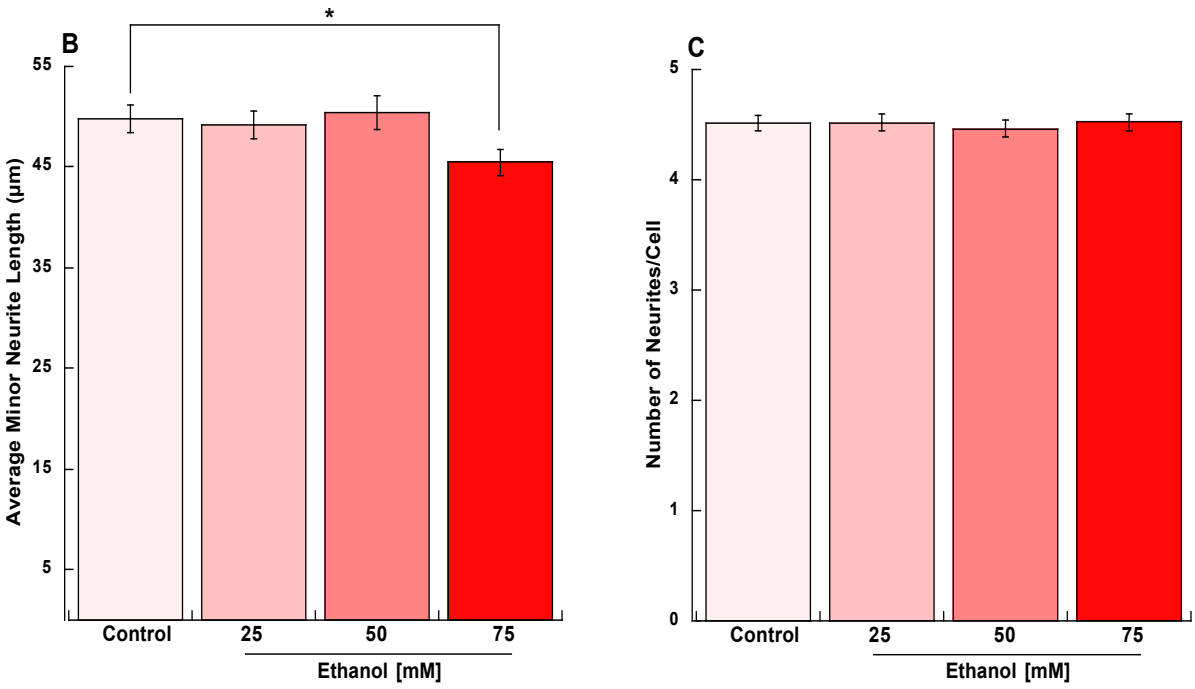


Figure 2.2. Effect of choline-treated astrocytes on neurite outgrowth of pyramidal neurons. Astrocytes plated on glass coverslips were treated for 24h in the presence or absence of choline (10, 50, or 100 mM); treatments were then washed out and replaced with fresh medium. 2h later, freshly isolated hippocampal neurons were plated on top of the astrocytes for an additional 16h, at the end of which neurons were fixed, labeled with a β-III tubulin antibody followed by a fluorescent secondary antibody, and morphometrically analyzed using the software Neurolucida. **A.** Length of the longest neurite. **B.** Average minor neurite length. **C.** Number of neurites/neuron.

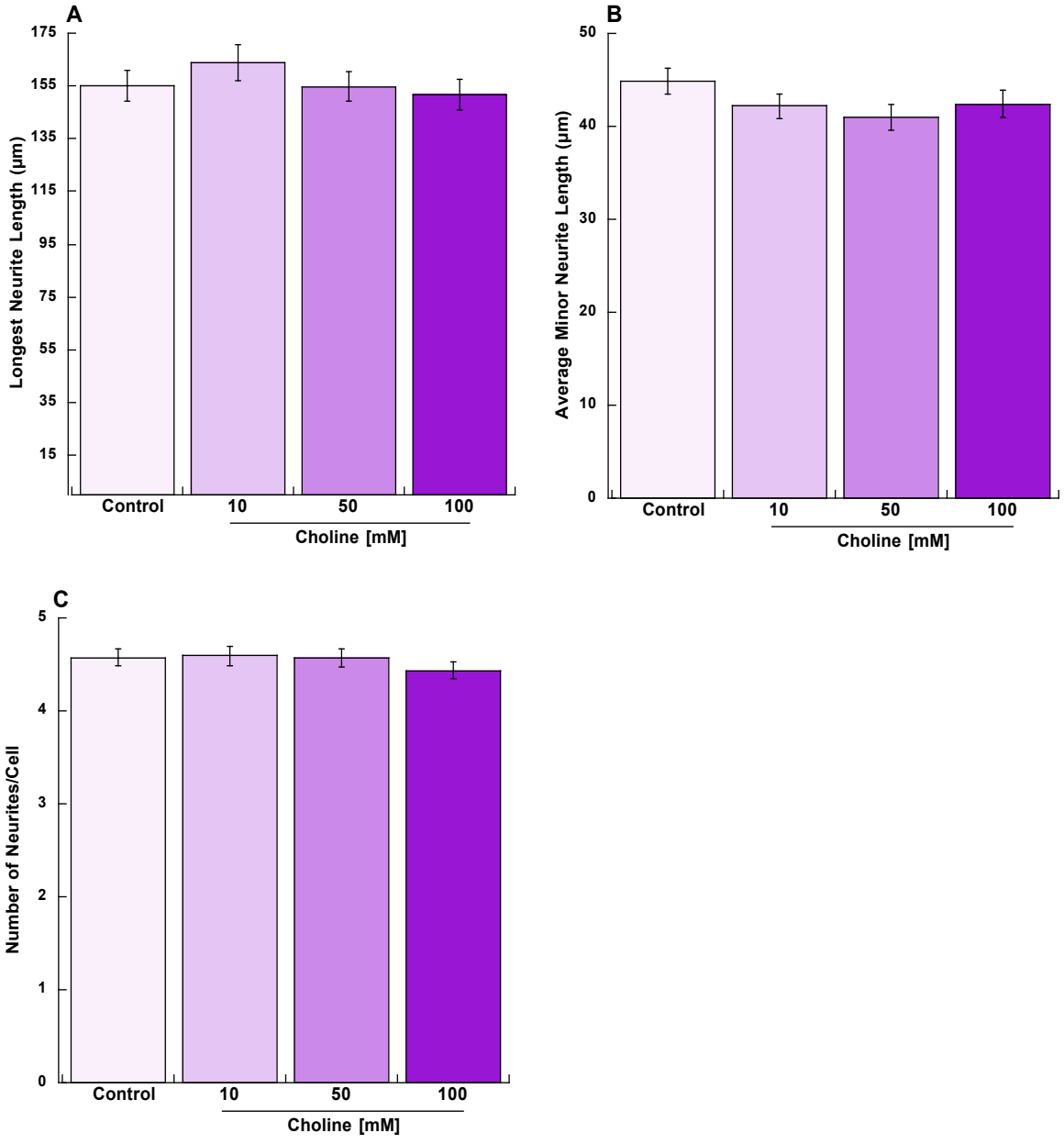


Figure 2.3. Effect of choline-plus-ethanol-treated astrocytes on neurite outgrowth of pyramidal neurons. Astrocytes plated on glass coverslips were treated for 24h in the presence or absence of choline (100 mM), ethanol (75 mM), or ethanol and choline together; treatments were then washed out and replaced with fresh medium. 2h later, freshly isolated hippocampal neurons were plated on top of the astrocytes for an additional 16h, at the end of which neurons were fixed, labeled with a β-III tubulin antibody followed by a fluorescent secondary antibody, and morphometrically analyzed using the software Neurolucida. **A.** Length of the longest neurite. **B.** Average minor neurite length. **C.** Number of neurites/neuron.

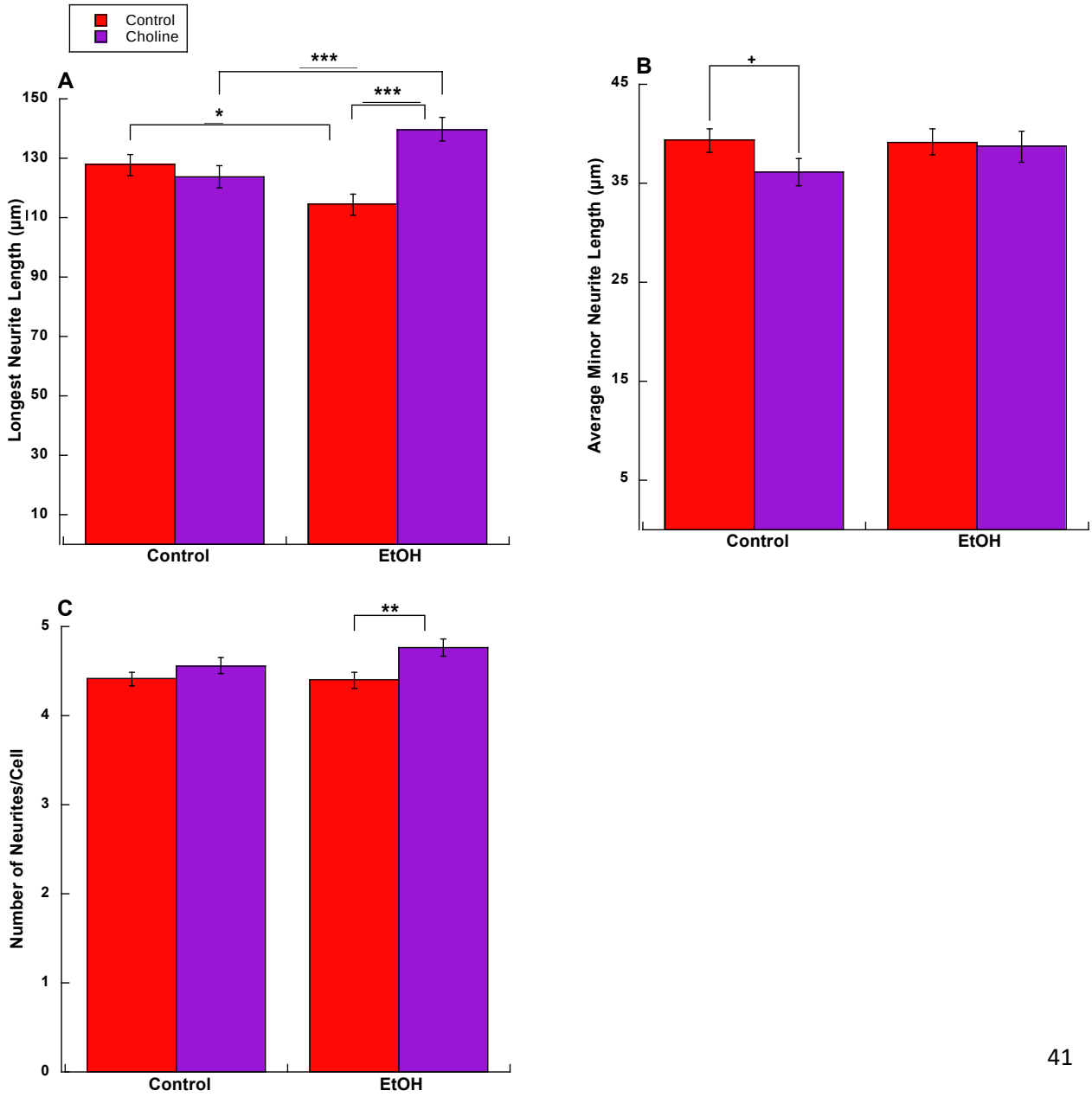


Table 2.4. Ethanol and choline treatment multilevel analysis and FDRs

Parameter	χ2		DF		p value		q value (FDR correction)	
	Interaction		Interaction		Interaction		Interaction	
Longest Neurite Length	15.373		1		8.83E-05		2.65E-04	
Average Minor Neurite Length	1.136		1		0.287		0.287	
Average Number of Neurites	1.603		1		0.206		0.287	
Parameter	χ2		DF		p value		q value (FDR correction)	
	EtOH	Choline	EtOH	Choline	EtOH	Choline	EtOH	Choline
Longest Neurite Length	7.329	0.175	1	1	0.007	0.676	0.010	0.676
Average Minor Neurite Length	0.712	2.131	1	1	0.399	0.144	0.399	0.433
Average Number of Neurites	8.225	0.901	1	1	0.004	0.343	0.010	0.514

Figure 2.4. Pyramidal neurons immunocytochemically stained with β-III tubulin antibody. Representative hippocampal neurons co-cultured with control (**A**), ethanol-treated (**B**), choline-treated (**C**) and ethanol-plus-choline-treated (**D**) astrocytes.

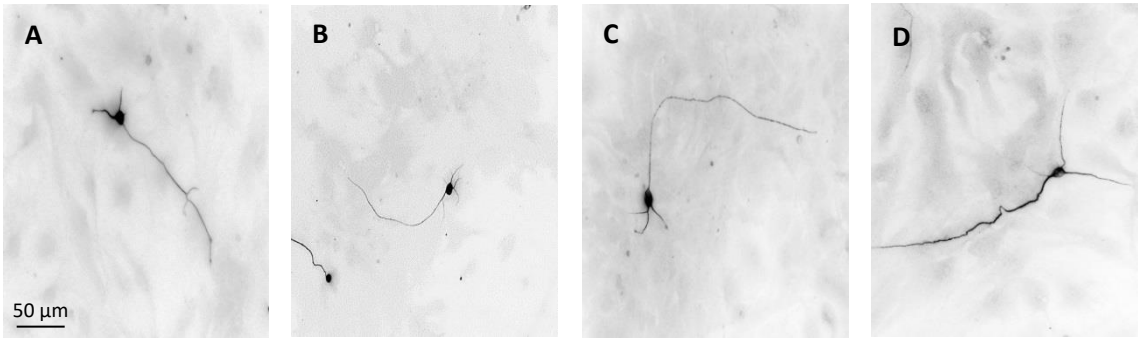
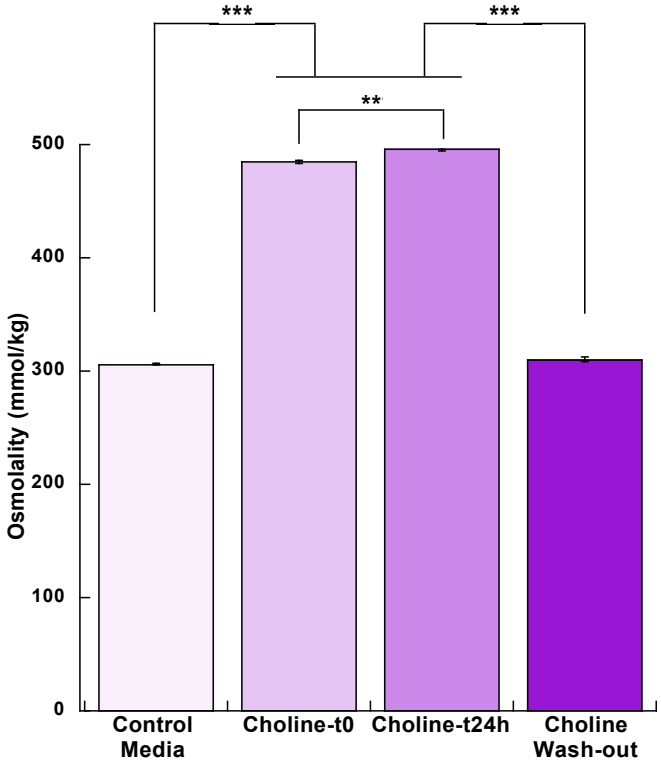


Figure 2.5. Effect of choline on osmolality. Osmolality was evaluated in treatment-free DMEM/0.1% BSA medium (control; left column), in the medium containing 100 mM choline chloride before (t0, second column from the left) and after (t24, third column from the left) 24h incubation on astrocytes. In some of the wells containing choline-treated astrocytes, the medium containing choline was removed and fresh DMEM/0.1% BSA was added to the cultures for 2h before being evaluated for osmolality (column on the right). **p<0.01, ***p<0.001.



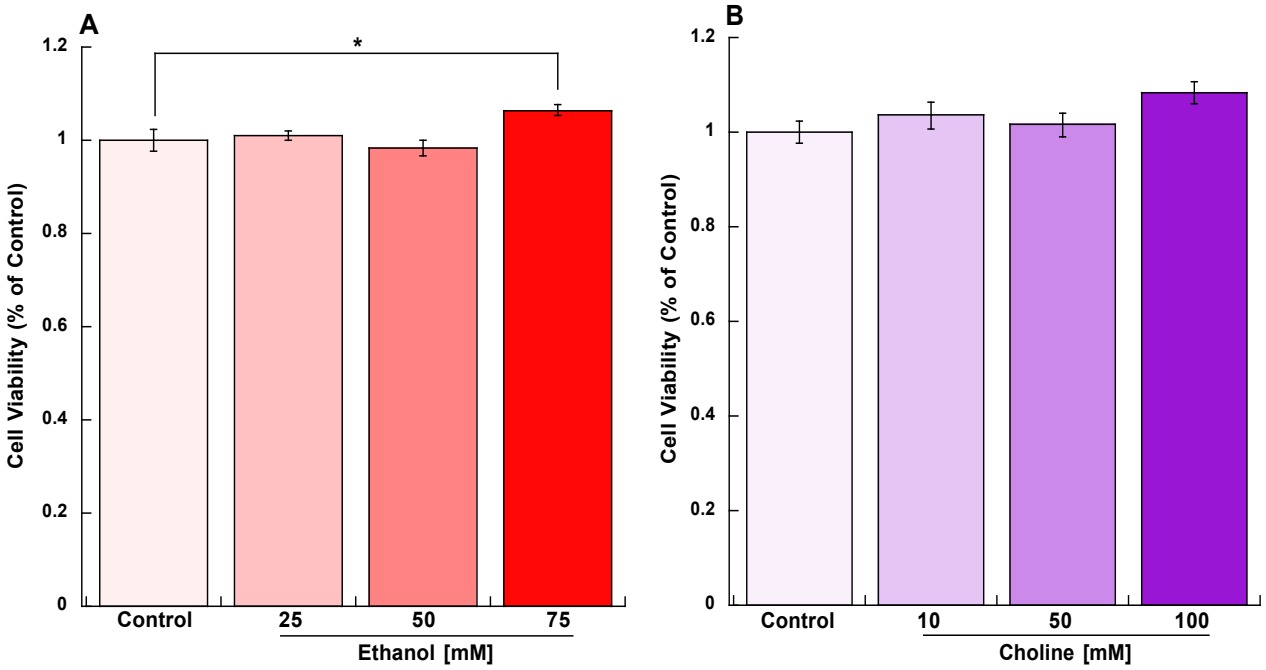
DISCUSSION

Cognitive and behavioral consequences of developmental ethanol exposure may, at least in part, be due to alterations in neuronal plasticity and connectivity, as evidenced by rodent preclinical studies and human imaging studies (Lebel et al., 2012; Medina, 2011; Wozniak et al., 2013). Our laboratory has been investigating the hypothesis that some of the effects of ethanol on neuronal plasticity may be mediated by astrocytes as reviewed in (Guizzetti et al., 2014) and (Wilhelm & Guizzetti, 2016). In culture, neurons develop faster and reach full morphological and functional maturation only when they are plated in the presence of astrocytes (Christopherson et al., 2005; Dotti et al., 1988; Kaech & Banker, 2007; Pfrieger & Barres, 1997), underscoring the role of astrocytes in neuronal development.

We optimized *in vitro* models to assess how astrocyte treatments with ethanol affect neuronal development (Guizzetti et al., 2010; Zhang et al., 2014b). We previously reported that neurons co-cultured on top of astrocytes pre-treated with 75 mM ethanol displayed reduced neurite outgrowth in comparison to neurons co-cultured with control astrocytes (Zhang et al., 2014b). In the present study, we expand on this observation to report that neurons cultured with ethanol-treated astrocytes had reduced longest neurite length (the one that will likely develop into the axon, (Dotti et al., 1988; Guizzetti et al., 2010)) starting at the lowest ethanol concentration used, 25mM (Fig. 2.1A, Table 2.2). The ethanol concentrations we used in this study, 25 mM, 50 mM, and 75 mM (corresponding to 0.115 g/dL, 0.23 g/dL, and 0.35 g/dL respectively) are clinically relevant, as they can be found in the blood of individuals after high ethanol intake (Adachi et al., 1991), and are within the range of concentrations recommended for *in vitro* studies (Deitrich & Harris, 1996). Furthermore, we verified that these ethanol concentrations did not reduce astrocyte viability (Fig. 2.6A), although there was an increase in astrocyte viability when astrocytes were exposed to 75 mM ethanol, this is likely not physiologically relevant.

Choline supplementation has been shown to ameliorate behavioral abnormalities in rodents developmentally exposed to ethanol, and is currently being investigated for its effectiveness in children

Figure 2.6. Effect of ethanol and choline on astrocyte viability. Astrocytes plated in 24-well plates were treated for 24h in the presence or absence of ethanol (25, 50, or 75 mM) (**A**) or choline (10, 50, or 100 mM) (**B**); treatments were then washed out and replaced with fresh medium for 2h. Cell viability was then measured by the MTT test. *p<0.05.



with FASD (Akison et al., 2018; Bearer et al., 2015; Ryan et al., 2008; Thomas et al., 2004a; Thomas et al., 2004b). An important goal of this study was to test whether choline supplementation in astrocytes ameliorates the effects of ethanol on neurite outgrowth. We report that, while morphometric parameters of neurons cultured with choline-treated astrocytes were not statistically different from the ones of neurons co-cultured with control astrocytes (Fig. 2.2, Table 2.3), data from neurons co-cultured with astrocytes exposed to both ethanol and choline together showed a protective effect of choline on the reduced length of the longest neurite caused by ethanol (Fig. 2.3, Table 2.4).

Due to the high concentrations of choline used in these studies, representative of other *in vitro* experiments (Parrish et al., 2008; Singh et al., 2017), we verified that the changes in choline levels do not affect astrocyte viability (Fig. 2.6A). We also verified that choline treatments in astrocytes do not change the osmolality of the medium during neuron co-culture (Fig. 2.4, compare the first column to the last column); this is an important consideration given the fact that changes in osmolality of the medium in which neurons are grown can affect neurite outgrowth (Cubillan et al., 2012). There is also an increase in osmolality when comparing the media before and after the 24h incubation. We hypothesize that this increase is due to the factors that are released by astrocytes during the 24h incubation. Together these data indicate that choline counters the changes induced by ethanol in astrocytes, which are therefore able again to foster neuronal development similarly to control astrocytes.

Choline is an essential nutrient and a precursor of several molecules involved in important cellular functions. Among the most important choline metabolites are betaine, which can be converted to methionine and subsequently to S-adenosylmethionine, the universal methyl donor used by DNA methyl transferases in DNA methylation reactions, and the neurotransmitter acetylcholine (Li & Vance, 2008; Niculescu & Zeisel, 2002). However, because the conversion of choline to betaine and then to methionine is catalyzed by enzymes present only in the liver and kidneys, and the conversion of choline to acetylcholine by choline acyltransferase occurs only in cholinergic neurons (Li & Vance, 2008), it is

reasonable to assume that these two metabolites do not play a role in the observed effect of choline as mediated by astrocytes. Another major metabolite of choline is phosphatidylcholine (PC), a major membrane phospholipid which accounts for about 95% of choline in most of the tissues (Fig. 1.1); the enzyme necessary for the biosynthesis of PC is present in all nucleated cells (Li & Vance, 2008). PC also plays an important role in signal transduction, as the hydrolysis of PC by the enzyme phospholipase D (PLD) generates phosphatidic acid (PA), a second messenger that can induce neurite outgrowth (Zhu et al., 2016).

It is well established that ethanol alters PA signaling. Indeed, ethanol competes with water as a substrate for PLD whereby PA production is inhibited and phosphatidylethanol (PEth) is formed instead (Guizzetti et al., 2004; Gustavsson, 1995; Klein, 2005). This mechanism may be involved in the protective effect of choline on the inhibition of neurite outgrowth mediated by ethanol-treated astrocytes. The reason why choline supplementation alone does not, on its own, have an effect on neuronal development may be that cellular homeostatic processes regulate the incorporation of choline into PC or other choline metabolites only when needed by the cell, as, for instance, when in the presence of ethanol.

In conclusion, we show that choline can prevent the effects of ethanol-treated astrocytes on neurite outgrowth. This study is the first to suggest that the beneficial effects of choline may be attributed to an effect not on neurons, but on astrocytes. Astrocyte involvement in brain development is now well established (Allen & Lyons, 2018); our research has been aimed at investigating the effects of ethanol on astrocyte functions that can then result in altered neuronal development. It is therefore not surprising that astrocytes can also be the target for possible interventions for the treatment of FASD, such as choline. Future studies will be aimed at characterizing the mechanisms by which choline ameliorates the effects of ethanol-exposed astrocytes on neurite outgrowth.

CHAPTER 3: NEONATAL ETHANOL AND CHOLINE TREATMENTS ALTER THE MORPHOLOGY OF DEVELOPING RAT HIPPOCAMPAL PYRAMIDAL NEURONS IN OPPOSITE DIRECTIONS

This chapter has been reformatted for inclusion in this dissertation from:

Goeke, C., M., Roberts, M., L., Hashimoto, J., G., Finn, D., A., Guizzetti, M. (2018), Neonatal ethanol and choline treatments alter the morphology of developing rat hippocampal pyramidal neurons in opposite directions, Neuroscience, 374: 13-24.

Support: This work was supported by VA Merit Review Award # I01BX001819 and by NIH/NIAAA R01AA021468, and R01AA022948 to MG and by facilities and resources at the VA Portland Health Care System (MG and DAF).

ABSTRACT

Some of the neurobehavioral deficits identified in children with Fetal Alcohol Spectrum Disorders (FASD) have been recapitulated in a binge model of gestational third trimester-equivalent ethanol exposure, in which Sprague–Dawley rats are intragastrically intubated between postnatal day (PD) 4 and PD9 with high doses of ethanol. In this model, the ameliorating effects of choline administration on hippocampus dependent behaviors altered by ethanol have also been extensively documented. In the present study, we investigated the effects of ethanol (5 g/kg/day) and/or choline (100 mg/kg/day) on morphometric parameters of CA1 pyramidal neurons by Golgi–Cox staining followed by Neurolucida tracing and analysis. We found that ethanol increased apical dendrite complexity in female and male pups neonatally exposed to ethanol. Ethanol did not significantly affect basilar dendrite parameters in female and male rats. Interestingly, choline treatments decreased basilar dendrites' length, number, and maximal terminal distance in male pups. When pups were co-treated with ethanol and choline, choline did not rescue the effect of ethanol. In conclusion, ethanol increases while choline decreases dendritic length and arborization of hippocampal CA1 pyramidal neurons in PD9 rats. We hypothesize that developmental ethanol exposure induces a premature maturation of neurons, leading to early restriction of neuronal plasticity, while choline treatments delay the normal program of neuronal maturation and therefore prolong the window of maximal plasticity. Choline does not prevent the effects of developmental ethanol exposure on hippocampal pyramidal neurons' morphology characterized in the present study, although whether prolonged choline administration after developmental ethanol exposure rectifies ethanol damage remains to be assessed.

INTRODUCTION

Ethanol abuse during pregnancy may lead to Fetal Alcohol Spectrum Disorders (FASD) characterized by structural brain abnormalities and compromised cognitive and behavioral functions (Hellemans et al., 2010; Riley et al., 2011). Clinical and preclinical studies indicate that neuronal

plasticity and connectivity are affected by *in utero* ethanol exposure; these alterations may play a major role in central nervous system (CNS) dysfunction present in individuals with FASD (Lebel et al., 2012; Medina, 2011; Wozniak et al., 2013).

Ethanol affects the development of the CNS throughout gestation (Rice & Barone, 2000). The third trimester of human gestation is characterized by functional maturation of several brain regions, including the hippocampus; this developmental stage in rats occurs mostly during the first nine postnatal days. Major events during this period include a massive increase in brain size (the brain growth spurt), proliferation of astrocytes and oligodendrocytes, and dendritic arborization (Rice & Barone, 2000). Ethanol exposure during this developmental stage induces microcephaly, cerebellar and hippocampal abnormalities, severe apoptotic neuronal death in the hippocampus and cerebral cortex, and behavioral dysfunctions (Bonthius & West, 1990, 1991; Ikonomidou et al., 2000; Patten et al., 2014). Of particular relevance to the present study is the fact that ethanol alters hippocampus-dependent behaviors in several rodent models of FASD, including models of gestational third trimester-equivalent ethanol exposure (Berman & Hannigan, 2000; Christie et al., 2005; Gianoulakis, 1990; Goodlett & Peterson, 1995; Johnson & Goodlett, 2002; Kelly et al., 1988; Patten et al., 2014; Popovic et al., 2006; Thomas et al., 2008; Thomas et al., 2010).

A substantial body of evidence derived from behavioral and neurochemical studies in rats indicates that choline improves hippocampal functions in the adult and aging brain, and that choline supplementation during gestation, as well as during the early postnatal period, improves memory performance throughout life (Zeisel & Niculescu, 2006). More relevant to the present study, choline has been consistently shown to ameliorate deficits in hippocampus-associated behaviors in rats exposed to ethanol during brain development (Thomas et al., 2000; Thomas et al., 2004a; Thomas et al., 2007; Thomas et al., 2009; Thomas et al., 2010). Additionally, a few studies explored how choline may ameliorate some of the effects of ethanol, such as ameliorating the deficits induced by ethanol on a

working memory variant of the Morris Water Maze (Balaraman et al., 2017; Otero et al., 2012; Tang et al., 2014). For these reasons, choline is currently being tested clinically for its effectiveness in treating FASD (Nguyen et al., 2016; Wozniak et al., 2015).

Ethanol causes long-lasting changes in dendritic arborization and/or number of dendritic spines in different populations of neurons after prenatal and/or neonatal exposure. Neonatal ethanol exposure decreases spine density and dendritic complexity of basilar dendrites, as well as dendritic spine density in apical dendrites of layer II/III pyramidal neurons of the medial prefrontal cortex (mPFC) in juvenile rats, an effect that is reversed by voluntary exercise (Hamilton et al., 2010; Hamilton et al., 2015; Whitcher & Klintsova, 2008). In addition, ethanol alters neuronal development, measured as neurite outgrowth, in hippocampal pyramidal neurons *in vitro* (see chapter 2) (Giordano et al., 2011; Guizzetti et al., 2010; Lindsley et al., 2002; Lindsley et al., 2003; Lindsley & Clarke, 2004; VanDeMark et al., 2009b; Yanni & Lindsley, 2000; Yanni et al., 2002; Zhang et al., 2014b). Together, this published literature supports the hypothesis that ethanol alters the proper development of neurons leading to altered brain connectivity.

We undertook the present study to investigate the effect of binge ethanol exposure and co-treatment with choline during the third trimester of gestation equivalent, between postnatal day (PD) 4 and PD9, on dendritic arborization of CA1 pyramidal neurons in pups euthanized two hours after the last ethanol exposure on PD9. Our rationale for exploring alterations in neuronal morphology occurring in developing neurons is that appropriate brain development requires developmental events to occur in a synchronized manner, so a delay or acceleration of any given event may have profound functional consequences that may persist throughout life.

METHODS

Animals

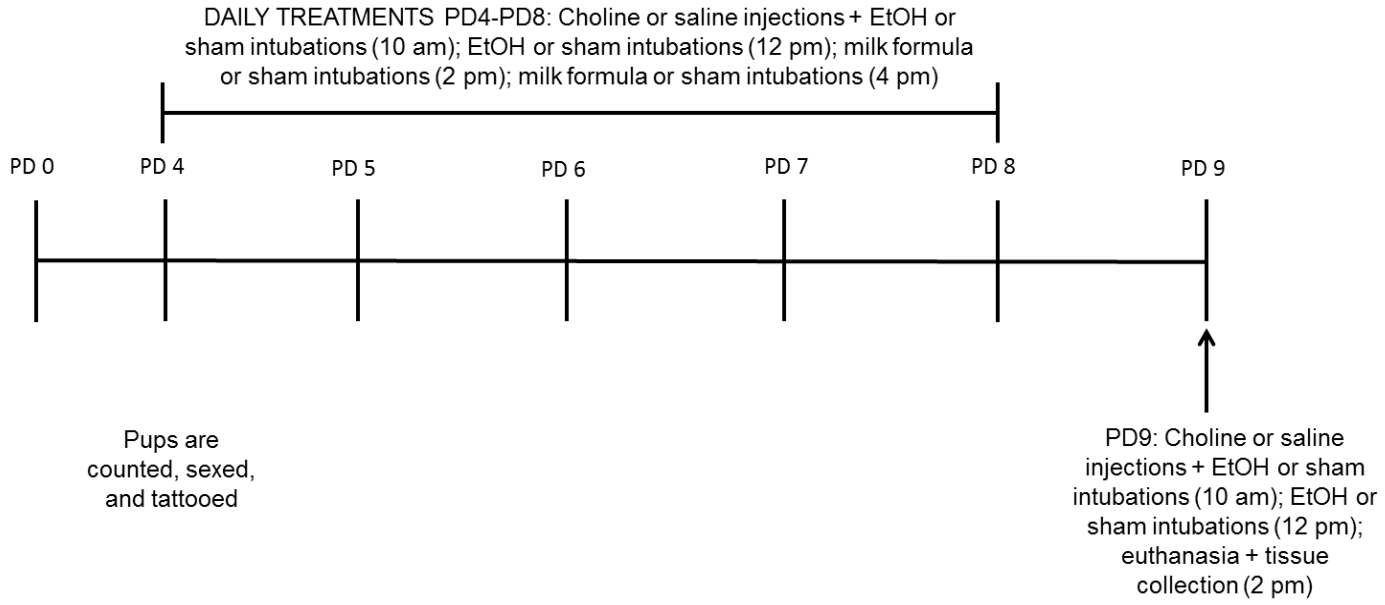
Timed-pregnant Gestational Day (GD) 15 Sprague-Dawley rats were purchased from Charles River (Wilmington, MA) and maintained at the VA Portland Health Care System Veterinary Medical Unit under a 12-h light/dark cycle (lights on from 6:00 to 18:00) at 22 ± 1°C. Pregnant animals had *ad libitum* access to water and food (chow diet). All animal procedures were approved by the VA Portland Health Care System Institutional Animal Care and Use Committee and followed US National Institutes of Health animal welfare guidelines.

In vivo neonatal ethanol and choline treatments

On PD4, animals were counted and their sex was determined; four litters of pups were used. When possible, the litters were culled to ten pups, five of each sex, and one animal/sex/litter was randomly assigned to one of the following conditions: (1) sham intubation and saline injection control (four female and four male pups), (2) sham intubation and choline injection (three female and four male pups), (3) ethanol intubation and saline injection (four female and four male pups), (4) ethanol intubation and choline injection (four female and four male pups), (5) untouched animals that remained with the dam (data not shown). In total, data presented in this study were obtained from the analysis of 31 pups derived from four different litters: four females and four males for conditions 1, 3, and 4; three females and four males for condition 2 (one of the litters had only three females).

Before the beginning of the treatments, pups were tattooed with subcutaneous injections of India Ink on their paws for identification. Between PD4 and PD9, pups were weighed and injected subcutaneously with saline or 100 mg/kg choline each day, followed by two ethanol in milk formula or sham intragastric intubations (Balaraman et al., 2017; Monk et al., 2012; Otero et al., 2012; Ryan et al., 2008; Schneider & Thomas, 2016; Thomas et al., 2007). Pups that were given ethanol were also given two intubations of milk formula without ethanol at two–hour intervals, starting two hours after the last ethanol intubation, to compensate for lack of suckling caused by inebriation; pups not receiving ethanol were sham-intubated at the same intervals (Fig. 3.1). Intragastric intubation was done by inserting

Figure 3.1. Schematic representation of the experimental design employed in this study. Before the beginning of the treatments on PD4, pups were counted, sexed, and tattooed on their paws for identification. Between PD4 and PD8, pups were injected subcutaneously with saline or 100 mg/kg choline each day, followed by two ethanol (in milk formula) or sham intragastric intubations, and two milk formula only or sham intubations two hours apart (at 10 am, 12 pm, 2 pm, and 4 pm, respectively). On PD9, pups were injected subcutaneously with saline or 100 mg/kg choline, followed by two ethanol or sham intragastric intubations (at 10 am and 12 pm); pups were then euthanized 2 h later (at 2 pm).



flexible tubing that was dipped into corn oil for lubrication into the esophagus of the neonatal rat. Animals in the ethanol and ethanol+choline groups received 5 g/kg/day ethanol in milk formula (Similac Advance Early Shield with iron) delivered in two separate feedings two hours apart, at a concentration of 11.9% ethanol in formula, and an intubation volume of 0.0278 mL/g. Rat pups were weighed daily.

During the intubation process, which took approximately 10 minutes/litter, rat pups were removed from their dam and placed on a heating pad. On PD9, two hours after the last ethanol intubation, animals were anesthetized by an intraperitoneal injection with a cocktail of Ketamine (500 mg/10 mL, 100 mg/kg), Xylazine (50 mg/10 mL, 10 mg/kg) and Acepromazine (10 mg/10 mL, 1 mg/kg) in 0.9% saline and decapitated. At the time of euthanasia, 2h after the second ethanol intubation, trunk blood was collected to determine ethanol concentration and the brains were collected for Golgi–Cox staining. Four litters were used in these experiments; all animals survived throughout the treatments.

Blood ethanol concentration (BEC) determination

Following euthanasia, 20 µL of trunk blood was collected from the animals and mixed into 500 µL of a matrix consisting of 4 mM n-propanol internal standard in distilled water. BECs were determined by head-space gas chromatography, as previously described (Finn et al., 2007).

Tissue collection and staining

All brains were stained with the Golgi–Cox solution (using the FD Rapid GolgiStain^{TM} Kit from NeuroTechnologies Inc., Columbia, MD, USA) according to manufacturer's instructions. Briefly, the tissue was rinsed in water and then immersed in the Golgi–Cox impregnation solution, and stored at room temperature for two weeks. The tissue was then transferred to Solution C and stored at 4^{o}C for 48h. The brains were then rapidly frozen and embedded in Tissue Freezing Medium before sectioning on the cryostat. Coronal sections (100 µm in thickness) were mounted on gelatin-coated microscope slides and dried at room temperature overnight, then stained the next day.

Microscopy

All pyramidal neurons were traced with the software Neurolucida (Version 11, MBF Bioscience, Williston, VT, USA) on a Leica DM500b microscope equipped with a DFC36 FX camera by a researcher blind to the treatments of the analyzed samples. Three or four slices/brain containing the central part of the hippocampus were selected for analysis. Twelve cells/brain (six cells/hippocampal hemisphere) were measured using a 40x objective. Only fully impregnated, CA1 pyramidal neurons clearly distinguishable from neighboring neurons were measured. Basilar and apical dendrites were analyzed separately using the software Neurolucida Explorer. For apical dendrites the following parameters were analyzed: complexity; total apical dendrite length (μm); sum of terminal orders; and number of ends. For basilar dendrites the following parameters were analyzed: complexity; total basilar dendrite length (μm); sum of terminal orders; number of ends; number of basilar dendrites/neuron; and maximal terminal distance. Complexity was calculated as (sum of terminal orders+number of ends)*(total dendrite length/number of primary dendrites).

Statistical analysis

BECs and body weights (Table 3.1) were analyzed by a two-way ANOVA with ethanol treatment and choline injection as the two independent variables. To account for the nested (dependent) data (12 neurons/brain from four or, in one condition, three different brains) from neuron morphometric analysis, we carried out linear mixed effect analysis (multilevel analysis) as previously described (Aarts et al., 2014). For the neuron morphometric parameters we used R (R Core; (Team & RC, 2017) and lme4; (Bates et al., 2015)) to perform a multilevel analysis including animal as the random effect to account for the multiple cells analyzed from each animal. As fixed effects, we used ethanol treatment and choline injection; p-values were obtained by likelihood ratio tests. In most of the cases (Figs. 3.2A–D; 3.3A, B; 3.4A–F; 3.5A–F) data were log transformed before statistical analysis to satisfy normality and homoscedasticity assumptions. Residual and Q–Q plots did not reveal any obvious deviations from homoscedasticity or normality following log transformation. The nominal p-values derived from

multilevel analyses of individual parameters (with the exception of complexity, which is a composite parameter including all the other analyzed parameters) were then corrected for multiple comparisons using the Benjamini–Hochberg approach (Benjamini & Hochberg, 1995) to adjust nominal p-values to False Discovery Rate (FDR, q-values). Significance was considered to be $p<0.05$ (for complexity) and $q<0.05$ (for all the other parameters investigated). All data are reported as mean ± the standard error from 48 neurons per condition (with the exception of the female sham intubation and choline injection group that had 36 neurons).

RESULTS

Treatment outcomes: body weights and blood ethanol concentrations

There were no significant differences in the weights of the pups assigned to the four different treatment groups at PD4; animals in all groups gained weight during the treatment window (between PD4 and PD9). However, there was a trend toward decreased body weight in the ethanol-treated groups of both sexes beginning on PD5; the reduction in weight was significant in females at PD9 and in males at PD7, PD8, and PD9 (Table 3.1). We did not measure brain weight in this cohort of animals. However, in other cohorts of animals treated in the same way, we observed a significant decrease in brain weight in the ethanol-treated groups, while choline did not have significant effects (not shown). Low body and brain weights are hallmarks of Fetal Alcohol Syndrome (FAS), and can be present in FASD (Riley et al., 2011). BECs measured 2h after the last intubation on PD9 by gas chromatography (Finn et al., 2007) ranged between 60 and 65 mM with no differences between females and males, and no differences between the ethanol and ethanol + choline groups (Table 3.1).

Effects of ethanol and choline on CA1 pyramidal neuron apical dendrite parameters of neonatal female and male rats

Table 3.1. Treatment outcomes. Trunk blood was collected from PD9 female and male rats after euthanasia and BECs were measured. Female and male pups were weighed daily between PD4 and PD9.

	Control	SEM	Choline	SEM	EtOH	SEM	EtOH + Choline	SEM
BEC (mM)								
Females	N/A	N/A	N/A	N/A	59.96	±4.75	62.42	±1.2
Males	N/A	N/A	N/A	N/A	64.27	±2.32	60.60	±1.16
Body Weights of Female Pups (g)								
PD4	9.52	±0.55	9	±1.39	9.28	±0.46	9.10	±0.71
PD5	11.3	±0.70	10.57	±1.64	10.50	±0.68	10.00	±0.81
PD6	13.43	±0.85	12.70	±1.84	11.82	±0.71	11.47	±0.87
PD7	15.40	±0.95	14.73	±2.23	13.40	±0.78	13.07	±0.86
PD8	17.65	±1.03	16.90	±2.45	15.30	±0.90	14.47	±0.79
*PD9	19.85	±1.20	19.27	±2.39	16.93	±0.90	16.52	±0.73
Body Weights of Male Pups (g)								
PD4	9.82	±0.62	9.28	±0.72	9.42	±0.83	9.57	±0.82
PD5	11.68	±0.65	11.10	±0.84	10.68	±0.85	10.62	±0.84
PD5	13.7	±0.67	12.95	±0.96	12.07	±0.91	11.93	±1.00
*PD7	15.85	±0.81	15.30	±1.18	13.45	±0.96	13.38	±0.98
*PD8	18.18	±0.87	17.45	±1.24	15.12	±0.94	15.05	±1.05
*PD9	20.1	±0.80	19.60	±1.11	17.10	±0.92	17.15	±1.06

* Two-way ANOVA revealed a main effect of ethanol in female pups at PD9 [$F(1,11)=5.01$, $p \leq 0.05$] and in male pups at PD7 [$F(1,12) = 4.7488$, $p \leq 0.05$], PD8 [$F(1,12) = 6.01$, $p \leq 0.05$], and PD9 [$F(1,12)=7.72$, $p \leq 0.05$]

As there was a significant main effect sex, such that female neuronal morphological parameters were statistically greater than males following corrections for multiple comparisons in all analyzed parameters of apical dendrite morphology, we analyzed the sexes separately. Pyramidal neurons have one large apical dendrite per neuron that emerges from the apex of the soma and branches several times; at the opposite side of the soma, pyramidal neurons have several relatively short basilar dendrites, which emerge from the base of the pyramidal cell's soma. Because the morphology of apical and basilar dendrites is very different, the two dendritic trees are analyzed separately.

We investigated the effect of ethanol on apical dendrite complexity, defined by the Neurolucida Explorer software as: (sum of terminal orders+number of ends)*(total dendrite length/number of primary dendrites). Multilevel analysis of log-transformed data revealed a significant upregulation of apical dendrite complexity in female and male rat pups neonatally exposed to ethanol (Figs. 3.2A; 3.3A Tables 3.2 and 3.3). Complexity is a composite measurement that includes four different measurements: the sum of terminal orders (i.e., the number of ‘‘sister" branches encountered from each end to the cell body); the number of ends; the total dendrite length; and the number of primary dendrites.

In order to examine the specific effects of ethanol on each of the measurements in the complexity equation (with the exception of the number of primary dendrites, which, in the case of the apical dendrite, is always one), we carried out multilevel analyses of each individual measurement with FDR corrections. We found that ethanol significantly increased each of the individual components of complexity (namely apical dendrite length, sum of terminal orders, and number of ends) in female pups (Fig. 3.2B–D; Table 3.2). In male pups, ethanol increased the sum of terminal orders and number of ends; the apical dendrite length also trended toward an increase, but, after FDR corrections, it did not reach statistical significance (Fig. 3.3B–D; Table 3.3). Together these results indicate that ethanol increases the complexity of apical dendrites in developing CA1 neurons by increasing their branching.

Fig. 3.2. Effects of neonatal ethanol exposure and choline treatments on morphometric parameters of apical dendrites of CA1 pyramidal neurons in PD9 female rats. Morphometric measurements of apical dendrites of CA1 pyramidal neurons from female PD9 rats exposed to 5 g/kg/day ethanol and/or 100 mg/kg/day choline between PD4 and PD9 were analyzed by Neurolucida Explorer. (A) Apical dendrite complexity (1000x); a composite measurement defined as [(sum of terminal orders+number of ends)*(total dendrite length/number of primary dendrites)]. (B) Apical dendrite length (in μm). (C) Apical dendrite sum of terminal orders (defined as the number of ‘‘sister” branches encountered from each end to the cell body). (D) Number of ends per apical dendrite. Shown in each graph is the mean ± the standard error from 48 neurons per condition (with the exception of the choline group that had 36 neurons). +$p < 0.05$ (before FDR corrections); *$q < 0.05$ (after FDR corrections).

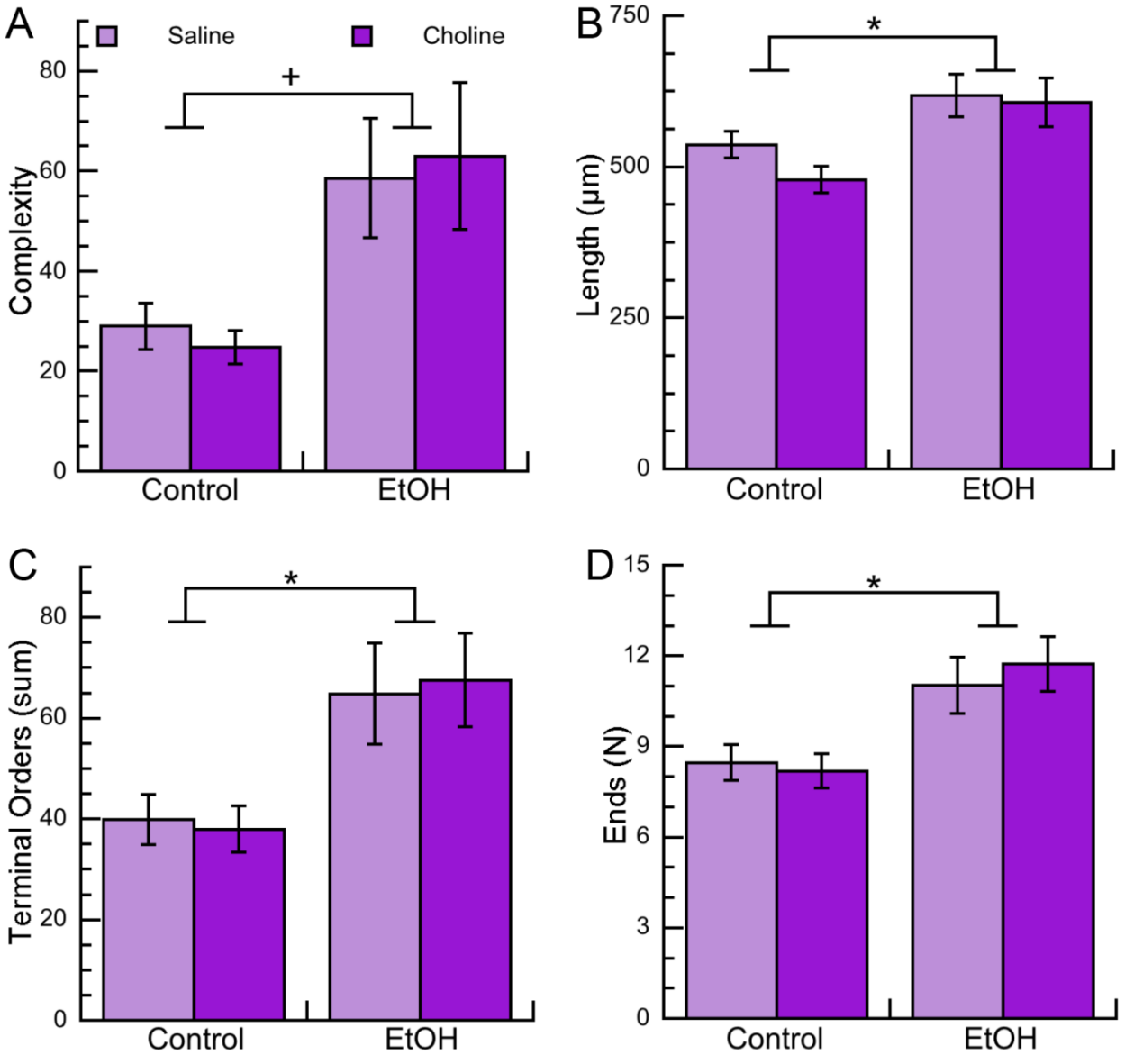
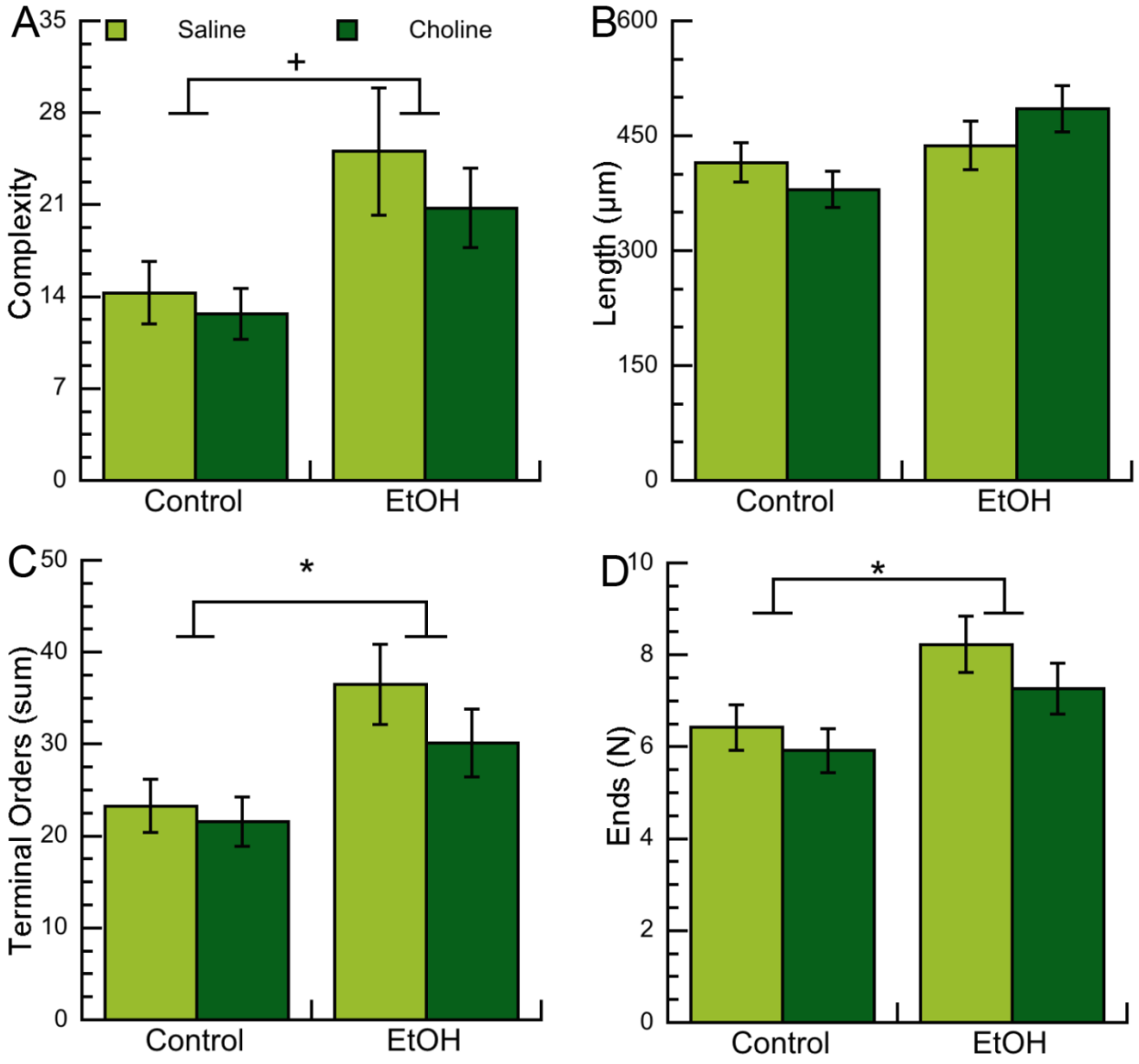

A
Saline
Choline
Complexity
80
60
40
20
0
+
Control
EtOH
B
750
500
250
0
Length (µm)
*
Control
EtOH
C
Terminal Orders (sum)
80
60
40
20
0
*
Control
EtOH
D
Ends (N)
15
12
9
6
3
0
*
Control
EtOH

Fig. 3.3. Effects of neonatal ethanol exposure and choline treatments on morphometric parameters of apical dendrites of CA1 pyramidal neurons in PD9 male rats. Morphometric measurements of apical dendrites of CA1 pyramidal neurons from male PD9 rats exposed to 5 g/kg/day ethanol and/or 100 mg/kg/day choline between PD4 and PD9 were analyzed by Neurolucida Explorer. (A) Apical dendrite complexity (1000x); a composite measurement defined as [(sum of terminal orders+number of ends)*(total dendrite length/number of primary dendrites)]. (B) Apical dendrite length (in μm). (C) Apical dendrite sum of terminal orders (defined as the number of ‘‘sister” branches encountered from each end to the cell body). (D) Number of ends per apical dendrite. Shown in each graph is the mean ± the standard error from 48 neurons per condition. Multilevel analysis was carried out on original (C, D) or log-transformed (A, B) data. +$p<0.05$ (before FDR corrections); *$q<0.05$ (after FDR corrections).


A
Saline
Choline
+
Complexity
35
28
21
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Control
EtOH
B
600
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150
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Length (µm)
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Terminal Orders (sum)
50
40
30
20
10
0
Control
EtOH
D
*
Ends (N)
10
8
6
4
2
0
Control
EtOH

Table 3.2. Female apical dendrite multilevel analysis and false discovery rate corrections.

Morphometric Parameter	χ^2		DF	p value		q value (FDR correction)	
	EtOH	Choline		EtOH	Choline	EtOH	Choline
Complexity*	5.4443	0.0152	1	0.0196	0.9020	N/A	N/A
Length (µm)*	4.1200	0.9476	1	0.0424	0.3303	0.042	N/A
Terminal Order*	4.8395	0.3830	1	0.0278	0.5360	0.042	N/A
Number of Ends*	6.2081	0.3911	1	0.0127	0.5317	0.038	N/A

*: Measurements were log-transformed prior to statistical analysis to satisfy the assumption of normal distribution of residuals.

Table 3.3. Male apical dendrite multilevel analysis and false discovery rate corrections.

Morphometric Parameter	χ^2		DF	p value		q value (FDR correction)	
	EtOH	Choline		EtOH	Choline	EtOH	Choline
Complexity*	5.1748	1.004	1	0.0229	0.31645	N/A	N/A
Length (µm)*	2.5249	0.0186	1	0.1121	0.8916	0.112	N/A
Terminal Order	6.6194	1.0833	1	0.0101	0.2980	0.030	N/A
Number of Ends	5.3213	1.2858	1	0.0211	0.2568	0.032	N/A

*: Measurements were log-transformed prior to statistical analysis to satisfy the assumption of normal distribution of residuals.

Surprisingly, no effects of choline were observed in any of the apical dendrite parameters analyzed in either females or males (Tables 3.2 and 3.3) indicating that six-day choline treatments did not affect apical dendrites in neonatal rats.

Effects of ethanol and choline on CA1 pyramidal neuron basilar dendrite parameters of neonatal female and male rats

Basilar dendrite morphometric parameters assessed were the same as the ones investigated for apical dendrites with two additions: the total number of dendrites (which, differently from the apical dendrite, is variable in basilar dendrites), and the maximal terminal distance. In PD9 female pups we did not observe effects of ethanol on any of the basilar dendrites morphometric parameters analyzed (Fig. 3.4A–F; Table 3.4); while in male animals we observed a trend toward increased basilar dendrite complexity, length, and terminal distance after multilevel analysis, but not after multiple comparison corrections (Fig. 3.5A, B, F; Table 3.5).

An interesting finding was that, in contrast to what was observed in apical dendrites, choline affected some basilar dendrite morphometric parameters. In females, we observed a trend ($p=0.096$) toward a decrease in basilar dendrite complexity (Fig. 3.4A; Table 3.4) and a decrease in the maximal terminal distance after multilevel analysis, but not after FDR corrections (Fig. 3.4F; Table 3.4). Furthermore, we observed that choline, but not choline plus ethanol, displayed a trend toward a reduction in every basilar dendrite parameter examined both in females and males. Because of this observation, we ran multilevel analyses followed by FDR corrections on sham control versus choline alone and found that, in females, maximal terminal distance was the only parameter significantly reduced by choline, but not significant after FDR corrections (Fig. 3.4F; Table 3.6). Interestingly, in males the effect of choline was more pronounced, as choline significantly reduced the total basilar dendrite length, the number of basilar dendrites, and the basilar dendrite maximal terminal distance (Fig. 3.5B, E,

Fig. 3.4. Effects of neonatal ethanol exposure and choline treatments on morphometric parameters of basilar dendrites of CA1 pyramidal neurons in PD9 female rats. Morphometric measurements of basilar dendrites of CA1 pyramidal neurons from female PD9 rats exposed to 5 g/kg/day ethanol and/or 100 mg/kg/day choline between PD4 and PD9 were analyzed by Neurolucida Explorer. (A) Basilar dendrite complexity (1000x); a composite measurement defined as [(sum of terminal orders+number of ends)*(total dendrite length/number of primary dendrites)]. (B) Basilar dendrite length (in μm). (C) Basilar dendrite sum of terminal orders (defined as the number of ‘‘sister” branches encountered from each end to the cell body). (D) Basilar dendrite number of ends. (E) Number of basilar dendrites per neuron. (F) Basilar dendrite maximal terminal distance (defined as the linear distance between the end of the farthest branch of the apical dendrite and the soma). Shown in each graph is the mean ± the standard error from 48 neurons per condition (with the exception of the sham intubation and choline injection group that had 36 neurons). +$p<0.05$ after multilevel analysis (before FDR corrections); p values approaching significance ($0.05<p<0.1$) are also reported.

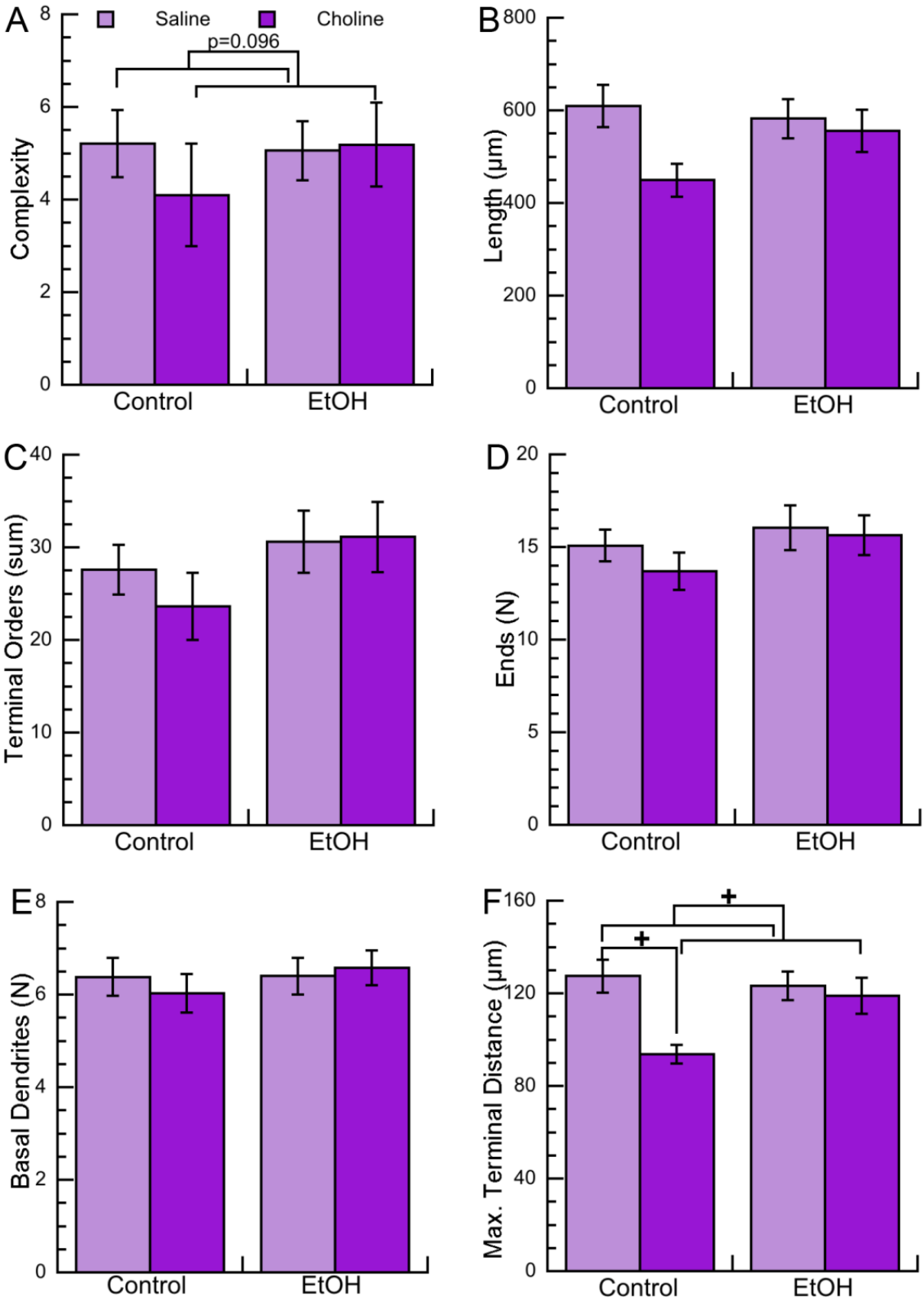

A
Saline
Choline
p=0.096
Complexity
0
2
4
6
8
Control
EtOH
B
Length (µm)
0
200
400
600
800
Control
EtOH
C
Terminal Orders (sum)
0
10
20
30
40
Control
EtOH
D
Ends (N)
0
5
10
15
20
Control
EtOH
E
Basal Dendrites (N)
0
2
4
6
8
Control
EtOH
F
Max. Terminal Distance (µm)
0
40
80
120
160
+
+
Control
EtOH

Fig. 3.5. Effects of neonatal ethanol exposure and choline treatments on morphometric parameters of basilar dendrites of CA1 pyramidal neurons in PD9 male rats. Morphometric measurements of basilar dendrites of CA1 pyramidal neurons from male PD9 rats exposed to 5 g/kg/day ethanol and/or 100 mg/kg/day choline between PD4 and PD9 were analyzed by Neurolucida Explorer. (A) Basilar dendrite complexity (1000x); a composite measurement defined as [(sum of terminal orders+number of ends)*(total dendrite length/number of primary dendrites)]. (B) Basilar dendrite length (in μm). (C) Basilar dendrite sum of terminal orders (defined as the number of ‘‘sister” branches encountered from each end to the cell body). (D) Basilar dendrite number of ends. (E) Number of basilar dendrites per neuron. (F) Basilar dendrite maximal terminal distance (defined as the linear distance between the end of the farthest branch of the apical dendrite and the soma). Shown in each graph is the mean ± the standard error from 48 neurons per condition. +$p < 0.05$ after multilevel analysis (before FDR corrections); *$q < 0.05$ (after FDR corrections); p values approaching significance ($0.05 < p < 0.1$) are also reported.

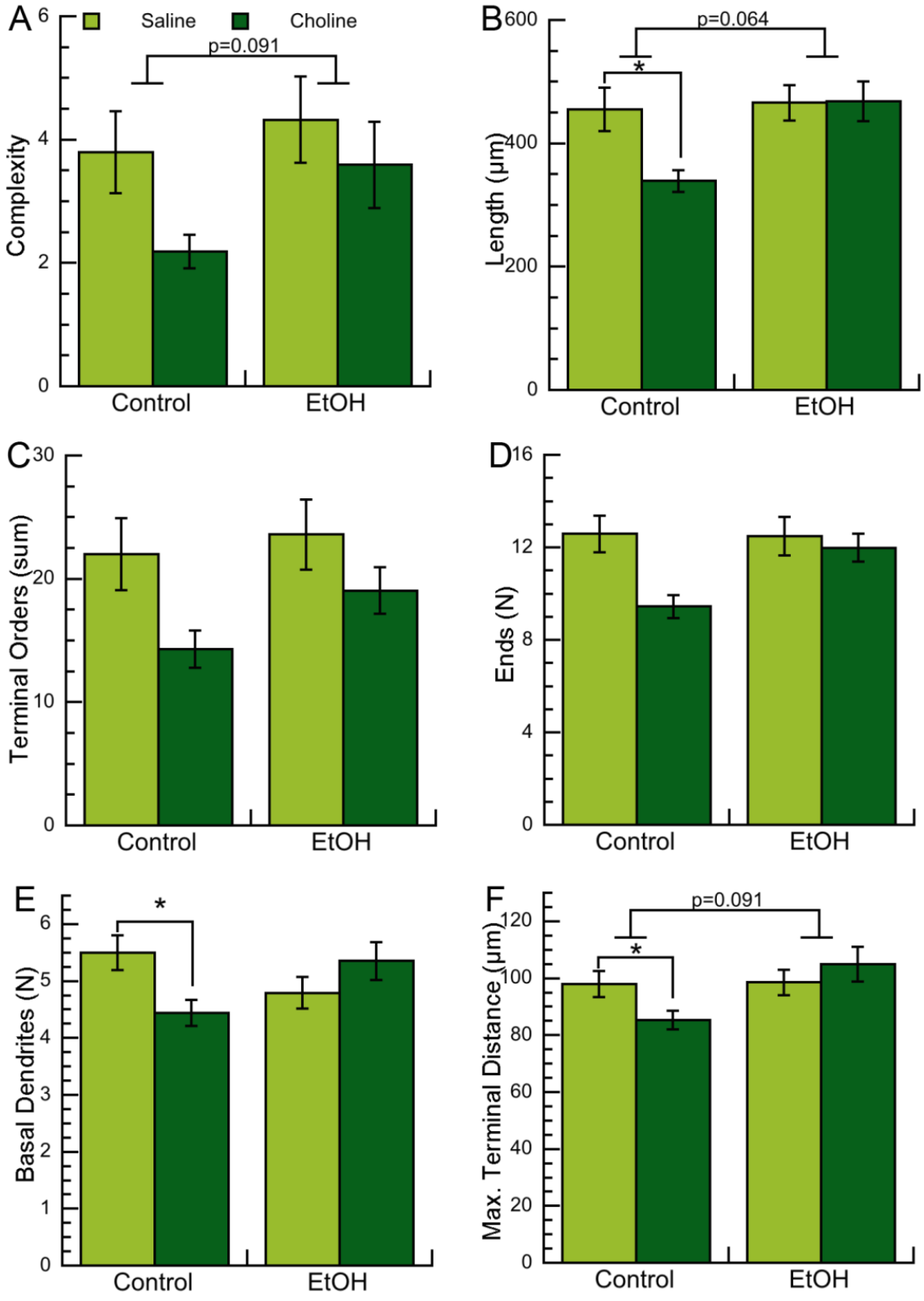

A
Saline
Choline
p=0.091
Complexity
0
2
4
6
Control
EtOH
B
p=0.064
*
Length (µm)
0
200
400
600
Control
EtOH
C
Terminal Orders (sum)
0
10
20
30
Control
EtOH
D
Ends (N)
0
4
8
12
16
Control
EtOH
E
*
Basal Dendrites (N)
0
1
2
3
4
5
6
Control
EtOH
F
p=0.091
*
Max. Terminal Distance (µm)
0
20
40
60
80
100
120
Control
EtOH

Table 3.4. Female basilar dendrite multilevel analysis and false discovery rate corrections.

Morphometric Parameter	χ^2		DF	p value		q value (FDR correction)	
	EtOH	Choline		EtOH	Choline	EtOH	Choline
Complexity*	0.2121	2.7733	1	0.6451	0.0958	N/A	N/A
Length (μm)*	0.0999	2.0041	1	0.7519	0.1569	N/A	0.392
Terminal Order*	0.6356	1.2496	1	0.4253	0.2636	N/A	0.439
Number of Ends*	0.3926	0.3678	1	0.5309	0.5442	N/A	0.680
Number of Basal Dendrites*	0.4184	0.0028	1	0.5177	0.9576	N/A	0.958
Terminal Distance*	1.0002	4.6575	1	0.3173	0.0309	N/A	0.155

*: Measurements were log-transformed prior to statistical analysis to satisfy the assumption of normal distribution of residuals.

Table 3.5. Male basilar dendrite multilevel analysis and false discovery rate corrections.

Morphometric Parameter	χ^2		DF	p value		q value (FDR correction)	
	EtOH	Choline		EtOH	Choline	EtOH	Choline
Complexity*	2.8630	1.1828	1	0.0906	0.2768	N/A	N/A
Length (μm)*	3.4234	2.0165	1	0.0643	0.1556	0.228	N/A
Terminal Order*	2.0250	0.7417	1	0.1547	0.3891	0.258	N/A
Number of Ends*	1.1684	1.5018	1	0.2797	0.2204	0.350	N/A
Number of Basal Dendrites*	0.0051	0.7132	1	0.9428	0.3984	0.943	N/A
Terminal Distance*	2.8500	0.7405	1	0.0914	0.3895	0.228	N/A

*: Measurements were log-transformed prior to statistical analysis to satisfy the assumption of normal distribution of residuals.

Table 3.6. Female basilar dendrite multilevel analysis and false discovery rate corrections of the effect of choline versus sham control.

Morphometric Parameters	$\chi 2$	DF	p value	q value (FDR correction)
Complexity*	2.3808	1	0.1228	N/A
Length (μm)*	1.7615	1	0.1844	0.319
Terminal Order*	1.7061	1	0.1915	0.319
Number of Ends*	0.4624	1	0.4965	0.621
Number of Basal Dendrites*	0.0447	1	0.8326	0.832
Terminal Distance*	4.0306	1	0.0447	0.223

*: Measurements were log-transformed prior to statistical analysis to satisfy the assumption of normal distribution of residuals.

Table 3.7. Male basilar dendrite multilevel analysis and false discovery rate corrections of the effect of choline versus sham control.

Morphometric Parameters	$\chi 2$	DF	p value	q value (FDR correction)
Complexity*	0.8150	1	0.3666	N/A
Length (μm)*	4.8686	1	0.0273	0.046
Terminal Order*	0.6581	1	0.4172	0.417
Number of Ends*	2.5605	1	0.1096	0.137
Number of Basal Dendrites*	7.2918	1	0.0069	0.035
Terminal Distance*	4.9383	1	0.0263	0.046

*: Measurements were log-transformed prior to statistical analysis to satisfy the assumption of normal distribution of residuals.

F; Table 3.7). In a similar manner, we also compared ethanol-treated animals with ethanol plus choline-treated animals; these analyses did not elicit any statistically significant results (not shown).

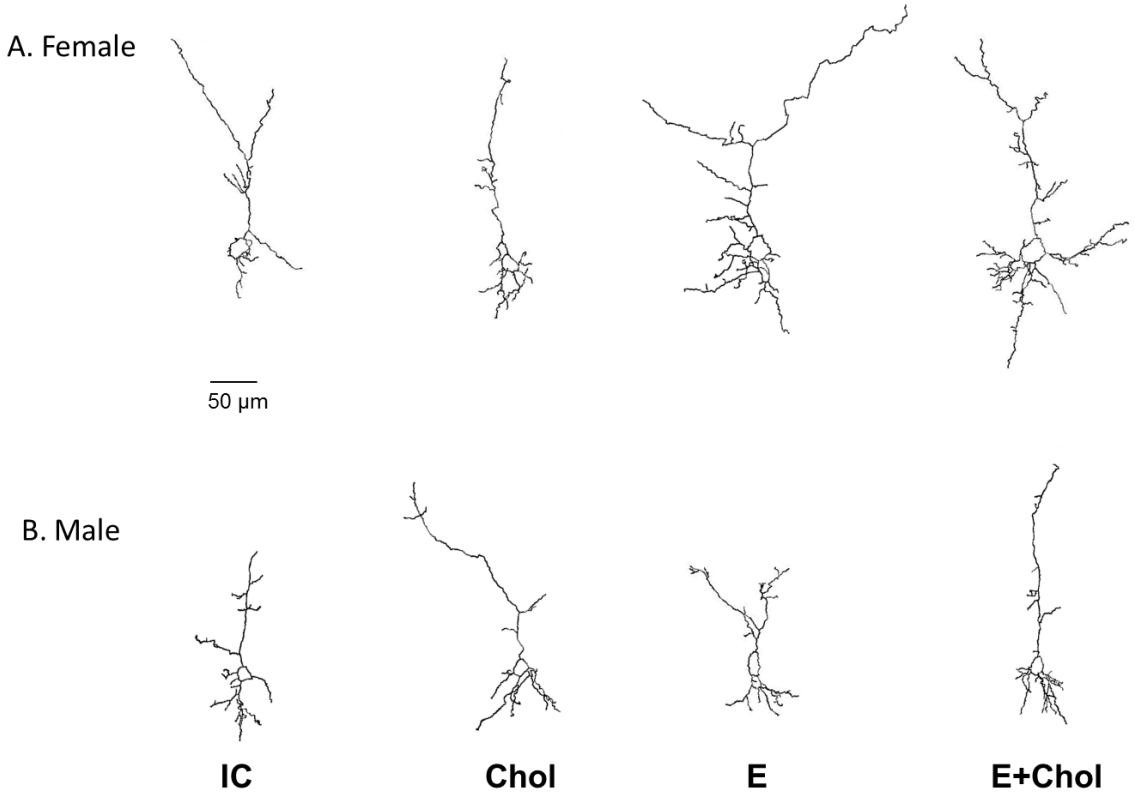
In summary, the effects of choline were always in an opposite direction compared to the effects of ethanol, as ethanol increased apical dendrite morphological parameters and choline decreased basilar dendritic morphological parameters. Choline therefore appeared to exert its effects mostly in the absence of ethanol, as when present together with ethanol, the effect of choline was abolished.

CA1 pyramidal neuron morphology at PD9

Representative tracings of CA1 pyramidal hippocampal neurons from female and male pups are shown in Fig. 3.6A and 3.6B respectively. Representative neurons were selected based on the criterion that complexity of the apical dendrite was close to the average apical complexity per each treatment and sex. CA1 pyramidal neurons from PD9 rats clearly display a simpler dendritic arbor compared to the arbor of hippocampal pyramidal neurons from adult animals, in agreement with the notion that at PD9 dendritic arborization is still ongoing. It has been reported that, in the adult rat, the combined length of all CA1 dendritic branches is between 12 and 13.5 mm, of which 36% (4.32–4.86 mm) is from basilar dendrites and 64% (7.68–8.64 mm) is contributed by apical dendrites (Spruston & McBain, 2007). We found that, in CA1 neurons at PD9, the total basilar dendrite length is 455.15 µm ± 35.3 in males and 609.4 µm ± 45.1 in females, corresponding to 53.3% and 53.18% of the total dendrite length respectively. The total length of the apical dendrite is 415.17 µm ± 47.7 in males and 536.5 µm ± 46.8 in females, corresponding to 47.7% and 48.8% of the total dendrite length respectively. The number of basilar dendrites at this developmental stage (6.38 ± 0.41 in females and 5.5 ± 0.305 in males) is comparable to that of adult animals (on average 5) (Spruston & McBain, 2007), indicating that all the dendrites have formed but the overall growth and arborization is incomplete.

DISCUSSION

Fig. 3.6. Representative Neurolucida tracings of female and male hippocampal CA1 pyramidal neurons. Shown are the tracings of female (A) and male (B) neurons from each of the four treatment conditions. IC: Intubation and injection control; Chol: choline injected; E: ethanol-intubated; E+Chol: ethanol intubated and choline injected animals. The displayed neurons were selected because their apical dendrites' complexity was the closest to the mean complexity value per treatment group per sex. Scale bar represents 50 µm.



Studies of children prenatally exposed to ethanol have shown significant deficits in hippocampus-mediated processes (Pei et al., 2008; Willoughby et al., 2008). Several preclinical studies of developmental ethanol exposure have also identified alterations in hippocampus-dependent behaviors (Berman & Hannigan, 2000; Christie et al., 2005; Gianoulakis, 1990; Goodlett & Peterson, 1995; Johnson & Goodlett, 2002; Kelly et al., 1988; Patten et al., 2014; Popovic et al., 2006; Thomas et al., 2008; Thomas et al., 2010); in some of these studies, choline supplementation improved the behavioral outcome of neonatal ethanol exposure (Schneider & Thomas, 2016; Thomas et al., 2004a; Thomas et al., 2007). Alterations in brain connectivity and processing after in utero ethanol exposure suggested by some recent human studies (Lebel et al., 2012; Wozniak et al., 2013) may be responsible for some of the behavioral and cognitive effects of developmental ethanol exposure. In support of this hypothesis, recent work found that alterations in structural plasticity and neuronal cytoarchitecture (measured as changes in dendritic arborization and neuronal morphology) in the prefrontal cortex of adult animals were associated with alterations in synaptic plasticity and behavior (Kolb & Gibb, 2015; McEwen, 2013).

Previous studies have examined the effects of developmental ethanol exposure on dendrite arborization and neuronal morphology of cortical pyramidal neurons and spiny neurons of the nucleus accumbens in juvenile or adult animals (Hamilton et al., 2010; Hamilton et al., 2015; Rice et al., 2012; Whitcher & Klintsova, 2008). However, a systematic investigation of parameters of structural plasticity of pyramidal neurons of the hippocampus, a region highly affected by developmental ethanol exposure, after neonatal ethanol exposure and/or choline intervention has not been carried out. Therefore, we undertook the present study to explore changes induced by ethanol and choline exposure during the third trimester equivalent of human gestation on CA1 pyramidal neuron morphometric parameters after Golgi–Cox staining of PD9 brains.

We decided to examine changes induced by ethanol immediately after exposure in PD9 animals, i.e., in animals at a developmental stage corresponding to the end of the third trimester of gestation in humans. This developmental window is characterized by, among others, a fast and massive increase in brain size, dendritic arborization, and glial cell proliferation and by the beginning of synaptogenesis. The rationale behind our approach is that deviations from the physiological program of neuronal morphological development occurring at this very critical developmental stage, when numerous events need to occur in a synchronized and coordinated manner, very likely lead to long-lasting alterations in brain connectivity even if these morphological differences are no longer detectable later in life. Altered morphology of neurons at this developmental stage is therefore likely to predict alterations in brain circuits and behavior in adolescence or adulthood.

The present study differs from previous studies in several ways: (1) nearly all the published studies analyzed neurons at a much later time-point in development (i.e., in juvenile or adult animals) (Hamilton et al., 2010; Rice et al., 2012; Whitcher & Klintsova, 2008); (2) previous studies analyzed morphological changes in different neuronal populations (i.e., in cortical pyramidal neurons or spiny neurons of the nucleus accumbens) (Hamilton et al., 2010; Rice et al., 2012; Whitcher & Klintsova, 2008); and (3) there are no published studies investigating the effect of choline on ethanol-induced changes in neuronal morphology. Because the dendritic tree of pyramidal neurons has two distinct domains: the basilar and the apical dendrites, which are morphologically very different (Spruston, 2008), we analyzed apical and basilar dendrites separately. Pyramidal neurons have one large apical dendrite per neuron that emerges from the apex of the soma and branches several times. The apical dendrite of CA1 pyramidal neurons occupies the *stratum radiatum* (proximal apical) and the *stratum lacunosum-moleculare* (distal apical) and extends to the hippocampal fissure (Spruston, 2008). At the opposite end, pyramidal neurons have several relatively short basilar dendrites, which emerge from the

base of the pyramidal cell's soma, occupy the *stratum oriens*, and reach toward the *alveus* of the hippocampus (Spruston, 2008).

We found that ethanol increases the complexity of apical dendrites (Figs. 3.2 and 3.3) without affecting the maximal terminal distance from the soma in both female and male pups (not shown) indicating that ethanol increases the arborization of apical dendrites without affecting their reach toward the hippocampal fissure. Ethanol did not affect basilar dendrites in female pups (Fig. 3.4). The observed trend (not statistically significant) toward an increase in basilar dendrite complexity, length, and maximal terminal distance induced by ethanol in male pups appears to be driven by the decrease in basilar dendrite arborization observed in the choline group (Fig. 3.5). A possible interpretation of our results is that developmental ethanol exposure induces a premature maturation of apical dendrites in hippocampal pyramidal neurons, which may lead to premature restriction in neuroplasticity and in the ability of developing neurons to respond to intrinsic and environmental signals, as well as to altered synaptic circuits.

In line with our findings, there is an extensive literature indicating increased dendritic arborization in pyramidal neurons of the adult rat mPFC after exposure to stimulant drugs, such as amphetamine, cocaine, nicotine and tetrahydrocannabinol (reviewed in (Kolb & Gibb, 2015). It has been hypothesized that this drug-induced increase in dendritic arborization may reduce the physiological plasticity of neurons in response to environmental enrichment (Kolb et al., 2003). Additionally, the observed ethanol-induced increase in dendritic arborization is in agreement with the hyperconnectivity of multisensory areas of the cortex reported in ferrets exposed to ethanol during the period equivalent to the third trimester of human gestation (Tang et al., 2017). This study is also in agreement with several other studies reporting altered neuroplasticity in animal models of FASD (reviewed in (Medina, 2011).

A second goal of this research was to investigate whether choline prevents the effects of ethanol on neuronal morphology, as it has been reported that choline ameliorates neonatal ethanol exposure-induced behavioral alterations (Schneider & Thomas, 2016; Thomas et al., 2004a; Thomas et al., 2007). We found that choline did not affect apical dendrites (Figs. 3.2 and 3.3), but decreased basilar dendritic arborization in males and, to a lesser extent, in females (Figs. 3.4 and 3.5).

Our results suggest that choline and ethanol both affect the structural plasticity of developing hippocampal neurons, albeit in opposite direction. Indeed, while ethanol increases dendritic arborization and complexity mostly in the apical dendrites, choline decreases length, number, and terminal distance in the basilar dendrites of male pups and displays a trend toward decreased complexity and terminal distance in female pups. It can be hypothesized that, in opposition to ethanol, choline slows down the process of neuronal differentiation, therefore allowing for prolonged plasticity in response to intrinsic and environmental factors during this period of brain development.

It should be pointed out that in our study choline, while effective in reducing basilar dendrite arborization when administered alone, did not prevent the effects of ethanol when co-administered. The reason for this may be that we carried out treatments with choline for only six days, while choline was reported to improve alterations in hippocampus-dependent behaviors induced by neonatal ethanol exposure after about 3 weeks of intervention (Schneider & Thomas, 2016; Thomas et al., 2007; Thomas et al., 2010). In the present study we did not carry out dendritic spine analysis because at this developmental stage few fully formed synaptic spines are present in the CA1 region of the hippocampus (Bourne & Harris, 2008), in agreement with the notion that the majority of synaptogenesis occurs postnatally in humans and during the third postnatal week in rodents (Semple et al., 2013).

In conclusion, our study investigated for the first time the effects of neonatal ethanol and choline treatments on CA1 pyramidal neuron dendritic arborization after Golgi–Cox staining of PD9 brains. Our results suggest that ethanol accelerates, while choline delays, the development of CA1

pyramidal neurons. Choline and ethanol appear to work through different mechanisms; indeed different morphometric parameters are affected by choline and ethanol, with ethanol increasing dendritic branching in apical dendrites of female and male pups and choline decreasing the total length, number of ends, and terminal distance of the basilar dendritic tree in male pups and trending toward a decrease in basilar dendrite complexity and maximal terminal distance in females. Although the effects of ethanol were not counteracted by the effects of choline in our study, this may be the result of the experimental design in which choline treatments were carried out for only six days (during ethanol treatments). The experimental design employed in this study, in which changes in dendritic arborization were analyzed in still-developing neurons, allowed us to identify ethanol-induced effects not previously reported that very likely lead to altered brain connectivity.

The alterations in dendritic arborization induced by ethanol in hippocampal pyramidal neurons of PD9 rats may be, at least in part, responsible for the behavioral and cognitive effects of developmental ethanol exposure. Our study indicates that choline supplementation does not prevent the effects of ethanol on the developing CA1 pyramidal neurons. However, further studies are necessary to investigate whether longer choline treatments after ethanol exposure can rectify the effects of ethanol on dendrite arborization.

CHAPTER 4: NEONATAL CHOLINE EXPOSURE CONTINUING UNTIL ADOLESCENCE HAS A SEX-SPECIFIC EFFECT ON THE NEURONAL MORPHOLOGY OF HIPPOCAMPAL CA1 PYRAMIDAL NEURONS IN RATS

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Support

This work was supported by NIH/NIAAA F31AA024950-01A1 to CG, by VA Merit Review Award # I01BX001819 and by NIH/NIAAA R01AA021468 and R011AA022948 to MG, and by facilities and resources at the VA Portland Health Care System.

ABSTRACT

Exposure of the developing fetus to ethanol during gestation can result in Fetal Alcohol Spectrum Disorders (FASD) (Riley et al., 2011). A number of rodent models have been developed to examine FASD in the laboratory, one of which (Goodlett & Peterson, 1995; Goodlett & Johnson, 1997; Johnson & Goodlett, 2002) targets the brain growth spurt (Dobbing & Sands, 1979). The brain growth spurt, which occurs primarily during gestation in humans, is a period of interest in FASD research, and occurs postnatally in rats (postnatal days 4-9). Therefore, we used intragastric intubation and subcutaneous injection to expose neonatal rats to ethanol and choline, respectively, stopping the ethanol exposure on postnatal day (PD) 9, but continuing choline exposure until PD30. We then examined the apical and basilar dendrites of CA1 hippocampal pyramidal neurons using Golgi-Cox staining, to quantify any morphological changes caused by neonatal ethanol exposure, and any possible amelioration by long-term choline exposure. Choline had a sex specific effect; male animals exposed to choline had decreased parameters of apical dendrite morphology when compared to both female animals exposed to choline and male control animals. We did not see any significant morphological effects of ethanol apical dendrites, although there was a trend toward decreased complexity and terminal orders in male apical dendrites following ethanol intubation, as well as a decrease in the sum of terminal orders in female basilar dendrites exposed to both ethanol and choline. These data indicate that the effect of choline exposure differs by sex, and that the long-term effects of developmental ethanol exposure are not as prominent in the adolescent hippocampus as they are in the neonatal brain.

INTRODUCTION

Drinking ethanol during pregnancy is very prevalent (Popova et al., 2017), despite the fact that ethanol is known to cross the placenta and the blood-brain barrier (BBB), and to have effects on the fetus. These effects are collectively known as Fetal Alcohol Spectrum Disorders (FASD) (May et al., 2014; Riley et al., 2011), and are characterized a variety of CNS dysfunctions (Medina, 2011; Riley et al., 2011).

Such dysfunctions include attention deficits and motor hyperactivity (Aragon et al., 2008), lower global intelligence (Lebel et al., 2012), impaired memory (MacIlvane et al., 2016; Waddell & Mooney, 2017), and difficulties in spatial learning (Wagner et al., 2014). In the United States alone, it is estimated that between 24 and 48 in 1,000 live births are individuals with FASD, a number which is considerably higher in other populations in the world, for example a population in South Africa, where it is estimated that between 68 and 89 in 1,000 live births are individuals with FASD (Popova et al., 2017). FASD is therefore considered to be the greatest cause of preventable intellectual disability.

The ethanol-induced effects on the brain that underlie the FASD are not fully understood. However, it is hypothesized that specific deficits and alterations seen in FASD may depend on the gestational timing of ethanol exposure. A key event during brain development is the brain growth spurt (Dobbing & Sands, 1979; Rice & Barone, 2000), which is characterized by a rapid increase in brain size and weight, as well as increased astrocyte proliferation and dendritic arborization (Rice & Barone, 2000). In humans, the brain growth spurt occurs, in part, prenatally, during the third trimester of gestation, and is therefore a target for the teratogenic effects of ethanol.

Another important area of FASD research is development of a treatment for individuals prenatally exposed to ethanol. There are a number of potential treatments that are being investigated, such as voluntary exercise. Voluntary exercise has been shown to reduce ethanol induced decreases in dendritic complexity of the prefrontal cortex (Hamilton et al., 2015), decrease ethanol-induced increases in levels of hippocampal BDNF, and increase ethanol-induced decreased levels of DNA methylation in the hippocampus (Boschen et al., 2016a). These data indicate that voluntary exercise is a potential treatment that can successfully ameliorate some of the effects of gestational ethanol exposure. However, the most prominent potential treatment for FASD is choline, which is an essential nutrient that is found in many foods, including liver and wheat germ (Zeisel & Niculescu, 2006). Like ethanol,

choline can cross both the placenta and the BBB (Allen & Lockman, 2003), and can therefore also affect the developing fetus.

A substantial amount of work has been done to assess the possibility of choline as a treatment for individuals with FASD. Such preclinical research has found that choline supplementation can reduce ethanol-induced hyperactivity (Thomas et al., 2007), decrease the amount of ethanol-treatment induced errors on reversal learning tasks (Thomas et al., 2004a), and decrease the number of trials needed to reach success on discrimination learning tasks (Thomas et al., 2000). However, the effects of choline appear to be selective, as there was no change following choline exposure on ethanol-induced motor deficits in coordination (Thomas et al., 2004b). Furthermore, many of the studies examining the effects of choline on ethanol have been done in only males (Bekdash et al., 2013; Hurtado et al., 2007; Li et al., 2004), and it is relatively unknown if there are any sex differences in the response to choline treatment.

As many of the behaviors changed by ethanol and ameliorated by choline are hippocampus-dependent, it is worth analyzing morphological changes in the hippocampus following ethanol exposure, and whether or not these changes are reversed by choline. Previously (chapter 3), we used a model of FASD in rodents (Goodlett & Johnson, 1997; Patten et al., 2014) to examine the morphological effects of ethanol and/or choline given to rats from postnatal day (PD) 4-9 (Goeke et al., 2018). PD4-PD9 is when rodents undergo their brain growth spurt (Dobbing & Sands, 1979), and this time period is therefore considered to be the equivalent to the third trimester of human gestation. In our previous study (Goeke et al., 2018) in chapter 3, we showed that ethanol intubation from PD4-PD9 significantly increased several measures of neuronal morphology in CA1 hippocampal pyramidal cells in a non sex-dependent manner. These parameters included the length, complexity and branching of the apical dendrites of these cells, indicating that ethanol may be inducing a premature maturation at this time point. We also observed that choline trended toward a decrease in several parameters in basilar dendrites; specifically, basilar dendrite length, dendrite quantity, and dendrite reach were decreased following choline

exposure. In the apical dendrites, the increase following ethanol exposure was seen more strongly in females, while the decrease in basilar dendrite parameters following choline was seen more strongly in males. Thus, not only did we see changes in opposite directions caused by ethanol and choline exposure, this change was specific to the area of the cell examined, and differed in strength by sex.

As there is a substantial body of literature indicating that choline may have a rescue effect on the changes caused by ethanol (Bearer et al., 2015; Bekdash et al., 2013; Idrus et al., 2016; Monk et al., 2012; Ryan et al., 2008; Schneider & Thomas, 2016; Tang et al., 2014; Thomas et al., 2000; Thomas et al., 2004a; Thomas et al., 2007; Thomas et al., 2008; Thomas et al., 2009; Thomas et al., 2010), but our previous study (chapter 3) did not find any rescue effect of short-term choline treatment (Goeke et al., 2018), we expanded the time period of choline exposure in this present study. Specifically, while we kept the ethanol exposure paradigm the same, from PD4-PD9, we continued the choline injections through to PD30, which is roughly around the beginning of rat adolescence (Semple et al., 2013). As with our previous study, we collected the brains for examination of parameters of neuronal morphology via Golgi-Cox staining. We saw no significant changes following ethanol intubation on apical dendrites. We did, however, see a sex-specific effect of choline. Male animals given choline had decreased parameters of apical dendrite morphology in hippocampal CA1 pyramidal neurons when compared to both female animals given choline, and male control animals. However, in female basilar dendrites, ethanol and choline in combination resulted in fewer terminal orders than choline alone; there was a trend toward this same pattern in female basilar ends.

The results of this paper, in combination with the results previously reported, support the idea that choline influences neuronal morphology, and in doing so may be a potential treatment for individuals with FASD. However, the efficacy of choline as a treatment may depend on both the timing of the choline supplementation, as well as sex. These results also shed light on morphological changes caused by ethanol; at PD9, it appeared that ethanol induced a premature maturation, where there were

few effects at PD30. We postulate that the premature maturation seen at PD9 may result in altered connections in these neurons at PD30, which in turn may underlie some of the behavioral and cognitive symptoms seen in individuals with FASD.

METHODS

Animals

Time-mated gestational day (GD) 15 Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Upon arrival, animals were maintained in the VA Portland Health Care System Veterinary Medical Unit at 22 ± 1°C under a 12-hour light/dark cycle. Pregnant animals had *ad libitum* access to food and water. All animal procedures were approved by the VA Portland Health Care System Institutional Animal Care and Use Committee, and followed US National Institutes of Health animal welfare guidelines.

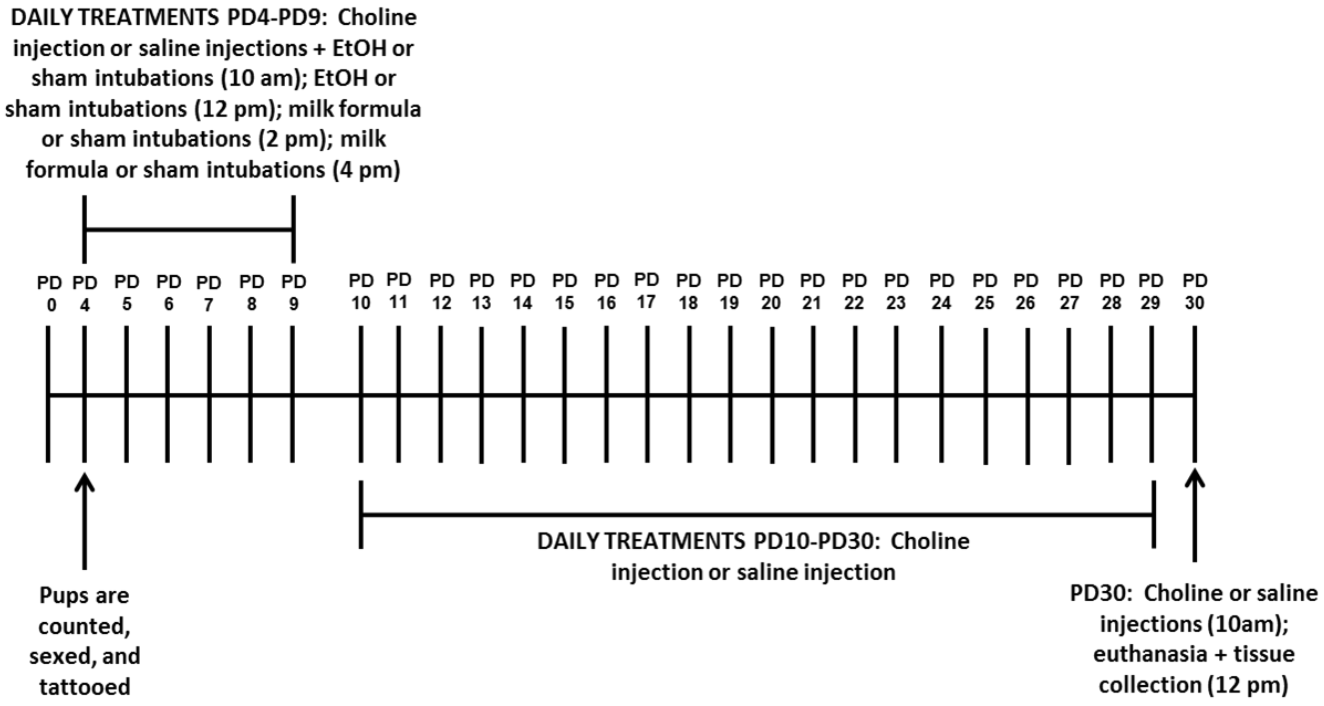
Ethanol Intubation and Choline Injections

On PD4, pups in each litter were counted, sexes were determined, and pups were pseudo-randomly assigned to experimental groups. When possible, litters were culled to 10 pups: 5 females and 5 males, to allow for 1 pup/litter/sex to be assigned to each group. Within a litter, no more than one pup of each sex was assigned to each group. India ink was injected subcutaneously into the pups' paws for identification. There were ten groups (5 female and 5 male): intubated control (sham intubation + saline injection), choline (sham intubation + choline injection), ethanol (ethanol intubation + saline injection), ethanol and choline (ethanol intubation + choline injection) and untouched animals. Untouched animals remained with the dam at all times, and analysis showed no difference between untouched animals and sham intubation + saline injection animals (data not shown). In total, for the results reported, there were 30 animals spread across the 4 treated groups: 4 animals for each of the groups, with the exception of the ethanol and choline, and the intubated control groups, which had only 3 females.

Every day from PD4-PD30, animals were weighed, and their weight was used to determine the volume of the injection/intubation to be administered. Once a day, animals were given a single injection of either saline or choline chloride (100 mg/kg) subcutaneously. Each animal was also intubated 4 times daily, from PD4-PD9, with all intubations being two hours apart. All intubations involved inserting flexible tubing, dipped into corn oil for lubrication, through the mouth and the esophagus, into the stomach of the rat. All sham intubations consisted of inserting the tube into the rat to replicate the physical effects of the ethanol intubation, but without any treatment. For the ethanol intubated groups, the first two intubations of the day consisted of ethanol (5 g/kg/day) in milk formula (Similac Advance Early Shield with Iron). These intubations were at a concentration of 11.9% ethanol and an intubation volume of 0.0278 mL/g. The second two intubations in the ethanol group were milk formula alone. This was done to offset the lack of suckling in the ethanol-intubated, intoxicated animals.

During the intubation process, which took approximately 10 minutes/litter, the animals were removed from their dam and placed on a heating pad, and pups were weaned at PD21. Sham and ethanol intubations finished on PD9. Between PD10 and PD30, the animals were still weighed daily, and continued receiving their subcutaneous injections of saline or choline, which took approximately 3-4 minutes/litter. On PD30, two hours after the injection, the animals were anesthetized with an intraperitoneal cocktail (Ketamine; 500 mL/10 mL, 100 mg/kg, Xylazine; 50 mg/10 mL, 10 mg/kg, Acepromazine; 10 mg/10 mL, 1 mg/kg) in 0.9% saline. Following confirmation of anesthesia via toe pinch, animals were decapitated and their brains were collected for Golgi-Cox staining (Das et al., 2013). Four litters were used, and initially all had 1 animal/sex/group. However, one female (ethanol intubation + choline injection) died during the study, and one female brain (sham intubation + saline injection) was lost during tissue staining. A timeline of these treatments can be seen in Figure 4.1.

Figure 4.1. Timeline of experimental design employed in this study. On PD4, all litters were counted, the sexes of the pups were determined, and the pups were assigned to treatment groups. Each pup was given a number, and tattooed for identification. From PD4-PD9, all pups were given a single subcutaneous injection of either saline or choline, along with either four sham intubations, or two intubations of ethanol in milk formula and two intubations of milk formula alone. All intubations were two hours apart (10 am, 12 pm, 2 pm, 4 pm). From PD10-PD30, the pups were only given a single injection of either saline or choline. On PD30, the animals were euthanized 2 hours after the injection and the brains were collected for Golgi-Cox staining. All animals were weighed once a day, every day from PD4-PD30.



Tissue Collection and Staining

Brains were stained with the Golgi-Cox Solution according to the manufacturer's instructions (FD Rapid GolgiStain™ Kit, NeuroTechnologies Inc., Columbia, MD, USA). Briefly, the brains were rinsed in water immediately following removal from the animal and immersed in the Golgi-Cox impregnation solution, where they remained for two weeks at room temperature. Following this, the brains were transferred to Solution C (from the same kit) and incubated for 48 hours at 4°C. The tissue was then rapidly frozen and embedded in Tissue Freezing Medium for sectioning on a cryostat. Brains were sliced coronally at a thickness of 100 µm, before being mounted on gelatin-coated microscope slides. Slides were dried at room temperature overnight.

Microscopy

Hippocampal pyramidal neurons from the CA1 region were digitally reconstructed using the microscopy software Neurolucida (Version 11, MBF Bioscience, Williston, VT) on a Leica DM500b microscope with a DFC30 FX camera. The researcher responsible for the digital reconstruction was blind to the treatment group of the neurons. 4-5 slices/brain containing the central part of the hippocampus were selected for analysis, and 12 cells/brain (six cells/hippocampal hemisphere) were traced. In order to be selected for tracing, pyramidal hippocampal neurons had to be fully impregnated with Golgi-Cox staining and clearly distinguishable from any neighboring cells. The apical and basilar dendrites of these cells were measured separately; all data were analyzed using the program Neurolucida Explorer. Four apical dendrite parameters were analyzed: complexity, total apical dendrite length (µm), the sum of the terminal orders, and the number of ends. Basilar dendrite analysis included the same four parameters as the apical dendrites, but also included the number of basilar dendrites/neuron. Complexity was calculated by Neurolucida as (sum of terminal orders + number of ends) * (total dendrite length/number of primary dendrites).

Statistical Analysis

Body weights were analyzed via a multilevel analysis accounting for day as a random effect, followed by Tukey's post-hoc analysis. For the digital reconstruction parameters, we used a nested, multilevel analysis (Aarts et al., 2014). We used linear mixed effect analysis (multilevel analysis), as previously described in chapter 3 (Goeke et al., 2018), using the statistical program R. The R package lme4 was used to perform the multilevel analysis (Bates et al., 2015; Team & RC, 2017), and accounted for the animal as a random effect, in order to account for the fact that multiple neurons were measured within a single animal. Intubation (ethanol vs. sham) and injection (saline vs. choline) were used as fixed effects, and p-values were obtained by likelihood ratio tests. All data were log-transformed before analysis in order to satisfy normality and homoscedasticity assumptions; following log-transformations, all Q-Q and Residuals plots indicated compliance with homoscedasticity and normality. Significance was $p \leq 0.05$. All data are reported as mean ± S.E.M. of either 48 neurons/group or 36 neurons/group.

RESULTS

Treatment Outcomes: Body Weights

Animals in all treatment groups were weighed once daily from PD4-PD30. There were significant differences in both female and male body weights (Table 4.1). These differences were not due to either choline or saline injections, but were due to ethanol intubations. Specifically, female sham intubated animals weighed more than female ethanol intubated animals from PD5-PD12 ($p<0.05$), and male sham animals weighed more than male ethanol intubated animals from PD5-PD14 ($p<0.05$). Following female PD12 and male PD14, there were no longer any significant differences in body weight due to treatment. We did not measure the blood ethanol concentrations of these animals, as the ethanol treatment was no longer ongoing at the time of euthanasia. However, previous studies (chapter 3) indicate that the expected blood ethanol concentrations at PD9 would be between 60 mM and 65 mM, with no difference between sexes (Goeke et al., 2018).

Table 4.1. Body weights. Pups of both sexes were weighed once daily from PD4-PD30. Ethanol intubation was from PD4-PD9 and choline injection was from PD4-PD30. Data were analyzed using a multilevel analysis, which showed a main effect of ethanol intubation [$\chi^2(1, N = 30) = 152.91$, $p<0.001$]. Subsequent Tukey's post-hoc analysis (ethanol vs. sham intubation) showed that females given ethanol weighed less than sham intubated females from PD5-PD12, and male animals given ethanol weighed less than sham intubated males from PD5-PD14. Data are given for each day as mean ± S.E.M. *p<0.05,Tukey's post-hoc analysis.

Body Weights of Female Pups (g)								
Day	Sham+ Saline	SEM	Sham+ Choline	SEM	EtOH+ Saline	SEM	EtOH+ Choline	SEM
PD4	10.35	0.50	10.03	0.24	9.58	0.43	10.13	0.30
PD5*	12.53	0.53	12.23	0.39	10.78	0.68	10.95	0.39
PD6*	14.50	0.63	14.25	0.47	12.00	0.75	12.70	0.57
PD7*	16.78	0.63	16.00	0.47	13.38	0.80	14.37	0.71
PD8*	18.83	0.68	18.28	0.70	15.00	0.91	16.13	0.98
PD9*	21.20	0.56	20.73	0.78	16.70	0.98	18.00	0.96
PD10*	23.75	0.55	23.03	0.77	18.80	1.08	20.00	0.99
PD11*	25.68	0.76	24.93	0.91	21.25	1.20	22.90	1.12
PD12*	27.60	0.90	26.45	0.89	22.85	1.09	24.67	1.19
PD13	30.23	0.80	29.03	0.70	25.83	1.35	27.43	1.45
PD14	32.53	0.81	30.88	0.76	28.03	1.57	30.13	1.51
PD15	34.73	1.13	33.25	0.98	29.98	1.49	32.30	1.48
PD16	37.60	0.88	35.05	1.09	31.83	1.48	34.43	1.66
PD17	39.85	0.88	38.10	1.14	34.45	1.42	36.97	1.47
PD18	42.50	1.27	40.75	1.36	36.43	1.54	39.57	1.07
PD19	45.83	1.38	43.90	1.61	39.15	1.87	42.40	1.31
PD20	50.13	1.82	48.13	2.52	42.98	2.73	46.63	2.34
PD21	54.03	2.09	51.55	2.91	46.18	2.86	50.13	2.08
PD22	57.25	2.30	55.08	3.68	49.40	3.45	53.83	2.31
PD23	62.80	2.07	60.48	3.72	54.58	3.95	59.30	2.05
PD24	69.38	3.08	67.05	3.84	60.95	3.77	64.20	2.31
PD25	75.30	2.74	72.20	4.04	66.35	4.15	69.67	2.16
PD26	81.03	3.56	77.48	4.14	70.83	4.32	74.23	1.94
PD27	87.90	4.21	84.23	4.75	77.75	4.52	81.07	2.26
PD28	94.25	4.30	89.95	4.34	83.80	4.58	86.03	1.99
PD29	100.03	5.63	95.93	5.03	89.33	4.92	91.00	2.57
PD30	105.65	5.60	101.35	5.83	94.95	5.84	96.53	2.92

Body Weights of Male Pups (g)								
Day	Sham+ Saline	SEM	Sham+ Choline	SEM	EtOH+ Saline	SEM	EtOH+ Choline	SEM
PD4	10.55	0.44	10.33	0.20	10.73	0.50	10.65	0.68
PD5	12.73	0.66	12.43	0.35	11.55	0.49	11.20	0.59
PD6*	14.83	0.69	14.30	0.32	13.20	0.50	12.63	0.75
PD7*	16.73	0.73	16.63	0.34	14.78	0.62	13.85	0.77
PD8*	19.15	1.04	18.63	0.54	16.20	0.80	15.60	0.94
PD9*	21.43	1.19	21.00	0.60	17.58	1.11	17.20	1.08
PD10*	24.33	1.37	23.15	0.56	19.70	1.26	19.25	1.26
PD11*	26.20	1.46	25.33	0.79	21.95	1.26	21.65	1.16
PD12*	27.70	1.31	26.65	0.69	23.80	1.23	23.38	0.99
PD13*	30.55	1.72	29.73	0.83	26.75	1.78	25.75	1.12
PD14*	32.98	1.76	31.68	1.01	28.95	1.72	27.95	1.20
PD15	34.70	1.60	34.25	1.08	31.45	2.13	29.90	1.72
PD16	37.03	1.43	36.00	1.12	33.43	2.17	32.18	1.91
PD17	39.35	1.46	38.80	1.42	36.10	2.23	35.00	1.55
PD18	41.75	1.96	41.63	1.48	38.28	2.32	37.28	1.82
PD19	44.90	2.45	44.35	1.55	41.25	2.52	39.75	2.18
PD20	49.35	2.93	48.65	2.22	45.33	3.09	44.03	2.81
PD21	53.68	2.79	52.08	2.22	48.98	3.60	47.53	3.34
PD22	58.08	3.16	55.93	2.44	53.40	4.20	52.15	3.74
PD23	64.80	3.66	61.70	2.40	59.38	4.29	57.48	3.84
PD24	71.33	3.90	67.88	2.76	65.10	4.84	63.50	4.13
PD25	78.58	4.15	73.70	2.12	71.28	4.35	68.68	3.98
PD26	84.50	4.14	79.30	2.94	76.73	5.26	74.80	4.40
PD27	91.95	4.69	85.55	3.33	83.45	5.20	81.85	4.64
PD28	99.65	4.79	91.75	3.17	90.08	5.85	88.23	4.84
PD29	107.35	4.74	99.65	3.40	98.00	6.15	95.83	5.10
PD30	113.78	5.10	104.98	3.39	104.10	6.79	102.00	5.68

Sex-Specific Effects of Choline Injections on Male Apical Dendrites

Following intubation with ethanol from PD4-PD9 and injection with choline from PD4-PD30, we examined several parameters of neuronal morphology in hippocampal CA1 pyramidal neurons. Pyramidal neurons consist of a single long apical dendrite and multiple shorter basilar dendrites. As these two portions of the dendritic arbor are highly distinct, we analyzed apical and basilar dendrites separately. Specifically, we carried out a three-way multilevel analysis of sex, ethanol, and choline, followed by corrections for multiple comparisons.

The first parameter analyzed was apical complexity, calculated as (sum of terminal orders + number of ends)*(total dendritic length/number of dendrites). Complexity takes into account a number of other parameters that measure the length and branching of the dendrite in question. Therefore, we also compared the individual parameters that are included in the calculation of complexity: the sum of the terminal orders (defined as the number of sister branches encountered when tracing a dendrite from the tip back to the cell body), the number of ends, the total dendritic length, and the number of dendrites. However, as there is only one apical dendrite, the number of dendrites was not included in this analysis.

When examining the apical complexity, length, sum of terminal orders, and number of ends, there was a significant interaction between sex and choline in all four variables, as well as a significant three-way interaction between ethanol, choline and sex in complexity (Fig 4.2A) length (Fig. 4.2B); this interaction approached significance in the sum of terminal orders (Fig. 4.3C), but was not significant in the number of ends (Fig. 4.2D, Table 4.2). Post-hoc analyses of these interactions showed that male animals given choline had reduced apical morphological parameters when compared to both female animals given choline, and male control animals. Furthermore, in the complexity and sum of the terminal orders, there was a trend toward a decrease in male apical dendrites following ethanol intubation.

These data show that there is a sex-specific effect of choline on apical dendrites, with a decrease in dendritic arborization and length in males given choline. Data also show that there is no longer the significant difference following ethanol exposure as was seen at PD9, as ethanol intubation only resulted in minor, non-significant differences in apical dendrites at PD30.

Basilar Dendrites

In basilar dendrites, we measured the same four parameters as in the apical dendrites: complexity (Fig. 4.3A), length (Fig. 4.3B), the number of ends (Fig. 4.3C), and the sum of the terminal orders (Fig. 4.3D). We also measured the quantity of basilar dendrites (Fig. 4.3E), which is the fourth portion of the complexity equation (Table 4.3). There was a trend toward a main effect of ethanol on basilar ends and sum of terminal orders and, when this was examined via post-hocs, it was found that female animals given both ethanol and choline had a significant reduction in the sum of terminal orders compared to females given choline alone, and that there was a trend toward this same pattern in female basilar ends.

CA1 Hippocampal Pyramidal Neuronal Morphology at PD30

Representative tracings of PD30 hippocampal CA1 pyramidal neurons of all groups for both sexes can be seen in Figure 4.4 (Fig. 4.4A: Female, Fig. 4.4B: Male). These representative neurons were selected due to their apical dendritic length being the closest to the average apical dendrite length for that group. When we examined the neurons from the PD9 animals via Golgi-Cox (Goeke et al., 2018), as seen in chapter 3, we found that the total female apical length was 536.5 µm ± 46.8 µm, and the total male apical length was 415.17 µm ± 47.7 µm, which were 46.8% and 47.7% of the total dendrite length (apical and basilar combined), respectively. By contrast, at PD30, the total female apical length was 3.66 mm ± 0.22 mm, and the total male apical dendrite length was 3.41 mm ± 0.22 mm, which were, respectively, 56.05% and 55.18% of the total dendrite length. Furthermore, at PD9, the female basilar dendrite length was 609.4 µm ± 45.1 µm, and the male basilar dendrite length was 455.15 µm ± 35.3

Table 4.2. Apical dendrite multilevel analysis and false discovery rate corrections.

Apical Morphometric Parameter	Main Effect χ^2			Interaction χ2				DF
	EtOH	Choline	Sex	EtOH-Sex	Choline-Sex	EtOH-Choline	EtOH-Choline-Sex	
Complexity	0.0222	0.6416	2.3699	0.1645	4.7927	0.8591	3.7967	1
Length	0.0038	0.8798	1.9096	0.0362	5.6384	0.7079	4.1852	1
Ends	0.1805	0.3080	1.6276	0.1497	4.8466	0.4923	2.4873	1
Terminal Order	0.0191	0.3394	2.0305	0.4037	3.9519	1.0134	2.7805	1
Apical Morphometric Parameter	**Main Effect p-value**			**Interaction p-value**				
	EtOH	**Choline**	**Sex**	**EtOH-Sex**	**Choline-Sex**	**EtOH-Choline**	**EtOH-Choline-Sex**	
Complexity	0.8814	0.4231	0.1237	0.6850	0.0286	0.3540	0.0514	
Length	0.9511	0.3483	0.1670	0.8490	0.0176	0.4001	0.0408	
Ends	0.6709	0.5789	0.2020	0.6988	0.0277	0.4829	0.1148	
Terminal Order	0.8901	0.5602	0.1542	0.5252	0.0468	0.3141	0.0954	

Apical Complexity	χ2	DF	p-value
Female			
Sham+Saline vs. Sham+Choline	1.8583	1	0.1728
Sham+Saline vs. EtOH+Saline	0.6865	1	0.4074
Sham+Choline vs. EtOH+Choline	0.0977	1	0.7546
EtOH+Saline vs. EtOH+Choline	0.0535	1	0.8172
Male			
Sham+Saline vs. Sham+Choline	5.2469	1	0.0220
Sham+Saline vs. EtOH+Saline	2.7568	1	0.0968
Sham+Choline vs. EtOH+Choline	1.8424	1	0.1747
EtOH+Saline vs. EtOH+Choline	0.0035	1	0.9528
Male vs. Female			
Sham+Saline vs. Sham+Saline	1.4986	1	0.2209
Sham+Choline vs. Sham+Choline	6.5634	1	0.0104
EtOH+Saline vs. EtOH+Saline	0.9546	1	0.3285
EtOH+Choline vs. EtOH+Choline	1.3746	1	0.2410

Apical Length	χ2	DF	p-value
Female			
Sham+Saline vs. Sham+Choline	1.5987	1	0.2061
Sham+Saline vs. EtOH+Saline	0.2648	1	0.6068
Sham+Choline vs. EtOH+Choline	0.6430	1	0.4226
EtOH+Saline vs. EtOH+Choline	0.0203	1	0.8868
Male			
Sham+Saline vs. Sham+Choline	6.7207	1	0.0095
Sham+Saline vs. EtOH+Saline	1.9129	1	0.1666
Sham+Choline vs. EtOH+Choline	3.0944	1	0.0786
EtOH+Saline vs. EtOH+Choline	0.0712	1	0.7895
Male vs. Female			
Sham+Saline vs. Sham+Saline	1.2267	1	0.2681
Sham+Choline vs. Sham+Choline	8.1566	1	0.0043
EtOH+Saline vs. EtOH+Saline	0.1721	1	0.6783
EtOH+Choline vs. EtOH+Choline	0.7427	1	0.3888

Apical Sum of Terminal Orders	χ2	DF	p-value
Female			
Sham+Saline vs. Sham+Choline	1.5337	1	0.2156
Sham+Saline vs. EtOH+Saline	0.8035	1	0.3700
Sham+Choline vs. EtOH+Choline	0.0005	1	0.9817
EtOH+Saline vs. EtOH+Choline	0.2175	1	0.6410
Male			
Sham+Saline vs. Sham+Choline	4.2245	1	0.0398
Sham+Saline vs. EtOH+Saline	2.8850	1	0.0894
Sham+Choline vs. EtOH+Choline	1.2586	1	0.2619
EtOH+Saline vs. EtOH+Choline	0.0061	1	0.9378
Male vs. Female			
Sham+Saline vs. Sham+Saline	1.5748	1	0.2095
Sham+Choline vs. Sham+Choline	4.5988	1	0.0320
EtOH+Saline vs. EtOH+Saline	1.0753	1	0.2998
EtOH+Choline vs. EtOH+Choline	1.4633	1	0.2264

Apical Ends	χ2	DF	p-value
Female			
Sham+Saline vs. Sham+Choline	1.7224	1	0.1894
EtOH+Saline vs. EtOH+Choline	0.2197	1	0.6393
Male			
Sham+Saline vs. Sham+Choline	4.8199	1	0.0281
EtOH+Saline vs. EtOH+Choline	0.0810	1	0.7760
Male vs. Female			
Sham+Saline vs. Sham+Saline	1.5446	1	0.2139
Sham+Choline vs. Sham+Choline	5.3906	1	0.0202
EtOH+Choline vs. EtOH+Choline	1.3614	1	0.2433

Figure 4.2. Effects of neonatal ethanol and choline exposure, and continuing choline exposure, on morphological parameters of apical dendrites of CA1 pyramidal neurons in PD30 female and male rats. Apical dendrites of CA1 pyramidal neurons from PD30 pups of both sexes given ethanol from PD4-PD9 and/or choline from PD4-PD30 were digitally reconstructed with Neurolucida and analyzed via Neurolucida Explorer. Examined were apical complexity (A), calculated via the equation (sum of terminal orders + number of terminals)*(total dendrite length/number of dendrites), the apical length (B), number of apical ends (C) and the sum of apical terminal orders (D) calculated as the number of sister branches encountered when tracing from the end of a dendrite back to the cell body. Graphs represent the mean ± S.E.M of either 48 neurons (all male groups, female ethanol intubation + saline injection, female sham intubation + choline injection) or 36 neurons (female ethanol intubation + choline injection, female sham intubation + saline injection). *$p \leq 0.05$, +$p < 0.1$.

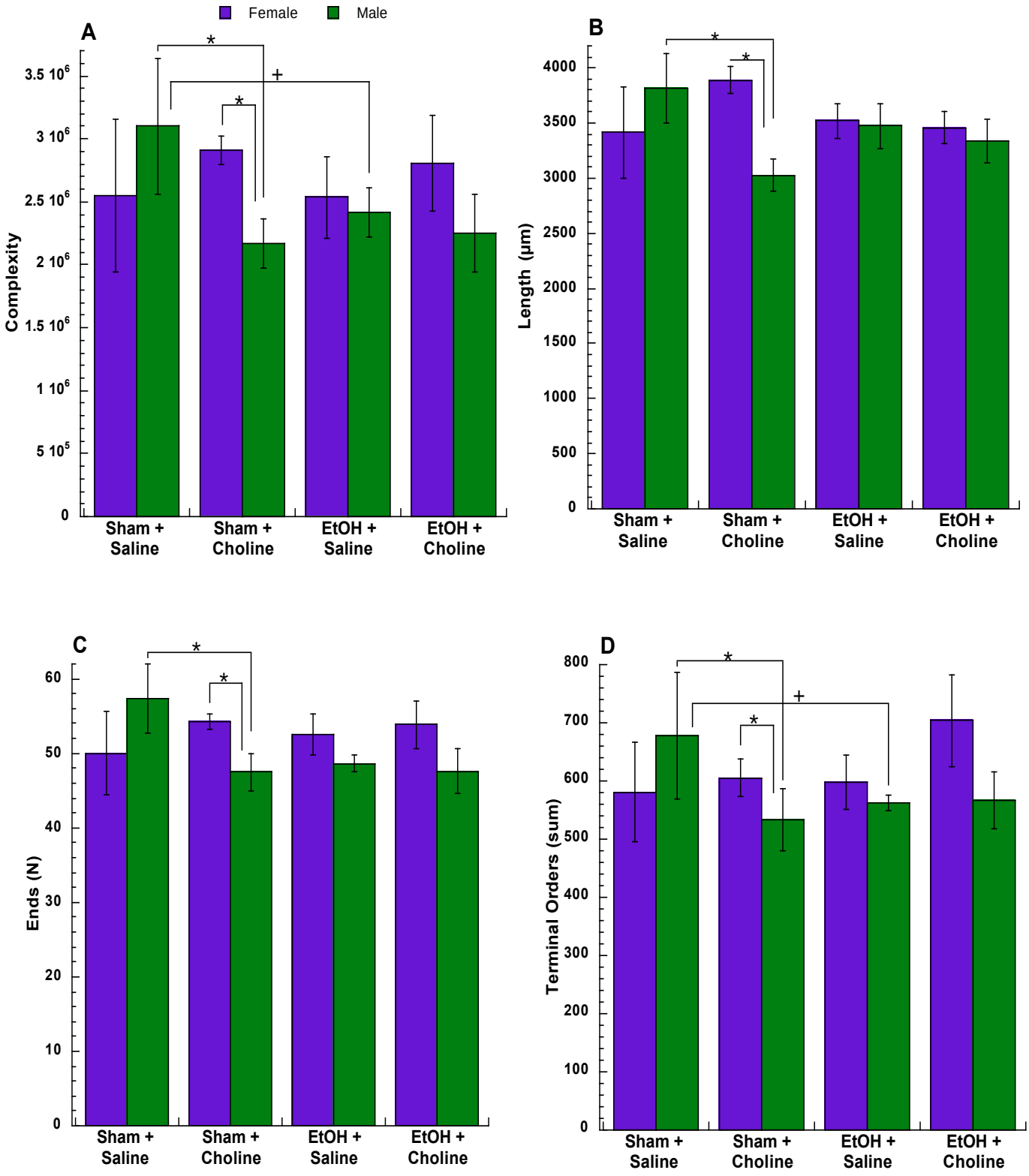

Female
Male
A
Complexity
3.5 10^6
3 10^6
2.5 10^6
2 10^6
1.5 10^6
1 10^6
5 10^5
0
Sham + Saline
Sham + Choline
EtOH + Saline
EtOH + Choline
B
Length (µm)
4000
3500
3000
2500
2000
1500
1000
500
0
C
Ends (N)
60
50
40
30
20
10
0
D
Terminal Orders (sum)
800
700
600
500
400
300
200
100
0

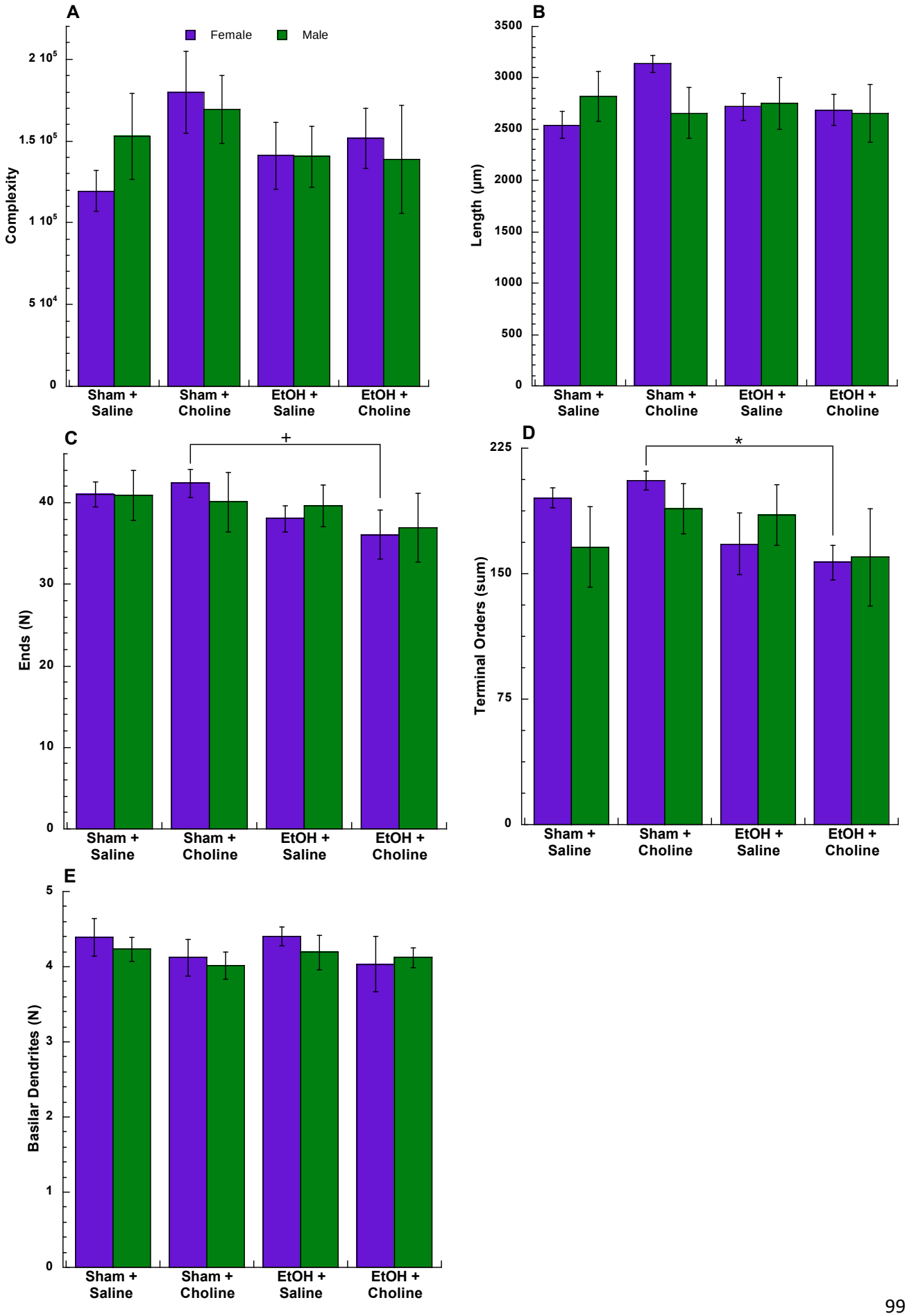
Table 4.3. Basilar dendrite multilevel analysis and false discovery rate corrections.

Basilar Morphometric Parameter	Main Effect χ^2			Interaction $\chi 2$				DF
	EtOH	Choline	Sex	EtOH-Sex	Choline-Sex	EtOH-Choline	EtOH-Choline-Sex	
Complexity	2.2348	0.0353	0.0355	0.0029	1.0272	0.3081	0.0016	1
Length	0.2255	0.1108	0.3997	0.0045	1.7315	0.4774	0.6235	1
Ends	3.4142	0.1029	0.2001	0.4473	0.7165	0.2925	0.1843	1
Terminal Order	3.3125	0.0981	0.2375	0.4333	0.6956	0.3447	0.1399	1
Quantity	0.0162	1.3830	0.3912	0.1908	0.4127	0.1878	1.5479	1
Terminal Distance	0.0269	0.4999	0.0509	0.2008	1.9112	0.7830	0.0120	1
Basilar Morphometric Parameter	**Main Effect p-value**			**Interaction p-value**				
	EtOH	**Choline**	**Sex**	**EtOH-Sex**	**Choline-Sex**	**EtOH-Choline**	**EtOH-Choline-Sex**	
Complexity	0.1349	0.8510	0.8505	0.9568	0.3108	0.5789	0.9680	
Length	0.6349	0.7393	0.5272	0.9467	0.1882	0.4896	0.4298	
Ends	0.0646	0.7484	0.6547	0.5036	0.3973	0.5886	0.6677	
Terminal Order	0.0688	0.7541	0.6260	0.5104	0.4043	0.5571	0.7084	
Quantity	0.8986	0.2396	0.5317	0.6623	0.5206	0.6647	0.2134	
Terminal Distance	0.8698	0.4795	0.8215	0.6541	0.1668	0.3762	0.9128	

Basilar Sum of Terminal Orders	χ2	DF	p-value
Female			
Sham+Saline vs. EtOH+Saline	1.3580	1	0.2439
Sham+Choline vs. EtOH+Choline	3.9357	1	0.0473
Male			
Sham+Saline vs. EtOH+Saline	0.6498	1	0.4202
Sham+Choline vs. EtOH+Choline	1.5319	1	0.2158

Basilar Ends	χ2	DF	p-value
Female			
Sham+Saline vs. EtOH+Saline	1.3537	1	0.2446
Sham+Choline vs. EtOH+Choline	2.8222	1	0.0930
Male			
Sham+Saline vs. EtOH+Saline	0.4750	1	0.4907
Sham+Choline vs. EtOH+Choline	0.4729	1	0.4917

Figure 4.3. Effects of neonatal ethanol and choline exposure, and continuing choline exposure, on morphological parameters of basilar dendrites of CA1 pyramidal neurons in PD30 female and male rats. Basilar dendrites of CA1 pyramidal neurons from PD30 pups of both sexes given ethanol from PD4-PD9 and/or choline from PD4-PD30 were digitally reconstructed with Neurolucida and analyzed via Neurolucida Explorer. Analyzed were basilar complexity (A), calculated via the equation (sum of terminal orders + number of terminals)*(total dendrite length/number of dendrites), the basilar length (B), number of basilar ends (C), sum of basilar terminal orders (D), calculated as the number of sister branches encountered when tracing from the end of the dendrite back to the cell body), and the quantity of basilar dendrites (E). Graphs represent the mean ± S.E.M. (see Figure 4.2 for number of animals). All data were log transformed before multilevel analyses. * $p \leq 0.05$, +$p < 0.1$.


A
Female
Male
Complexity
2 10^5
1.5 10^5
1 10^5
5 10^4
0
Sham + Saline
Sham + Choline
EtOH + Saline
EtOH + Choline
B
Length (µm)
3500
3000
2500
2000
1500
1000
500
0
C
+
Ends (N)
40
30
20
10
D
*
Terminal Orders (sum)
225
150
75
E
Basilar Dendrites (N)
5
4
3
2
1

μm, 53.2% and 52.3% of the total dendrite length, respectively. At PD30, the total female basilar dendrite length was 2.87 mm ± 0.16 mm, and the total male basilar dendrite length was 2.77 mm ± 0.28 mm, which were 43.95% and 44.82% of the total dendrite length, respectively.

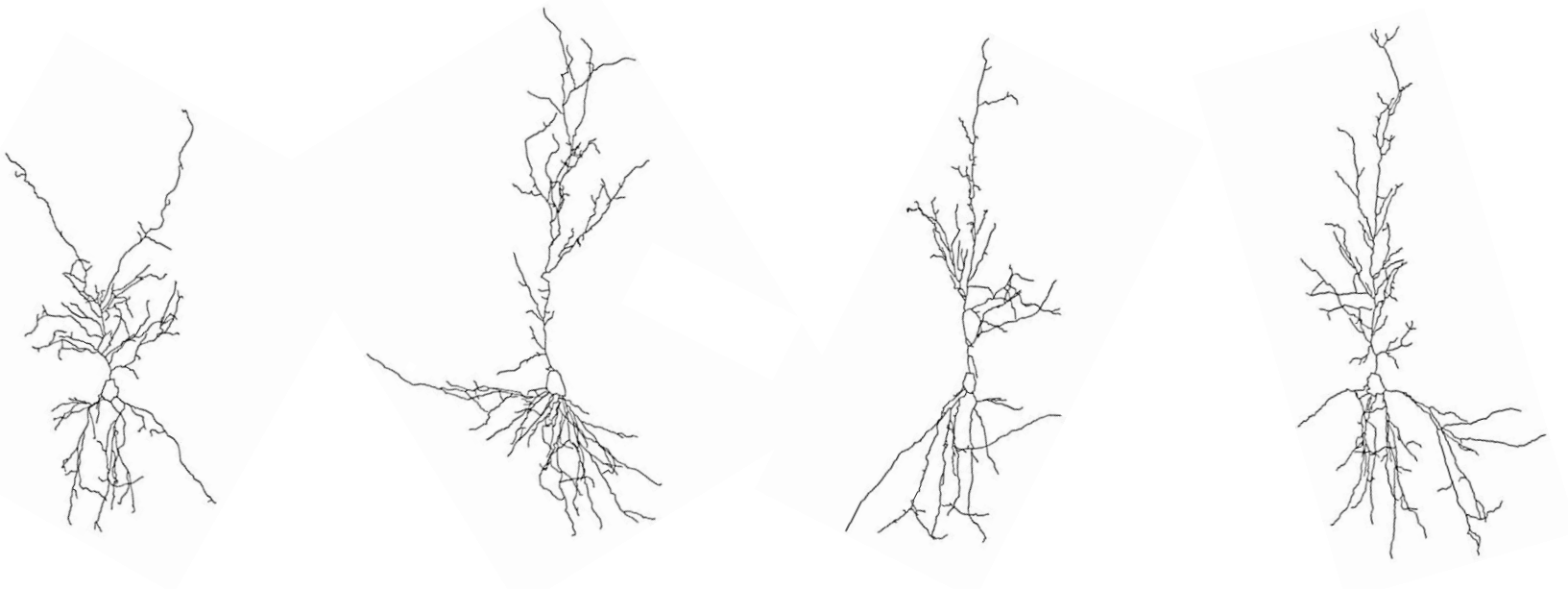
The combined length of all dendritic branches of an adult rat CA1 hippocampal neuron is usually between 12.0 mm and 13.5 mm (Spruston & McBain, 2007). Within this, the basilar dendrites are 4.32 mm – 4.86 mm (36% of total length), and the apical dendrites are 7.68 mm – 8.64 mm (64% of total length). Adult rats have been shown to have an average of 5 basilar dendrites per cell (Spruston & McBain, 2007). At PD30, females had 4.27 ± 0.24 dendrites, and males had 4.20 ± 0.16 dendrites. This too, is changed from the PD9 data, as animals at this time point had a different number of basilar dendrites: PD9 females had 6.38 ± 0.41 and PD9 males had 5.5 ± 0.305. These changes in distribution of dendrite length and dendrite number are indicative of later-life pruning and development, indicating that PD30 neurons are more developed than PD9 neurons, but are not yet at the developmental level of adult neurons.

DISCUSSION

Studies examining FASD have shown that gestational ethanol exposure can have a variety of morphological effects, such as decreasing hippocampal dendritic arbor size (Yanni & Lindsley, 2000) and simplifying dendrites of medium spiny neurons in the caudate putamen (Susick et al., 2014). FASD also presents with a number of behavioral changes in both humans and in animal models; many of which are associated with deficits in hippocampus-dependent learning and associated behaviors (Idrus et al., 2016; Thomas et al., 2000; Thomas et al., 2004a; Thomas et al., 2007). Therefore, this study examined the morphological effect of ethanol on the hippocampus, as well as the potential protective effect of choline.

Figure 4.4. Representative Neurolucida tracings of female and male hippocampal CA1 pyramidal neurons. Tracings represent neurons with apical dendrite length closest to the mean length of the apical dendrite per treatment group, per sex. Female (A) and male (B) neurons are shown from all four groups: sham intubation + saline injection, sham intubation + choline injection, ethanol intubation + saline injection, ethanol intubation + choline injection. Scale bar represents 100 μm.

A. Female



Sham+Saline | Sham+Choline | EtOH+Saline | EtOH+Choline

B. Male

100 µm

Choline is an essential nutrient that has been shown to have an ameliorating effect on ethanol-induced alterations following gestational exposure. Specifically, choline has been shown to reverse ethanol-induced disrupted performance on working memory and cognitive flexibility in rats (Waddell & Mooney, 2017), ameliorate ethanol-induced spatial learning deficits (Thomas et al., 2007) and attenuate ethanol-induced decreases in brain weight (Thomas et al., 2009). However, we recently determined that short-term choline treatment did not rescue the ethanol-induced changes in rat hippocampal neuronal morphology following neonatal exposure to ethanol and/or choline (Goeke et al., 2018), as seen in chapter 3. Therefore, in this study, we extended the period of choline exposure until early rat adolescence (Rice & Barone, 2000; Wagner et al., 2014), to view the long-term changes that neonatal ethanol exposure and continuing choline exposure had on neuronal morphology of hippocampal CA1 pyramidal neurons. We analyzed both the apical (Fig. 4.2) and basilar (Fig. 4.3) dendrites of hippocampal pyramidal CA1 neurons, and found that choline exposure decreased parameters of male apical dendrite morphology, compared to both male sham animals and female animals exposed to choline (Table 4.2), and non-significantly decreased complexity and the sum of terminal orders in male animals intubated with ethanol, when compared to male sham intubated animals. In the basilar dendrites, female animals given both ethanol and choline had significantly fewer terminal orders than female animals given choline only, and trended toward this pattern in ends (Table 4.3).

Choline therefore had a significant impact on neuronal morphology, and there are several ways it could be doing this. In particular, choline can be converted into the neurotransmitter acetylcholine, into betaine, through which it can influence DNA methylation (Niculescu & Zeisel, 2002), or into phosphatidylcholine (PC), a major membrane phospholipid (Fig. 1.1) (Li & Vance, 2008). Due to the differing responses to choline supplementation the sexes showed in this study, it is possible that the sexes are metabolizing choline in different ways. This is in line with previous studies, which have indicated a sex difference in response to choline, with females and males responding differently to

choline supplementation on tasks testing ethanol-induced balance deficits (Bearer et al., 2015), cognitive and working memory deficits (Waddell & Mooney, 2017) and alterations in miRNA variability (Balaraman et al., 2017). Studies have also shown that other brain areas, such as the medial prefrontal cortex, demonstrate sex specific morphological changes in response to treatments, such as stress (McEwen & Morrison, 2013).

In this study, males may be preferentially converting choline into acetylcholine in cholinergic neurons, or into betaine, in the liver. This would decrease the amount of choline available for conversion in the brain to PC, limiting the amount of PC available for use in membrane biogenesis, and potentially resulting in the decreased dendritic complexity seen in PD30 males given choline. Alternatively, increased amounts of choline may also be stimulating the developing choline system to the point where more acetylcholine is needed. When there is an increased need for acetylcholine, PC can be removed from the cell membrane and converted back into choline (Li & Vance, 2008), which would the allow for synthesis of more acetylcholine (Meck & Williams, 2003). Both of these potential pathways, utilizing choline to affect either DNA methylation or as a neurotransmitter, could potentially result in the alterations of dendritic arborization seen in males, as it would alter the amount of PC present in/available for the cell membrane.

However, choline did not cause any changes in female apical dendrites, also indicating a potential sex-specific manner of metabolizing choline. One possible explanation is that females may be using the acetylcholine metabolized from the choline treatments in a different manner than males. For example, an increased amount of acetylcholine in one sex could be preferentially activating muscarinic acetylcholine receptors, while an increase in acetylcholine levels in the other sex could be preferentially activating nicotinic acetylcholine receptors, as these two receptors are differentially expressed in the brain (Abreu-Villaca et al., 2011). This could then result in the sex difference seen following choline injections.

It is also possible that, as female and male brains develop at different rates (Fontaine et al., 2016; Lenz et al., 2012), the effects of ethanol and/or choline may manifest at different times in different sexes. It is possible that, had the animals been examined later in life, when they were further developed, females and males may have shown a similar response to the treatments (McEwen & Morrison, 2013). There are also similarities between the PD9 animals (chapter 3) and the PD30 animals analyzed here. In PD9 animals, choline induced a decrease in parameters of basilar dendrite morphology, more strongly in males. While choline affected basilar dendrites in PD9 animals, and apical dendrites in PD30 animals, both choline treatments induced a decrease in neuronal morphological parameters, one that was seen more strongly with males. Overall, however, the mechanisms behind the sex-specific effect of choline seen in these studies are unclear, and more research is needed before any concrete conclusions are finalized.

By contrast to the results seen following choline exposure, as well as the results seen in chapter 3 regarding PD9 rats (Goeke et al., 2018), there were only small effects of ethanol at PD30. The only significant effect of ethanol was on female basilar dendrites, as females given both ethanol and choline had fewer terminal orders than female animals given choline alone. PD9 rats exposed to ethanol from PD4-PD9 had a significant increase in apical dendrite morphological parameters in both females and males, which we hypothesized may be due, in part, to an induction of premature maturation. Due to ethanol intubation resulting in more complex and longer neurons at PD9, we hypothesize that an increase in maturation would result in hippocampal neurons making altered connections with other brain regions at PD30, resulting in the relatively few visible effects of ethanol seen at PD30.

Altered connections due to developmental ethanol exposure could be the underlying mechanisms for some of the cognitive and behavioral dysfunctions seen in FASD, in particular, connectivity differences between FASD and control cohorts (Wozniak et al., 2013). Therefore, even though there is no longer any morphological effect of ethanol seen at PD30, it is possible the changes

seen in PD9 animals are having a long-term effect, and that these long-term effects may be altered connections, which could be an underlying cause of some of the behavioral and cognitive symptoms seen in individuals with FASD. It is interesting to note that there was no effect of choline on apical dendrites when animals were given ethanol and choline together. A possible reason is that the presence of ethanol could be altering the way the hippocampus metabolizes or utilizes an increased amount of choline, thereby preventing any effects, deleterious or beneficial, of choline supplementation.

In conclusion, this study examined how neonatal ethanol intubation and choline injections, with choline injections continuing until adolescence in the rat, could alter the neuronal morphology in CA1 hippocampal pyramidal neurons. These results indicate that choline can alter the morphology of the hippocampus in a sex-specific manner. Therefore, the efficacy of choline as a treatment for humans with FASD may differ by sex. The choline exposure in this study is also equivalent to years of treatment in humans, indicating that prolonged treatment may be needed for a successful response to treatment. Furthermore, due to the differences in neuronal morphological alterations seen when comparing PD9 and PD30 animals, as well as the difference in levels of development, continuing ethanol and/or choline exposure for longer periods may result in a more profound effect in neuronal morphology later in life. Future research into choline as a treatment for FASD should therefore keep in mind that choline may need to be taken for a long period of time to have an effect, and that sexes may respond to choline as a treatment differently.

CHAPTER 5: ROLE OF TISSUE PLASMINOGEN ACTIVATOR UPREGULATION IN ETHANOL-TREATED ASTROCYTE-MEDIATED INHIBITION OF HIPPOCAMPAL NEURON NEURITE OUTGROWTH *IN VITRO*

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Support:

This work was supported by the NIH/NIAAA F31AA024950-01A1 to CG, and by NIH/NIAAA R01AA021468, R01AA022948 and VA Merit Review Award I01BX001819 to MG, and by facilities and resources at the VA Portland Health Care System.

ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD) results from *in utero* exposure to ethanol, and presents with many cognitive and behavioral symptoms. Little is known about the exact mechanisms behind these symptoms, necessitating research into exactly how ethanol can impact the brain. Studies have indicated that ethanol may upregulate tissue plasminogen activator (tPA), both *in vivo* and *in vitro*. tPA can convert plasminogen into plasmin, a serine protease that can degrade the extracellular matrix (ECM). In this study, we examined the possibility that one of the ways ethanol alters neuronal morphology is via upregulation of tPA. We exposed cortical astrocytes to ethanol, siRNA *Plat* (the gene that encodes for tPA), and/or a recombinant tPA protein. We then cultured hippocampal neurons with these treated astrocytes in order to determine the impact of the treatments on neuronal morphology, specifically as mediated by astrocytes. Ethanol exposure in astrocytes decreased the neurite outgrowth of neurons cultured with these astrocytes. Treatment of astrocytes with siRNA *Plat* also decreased neurite outgrowth, indicating that decreasing astrocytic *Plat* may be preventing movement of the growth cone in neurons. However, astrocytes treated with ethanol and siRNA *Plat* in combination resulted in neurite lengths similar to the length of control neurites. Increasing tPA levels in astrocytes via recombinant tPA replicated the effects of ethanol by decreasing neurite outgrowth, possibly through degradation of ECM proteins. These results support the idea that one of the ways that ethanol alters neuronal morphology may be via astrocyte-mediated increases in tPA. More research is needed to determine precisely how tPA is involved in the brain's response to gestational ethanol exposure, as well as if increasing and/or decreasing tPA levels, and their subsequent effects through different mechanisms, could alter the effect of ethanol.

INTRODUCTION

Ethanol can cross both the placenta and the blood-brain barrier (BBB), and as such, if a woman drinks ethanol during pregnancy, there can be very severe effects on the developing fetus, leading to

Fetal Alcohol Spectrum Disorders (FASD) (May et al., 2014; Riley et al., 2011). FASD is characterized by life-long cognitive and behavioral deficits, and is often associated with mental illnesses (Hellemans et al., 2010). The development of FASD has been shown to involve many different mechanisms and molecules, including tissue plasminogen activator (tPA).

tPA is a serine protease glycoprotein expressed by many different cell types, including both astrocytes and neurons (Hebert et al., 2015). It is involved in many different brain processes, including long-term potentiation (Baranes et al., 1998; Madani et al., 1999), reduction of NMDA receptors and dendritic spines following stress (Bennur et al., 2007; Pawlak et al., 2005a), and the conversion of plasminogen into plasmin, a secondary serine protease that can degrade the extracellular matrix (ECM) (Melchor & Strickland, 2005; Skrzypiec et al., 2009). tPA is also involved in the brain's response to ethanol, as $tPA^{-/-}$ neurons are resistant to neurodegeneration following ethanol withdrawal, and tPA activity in wild type mice is upregulated by ethanol withdrawal (Skrzypiec et al., 2009). $tPA^{-/-}$ mice have also been shown to be resistant to ethanol-withdrawal induced neurodegeneration in both the thalamus and the cortex, and were protected from an ethanol-induced reduction in freezing behavior in contextual fear conditioning seen in wild-type mice (Noel et al., 2011).

Furthermore, tPA itself is upregulated by ethanol, both *in vitro* via 75 mM ethanol (Zhang et al., 2014a), and *in vivo* via induction of physical dependence, accomplished by increasing ethanol doses up to 10% volume/volume over 14 days (Pawlak et al., 2005b). As tPA can convert plasminogen into plasmin, which could degrade the ECM, and ethanol itself has been shown to decrease secretion of astrocytic ECM proteins, such as proteoglycans and laminin subunits (Trindade et al., 2016; Wilhelm & Guizzetti, 2016), it is possible that an ethanol-induced increase in tPA and subsequent increase in plasmin (Wiera & Mozrzymas, 2015) could be degrading ECM proteins (Hebert et al., 2015). This degradation could alter the capacity of the neuronal environment to support neurite outgrowth, and therefore, ethanol-induced upregulation of tPA may play a role in the pathophysiology of FASD.

This role of tPA in the brain's response to ethanol likely involves astrocytes, as astrocytes have been shown to involved in the tPA/plasmin system in the CNS (Xin et al., 2010), and are involved in tPA's upregulation by ethanol (Zhang et al., 2014a). Astrocytes also regulate plasmin (Briens et al., 2017), secrete tPA (Hebert et al., 2015), express more *Plat* (the gene that encodes for tPA) than neurons (Wilhelm et al., 2018), and regulate ethanol's impacts on the brain (Zhang et al., 2014b). For example, *in vitro* exposure of astrocytes to ethanol (25 mM, 50 mM, 75 mM) resulted in an inhibition of muscarinic receptor signaling and a reduction in several parameters of neuronal morphology in neurons cultured with these treated astrocytes (Guizzetti et al., 2010; Zhang et al., 2014a; Zhang et al., 2014b). Astrocytes also mediated the effect of ethanol through secreted factors, as neurons exposed to the media of astrocytes treated with ethanol also showed reduced neuronal growth (VanDeMark et al., 2009b). This indicates that astrocyte-secreted factors, such as tPA (Hebert et al., 2015), may be involved in how astrocytes influence neurons and neuronal morphology in response to ethanol.

Therefore, in this study, we used an *in vitro* astrocyte-neuron co-culture model (Zhang et al., 2014b), similar to the one utilized in chapter 2, whereby we exposed astrocytes to ethanol, siRNA *Plat* to decrease tPA mRNA, and/or recombinant tPA to increase levels of tPA protein, and examined how astrocytes mediated the effects of these treatments on neurite outgrowth. We found that astrocyte treatment with ethanol alone decreased neurite outgrowth, and that siRNA *Plat* treatment alone also decreased neurite outgrowth. However, siRNA *Plat* in combination with ethanol exposure brought the levels of neurite outgrowth back up to the levels of control cells. Furthermore, incubation of astrocytes with a recombinant tPA protein replicated the effect of ethanol by decreasing neurite outgrowth. These results suggest that astrocyte-released tPA, which is upregulated by ethanol, plays a role in ethanol-treated astrocyte-induced inhibition of neurite outgrowth in hippocampal pyramidal neurons.

METHODS

Animals

Timed-pregnant Sprague Dawley rats at Gestational Day (GD) 15 were purchased from Charles River (Wilmington, MA). Pregnant animals were housed and maintained at the VA Portland Health Care System Veterinary Medical Unit, under a 12h light/dark cycle at 22 ± 1°C with *ad libitum* access to a chow diet and water. All animal procedures were approved by the VA Portland Health Care System Institutional Animal Care and Use Committee, and followed US National Institutes of Health animal welfare guidelines.

Cortical Astrocyte Primary Cultures

Cortical astrocyte cultures were prepared from GD21 fetuses, as previously described in chapter 2 (Guizzetti et al., 2010; Zhang et al., 2014b). Fetuses were separated by sex; astrocytes derived from female and male brain tissue were plated in separate flasks and cultured until confluence (10-16 days), in Dulbecco's Modified Eagle's Medium containing 10% Fetal Bovine Serum (DMEM/10% FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin (all from Gibco, Grand Island, NY) in a humidified incubator under a 37°C, 5% CO_2/95% atmosphere. Astrocytes from an equal number of female and male flasks were trypsinized, pooled, and plated onto circular glass coverslips placed in 24-well plates, at a concentration of $1x10^5$ cells/well, and cultured for three additional days in DMEM/10% FBS.

qRT-PCR

RNA was isolated from astrocytes using Trizol (Thermo-Fisher Scientific) and Direct-zol RNA MiniPrep Plus Kit (Zymo Research, Orange, CA), and quantified using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA), as previously described (Wilhelm et al., 2018). Primers were designed via NCBI Primer-Blast software, based on previously published primer sequences (Zhang et al., 2014a).

Cortical Astrocyte-Hippocampal Neuron Co-Cultures

Hippocampal neurons were prepared from an equal number of female and male GD21 fetuses, as previously described in chapter 2 (Guizzetti et al., 2008; Guizzetti et al., 2010; Zhang et al., 2014b).

Neurons were plated on top of the treated astrocytes 2h after treatment removal, at a concentration of $1x10^4$ neurons/coverslip. The co-cultures were then incubated for 16h before immunocytochemistry.

Ethanol Treatments and siRNA Transfection

Astrocytes plated on glass coverslips in 24-well plates were transfected with either a non-target siRNA, an siRNA *Plat*, or treated with transfection reagents only. Immediately prior to transfection, the DMEM/10% FBS medium was replaced with serum free DMEM medium supplemented with 0.1% Bovine Serum Albumin (DMEM/0.1% BSA; BSA was purchased from Sigma Aldrich, St. Louis, MO) without antibiotics. Transfection was performed by mixing Lipofectamine RNAiMAX (catalog #: 13778030, Thermo-Fisher Scientific, Waltham, MA) with Opti-MEM I Reduced Serum Media (Gibco, Grand Island, NY) and adding siRNA (*Plat* or non-target) to a final concentration of 50 nM (siRNA *Plat* catalog #: 1330001, Thermo-Fisher Scientific, siRNA non-target catalog #: 462001, Invitrogen, Carlsbad, CA). This mixture was allowed to sit for 10 minutes, so that the transfection complexes could form, and then added to the astrocytes for 24h. Following the 24h incubation, the transfection reagents were washed out and replaced with DMEM/0.1% BSA medium with antibiotics. Due to the results of previous research (chapter 2), astrocyte cultures were exposed to either control medium or medium containing 75 mM ethanol, and were then placed in sealed chambers designed to prevent ethanol evaporation. These chambers also contained a dish of water with the same concentration of ethanol as the treatment, in order to keep the environment constant and prevent ethanol evaporation (Chen et al., 2013; Zhang et al., 2014b). A gas mixture (5% CO_2/95% air) was run through the chambers for four minutes, and the astrocytes were exposed to ethanol for 24h, for a total of 48h of treatment (see chapter 2).

rtPA Treatments

Astrocyte cultures were switched to DMEM/0.1% BSA with antibiotics for 24h prior to treatments with 50 nM active recombinant rat tPA (rtPA, catalog #: 25692, Molecular Innovations, Novi,

MI) or without (control), in DMEM/0.1% BSA medium. As it was possible that increases in levels of astrocytic tPA protein would be transient, we tested two different timings of exposure to rtPA; 4h and 16h. This tested whether the effects of increased tPA were relatively long-lasting, or if they were limited to the period shortly after rtPA application.

Ethanol Concentration Determination

20 µL of ethanol-containing medium was collected at the beginning and end of each experiment and mixed with 500 µL of 4 mM n-propanol internal standard in distilled water. Ethanol concentrations in the medium were determined via head-space gas chromatography, as previously described (Finn et al., 2007). There was no significant evaporation of ethanol in any condition during the exposure period (data not shown).

Cell Viability

Astrocyte viability following treatment was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetraxzolium bromide (MTT) assay (Sigma Aldrich, St. Louis, MO), as previously described (Gavin et al., 2016; VanDeMark et al., 2009a, 2009b). Astrocytes were incubated with 500 µL MTT (0.5 mg/mL) in DMEM/0.1% BSA for 2h. After the removal of MTT-containing medium, 500 µL of DMSO was added, and the resulting solution was read on a spectrophotometer at 570 nm. Data are expressed as a percent of control.

Immunocytochemistry

After the 16h incubation, astrocyte-neuron co-cultures were fixed (4% paraformaldehyde in phosphate buffered saline) and immunostained with a neuron-specific anti-β-III tubulin antibody (1:150, catalog #: MAB1637, Millipore, Burlington, MA), followed by a goat anti-mouse Alexa Fluor 488 secondary antibody (1:300, catalog #: A11001, Thermo-Fisher Scientific, Waltham, MA), as previously described (Zhang et al., 2014a). The glass coverslips were then mounted on microscope slides.

Microscopy

The neurons were examined on a Leica DM500b microscope with a DFC36 FX camera, using the program Neurolucida (Version 11, MBF Bioscience, Williston, VT, USA). Selection of neurons to be measured and analyzed was performed by a researcher blind to the experimental treatments. Two pictures were taken of each circular glass coverslip at a 5x objective. Cells were selected as measurable if they 1) had three or more neurites that were all longer than the cell body, 2) did not overlap with other neurons, 3) were fully on top of the astrocyte monolayer, 4) were pyramidal neurons, and 5) had no breakage of the cell and/or neurites. The selected neurons were then located with a 40x objective, fluorescent pictures were taken, and all neurites were traced using Neurolucida. The data files produced from these tracings were analyzed with the program Neurolucida Explorer. Three parameters were analyzed from each cell: the length of the longest neurite, the average length of the minor neurites, and the number of neurites.

Statistics

Data were analyzed with the statistical program R (Team & RC, 2017) and the package lme4 (Bates et al., 2015) using a multi-level analysis, following outlier identification with a value equal to 1.5 of the Inter-Quartile Range. As the neurons within a single coverslip were dependent on each other and the different coverslips were independent of each other, we used a nested multilevel analysis (Aarts et al., 2014) (See Table 5.1 for sample sizes of experiment number, coverslips/condition and cells/condition). Benjamini-Hochberg tests were run to correct for multiple comparisons and to control for the false discovery rate (FDR), as three different parameters were analyzed for each experiment (length of the longest neurite, average length of the minor neurites, average number of neurites). ANOVA were run for parameters other than neuronal morphology and Tukey's post-hoc tests were run when appropriate. A post-hoc p-value of <0.05 and an FDR q-value of <0.05 were considered significant. Data are reported as mean ± S.E.M.

Table 5.1. Neuron morphometric analysis experimental sample size

Experiment Type	Treatment	Number of Experiments	Coverslips/ Condition	Cells/ Condition
siRNA *Plat* + EtOH Treatment (Fig 5.3)	Control	4	11	285
	siRNA *Plat*		10	278
	EtOH		10	313
	siRNA *Plat* + EtOH		12	246
rtPA Treatment (Fig. 5.5)	Control 4 hours	3	9	297
	rtPA 4 hours		8	382
	Control 16 hours		9	334
	rtPA 16 hours		8	400

RESULTS

Ethanol increases expression of Plat in astrocytes, which is reversed by siRNA Plat

In order to confirm the effect of ethanol and/or siRNA *Plat* on *Plat* expression, astrocytes were exposed to ethanol and/or transfected with siRNA *Plat*. Ethanol increased astrocytic expression of *Plat* (55% increase compared to control), and siRNA *Plat* decreased astrocytic expression of *Plat* (97% decrease compared to control). A combination of siRNA *Plat* and ethanol together also significantly decreased *Plat* expression [ANOVA, interaction: $F(1,6) = 69.46$, $p<0.001$, effect of ethanol: $F(1,6) = 68.13$, $p<0.001$, ANOVA, effect of transfection: $F(1,6) = 1407$, $p<0.001$, non-target vs. ethanol, non-target vs. siRNA *Plat*, ethanol vs. ethanol + siRNA *Plat*, all $p<0.001$, Figure 5.1].

siRNA Plat alone and ethanol alone decreased neurite outgrowth, an effect which was reversed when astrocytes were treated with siRNA Plat and ethanol together

Ethanol has been shown to upregulate astrocytic expression of the gene *Plat* and alter neurite outgrowth in an astrocyte-mediated manner (Zhang et al., 2014b). Therefore, the first experiment transfected astrocytes with siRNA *Plat* in combination with ethanol, to determine how decreasing *Plat* alters the effect of ethanol on neurite outgrowth. Astrocytes were exposed to siRNA *Plat*, 75 mM ethanol, both in combination, or neither, after which treatments were removed, hippocampal neurons were cultured on the treated astrocytes, and neurite outgrowth was measured. We analyzed the longest neurite length, the average length of the minor neurites and the average number of neurites in hippocampal pyramidal neurons. A nested multilevel analysis with corrections for multiple comparisons showed a main effect of both ethanol exposure ($q=0.016$) and siRNA *Plat* ($q=0.013$) on the longest neurite length, as well as an interaction ($q=0.002$, Fig. 5.2A, Table 5.2). Specifically, there was a significant decrease in the longest neurite length following transfection with siRNA *Plat* ($q=0.013$, 16.67% decrease compared to control), as well as a significant decrease following ethanol exposure ($q=0.008$, 17.02% decrease compared to control).

Figure 5.1. ***Plat*** **expression following transfection and ethanol exposure**. Expression of *Plat* in astrocytes was examined using qRT-PCR following transfection with 50 nM siRNA *Plat* and/or exposure to 75 mM ethanol. Ethanol exposure increased expression of *Plat,* while siRNA *Plat* transfection decreased expression of *Plat,* both with and without ethanol. Data were analyzed using a two-way ANOVA, followed by Tukey's for post-hoc. ***p<0.001.

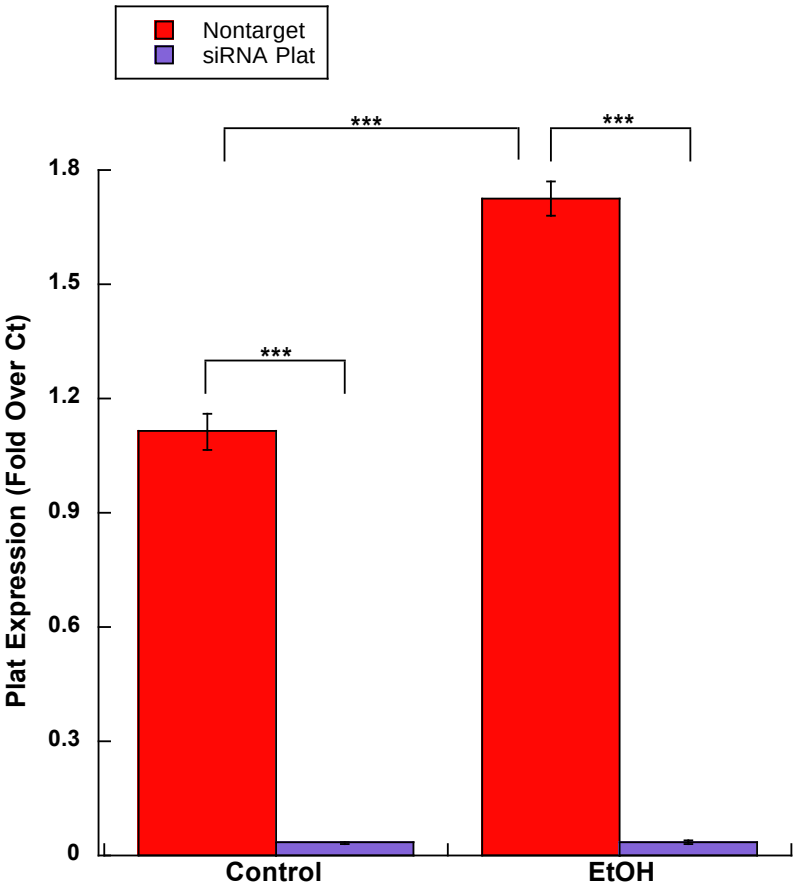


Figure 5.2. Effects of astrocytic siRNA *Plat* transfection and ethanol exposure on neurite outgrowth. **A.** Ethanol exposure (75 mM) alone and 50 nM siRNA *Plat* transfection alone both decreased the longest neurite length, while siRNA *Plat* in combination with ethanol had levels of neurite outgrowth similar to controls. **B.** Ethanol exposure alone and siRNA *Plat* exposure alone both decreased the average minor neurite length. **C.** There were no changes in the number of neurites. All data were analyzed using a nested multilevel analysis and the Benjamini-Hochberg approach to adjust nominal p-values to FDR. *q<0.05 (after FDR corrections), #p<0.05 (before FDR corrections)

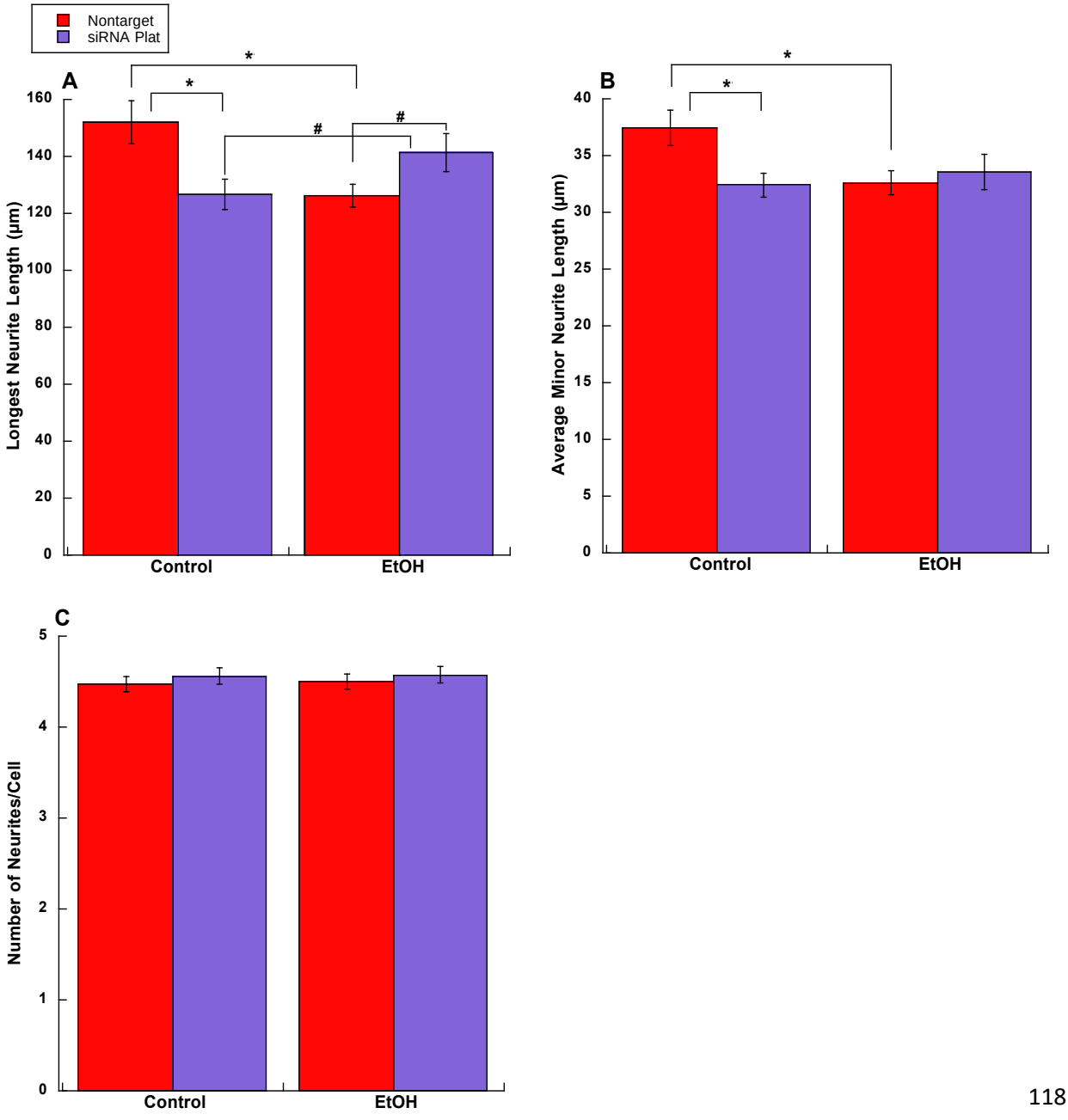
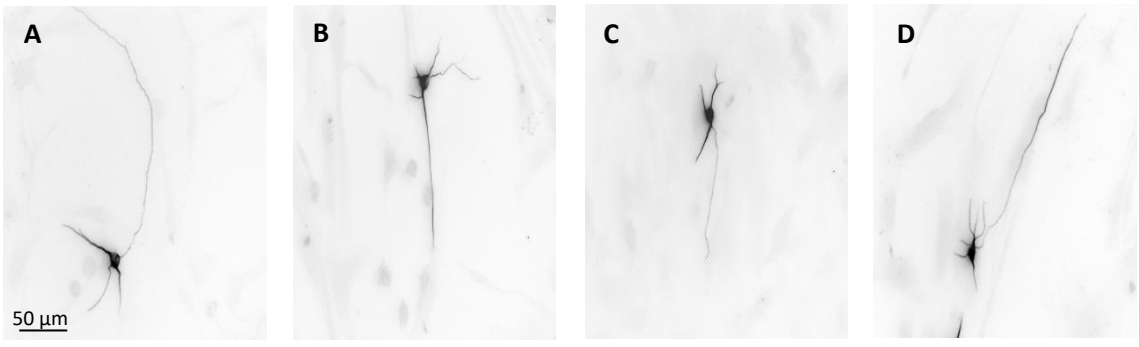


Table 5.2. siRNA *Plat* and EtOH treatment multilevel analyses and FDRs

Table 2. siRNA *Plat* and EtOH Treatment Multilevel Analyses and FDRs								
	χ^2		DF		p value		q value (FDR correction)	
Parameter	Interaction		Interaction		Interaction		Interaction	
Longest Neurite Length	11.895		1		0.001		0.002	
Average Minor Neurite Length	5.160		1		0.023		0.035	
Average Number of Neurites	0.003		1		0.956		0.956	
	χ^2		DF		p value		q value (FDR correction)	
Parameter	EtOH	siRNA *Plat*	EtOH	siRNA *Plat*	EtOH	siRNA *Plat*	EtOH	siRNA *Plat*
Longest Neurite Length	6.585	7.534	1	1	0.010	0.006	0.016	0.013
Average Minor Neurite Length	6.480	6.927	1	1	0.011	0.008	0.016	0.013
Average Number of Neurites	0.047	0.693	1	1	0.829	0.405	0.829	0.405

	χ2	DF	p-value	q-value
Longest Neurite Length				
EtOH vs. siRNA *Plat* + EtOH	4.169	1	0.041	0.124
siRNA *Plat* vs. siRNA *Plat + EtOH*	4.051	1	0.044	0.132

Figure 5.3. Representative images of pyramidal neurons cultured with transfected astrocytes. A. Control, **B.** siRNA *Plat*, **C.** Ethanol, **D.** siRNA *Plat* + Ethanol.



However, when astrocytes were treated with both siRNA *Plat* and ethanol together, the longest neurite length was not different from control length. The combination treatment did result in neurite lengths longer than both the ethanol alone (siRNA *Plat* + ethanol 10.73% longer) and the transfection alone (siRNA *Plat* + ethanol 10.36% longer) treatments; this was not significant after corrections for multiple comparisons (before corrections, vs. transfection p=0.04, vs. ethanol p=0.04, Table 5.2).

There was also an interaction between siRNA *Plat* and ethanol exposure in the average minor neurite length (q=0.035), as well as a main effect of both ethanol (q=0.016) and siRNA *Plat* (q=0.013, Fig. 5.2B). Transfection of control astrocytes with siRNA *Plat* induced a significant decrease in the minor neurite length compared to control (q=0.037, 13.42% decrease). Ethanol treatment of astrocytes induced a similar decrease when compared to controls (q=0.039, 12.91% decrease). However, there was no rescue effect of the combination treatment of siRNA *Plat* with ethanol. There was no change caused by any treatment on the number of neurites (Fig. 5.2C). Representative images of neurons in all four conditions can be seen in Figure 5.3 (A: Control, B: siRNA *Plat*, C: Ethanol, D: siRNA *Plat* + Ethanol, scale bar = 50 μm).

rtPA decreased neurite outgrowth

Because changing the levels of *Plat* altered the effect of ethanol on neurite outgrowth, and ethanol exposure increased astrocyte expression of *Plat,* the next experiment exposed astrocytes to recombinant (r) tPA, to determine if directly increasing tPA protein levels would have a similar effect on neurite outgrowth as ethanol exposure. Astrocytes were exposed to media containing 50 nM rtPA or control media, for either 4h or 16h. There was a main effect of rtPA on the longest neurite length (q<0.001, Fig. 5.4A, Table 5.3). This was due to a decrease in the length of the longest neurite following both 4h (q=0.002, 14% decrease) and 16h (q=0.005, 11.57% decrease) rtPA exposure. There was also a main effect of rtPA exposure on the average minor neurite length (q<0.001, Fig. 5.4B). As with the longest neurite length, exposure to astrocytes treated with rtPA for both 4h (q=0.025, 12.91% decrease)

Figure 5.4. Effects of astrocytic rtPA exposure on neurite outgrowth. **A.** Exposure to 50 nM rtPA, for either 4h and 16h, significantly decreased the length of the longest neurites when compared to controls. **B.** Exposure to rtPA, for either 4h and 16h, significantly decreased the length of the average minor neurites when compared to controls. **C**. There was no change in the number of neurites. All data were analyzed using a nested multilevel analysis and the Benjamini-Hochberg approach to adjust nominal p-values to FDR. *q<0.05, **q<0.01.

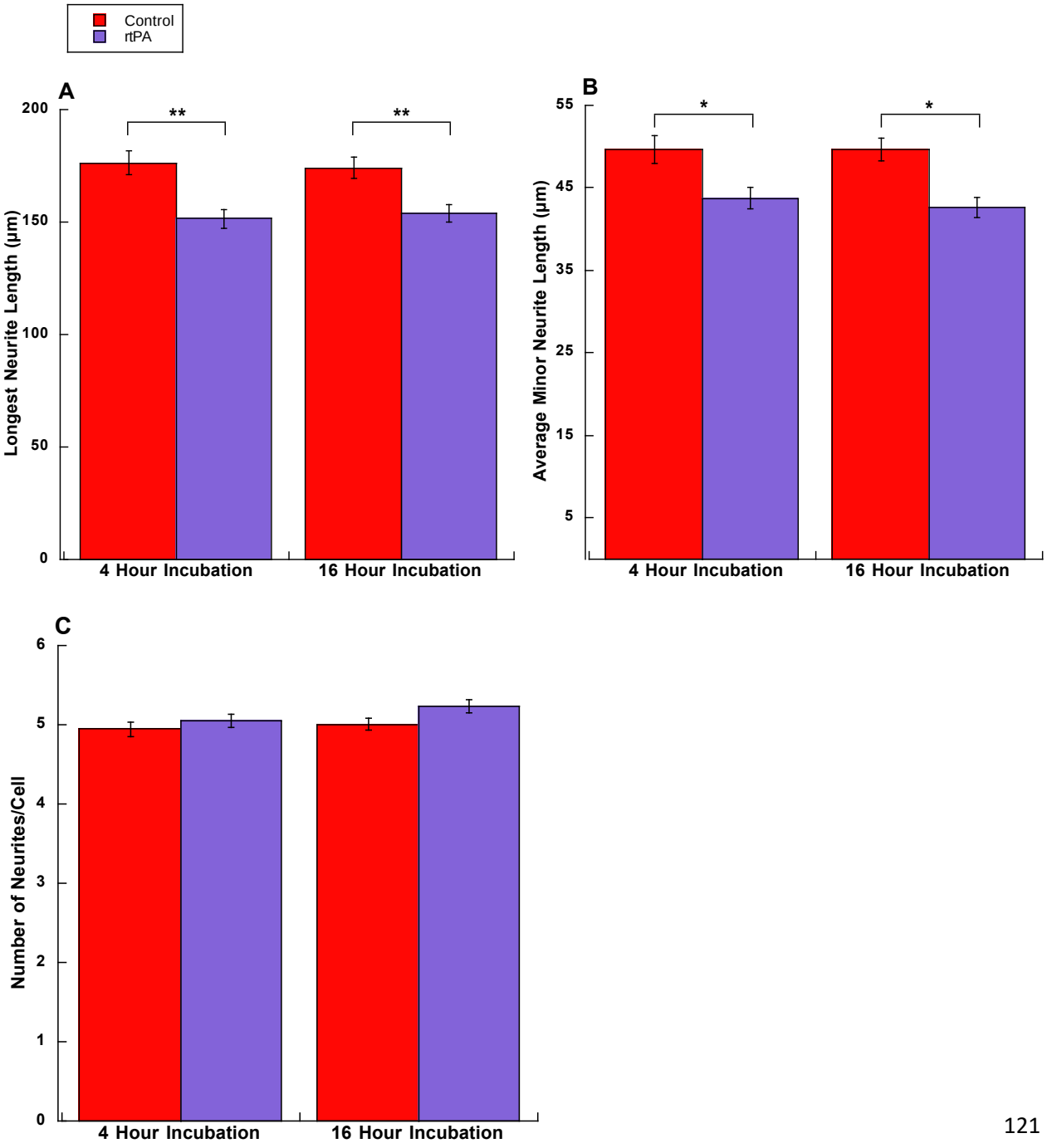
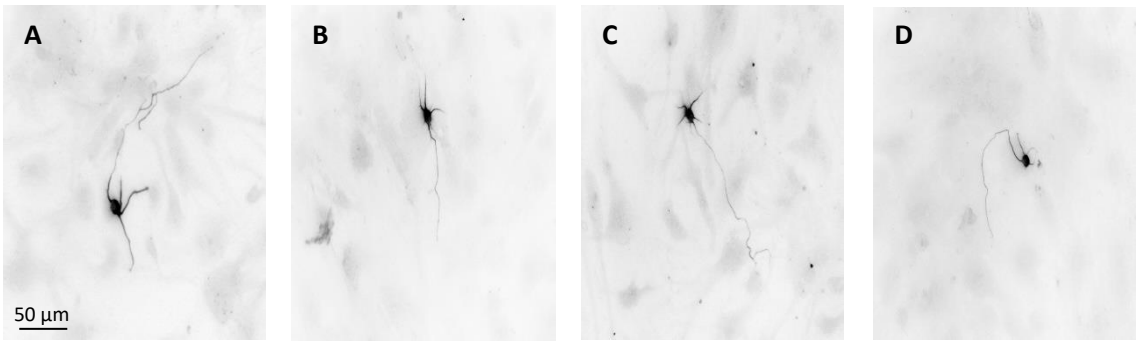


Table 5.3. rtPA treatment multilevel analyses and FDRs

Table 3. rtPA Treatment Multilevel Analyses and FDRs				
Parameter	χ^2	DF	p value	q value (FDR correction)
Longest Neurite Length	29.489	1	5.62E-08	1.69E-07
Average Minor Neurite Length	27.512	1	1.56E-07	2.34E-07
Average Number of Neurites	3.965	1	0.046	0.046

Figure 5.5. Representative images of pyramidal neurons cultured with astrocytes exposed to rtPA. A. Control 4h, **B.** rtPA 4h, **C.** Control 16h, **D.** rtPA 16h.



and 16h (q=0.001, 14.05% decrease) resulted in decreased average minor neurite length. There was a main effect of rtPA exposure on the number of neurites (q=0.046, Fig. 5.4C), however, this was not due to any physiologically relevant differences (4h Control vs. 16h rtPA). Representative images of neurons in all four conditions can be seen in Figure 5.5 (A: Control 4h, B: rtPA 4h, C: Control 16h, D: rtPA 16h, scale bar = 50 μm).

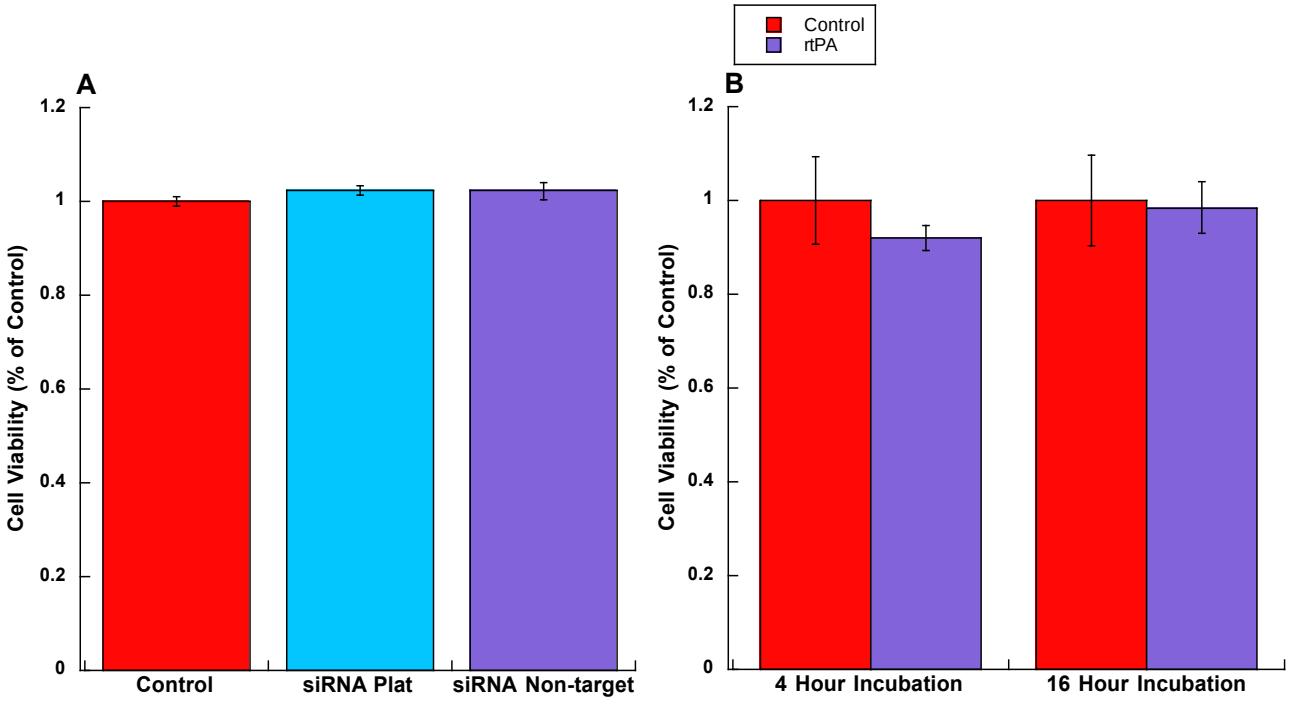
Changes in neurite outgrowth are not due to changes in astrocyte viability

As tPA has the potential to be neurotoxic (Hebert et al., 2015; Tsirka et al., 1995), and a lack of astrocytes would change neurite outgrowth (Christopherson et al., 2005; Dotti et al., 1988; Kaech & Banker, 2007; Pfrieger & Barres, 1997), we used the MTT cell viability assay to determine if either siRNA *Plat* or rtPA caused cytotoxicity in astrocytes. Astrocytes were transfected with siRNA *Plat*, a non-target siRNA control, a non-transfection control, or 50 nM rtPA for 4h or 16h, with appropriate controls, as described above. All experiments followed the same timing as the astrocyte-neuron co-culture. After these treatments, the MTT assay was run on the astrocytes. There was no change in the astrocyte viability following either transfection (Fig. 5.6A) or rtPA exposure (Fig. 5.6B).

DISCUSSION

Ethanol can impact the developing fetus *in utero*, and the resulting spectrum of symptoms is termed 'Fetal Alcohol Spectrum Disorders' (FASD) (Guizzetti et al., 2014; Riley et al., 2011). FASD presents with many cognitive and behavioral deficits, including decreased global intelligence (Aragon et al., 2008), difficulty with attention, executive functioning and motor deficits (Klintsova et al., 2013), and deficits in visuo-spatial learning (Thomas et al., 2007). Tissue plasminogen activator (tPA) is both involved in FASD (Noel et al., 2011), and upregulated by ethanol exposure (Zhang et al., 2014a). Furthermore, previous studies have shown that astrocytes express *Plat,* the gene that encodes for tPA (Hebert et al., 2015), and that *in vitro* astrocytes express more *Plat* than a whole brain homogenate (Wilhelm et al., 2018), indicating that astrocytes likely express more *Plat* than other cell types in the

Figure 5.6. siRNA *Plat* and rtPA changes in astrocyte viability. Transfection with siRNA *Plat* or controls did not change astrocyte viability (**A**). Exposure to rtPA did not change astrocyte viability, regardless of exposure timing (**B**). Data were analyzed using a one-way ANOVA, followed by a Dunnett's post-hoc test (**A**) and a two-way ANOVA, followed by a Tukey's post-hoc test (**B**). Data are expressed as percent of control.



brain. Therefore, the increase in *Plat* and tPA seen following ethanol exposure is likely mediated by astrocytes. Specifically, astrocytes may be mediating the effect of increased tPA on neurite outgrowth, as previous studies have shown that astrocytes mediate the effects of other treatments, such as ethanol and the cholinergic agonist carbachol, on neurite outgrowth (Chen et al., 2013; Giordano et al., 2009; Giordano et al., 2011; Guizzetti et al., 2008; Zhang et al., 2014b).

Therefore, in this study we treated astrocytes with an siRNA to decrease *Plat* in combination with ethanol, and cultured neurons with these astrocytes. We also exposed astrocytes to recombinant (r) tPA, culturing neurons with these astrocytes in the same manner, in order to determine how changes of tPA levels in astrocytes is involved in hippocampal neuron neurite outgrowth. Decreasing astrocytic *Plat* via the siRNA resulted in decreased neurite lengths in neurons cultured with these astrocytes. Astrocytes exposed to ethanol also decreased neurite lengths. However, a combination treatment of siRNA *Plat* and ethanol had a mild reversal effect, whereby neurons cultured with astrocytes exposed to a combination treatment had neurite lengths that were similar to control lengths. The combined treatment also resulted in neurite lengths that were longer than the siRNA *Plat* alone and ethanol alone conditions, although this was no longer significant following corrections for multiple comparisons (Fig. 5.2, Table 5.2). Furthermore, neurons cultured with astrocytes exposed to rtPA also had decreased neurite outgrowth, similar to the decrease seen following ethanol exposure (Fig. 5.4, 5.5). None of these changes were due to astrocyte cell death (Fig. 5.6).

These data show that astrocytes mediate the effects of both increases and decreases in levels of tPA on neurite outgrowth, although likely through different mechanisms. One potential way through which siRNA *Plat,* which decreases expression of the gene that encodes for tPA, could decrease neurite outgrowth may be due to interference with the growth cone. As tPA is needed for the movement of the growth cone (Garcia-Rocha et al., 1994; Pittman et al., 1989), and tPA can be secreted by astrocytes (Zhang et al., 2014a), it is possible that by decreasing astrocytic *Plat* expression, these treatments are

decreasing the amount of astrocytic tPA. A lack of astrocytic tPA would prevent normal movement of the growth cone in neurons cultured with the treated astrocytes, resulting in reduced neurite outgrowth.

Ethanol, which has been shown to increase levels of tPA in astrocytes (Zhang et al., 2014a), also decreased neurite outgrowth (Zhang et al., 2014b). The effect of ethanol on neurite outgrowth may be, in part, due to this increase in tPA. In particular, ethanol-induced tPA could be degrading extracellular matrix (ECM) proteins (Hebert et al., 2015). Aside from secreting tPA, astrocytes can also secrete ECM proteins, such as fibronectin and laminin (Guizzetti et al., 2008; Moore et al., 2009), and a reduction of these ECM proteins could alter neuronal morphology and the neuronal environment (Guizzetti et al., 2010). Ethanol exposure has also been shown to change the astrocytic expression and secretion of these ECM proteins (Trindade et al., 2016; Zhang et al., 2014a). Therefore, ethanol's increase in astrocytic tPA could be degrading ECM proteins, or even altering secretion of these proteins from astrocytes, decreasing support for neurite outgrowth. Interestingly, decreasing *Plat* expression in combination with ethanol exposure resulted in a partial amelioration of the effects of both of these treatments alone, as the combination treatment resulted in neurite lengths similar to those of controls. This further indicates that tPA is involved in the response to ethanol exposure, as changing the levels of *Plat* changed the response of the cells to ethanol.

The experiments exposing astrocytes to rtPA, assumed to directly increase levels of astrocytic tPA protein, also supports the hypothesis that tPA is involved in the brain's response to ethanol. Ethanol, which has been shown to increase tPA levels, decreased neurite outgrowth, an effect which was replicated by directly increasing tPA protein levels (Zhang et al., 2014a; Zhang et al., 2014b). This further indicates that the increase in tPA seen following ethanol exposure could be responsible for some of the changes in neurite outgrowth seen in neurons cultured with ethanol treated astrocytes. As with

the transfection experiments, rtPA-induced changes in neurite outgrowth were not due to astrocyte cytotoxicity induced by increases in tPA.

In conclusion, these experiments examined ethanol induced alterations of neuronal morphology as mediated by astrocytes, and the role played by tPA in these alterations. Some of the cognitive and behavioral symptoms seen following *in utero* ethanol exposure may be due these morphological alterations. Specifically, ethanol-induced increases in tPA, and subsequent degradation of the neuritogenic ECM proteins released by astrocytes, may be involved in ethanol-induced changes in neuronal morphology. Therefore, in accordance with previous research that also indicates tPA plays a role in the response of the brain to ethanol (Noel et al., 2011; Pawlak et al., 2005b; Zhang et al., 2014a), the data of these experiments support the hypothesis that changes in astrocytic tPA following ethanol exposure may be an underlying cause of some of the neuronal morphological alterations seen in FASD.

CHAPTER 6: GENERAL DISCUSSION

Summary of Data

The hypothesis of this dissertation was that ethanol alters neuronal morphology, and that this may, in part, be through an astrocyte-mediated, ethanol-induced increase in tissue plasminogen activator (tPA). A secondary hypothesis was that astrocytes would also mediate a rescue effect of choline on the effects of ethanol on alterations in neuronal morphology as choline is hypothesized to be a potential treatment for individuals with Fetal Alcohol Spectrum Disorders (FASD). Experiments testing this hypothesis demonstrate that, *in vitro*, astrocytes modulate an effect of ethanol that is inhibitory to neurite outgrowth, as well as a rescue effect of choline. *In vitro* experiments also demonstrated that tPA is involved in the alteration of neuronal morphology following ethanol exposure, and is also mediated by astrocytes. Examining the effect of ethanol and choline *in vivo* demonstrated that neonatal ethanol exposure increased apical dendritic complexity in postnatal day (PD) 9 hippocampal CA1 pyramidal neurons, an effect which was no longer seen in adolescent rats. Choline, by contrast, demonstrated a sex-specific effect in adolescence (PD30), whereby females showed no change following choline exposure, while males had significant decreases in morphology following choline exposure. At PD30, ethanol only had a small effect, as it decreased terminal orders in female basilar dendrites in choline treated animals. These data further elucidate the underlying mechanisms and morphological changes of FASD, including the role of astrocytes, tPA, and the possibility of choline as a treatment.

Astrocytic Modulation of Neurite Outgrowth *In Vitro*

The experiments in chapters 2 and 5 were done to establish if and how astrocytes mediated the effect of ethanol, choline, and changes in the level of tPA on neurite outgrowth *in vitro*. Astrocytes mediated an effect of ethanol and both decreases and increase of tPA on neurite outgrowth. However, astrocytes mediated a rescue effect of choline on ethanol-induced alterations of neurite outgrowth, but no effect of choline alone on neurite outgrowth.

There are several potential ways astrocytes could be mediating the decreases in neurite outgrowth seen following ethanol exposure (chapter 2). One potential way is through astrocytic secretion of ECM proteins, as levels of these proteins are decreased by ethanol exposure (Giordano et al., 2011; Guizzetti et al., 2008; Guizzetti et al., 2010; McKeon et al., 1995; Moore et al., 2009; Silver & Miller, 2004; Trindade et al., 2016). An ethanol-induced decrease in ECM proteins, such as fibronectin and laminin, could alter the cellular environment and the growth supporting structure normally provided by astrocytes, resulting in the observed decrease in neurite outgrowth (Giordano et al., 2011).

Ethanol has been shown to decrease astrocytic ARSB, thereby increasing sGAGs (Zhang et al., 2014b). Increased sGAGs are inhibitory to the growth of neuronal processes (Wang et al., 2008), making this a potential way through which astrocytes could be mediating the decrease in neurite outgrowth caused by ethanol exposure. Astrocytes also express a variety of neurotrophic factors that induce and support neurite outgrowth; in some situations ethanol exposure reduces secretion of these factors, resulting in altered neurite outgrowth (Kim & Druse, 1996). Another potential mechanism is via astrocytic muscarinic receptors, which are involved in neurite outgrowth (Guizzetti et al., 1996; Guizzetti et al., 2008). In particular, in control conditions, carbachol activates astrocytic muscarinic receptors, increasing neurite outgrowth, an effect which was inhibited by ethanol (Giordano et al., 2011; Guizzetti et al., 2010). Thus, there are many ways astrocytes may be mediating the effects of ethanol.

Astrocytes also mediated the rescue effect of choline exposure on the effect of ethanol on neurite outgrowth. Astrocytes may be mediating this rescue effect via the metabolites of choline. While choline can be converted into the neurotransmitter acetylcholine, or converted into betaine, then to methionine and S-adenosylmethionine, influencing DNA methylation, neither of these metabolites are converted in astrocytes (Li & Vance, 2008; Niculescu & Zeisel, 2002). However, choline can also be converted into phosphatidylcholine (PC) (Guizzetti et al., 2014), which may be how the astrocytes are utilizing the choline in these experiments to rescue the effect of ethanol on neurite outgrowth. PC is a

major membrane phospholipid made in all mammalian nucleated cells, in both cell bodies and axons, and is therefore almost globally available throughout the body, including in the brain (Li & Vance, 2008). PC can alter the integrity and structure of cell membranes, and increase neurite lengths (Rema et al., 2008; Zhu et al., 2016), making it especially important in the developing brain, when processes are growing and forming connections (Jacobson et al., 2018; Jiang et al., 2014; Rice & Barone, 2000). PC can also be a precursor for signaling molecules (Li & Vance, 2008; Tang et al., 2014), which may have their own effects on neurite outgrowth.

Specifically, PC can be hydrolyzed by phospholipase D (PLD) to generate phosphatidic acid (PA) (Guizzetti et al., 2004; Klein, 2005), a lipid second messenger that can impact cell proliferation, cytoskeletal reorganization and vesicular trafficking (Guizzetti et al., 2014; Zhu et al., 2016). However, the presence of ethanol alters this pathway, as, when ethanol is present, PLD can instead transphosphorylate PC into choline and phosphatidylethanol (PEth) (Guizzetti et al., 2004; Klein, 2005). Therefore, in the presence of ethanol, astrocytes may be preferentially converting PC into PEth and choline, resulting in decreased production of PA, and a reduction in the downstream effects of PA that increase neurite lengths and support cell proliferation. This is also potentially how astrocytes are mediating the rescue effect of choline; an increased amount of choline in astrocytes may result in astrocytic PLD primarily creating PA instead of creating PEth, subsequently increasing neurite outgrowth due to the increased presence of PA, instead of decreasing neurite outgrowth via PEth.

An interesting finding was that astrocytes only mediated the effects of choline when ethanol was present; there was no change in neurite lengths when astrocytes were treated with choline alone. This is potentially due to a ceiling effect, whereby elevating choline levels past a certain point no longer has an effect (Klein et al., 1991; Tang et al., 2014). This ceiling effect may be due to a rapid metabolism of excess choline that prevents choline past a certain concentration from having an effect (Klein et al., 1991). Furthermore, ethanol has been shown to decrease intracellular choline (Tang et al., 2014). Due

to this, choline may be having an effect when given with ethanol as, due to the decrease in choline concentration seen with ethanol, choline and ethanol in combination would not result in a level of choline considered to be an excess. It is also possible that cell types other than astrocytes mediate the effect of choline alone, as previous experiments using non-astrocytic cell types have shown an effect of choline-only treatments (Li et al., 2004; Otero et al., 2012; Rema et al., 2008). Therefore, there are several ways choline could be impacting neurite outgrowth, especially as mediated by astrocytes.

Astrocytes also mediated the effects of tPA, whereby decreasing astrocytic *Plat*, the gene that encodes tPA, resulted in reduced neurite outgrowth in neurons cultured with these treated astrocytes. However, astrocytes treated with a combination of ethanol and decreased *Plat* partially ameliorated the decrease in neurite length induced by ethanol alone and decreased by *Plat* alone. Furthermore, astrocytes treated with recombinant (r) tPA protein also induced a decrease in neurite lengths. Astrocytes therefore mediated a similar effect on neurite outgrowth with bidirectional manipulations of tPA.

Astrocytes could be mediating the decrease in neurite outgrowth following an increase in tPA via ECM degradation. tPA has been shown to degrade ECM proteins, some of which, such as fibronectin and laminin, are secreted by astrocytes (Guizzetti et al., 2008; Hebert et al., 2015). As degradation of ECM proteins has been shown to decrease neurite outgrowth (Giordano et al., 2009; Guizzetti et al., 2008), it is possible that ECM degradation by tPA is the reason for the decrease in neurite outgrowth seen in chapter 5 following rtPA treatments, as it may be creating a neuronal environment unsuitable for neurite outgrowth. In particular, tPA may be degrading the ECM via conversion of plasminogen into plasmin, as an increase in plasmin has been shown to increase degradation of ECM proteins (Skrzypiec et al., 2009).

Another way astrocytes could be mediating the effects of tPA on neurite outgrowth is through regulation of the amount of tPA and plasmin available. For example, the astrocyte plasma membrane

can provide a surface for tPA's activation of plasmin, and can take up both plasminogen and plasmin via cell surface actin mediated endocytosis (Briens et al., 2017). Astrocytes can also endocytose extracellular tPA via the low-density lipoprotein-related protein receptor, and, via a different mechanism, exocytose tPA in a manner dependent on the levels of extracellular glutamate (Casse et al., 2012; Goto et al., 2007; Polavarapu et al., 2007). Thus, astrocytes can alter the amount and availability of tPA and plasmin, thereby mediating their effects.

Furthermore, astrocyte mediation of the effect of tPA on neurite outgrowth may also be specifically involved with the effect of ethanol exposure. Specifically, tPA mRNA and protein levels are upregulated following ethanol exposure, both *in vivo* and *in vitro* (Wilhelm et al., 2018). Astrocytes also likely produce more tPA than other cell types in the brain, as there were higher levels of *Plat* expression and higher levels of tPA protein in astrocyte cultures than in whole brain homogenates. This increase in tPA may be due to an ethanol-induced decrease of DNA methylation on the gene that encodes for tPA (Guizzetti, 2015; Zhang et al., 2014a), an effect specifically seen in astrocytes. An alternative explanation may be that, in condition conditions, activation of astrocytic muscarinic receptors upregulates plasminogen activator inhibitor 1 (PAI-1), which inhibits tPA. However, ethanol inhibits this upregulation of PAI-1, resulting in a downstream increase of tPA (Guizzetti et al., 2014). Thus, there are data supporting the hypothesis that astrocytes mediate the effect of tPA in the response to ethanol.

An interesting result of experiments examining astrocytic modulation of ethanol and tPA was that different levels of tPA had similar effects. Specifically, increasing and decreasing tPA and *Plat*, respectively, in astrocytes both decreased neurite outgrowth. As discussed above, an increased amount of tPA could be decreasing neurite outgrowth due to ECM degradation. By contrast, the decrease in neurite outgrowth seen following transfection with siRNA *Plat* could potentially be due to tPA's involvement in the growth cone (Garcia-Rocha et al., 1994; Pittman et al., 1989; Seeds et al., 1999). Specifically, decreasing *Plat* to the extent seen in chapter 5 could eliminate astrocytic tPA to insufficient

levels when neurons are added to assist in the movement of the growth cone, resulting in the observed decrease in neurite lengths.

By contrast, a combination treatment of decreased *Plat* and increased ethanol resulted in neurite lengths that were not significantly different from the lengths of control neurons. This may be due to tPA being needed in order for ethanol exposure to have an impact. For example, $tPA^{-/-}$ mice lacked the ethanol withdrawal induced neurodegeneration seen in wildtype mice, indicating that this neurodegeneration may be dependent on the tPA/plasmin system (Noel et al., 2011; Skrzypiec et al., 2009). Therefore, the reduction in *Plat* expression seen following transfection may have prevented the effects of ethanol that depend on the ethanol-induced increase in *Plat* and tPA. However, more research is needed to determine the specific mechanisms behind this interaction, as well as why ethanol exposure altered the effect of decreased *Plat*.

A potential physiological interpretation of the changes in neurite outgrowth seen in the *in vitro* experiments may be that these alterations in neurite outgrowth mediated by astrocytes could result in altered connectivity. By altering neurite length, the treatments may be changing how the neuron would respond to signals from other brain areas or cells. For example, reducing neurite length could reduce or alter the influence of the projection dependent on that neurite (Hamilton et al., 2015). This could then result in alteration or dysregulation of the information synthesis and processing of this neuron. There are also potential electrophysiological consequences of changes in neurite outgrowth, as altering the size of the neurite could change the input resistance, potentially altering the integration of inputs into a coherent signal (Li et al., 2004).

Overall, these experiments showed that astrocytes mediate the effects of ethanol, choline, increased tPA, and decreased *Plat in vitro*. These experiments also support the hypothesis that, *in vitro*, tPA is involved in astrocytic-mediation of the effect of ethanol on neurite outgrowth, and the hypothesis

that choline could potentially be a treatment for the effects of ethanol exposure, again as mediated by astrocytes.

Effects of Choline and Ethanol *In Vivo*

The experiments done in chapter 3 and 4 expanded upon the *in vitro* research examining the effect of ethanol and choline by utilizing an *in vivo* rat model of FASD. This model examined the rat equivalent to the human third trimester of gestation, in order to specifically target the brain growth spurt (Dobbing & Sands, 1979; Driscoll et al., 1990; Goodlett & Peterson, 1995; Goodlett & Johnson, 1997; Johnson & Goodlett, 2002; Kelly et al., 1988). The brain growth spurt is a period of development during which the brain undergoes a large increase in size and weight, along with astrocyte and oligodendrocyte proliferation, synaptogenesis and dendritic arborization (Guerri, 1998). As the brain growth spurt occurs primarily prenatally in humans, it is highly susceptible to the teratogenic effects of ethanol (Dobbing & Sands, 1979), and is therefore of interest to FASD research.

In the experiments of chapter 3, rats were intragastrically intubated with ethanol and subcutaneously injected with choline during their brain growth spurt, from PD4 to PD9. In the experiments in chapter 4, the choline exposure was extended until PD30, although the ethanol exposure was still limited to PD4-PD9 (Goodlett & Peterson, 1995; Goodlett & Johnson, 1997; Johnson & Goodlett, 2002; Kelly et al., 1988; Klintsova et al., 1998; Klintsova et al., 2002; Wagner et al., 2014; Zhang et al., 2014b). In both sets of experiments, brains were stained with Golgi Cox (Das et al., 2013), and the morphology of hippocampal CA1 pyramidal neurons was examined.

In PD9 rats exposed to ethanol and choline from PD4-PD9, ethanol intubation increased parameters of neuronal morphology, including length and complexity. This increase following ethanol intubation was primarily seen in apical dendrites, and was more strongly seen in female animals (chapter 3). By contrast, choline injections resulted in a minor decrease in basilar dendrite morphology, an effect which was stronger in males. As these experiments did not show any rescue effect of choline,

despite previous research showing a rescue effect of choline supplementation on ethanol-induced deficits, both behaviorally and biologically (Balaraman et al., 2017; Monk et al., 2012; Otero et al., 2012; Ryan et al., 2008; Schneider & Thomas, 2016; Thomas et al., 2000; Thomas et al., 2004a; Thomas et al., 2007; Thomas et al., 2009; Thomas et al., 2010), the experiments in chapter 4 continued the choline exposure until rat early adolescence (PD30). In these animals, there was only a small effect of ethanol exposure, on female basilar sum of terminal orders, but there was a sex-specific effect of choline, whereby choline decreased male apical dendrite morphological parameters, but did not have any effect in females, or on basilar dendrites.

As mentioned in chapter 3, the effects of ethanol on hippocampal pyramidal neurons at PD9 may represent a premature maturation. This premature maturation is potentially due to ethanol increasing neurotrophic factors (Boschen et al., 2015; Heaton et al., 2000). For example, rats intubated with ethanol (5.25 g/kg/day, BECs: 80 ± 20 mM) from PD4-PD9 had an increase in BDNF protein in the hippocampus seen at PD10 (Boschen et al., 2015). Another study, in which rats were exposed to ethanol via vapor inhalation from PD10-PD15 (BEC of 73.04 ± 5.58 mM), ethanol also induced an increase in BDNF mRNA in the hippocampus, an effect which was still seen at both PD16 and PD20 (Miki et al., 2008). An increase in BDNF was also seen in the hippocampus and cortex/striatum in rats given ethanol from PD4-PD10 via vapor inhalation (BEC of 34.95 ± 3.91 mM) and examined at PD10 (Heaton et al., 2000). Lastly, ethanol (11% volume in water, given throughout gestation and until weaning) has also been shown to elevate levels of nerve growth factor (NGF) in the cortex and the hippocampus (Ceccanti et al., 2012). Therefore, it is possible that the increased apical length and complexity seen in PD9 animals given ethanol is due to ethanol-induced increases in neurotrophic growth factors, causing alterations in the rate of maturation. It is also possible that these alterations in dendritic length and complexity would result in altered connections with other areas of the brain (Cook & Wellman, 2003;

Hamilton et al., 2010), which could potentially underlie some of the CNS dysfunctions seen in FASD (Wozniak et al., 2013).

Also at PD9, choline altered neuronal maturation, albeit in the opposite direction as ethanol, as parameters of basilar dendrite morphology were decreased. As mentioned previously, choline can be converted into either betaine, in the liver and kidneys, acetylcholine, in cholinergic neurons, or PC, the membrane phospholipid (Zeisel & Niculescu, 2006). The effects of choline injections may be due to levels of acetylcholine present in the brain acting as a factor influencing the amount of PC present in the cell membrane. Specifically, if there is a lack of acetylcholine, PC can be removed from the membrane and converted back into choline, which can in turn be converted into acetylcholine (Rema et al., 2008; Ulus et al., 1988). A removal of PC from the cell membrane to synthesize acetylcholine could potentially alter dendrite complexity and length, as seen following choline supplementation in these animals.

A lack of acetylcholine induced removal of PC from the cell membrane may be potentially due to choline supplementation altering the development of the cholinergic system. The cholinergic system in rats is developmentally regulated (Abreu-Villaca et al., 2011), undergoing significant growth in the first postnatal week (Nyakas et al., 1994). Altered development of the cholinergic system could be increasing the need for acetylcholine and decreasing the amount of PC present in the cell membrane, as well as decreasing the amount of choline supplementation that is converted to PC, which could result in the alterations in dendritic morphology as seen in chapter 3.

Acetylcholine being the primary fate of choline supplementation at PD9 may also be a potential reason for why, unlike *in vitro* choline treatments, choline supplementation at PD9 did not rescue the effects of ethanol. *In vitro,* the only possible fate of choline is PC, as conversion to betaine occurs in the liver and kidneys, and conversion to acetylcholine occurs in cholinergic neurons (Li & Vance, 2008). However, *in vivo,* all pathways are equally possible. The rescue effect shown by choline *in vitro* could be a result of the increased amount of choline stimulating production of PA in lieu of PEth (Guizzetti et al.,

2014). *In vivo,* choline may no longer be increasing the amount of PA, as it could be acting through one of the other pathways of choline and, as a result, there would be no rescue effect of choline on the morphological alterations induced by ethanol, unlike what was seen *in vitro*.

Alternatively, the development of the cholinergic system at this developmental stage could mean that there is already an adequate supply of acetylcholine already present in PD9 rats. As one of the metabolites of choline is betaine, which is converted to methionine and then to S-adenosylmethionine, the universal methyl donor for DNA methylation reactions (Li & Vance, 2008; Niculescu & Zeisel, 2002), choline supplementation may be altering DNA methylation. This is in line with previous research, which has shown that rats treated with choline alone from PD2-PD20 had increased global methylation in the hippocampus and the PFC (Otero et al., 2012). More research is therefore needed regarding the functional results of choline supplementation induced changes in methylation.

However, continuing choline exposure until PD30 had a different effect than stopping choline exposure at PD9. PD30 animals showed a decrease in apical dendrite morphology following choline exposure, an effect that was seen only in males. This is in contrast to the PD9 animals, in which the effects choline, while still primarily seen in males, altered the basilar dendrites. The sex-specific effect of choline injections indicates that the sexes may have different manners of handling increased amounts of choline during development. As mentioned above, there are three metabolites of choline (Li & Vance, 2008), and the different sexes could be preferentially converting choline into different metabolites, resulting in different effects of the same treatment. Alternatively, even if the sexes were converting choline to the same metabolite, they could be utilizing it in different ways. For example, acetylcholine can act on either muscarinic receptors, which can assist in neuronal differentiation, or nicotinic receptors, which can assist in regulating neurite outgrowth, which could produce different effects if the primary receptor activated differs by sex (Abreu-Villaca et al., 2011).

Interestingly, at both PD9 and PD30, the apical and basilar dendrites of the same cell responded differently. At PD9, ethanol affected the apical dendrites, while choline affected the basilar dendrites. At PD30, only a small effect of ethanol on female terminal orders was seen in basilar dendrites, but choline affected only the apical dendrites. Apical and basilar dendrites responding differently may be due to the different connections they have (Spruston, 2008). Basilar dendrites primarily receive inputs from the CA3 through the Schaffer Collaterals, while the apical dendrites receive inputs from the entorhinal cortex and the thalamic nucleus reuniens through the perforant path (Spruston & McBain, 2007). As these two types of dendrites have different inputs and connections, they are likely morphologically different, which may be the cause of their differing response to treatments. For example, decreasing basilar dendrite complexity could lessen the influence that the inputs received by the basilar dendrites would have, i.e. decreasing the amount of connections, thereby decreasing the signaling of this dendrite (Hamilton et al., 2010). Treatments specifically affecting different portions of the same cell in different ways indicates that the ethanol and/or choline may preferentially be affecting specific pathways in the brain, instead of having global effects.

Furthermore, a potential effect of altering dendritic morphology is altered connections. It could be that the alterations of connections seen following ethanol and choline exposure may also differ based on the pathways particular to dendritic type. Other studies have also indicated that there are distinct sensitivities to treatments in different areas of the cells and regions of the brain (Susick et al., 2014), as well as differences between females and males in development of connectivity (Keil et al., 2017). More research would be needed to determine precisely what these altered connections would be, and whether the effect of choline changes from targeting the basilar dendrites to targeting the apical dendrites at different developmental ages.

However, unlike the significant effects of ethanol seen at PD9, an increase in apical morphological parameters, the only significant effect of ethanol seen at PD30 was a decrease in female

basilar terminal orders in choline treated animals. Differing effects of ethanol treatments may be due to the fact that rats at PD30 are at a different developmental stage than at PD9 (Rice & Barone, 2000). The PD9 rat brains were collected 2 hours after ethanol exposure had ended. Their brains were therefore responding to relatively recent ethanol treatment, one specifically given during their brain growth spurt. At PD30, the rats had not been exposed to ethanol for 21 days, and were no longer in the brain growth spurt phase of development (Dobbing & Sands, 1979). As previous studies have shown neurons can recover from treatments, such as a reversal of apical dendritic arbor shrinkage in mPFC pyramidal neurons of layer III following stress (McEwen & Morrison, 2013; Radley et al., 2005), it is possible that the neurons in the hippocampus of the PD30 animals had recovered to an extent from the morphological effects of PD4-PD9 ethanol treatments.

It is also possible that the multiple developmental stages occurring between PD9 and PD30 (Semple et al., 2013) provided opportunities to normalize activity of particular developmental events in the ethanol treated animals when compared to control animals, despite the increased complexity seen at PD9. However, this does not mean that there are no longer any effects of ethanol at PD30; it is possible that the effects of ethanol present as the altered connections mentioned above; neurons being longer and more complex at PD9 may have resulted in different connections with other areas of the brain.

There is a lack of research as to why there are different effects of choline in PD30 rats when comparing choline alone treatments to choline given when ethanol is present. It is possible that ethanol exposure is altering the phenotype of the neuron (Otero et al., 2012). An ethanol induced neuronal alteration may prevent choline from having the same effect as in animals given choline alone. While this theory is extremely tentative and speculative, it is clear that the presence of ethanol alters the effects of choline exposure, and that this is a future avenue for research. Interestingly, pups born to choline

deficient dams had more severe deficits related to gestational ethanol exposure (Idrus et al., 2016), indicating that ethanol and choline can modify the effects of each other.

The alterations of neuronal morphology by ethanol and choline *in vivo* could have functional consequences. One potential consequence is altered connectivity (Cook & Wellman, 2003), which has been seen in humans with FASD (Wozniak et al., 2013). By changing the length and complexity of the dendrites, the inputs to and from these neurons would be altered, changing the circuits connected to the hippocampal pyramidal neurons (Hamilton et al., 2010). Changing the dendritic morphology and complexity could also alter information processing within these dendrites (Rema et al., 2008), and potentially change the surface area available for eventual production of dendritic spines (Rice et al., 2012).

Ethanol and choline can therefore alter hippocampal pyramidal neuronal morphology in a variety of ways. Specifically, both treatments alter hippocampal pyramidal neurons in a manner dependent on the timing of the exposure, the area of the cell examined, and the age and sex of the animals. Ethanol and choline's capacity to alter neuronal morphology therefore deserves more examination, especially as there are morphological and behavioral indications that choline can reverse or ameliorate some of the alterations caused by gestational ethanol exposure (Thomas et al., 2004a; Thomas et al., 2007; Thomas et al., 2009; Thomas et al., 2010).

Discrepancies Between *In Vivo* and *In Vitro* Experiments

One point of interest is that the results presented in this dissertation differed between *in vitro* and *in vivo* experiments. *In vitro*, choline alone had no effects, but when given in combination with ethanol, choline prevented the ethanol-induced, astrocyte-mediated decrease in neurite outgrowth (chapter 2). By contrast, choline alone *in vivo* (chapters 3, 4) had an effect at both PD9 and PD30; a decrease in basilar parameters and a sex-specific decrease of apical parameters, respectively. This incongruence is potentially due to the conversion of choline into PC vs. into acetylcholine or betaine, as

mentioned above (Jiang et al., 2014). In the *in vitro* experiments of this dissertation, astrocytes are the only cell type treated with choline. As acetylcholine is primarily synthesized by cholinergic neurons (Abreu-Villaca et al., 2011; Radziejewska & Chmurzynska, 2019), and the conversion of choline into betaine primarily happens in the liver and kidneys (Li & Vance, 2008; Niculescu & Zeisel, 2002), the most likely fate of choline supplementation *in vitro* is conversion into PC. However, all three pathways are equally possible *in vivo*, and therefore, the differences seen in alterations of neurite outgrowth/neuronal morphology following choline exposure could be due to differences in the fate of choline.

Ethanol exposure also had different results *in vivo* vs. *in vitro*, modifying length and complexity in opposite directions (a decrease *in vitro*, an increase at PD9 *in vivo*, and a decrease at PD30). This is potentially due to the morphological differences between hippocampal neurons *in vivo* vs. *in vitro*, which may also be influencing the distinct results seen in choline exposure (Spruston, 2008). *In vitro*, the hippocampal pyramidal neurons measured have major and minor neurites which, if left to grow, would likely become the axon and dendrites respectively (Dotti et al., 1988). However, at the point in culture at which they were examined, the neurites have not yet become axons or dendrites. By contrast, in the Golgi-Cox stained brains, the hippocampal pyramidal neurons have both apical and basilar dendrites, indicating that these cells are at a different developmental stage than the cells in culture (Keil et al., 2017).

Furthermore, the *in vivo* ethanol intubation and choline injections also constitute a different paradigm than the *in vitro* culture, as in the culture system the only cells exposed were astrocytes, while the *in vivo* experiments exposed the whole animal to ethanol and choline. The ethanol exposure *in vivo* was also given as a single dose that was metabolized by the body and diminished over time. However, *in vitro*, the concentration of ethanol did not change following the incubation indicating that, unlike *in vivo,* astrocytes were continually being exposed to a constant concentration of ethanol. Lastly, the *in*

vitro experiments cultured hippocampal neurons on top of cortical astrocytes. It is possible that, had the astrocytes also been cultured from the hippocampus, the response to ethanol and/or choline exposure *in vitro* may have replicated more closely the effects of ethanol and choline *in vivo*.

Another potential reason for the differences seen is that astrocytes do not metabolize ethanol. As the animals *in vivo* do metabolize ethanol, this results in a different exposure paradigm between *in vivo* and *in vitro*. Specifically, the astrocytes are being continually exposed to a constant concentration of ethanol, as there was no evaporation (chapter 2, 5), while the animals would be exposed to a gradually decreasing dose of ethanol due to the metabolism over time.

Overall, despite the differences in results seen *in vivo* vs. *in vitro*, both sets of experimental results are equally valid, and both further elucidate the effects of choline and ethanol on hippocampal neuronal morphology. However, due to the differences described above, they cannot be directly compared, and each set of results must be interpreted separately.

Limitations

There are several limitations to the experiments described here. One such limitation is that it is relatively unknown exactly how well results seen in animal models, in this case rats, can be generalized to humans, as there are studies showing a species-specific response to certain treatments, including expression of tPA in astrocytes, and comparisons of FASD models between humans and animals (Driscoll et al., 1990; Tjarnlund-Wolf et al., 2014). This is compounded as, due to the period of development of interest occurring postnatally in rats, the animals in the *in vivo* studies were treated postnatally. This does not accurately reflect humans with FASD, as the ethanol exposure in humans occurs entirely prenatally. Thus, our experimental paradigms do not account for the biology or behavior of the mother, both of which would change while the mother was intoxicated. These *in vivo* experiments also only utilized on strain of rats, Sprague-Dawley. As this strain is an inbred rat strain, it likely does not completely reflect the genetic heterogeneity that is found in humans, which may, in turn,

be influencing the effects of gestational ethanol exposure. This *in vivo* model is also designed to mimic a specific form of drinking, binge drinking, as the pups are exposed to a relatively high concentration of ethanol for a relatively short period of time. Therefore, the results in these experiments may not reflect the alterations seen by different ethanol exposure paradigms.

Furthermore, in the *in vivo* studies, stress is possibly a factor that could influence changes in neuronal morphology (Bennur et al., 2007; Cook & Wellman, 2003; Jakubowska-Dogru et al., 2017; McEwen, 2013; Pawlak et al., 2005a). Not only are the pups separated from the mother, albeit for only a short period of time, they are also undergoing intragastric intubation and/or subcutaneous injections. These experiences, while temporally brief, could, over time, result in changes in the hippocampus due to the stress induced by these treatments. Another consideration is that, at PD9, we elected to analyze females and males separately, thus reducing our statistical power. This may have resulted in a group sizes not adequate for analyzing sex differences, limiting our interpretations, and a greater sample size would be required for more thorough analyses. Also *in vivo*, as certain metabolites of choline are metabolized in areas other than the brain, specifically conversion to betaine, which happens in the livers and kidneys, it is not known how much of the *in vivo* choline treatments passed the BBB and directly affected the brain.

Future Directions

There are many future experiments that could be done to expand upon the results from the experiments presented here. First, the effects of tPA were only examined *in vitro*; thus, a natural extension of these experiments would be to examine the effects of tPA *in vivo*. This could be done via stereotactic injections into the CA1 of the hippocampus, which was the area of interest examined in the *in vivo* studies presented here. These injections would ideally encompass both increases and decreases in tPA levels, along with ethanol intubation, to replicate the experimental conditions utilized *in vitro* in this dissertation. It would be particularly interesting to see the effect of altered levels of tPA *in vivo*, due

to the above-mentioned involvement of tPA in the growth cone (Garcia-Rocha et al., 1994). As the astrocyte-neuron co-culture system is a simplified version of the whole brain, and indeed, the experiments presented here show different results following ethanol and choline exposure *in vivo* vs. *in vitro*, examining the effects of altered tPA *in vivo* could greatly expand upon the proposed role of tPA in the development of FASD.

Furthermore, future experiments could also examine the effect of choline. Specifically, the *in vitro* experiments did show a rescue effect of choline, mediated by astrocytes, but this same experimental setup did not exhibit an effect of choline alone. As there are *in vivo* studies that show changes in non-ethanol treated animals following choline exposure (Otero et al., 2012; Thomas et al., 2000; Waddell & Mooney, 2017), a possible avenue to pursue is how the effects of choline exposure are mediated by non-astrocytic cell types. A way to examine this would be to expand the co-culture system presented in chapters 2 and 5. These experiments specifically isolated and examined neurons and astrocytes, but full understanding of the effects of choline could also examine other cell types, such as oligodendrocytes or microglia. This would elucidate choline's effects on the brain, especially when given alone and not in combination with ethanol. Given that a potential way in which choline could be having the effects seen *in vivo* is through acetylcholine (Li & Vance, 2008), another future experiment could be to treat astrocyte-neuron co-cultures with acetylcholine *in vitro*, to see how treatment with a specific metabolite of choline alters neurite outgrowth, especially in comparison with treatment with choline directly. *In vitro* experiments examining acetylcholine could also utilize an antagonist for specific types of acetylcholine receptors, in order to specifically isolate which receptor is potentially mediating these effects.

Another possible avenue of analysis would be to probe the sex differences seen in the *in vivo* experiments; specifically, at PD30, male rats showed a difference in neuronal morphology following choline injection, while female rats did not. While there have been some indications of minor

differences between females and males in humans with FASD (Fontaine et al., 2016; Lebel et al., 2012), most human studies have examined the sexes together, focusing on the differences between non-FASD groups and FASD groups, rather than including sex as a variable. However, the results of this dissertation indicate that there may be more sex differences in FASD and response to treatments than previously assumed, especially morphologically. There is therefore a lack of evidence regarding potential sex differences and their underlying mechanisms. Therefore, sex differences are a possible area of future research.

A further potential experiment could be to examine astrocytes *in vivo*, such as via immunohistochemistry to visualize location of astrocytes in relation to the dendrites, to analyze if astrocytes are potentially involved in the alterations of neuronal morphology seen in chapters 3 and 4. *In vivo* experiments could also vary the timing of the exposures. In both *in vivo* experiments presented here, choline was given simultaneously with ethanol. A future experiment could be to give ethanol from PD4-PD9, as was done in these experiments, but to only give choline after the ethanol treatment has ended, to replicate humans being treated with choline after birth. Choline exposure could also be given later in life, such as starting in adolescence, in order to further elucidate how the timing of choline exposure, especially in relation to the timing of ethanol exposure, changes the effects of choline and ethanol. In relation to this, the oldest animals examined in these experiments were rats at PD30. Other experiments could therefore examine animals at later ages, including adulthood, or even old age, to determine long-term effects of treatments. Golgi-Cox examination of choline and ethanol exposure could also include analysis of dendritic spines, or other forms of neuronal morphology not presented here.

Lastly, work needs to be done to determine why the apical and basilar dendrites in the *in vivo* studies showed different morphological changes in response to the same treatments. Some research has been done into how these two types of dendrites differ (Spruston & McBain, 2007; Spruston, 2008).

In particular, the apical dendrites primarily receive input from the entorhinal cortex and the thalamic nucleus reuniens, while the basilar dendrites receive inputs from CA3 neurons through the Schaffer Collaterals (Spruston & McBain, 2007; Spruston, 2008). Apical dendrites also respond to activation of NMDA receptors resulting from voltage dependent relief of the Mg^{2+} block, while basilar dendrites do not (Spruston, 2008). The main apical dendritic shaft also propagates calcium waves evoked by coincident action potential firing and activation of either metabotropic glutamate receptors or metabotropic acetylcholine receptors, an effect which was not seen in basilar dendrites (Spruston & McBain, 2007). Therefore, there are some known differences between apical and basilar dendrites on CA1 pyramidal neurons. However, not enough is known about the mechanical, electrophysiological, and functional differences to determine why apical and basilar dendrites belonging to the same cell have different responses to the same treatment.

In conclusion, the experiments of this dissertation indicate that some of the CNS and behavioral dysfunctions seen in individuals gestationally exposed to ethanol may be due to subtle and complex alterations in neuronal morphology of the hippocampus. Some of these alterations may be a result of an ethanol-induced increase in the levels of tPA, one that may be mediated by astrocytes. These experiments also indicate that astrocytes can mediate a rescue effect of choline *in vitro*, and that choline can alter the morphology of hippocampal neurons *in vivo*. The alterations of morphology induced by these treatments depend on a number of factors, including the age and sex of the animal, *in vivo* vs. *in vitro* exposure paradigms, and the area of the cell examined. In total, astrocytes and ethanol-induced increases of tPA may be involved in the changes in the brain caused by gestational ethanol exposure, and, while research has indicated that choline may be a treatment for FASD (Thomas et al., 2000; Thomas et al., 2004a; Thomas et al., 2007; Thomas et al., 2009; Thomas et al., 2010), the efficacy of this treatment will likely be dependent on both the sex of the individual and the timing of choline

exposure. These results, therefore, open many new areas of potential research regarding both the development of and treatments for FASD.

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