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The Effects of Fetal Alcohol Exposure on Gene Expression and Associated Behavioral Markers in Sprague Dawley Rats

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THE EFFECTS OF FETAL ALCOHOL EXPOSURE ON GENE EXPRESSION AND ASSOCIATED BEHAVIORAL MARKERS IN SPRAGUE DAWLEY RATS

By Sarah Willey

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ABSTRACT

THE EFFECTS OF FETAL ALCOHOL EXPOSURE ON GENE EXPRESSION AND ASSOCIATED BEHAVIORAL MARKERS IN SPRAUGE DAWLEY RATS

By Sarah Willey

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Alcohol is a dangerous recreational substance because of its availability and the economic, social, and biological problems that it can cause. Fetal alcohol exposure, although preventable, is particularly harmful to a developing embryo. This exposure often causes fetal alcohol spectrum disorder (FASD), the symptoms of which include learning disabilities and psychological hardships with comorbidities such as anxiety, depression, and attention deficit disorder. One of the main teratogenic effects of alcohol is the effect on epigenetic modifications and altered gene expression during development. These modifications involve changes in the methylation and condensation of DNA that then alters the expression of specific genes into proteins.

The first trimester is a particularly important timeframe of focus since this is a critical time in embryonic development and also a time when women may consume excessive amounts of alcohol before they know they are pregnant. In my Honors Thesis project I aimed to examine the effects of prenatal ethanol exposure on both behavior and gene expression in a rat model system. I used quantitative reverse transcriptase PCR to study the expression of two DNA methyl transferase genes critical for epigenetic modification and another gene associated with neuronal function and psychiatric disorders. Expression levels of these genes were altered in response to prenatal alcohol exposure. Behavior studies involving the Morris Water Maze and

forced swim test to study to spatial learning and depression, respectively, suggested that alcohol does affect behavior, although these differences were not statistically significant. This is the first study to analyze the gene expression and behavioral effects that a realistic level of alcohol consumption has on a developing embryo during the first trimester.

INTRODUCTION

Fetal alcohol spectrum disorder (FASD) encompasses a large range of clinical abnormalities associated with prenatal alcohol exposure. FASD is characterized by cognitive deficits, however it can be comorbid with other psychiatric illness including depression (Popova et al., 2016) as well as spatial learning and memory deficits (Toso et al., 2006). Fetal Alcohol Syndrome (FAS) exists at the most severe end of this spectrum and is characterized by growth deficiencies, central nervous system dysfunction, and cranio-facial disfigurement (Jones & Smith, 1973). Prenatal exposure to alcohol is of great economic and social concern to our country and the world. It is a leading preventable cause of neurodevelopmental disorders, intellectual disabilities, and birth defects (Guerri et al., 2009). Based on physical examinations, experts think that the full range of FASD in the United States and some Western European countries could be as high as 2 to 5 cases per 100 school children (May et al., 2014). The lifetime cost for one individual with FAS was estimated at $2 million in 2002. This estimate does not include data on people with other FASDs (Lupton et al., 2004).

The majority of women discover they are pregnant between weeks four through seven, however this timeframe can extend well through the first 12 weeks of pregnancy (American Pregnancy Association). Thus, women could be consuming alcohol without know that they are pregnant. Because ethanol readily crosses the placenta and accumulates in the embryo and fetus at concentrations proportionate to maternal blood levels within the first hour of alcohol consumption (Lo et al., 2017), an embryo's chance of being exposed to alcohol is increased during the first trimester. Thus, an embryo's chance of being exposed to alcohol is increased during the first few months of pregnancy when many women have not yet realized they are pregnant at this time and consume alcoholic beverages.

Even at low levels, maternal alcohol consumption has been adversely related to child behavior. Consumption as low as one drink per week has produced deleterious effects on child behavior such as externalizing and aggressive behaviors (Sood et al., 2001). Low alcohol consumption is not a realistic assumption for women of reproductive age. The Behavioral Risk Factor Surveillance System (BRFSS) defined binge drinking as having "consumed four or more drinks on an occasion at least one time in the past 30 days." Data collected from the BRFSS found that the percentage of binge drinkers among women of reproductive age (18-44 years) who reported any alcohol use in 2013 ranged from 23.5% to 42.6% on a state-by-state basis. Of those women surveyed, one in five nonpregnant women reported binge drinking in the past 30 days. Binge drinking behavior and associated withdrawal effects have been replicated in rats through self-administration of ethanol, thus rats have proven time and time again to be reliable and accurate animal models of binge alcohol exposure. Physical dependence can be so strong in rats that withdrawal can cause death from tonic-clonic seizures (Falk et al., 1972). Binge alcohol exposure during the first trimester can have extremely deleterious effects on a developing fetus due to the crucial developmental milestones that occur during this timeframe.

The first trimester is a crucial developmental milestone for neural tube development and cell differentiation. Ethanol exposure during this delicate developmental timeline may increase a child's risk for developing FASD. As previously mentioned, the majority of pregnant women discover they are pregnant between weeks four through seven. Week five marks the initial specification of the tissue that will become the brain, spinal cord, and heart in the developing embryo. During week six, the neural tube closes and the heart begins to pump blood and by week seven the embryo's brain and face are rapidly developing (Gilbert, 2014).

Neural tube formation is crucial for embryonic development. For this to occur, the neural plate must move inside the embryo to form the neural tube. Primary neurulation and secondary neurulation are the steps by which this occurs (Figure 1). During primary neurulation in the anterior side of the embryo, mesodermal cells of the notochord signal to the cells surrounding the neural plate to fold and separate from the surface to form a hollow tube (Gilbert, 2014). The expression of various cell adhesion molecules mediates the separation of the neural tube from the surface ectoderm. Ectodermal cells adjacent to the neural plate become neural crest cells, which are mesenchymal and produce a variety of cell types including neurons and glia of the cranial glia, cranial cartilage and bone, connective tissue, sensory neurons and glia, and pigment cells. The posterior neural tube then arises from a collection of mesenchyme cells during secondary neurulation. This cluster of cells forms into a solid cord that forms cavities that come together to create a hollow tube (Figure 1**)**. Primary neurulation generally makes the anterior portion of the neural tube, which will eventually form the brain and anterior spinal cord, while secondary neurulation makes the posterior portion. Neural tube closure is initiated at several different places along the anterior-posterior axis in mammals.

These key nervous system developmental milestones occur during the period when women are most likely to find out they are pregnant and when they are most likely to binge drink without knowledge of their pregnancy. Although studies linking alcohol and neural tube defects have been inconsistent (Leng et al., 2016), there are clearly effects of alcohol exposure on neurodevelopmental processes that manifest later in development and adulthood. The phenotype of FASD suggests particularly that prenatal ethanol exposure disturbs neural crest cell development (Cartwright & Smith, 1995). The neural crest cell migration paths, which are affected by signals from the neural tube and adjacent, determine the subsequent differentiation

and localization of these cells (Gilbert, 2014). Although the cellular and molecular mechanisms responsible for ethanol's teratogenicity remain largely unknown, craniofacial malformations most likely related to neural crest cells are a common symptom of FASD. Malformations have been previously connected to ethanol-induced cranial neural crest cell (NCC) damage in mammalian embryos (Chen & Sulik, 1996). NCCs are of particular interest to the study of craniofacial malformations due to their susceptibility to free radical damage. Exposure of NCCs to ethanol stimulates the production of superoxide anion radicals, which suggests that ethanol-induced NCC injury is mediated through the generation of free radicals. Ethanol induced free radicals may also play a large role in neural degeneration, possibly more so than the NCCs (Chen & Sulik, 1996).

Alcohol exposure affects animals and humans in a similar way. The rat brain and the human brain have strong correlations between structure, function, and behavioral abilities (Otero et al., 2012). These similarities have made it possible for researchers to use animal models to study FASD. Sprague Dawley rats, *Rattus norwegicus,* are among the most commonly used models to study the effects of FASD due to their size and complex behaviors. Growth retardation, structural abnormalities, central nervous system dysfunction, and cognitive deficits have been previously observed in rat models of FASD (Patten et al., 2014). Due to alcohol's wide spread effects on the developing hippocampus, Animal research has established that the hippocampus is particularly sensitive to the teratogenic effects of prenatal alcohol exposure (Livy et al., 2003; Maier & West, 2001).

The Role of the Hippocampus

The hippocampus plays an important role in the processing and memory of spatial and contextual information. Rats without a hippocampus show impaired performance on tasks that require the utilization of spatial and contextual information (Jarrard, 1993). Clinical studies have found learning and memory deficits in children with FASDs (Hamilton et al., 2012). The hippocampus is also associated with serotonin function (Dale et al., 2016). Numerous studies have associated changes in the hippocampus to depression and major depressive disorder (O'Connor & Paley, 2006). These deficits are complex and numerous and include both verbal and nonverbal skills. Hippocampal alterations in children with FASD have also been associated with an increased incidence of depression. O'Connor and Paley (2006) found that higher levels of prenatal alcohol exposure correlated with increased depressive symptoms. Interestingly, this relationship was mediated by mother-child interactions that occur over time suggesting that lower levels of maternal emotional support increase depressive symptoms experienced by children exposed to alcohol while in the womb.

Evidence suggests that psychiatric disorders and behavioral deficits of children with FASD persist into adulthood (Barr, 2006; Spohr, 2007). The prevalence of deficits into adulthood creates social and economic hardship for people with FASD and their families. It is important to evaluate if rats that have been prenatally exposed to alcohol maintain or decrease cognitive brain function as they age in order to develop a way to ease the effects of FASD in humans. Two predominant effects of fetal alcohol exposure found into adulthood are depression and spatial learning and memory deficits. The Morris Water Maze is used in rodent models for identifying spatial learning and memory deficits. In animal models, the Porsolt forced swim test is used as a possible indicator of depression.

The aim of this study is to evaluate the effects that first trimester ethanol exposure in rats could have on the development of FASD associated behaviors such as depression and spatial learning and memory deficits as well as the underlying mechanisms related to the expression of specific genes, namely *HTR2A* (serotonin receptor) and DNMTs (DNA methyl transferases).

I. FASD and Depression

Although the most common manifestations of FASD are deficits in cognitive ability, FASD is also associated with various psychiatric illnesses including depression. Prenatal alcohol exposure has been associated with an increased incidence of depression (Caldwell et al., 2008). Adults with FASD most commonly experience drug or alcohol dependence, depression, and psychotic disorders (Famy et al., 1998). In animal models, the Porsolt forced swim test is used as a possible indicator of depression.

Reliable animal models of FASD have allowed researchers to explore the genetic mechanisms involved in prenatal alcohol exposure and its associated behavioral and psychiatric disorders. Behavioral epigenetics has gained popularity in the past decade for its ability to provide new insights into a biological basis of the behavioral effects of gene-environment interactions. Epigenetic alterations can change gene activity in response to environmental adversity. DNA methylation has emerged as the central pathway linking these gene-environment interactions (Roth, 2013). Prenatal ethanol induced epigenetic changes of *HTR2A* in hippocampal tissue may be a large contributor to depressive behaviors experienced by people with FASD.

Regulation of 5-Hydroxytryptamine Receptor 2A (*HTR2A*)

Disturbances of the serotonergic pathway have been linked to depressive disorders in humans. Serotonin (5-HT) is a monoamine neurotransmitter involved in the regulation of body temperature, sleep, pain, mood, and energy. Serotonin is crucial for numerous stages of embryogenesis (neuronal development and differentiation, left/right axis modeling in vertebrates) and placentation (early gastrulation, blastocyst adhesion, and adhesion) (St.-Pierre et al., 2016). Several 5-HT receptors exist, each with different subtypes and biological roles. Serotonin 2A receptor (5-HT_{2A}), is a G-protein-coupled receptor that's defects have been linked to the pathogenesis of major psychiatric disorders including depression (Raote, Bhattacharya, & Panicker, 2007).

HTR2A is the gene that encodes the 5-HT_{2A} receptor and is involved in the regulation of serotonergic neurotransmission and the hypothalamic-pituitary-adrenal (HPA) axis (Falkenberg, 2011). Functional regulation of the 5-HT_{2A} receptor involves internalization and recycling of the receptor. These two phases regulate the desensitization and resensitization processes that help to prevent overstimulation and allow the receptor to recover signaling ability. Desensitization and resensitization are common processes of G-protein-coupled receptors.

The placenta is a plastic organ that can quickly adapt to maternal environmental changes, for example, through epigenetic mechanisms. Ethanol exposure is associated with placental dysfunction, decreased placental size and impaired blood flow and nutrient transport (Burd, 2006). Disrupted placental serotonin signaling can affect infant brain development and cause long-term neurobehavioral issues (Paquette et al., 2014). Therefore, prenatal ethanol exposure's deleterious effects on placental integrity and function could cause an imbalance in fetal serotonin regulation and lifelong behavioral disorders such as depression.

The expression of Tryptophan hydroxylase (TPH)-1 in the peripheral nervous system and THP-2 in the central nervous system allow for the synthesis of serotonin from L-tryptophan. Monoamine oxidases A and B (MAOA and MAOB) degrade the serotonin after it is synthesized. The complexity of this system and the widespread use of serotonin could possibly explain why serotonin disruption is involved in a large variety of diseases. The pattern of serotonin system development *in utero* in humans is relatively similar to early embryo development in rodents. In rodents, MAOA and SERT (serotonin transporter) are present in the developing brain before the appearance of the first serotoninergic neuron (Booij et al., 2015). SERT appears at week 8 of pregnancy in humans, followed by the formation of clustered serotonin cell bodies in the raphe nuclei at week 15. The results from Booij et al. (2015) suggest that the fetus depends on an external source of serotonin that allows the serotonin system to develop due to the fetus's lack of independent synthesis. Bonnin et al. (2011) used a mouse model to show that the placenta synthesizes its own serotonin from the mother's L-tryptophan by way of the placental TPH-1 enzyme. Booij et al. (2015) also noted that MAOA stabilizes early in life. Therefore, deregulation in serotonin homeostasis during early crucial neural developmental windows *in utero* could alter fetal short-term development and lifelong health.

The *HTR2A* gene has been directly associated with major depressive disorder (Mandelli, 2013). Studies have shown decreased *HTR2A* expression in the brains of patients who have committed suicide. *HTR2A* methylation has been associated with decreased expression of *HTR2A* and occurs in higher rates in patients with psychiatric disorders such as depression and schizophrenia (Abdolmaleky et al., 2011). Studies have shown that antipsychotic drugs decrease *HTR2A* methylation and increase *HTR2A* expression. This study aims to look at the association between *HTR2A* expression in hippocampal tissue and depressive behaviors in rats under the

assumption of an inverse association between DNA methylation and gene expression (Abdolmaleky et al., 2011; Mandelli & Serretti, 2013). Abdolmaleky et al. (2011) matched DNA derived from post-mortem brains of patients with schizophrenia and bipolar disorder to control subjects. Bisulfite DNA sequencing was used to screen and quantify cytosine methylation in the *HTR2A* promoter and corresponding gene expression was analyzed by qRT-PCR. They found that epigenetic down-regulation of *HTR2A* was associated with early age of disease onset in schizophrenia and bipolar disorder. Conversely, Mandelli and Serretti (2013) used a review approach to investigate the literature regarding the interaction between genes modulating brain functions in the psychopathology of major depression and suicidal behaviors. Their literature review found that environmentally induced DNA methylation was inversely associated to gene expression in the case of major depression and suicidal behaviors. They emphasized the usefulness of studying genetic-environmental interactions for elucidating the effect of genes on the risk of developing major depression and suicidal behaviors.

Possible Molecular Mechanisms related to HTR2A expression and FASD

Epigenetic regulation in eukaryotes relies on DNA methylation. Preimplantation embryos represent one of crucial developmental periods when genomic methylation patterns are established. DNA methyltransferases (DNMTs) contribute to gene regulation by maintaining DNA methylation patters at specific regions of the genome. DNMTs establish and maintain cytosine methylation patterns throughout cell replication. DNMTs are responsible for the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to the 5' position on the cytosine pyrimidine ring and are vital for mammalian development (Morris & Monteggia, 2014). The addition of a methyl group in mammals is catalyzed by three different DNA methyltransferases: Dnmt1, Dnmt3a, and Dnmt3b. The current study focused on the regulation of Dnmt1 and

Dnmt3a. Dnmt1 is known as the maintenance Dnmt and is responsible for stabilizing methylation patterns that were previously established. Dnmt3a executes initial methylation patterns on unmethylated DNA. These DNMT's establish and maintain the cytosine methylation patterns throughout cell replication (Denis, 2011). Most Dnmt3a in neural stem cells is located within gene bodies and intergenic regions. This finding is consistent with the recent discovery that DNA methylation levels are high in the gene bodies of actively transcribed genes (Wu et al., 2010).

Abnormal DNA methylation patterns are thought to play a vital role in the mechanism by which prenatal alcohol alters a growing fetus. Prenatal alcohol exposure during embryonic development affects the transfer of folate from the mother to the embryo (Laufer et al., 2016). Folate is a methyl donor and is essential for establishing and maintaining DNA methylation. The lack of folate can potentially cause altered epigenetic patterns in the developing embryo and possibly lead to behavioral deficits and psychiatric disorders.

As the primary maintenance methyltransferase, Dnmt1 preserves methylation patterns during cell division. Dnmt1 localizes to DNA replication foci during the S phase and proceeds to preferentially methylate hemimethylated CG dinucleotides by interacting with UHRF1 (Denis, 2011). The exact methods underlying Dnmt1 regulation are poorly understood. Du et al. (2010) aimed to illuminate one pathway that regulates Dnmt1 protein stability. Their model suggests that Dnmt1, HASP, Tip60, UHRF1, HDAC1, and PCNA form a macromolecular protein complex that binds at the DNA replication fork to regulate the functional availability of Dnmt1. Du et al. (2010) claim that first, Tip60 acetylates Dnmt1. This acetylation triggers UHRF1 to ubiquitinate Dnmt1 and therefore leads to proteasomal degradation. HDAC1 and HAUSP then use deacetylation and deubiquitination to protect Dnmt1 from degradation. HAUSP and HDAC1

are the protectors and Tip60 and UHRF1 are the functional destroyers and together they form a multifunctional protein complex with Dnmt1. Parts of this newly formed complex localize to the replication fork through their association with PCNA. Therefore, the efforts of the functional destroyers and the protectors maintain the proper abundance of Dnmt1 at different stages during the cell cycle. Dnmt1 increases in S phase, decreases after S phase, and is lowest in G_1. These results suggest Dnmt1 abundance is decreased after DNA replication by Tip60- and UHRF1-mediated proteasomal degradation.

Many unknowns exist surrounding the structures and mechanisms of Dnmts. For example, the crystal structure of Dnmt1 bound to hemimethylated DNA must be solved before Dnmt1 substrate recognition can be understood. A primary question in this mystery is what defines the intrinsic preferences of DNMTs for certain DNA sequences. Understanding DNMTs preference to hemimethylated CG, unmethylated CG, or non-CG substrates may rely on determining the crystal structure of Dnmt1. For example, Dnmt1 has shown dependence on the SRA domain of UHRF1 for its recruitment to hemimethylated CG sites. Dnmt3 enzymes, on the other hand, are responsible for *de novo* methylation during embryonic development. The way in which they target to genomic regions has been proposed in several models. These models focus on the influence that interacting partners have on the Dnmt3 enzymes and their inherent properties. These models suggest that the global *de novo* methylation performed by Dnmt3 enzymes depends on the innate sequence preferences of Dnmt3 (Jurkowska et al., 2011). The importance of sequence preference by *de novo* DNMTs in human genomic methylation patterns is further supported by the catalytic activities of Dnmt3a and Dnmt3b. Sequences next to the target CG sites have been found to influence these catalytic activities.

Many recent studies have been performed on the chromatin-mediated mechanisms of DNMTs. By studying the interaction between DNMTs and native nucleosomes, Jeong et al. (2009) found that Dnmt3a and Dnmt3b were mostly found stably anchored to nucleosomes with higher levels of methylated DNA in somatic cells. These results suggest that cues from the chromatin component, in addition to hemimethylation, are required for the inheritance of DNA methylation in certain cell types. DNA methylation works with local chromatin conformations to regulate certain methylation patterns that control gene transcription. DNMTs' ability to "read" histone modifications allows them to recruit nucleosomes carrying specific marks.

DNA Methyltransferase and Prenatal Ethanol Exposure

DNA methylation is known to play a role in the regulation of gene expression during embryogenesis and prenatal ethanol exposure causes changes in DNMT activity. DNA methylation changes are highly organized during rodent embryo development. A cycle of demethylation occurs before implantation, which is proceeded by a wave of *de novo* methylation. These steps establish embryonic methylation patterns critical for assigning somatic DNA methylation patterns. This pattern of epigenetic reprogramming is crucial for normal embryonic development. Disruption of the cycle of methylation that occurs during early development will cause neural tube deficits (Liu et al., 2009) and may cause the cognitive deficits associated with FASD. Garro et al. (1991) found that acute ethanol administration to pregnant mice caused widespread hypomethylation of fetal DNA. Nuclei isolated from the ethanol-exposed mouse fetuses had lower levels of methylase activity than the control fetuses even in the presence of excess S-adenosylmethionine (methyl donor for DNMTs). These ethanol-associated changes in fetal DNA methylation could contribute to the developmental deficits children with FAS experience. Alcohol has been known to inhibit folate-mediated methionine synthesis in animals.

This inhibition interrupts critical methylation processes that are mediated by s-adenosyl methionine. S-adenosyl methionine is the activated form of methionine and the substrate for biologic methylation. Although fetal gene expression patterns have been altered after alcohol exposure, the mechanism by which alcohol causes changes in gene expression remains to be found.

DNA methylation has been identified as a major factor involved in the epigenetic regulation of learning and memory (Roth et al., 2013). Hippocampal changes in DNMT regulation may be the cause of spatial learning and memory deficits in fetuses exposed to alcohol during the first trimester.

Forced Swim Test

The Porsolt forced swim test, initially described by Porsolt et al. (1977, 1978), is a well-established model of behavioral despair in rodents and is used to quantify depressive behavior. The forced swim test was developed based on the notion that when placed in a cylinder of water, rodents that stop active escape behaviors will become immobile. An increase in immobility is considered as an increase in behavioral despair. Animals are placed in a cylindrical tank of water (deep enough so the tail cannot touch the bottom) for five minutes. In this study, the time spent performing climbing behavior was recorded and subtracted from the total time in the water. Climbing behavior is defined as actively scratching the side of the tank. Antidepressants have been shown to decrease immobility (Cryan et al., 2005).

Hellemans et al. (2010) found that males that were prenatally exposed to alcohol did not differ from their control counterparts in the Porsolt forced swim test, while females that were prenatally exposed to alcohol showed greater immobility than control females. This finding

suggests that prenatal alcohol exposure selectively increases depressive-like behaviors in female offspring in the forced swim test (Hellemans et al., 2010). However, Slone & Redei (2001) found no gender differences in males and females who were prenatally exposed to ethanol and underwent the forced swim test. These conflicting conclusions demonstrate the hypersensitivity of results to experimental design and execution when studying fetal alcohol exposure induced depression in any animal model. The forced swim test was used to measure helplessness in rats that received either the prenatal control treatment or prenatal ethanol treatment.

II. FASD and Spatial Learning and Memory Deficits

Children with FASD have been shown to have an impaired ability to learn and remember spatial locations. Little is known about the cause of this spatial learning and memory deficit, however researchers think that modifications in the hippocampus could be the cause. In a 2003 paper published in the journal of Behavioral Brain Research, Hamilton et al. used a computerized version of the Water Maze test. This virtual test was sensitive to hippocampal functioning and tested spatial learning in male children with FAS. Hamilton et al. (2003) came to the conclusion that children with FAS demonstrate difficulty with spatial learning. Hamilton et al. correlate fetal alcohol exposure in humans and animal models to spatial learning and memory deficits. Several studies outlined by Morris and Monteggia (2014) found that disrupted homeostatic expression of DNA methyltransferases contributes to learning and memory deficits.

Morris Water Maze

Richard G. Morris first used the Morris Water Maze (MWM) test in 1981 to test hippocampal-dependent learning. The MWM is unique because it reduces odor trail interference and can test spatial and non-spatial conditions in order to analyze acquisition of spatial memory and long-term spatial memory (Bromley-Brits et al., 2011). The maze consists of a large, circular pool filled with opaque water and a platform that is submerged just below the water's surface. Rats start from a randomized location and use a series of visual cues placed on the wall to navigate a pool of water in search for the submerged platform. After a number of trials, rats begin to learn where the platform is located by using the visual cues on the walls. Several studies have used the MWM to test the effects of fetal alcohol exposure on spatial learning and memory. The MWM has been shown to be sensitive to the neurobehavioral teratogenicity of fetal alcohol exposure in rodent models (Johnson & Goodlett, 2002; Richardson et al., 2002; Savage et al., 2002). Johnson and Goodlett (2002) exposed rats to ethanol during postnatal days (PD) 7-9 (5.25g/kg/day) and observed whether it would produce selective spatial learning deficits that endured into adulthood. The rats were tested on PD70 in the Morris Water Maze. They found that neonatal alcohol treatment significantly impaired acquisition and reduced place biases in the probe trial. Richardson et al. (2002) hypothesized that chronic prenatal ethanol exposure through maternal ethanol ingestions would results in spatial learning impairments and short- and long-term plasticity in the CA1 region of the postnatal guinea-pig hippocampus. Pregnant guinea pigs were treated with 4g/kg of ethanol per day throughout gestation. These offspring were studied between PD 40 and 80 in the Morris Water Maze. They found that chronic prenatal ethanol exposure impairs spatial performance and long term potentiation in CA1 neurons. Due to these results Richardson et al. (2002) concluded that hippocampal dysfunction could contribute to the

cognitive and behavioral deficits that results from chronic prenatal ethanol exposure. Savage et al. (2002) allowed pregnant rats to consume 2%, 3%, or 5% liquid ethanol diet throughout gestation. They tested adult offspring for either evoke [3H]-D-aspartate (D-ASP) release from hippocampal slices or spatial learning using the Morris Water Maze. Offspring whose mothers' consumed 3% or 5% liquid ethanol diet experienced significantly difference results in the one-trial learning on a moving platform version of the Morris Water Maze and the activity-dependent potentiation of evoked D-ASP release from slices of hippocampal tissue compared to the control. This study shows that the threshold for eliciting learning deficits with prenatal alcohol exposure is less than 30/mg/dL, which translates to the equivalent of drinking 1 to 1.5 ounces of ethanol per day.

Study questions and hypotheses

The present study aims to elucidate the effects that binge alcohol exposure during the first trimester of pregnancy could have on behaviors and gene expression in their progeny using a rat model. I hypothesized that behaviors such as depression and spatial learning deficits would be higher in animals prenatally exposed to alcohol. Based on previous published results, several genes were chosen to examine the effects of alcohol on the expression of specific genes associated with depression and spatial learning, namely DNA methyl transferases critical for epigenetic regulation, and HRT2A, a critical component of the serotonergic pathway. This study used RT-qPCR to analyze Dnmt3a and Dnmt1 regulation in hippocampal tissue of rats that were exposed to ethanol *in vivo*. It was hypothesized that expression of these genes would be affected in response to prenatal ethanol exposure. More specifically, the expression *HTR2A* will be downregulated and Dnmt1 and Dnmt3a will be upregulated. The rats treated with ethanol in

utero will also experience increased immobility times in the forced swim test and increased latency times in the Morris Water Maze compared to control rats. An increase in immobility time should be associated with the downregulation of *HTR2A* and upregulation of the Dnmts. These two factors together may be an indicator of prenatal ethanol induced depression. Increased latency times should be connected to general hypermethylation and increased DNMT expression in rats treated with ethanol.

METHODS

Fetal Alcohol Exposure in Rattus norwegicus

Dams were assigned to one of four groups: postnatal day (PD) 30 control, PD30 ethanol, PD60 control, or PD60 ethanol. Dams were received on gestational day (GD) 2 and received ad libitum access to food and water throughout gestation. Dams were subcutaneously injected with either 1.5g/kg of 20% ethanol in saline or 1.5g/kg of 0.9% saline from GD3 to GD10. This time frame is equivalent to the first trimester of human pregnancy. All injections were performed two days in a row with a rest day in between. Injection schedule is as follows: GD3-GD4, GD6-GD7, GD9-GD10. All dams did not receive injections on the days in-between the above injection days.

On PD1 pups were weighed as a litter and an average weight of each pup was calculated from the total litter weight. The pups were given ad libitum access to the mother's milk. On PD21 pups were weaned from the dam and separated by gender.

Pups had ad libitum access to food and water after weaning and were euthanized PD30 or PD60.

Behavioral Despair Tests

Half of each of the four litters (PD30 control, PD30 ethanol, PD60 control, and PD60 ethanol) did not participate in behavioral despair tests in order to evaluate whether the behavioral tests had an effect on the genetic results. Rats that completed behavioral tests did so from either PD26 to PD30 or from PD56 to PD60. All rats were euthanized on the last day of the behavioral studies despite whether they participated in the behavioral tests or not.

Forced Swim Test

The forced swim test was performed on PD26 and PD56 depending on the group assignment. The forced swim test was used to assess behavior that could indicate depression. Animals were placed in a plastic tank (30cm diameter) filled with water (23-25 ºC) to a height of 50-60cm for 5 minutes. Animals were scored for climbing as defined by thrashing movements along the sides of the water tank measured in number of seconds. Any animal that was in danger of drowning would have been immediately removed from the water, however this did not occur.

Morris Water Maze Test

Morris water maze tests were performed over four days in order to assess possible spatial learning and memory deficits. A small pool (divided into four quadrants: north, south, east, west) with a platform was filled so the platform was just submerged. The water was made opaque by adding white tempura paint. Rats were given 60 seconds to find the platform. Rats were directed to the platform and left on it for 15 seconds if they did not find the platform in the alloted 60 seconds. They were allowed to sit on the platform for 15 seconds in order to observe, and hopefully remember, the visual cues on the walls. When placed in the pool, rats swam until they found the platform.

Rats were placed in the pool four times a day for four days (PD27-PD30 and PD57-PD60). The length of time it took the rat to find the platform was compared over successive trials to determine spatial learning. On the final trial of the Morris Water Maze, the platform was removed and a full 60 second trial for each rat was recorded to evaluate how long the rat spent in the quadrant where the platform was previously. All rats were euthanized by CO_2 exposure 30 minutes following the final Morris Water Maze test.

Gene Expression Analysis

Tissue Collection

All rats were euthanized by CO_2 exposure 30 minutes following the final Morris Water Maze test in order to allow for the transcription of c-Fos for possible future studies. The rats' brains were removed without profusion with parafermaldehyde in order to maintain the integrity of the RNA. The frontal cortex, entire hippocampus, and raphe nuclei were removed from each brain and immediately placed in separate microcentrifuge tubes containing 150µL of RNALater (QIAGEN). However, only the hippocampal tissue was used for RNA isolation, cDNA synthesis and quantitative RT-PCR analysis. Tissue samples were stored at 4 ºC until RNA was isolated (no longer than 90 days).

RNA isolation and cDNA synthesis

Due to time and resource constraints, only female hippocampal tissue was analyzed for gene expression. The results of the Morris Water Maze indicated that the female rats were slightly more affected, although not significantly so, by ethanol than the males. RNA was extracted from hippocampal tissue samples using the QIAGEN RNA-Easy kit. The tissue was removed from the RNAlater and placed in 600 µL of Buffer RLT. Tissue was homogenized by grinding and then by passing the solution through a 20 gauge needle 6-7 times. The solution was centrifuges for 3 minutes at 1300rpm and the supernatant was transferred to a clean microcentrifuge tube. 600µL of 70% ethanol was added to the lysate and mixed by gently pipetting. The sample was placed in an RNAeasy mini column and centrifuged for 15 seconds at 1300rpm. The flowthrough was discarded and 700µL of Buffer RW1 was added to the column and centrifuged again. This process was repeated twice with 500µL of Buffer RPE. The silica-gel membrane was then dried by centrifuging the tube for 2 minutes. The RNA was eluted by adding

50μL of RNase free water to the tube and centrifuging for 1 minute. The RNA that was eluted into the microcentrifuge tube was stored at -20°C.

First strand cDNA synthesis was performed from the isolated RNA using Oligo(dT) primers and Superscript II reverse transcriptase (Life Technologies). 10μL of the isolated RNA was combinted with 1μL of Oligo(dT) (500μg/mL) and 1μL of dNTP mix (10mM each). This solution was then heated to 65°C for 5 minutes, chilled on ice, and centrifuged for 15 seconds. 4μL of 5X First-Strand Buffer and 2μL of 0.1MM DDT were added to the centrifuged solution. These contents were then mixed and incubated at 42°C for 2 minutes. 1μL of SuperScript II RT was added and mixed by gently pippetting. This solution was then incubated at 42°C. After 50 minutes, the solution was heated to 70°C for 15 minutes. Isolated cDNA was stored at -20°C.

Quantitative real time PCR

Primer sequences related to this project can be found in Table 1

cDNA primers for Dnmt3a, Dnmt1, and *HTR2A* were designed using primer3 and acquired from invitrogen (Thermo). β-*actin* was used as the reference gene for this experiment to normalize expression levels between samples. Each primer was tested using a dilution series of cDNA. cDNA was diluted four times by a factor of 10 yielding samples with concentrations of 0.1, 0.01, 0.001, and 0.0001 μg/μL. Quantitative RT-PCR (qRT-PCR) analysis of each dilution series was run using iQ SYBR® Green master mix from BioRad in 96 well plates on an iCycler running Real Time Detection System Software, version 3.1 (Bio-Rad). Each reaction contained 10μL of iQ SYBR Green supermix, 8μL of water, 1μL of primer mix (10μM each), and 1μL of cDNA sample.

Two of the initially designed primers did not produce a sufficient reaction efficiency or standard curve (DNMT1, DNMT3a). Another primer for Dnmt1 was then designed and produced

a satisfactory efficiency (99.4%). An alternative Dnmt3a primer was previously ordered in the initial batch of primers, which was then tested and used for the final tissue analysis (E: 83.1%). After each primer showed a consistent efficiency over the course of several quantitative RT-PCR analyses, cDNA isolated from the hippocampal tissue was analyzed using the same quantitative RT-PCR reactions with ExiLENT SYBR® Green master mix from Exiqon as mentioned above. Initial qRT-PCR analysis revealed extremely high expression of *HTR2A*. Because of this, and a concern regarding the limited cDNA that was isolated, cDNA samples were diluted 10 fold and used for analysis. Dilutions were made to avoid isolating more cDNA and possibly creating another variable among cDNA homogeny within individual samples.

Quantitative real time PCR Results Analysis

Gene expression was analyzed using the Real Time Detection System Software, version 3.1 (Bio-Rad). The log of the concentrations of the standard genomic DNA was graphed as a function of threshold cycle (the cycle at which fluorescent intensity increases above background fluorescence). The levels of. β-*actin* expression in the different cDNA samples were used to normalize the concentration of *HTR2A,* Dnmt1, and Dnmt3a.

RESULTS

Behavioral Analysis of Prenatal Exposure to Ethanol

Birth Weight

Average individual birth weights were calculated by dividing the total litter weight by the number of pups in the litter. An independent samples t-test was used to analyze birth weights. Prenatal ethanol exposure significantly increased the birth weights of the rat pups compared to the control rat pup birth weights ($p < 0.001$) (Figure 2). The number of pups in each litter was not significantly different between control pups and ethanol-exposed pups.

Depression

The forced swim test is an established test for quantifying a possible feeling of hopelessness in the rats, which is a measurement of depression. Feeling of hopelessness was correlated with increased immobility times. In utero exposure to ethanol did not cause any significant differences in the times that rats spent exhibiting clawing behavior during the forced swim test. However, six out of the eight postnatal day (PD) 30 ethanol exposed rats had longer immobility times than their control counterparts (Figure 3). PD60 rats did not show this same trend, all of the control and ethanol rats from both genders had very similar immobility times.

Spatial Learning and Memory

The Morris Water Maze was used to evaluate the effect of prenatal ethanol on the rats' spatial learning and memory abilities. The latency time is the time in seconds that the rat took to find the platform. Independent sample t-tests were used to analyze the results from the Morris Water Maze. Trends in the data were observed but no statistically significant differences were detected. Age did not significantly change the times of rats prenatally exposed to ethanol. PD30 ethanol

rats did not find the platform any faster or slower than PD60 ethanol rats (Figure 4). The same lack of a difference was observed in the PD30 and PD60 control rats (Figure 4)

PD30 control males and females were not significantly different from one another but they have more similar latency time than PD30 ethanol males and females did (Figure 5). As before, due to the insignificant differences of gender on latency times in PD30 rats, data from both males and females was plotted together to compare the effects of ethanol. In PD30 rats, prenatal ethanol treatment did not significantly affect latency times when compared to the control treatment group's latency times (Figure 6).

The mean latency times of PD60 males and females under control and ethanol conditions were also calculated. The difference between control males and control females at PD60 was almost statistically significant ($p = 0.058$). In this case, the PD60 control females started with higher latency times that declined more rapidly over the 16 trials compared to the males that started with lower times that declined less rapidly. Ultimately, the males ended with shorter latency times in trial 16 (Figure 7). This trend was slightly less dramatic in the PD60 ethanol group of males and females but the results were inconclusive ($p > 0.05$). PD60 ethanol males followed a steadier decline of latency times compared to the PD60 ethanol females, which tended to have sharp changes in mean latency times from trial to trial. Both males and females had very similar latency times for trial 16 (Figure 7). Due to the statistically insignificant differences of gender on latency times in PD60 rats, data from both males and females was plotted together to compare the effects of ethanol. The latency times of both males and females combined were not significantly affected by prenatal ethanol treatment.

The latency times from each individual day for the ethanol and control PD60 rats were analyzed using an independent sample t-test. Day 3 was the only day that came close to having a

significant difference between rats exposed to ethanol and control rats ($p = 0.070$), however the latency times were still not significantly different. This analysis process was repeated for PD30 rats. None of the days represented a significant difference between ethanol rats' and control rats' latency times ($0.787 > p > 0.920$).

Effects of Prenatal Ethanol Exposure on Gene Expression

Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR) was used to quantify the expression of *HTR2A,* Dnmt1, and Dnmt3a in RNA isolated from female rat hippocampal tissue. The efficiencies determined for primers for Dnmt1, Dnmt3a, and *HTR2A* were 99.4%, 83,1%, and 97.9%. β-*actin* was used as a reference gene to normalize expression levels. Four litters of rats in total were used for this study. Two of them were raised until postnatal day (PD) 30 and two litters were raised until PD60. One litter from each age group performed behavioral testing in the four days leading up to ethanasia. Half of all rats were withheld from behavioral testing in order to analyze the effects that the behavioral testing could have on the expression of our target genes. Tissue from both non-behavioral and behavioral rats was obtained at postnatal day (PD) 30 and PD60.

Prenatal ethanol exposure caused an 88-fold upregulation of *HTR2A,* from 0.042 to 3.681 normalized expression levels, ($p<0.001$) in PD30 non-behavioral ethanol exposed rats compared to PD30 non-behavioral saline controls (Figure 10). Multiple, but not all, interactions of rat age (PD30 and PD60) and treatment (ethanol vs control) showed significant differences in relative normalized expression of *HTR2A.* The gene study of rats euthanized on PD60 reveals that rats prenatally treated with ethanol have a lower expression of *HTR2A* than PD60 control rats, however this result is not statistically significant (Figure 10). PD60 rats that received prenatal

saline had a significant 17-fold upregulation of expression of *HTR2A* compared to PD30 control rats, from 0.042 to 0.731 normalized expression levels ($p<0.001$) (Figure 10). Prenatal ethanol exposure affected this age-related expression pattern. Rats prenatally treated with ethanol and allowed to age to 60 days old showed a significant decrease, from 3.681 to 0.707 normalized expression levels, ($p<0.001$) in *HTR2A* expression compared to ethanol treated rats raised to only 30 days (Figure 10). PD30 ethanol rats showed a 5-fold upregulation of *HTR2A*, from 0.707 to 3.681 normalized expression levels, compared to PD60 ethanol rats. Exposure to behavioral testing decreased the normalized expression of *HTR2A* in PD30 rats prenatally exposed to ethanol compared to the PD30 ethanol rats that did not experience behavioral testing, from 3.681 to 0.187 normalized expression levels (Figure 10). Conversely, behavioral testing on the PD60 ethanol group significantly increased *HTR2A* expression by 2 fold, from 0.707 to 2.018 normalized expression levels. Behavioral testing also significantly increased *HTR2A* expression in PD60 control rats by 5.5-fold, from 0.731 to 4.047 normalized expression levels. Behavioral testing did not cause a significant affect on *HTR2A* expression in PD30 control rats (Figure 10).

Dnmt3a was significantly upregulated 53 fold, from 0.126 to 6.693 normalized expression levels ($p<0.0001$) in the hippocampal tissue of non-behavioral 30 day old rats that were prenatally exposed to ethanol during the first trimester of pregnancy compared to the non-behavioral 30 day old rats exposed to saline (Figure 11). Dnmt3a expression was significantly downregulated in non-behavioral PD60 rats prenatally exposed to ethanol compared to non-behavioral PD60 control rats, from 0.531 to 1.942 normalized expression levels. Dnmt3a expression in non-behavioral PD60 control rats was significantly upregulated 15-fold compared to non-behavioral PD30 control rats, from 0.126 to 1.942 normalized expression levels ($p<0.0001$). Dnmt3a expression of PD60 ethanol rats was significantly downregulated compared

to PD30 ethanol rats, from 6.693 to 0.531 normalized expression levels (Figure 11). Behavioral protocol had no affect on Dnmt3a expression in the PD30 control rats. However, PD60 control rats that participated in behavioral studies had a significantly 3.6-fold upregulation of Dnmt3a expression, from 1.942 to 6.984 normalized expression levels, compared to PD60 control rats that did not undergo behavioral testing (Figure 11). The behavioral protocol significantly downregulated Dnmt3a expression in PD30 rats prenatally exposed to ethanol compared to PD30 rats that were prenatally exposed to ethanol but did not participate in behavioral studies, from 6.693 to 0.346 normalized expression levels. Conversely, the behavioral protocol caused a 13-fold upregulation of Dnmt3a expression, from 0.531 to 6.985 normalized expression levels, in PD60 rats prenatally exposed to alcohol compared to PD60 ethanol rats.

Dnmt1 expression was significantly upregulated by 17.6 fold, from 0.007 to 0.120 normalized expression levels, in non-behavioral PD30 ethanol rats compared to non-behavioral PD30 control rats (Figure 12). This significant upregulation was not observed in PD60 ethanol rats compared to PD60 control rats due to large error bars. Dnmt1 expression in PD60 control rats was significantly upregulated by 4.5-fold, from 0.007 to 0.031 normalized expression levels, compared to PD30 control rats (Figure 12). This significant upregulation was not observed in PD60 ethanol rats compared to PD30 ethanol rats due to large error bars. The behavioral protocol significantly downregulated Dnmt1 expression in PD30 ethanol rats, from 0.011 to 0.120 normalized expression levels, and upregulated expression in PD30 control rats by 2 fold, from 0.007 to 0.014 normalized expression levels. Participation in the behavioral protocol did not significantly affect Dnmt1 expression in PD60 ethanol rats or PD60 control rats (Figure 12).

DISCUSSION

This study examined the effects of a realistic model of binge drinking during the first trimester of pregnancy on the expression of genes related to FASD and their possible associated behavioral markers. Changes in *HTR2A,* Dnmt1, and Dnmt3a expression have been linked to the molecular impact of prenatal ethanol exposure. Although the behavioral data was not significant, the results shed light on trends associated with ethanol exposure and possible sex-related effects. Pre-existing sex-differences for spatial learning and memory and risk for depression could make prenatal alcohol exposure especially harmful to some individuals. Prenatal alcohol exposure generally upregulated gene expression earlier in life compared to the downregulation that occurred in the older rats. Participation in behavioral testing significantly impacted the gene expression of all target genes in rats prenatally exposed to alcohol.

In order to gain a more comprehensive understanding of the effects of first trimester prenatal alcohol exposure, it is important to understand how alcohol influences DNA methylation. This study is important to the field of fetal alcohol exposure related epigenetics because a ratio of alcohol to body mass realistic to an adult female was administered to pregnant rats during a time when women may not know they are pregnant. Changes in DNA methyltransferase expression may predict if this pattern of prenatal ethanol exposure leads to hypermethylation.

I investigated the expression of Dnmt1, Dnmt3a, and *HTR2A* in response to prenatal ethanol or prenatal saline exposure. These molecular markers have been associated with the psychopathology and cognitive deficits of FASD. Both behavioral and gene expression tests were run in order to elucidate these effects. The forced swim test was used to observe the effect of prenatal alcohol exposure on levels of hopelessness, measured by the time that the rats spent

actively trying to escape subtracted from the total time in the tank of water. Theoretically, signs of increased hopelessness in the rats that prenatally received ethanol could be connected to hippocampal hypermethylation, which would cause an upregulation of DNA methyltransferases and the subsequent down-regulation of *HTR2A.* Prenatal alcohol exposure has been associated with these broad hypermethylation patterns It is important to note the possible evolutionary benefits that offspring can receive from epigenetic modification *in utero*. The fetal origins of adult disease hypothesis states that common adult diseases might originate during fetal development. This hypothesis is based on the concept of fetal programming, which was originally developed from a large pool of data that showed low birth weights were associated with increased risk for various diseases such as hypertension (Hellemans et al., 2010). Evolutionarily, the function of fetal programming could be adaptive, preparing offspring to experience an environment after birth that is similar to the one experienced during gestation. However, when a mismatch between prenatal and postnatal environments occurs outcomes such as depression may occur. The level of specificity in which DNA methyltransferases regulate fetal brain development under homeostatic conditions lacking environmental stress opens the doors for prenatal ethanol exposure to cause hypermethylation and cognitive deficits. Prenatal ethanol exposure has been shown to upregulate Dnmt1 and Dnmt3a in the hippocampus (Morris, 2014). Spatial learning and memory deficits in rats exposed to ethanol in the womb would be evident in the Morris Water Maze and in the levels of Dnmt3a and Dnmt1 expression. Animal models of FASD have found that rats that experienced ethanol during fetal development took longer to navigate the Morris Water Maze task (Berman & Hannigan, 2000). In order for the memory formation to occur that allows the rats to navigate the maze, precise and temporally coordinated changes in transcription factor activation and inactivation, gene expression, and

bidirectional changes in the expression and activity of chromatin and DNA modifying enzymes must take place (Morris & Monteggia, 2014). The precision necessary for spatial memory formation allows for enormous destruction by environmental stressors including ethanol-induced DNA methylation.

Birth Weight

This study found that prenatal ethanol exposure significantly increased the birth weight of pup compared to the control pups. The number of pups per litter was not significantly different between the treatment groups, however the two control rats gave birth to 17 pups and 13 pups while the two ethanol rats gave birth to 13 pups and 11 pups. Even though this difference in litter size was not significant, fewer pups in the ethanol-exposed litters could have allowed the pups to grow larger in utero compared to the control pups. There is no literature that supports an increased birth weight with prenatal ethanol exposure.

Depression

Although the forced swim test did not produce any statistically significant results, six out of the eight PD30 ethanol rats had higher immobility times than their PD30 control counterparts. This trend suggests that the younger rats that received prenatal ethanol may have a behavioral marker for depression. Animal models of depression have shown that treatment using selective serotonin reuptake inhibiting anti-depressants decreases immobility time in the forced swim test, therefore decreasing the possible "depression" that the rodents are experiencing (Castagne et al., 2010; Mague et al., 2003). While not statistically significant, these results agree with previous studies that have found an association between fetal alcohol spectrum disorder and depression (Famy et

al., 1998; Streissguth et al., 1996). One study revealed that 94% of participants with FASD experienced mental health problems. Of these participants, 23% of adults had attempted suicide, and 43% had threatened to commit suicide (Kellerman, 2003).

The forced swim test is a highly debated method of measuring depression and hopelessness in rodents. Arguments against the behavioral test note that there is no concrete method of knowing whether or not the rats are experiencing depression or hopelessness. Many protocols also exist for the forced swim test, all with different positive and negative aspects. The results of the forced swim test performed in this study may not have been statistically significant due to human error. In this study, six out of the eight PD30 ethanol rats showed increased immobility times compared to their control counterparts.

Spatial Learning and Memory

The Morris Water Maze is a test for spatial learning and memory ability in rodents. Deficits can be detected by analyzing how long a rat takes to find the platform that is hidden directly under the surface of the water and how these times change over the course of 16 trials. Prenatal alcohol exposure in rats has been shown to increase latency times in the Morris Water Maze Test (Westergren, 1996). The hippocampus is believed to be at the heart of the complicated process of acquiring and storing spatial memory. Therefore, any defects in the hippocampus could affect the processing and storing of spatial memories. Prenatal ethanol exposure has a known negative impact on hippocampal functions, including spatial memory (Scott-Goodwin et al., 2016). It is appropriate to perform the Morris Water Maze test on rats that have experienced prenatal alcohol exposure because spatial memory deficits are common cognitive alterations present in FASD

(Scott-Goodwin et al., 2016). Slight trends were observed and described in the results section, however no dramatic changes in latency times occurred.

A larger sample size may have lead to significant data in the Morris Water Maze. The water maze has even more room for slight differences between rats from rat handling, placement in the pool, latency timing, the time the rat is allowed to stay on the platform before being taken out of the pool, to even how long that each rat was dried off. The results of the water maze could be compromised if the rats were handled even slightly different from each other at such a crucial developmental age. More experience or larger sample size can overcome these issues.

Upregulation of Dnmt3a and Dnmt1 Suggests Alcohol-Induced Epigenetic Modifications

Many environmental factors, including alcohol, are known to alter the intrauterine environment in such a way that contributes to abnormalities in fetal development. The relationship between fetal alcohol exposure and FASD has been a useful model in studying the epigenetic pathway between environment and phenotype. Several studies have found altered DNA methylation in multiple models of FASD (Resendiz et al., 2013). Some researchers believe that alcohol-induced epigenetic alteration has achieved a dysregulation of *S*-adenosylmentionine (SAM), an active methyl donor that is recruited by DNA methyltransferases to transfer a methyl group to the cytosine. Homeostatic DNA methylation includes hyper- and hypomethylation in the embryonic genome during neurulation and in neural stem cell differentiation. Alcohol's affect on DNA methylation can be locus specific and bidirectional so any significant change in Dnmt3a expression suggests epigenetic alterations of the genome. Downregulation of Dnmt3a has been associated with anti-depressant drugs, suggesting that lowing levels of Dnmt3a would be beneficial when treating depression (Melas et al., 2012)

Dnmt3a and Dnmt1 were significantly upregulated in rats prenatally treated with ethanol during the first trimester. Dnmt3a could be upregulated through a regulatory transcription factor. Dnmt3a may then methylate other genes to decrease their expression.

Downregulation of HTR2A in Response to Prenatal Alcohol Exposure

The relationship between anti-depressant drug efficacy, depression, and *HTR2A* polymorphisms is well known across many studies. However, only few connections have been made between how *HTR2A* expression affects the likeliness of developing depressive disorders. It has been suggested that *HTR2A* is downregulation in certain areas of the brain may contribute to depression but more research needs to be done to validate this limited research.

Prenatal ethanol treatment significantly increases *HTR2A* expression early in life compared to the control. This increased expression balances out by PD60, where there is no significant difference between *HTR2A* expression in ethanol rats compared to control rats. *HTR2A* expression in the ethanol treated rats was significantly downregulated from 30 days old to 60 days old. Conversely, *HTR2A* expression was upregulated from PD30 to PD60 in the control rats. Ethanol appears to have reversed this natural age-related upregulation of *HTR2A* that is observed in the control rats. These results indicate that prenatal ethanol exposure may initially cause an increase in *HTR2A* expression, but as aging occurs prenatal ethanol exposure ultimately decreases the normal regulation of *HTR2A.* An increase in DNA methylation after PD30 in response to prenatal alcohol exposure may have caused the ultimate down-regulation of *HTR2A.* Epigenetic downregulation of *HTR2A* has been associated with schizophrenia and bipolar disorder (Abdolmaleky et al., 2011). *HTR2A* has also been associated with placental mitogenesis and DNA methylation of placental *HTR2A* may alter fetal serotonin signaling (Paquette, 2014). The hypermethylation of *HTR2A* caused by prenatal ethanol exposure may

have caused psychological effects in the rats that were not properly tested with the forced swim test.

Behavioral Tests Down-regulated HTR2A

In healthy individuals, stress may induce expression of *HTR2A* as an adaptive function (Cheah et al., 2017). However, in ethanol treated rats with already hypermethylated *HTR2A*, the stress of the behavioral testing caused counterintuitive *HTR2A* expression. The behavioral testing down-regulated *HTR2A* in the rats prenatally exposed to ethanol but had no effect on *HTR2A* regulation in the control rats. The behavioral tests did not affect *HTR2A* expression in control rats. Ethanol magnified the effects of the stress past the control genetic response to stress to the point where behavioral testing caused a significant decrease in *HTR2A* expression. This result suggests that the alcohol genetically magnified the stress of behavioral testing.

Conclusion and Future Research

The importance of educating women on the effects of alcohol consumption during the first trimester of pregnancy is invaluable. It is important to explore alcohol's effects during the first trimester because this is when women may not know that they are pregnant. In order for that communication to begin, researchers must know exactly how prenatal alcohol exposure affects a developing embryo during the first trimester. Past studies have either focused on alcohol's effects throughout the whole pregnancy, or on specific snapshots of alcohol exposure. These studies have found that alcohol can have significant epigenetic effects on a developing fetus, which can lead to the phenotypes associated with FASD. The exact molecular mechanisms of this epigenetic alteration are still largely unknown.

First and foremost, the male rat hippocampal tissue and the rest of the female hippocampal tissue should be genetically analyzed to determine if the genetic trends observed in this study are consistent with the rest of the rats. Replicating results is always important in any study. Although three runs of each genetic expression qRT-PCR analysis were run per gene, more replicates of the data would hopefully lead to smaller error bars.

Epigenetics is a rapidly growing field that has the ability to explore nearly any environmental impact dozens of aspects of genetic expression and methylation. Examining the epigenetic effects of ethanol is a specific step towards a more concentrated problem. The epigenetics of prenatal ethanol exposure during the first trimester further specifies the problem, however there are numerous aspects to this process that are still unknown or may be known but not completely understood.

Future studies of first trimester specific ethanol exposure should focus on the role that brain-derived neutrophic factors (BDNFs) play in the psychological and cognitive abnormalities

caused by FAS. The original plan of this study was to study BDNF expression; however adding BDNF expression to the genes already being tested was not realistic with the time restrictions. BDNFs are already connected to spatial learning and memory and depression; therefore it would be interesting to study the effects of a realistic binge drinking behavior during the first trimester of pregnancy on BDNFs and fetal development. Decreased BDNF protein levels have been associated with depression in mice prenatally exposed to alcohol (Caldwell et al., 2008).

Ten-eleven translocation (Tet) proteins are interesting candidates as molecular agents of demethylation. Tet1, Tet2, and Tet3 are known to be the mediators of 5mC demethylation in plants. In mammalian tissue, they show a strong preference for CpG-rich motifs. Tet1 knockouts have impaired spatial memory formation and abnormally enhanced long-term depression triggered in the hippocampus (Morris & Monteggia, 2014). Tet proteins could represent yet another pathway that prenatal ethanol exposure disturbs.

Bisulfite sequencing would be the next step in examining the epigenetic mechanisms and methylation patterns of genes associated with FASD. Bisulfite sequencing is the bisulfite treatment of DNA to determine the pattern of methylation. This treatment converts cytosine residues to uracil and leaves the 5-methylcytosine residues unaffected. DNA that has been treated with bisulfite retains only methylated cytosines.

The neurodegenerative apoptotic properties of ethanol have been studied but the exact mechanisms are largely unknown. Ikonomidou et al (2000) proposed that ethanol works as a blockade of *N*-methyl-D-aspartate (NMDA) glutamate receptors and excessively activates $GABA_A$ receptors. This dual mechanism triggers widespread apoptotic neurodegeneration in the developing rat forebrain. The neurodegeneration that prenatal ethanol exposure causes is particularly damaging to hippocampal neurons. Hippocampal neurodegeneration could possibly

be the cause of spatial learning and memory deficits in children with FASD experience. Ikonomidou et al. (2000) believe that ethanol's effects on the hippocampus are most potent during the period of synaptogenesis. This stage of development occurs during the last trimester of pregnancy in humans to several years after birth. Due to this timeframe of particular vulnerability, Ikonomidou et al. (2000) designed their experiment to only target the third trimester equivalent of pregnancy in rats. While administering alcohol during the third trimester led them to support ethanol's dual mechanism, it is not a realistic representation of human females' consumption of alcohol during pregnancy. Ikonomidou et al. (2000) concluded that exposure of the developing rat brain to ethanol during synaptogenesis induces an apoptotic neurodegenerative reaction that deletes large numbers of neurons from several crucial brain regions, including the hippocampus. They concluded that ethanol's proapoptotic effects come from the blocking action at NMDA glutamate receptors and its positive modulatory action at $GABA_A$ receptors. Although Ikonomidou et al. (2000) used a concentration of ethanol with the rats that is extremely unrealistic for women, their results shed light onto the poorly studied apoptotic mechanisms of prenatal ethanol exposure.

This study makes a significant contribution to understanding the epigenetic impacts that a realistically high amount of binge drinking in an adult woman has on a fetus. A large portion of the studies analyzing the effects of prenatal alcohol exposure on developing fetuses use severely unrealistically high levels of alcohol for an adult woman, or for any person. This study did not tailor the alcohol dosage to the desired genetic result. Instead, it used a realistic binge drinking dosage and protocol and showed a significant upregulation of *HTR2A* accompanied by an upregulation of Dnmt3a and Dnmt1.

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TABLES AND FIGURES

Table 1: Sequences of the primers used in this study

Gene	Sequence
HTR2AL	TCATCATGGCAGTGTCCCTA
HTR2AR	ACAGGCATGACAAGGAAACC
Dnmt3aL	ACCAGGCCACCTACAACAAG
Dnmt3aR	AGCCTTGCCAGTGTCACTTT
DNMT1L	CCAGATACCTACCGGTTATTCG
DNMT1R	TCCTTTAACTGCAGCTGAGGC
DNMT3aL	CTGAAATGGAAAGGGTGTTTGGC
DNMT3aR	CCATGTCCCTTACACACAAGC
b-actinL	CGAGTACAACCTTCTTGCAGC
b-actinR	ACCCATACCCACCATCACAC
Dnmt1L	GCGCTCATTGGCTTTTCTAC
Dnmt1R	CTCGACCACAATCTTGCTGA

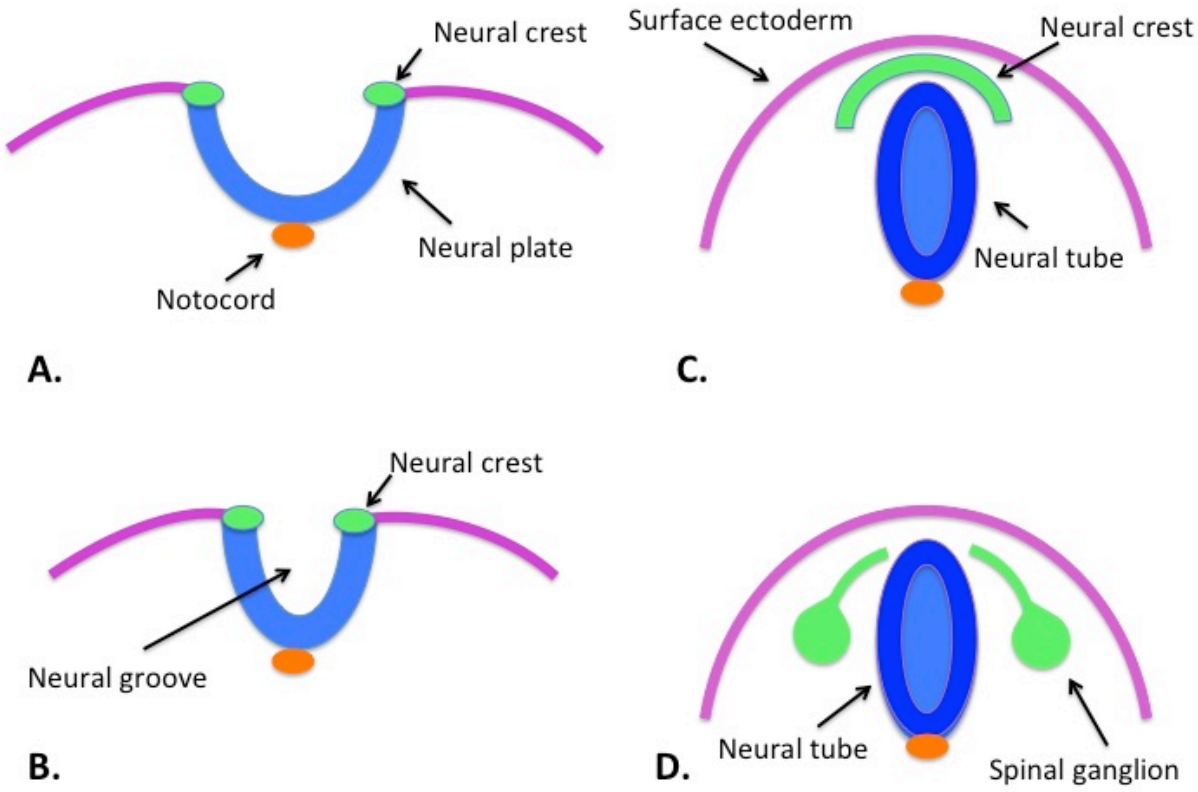


Figure 1: Neural Tube Development. A) Neuroectodermal tissues thicken into the neural plate after differentiation from the ectoderm. The neural crest is created from the neural plate borders as the neural plate bends dorsally. **B)** The neural plate bends dorsally and creates the neural groove. The two ends of the neural plate eventually join at the neural crests. **C)** The neural crest is disconnected from the epidermis after the closure of the neural tube. Neural crest cells differentiate to form the majority of the peripheral nervous system. **D)** The notochord begins to degenerate and exists as the nucleus pulposus. The spinal ganglion form from the neural crest.

Illustration design adapted from Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013.

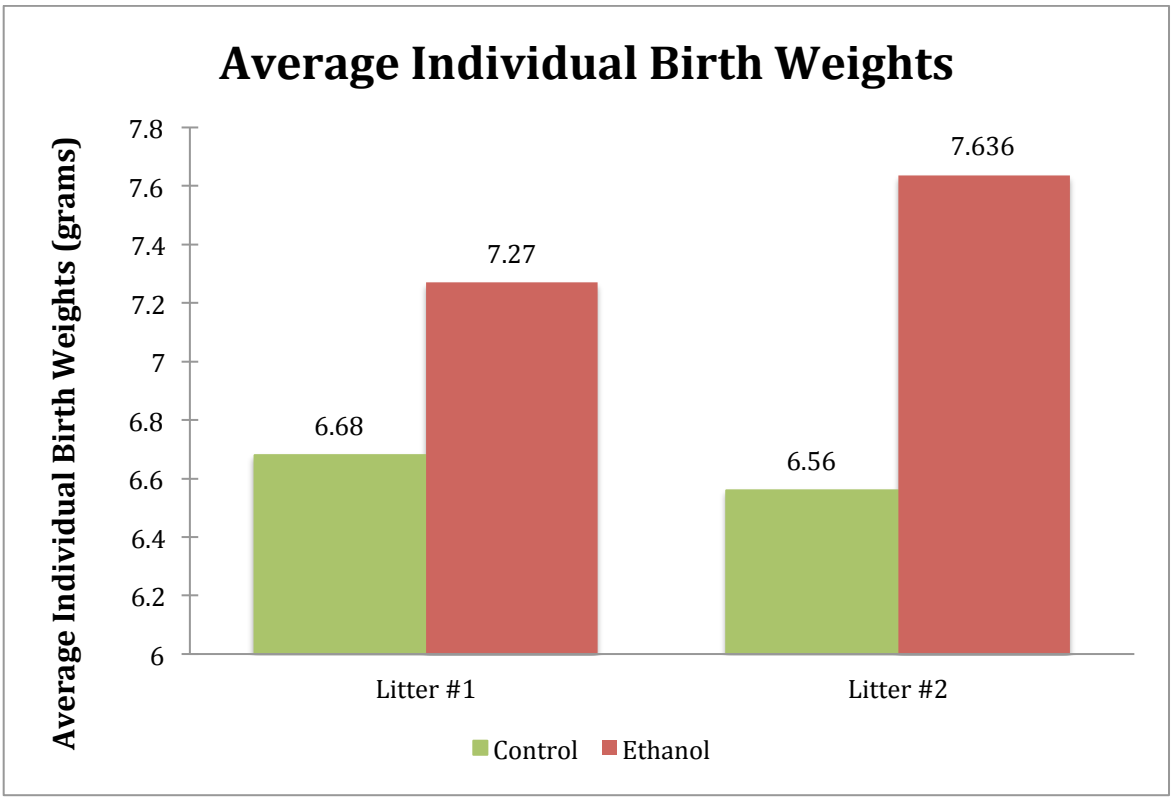


Figure 2: Birth Weights of Control and Ethanol Exposed Pups. This graph shows that for both litter 1 and litter 2 the pups prenatally exposed to ethanol on average weighed significantly more than the pups prenatally exposed to saline.

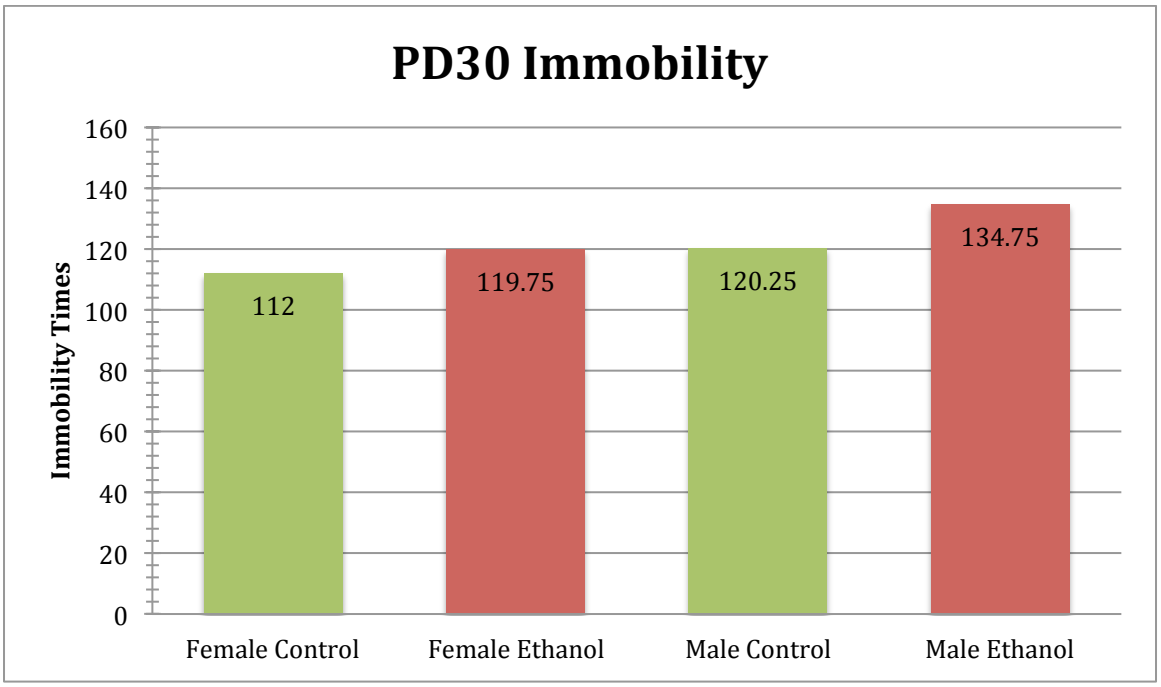
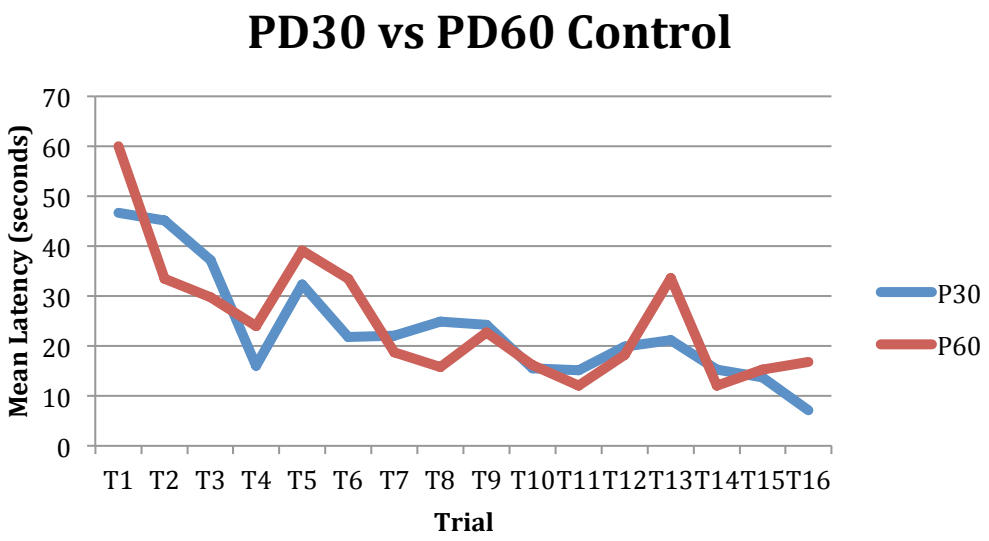


Figure 3: Immobility Time Results from Forced Swim Test. The graph displays the immobility times of PD30 ethanol and control rats. Six out of the eight PD30 ethanol rats had higher immobility times than their control counterparts however the difference in times was not significant. The numbers in each bar are the average immobility times of each treatment group.

A.



B.

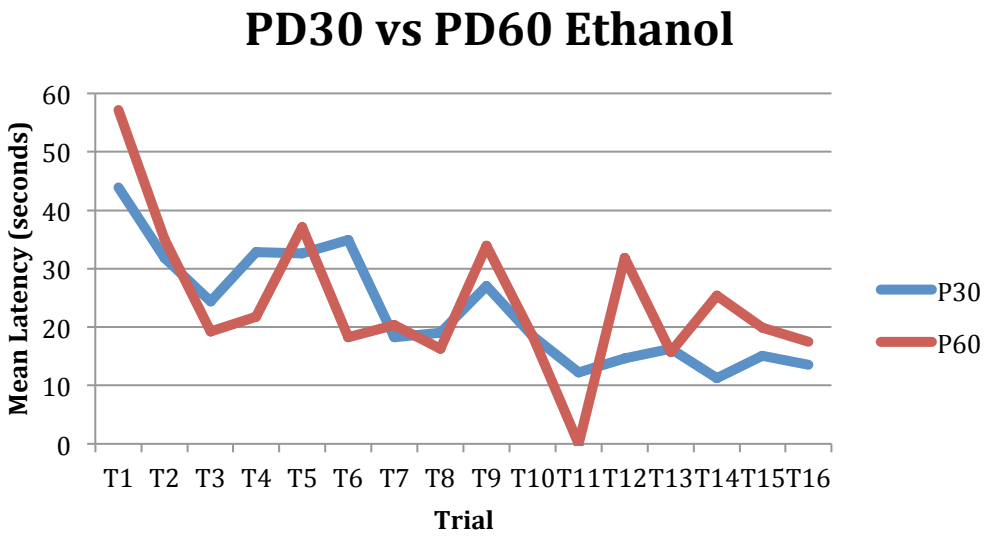
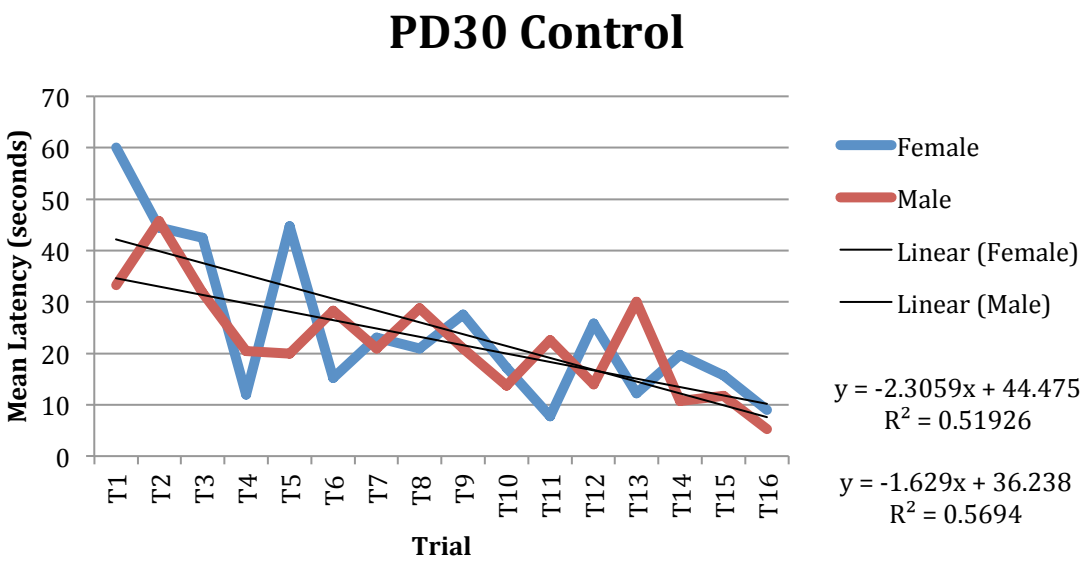


Figure 4: Change in time from latency times of PD30 to PD60 rats. These plots show the change in latency times with age of control (A) and ethanol (B) rats. No significant differences were seen.

A.



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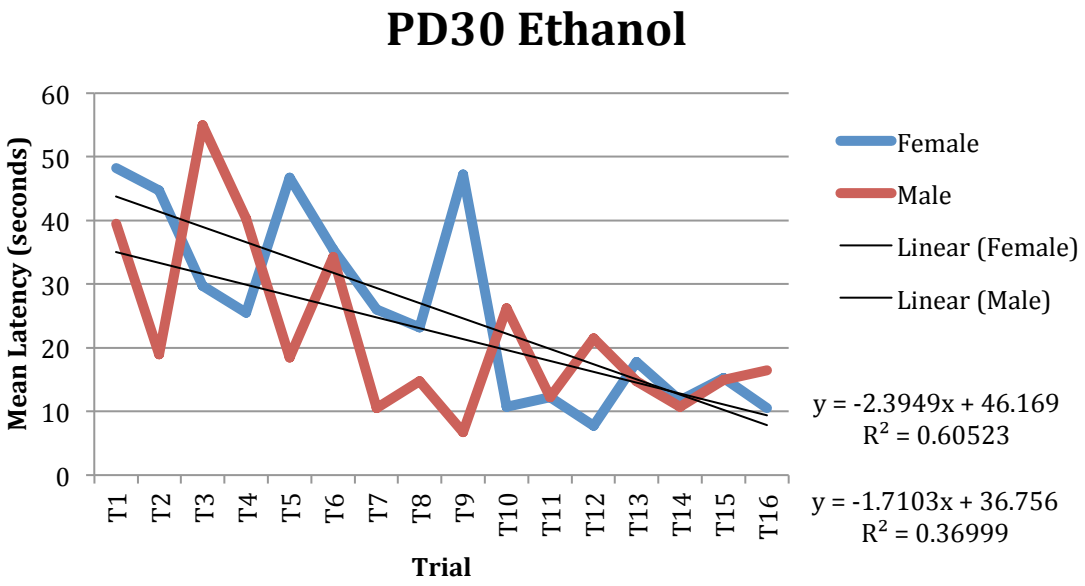


Figure 5: Latency Time Results for PD30 rats from Morris Water Maze. The plot displays the latency time trends from trial 1 to trial 2 in PD30 control (A) and ethanol (B) rats separated by gender. No significance was found but trends can be seen.

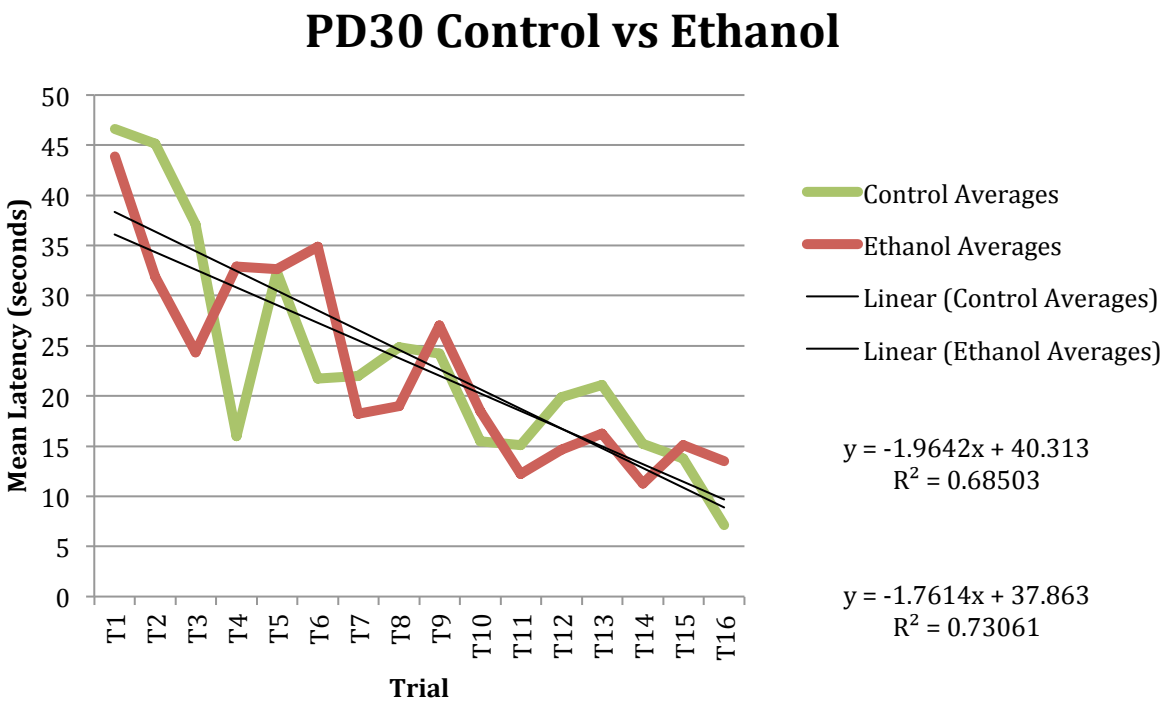
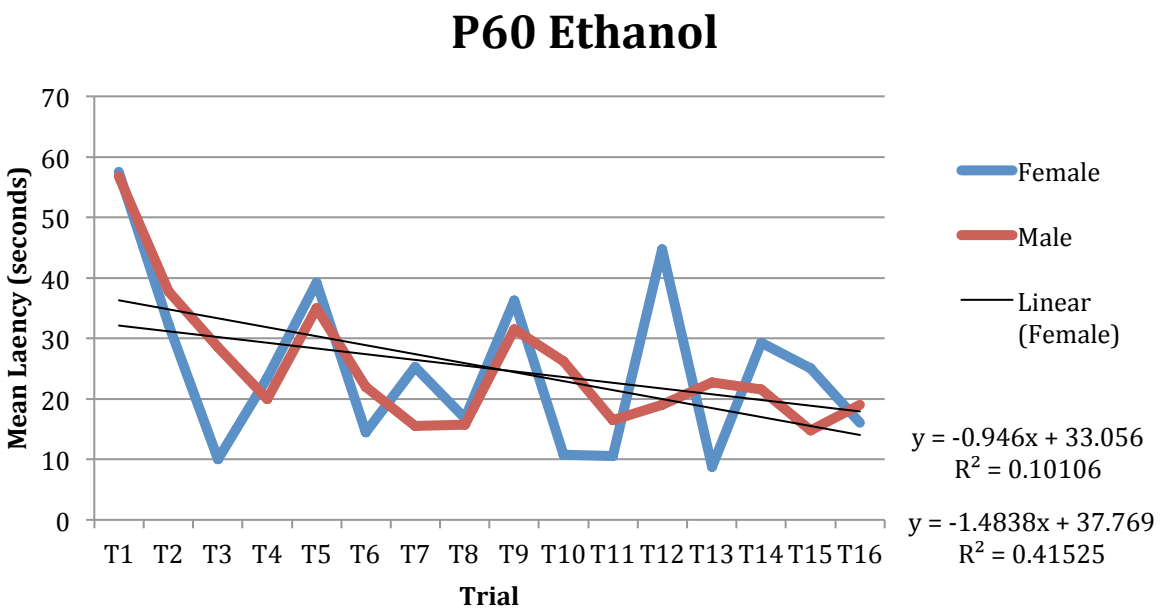


Figure 6: Latency Time Results for PD30 rats from Morris Water Maze. This graph shows the latency times of PD30 ethanol rats plotted with the PD30 control rats. No significant differences were seen.

A.



B.

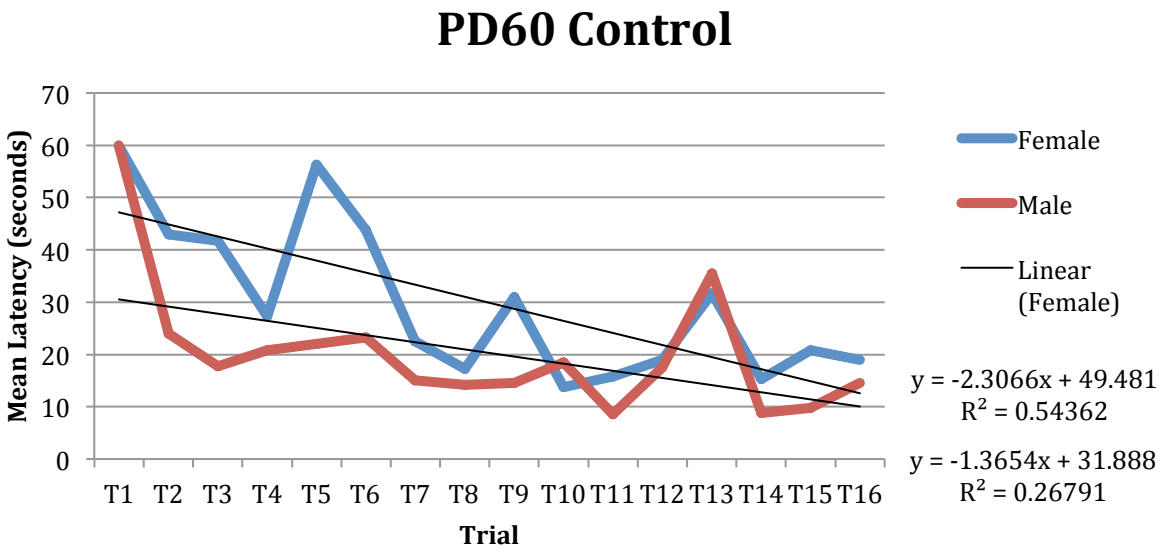


Figure 7: Latency Times from Morris Water Maze test of PD60 rats. This graph shows PD60 ethanol (A) and PD60 control (B) rats separated by gender. No significant differences were seen.

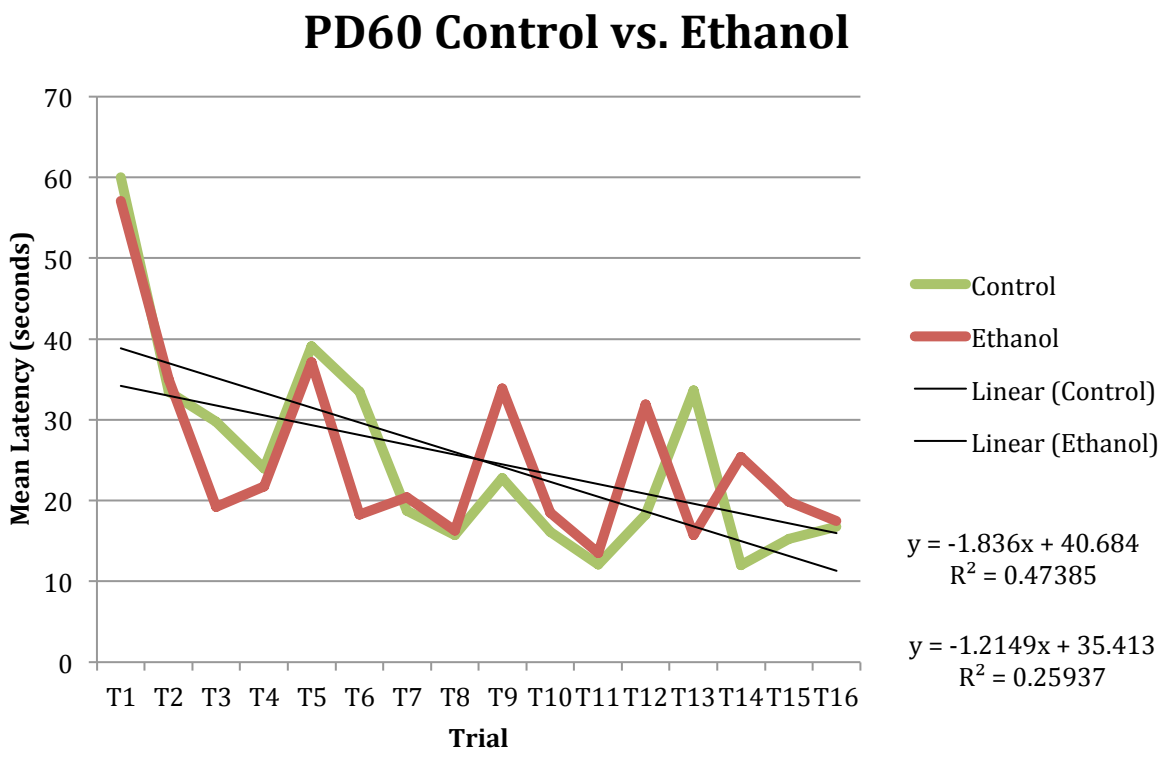
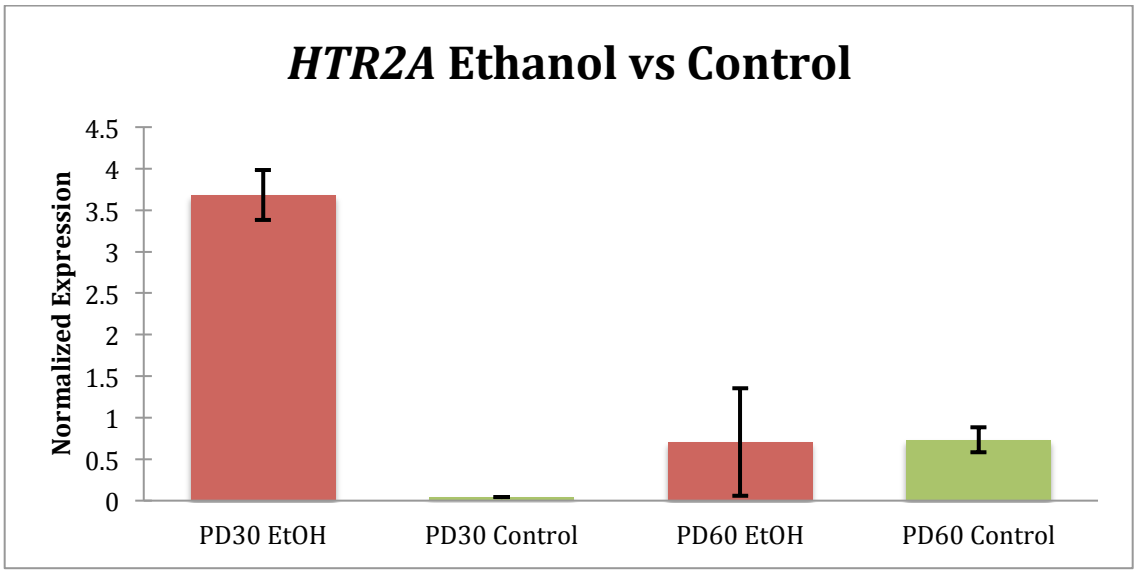


Figure 8: Latency Times from Morris Water Maze Test of PD60 ethanol and control rats. This plot shows PD60 control vs ethanol rats. Ethanol slightly decreased the slope of learning. No significant differences were seen.

A.



B.

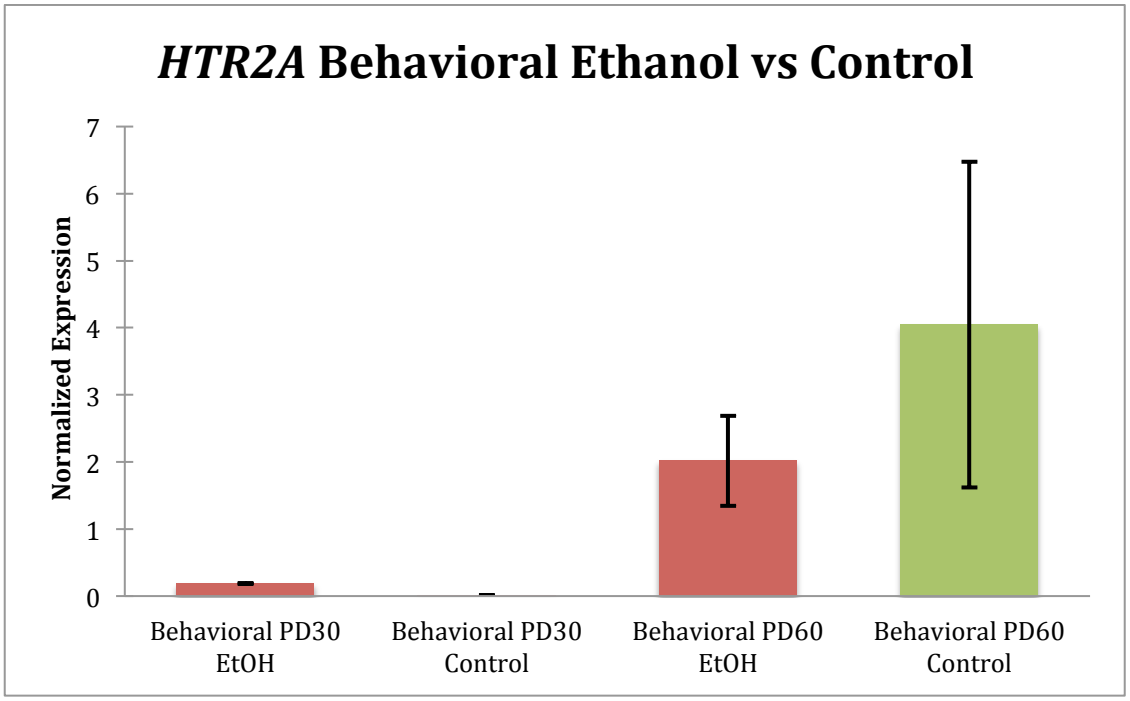
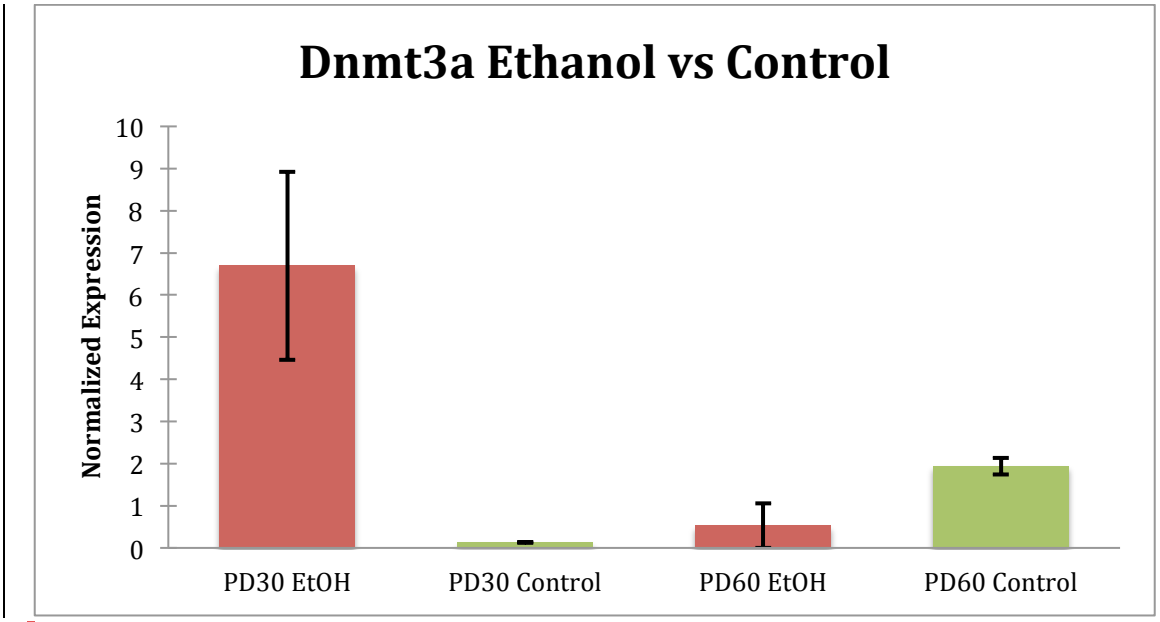


Figure 10: Quantitative RT-PCR data showing *HTR2A* Normalized Expression in Hippocampal tissue. cDNA was isolated from female hippocampal tissue of individuals that did not participate in behavioral testing (A) and those that did (B). Expression levels were calculated with normalizing to β-actin.

A.



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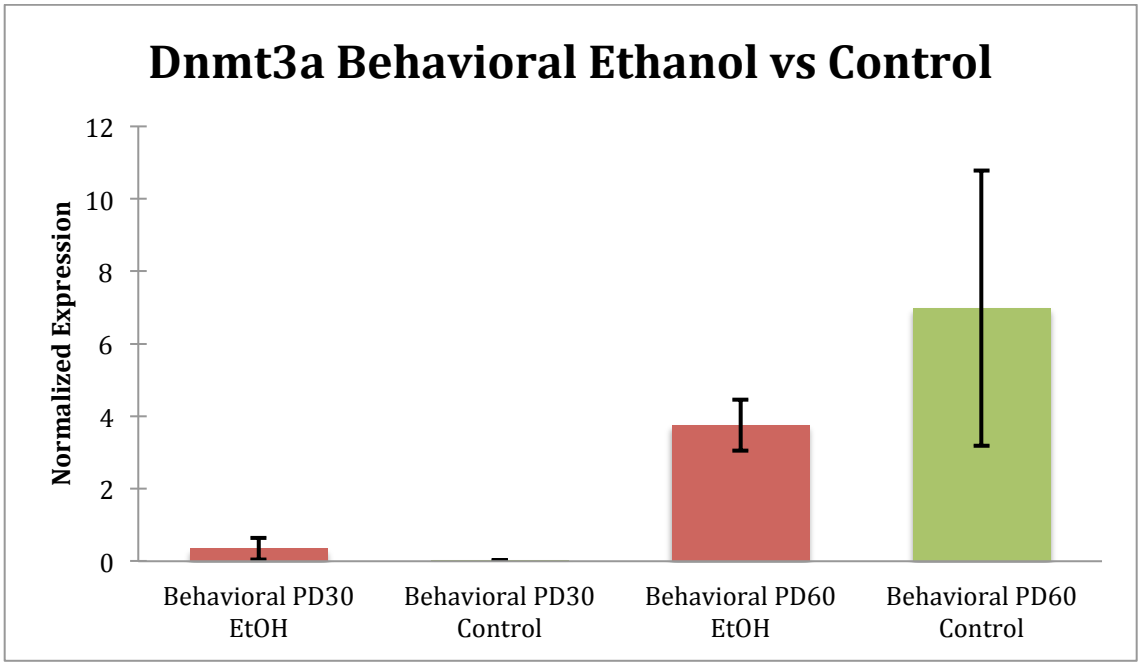
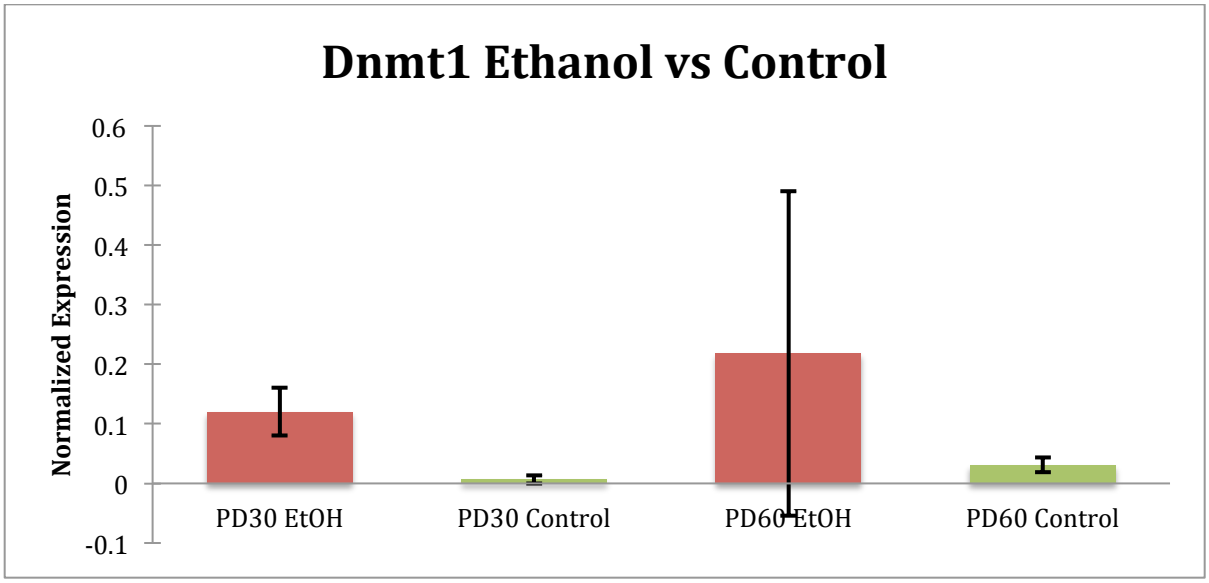


Figure 11: Quantitative RT-PCR data showing Dnmt3a Normalized Expression in Hippocampal tissue. cDNA was isolated from female hippocampal tissue of individuals that did not participate in behavioral testing (A) and those that did (B). Expression levels were calculated with normalizing to β-actin.

A.



B.

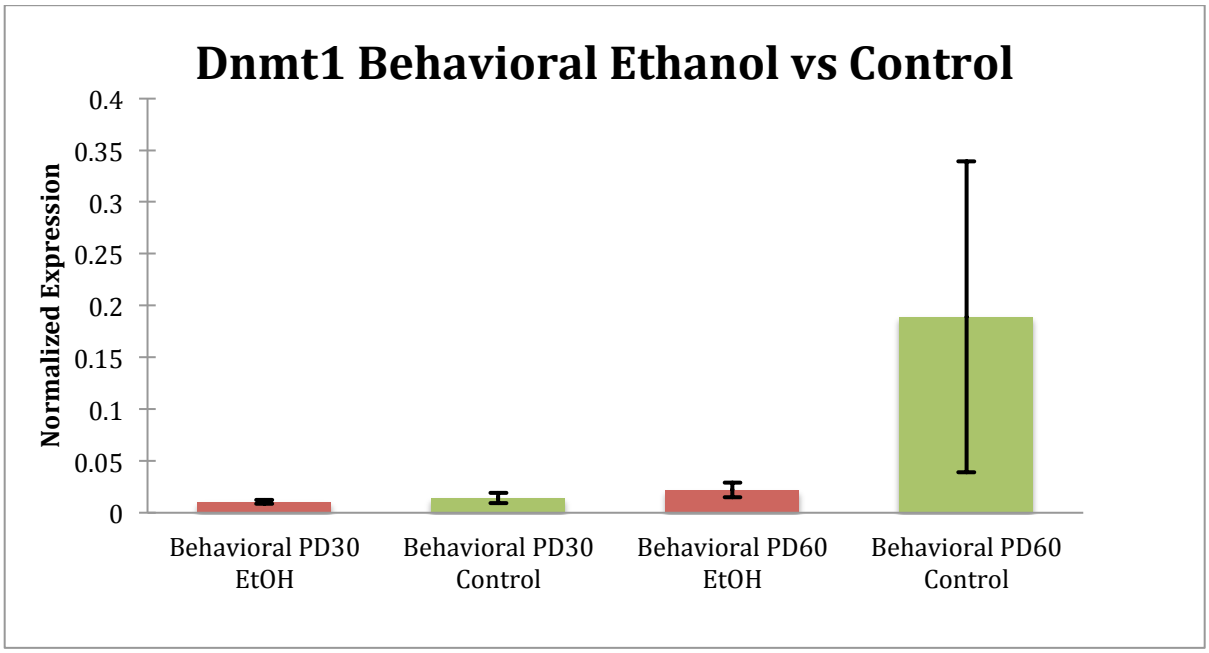


Figure 12: Quantitative RT-PCR data showing Dnmt1 Normalized Expression in Hippocampal tissue. cDNA was isolated from female hippocampal tissue of individuals that did not participate in behavioral testing (A) and those that did (B). Expression levels were calculated with normalizing to β-actin.