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Choline Supplementation and Dna Methylation in the Hippocampus and Prefrontal Cortex of Rats Exposed to Alcohol During Development

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CHOLINE SUPPLEMENTATION AND DNA METHYLATION IN THE HIPPOCAMPUS AND PREFRONTAL CORTEX OF RATS EXPOSED TO ALCOHOL DURING DEVELOPMENT

by

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DEDICATION

I would like to dedicate this paper to my two children, Vincente and Kalena, and to my future children. You are too young now, but as you get older, I hope that this paper will inspire you to always bring out the best that is in you in whatever you do in life. I hope that it encourages you to always keep on going, with your head held high, no matter what obstacles may come your way. I hope that it gives you the faith to believe that, with God's grace, anything is possible. And I hope that it motivates you to always strive for excellence and to be successful in whatever endeavors you choose to follow. May the Good Lord guide you and see you through years of happiness and prosperity, and in difficult moments, may He fill your heart with understanding, patience and humility.

Love Always, Mom.

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ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD) refers to the effects of alcohol exposure during development on individuals. Some of the most frequent deficits seen in children with FASD and in animal models of FASD are spatial memory impairments and impaired executive functioning, which are likely related to alcohol-induced alterations of the hippocampus and medial prefrontal cortex (mPFC), respectively. Choline, a nutrient supplement, has been shown in a rat model to ameliorate some of alcohol's teratogenic effects and this effect may be mediated through choline' effects on DNA methylation. This study used a rat model of FASD to investigate the underlying mechanisms of alcohol-induced deficits and the impact of choline on these deficits by examining changes in DNA methylation in the PFC and hippocampal areas. Alcohol was given by intragastric intubation to rat pups during the neonatal period (postnatal days 2-10) (ET group), which is equivalent to the third trimester in humans and a period of heightened vulnerability of the brain to alcohol exposure. Control groups included an intubated control group given the intubation procedure without alcohol (IC) and a non-treated control group (NC). Choline or saline was administered subcutaneously to each subject from postnatal day 2 to 20. On postnatal day 21, the brains of the subjects were removed and processed for relative optical density (ROD) of immunohistochemical staining for neuronal-specific enolase (NSE) and global DNA methylation as measured by

chemiluminescence using the cpGlobal assay. In the mPFC, choline supplementation led to significant increases in ROD of staining for NSE, regardless of treatment or sex. No differences in NSE staining were found in the CA1 region of the hippocampus. Alcohol exposure caused hypermethylation in the hippocampus and mPFC, which was significantly reduced after choline supplementation. In contrast, control animals showed increases in DNA methylation in both regions after choline supplementation, suggesting that choline supplementation has different effects depending upon the initial state of the brain. This study is the first to show in a rat model of FASD changes in global DNA methylation of the hippocampus CA1 region and mPFC after neonatal alcohol exposure with and without choline supplementation. Molecular mechanisms of alcohol's teratogenesis leading to a state of hypermethylation are proposed based on alcohol's and choline's effects in the folate/choline path to DNA methylation.

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I. INTRODUCTION

Investigating the teratogenic effects of alcohol exposure during development has been an intensive area of research for many years. While the major characteristics of children born to alcoholic mothers were described in 1968 (Lemoine, Harousseau, Borteyru & Menuet, 1968), it was not until 1973 that these characteristics were given the term Fetal Alcohol Syndrome (FAS) (Jones & Smith, 1973). Most recently, the term of Fetal Alcohol Spectrum Disorders (FASD) has been used to describe the wide spectrum of teratogenic effects of alcohol on individuals who are exposed to the drug during fetal development. On the most severe end of this spectrum is FAS, which is characterized by growth deficiencies, with body weight usually at less than the 10^{th} percentile, cranio-facial dysmorphology such as a short palpebral fissures, flat midface and thin upper lip, and central nervous system (CNS) dysfunction manifested as hyperactivity, attention deficits, and cognitive impairments in learning and memory (Jones & Smith, 1973; Streissguth & O'Malley, 2000). In a study examining the different diagnostic methods and criteria used to measure prevalence rates of FASD, it was estimated that FASD can affect up to 10 per 1000 live births (May & Gossage, 2001).

Some of the most characteristic deficits seen in children with FASD and animal models of FASD are spatial memory impairments (Blanchard, Riley & Hannigan, 1987; Goodlett, Kelly & West, 1987; Kelly, Goodlett, Hulsether & West, 1988; Reyes, Wolfe & Savage, 1989; Gianoulakis, 1990; Zimmerberg, Sukel & Stekler, 1991; Streissguth, Sampson, Olson, Bookstein, Barr et al., 1994; Goodlett & Peterson, 1995; Minetti, Arolfo, Virgolini, Brioni &

Fulginiti, 1996; Westergren, Rydenhag, Bassen, Archer & Conradi, 1996; Kim, Kalynchuk, Kornecook, Mumby, Dadgar et al., 1997; Nagahara & Handa, 1997; Mattson & Riley, 1998; Uecker and Nadel, 1998; Cronise, Marino, Tran & Kelly, 2001), which may be dependent upon alterations in the hippocampus (Barnes & Walker, 1981; Davies & Smith, 1981; West, Hodges & Black, 1981; Abel, Jacobson & Sherwin, 1983; West & Hodges-Savola, 1983; West & Pierce, 1984; Ferrer, Galofre, Lopez-Tejero & Llobera, 1988; Smith & Davies, 1990; Wigal & Amsel, 1990; Perez, Villanueva & Salas, 1991; Greene, Diaz-Granados & Amsel, 1992; Diaz-Granados, Greene & Amsel, 1993; Miller, 1995; Ba, Seri & Han, 1996; Tanaka, 1998; Uecker and Nadel, 1998; Riikonen, Salonen, Partanen & Verho, 1999; Berman and Hannigan, 2000; Archibald, Fennema-Notestine, Gamst, Riley, Mattson et al., 2001; Autti-Ramo, Autti, Korkman, Kettunen, Salonen et al., 2002; Tran & Kelly, 2003; Sowell, Lu, O'Hare, McCourt, Mattson et al., 2007; Willouhgby, Sheard, Nash & Rovet, 2008), and impaired executive functioning (Connor, Sampson, Bookstein, Barr & Streissguth, 2000; Kodituwakku, Kalberg & May, 2001), which is likely the result of damage to the prefrontal cortex (PFC) (Inomata, Nasu & Tanaka, 1987; Nagahara & Handa, 1995; Sowell, Thompson, Mattson, Tessner, Jernigan et al., 2002; Whitcher & Klintsova, 2008). Choline – a nutrient supplement – has been recently shown in a rat model to ameliorate some of these behavioral deficits observed in subjects pre- or neonatally exposed to alcohol (Thomas, La Fiette, Quinn & Riley, 2000; Thomas, Garrison & O'Neill, 2004; Thomas, Biane, O'Bryan, O'Neill & Dominguez, 2007; Ryan, Williams & Thomas, 2008). Choline has been suggested to have effects on behavior by affecting the epigenome, most particularly through changes in DNA methylation (Jhaveri, Wagner & Trepel, 2001; Waterland & Jirtle, 2003; Niculescu, Yamamuro & Zeisel, 2004; Niculescu, Craciunescu & Zeisel, 2006; Waterland, Dolinoy, Lin, Smith, Shi et al., 2006; Kovacheva, Mellott, Davison, Wagner, Lopez-Coviella et

al., 2007; Pogribny, Karpf, James, Melnyk, Han et al., 2008; Davison, Mellott, Kovacheva & Blusztajn, 2009; Mehedint, Niculescu, Craciunescu & Zeisel, 2010). Thus, in the present study, a rat model of FASD was used to investigate the underlying mechanism of alcohol-induced deficits by looking at changes in DNA methylation in the prefrontal and hippocampal areas. In addition, this study also examined changes in DNA methylation when subjects were supplemented with choline during and after early neonatal alcohol exposure.

In order to provide the appropriate background underlying the current study, both the clinical and animal literatures that have examined spatial learning, executive functioning, and memory deficits related to developmental alcohol exposure are covered. Also, studies examining possible treatment options to ameliorate such alcohol-induced deficits, including the recent use of choline supplementation, are also discussed. This is then followed by a review of choline's mechanisms of action in the brain. Finally, DNA methylation and the idea of how this epigenetic process may be a mechanism underlying alcohol-induced deficits in spatial learning and memory, and the impact of choline on these deficits, is also be discussed.

1.1 Learning Deficits in People with FASD

Children with FASD may not show any physical abnormalities or may show physical abnormalities that can disappear with age. However, with or without the physical features associated with fetal alcohol exposure, high levels of prenatal alcohol exposure may still lead to behavioral anomalies and intellectual deficits in newborns and young children (Landesman-Dwyer, Keller & Streissguth, 1978; Mattson, Riley, Gramling, Delis & Jones, 1997). For instance, by using a set of neuropsychological tests, Mattson, Riley, Gramling, Delis and Jones, (1998) examined 3 groups of children ranging from 5 to 16 years of age. The groups included: 1)

children with FAS, 2) children without FAS who were born to alcohol-abusing women, and 3) typical controls. The results of the study showed that, compared to controls, both groups of children who were exposed to ethanol *in utero* were impaired on tests of language, verbal learning and memory, academic skills, fine-motor speed, and visual-motor integration, regardless of whether they showed physical features associated with the diagnosis of FAS or not.

The effect of alcohol exposure during development on learning and memory has been widely studied and is one of the more commonly reported neuropsychological deficits both in the human literature and in animal models. Clarren and Smith (1978) found that in a sample of 126 FAS offspring, over 85% scored more than 2 standard deviations below the mean on IQ tests. Streissguth, Herman and Smith (1978) have also looked at performance on IQ tests in sample populations of children and young adults born with FAS and found an average IQ level of 65 (with a range from 16 to 105). It was further found in this latter study that IQ scores did not change, but remained fairly stable, when participants were re-tested 1 – 4 years later. Later studies have also found that children with a history of prenatal alcohol exposure show deficits in several areas of learning and memory, including spatial memory (Mattson & Riley, 1998; Streissguth et al., 1994). What is more, within a sample of Native-American FASD children with an average age of 10 years, spatial memory was significantly impaired whereas performance on an object recognition task was not different from that of age- and gender-matched control children (Uecker and Nadel, 1998).

More recent clinical studies also show that children born with FASD exhibit deficits in verbal learning, spatial memory and hippocampal development. Willouhgby et al. (2008) investigated verbal learning and verbal and spatial recall in children with FASD. Through the use of a clinical neuropsychological battery of learning and recall tests, as well as structural magnetic

resonance imaging (MRI), both behavioral and neuroanatomical deficits were examined. FASD children showed significant impairment in intellectual ability, everyday spatial recall abilities, delayed reproduction of a spatial figure, as well as poorer performance in tasks involving immediate and delayed recall, delayed recognition and verbal learning. Neuroanatomically, FASD children showed significant volume reduction in the left hippocampus. Similar studies also showed impairments in both immediate and delayed recall memory, which was directly related to how poorly these children were in the acquisition of new information (Willford, Richardson, Leech & Day, 2004) or to pervasive deficits observed in verbal learning (Mattson, Riley, Delis, Stern & Jones, 1996; Mattson et al., 1998; Mattson & Roebuck, 2002). Relatively few studies have directly studied spatial memory abilities in children with FASD. However, in a review by Uecker & Nadel (1996) it was highlighted that FAS children showed impaired recall for spatial locations. These children not only showed difficulty reproducing previously viewed spatial arrangements immediately and after a delay, but they also made more distortions in their reproductions of the spatial arrangements relative to their control counterparts.

Using several neuroimaging techniques, studies have been able to pinpoint brain changes, specifically in the hippocampal and prefrontal areas, which are the result of developmental alcohol exposure. For instance, Archibald et al. (2001) found small reductions in hippocampal volume, an effect that was more pronounced in FAS children than in FASD children who showed no facial dysmorphology. In addition to decreased volume, FASD children also showed greater hippocampal asymmetry such that the left hippocampus was smaller than the right (Riikomen et al., 1999) and left (but not right) hippocampal thinning (Autti-Ramo et al., 2002). In examining brain areas associated with deficits in learning among FASD children, Sowell et al. (2007) used functional MRI (fMRI) techniques to observe brain activation in children

performing a verbal learning task. While healthy children showed unilateral activation of the hippocampus and parahippocampal gyri, FASD children showed a complete absence of activation in these same brain regions. Thus, clinical studies have shown that one of the most observed cognitive deficits related to developmental alcohol exposure in children whose mothers drank while pregnant involves learning and memory impairments. Notably, studies have found that deficits in spatial learning and recall are more prominent than other forms of learning and memory deficits such as object recognition. What is more, such spatial deficits have been congruent with neurological findings of alcohol-induced structural and functional changes in the hippocampus, an area of the brain involved in learning and memory abilities. Although clinical studies directly relating the teratogenic effects of alcohol on spatial performance with an impaired PFC have not been found, the hippocampus has been suggested to have connections with areas of the PFC, which is involved in executive functioning and working memory (Becker, Olton, Anderson & Breitinger, 1981).

There have also been clinical studies directly examining executive functioning in FASD. One particular study used a battery of behavioral tests that measured executive functioning in adult males diagnosed with FAS or fetal alcohol effects (FAE) (Connor et al., 2000). According to the results of this study, there were significant decrements in executive functioning ability among the FAS individuals compared to otherwise healthy controls. Tests that showed deficits included Stroop scores, Trails scores, Wisconsin Card Sorting scores, Ruff's Figural Fluency scores, and Consonant Trigrams scores. In a similar clinical study (Kodituwakku et al., 2001) examining executive functioning in individuals prenatally exposed to alcohol, executive functioning performance was further categorized into two domains (cognition-based and emotion-related). In support of the dichotomy of this cognitive ability, damage to the

orbitofrontal cortex has been shown to cause deficits in emotion-related executive functioning (Rolls, Hornak, Wade & McGrath, 1994), while lesions to the lateral PFC led to deficits in cognition-based executive functioning (Dias, Robbins & Roberts, 1996). In the Kodituwakku et al. (2001) study, prenatal alcohol exposure led to impaired performance in tests measuring both types of executive functioning.

In an imaging study (Sowell et al., 2002), investigators examined prenatal alcohol-induced shape abnormalities in regional brain areas in children and adolescents exposed to large quantities of alcohol during early gestational development. Results showed that in these subjects, whose ages ranged from 8 to 22 years, one of the most affected brain areas was the frontal lobes. The ventral aspects of the frontal area showed a significant decrease in brain surface or a reduction in brain growth in alcohol-exposed individuals compared to otherwise healthy controls.

1.2 Learning Deficits in Animal Models of FASD

Because there is a significant amount of congruence between animals and humans in the effects of alcohol exposure (Driscoll, Streissguth & Riley, 1990; Hannigan and Abel, 1996; Hannigan, 1996), animals have been used to further examine the effects of developmental alcohol exposure on brain and behavior. The main advantage to using animal models, as outlined in Abel (1981), is the control over factors such as maternal health, poly-drug use, stress, blood alcohol concentration (BAC) levels, critical periods of exposure, and effects related to alcohol's caloric intake. Also, according to Hannigan (1996), the major goals of using animal models of FAS include studying the nature of common reactions to alcohol, identifying risk factors, discovering mechanisms and exploring potential treatments. Thus, animal models of FASD,

specifically rodent models, have been used extensively to further examine both the behavioral deficits and underlying mechanisms involved in alcohol's effects on learning and memory. For example, adult offspring of dams which were either intubated with 4 or 6 g/kg/day of alcohol (Abel, 1979) or consumed a liquid diet containing 35% ethanol-derived calories (EDC) (Bond & DiGiusto, 1978) showed impaired learning in a two-way shock avoidance task. Animals in both studies were fostered and cross-fostered, showing that these deficits in learning were not due to maternal factors, but to the prenatal influences involved in alcohol exposure.

In particular, many studies examining the effects of pre- and/or postnatal alcohol exposure on spatial learning abilities in the rodent model have used different procedural measures in their assessment. Such differences include alcohol exposure at different gestational time points, different doses of alcohol, different routes of alcohol administration to the dams and/or the pups, and different types of spatial learning tasks used. The differences in procedure and gestational period of alcohol exposure among these studies have probably led to varied results such as greater effects in males (Zimmerberg et al., 1991; Goodlett & Peterson, 1995), or in females (Blanchard et al., 1987; Kelly et al., 1988; Minetti et al., 1996), or no gender differences (Gianoulakis, 1990; Goodlett et al., 1987; Reyes et al., 1989; Goodlett & Peterson, 1995), long-lasting effects seen up to adulthood (Nagahara & Handa, 1997; Gianoulakis, 1990), effects observed only in juveniles (Westergren et al., 1996; Cronise et al., 2001), or no age difference (Kim et al., 1997; Reyes et al., 1989; Zimmerberg et al., 1991) in tasks involving measures of spatial learning and memory abilities. Nevertheless, all of these studies have shown to some degree an effect of developmental alcohol exposure on spatial learning abilities, which can be related to hippocampal damage, an area of the brain that is particularly important for spatial learning and memory.

One of the earliest studies examined spatial performance in a Morris water maze (MWM) task among juvenile offspring of dams which were given a liquid diet consisting of 35% EDC during gestation day (GD) 6 to 20 (Blanchard et al., 1987). In the MWM task (Morris, Garrud, Rawlins & O'Keefe, 1982), animals are required to locate a platform hidden just beneath the surface of a pool of opaque water. Locating the platform is based on distal spatial cues relative to the location of the platform. These spatial cues are usually defined as posters, furniture and other physical stimuli located in the testing room. In the Blanchard et al. (1987) study, measures included latency and distance traveled to reach the hidden platform. Results showed that during the 3 days of testing, alcohol-exposed females had longer latencies to reach the platform than controls on days 1 and 2, suggesting that prenatal alcohol exposure delays spatial learning. Among alcohol-exposed males, the latency to reach the platform was evident on day 2, but by day 3, these animals were not different from their controls. This further suggests that not only do alcohol-exposed animals take longer to learn the task, showing less improvement in performance than controls from day 1 through day 3, but that FASD females take longer, and thus are more affected, than their male counterparts.

Gianoulakis (1990) also examined long-term deficits in spatial memory after prenatal alcohol exposure. Dams were fed an ethanol-containing diet throughout gestation, and their offspring were tested in the MWM at postnatal day (PD) 40, 60 and 90. Ethanol exposure caused deficits in spatial learning where animals exposed to alcohol *in utero* took longer to perform the task, swam longer distances before locating and climbing onto the platform, and searched for the platform in all 4 quadrants during the probe trial. These deficits were seen up to adulthood, suggesting that the teratogenic effects of ethanol are long-lasting. Similar to this, Westergren et al. (1996) examined FASD rats that were exposed *in utero* to a liquid diet containing ethanol

from GD 11 to 21, at 6 months of age in the MWM. According to the data, FASD rats only showed marginally significant impairments such that alcohol-exposed animals showed a tendency towards lowered performance in the acquisition phase. Minetti et al. (1996), using a single dose (2.4 g/kg) of ethanol administered via 2 intraperitoneal (*i.p.*) injections on GD 8, showed that only adult female offspring showed deficits in escape latencies during the MWM probe trials. This suggests that at a low exposure, females are still sensitive to the effects of early alcohol exposure on spatial abilities when tested in adulthood.

In a comparison of the teratogenic effects of ethanol in a spatial versus non-spatial task, Kim et al. (1997) gave pregnant rat dams a diet containing 36% EDC throughout gestation up until GD 21. Their offspring were then tested during the adult stages on both an object-recognition delayed-nonmatching-to-sample (non-spatial) task and the MWM (spatial) task. While ethanol-exposed rats showed no deficits in the non-spatial task compared to controls, prenatal ethanol led to significant deficits in spatial learning. According to the authors, this finding suggests that the underlying mechanisms involved in spatial cognitive abilities seemed to be more vulnerable to developmental ethanol effects than those mechanisms related to object recognition. By increasing the delay period between training and testing in the MWM task from 1 day to 3 days Matthews & Simson (1998) showed that adult animals exposed to 3 g/kg or 5 g/kg ethanol *in utero* from GD 8 to 20 showed impaired spatial memory when the interval was 3 days, but not 1 day.

Other studies, as in Goodlett et al. (1987), used an artificial rearing procedure where rat pups were administered alcohol during PD 4-10. This early postnatal period in the rat represents the 3rd trimester of human pregnancy and is a period of rapid brain growth (Dobbing & Sands, 1979; Bayer et al., 1993). In this procedure, pups are taken from their dams, lightly anesthetized

and implanted with feeding tubes and placed in artificial holders (which are aerated and warm). A timer-controlled infusion pump is used to administer milk formula to the pups. In the Goodlett et al. (1987) study, pups were assigned to 4 treatment groups – suckle controls, gastrostomy controls, uniform alcohol exposure (pups received 2.5% (v/v) ethanol in milk solution for 12 feedings in a 24-hour period), and condensed alcohol exposure (pups received 7.5% (v/v) ethanol in milk solution for 4 feedings within an 8-hour period). From PD 19 – 30, pups were tested for spatial performance on the MWM. Compared to the two control groups, pups in the condensed condition showed severely impaired performance, while the uniform condition group showed mild effects in the development of spatial abilities.

A later study (Kelly et al., 1988) compared the effect of a condensed versus a uniform pattern of ethanol exposure from PD 4 to 10, during the time of the brain growth spurt, on spatial abilities in the MWM task in adult rats. Using the artificial rearing procedure, the condensed alcohol-exposed group was fed a milk solution that was 7.5 % (v/v) ethanol for a total of 4 feedings per day, while the other 8 feedings comprised of just a milk solution. On the other hand, the uniform alcohol-exposed group was given a milk solution containing 2.5% (v/v) ethanol for all 12 feedings per day. During the adult period beginning on PD 90, animals were then tested for spatial performance. Using distal cues, results showed disruption in performance in distance traveled due to condensed alcohol exposure, but only in the female rats. Female rats in the condensed group swam greater distances compared to all other groups. Since the condensed alcohol group showed higher BAC levels than the uniform alcohol group, this finding suggests that high BAC levels during a time critical for brain growth spurt leads to long-term deficits in spatial performance in female adult rats.

To examine the level of perinatal alcohol that is required to show deficits in spatial learning, Tomlinson, Wilce and Bedi (1998) exposed rat pups to 2, 4, or 6 g/kg alcohol via intragastric cannula over an 8-hour period on PD 5. Using the MWM task, animals were tested during adulthood (PD 61 to 64) and measures of escape latency – the time it took for the animal to locate and climb onto the submerged platform – were taken. Whereas rats exposed to 2 or 4 g/kg body weight of alcohol did not show a significant difference in performance compared to controls, rats administered 6 g/kg body of alcohol showed significant deficits in escape latency, suggesting that ethanol exposure greater than 4 g/kg body weight given over an 8-hour period in 5 day old rats leads to long-term spatial deficits. Similarly, Pauli, Wilce and Bedi (1995) exposed rats to 7.5 g/kg of ethanol, also by intragastric cannula, over an 8-hour period, on either PD 5 or PD10 of neonatal life. From PD 41 to 54, subjects were then tested on the MWM task. Statistical analyses showed that spatial learning ability was significantly impaired in both PD 5 and PD 10 alcohol-exposed subjects, with no significant difference observed in the degree of impairment due to alcohol exposure between the two time points.

In looking at ethanol exposure during the period of the brain growth spurt in rats, Goodlett and Peterson (1995) examined whether a more limited (3-day) binge-like exposure would induce place learning deficits, and whether these deficits depended on the developmental timing of the alcohol exposure. Using the artificial rearing procedure, pups were given 4.5 g/kg/day ethanol in a milk solution during one of three periods – from PD 4 – 6, from PD 6 – 9, or from PD 4 – 9. Animals were then tested during the juvenile period, from PD 26, in the MWM task. Results revealed that while both male and female subjects given ethanol from PD 4 – 9 showed deficits in both the acquisition and probe trials, only the males given ethanol from PD 6 – 9 showed similar deficits. Females showed no deficits when alcohol exposure was limited to

only PD 4 – 6 or PD 6 – 9. According to the authors, this finding suggests that alcohol exposure during the latter phase of the brain growth spurt constitute a sex-specific period of enhanced vulnerability, at least among juveniles, in tasks requiring spatial performance.

Some studies, such as in Goodlett and Johnson (1997), determined the effects of developmental alcohol exposure on spatial learning using an intragastric intubation procedure (fully described in Kelly & Lawrence, 2008). This method, which will be employed in the present study, involves gently inserting a measured length of PE-10 tubing, lubricated with corn oil and attached to a syringe filled with the desired amount and dose of alcohol mixed in a milk solution, into the mouth of the pup, down the esophagus and into the stomach. Once the tubing is in place, the milk/ethanol solution from the adjoining syringe is directly administered. Once this is done, pups are placed back with their dams, and the procedure is repeated at least 2 hours later, but with a milk solution only, to control for the intoxicating effects of the ethanol that may prevent the pups from nursing (Marino, Cronise, Lugo & Kelly, 2002). In the Goodlett and Johnson (1997) study, pups were intubated either on PD 4-6, PD 7-9, or PD 4-9 and then tested during the juvenile period (PD 26-31) on the MWM task. Results showed that early developmental alcohol exposure, particularly PD 7-9, led to significant learning deficits and the deficits depended on both the period of exposure and the BAC level.

A more recent study (Cronise et al., 2001) looked at the effects of alcohol exposure at different gestational time points on a spatial learning and passive avoidance task in juvenile and adult rats. Animals were exposed to alcohol via intragastric intubation either during gestational days (GD) 1-10 or 11-22, or PD 2-10, or all 3 time periods (equaling all three trimesters of human pregnancy). The results showed no effect of alcohol on the passive avoidance task among either juveniles or adults. Contrary to the findings by Goodlett and Johnson (1997), in the MWM

spatial task, only the juveniles exposed to alcohol during all three periods showed significant impairment compared to controls. Adult animals showed no impairment. This finding showed that prolonged exposure to alcohol is both necessary and sufficient to cause spatial memory deficits, and that these deficits are transient, meaning that they were not observed in adulthood.

Other studies looked at spatial deficits in animal models of FAS/FASD using different spatial tasks. For instance, several studies used the T-maze paradigm. The general description of this apparatus includes an alley way, which contains a start box with a guillotine door, and which leads to two arms of the maze, one arm on the left and the other on the right – in such a way that the alley and arms together form the shape of the letter "T". Lochry and Riley (1980) were among the first to explore effects of prenatal alcohol exposure using the T-maze. In this study pregnant rat dams received a liquid diet containing 35, 17.5 or 0% EDC from GD 5 to 20. Offspring were then tested right after weaning, from PD 22 to 25, to test for retention capabilities in the T-maze. The floor of the apparatus consisted of steel bars from which distributed shock (0.25 mA) was delivered. At the start of each trial, subjects were placed in the start box facing away from the door. When the door was raised, this activated a shock source from which the animal had to escape/terminate by either running to their preferred arm or to the non-preferred arm. Errors were defined as subjects moving past the choice point with all 4 paws facing toward the incorrect goal. Results showed that animals exposed prenatally to alcohol made more errors, a deficit that was also dose-dependent, since animals exposed to 35% EDC showed more errors than those exposed to 17.5% EDC *in utero*.

Abel (1982) used rectangular chambers to examine the hypothesis that *in utero* alcohol exposure, via a liquid diet containing 35% or 17% EDC, causes delay in maturation in female rat offspring. On PD 16, PD 40-42 and PD 114, animals were tested in mazes of appropriate size for

passive avoidance. One side of the chamber was covered with black, while the other side was covered with white colorboard. The floors on both sides had a grid floor of stainless steel bars, from which a pulse of shock was delivered to the animals. A light was located on the far end of the white chamber to indicate that area as the 'safe' chamber. Data showed a main effect of group and a main effect of age. Younger animals (PD 17) exposed to 35% EDC *in utero* required more trials to learn the passive avoidance response. By PD 40-42 animals exposed to either dose of ethanol required more trials. By PD 114, even though the animals from both alcohol groups still required more trials, the deficit was not significantly different from controls. This inability to learn the task was the result of increased perseverative behavior in the spontaneous alternation task observed in the alcohol-exposed animals. This further suggests deficits in or delayed maturation of inhibitory mechanisms since the task requires animals to inhibit the dominant perseverative response that is usually seen at an early age. Nagahara and Handa (1997) also examined the deficits due to prenatal alcohol exposure via a 35% EDC liquid diet given during the last week of gestation on a T-maze alternation task using animals at three different ages - juveniles (PD 38 to 44), young adults (PD 82 to 89)**,** and adults (PD 173 to 180). In this study, the alternation task included delay periods which ranged from 0 seconds, 10 seconds, 30 seconds, and 60 seconds. Compared to controls, the results showed that while only the alcohol-exposed juveniles showed deficits during the 10-second delay, alcohol-exposed animals at all three ages showed deficits for the 30- and 60-second delays.

In a water version of the T-maze task (Wainwright, Ward, Winfield, Huang, Mills et al., 1990), animals were required to show reversal learning. Initially, animals had to learn which arm of the maze contained a barrier and which arm contained an escape platform. After learning to escape by turning in one direction to get to the escape platform, animals prenatally exposed to

liquid diets containing 35% EDC showed deficits in learning the reverse – when the escape platform was switched to the other arm. Zimmerberg et al. (1991) tested rats for both spatial reference and spatial working memory abilities using the T-maze. In the spatial reference test, animals were required to always go to the right arm for the escape platform. In the spatial working memory test, animals first went to one arm to escape, and then on the test trial had to go to the opposite arm to find the platform. Animals prenatally exposed to alcohol showed deficits in both tests, with only males showing impairment in spatial working memory. A similar spatial alternation test was used to examine rats exposed to binge-like alcohol at one of two time points during the neonatal period – on PD 4 to 5, on PD 8 to 9, or at these two time points combined. Rats that were exposed to alcohol on all four postnatal days (the combined group) showed deficits in their rate of acquisition for conditional alternation. Rats exposed to alcohol on PD 8 to 9 and the combined group showed significantly more within-trial errors (Thomas, West & Goodlett, 1996).

Another type of maze, the radial arm maze, has also been used to determine spatial deficits related to developmental alcohol exposure. This maze usually consists of a total of 8 arms, all of the same color and equal dimensions, protruding from a central area or platform. In a spatial test, animals are usually required to determine which of the 8 arms are baited with a reward based on specific intramaze cues. Other times, all 8 arms are baited with a reward and the animals are required to remember, based on distal cues spatially located in the testing room, which arms were already entered on a given trial. This latter task requires more spatial working memory. One of the first studies looking at deficits in performance using the radial arm maze (Reyes et al., 1989) found that adult male and female rats exposed to 17% or 35% EDC *in utero* either failed to reach criterion or took significantly more (twice as many) training trials to reach

criterion, compared to controls. With all 8 arms baited with a food reward, these deficits were based on two measures – 1) the length of time it took for each rat to receive all 8 rewards, and 2) the number of times the rat re-entered an arm in which an award was already obtained on the given trial. Mice exposed to neonatal ethanol exposure from PD 2 to 14, via subcutaneous injections of 3 g/kg ethanol, also showed deficits in the radial arm maze (Pick, Cooperman, Trombka, Rogel-Fuchs & Yanai, 1993). Sessions in the maze were completed when all 8 arms were visited, or when a total of 16 arm entries were made. Criterion was defined as having achieved 8 correct entries out of the first 8 entries for 2 consecutive days. Neonatal ethanol exposure in the mice caused a significant decrease in the number of successful entries in the first 8 trials, as well as significantly more trials needed to enter all 8 arms.

To highlight the deficits in spatial learning caused by developmental alcohol exposure, Berman and Hannigan (2000) compared performance in FASD rats using both visual and spatial cues using a radial arm maze with one of three colors – white, grey or black. The visual cue task required rats to find food in the three black arms of the maze, which changed position daily so that spatial positioning of the arms was irrelevant. Subsequently, the rats were required to switch criteria and find food in the white arms of the maze. In the spatial task, the spatial position rather than the color of the rewarded arms was important. The results showed no significant effect of prenatal alcohol exposure on the visual task. However, compared to controls, prenatal alcohol caused more deficits in the spatial task where FASD rats showed significantly longer latencies to locate food in the three baited arms, and made more errors when the contingency shifted from the visual task to the spatial task.

Thus, congruent with findings from the clinical studies, animal models of developmental alcohol exposure have also found impairments in learning and memory performance caused by

pre- and/or postnatal alcohol exposure. Specifically, deficits in the spatial aspects of learning and memory are more evident, suggesting that this type of cognitive impairment is one of the most vulnerable to developmental alcohol insults compared to other non-spatial tasks such as object recognition or visual cue performance. Also, while many studies have found spatial deficits caused by alcohol exposure during various developmental time points, the period equivalent to the third trimester of human pregnancy in the rodent model – a period of rapid brain growth – is also especially sensitive to the debilitating effects of binge-like alcohol exposure on spatial performance.

These findings in the behavioral data highlight deficits in spatial learning and memory in animal models of FASD. Similar findings in the clinical literature not only show deficits in the spatial memory functioning, but also hint at associated neuroanatomical deficits to the PFC and hippocampus due to prenatal alcohol exposure. Given this, several studies have begun to examine changes in the formation and functioning of the hippocampus and PFC in animal models of FASD and how these differences may be related to the alcohol-induced changes in spatial learning and memory performance.

1.3 Alcohol-induced Changes in the Hippocampus

A review by Berman and Hannigan (2000) show that several behavioral, neuroanatomical and electrophysiological studies all support the hypothesis that exposure to alcohol *in utero* leads to deficits in the development and functioning of the hippocampus – a brain area critical to spatial learning and memory abilities and particularly vulnerable to alcohol exposure in both animals and humans. Adult (60-day old) rats, whose dams received an ethanol containing liquid diet from GD 10 to 21, showed a 20% decrease in the number of pyramidal cells in the dorsal

hippocampus area (Barnes & Walker, 1981). This same study, however, failed to show any effects on the number of granule cells in the dentate gyrus (DG) area of the hippocampus. On the other hand, a similar study (West et al., 1981), in which rats were exposed to alcohol for the entire gestation period via a liquid diet containing 35% EDC given to their dams, showed significant topographic differences in hippocampal mossy fibers among FASD animals when compared to control counterparts. Since mossy fibers form the main input from the DG to the CA3 region of the hippocampus, the study further suggests that gestational alcohol disrupts this transfer of information between the two hippocampal areas, and thus disrupts hippocampal functioning. A later study (Perez et al., 1991) showed that even a single day (GD 7) of ethanol exposure during the gestation period can result in neuroanatomical changes to the hippocampus. In this study, rat dams were administered 4 *ip* injections of ethanol over the course of GD 7, resulting in a total dose of 12 g/kg body weight. This one day of ethanol exposure caused a significant decrease in both the number of neuronal cell bodies of the hippocampus and the number of apical and basal dendritic spines of the hippocampal pyramids.

Similarly, Davies and Smith (1981), in examining the hippocampus of 14 day old mice exposed to ethanol during late gestation and early neonatal development (perinatal exposure), found marked reductions in the length of the basilar dendrites of the pyramidal neurons. In a study done by Ferrer et al. (1988), rats prenatally exposed to alcohol showed reductions in dendritic spine number on the secondary apical dendritic branches and basilar dendrites of the CA1 and CA3 regions; however, the effects were only seen on PD 15, and not at PD 90. The authors suggest that the effects of prenatal alcohol on dendritic spine number might be transient, yet other studies have found this effect occurring up to the adult stages (Abel et al., 1983; Perez et al., 1991). Other structural changes found in the adult rat resulting from perinatal alcohol

exposure (from GD 12 – PD 9) include densely packed pyramidal neurons in the CA1, less elaborate (and hence more immature) dendritic arbors of the pyramidal cells and fewer dendritic spines (Smith & Davies, 1990).

A later study (Ba et al., 1996) also examined changes in the hippocampus, but looked at the effects of longer periods of alcohol exposure. Female rats were given 12% ethanol in water as the only available drinking fluid for a period of 60 days prior to mating. Once pregnancy was determined, the ethanol solution was given to the dams throughout both gestation and lactation. The same treatment was delivered to the offspring when they were weaned from their dams, up until PD 45. Data analyses showed that ethanol exposure caused a significant reduction in cell number in the CA3 region of the hippocampus, along with altered width of the soma in the CA3 pyramidal cells compared to controls. Even though this provides quite compelling effects of ethanol, as was pointed out in Berman & Hannigan (2000), attribution of the ethanol effects on the CA3 region of the hippocampus is difficult to determine given the huge window in which alcohol was given to the mother before pregnancy, during pregnancy and lactation, and then to the offspring up until PD 45.

Using a more specific time period, Greene et al. (1992) examined hippocampal damage in rats exposed to alcohol from PD 4 – 10. Two daily schedules of ethanol administration were used – one schedule produced high-peak BAC levels while the other schedule produced low-peak BAC levels. Compared to controls, animals with high-peak BAC levels showed significant decreases in the number of CA1 pyramidal cells, as well as a decreased CA1 area. Animals from the low-peak BAC levels group only showed increased mature granule cell density.

A study by Wigal & Amsel (1990) failed to support this finding, but rather, found reduced numbers in CA1 cell count after prenatal and not neonatal alcohol exposure. However,

Diaz-Granados et al. (1993) found similar results to that found by Greene et al. (1992). That is to say, reductions in the number of CA1 pyramidal cells and reduction in CA1 area were caused by early neonatal but not prenatal alcohol exposure. Also, pyramidal cell density was significantly more reduced in females receiving both pre- and postnatal alcohol treatment when compared to females that received alcohol treatment only during the early postnatal period, suggesting a higher susceptibility of pyramidal density in the CA1 region to combined ethanol effects. Lobaugh, Wigal, Greene, Diaz-Granados and Amsel (1991) also failed to show significant reductions in the number of pyramidal cells in the CA1 region after prenatal alcohol exposure to 35% EDC – this finding was found for both juveniles (PD 21) and adults (PD 180).

However, to attempt to account for disparities in the findings of hippocampal CA1 effects due to pre- and/or postnatal alcohol exposure, unbiased stereological counting of cells in the hippocampal area might be more useful. Miller (1995), in an attempt to test the hypothesis of differential effects on the hippocampus due to prenatal versus postnatal ethanol exposure, used rigorous stereological procedures for measures of neuronal count and neuronal density. While prenatal alcohol exposure caused decreased numbers in neurons of the CA1 region, but not in the DG, postnatal alcohol exposure only showed reductions in CA1 due to high BAC levels (339 ± 17 mg/dl). In addition, postnatal alcohol exposure led to significant increases in the total number of neurons in the DG, as well as neuron density. This 'hyperdevelopment' caused by early alcohol exposure is also seen in hippocampal mossy fibers after prenatal alcohol insult (West & Hodges-Savola, 1983; West & Pierce, 1984), as well as an increase in area of the CA4 region due to combined pre- and neonatal or just neonatal alcohol exposure (Wigal & Amsel, 1990).

In a series of studies done by Tanaka and colleagues (see Tanaka, 1998), rat models of FASD were produced by feeding dams 5-30% ethanol as drinking fluid before and after

pregnancy. This method produced offspring that showed postnatal signs of CNS abnormalities, in particular morphological changes and decreased density of synaptic formation in the hippocampus. The consistent effect of prenatal ethanol on hippocampal synaptic formation led to the hypothesis that this measure is not only one of the most vulnerable to ethanol's teratogenic effects, but also is associated with impaired learning and memory (Tanaka, 1998).

In a more recent study (Tran & Kelly, 2003), critical periods for the effects of ethanol on hippocampal cell loss were determined. In this study, animals were exposed to alcohol at different gestational time points with BACs equated across time periods. At adulthood, stereological estimates of the total number of hippocampal CA1 and CA3 pyramidal cells, as well as DG granule cells were determined. Results from this study revealed a significant reduction in cell number in the CA1 regions of animals exposed to ethanol either during the postnatal period (3rd trimester human equivalent) or during all 3 trimester equivalents. Since the present study will be looking at alcohol exposure during the 3rd trimester equivalent to human pregnancy using a rat model (PD 2 to 10), the hippocampal area and specifically the CA1 region of the hippocampus will be looked at for changes in both global DNA methylation and neuronal density (discussed below).

Given the animal research showing damage to the hippocampus due to perinatal alcohol exposure, it has been speculated that this damage underlies the deficits in spatial memory performance in animal models of FASD (Uecker & Nadel, 1998). The first study to examine a possible association between behavior and neuroanatomical changes due to prenatal alcohol exposure was Abel et al. (1983). Pregnant dams were given 3 g/kg ethanol twice daily throughout gestation via the intubation procedure. Controls received isocaloric sucrose, and were pair-fed and pair-watered to the ethanol-treated animals. Offspring of the dams were then

fostered to surrogate dams that were not part of the experimental treatment. During adulthood, animals were tested on a two-way shock avoidance task, after which animals were sacrificed to obtain their brains. The apical and basilar dendritic spines in the CA1 hippocampal pyramidal neurons were examined and counted. Results showed that prenatal alcohol led to behavioral deficits where ethanol-exposed animals made significantly fewer incidences of avoidance than controls. Prenatal alcohol also led to significant decreases in both apical and basilar dendritic spine number. It was thus suggested that these neuroanatomical changes that occurred due to prenatal alcohol exposure may provide the structural basis for observed learning deficits.

Thus, rodent models of early alcohol exposure have reliably been shown to lead to deficits in the hippocampus. These findings are of particular interest to the present study, which will examine changes in DNA methylation in the hippocampal region after exposure to alcohol during the early neonatal period. Since the CA1 region is a hippocampal area most sensitive to early alcohol insult (Greene et al., 1992), neuronal density will also be measured in the CA1 region of the hippocampus as a positive control in the present study.

1.4 Alcohol-induced changes in the Prefrontal Cortex

Another brain area related to learning and memory, the PFC, has also been shown to be affected by developmental alcohol exposure in animal models of FASD. Many of the deficits detected in animal models of FASD utilize behavioral tests that not only involve the hippocampus but also involve the PFC (Inomata et al., 1987; Nagahara & Handa, 1995; Whitcher & Klintsova, 2008). Furthermore, there is evidence of neuronal changes in the PFC of animals exposed to alcohol during development. Because of these findings, the present study will examine changes in neuronal density in the CA1 and medial PFC (mPFC) brain regions and also

changes in global DNA methylation in the hippocampus and PFC due to early neonatal ethanol exposure and choline treatment.

One of the earliest studies examining the effects of developmental alcohol on the PFC (Inomata et al., 1987) exposed pregnant rats to 20% ethanol diluted in drinking water. The brains of fetal offspring on GD 21 were examined. While the actual structure of the synaptic junction showed no significant differences between alcohol-exposed and control brains, alcohol exposure reduced numbers of synapses to only one third of that of the controls. Another study (Nagahara & Handa, 1995) examined the effects of prenatal alcohol on the inductions of several immediate early genes (a useful measure of the level of neuronal activation) in various areas of the brain. Adult offspring of rat mothers that were fed a 35% EDC diet during the last week of gestation were tested on a T-maze involving an alternation task with delay increments of 0, 30 and 60 seconds. On the last day of testing, a subset of animals was tested and then killed 30 minutes after the test period in order to obtain levels of expression of the immediate early genes, which included *c-Fos, jun B* and *zif268.* Results showed memory deficits at the 60-second delay among alcohol-exposed animals. In addition, alcohol-exposed rats showed significantly reduced expression of the *c-Fos* and *jun B* mRNA levels in the orbital, prelimbic and anterior cingulated regions of the PFC. The authors of this study interpret their findings as showing fetal alcohol changes in the adult PFC that may contribute to the observable deficits seen in the alternation task of the T-maze.

Most recently, binge-like alcohol exposure during the early neonatal period equivalent to the brain growth spurt (PD 4 to 9) has also been shown to lead to neuronal changes in the mPFC (Whitcher & Klintsova, 2008). In this study, neonatal rat pups received a dose of 5.25 g/kg/day of ethanol in milk formula via intubations. At adolescence (PD 26 to 30), dendritic analysis of

layer III pyramidal neurons in the mPFC was performed by using a computer-based neuron tracing system. While early neonatal alcohol exposure did not affect depth (the distance from the cortical surface to the center of the soma) of the neurons, or length of dendritic complexity (measured as the mean number of intersections with each concentric sphere surface), it caused significant reductions in apical spine density in the mPFC. The finding of an effect in dendritic density but not dendritic tree structure is rare. According to the authors, one possible explanation for such a finding is that the neonatal alcohol exposure is influencing the afferents of these neurons, which include glutamatergic pathways such as the hippocampal-cortical pathway. Another possible explanation given by the authors is that neonatal alcohol is causing a reduction in the functioning of the mPFC neurons, since a change in spine density likely affects cell function via reduced excitability.

1.5 Ameliorating FAE on Learning and Memory

Despite such overwhelming evidence of alcohol's negative effects on learning and memory capabilities, as well as on impaired functioning of brain areas such as the hippocampus and PFC, alcohol consumption during gestation remains a societal problem. Thus, studies have begun to look at treatment options that can, at least, attenuate some of the cognitive deficits related to prenatal alcohol exposure. Potential treatments that have been investigated include zinc supplementation, NMDA receptor antagonist treatment, use of Vitamin E, and the use of growth factors and hormones such as estrogen (Es). Treatments used during postnatal periods include exposure to an enriched environment and voluntary exercise. More recently, pre- and/or postnatal treatment with choline supplementation have shown mitigating effects specifically on spatial learning and memory deficits.

Assadi and Ziai (1986) found that human infants exposed to alcohol *in utero* showed decreased plasma zinc levels. Congruent with this finding, Tanaka (1998) discussed the possible role of zinc supplementation as a repair mechanism since the hippocampus is not only highly vulnerable to prenatal ethanol exposure, but is also rich in zinc, a component found to be deficient in the hippocampal area in children born with FAS. However, while co-administration of zinc with ethanol during gestation led to significant increases in cerebral weight, RNA and protein content (Tanaka, Inomata & Arima, 1983; Tanaka, Nasu & Inomata, 1991), as well as number of dendritic branches per neuron in the PFC (Tanaka, Iwasaki, Nakazawa & Inomata, 1988), these ameliorative effects never reached similar levels seen in control animals that were not exposed to the prenatal ethanol. Zinc supplementation in alcohol-exposed rats did not impact synaptic formation in the hippocampus (Tanaka et al. 1991).

Thomas, Weinert, Sharif and Riley (1997) examined whether MK-801, an antagonist of the NMDA receptor that protects against NMDA receptor-mediated excitotoxicity, could effectively block the teratogenic effects of alcohol on behavior. Rat pups were exposed to ethanol on PD 6 via the artificial rearing procedure. One group was then treated with MK-801 21 hours after alcohol exposure, and another group was treated with MK-801 only. On PD 40, animals were then tested in a water version of the T-maze in which they were required to find the escape ladder in the non-preferred arm (discrimination), and then later in the previously non-reinforced arm (reversal trial). While neonatal ethanol exposure led to deficits in both tests, combined treatment with the MK-801 significantly ameliorated these deficits in the reversal learning task. Treatment with MK-801 alone had no significant effect on performance in controls. According to the authors, this finding suggests that MK-801during the ethanol withdrawal period can protect against alcohol-related deficits in spatial reversal learning.

The use of vitamin E (Marino, Aksenov & Kelly, 2004) was also examined as a potential therapeutic effect for developmental alcohol deficits, particularly oxidative stress in the hippocampus. In this study, rat pups were administered 5.25 g/kg of ethanol mixed in a milk solution via intragastric intubation during the early neonatal period (PD 7, 8 and 9). A proportion of these animals were also co-administered 2 g/kg Vitamin E mixed with a milk solution beginning on PD 6. During the juvenile to early adolescent period, from PD 22 to 29, subjects were tested on the MWM task, after which stereological analysis of hippocampal CA1 neurons was performed. For the distal cue condition of the MWM, early neonatal ethanol caused a significant increase in escape latency, a deficit that was not ameliorated by the vitamin E treatment. However, ethanol-exposed animals showed a significant decrease in neuronal number and significantly increased carbonyl levels in the CA1 region of the hippocampus, deficits that were ameliorated by vitamin E treatment. According to the authors, these findings together suggest that the oxidative stress occurring in the hippocampus due to developmental ethanol exposure may not be the only explanation of the observed behavioral deficits in spatial learning and memory.

Another more recent study (McGough, Thomas, Dominquez & Riley, 2009) examined the possible role of insulin-like growth factor-I (IGF-I) in mitigating the effects of third trimester equivalent alcohol exposure using a rat model. Rat pups were given 5.25 g/kg/day of ethanol via the intubation procedure from PD 4 to 9, treated with 10 µg/day of IGF-I from PD 10-13, and then tested on 3 behavioral tasks, which included tests of open field activity from PD 18-21, a parallel bar to test for motor coordination from PD 30 – 32, and the MWM to test for spatial memory from PD 45 – 52. Results showed that while neonatal alcohol exposure led to deficits in

all three behavior tasks, treatment with IGF-I showed ameliorating effects only for those deficits observed in motor coordination.

Another study (Barclay, Hallbergson, Montague & Mudd, 2005) examined cortical neurons *in vitro* taken from fetal rats on GD 16 with or without different growth factors and Es. After ethanol administration to the tissue for the first 4 days *in vitro*, factors including IGF-I (10 ng/ml), IGF-II (10 ng/ml), basic fibroblast growth factor (bFGF, 5 ng/ml), nerve growth factor (NGF, 100 ng/ml), and Es (10 ng/ml) were administered on the 4^{th} and 5^{th} *in vitro* days. According to the results, while IGF-I and bFGF reduced ethanol's toxic effect on neuronal survival, Es, bFGF, and NGF increased total neurite length after ethanol treatment. None of the treatments showed any statistically significant effect on the mean number of primary neurites, that is, on the number of projections from the neuronal cell body. However, all the treatments were found to cause a statistically significant increase in the mean number of secondary neurites per cell (a measure of neuronal branching), compared to the tissue that was treated with ethanol alone.

Other studies have also looked at how the environment may play a role in attenuating the effects caused by prenatal alcohol exposure. The rationale for such studies stemmed from research showing that, in an animal model, an enriched environment prompted nervous system development, facilitated the nervous system in recovery after injury, as well as improved memory and learning (Markham & Greenough, 2004; Will & Kelche, 1992). For instance, according to a review by Will and Kelche (1992), greater differences have been seen between impoverished and enriched groups with brain damage than the differences found between groups with no brain damage. This finding suggested that exposure to an enriched environment may have more beneficial effects in the compromised brain. This review also further highlighted that

the impact of environmental enrichment depends on the type of brain damage, and is particularly beneficial when the damage is to the hippocampal region.

With this in mind, studies began looking at how manipulating the postnatal environmental conditions can influence changes seen in animals prenatally exposed to alcohol. In one such study (Hannigan, Berman & Zajac, 1993), animals were exposed to alcohol from GD 13 to 19 via intragastric intubation. After weaning at PD 21, animals were either housed alone (the isolated condition) or group housed in large cages containing toys (enriched condition). During adulthood, animals were tested for locomotor activity, rearing and stereotypy in an open-field box, for spatial learning in the MWM and radial-arm maze tasks, as well as for hind limb gait. While the results showed no differences between groups in the open-field box or on hind limb gait, environmental enrichment led to improvements in performance in both the Morris water and radial-arm mazes, regardless of prenatal treatment. In another study that also found improved performance in the MWM due to an enriched environment in animals prenatally exposed to alcohol (Wainwright, Levesque, Krempulec, Bulman-Fleming & McCutcheon, 1993), brain growth was also examined. Results showed that while ethanol led to decreased brain growth in the occipital lobe (with no effects on growth of the frontal or parietal areas), postnatal environment did not have any impact on changes observed.

A later study (Berman, Hannigan, Sperry & Zajac, 1996) looked at the effects of an enriched environment in alcohol-exposed rats on dendritic spine density in the hippocampus CA1 region. However, while an enriched environment led to increased apical and basilar spine densities in control animals, rats exposed to prenatal alcohol did not show significant increases after being reared in an enriched environment. Later analyses of the same brains also failed to show significant differences due to environmental manipulation in spine densities in the CA3

region – an area shown to be highly sensitive to memory impairments observed after prenatal alcohol exposure (Tanaka, 1998). Given that environmental enrichment has been shown to ameliorate some of the effects of prenatal alcohol exposure on the behavioral level and not at the level of the brain, this may suggest that this form of 'treatment' is not long-term or that the key impact is not on neuronal morphology.

Voluntary exercise has also been shown to improve learning and memory capabilities, as well as hippocampal structure and functioning. For example, in rodent models, voluntary exercise has been shown to improve cholinergic hippocampal functioning (Fordyce & Farrar, 1991), increase the expression of neurotrophic factors (Farmer, Zhao, van Praag, Wodtke, Gage et al., 2004) and acetylcholine (Ach) release (Dudar, Whishaw & Szerb, 1979), as well as increase long-term potentiation (LTP) and neurogenesis (van Praag, Christie, Sejnowski & Gage, 1999; Farmer et al., 2004). It has also been shown to alter the levels of gene transcripts involved in neuronal activity, synaptic structure and neuronal plasticity (Tong, Shen, Perreau, Balazs & Cotman, 2001), as well as those involved in synaptic trafficking, signal transduction pathways, and transcription regulation (Molteni, Ying, & Gomez-Pinilla, 2002). Behaviorally, voluntary exercise has been observed to improve performance in spatial learning tasks such as the MWM (van Praag et al., 1999; Alaei, Moloudi, Sarkaki, Aziz-Malekabadi & Hanninen, 2007), and radial arm maze (Anderson, Rapp, Baek, McCloskey, Coburn-Litvak et al., 2000). Given these enhancing effects of voluntary exercise, particularly on the hippocampus and on hippocampus-dependent spatial learning and memory, studies began to examine the possible mitigating effects of this factor on deficits caused by developmental alcohol exposure.

For instance, Christie, Swann, Fox, Froc, Lieblich et al. (2005) provided offspring of rat dams who were exposed to a liquid diet containing 35% EDC during gestation either a standard

home cage after weaning or a home cage containing a running wheel. During adulthood (from PD 60), these offspring were tested for spatial performance in the MWM task. According to the results, prenatal alcohol exposure led to spatial memory deficits. However, alcohol-exposed animals with free access to voluntary exercise in the running wheel showed enhanced performance in the MWM that was not different from controls. Similarly, in Sim, Kim, Shin, Chang, Shin et al. (2008), 21-day old rat pups exposed to alcohol from GD 15 to birth showed increased *c-Fos* expression (an immediate early gene that represents neuronal activity and which also plays a role in the development of the brain) in the hippocampus after postnatal treadmill exercise. This was significantly different from the depressed *c-Fos* expression that was observed in alcohol-exposed pups that were not exposed to the treadmill exercise.

The ameliorating effects of cholinergic drugs and cholinergic-rich transplants have been examined in rats consuming alcohol during adulthood and so the findings may not be relevant to animal models of FASD. Nevertheless, these studies examining postnatal alcohol consumption and withdrawal show similar behavioral and neural effects related to learning and memory that are also observed after developmental alcohol exposure. For instance, both in humans and in animal models, chronic postnatal ethanol exposure have been shown to produce cognitive deficits that are observed in behavioral tests (Victor 1994; Walker, Hunter & Abraham, 1981; Arendt, Henning, Gray & Marchbanks, 1988), which parallel the neural deficits that are found. These deficits include neuronal and synaptic loss, dendritic atrophy, neurotransmitter system dysfunction, and, of interest to the present study, reductions in the cholinergic innervations of the hippocampus (Walker, Barnes, Zornetzer, Hunter & Kubanis, 1980; Walker et al., 1981; Paula-Barbosa, Brandao, Madeira & Cadete-Leite, 1993; De Witte, 1996; Fadda & Rossetti, 1998; Arendt et al., 1988; Arendt, Allen, Marchbanks, Schugens, Sinden et al., 1989; Casamenti, Scali,

Vannucchi, Bartolini & Pepeu, 1993; Cadete-Leite, Andrade, Sousa, Ma & Ribeiro-Da-Silva, 1995). Arendt et al. (1988; 1989) showed a progressive decline in both cortical and hippocampal cholinergic, noradrenergic and serotonergic markers, which paralleled the deficits found in both spatial and associative memory performance in the radial arm maze task. These deficits were significantly improved after cholinergic-rich transplants of fetal tissue into the cortex and/or hippocampus regions, with transplanted alcohol-treated subjects showing marked improvements in spatial memory. Using a similar methodology, Hodges, Allen, Sinden, Mitchell, Arendt et al. (1991) showed that while alcohol consumption, even after a full year of alcohol withdrawal, led to impaired acquisition and performance in the maze tasks, treatment with cholinergic agonists and cholinergic transplants from the basal forebrain and ventral mesencephalon areas led to improved performance. These effects were more prominent for working than reference memory, and coincided with neural findings of reduced choline acetyltransferase activity in the cortex and hippocampus and reduced acetylcholinesterase (AchE) cell counts in areas related to the forebrain cholinergic projection system of alcohol-treated rats – an effect that was not observed in alcohol-treated rats that received cholinergic-rich transplants.

Related to the cholinergic system is a nutrient, choline, that has been shown to enhance learning and memory performance, as well as structural and functional efficacy of several brain areas, most notably the hippocampus (see below). Current research has begun to focus on the use of this nutrient as a supplement to ameliorate the learning and memory deficits seen in rodents exposed to developmental alcohol exposure. Several studies thus far have shown how this nutrient can not only reverse the effects on learning and memory in animal models of FASD, but that the short-term administration of choline produces long-term beneficial effects.

1.6 Choline Supplementation on Learning and Memory in FASD Animals

In a series of animal studies done by Thomas and colleagues, choline supplementation in rats exposed to alcohol either during the prenatal or early postnatal period led to improved spatial performance on the T-maze or MWM tasks. In one such study (Thomas et al., 2000), rats were exposed to alcohol from GD 6 to 20 by feeding pregnant dams a liquid diet containing 35% EDC, and then treated with choline supplementation, via intubation, during the neonatal period, from PD 2 to 21. From PD 45, which represents the periadolescent period, animals were tested in a T-maze apparatus to assess visuo-spatial discrimination abilities. According to the results, while prenatal alcohol exposure led to deficits in learning, animals that were prenatally exposed to alcohol and then treated with choline during the early postnatal period showed similar performance in the task compared to the controls. This finding suggested that choline significantly attenuated the learning deficits associated with prenatal alcohol exposure.

Another similar study also found that choline supplementation reversed the effects of alcohol exposure during the brain growth spurt (PD 4 to 9) (Thomas et al., 2004), a period in the rat model that represents the third trimester of human pregnancy. In this study, pups were given alcohol via the artificial rearing procedure, and choline was administered from PD 4 to 30. Choline supplementation not only reduced learning deficits on a spatial discrimination reversal learning task using a water version of the T-maze, but also led to decreased hyperactivity when animals were tested in an automated open-field. Choline has also been shown to be effective in relieving the learning deficits caused by neonatal alcohol exposure when given after (from PD 10 to 30), rather than during, this period of brain growth spurt (Thomas et al., 2007), suggesting that choline supplementation can alter or enhance functioning in an already compromised brain.

In a study to determine the most effective time period for choline administration, it was found that choline treatment from PD 21 to 30 did not mitigate the deficits on performance seen in animals exposed to neonatal alcohol when these animals were tested in the acquisition phase of the MWM task. However, performance of alcohol-exposed animals given choline treatment from PD 11 to 20 or PD 11 to 30 did not differ significantly from controls or the other ethanol groups. During the probe trials, choline administered early (PD 11 to 20) and later (PD 21 – 30) led to attenuation of ethanol-induced effects on performance. According to the authors, these findings provide a 'window' in which choline supplementation may significantly and effectively reduce some of the learning impairments, such as spatial memory deficits, caused by developmental alcohol exposure (Ryan et al., 2008).

In these studies, one major recurring topic that is addressed is the need to determine the underlying mechanisms of choline's action in the compromised brain, specifically in the hippocampus. Several mechanisms, outlined in Ryan et al. (2008), through which choline may be acting include the increase in CA1 pyramidal and DG cell spine densities when given to an otherwise healthy rat from PD 16 to 30 (Meck, Williams, Cermak & Blusztajn, 2008); its influence in the structural integrity and signaling functions of cell membranes (Zeisel, 2006); actions as a methyl donor to the methionine-homocysteine cycle (Zeisel & Niculescu, 2006); and its influence in DNA methylation and subsequent gene expression (Niculescu et al., 2006). Since the current study focuses on the mechanism of choline, a more detailed discussion of actions of choline follows.

1.7 Mechanisms of Action of Choline

Choline is a nutrient that has been shown to be important in brain formation and functioning in both humans and mammals (Blusztajn, Cermak, Holler & Jackson, 1998; Ziesel, 2004; 2006), especially during the prenatal and early postnatal periods. Studies have examined both the neurochemical (Cermak, Holler, Jackson & Blusztajn, 1998; Cermak, Blusztajn, Meck, Williams, Fitzgerald et al., 1999) and neurobehavioral (Meck, Smith & Williams, 1989; Meck & Williams, 1997a; 1997b; 1997c) actions of choline. It is involved in a myriad of molecular events (see Figure 1) such as the formation of cell membranes and their functioning, the metabolism of methyl groups, the synthesis of signaling molecules such as phosphatidylcholine and sphingomyelin, as well as in neurotransmission via the synthesis of the neurotransmitter Ach (Zeisel and Blusztajn, 1994; Blusztajn, 1998, Zeisel & Niculescu, 2006). Through its metabolite betaine, choline becomes a methyl group donor, altering gene and histone methylation processes, and is involved in the homocysteine methylation to form methionine (Finkelstein, 2000). Methionine is a precursor for S-adenosylmethionine (SAM), the physiological methyl group donor for protein, RNA and DNA methylation (Jeltsch, 2002). This last study also reported that choline caused methylation of the cytosines within cytosine-guanosine sites (CpG sites) of the promoter region of genes regulates gene expression (epigenetic regulation).

Several lines of research have shown that choline availability during early development has profound effects on behavior and neural functioning that persist well into adulthood. Specifically, and of particular interest to the present study, choline supplementation increases the levels of choline in the brain and is believed to be involved in the amelioration of memory functioning (Klein, Koppen & Loffelholz, 1998; Zeisel, 2000). More specifically, choline impacts the formation and functioning of the hippocampus. The hippocampus is rich in

cholinergic neurons, which rely on choline for the synthesis of the neurotransmitter Ach (Blusztajn et al., 1998). Studies using rodent models have shown that choline supplementation during the gestation period critical for hippocampal development led not only to significant increases in cell number (Albright, Friedrich, Brown, Mar & Zeisel, 1999), but also to increases in cell size, cell structure, and hippocampal functioning (Williams, Meck, Heyer & Loy, 1998; Li, Guo-Ross, Lewis, Turner, White et al., 2004). Prenatal choline has also been shown to increase Ach release (Cermak et al., 1998), decrease the activity of AchE (Cermak et al., 1999), enhance cholinergic tone (Montoya, White, Williams, Blusztajn, Meck et al., 2000), and increase NMDA-receptor mediated transmission (Montoya & Swartzwelder, 2000). Such enhancements in functioning include improved visuospatial and auditory memory (Williams et al., 1998; Meck, Smith & Williams, 1988; Meck & Williams, 1997; 1999; 2003), and LTP (Jones, Meck, Willliams, Wilson & Swartzwelder, 1999; Pyapali, Turner, Williams, Meck & Swartzwelder, 1998; Montoya et al., 2000). What is more, choline supplementation during these early developmental stages show long-term and permanent enhancement in hippocampal functioning throughout postnatal development (Meck et al., 1989; Zeisel, 2006). Studies have examined a dose response effect of choline in which choline deficiency leads to decrements in cell number, cell structure and functioning in the hippocampus region (Albright et al., 1999; Jones et al., 1999; Meck & Williams 1999).

There are critical periods of early development during which choline supplementation is effective (Zeisel, 2006). For example, Meck and Williams (2003) have found that the most effective postnatal period in which choline availability causes significant changes in the hippocampal region include PD 16 to 30. Particular to choline's enhancing effect on the hippocampus, the critical period for hippocampal development in rodent models is GD 11 to 18.

Thus, studies have shown that choline supplementation during this critical period of hippocampal development leads to significant increases in cell proliferation, as well as significant reductions in cell apoptosis (see Zeisel, 2006).

Choline supplementation has also been found to protect against the neurotoxic effects of an NMDA receptor antagonist, MK-801 (Guo-Ross, Clark, Montoya, Jones, Obernier et al., 2002; Guo-Ross, Jones, Shetty, Wilson & Swartzwelder, 2003). NMDA receptors in the hippocampus have been posited to be involved in LTP, which may mediate learning and memory – specifically the learning of contextual cues in Pavlovian fear conditioning. Activation of the NMDA receptor is followed by a host of cellular events including calcium influx, phosphorylation, alterations in receptor and channel phosphorylation, and conductance - all eventually leading to changes in gene expression (Bliss & Collingridge, 1993). Along with this finding, Alkondon and Alburquerque (2006) showed that an *in vitro* 10-minute bath exposure of rat hippocampal slices to various concentrations of choline caused a concentration-dependent decrease in the magnitude of Ach-induced NMDA excitatory postsynaptic currents (EPSCs). According to the authors, this finding suggests that choline is a strong inhibitor of the nicotinic acetylcholine receptor (nAChR), specifically the type III nAChR. Also, it is known that the choline uptake system is not yet fully matured in the developing brain (Klein, Weichel, Ruhr, Dvork & Loffelholz, 2002), likely leading to the presence of excess free choline during the fetal and early postnatal periods. Given this, and that the nAChRs in the brain are not only important for learning and memory (Arendash, Sengstock, Sanberg & Kem, 1995; Meyer, Tay, Papke, Meyers, Huang et al., 1997) but are also exposed to choline for prolonged periods, it was further suggested that the density and/or activity of the nAChRs can be increased by such high levels of choline exposure, especially during early development. In fact, electrophysiological studies have

shown conclusive evidence that choline is a complete and selective agonist at the alpha 7 subunit of nAChRs (Alkondon, Pereira, Cortes, Maelicke & Albuquerque, 1997; Albuquerque, Pereira, Braga & Alkondon, 1998; Alkondon, Pereira, Eisenberg & Albuquerque, 1999; Uteshev, Meyer & Papke, 2003). Selective alpha 7 subunit antagonists have been shown to block choline-induced electrophysiological responses from single cells or hippocampal slices (Alkondon et al., 1997; Albuquerque et al., 1998; Fayuk & Yakel, 2004).

The nAChRs could be mediating the memory-enhancing effects of choline when the nutrient is supplemented during the prenatal and postnatal periods (Alkondon & Alburquerque, 2006). Also, studies looking at LTP have found that choline availability during early development in rats facilitates hippocampal LTP that is seen during young and middle adulthood (Jones et al., 1999; Pyapali et al., 1998). Choline given during early development has been shown to increase basal levels of adult hippocampal neurogenesis, a process found to be involved in certain types of memories. Choline supplementation is accompanied by increases in brain-derived neurotrophic factor (BDNF) (Glenn, Gibson, Kirby, Mellott, Blusztajn et al., 2007; Wong-Goodrich, Mellott, Glenn, Blusztajn & Williams, 2008a), nerve-growth factor (NGF) (Sandstrom, Loy & Williams, 2002; Wong-Goodrich et al., 2008a), IGF-1 (Wong-Goodrich et al., 2008a; Wong-Goodrich, Glenn, Mellott, Blustzajn, Meck et al., 2008b) and IGF-2 (Napoli, Blusztajn & Mellott, 2008), which are proliferation/growth factors that normally show reduced concentrations in the hippocampus with age (Kuhn, Dickinson-Anson & Gage, 1996; Nacher, Alonso-Llosa, Rosell & McEwen, 2003; Rao, Hattiangady, Abdel-Rahman, Stanley & Shetty, 2005; Bimonte-Nelson, Granholm, Nelson & Moore, 2008; Hattiangady, Rao, Shetty & Shetty, 2005; Shetty, Hattiangady & Shetty, 2005).

Given choline's major role in the functioning of the hippocampus, research has also focused on improvements in behaviors such as learning and memory as a function of choline supplementation. Choline given for a period of 14 or 28 days in adolescent rats did not lead to improved performance in the MWM task, compared to controls (Guseva, Hpokins & Pauly, 2006). However, choline supplementation for 14, but not 28 days, showed significant increases in the α7 nAChR subtype density in the hippocampus and other brain areas. It is suggested that even though choline caused an increase in the α7 nAChR, the up-regulation of these receptors alone may not be sufficient for cognitive enhancement. The lack of behavioral improvement could also be due to route of administration of the choline (in food diet), the age of administration, the task used, or the combination of all 3 factors.

In another study that sought to determine whether postnatal choline administration aids in long-term memory (LTM) consolidation, (Gossell-Williams, Simon, Young & West, 2006), female rats were given choline supplementation during young adulthood (8 to 10 weeks old) at a dose of 100 mg/ml solution for a period of 14 days. Supplementation was administered via *i.p.* injections. Following treatment, animals were tested in a passive avoidance task using a T-maze, in which an electric foot shock was given upon entry into the previously determined preferred arm (usually the dark area). While short term memory of the aversive event was measured within 15 minutes after the shock, intermediate LTM of the event was measured 24 hours after the shock. Memory of the event was determined by reduced exploratory behavior and either avoiding entry into or longer latency to enter into the dark area. Results showed that while both control and choline-supplemented animals retained memory of the aversive event 15 minutes after the event, only choline-supplemented rats retained memory of the event 24 hours later. It was thus suggested that while there is short-term memory of an aversive stimulus regardless of

treatment, choline supplementation increased the ability to form intermediate LTM (thus consolidation of short-term memory into LTM) of the avoidance procedure.

Even though choline given during the postnatal periods between adolescence and early adulthood show some enhancing effects, choline given during early development – that is, pre- and/or early postnatal – shows more robust enhancement in cognitive behavior and impact on hippocampal structure and functioning. For instance, choline supplementation during the prenatal period has been shown to lead to long-term benefits in exploratory behavior and increases in hippocampal plasticity in aged rats (Glenn, Kirby, Gibson, Wong-Goodrich, Mellott et al., 2008). In this study, pregnant dams were given choline supplementation from GD 12 to 17 and their offspring were tested during adolescence (about 1 month of age) and then again during old age (about 24 months of age). Results showed increased object exploration in pre-pubertal females only, suggesting not only an age-dependent difference, but also a sex dependent difference in the effects of prenatal choline supplementation on such behavior in rats. This study also found that choline-supplemented rats showed higher basal levels of hippocampal cell proliferation (specifically in the DG) at 25 months of age compared to controls.

This latter finding supports a previous study showing that prenatal choline supplementation in rats during the same gestational period leads to increased hippocampal neurogenesis in the adult offspring (Glenn et al., 2007). According to the authors, this effect of prenatal choline supplementation regulates a series of neural changes leading to preservation of cognitive functioning well into old age. Other studies have also looked at prenatal choline supplementation using a peak-interval timing procedure in which a food reward is given when the rat presses a lever after a certain amount of time. In these studies, choline supplementation was shown to enhance attention and precision in the temporal control of responding (Meck &

Williams, 1997a; 1997b) and facilitate timing by reducing impulsive responding early in the interval (Cheng & Meck, 2007) compared to controls.

Of relevance to the present study, a number of studies using a rat model have also focused on increased memory capacity and functioning due to choline supplementation during gestation which also leads to the formation of more enduring memories well into adulthood. For instance, from GD 11 to 17 , a period when changes in choline availability are most effective on the development of the hippocampus (Meck & Williams, 2003), dams supplemented with a choline-rich diet produced offspring with a significant enhancement of spatial memory during adulthood (Meck & Williams, 1997a; Meck et al., 1988; 1989; Schenk & Brandner, 1995; Tees & Mohammedi, 1999). What is more, this choline availability during the same gestational period has also been shown to prevent, or at least significantly slow down, memory declines that are naturally observed with age (Meck et al., 2008; Meck & Williams, 2003).

Other studies have also looked at the enhancing or protective effects of choline supplementation on memory in the face of seizure-induced memory deficits (Holmes, Yang, Liu, Cermak, Sarkisian et al., 2002; Yang, Liu, Cermak, Tandon, Sarkisian et al., 2000), on motor deficits in a mouse model of Rett syndrome (Nag & Berger-Sweeney, 2007) and on sensory gating in a mouse model of schizophrenia (Stevens, Adams, Yonchek, Hickel, Danielson et al., 2008). Guseva, Hopkins, Scheff and Pauly (2008) tested the hypothesis that dietary choline supplementation can alleviate the cognitive deficits observed after traumatic brain injury (TBI). Using a rat model, subjects were given 2% choline in their diet for a period of 14 days, after which they were subjected to a controlled cortical impact brain injury. Eight days after brain injury, subjects were tested for spatial memory via the MWM task. Results showed significantly improved performance on both the acquisition and memory retention phases in TBI rats that

were on the choline-supplemented diet. Whereas TBI lead to a significant reduction in α7 nicotinic receptor binding in the CA1 region of the hippocampus (along with other brain areas), choline supplementation was shown to reverse these binding deficits, specifically in CA1.

The long-term changes after choline supplementation on the structure and functioning of brain regions such as the hippocampus and behaviors related to hippocampal functioning such as learning and memory suggest that choline may be altering the epigenetic information related to hippocampal functioning. Most recently, Niculescu et al. (2006) conducted a study looking at mechanisms mediating the effects of choline on neuronal proliferation. It was hypothesized that these mechanisms likely involve changes in global DNA methylation and hypomethylation of the promotor region of the cyclin-dependent kinase inhibitor 3 gene. The hippocampi in fetal mice, whose dam was fed a choline-deficient diet, were isolated and changes in protein levels of two cyclin-dependent kinase inhibitors (Kap and p15INK4b) and in calretinin (a calcium binding protein expressed as neurons differentiate) were determined. These changes were then related to changes in DNA methylation of their respective genes (*Cdkn3, Cdkn2b*, and *Calb2*). This study found that a choline-deficient diet during gestation led to decreased DNA global methylation in the fetal hippocampus region. The authors suggest that such changes in gene methylation could mediate the expression of a cell cycle regulator, thus altering brain development.

Since choline supplementation leads to reversal effects of prenatal alcohol insults, and these effects are observed after choline treatment is complete, choline may be working through epigenetic changes in the alcohol-affected brain. This possibility will be explored in the present study. Epigenetic changes can be observed by first looking at possible changes in global DNA methylation. It is hypothesized that early alcohol exposure will lead to global DNA hypomethylation in the PFC and hippocampus – the two main areas of interest in the present

study. It is also hypothesized that choline supplementation will ameliorate the hypomethylation state of the DNA, returning the pattern of the methylation to normal states, similar to that of controls.

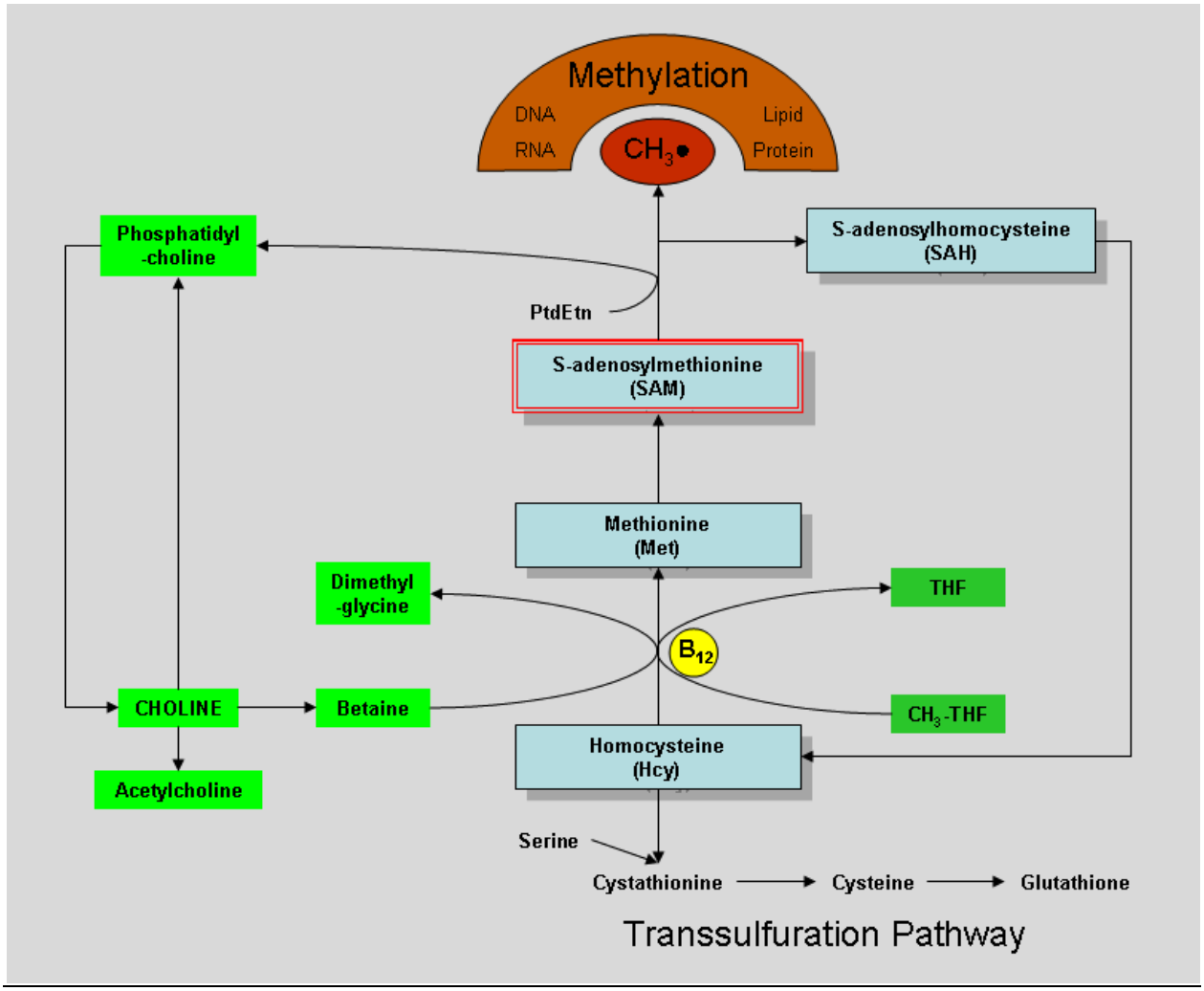


Figure 1. A Visual Illustration of Choline Metabolism in the Brain (by Niculescu, M. for release into the public domain through Wikipedia)

1.8 Epigenetics and DNA Methylation

The term epigenetics refers to the various mechanisms involved in the regulation of DNA expression and gene activity. Epigenetic mechanisms determine when and where during the course of development that a gene is expressed and also phenotype of a given cell without changing the actual DNA sequence (Robertson, 2005). The most well-studied epigenetic mechanism in determining gene expression is the pattern of DNA methylation that occurs within the specialized cell. DNA methylation regulates gene activity and is involved in the maintenance of stable phenotypes of differentiated cells (Holliday, 2005). The process is made up of two main constituents – DNA methyltransferases (*DNMT*s), which are enzymes that establish and maintain methylation patterns within DNA; and methyl-CpG binding proteins (MeCPs), which play a role in the normal functioning of the cells (Robertson, 2005). DNA methylation promotes normal functioning and development of an organism by acting as a 'silencer' of gene expression, thus repressing transcription of the gene. In this way DNA methylation controls gene expression and supports stability within the genome against large quantities of repetitive DNA (Robertson, 2005). Epigenetic changes that occur in the DNA are described as changes in the methylation process, such as hypo- and hypermethylation, of cytosine at position C5 in the CpG dinucleotides (Bird, 2002). Whereas hypomethylation leads to loss of growth control within the genome, hypermethylation can lead to the silencing of genes involved in cell-cycle regulation, tumor cell invasion, DNA repair, chromatin remodeling, cell signaling, transcription, and apoptosis (Robertson, 2005).

DNA methylation has been shown to be more stable than other epigenetic factors, making it a more likely candidate regulating various disorders (Beck, Olek & Walter, 1999). Abnormalities or disruptions in DNA methylation are widely observed in the development of

diseases such as: autism (Muhle, Trentacoste & Rapin, 2004); Beckwith-Wiedemann syndrome, characterized by perinatal overgrowth and embryonic tumors; Prader-Willi syndrome, which includes an inability to thrive in infancy, hyperphagia, obesity, mental retardation, and behavioral problems; Albright hereditary osteodystrophy, pseudo-hypoparathyroidism (PHP) type Ia, and PHP-Ib, both of which involve mental retardation and subcutaneous ossification; Angelman syndrome, which includes mental retardation, speech impairment, and behavioral abnormalities; Transient neonatal diabetes mellitus, which is a rare form of diabetes; and Fragile X syndrome, marked by mental retardation; and Systemic lupus erythematosus – an autoimmine disease (see Robertson, 2005; Jiang, Bressler & Beaudet, 2004). In the past few years, DNA methylation processes have become targeted as a mechanism for treatment in the study of cancer (Feinberg & Vogelstein, 1983; Feinberg, 2004; Ehrlich 2002, 2005, 2006; Shao, Lacey, Dubeau & Ehrlich, 2009; Irizarry, Ladd-Acosta, Wen, Wu, Montano et al., 2009) because epigenetic changes have been shown to be more readily reversible than changes in genetic sequences (Karpf & Jones, 2002). Studies have found that both hypo- and hypermethylation of DNA are involved in the development of cancer. For instance Jones and Baylin (2002) showed that tumor suppressor gene promotors can become hypermethylated, resulting in their silencing, and thus leading to the cell's inability to suppress tumor growth.

Changes in DNA methylation have also been implicated in declines in learning and memory capabilities that are observed as one ages, such as in late onset Alzheimer's disease (Wang, Oelze & Schumacher, 2008). Wang et al. (2008) examined DNA methylation in the brain tissue of already deceased Alzheimer's patients. Although there was no significant difference in methylation compared to otherwise healthy controls, Alzheimer's patients showed an unusual methylation pattern when compared to the overall pattern observed in controls. This finding

suggested a possible role of epigenetic deregulation in age-dependent Alzheimer's disease. It has been recognized that there may exist a relationship between a genome-wide decline in global DNA methylation and functional declines in learning and memory with age (Liu, van Groen, Kadish & Tollefsbol, 2009a).

In the review by Liu et al. (2009a), it was noted that reductions in the enzyme *Dnmt1*, a DNA methyltransferase that stabilizes and maintains methylation patterns early in development (Li, Bestor & Jaenisch, 1992), in neuronal precursors causes hypomethylation in neurons and glial cells that are quickly depleted postnatally (Fan, Beard, Chen, Csankovszki, Sun et al., 2001). Since this depletion does not have an impact on postmitotic neuronal survival, it is suggested that DNA methylation is important in the regulation of neurogenesis. It has been found that neurogenesis occurs postnatally both in humans and rodents (Eriksson, Perfilieva, Bjork-Eriksson, Alborn, Nordborg et al., 1998; Cameron & McKay, 1999) and that one of the two brain areas in which this adult neurogenesis occurs is the hippocampus (Gage, 2000; Lois & Alvarez-Buylla, 1994;). Also, neurogenic activity declines with age (Kuhn et al., 1996), coinciding with age-related decline in global DNA methylation (Wilson, Smith, Ma & Cutler, 1987). Given this, Liu et al. (2009a) proposes that this loss of DNA methylation as one gets older may lead to the reduction in neurogenesis and thus explain age-related declines in learning and memory performance.

Similarly, Holliday (1999; 2005) hypothesized that the pattern of DNA methylation in neurons plays an integral role in the coding of memory, in particular LTM. The author suggests that since LTM, which is retained for several years or a lifetime, requires the setting up and maintenance of neuronal communication and circuitry, this necessary stability of LTM may be dependent on enzymatic modification of cytosine in DNA to 5-methyl cytosine. Exactly how this

occurs is not yet understood, but it is proposed that the formation and maintenance of memory occur at specific sites in the DNA of neurons that are either methylated or unmethylated at first. Experience with a stimulus or event in the environment, which is to be remembered, changes the initial methylation state of the DNA, which in turn changes the phenotype of the neuron itself. With this modification in the activity or expression of the neuron, when the same stimulus or event is experienced again, the neuron responds differently – by sending an electrical signal to other neurons in its network. Since the process of hypomethylation results in activating a silent gene, Holliday (1999) further proposes that the environmental stimulus influences a demethylated change in the DNA of a neuron, leading the neuron to become primed to respond to that stimulus again.

Changes in epigenetic programming seem to be particularly sensitive to exposure to environmental stimuli during the prenatal and/or early postnatal periods, leading to phenotypic differences of an individual later in life. As was highlighted in a review by Szyf (2001), the dynamic and potentially reversible characteristic of epigenetic mechanisms such as DNA methylation may provide a basis for therapeutic intervention. Alterations in the epigenome have been shown to be caused by dietary supplementation, such as choline (see above) and other methyl supplementation nutrients. For instance, Cooney, Dave and Wolfe (2002) provided pregnant mice with one of three levels (control, mid-range and high) of methyl supplemented diets. According to the results, methyl supplementation in the diet of the pregnant mice led to enhanced DNA methylation levels in the offspring, as well as changes in the phenotype characteristic of increased health and longevity. In a similar study, Waterland and Jirtle (2003) showed that dietary methyl supplementation of agouti strain mice dams with extra folic acid, vitamin B12, choline and betaine resulted in phenotypic changes (measured by coat color) in the

viable yellow agouti mice (A*vy*/a) offspring as a result of increased CpG methylation. To test the hypothesis that early nutrition affects epigenetic gene regulation of the agouti allele, non-agouti (a/a) dams in this study were fed either a normal diet or a diet containing methyl supplementation two weeks prior to mating with A*vy*/a males, as well as throughout gestation and lactation. When examined at PD 21, it was found that the dietary supplementation shifted the coat color distribution of the A*vy*/a offspring towards a more brown (pseudoagouti), than yellow, phenotype. Congruent with this finding, maternal supplementation also led to increased methylation at the A*vy* CpG site.

A key environmental factor that has been shown to alter epigenetic programming, leading to long-term behavioral and neural effects is maternal care. For example, in examining the impact of maternal care on the epigenome, Weaver, Cervoni, Champagne, D'Alessio, Sharma et al. (2004) found that increased mothering behavior such as pup licking and grooming, and arched back nursing led to stable changes in DNA methylation and chromatin structure at a glucocorticoid receptor gene promotor in the hippocampus. What is more, the epigenetic differences seen in offspring of dams who showed high mothering behavior versus those who showed low mothering behavior were observed within the first week of life, were reversed by cross-fostering, and persisted into adulthood. This ability to show reversal in DNA methylation is highlighted in a review by Holliday (1989) that reveals how the phenotypes of many enzyme deficient rodent cell lines can be reactivated to wildtype after treatment with a potent demethylating agent called 5-azacytidine. This finding not only shows that the phenotypes are not a result of a structural gene mutation but rather by the inactivation of these genes via alterations in DNA methylation, but also that these altered methylation patterns are heritable (Holliday 1989; 2006). In support of the hypothesis of inheritance of altered methylation patterns

from one generation to the next, Anway, Cupp, Uzumcu and Skinner (2005) showed that estrogenic and/or antiandrogenic endocrine disruptors administered to gestating rat dams altered sperm DNA methylation (both hypo- and hypermethylation) in the male offspring producing male infertility, an effect that was also reliably observed in subsequent generations of male offspring.

The ability to reverse the pattern of DNA methylation is particularly interesting to the present study, which seeks to determine possible reversal in the DNA methylation patterns that may be observed in FASD subjects after receiving choline treatment. This finding will help explain the underlying mechanism through which choline is working to alleviate the effects on learning and memory that is observed in rat models of FAS (see above).

1.9 Measurement of DNA Methylation

There are several approaches to measuring DNA methylation in the genome. For instance, some techniques include radio-labeling the CpG sites using bacterial CpG methylase (Balaghi & Wagner, 1993) or a methyl-C antibody (Piyathilake, Johanning, Frost, Whiteside, Manne et al., 2000), bisulfate treatment of DNA and polymerase chain reaction (PCR) amplification of repetitive DNA elements (Yang, Estecio, Doshi, Kondo, Tajara et al., 2004), pyrosequencing (Karimi, Johansson & Ekstron 2006a; Karimi, Johansson, Stach, Corcoran, Gradner et al., 2006b), and the use of methyl sensitive restriction enzymes (Pogribny, Yi & James, 1999; Fujiwara & Ito, 2002; Karimi et al., 2006a; 2006b). The more common approach involves the use of chromatography techniques, and include reversed-phase high performance liquid chromatography (RP-HPLC) (Kuo, McCune, Gehrke, Midgett & Ehrlich, 1980), two dimensional thin layer chromatography (2D-TLC) (Wilson, Smith, Autrup, Krokan, Musci et al.,

1986), high performance liquid chromatography-mass spectrometry (HPLC-MS)(Annan, Kresbach, Giese & Vouros, 1989), high performance capillary electrophoresis (HPCE) (Fraga, Uriol, Borja Diego, Berdasco, Esteller et al., 2002; Stach, Schmitz, Stilgenbauer, Benner, Dohner et al., 2003), and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (Song, James, Kazim & Karpf, 2005). However, according to Anisowicz, Huang, Braunschweiger, Liu, Giese et al. (2008), even though this latter set of measures using chromatography techniques represent the widely-accepted standard and definitive way of measuring global DNA methylation, it is expensive, requires very specialized skill sets, and lacks the capacity to analyze multiple samples at the same time.

Using a more recently developed assay known as CpGlobal, which will be used in the present study (see methods section for protocol), Anisowicz et al. (2008) showed how this method of using methyl sensitive restriction enzymes is easy to use, while at the same time produces results that make this method a very good alternative to HPCE (mentioned above). Methyl sensitive restriction enzymes are effective in measuring global DNA methylation in such a way that when applied to genomic DNA (gDNA), they produce a set of digested fragments that can be measured and translated into how much DNA methylation is actually present in the genome. In this study, several tests were carried out to determine the efficacy of the CpGlobal design, as well as the accuracy of its measures. For the design, the analytical sensitivity of the assay was determined via the methylation of the entire genome of Lambda DNA using a bacterial CpG methylase (M.SssI), which served to methylate all the cytosine residues that resided within the CpG dyad. Another test examined the efficiency of digestion and end-fill reactions for DNA using a methyl-insensitive restriction enzyme (MspI) and a range of DNA concentrations. The results provided a technique that allowed for multiple samples to be

analyzed at the same time (high-throughput), as well as for all the necessary steps involved in the measures of global DNA methylation – digestion, end-fill with biotinylated nucleotides, attachment to the surface of the well and chemiluminescence – to occur in a fixed location (that is, in the well of the microtiter plate). Next, the accuracy of the CpGlobal assay and the most effective methyl sensitive restriction enzymes that could be applied to the assay were determined. A series of mixtures with 5% increases in ratios of unmethylated to methylated Lambda DNA were developed and quantitated using 5 different methyl-sensitive restriction enzymes – AciI, BstUI, HpaII, HinP1I and HypCH4IV. Results showed that the enzymes HpaII, HinP1I and to a lesser extent, HypCH4IV were the most effective indicators for global DNA methylation using the CpGlobal technique. In addition to this, measures using CpGlobal was compared to those using HPCE by applying the technique to a selection of cancer cell lines and the results showed a good linear fit (a strong correlation) in the data from both types of assays.

II. PURPOSE OF STUDY

Early ethanol exposure, especially during the brain growth spurt (during the 3rd trimester equivalent of human pregnancy), consistently leads to deficits in learning and memory, specifically in spatial learning and memory. Concurrent with this, the hippocampus and the PFC are among the most vulnerable brain areas to be affected by ethanol's teratogenic effects. Given the findings from Thomas and colleagues that choline supplementation during early development alleviates the deficits in spatial learning in a rat model of developmental alcohol exposure, and that choline alters global methylation in the brain, it is suggested that this nutrient may impact alcohol-induced effects on learning and memory via changes in DNA methylation.

Few studies have examined DNA methylation changes when choline supplementation is administered to subjects exposed to ethanol during development. In addition to this, only a few studies have looked at possible global DNA methylation changes due to exposure to alcohol during the developmental period. In Garro, McBeth, Lima and Lieber (1991) study, a mouse model was used to determine the effect of maternal alcohol consumption on DNA methylation and DNA methyltransferase activity in the fetus. From GD 9 to 11, pregnant mice were administered 3g/kg of ethanol twice daily (except on GD 11 when the animals were given the dose only once during the morning). On GD 11, 5 hours after ET administration, the fetuses were retrieved in order to measure changes in DNA methylation and DNA methyltransferase levels. Measures of the methyl-accepting capacity of the fetal DNA were determined using *Hpa*II methylase – a bacterial methylase that recognizes the 5´-CCGG´-3, and methylates CpG

cytosines that are normally methylated by the DNA methyltransferase enzyme. Results showed that acute ethanol exposure *in utero* led to a decrease in methylation of the fetal DNA, signified by the finding that the ethanol-exposed fetal DNA was a significantly better methyl acceptor compared to that of control fetuses. Fetal nuclear methylase activity was also shown to be significantly lower than that of controls. An *in vitro* analysis showed that this decrease or inhibition in DNA methyltransferase activity was the result of exposure to acetaldehyde – the first metabolite of ethanol, and not ethanol itself. Since changes in DNA methylation patterns are associated with changes in gene expression, the authors suggest that the acute ethanol-induced hypomethylation observed may contribute to the developmental abnormalities observed in FASD.

This hypothesis is echoed in a review by Haycock (2009) that examined the possible relationship between epigenetic factors and reprogramming, and ethanol teratogenesis. Given that there are significant and dramatic epigenetic reprogramming taking place during development, from pre-implantation to gastrulation, that are critical for cell differentiation and later maintenance of cell functioning, Haycock suggests that ethanol-induced abnormalities could be the result of ethanol disruption of these reprogramming events. However, the main focus of this review was on ethanol's effects on the CNS via epigenetic changes that occur during the preconception and pre-implantation periods and effects on sperm and the placenta, including associations with effects on the offspring due to paternal alcohol consumption. The focus of the present study is on alcohol-induced methylation changes with alcohol consumption during the early postnatal period.

A study (Liu, Balaraman, Wang, Nephew & Zhou, 2009b) examining methylation changes in mice whole embryo after in vitro ethanol exposure determined differences in alcohol-

induced alterations in genes based on the levels of cytosine and guanosine content, as well as whether the affected embryo had a closed or opened neural tube phenotype. On GD 8, pregnant mice were sacrificed to obtain their embryos. The embryos were then placed in a medium containing 6 µl/ml of 95% ethanol for a period of 44 hours. gDNA was then extracted from the embryos to measure methylation changes according to the neural tube open versus the neural tube closed phenotypes, as well as density levels of the CpG content of the genes. According to the results, alcohol-induced hypomethylation was more pronounced in genes with low levels of CpG density, while hypermethylation was more evident among genes with high levels of CpG content. What is more, embryos with the neural tube opened showed a significant state of demethylation, while hypermethylation was more evident in those with the neural tube closed.

One concern with the Garro et al. (1991) and Liu et al. (2009b) studies is the ability to generalize the findings to the different teratogenic effects of alcohol. Alcohol is administered during early gestation for a short period of time, and given the large amount of literature on developmental alcohol effects, it is well known that the type of behavioral and neural deficits seen are sensitive to the time period in which alcohol is given. Although these two studies have shown that alcohol exposure during early development causes changes in DNA methylation, and in the case of the Liu et al. (2009b) study, these changes are dependent on CpG content and neural tube phenotype, these changes can only be compared to developmental abnormalities that may be observed due to alcohol exposure during the given time periods in which alcohol was administered. Also, developmental alcohol exposure affects a large number of CNS regions. In these two studies, measures of DNA methylation and DNA methyltransferase were done on the fetus or embryo as a whole, and not on any specific CNS region. As was pointed out by Haycock (2009), the effects of *in utero* alcohol exposure involve a wide range of morphological and

physiological abnormalities, suggesting that the etiology of FASD includes "a potentially bewildering array of heterogeneity".

Thus, it still leaves open the question of how changes in DNA methylation in one area versus another can help explain changes that are observed both at the neural and behavioral levels. Also, knowing which brain areas are more affected by alcohol-induced changes in DNA methylation might further enable both the development of intervention strategies and the determination of critical windows for treatment to be most effective. Nevertheless, the Garro et al. (1991) study provides a very useful start in looking at how developmental alcohol and its effect on changes in DNA methylation could explain the deficits seen in those suffering from fetal alcohol exposure.

The present study is the first to determine differences in overall DNA methylation specifically in the hippocampus and PFC using a third-trimester rat model of FASD. Possible changes observed in these specific brain areas will help piece together underlying mechanisms responsible for some of the cognitive inefficiencies seen in adolescence, and sometimes up to adulthood, after developmental alcohol exposure. This study is also the first to explore whether there are changes in overall DNA methylation when ET subjects are given choline supplementation during early postnatal development. Animals were exposed to alcohol during the third trimester equivalent of human pregnancy (Bayer, Altman, Russo & Zhang, 1993) and then treated with choline supplementation from the early postnatal period (PD 2) up to PD 20 (Ryan et al., 2008). On PD 21, animals were sacrificed to obtain the hippocampus and PFC regions to measure levels of global DNA methylation. Since the CA1 region of the hippocampus and the mPFC are two brain regions that have shown reliable deficits due to developmental alcohol insult, these areas were also measured for neuronal density using neuron-specific enolase

(NSE) staining. This measure was used to determine whether the subjects in this study showed alcohol-induced effects and will serve as a positive control.

It is hypothesized that alcohol exposure during the 3rd trimester equivalent of human pregnancy will lead to reductions in global DNA methylation in the hippocampus and PFC areas. It is also expected that choline supplementation given to ethanol-exposed subjects will lead to levels of global DNA methylation in these two brain regions similar to levels of control animals. In parallel to these expected findings, it is hypothesized that while ET-exposed subjects will show reductions in neuronal density in the CA1 region of the hippocampus and in the mPFC, ET-exposed subjects given choline supplementation will show no significant difference in neuronal density compared to controls.

III. METHODS

3.1 Subjects

All animal procedures followed institutional guidelines outlined by the American Association of Laboratory Animal Care (AALAC) and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina. All subjects were born and bred in the animal colony at the Department of Psychology, University of South Carolina, Columbia. Housing conditions were maintained at 22ºC with a 12-hr light-dark cycle, beginning at 0700 hr. Female Long-Evans rats were housed overnight with proven breeder Long-Evans males. The following morning, cotton swabs (moistened with distilled water) were used to put a vaginal smear on culture slides, and a microscope used to determine the presence of sperm. For any given breeder female, the first day on which sperm was present on the culture slide was designated GD 1. From the first day of pregnancy until GD 22, pregnant rats (dams) were singly housed in polypropylene cages with wood shavings, and received no treatment or handling other than the recording of their weights on GD 1, 5, 10, 15 and 20. Food and water were provided *ad libitum* to all dams.

After birth, the pups in each litter were quasi-randomly assigned to one of three treatment groups – an ethanol-treated (ET) group, an intubated control (IC) group, and a non-treated control (NC) group. Each pup from each litter were also quasi-randomly assigned to either the choline-supplemented condition or the placebo (saline) condition. Thus, this is a 3 (group) X 2 (sex) X 2 (choline) design. There were no more than one animal from a litter assigned to a cell.

In order to detect a medium effect size (0.5, Cohen's d) and a statistical power of .80 at alpha (α) = .05, 10 males and 10 females per group were required (Faul, Erdfelder, Lang & Buchner, 2007). Thus, a total of 120 subjects were used in this study. Pups remained in the same cage as their dam until PD 21, after which they were sacrificed and their brains retrieved for further analyses.

3.2 Postnatal ET Treatment

Dams usually give birth on GD 23, and this day was designated as PD 1 for the pups. All dams and their pups were left undisturbed on this day. From PD 2 to PD 10, equaling the third trimester of human pregnancy (Bayer et al., 1993; Dobbing & Sands, 1979), litters were culled to 10 pups (5 males and 5 females) where possible. Pups were weighed and, initially, colored with nontoxic marker for identification.

For the ET group, the procedure consisted of the administration of ethanol via intragastric intubation from PD 2 to 10. Intubation involved placing a PE10 Intramedic tubing, attached to a syringe and dipped in corn oil for lubrication, down the esophagus of the pup and injecting the solution directly into the stomach. Each pup from the ET group were given a 3.0 g/kg dose of ethanol in a volume of 27.8 ml/kg of milk solution (West, Hamre & Pierce, 1984) in order to obtain peak BACs between 300-400 mg/dl (Marino et al., 2002). Two hours after ET administration, the ET pups were intubated a second time, but just with 27.8 ml/kg of milk solution which compensates for any reduction in milk intake from the dam due to intoxication (Tran & Kelly, 2003). The IC pups received the same treatment procedure, twice per day, but were not given any solution. The NC pups were weighed daily, marked for permanent identification with India ink on PD 7, and given choline or saline treatment. All pups were

permanently marked on one or more of their paws for identification using the AIMS Animal Tattoo Identification kit (Serial # NEO9001000). Each paw to be marked was sterilized, and then tattooed using a standard tip tattoo needle dipped in India ink. The tattoo needle is machine operated and was controlled by the user via a foot pedal.

3.3 Blood Alcohol Concentration (BAC)

On PD 10, samples of 10 µl of blood from each pup in the ET and IC conditions were collected via a nick to the tail, 2 hours after ethanol administration, in order to assess maximum BAC levels (Marino et al., 2002). No blood was taken from the NC pups. Blood samples from the ET group only were placed in 190 µl of 0.53N perchloric acid, neutralized with 200 µl of 0.30 M potassium carbonate, vortexed, and centrifuged for 15 minutes in order to separate out the plasma. Samples were then frozen at −80 °C until time of assay. To control for any variability that might occur due to differences in the time spent frozen, a set of alcohol standards were made in parallel to the blood samples. These standards included concentrations of 0, 50, 100, 200, 300, 400, 500, and 600 mg/dl alcohol in water. BACs were analyzed using an enzymatic procedure. Blood samples were thawed to room temperature and centrifuged again for 15 minutes. In separate reaction tubes the following mixture was made: 400 µl of 1.875mM tris-β-nicotinamide adenine dinucleotide (tris-NAD) stock, 50 µl alcohol dehydrogenase (ADH) in distilled water (89.25 unit/ml), and 50 µl of supernatant from the blood sample or the standard. These reaction tubes were then incubated at room temperature for 1 hour. To determine BAC levels, absorbance values for the blood samples and standards were obtained via a spectrophotometer (Shimadzu UV-1601) set at 340 nm. By plotting the absorbance values of the alcohol standards on the Y-axis and their known concentrations on the X-axis, a standard curve

was generated. BACs for the samples of pup blood were then calculated using the equation of the standard curve.

3.4 Postnatal Choline Supplementation

From PD 2 to 20, each pup in each litter was quasi-randomly assigned to one of two supplemental conditions. Animals in the choline-supplement condition received subcutaneous (*s.c.*) injections of 100 mg/kg/day choline chloride dissolved in a volume of 6.66 ml/kg of saline, while the animals in the other saline condition (control condition) received s.c. injections of saline vehicle. Subcutaneous injections involved using a needle to administer the solution just beneath the skin of the animal. The skin of the animal (usually at the nape of the neck or upper back area) was pinched upwards and the needle was inserted into the raised skin. Subjects from all groups were weighed daily throughout treatment to monitor weight gain. Pups remained with their dam until PD21, at which time they were weighed and then sacrificed to obtain the hippocampus and PFC.

3.5 Tissue Collection

On PD 21, subjects were sacrificed via decapitation. Immediately after retrieval, the brain of each subject was divided sagittally into the left and right hemispheres. One half of the brain containing the hippocampus and PFC was used for measures of DNA methylation, and the other half was used to stain for NSE – an enzyme that binds to cultured neocortical neurons, in a calcium-dependent manner, and promotes cell survival (Kirino, Brightman, Oertal, Schmechel 7 Marangos, 1983). To control for hemispheric bias, where the left or right hemisphere may be more affected by ethanol and/or choline treatment, for each of the analyses done -DNA

methylation and NSE staining - brain tissue was semi-randomly obtained from the left or right hemisphere across all subjects, providing an even distribution of both left and right hemispheres for each measure. For DNA methylation analysis, the PFC was cut coronally from the designated brain half and immediately frozen in a container of isopentane kept chilled with dry ice. The entire hippocampus was extracted from the same tissue, using the white band of corpus callosum as a reference point, and was also immediately frozen with isopentane. After freezing, the tissue was placed in labeled tubes and kept frozen at −80 °C until time of assays. Brain halves that were obtained for NSE staining analysis were immediately immersed in 4.0% paraformaldehyde in 0.10 M phosphate buffer (pH 7.4; PB) firstly for 4 hours at room temperature, and then overnight at 4°C. Once fixed, the tissue was washed in solutions of 15% and then 30% sucrose in 0.10 M PB at 4°C (Mooney, Siegenthaler & Miller, 2004). Tissue was then frozen at −80 °C until time of assays.

3.6 DNA Methylation

Genomic DNA Isolation. gDNA was isolated from approximately 30 mg of rat brain tissue following the protocol of the manufacture (Promega # A1120). The quantity and quality of the gDNA were assessed by spectrophotometer (UV absorbance) and agarose gel electrophoresis respectively.

Hpa II and Msp I Digestion. DNA was digested with *Hpa*II (methylation sensitive/dependent) and *Msp*I (methylation insensitive) restriction enzymes. *Hpa*II has been widely used to define the methylation status of the entire genome, as well as of specific regions (Pogribny et al., 1999; Fujiwara et al., 2002; Karimi et al., 2006a; 2006b). It is a bacterial methylase that recognizes the sequence 5'-CCGG'-3 (Mann & Smith, 1977). When added to the

DNA it produces a set of digested fragments, which can be measured and used to determine the amount of methylation that is present (Anisowicz et al., 2008). *Msp*I is an isoschizomer of *Hpa*II (specific to the same recognition sequence, but is methylation insensitive) and was used to normalize the data collected from the methyl-sensitive restriction enzyme. This step aided in the removal of any intrinsic variations introduced through slight differences in DNA concentration, and digestion and end-fill reactions (Anisowicz et al., 2008).

Aliquots of 150 ng gDNA for each sample were placed into 9 wells of the 96-well MaxiSorp™ plates (Nunc #436110) (see Fig. 2). This method provided a high-throughput approach in which multiple samples were analyzed simultaneously. The DNA was digested with 1 unit of *Hpa*II (NEB # R0171S) added to the first three wells, 1 unit of *Msp*I (NEB # R0106S) added to the second three wells, and buffer control (NEBuffer1), with no enzyme, added to the last three wells. The total volume of the reactions was 30 ul. The plate was then incubated in 37 °C incubator for 1 hour.

End-fill Reaction. After DNA digestion, restriction enzymes were heat-inactivated at 80°C for 10 minutes, then 20 ul of the end-fill reaction mix was added to each well containing 0.1 uM Biotin-11-dCTP (PerkinElmer # NEL538001EA), 0.1 uM Biotin-11-dGTP (PerkinElmer # NEL541001EA), and 0.1 units Sequenase (USB 70775Y). The plate was then incubated in 37 °C incubator for 2 hours.

Coating Reaction. After end-fill with biotinylated nucleotides, 100 ul of Reacti-Bind DNA coating solution (Pierce # 17250) was added to each well of the plate. The plate was then incubated overnight at room temperature with shaking at 150 RPM.

HRP-Neutravidin Reaction. After coating, the plate was washed 4 times with TBS (10 mM Tris-HCL pH 8.0, 150 mM NaCl), then 200 ul of the Detector Block solution (KPL #

71-83-00) added to each well, after which the plate was incubated at room temperature for 1 hour. 150 ul of

Detector Block containing 2 ug/ml of HRP Neutravidin (1: 500 dilution) (Pierce # 31030) was added to each well after removing the Detector Block solution. The plate was again incubated at room temperature for 2 hours.

Luminescence Measurement. The Dector Block/HRP Neutravidin solution was removed from the plate and 1X Biotin wash solution (KPL # 50-63-06) was used to wash the plate 2 times. Then 150 ul of LumiGlo chemiluminescence substrate (KPL # 54-61-00) was added to each well. After 4 minutes, the luminescence was measured by GENios at 440 nM. This net luminescence was used to determine the global DNA methylation index (GDMI). The higher the index values for a given sample, the greater the loss of methylation that was measured (demethylation).

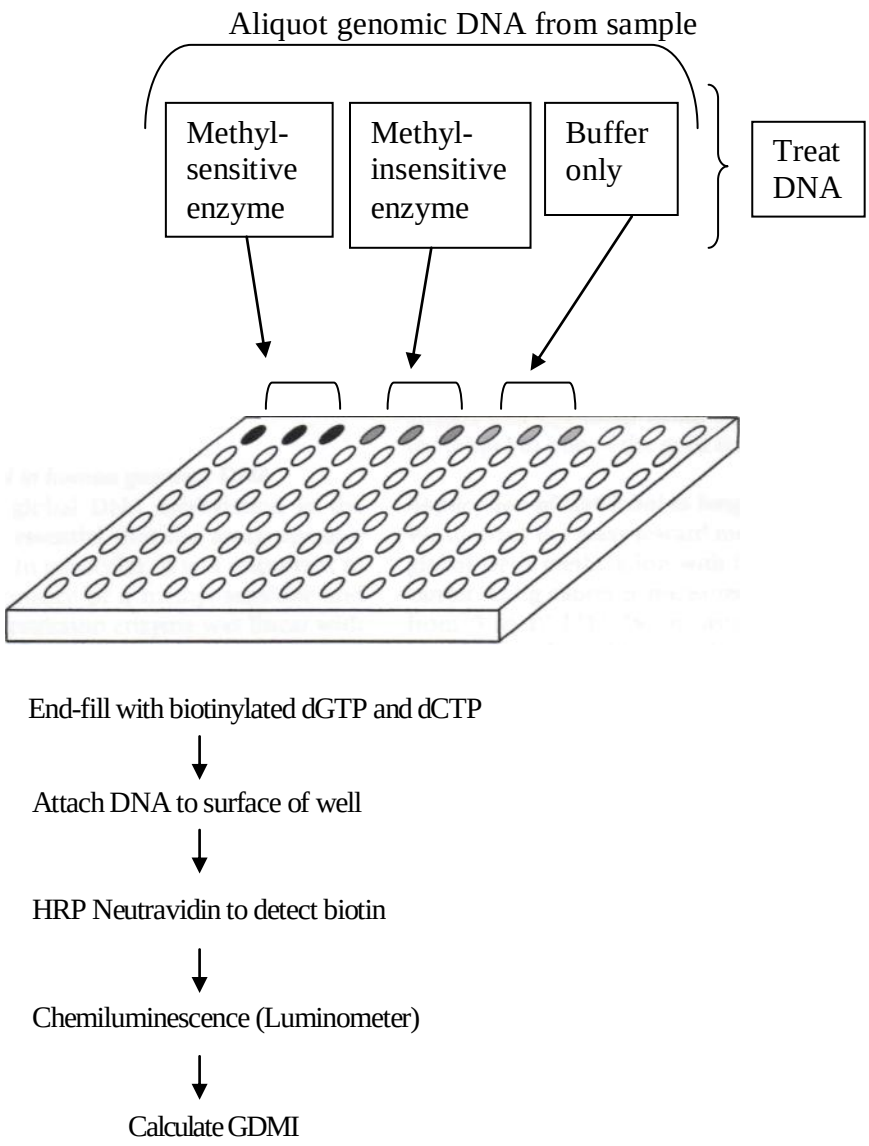


Figure 2. Visual example of the 96-well plates, taken from Anisowicz et al. (2008)

3.7 Neuron-Specific Enolase (NSE) Immunohistochemistry

For the NSE immunostaining, 40 µm serial coronal sections were cut through areas containing the CA1 area of the hippocampus and mPFC using a Reichert sliding microtome, and each area was sectioned from the most rostral to the most caudal. The desired areas were determined using atlas coordinates: CA1 – Bregma -2.28 mm to -6.84 mm; mPFC – Bregma 5.16 mm to -1.56 mm (Paxinos & Watson, 5th ed., 2005). Each section was placed, in serial order, in a micro-tube filled with anti-freeze. There were 8 micro-tubes such that every eighth of an area of brain tissue containing the CA1 of the hippocampus or mPFC was in each tube.

Free-floating tissue was stained in tissue culture wells to maintain serial sections. Tissue sections were washed 5 times for a period of 15 minutes per wash in tris-buffered saline (TBS) and then 15 minutes in methanol peroxide. The tissue was then blocked for 30 minutes in TBS with 3% Normal goat serum (NGS) (matched to host of 2º antibody – goat anti-rabbit IgG, peroxidase conjugated; Chemicon) & Triton. Sections were then incubated in primary antibody – polyclonal rabbit anti-NSE (AB951; Chemicon) used at 1:250 dilution in TBS with 1% NGS, without Triton, for 2 hours at room temperature followed by overnight incubation at 4°C. Sections were then washed 3 times at 15 minutes each in TBS. Sections incubated in secondary antibody were diluted 1:1000 for 1.5 hours in TBS with 1% NGS, and then washed again 3 times at 15 minutes each in TBS, after which it was incubated for 1 hour at room temperature in tertiary antibody – horseradish peroxidase-conjugated streptavidin at 1:1600 in TBS+ with Triton X-100 (no serum). The tissue was washed twice for 15 minutes per wash in TBS, and then once for 10 minutes in Tris buffer (no saline). NSE was detected using diaminobenzidine tetrahyrodchloride (DAB) reaction with 0.06% NiCl added to the sections for intensification. To kill the reaction, a final set of washes included four to five washes in TBS.

Sections were then mounted onto gelatin-coated slides, air dried, rinsed in distilled water, dehydrated and cover-slipped under cytoseal. Once cover-slipped, the sections were assessed for relative optical density (ROD) using computerized image analysis software. Density was determined as the total area covered by NSE stained neurons within a sampling area. Density was measured using MCID Image (Interfocus imaging, UK) attached to a light microscope (Nikon Optiphot) with a high-resolution device camera (Photometrics Cool Snap), and involved measurements of the light passing through the NSE-stained sampling areas. Each slide was placed under the microscope at 50 X magnification and the desired brain structures were defined using surrounding structures as markers. The CA1 region was defined as the dorsal most area of densely packed pyramidal cells within the hippocampus proper, recognized as a 'dorsal hump', just ventral to the corpus callosum. The area of the mPFC included the anterior cingulate cortex, prelimbic cortex and infralimbic cortex, and was determined as the innermost, medial to dorsal, regions of the brain tissue. Density was then determined by placing an open shaped cursor around a sample of the area of interest. The size of the shape was maintained by saving the settings in a file on the MCID program. A total of 4 samples per region were measured and the average of these 4 samples was used to determine ROD. Samples were determined based on quality of the staining for a given piece of tissue, as well as on how close the ROD values were to one another. For instance, an ROD value that was too high or too low compared to the values obtained from other samples in a given subject were not used. Otherwise, sampling was random. The ROD value, which is characteristic of how dense the staining is for each brain region, was thus determined by the MCID program. Values for these are shown as means per group ± SEM.

IV. DATA ANALYSIS

The statistical significance level was set at $\alpha = .05$ and non-significance was also reported in the results. Figures were only drawn for significant results. Calculation of the means and standard error, as well as the production of graphs, was performed using Microsoft Excel 2007, and data analyses were performed using the Statistical Package for the Social Sciences (SPSS) 16.0 for Windows.

4.1 Blood Alcohol Concentrations

A 2-way ANOVA (Supplement*Sex) was performed to determine whether these two factors have any effect on BAC levels.

4.2 Body Weights

Tests of within-subjects effects were used to measure the body weight of the dams on GD 1, 5, 10, 15, and 20. Body weight data for the pups were separated by sex and repeated measures ANOVA was performed on the pups' body weights from PD 2 to PD 21. A 3 x 2 ANOVA (ET Treatment*Supplement) was performed on the percent change in body weight of all animals from the first day of treatment to the day after the supplement was terminated.

4.3 Relative Optical Density

To determine differences in ROD among the groups, a 3-way ANOVA (ET treatment X supplement X sex) was performed on the calculated means, and this was used to analyze main and interactive effects in the CA1 and mPFC separately. Simple main effects were used to analyze any interactions, and Tukey's HSD *post hoc* tests were done where appropriate.

4.4 DNA Methylation

To determine the net luminescence, the average of the 3 values obtained from the no-enzyme (buffer) control was subtracted from the average of the 3 values obtained from the HPaII and MSP I enzyme values of each subject. To calculate GDMI, the average net luminescence value obtained for the methylation sensitive enzyme (HPAII) was divided by the average net luminescence value obtained for the methylation insensitive enzyme (Msp I) of each subject. The GDMI values for each subject was then averaged based on experimental group, and these means were used when analyzing group differences. A 3-way ANOVA (ET treatment X supplement X sex) was used to determine the main and interactive effects on differences in the mean GDMI values of each experimental group. Simple main effects were used to analyze any interactions, and Tukey's HSD *post hoc* tests were done where appropriate.

V. RESULTS

5.1 Physical Parameters

For all repeated measures analyses, Mauchly's test of sphericity showed that the assumption of equal variances was violated (p's < .01). Thus the degrees of freedom were corrected using the Greenhouse-Geisser estimates of sphericity (epsilon < .75).

Blood Alcohol Concentrations (BACs). There were no significant differences found in the average BAC levels between male and female subjects, as well as between subjects receiving the choline supplement and those receiving saline. These data are shown in Table 1.

Dam Body Weights. Tests of within-subjects effects showed a significant main effect of gestational day (GD) on body weight, $F(1.46, 26.35) = 271.56$, $p < .01$. This main effect was due to normal weight gain during pregnancy (data not shown).

Male Body Weights (PD 2 – 21). A repeated measures ANOVA, with Treatment and Supplement as between factors and Day from PD 2 to 21 as the repeated measure, was performed on the body weights across days. Tests of within-subjects effects showed a significant main effect of Day, $F(1.7, 91.97) = 4810.45$, $p < .01$ on body weight, and a significant Day x Treatment interaction, $F(3.41, 91.97) = 3.175$, $p < .05$. *Post-hoc* analyses revealed the ET group weighed significantly less than the NC groups from PD 4 to 21 ($p < .05$), and marginally less than the IC group only on PDs 11 and 12 ($p = .067$ and .054 respectively). This suggests that stress (specifically of the administration procedure) may account for some of the effects seen.

Body weight data for the males are shown in Table 2. Importantly, there was no impact of supplement on body weight in males.

A 3 x 2 ANOVA was performed on the percent change in body weight from the first day of treatment to the day after the supplement was terminated - body weight on PD 21 minus body weight on PD 2, divided by the body weight on PD 2, with the final result X 100. Test of between-subjects effects revealed a significant main effect of treatment, $F(2, 54) = 5.397$, $p < .01$. Post-hoc analyses further showed that the ET groups continued to gain weight at a slower rate than the NC groups ($p < .01$), while the ET groups only marginally differed from the IC groups ($p = .065$). The two control groups did not differ from each other. Data for percent change in body weight are shown in Figure 3.

Female Body Weights (PD 2 – 21). A repeated measures ANOVA, with Treatment and Supplement as between factors and Day from PD 2 to 21 as the repeated measures, was performed on body weight across days. Tests of within-subjects effects showed a significant main effect of Day, $F(1.67, 89.94) = 6272.28$, $p < .01$, on body weight, and a significant Day x Treatment interaction, $F(3.33, 89.94) = 3.982$, $p < .01$. *Post-hoc* analyses revealed the ET groups weighing less than the NC groups from PD 4 to 21 (p's $< .05$), and the IC groups from PD 4 to 14, PD 16 and PD 20 (p's $< .05$). Body weight data for the females are shown in Table 3. There was no impact of supplement on body weight in females.

A 3 x 2 ANOVA was performed on the average percent change in body weight from the first day of treatment to the day after the supplement was terminated (body weight on PD 21 minus body weight on PD 2, divided by the body weight on PD 2, with the final result X 100)). Test of between-subjects effects revealed a significant main effect of treatment, $F(2, 54) = 7.143$, $p < .01$. Post-hoc analyses further showed that the ET groups continued to gain weight at a

slower rate than both control groups ($p < .01$). The two control groups did not differ from each other. Data are shown in Figure 4.

5.2 Density of NSE Staining

Region mPFC: Figure 5 (a) displays ROD levels after NSE staining in the mPFC in an ET male subject supplemented with choline. The legend to the right shows the optical density by color. Figure 5 (b) shows the ROD levels in the mPFC brain region in an IC male subject supplemented with saline.

A 3-way ANOVA (Treatment X Supplement X Sex) was performed on the ROD values of the mPFC. Tests of between-subject effects revealed a significant main effect of Supplement on the average ROD, $F (1, 108) = 4.374$, $p < .05$ where subjects supplemented with choline showed a higher average density value than those supplemented with saline, regardless of sex and treatment group. These data are shown in Figure 7.

There were also trends towards significant main effects of Sex, $F (1, 108) = 3.623$, $p = .06$, and Treatment, $F (2, 108) = 2.779$, $p = .067$, in the mPFC. Males showed a marginally higher density value than the females, regardless of treatment and supplemental condition (see Figure 8). Fisher's LSD showed that subjects in the ET groups had significantly lower density value than those in the NC groups ($p < .05$), while the density values of the IC groups were marginally lower than those of the NC groups ($p = .06$). The ET and IC groups did not significantly differ from each other (see Figure 9).

Region CA1: Figure 6 (a) represents ROD levels in the CA1 region in an NC male subject supplemented with choline while Figure 6 (b) shows the same region in an ET male

subject supplemented with saline. A 3-way ANOVA (Treatment X Supplement X Sex) performed on the average ROD values of the CA1 region showed no main or interactive effects.

5.3 Methylation

A 3-way ANOVA (Treatment x Supplement x Sex) was performed on the GDMI for all treatment and supplement groups and found no significant differences between the sexes or the two control groups. Thus, analyses were further performed with sex and control groups combined.

Region PFC: A 2 x 2 ANOVA (Treatment x Supplement) revealed a main effect of Treatment on GDMI in the mPFC, $F(1, 55) = 5.899$, $p < .05$, where ET subjects showed significantly lower GDMI values compared to controls, regardless of supplemental condition. These lower values mean that ET-treated animals showed an increased state of methylation (hypermethylation) compared to control animals. There was also a significant Treatment x Supplement effect in the PFC, $F(1, 55) = 10.718$, $p < .01$. Post hoc analysis showed that, compared to the saline-supplemented ET subject, choline supplementation significantly increased GDMI values in the ET-treated subjects (reflecting decreased methylation). However, there was a decrease in GDMI values (thus, increased methylation) in control subjects receiving choline supplementation, which was significantly different from control subjects receiving saline. These data are shown in Figure 10a.

Region Hippocampus: A 2 x 2 ANOVA (Treatment x Supplement) revealed a main effect of treatment on GDMI in the hippocampus, $F(1, 55) = 9.048$, $p < .01$. ET subjects showed significantly lower GDMI values compared to controls, regardless of the supplemental condition, indicating an increased state of methylation (hypermethylation) compared to control animals.

There was also a significant Treatment x Supplement effect in the hippocampus, $F(1, 55) = 5.698$, $p < .05$. Post hoc analyses showed that choline supplementation significantly increased GDMI values in the ET-treated subjects (reflecting decreased methylation), but decreased GDMI values in controls, all compared to saline-supplemented subjects. These data are shown in Figure 10b.

Table 1. Mean BACs (mg/dl) and SEMs for ET treated male and female subjects receiving either choline or saline supplement.

Gender	**Supplement**	
	Saline	Choline
Male	358.2 ± 18.2	367.6 ± 17.6
Female	365.1 ± 22.5	365.3 ± 25.9

Table 2. Mean body weight (g) and SEMs in male subjects across treatment and supplement. The * and ** indicate significant difference from the NC group at p's < .05 and .01 respectively; the ° and °° indicate significant difference from the IC group at p's < 05 and .01 respectively

CHOLINE											
	PD 2	**PD 3**	**PD 4**	**PD 5**	**PD 6**	**PD 7**	**PD 8**	**PD 9**	**PD 10**	**PD 11**	**PD 12**
ET	7.3 ± 0.2	7.9 ± 0.3	8.9 ± 0.4*	9.9 ± 0.5*	11.6 ± 0.6*	13.2 ± 0.6*	14.9 ± 0.7*	17.0 ± 0.8*	18.9 ± 0.9*	21.1 ± 0.8*	23.2 ± 0.8*
IC	7.1 ± 0.3	8.3 ± 0.4	9.6 ± 0.4	11.2 ± 0.5	12.9 ± 0.6	14.7 ± 0.7	16.5 ± 0.7	19.0 ± 0.8	21.0 ± 0.9	23.5 ± 1.0	25.7 ± 1.0
NC	7.5 ± 0.2	8.6 ± 0.3	10.1 ± 0.3	11.7 ± 0.4	13.6 ± 0.5	15.5 ± 0.5	17.6 ± 0.5	19.8 ± 0.5	22.2 ± 0.5	25.0 ± 0.5	27.0 ± 0.4
	PD 13	**PD 14**	**PD 15**	**PD 16**	**PD 17**	**PD 18**	**PD 19**	**PD 20**	**PD 21**		**% Change PD 2 -21**
ET	25.7 ± 0.8*	28.5 ± 0.9*	30.6 ± 0.9*	32.9 ± 1.0*	35.0 ± 1.1*	37.0 ± 1.1*	39.9 ± 1.4*	44.1 ± 1.6*	48.1 ± 1.9*		560.4 ± 12.6**
IC	28.0 ± 1.0	30.4 ± 1.0	32.2 ± 1.0	34.8 ± 1.0	36.8 ± 1.0	38.7 ± 1.1	41.3 ± 1.3	45.6 ± 1.5	50.1 ± 1.5		605.6 ± 14.3
NC	29.3 ± 0.4	31.9 ± 0.5	34.1 ± 0.6	36.2 ± 0.6	38.3 ± 0.7	40.5 ± 0.7	43.7 ± 0.8	48.2 ± 1.0	52.5 ± 1.4		602.7 ± 9.9
SALINE											
	PD 2	**PD 3**	**PD 4**	**PD 5**	**PD 6**	**PD 7**	**PD 8**	**PD 9**	**PD 10**	**PD 11**	**PD 12**
ET	7.1 ± 0.2	7.9 ± 0.2	9.0 ± 0.2*	10.0 ± 0.2*	11.6 ± 0.3*	13.3 ± 0.3*	15.3 ± 0.4*	17.4 ± 0.4*	19.1 ± 0.4*	21.5 ± 0.4*	23.5 ± 0.4*
IC	7.1 ± 0.1	8.0 ± 0.3	9.3 ± 0.4	10.8 ± 0.6	12.4 ± 0.7	14.4 ± 0.7	16.2 ± 0.8	18.3 ± 1.0	20.4 ± 1.2	23.0 ± 1.2	25.0 ± 1.2
NC	7.1 ± 0.2	8.4 ± 0.4	9.9 ± 0.4	11.5 ± 0.6	13.4 ± 0.7	15.1 ± 0.7	17.1 ± 0.7	19.4 ± 0.7	21.7 ± 0.9	24.5 ± 0.9	26.5 ± 1.0
	PD 13	**PD 14**	**PD 15**	**PD 16**	**PD 17**	**PD 18**	**PD 19**	**PD 20**	**PD 21**		**% Change PD 2 -21**
ET	25.7 ± 0.5*	28.3 ± 0.5*	30.4 ± 0.6*	32.7 ± 0.6*	34.8 ± 0.7*	36.6 ± 0.8*	39.0 ± 0.9*	42.7 ± 0.8*	47.2 ± 0.9*		561.6 ± 8.4**
IC	27.0 ± 1.1	29.7 ± 1.2	31.6 ± 1.2	34.0 ± 1.2	36.0 ± 1.3	37.8 ± 1.3	40.7 ± 1.4	44.5 ± 1.5	48.9 ± 1.8		594.6 ± 27.9
NC	28.5 ± 1.0	31.1 ± 1.1	33.4 ± 1.2	35.5 ± 1.3	37.7 ± 1.4	39.3 ± 1.5	42.2 ± 1.5	46.8 ± 1.8	51.7 ± 2.0		627.9 ± 20.8

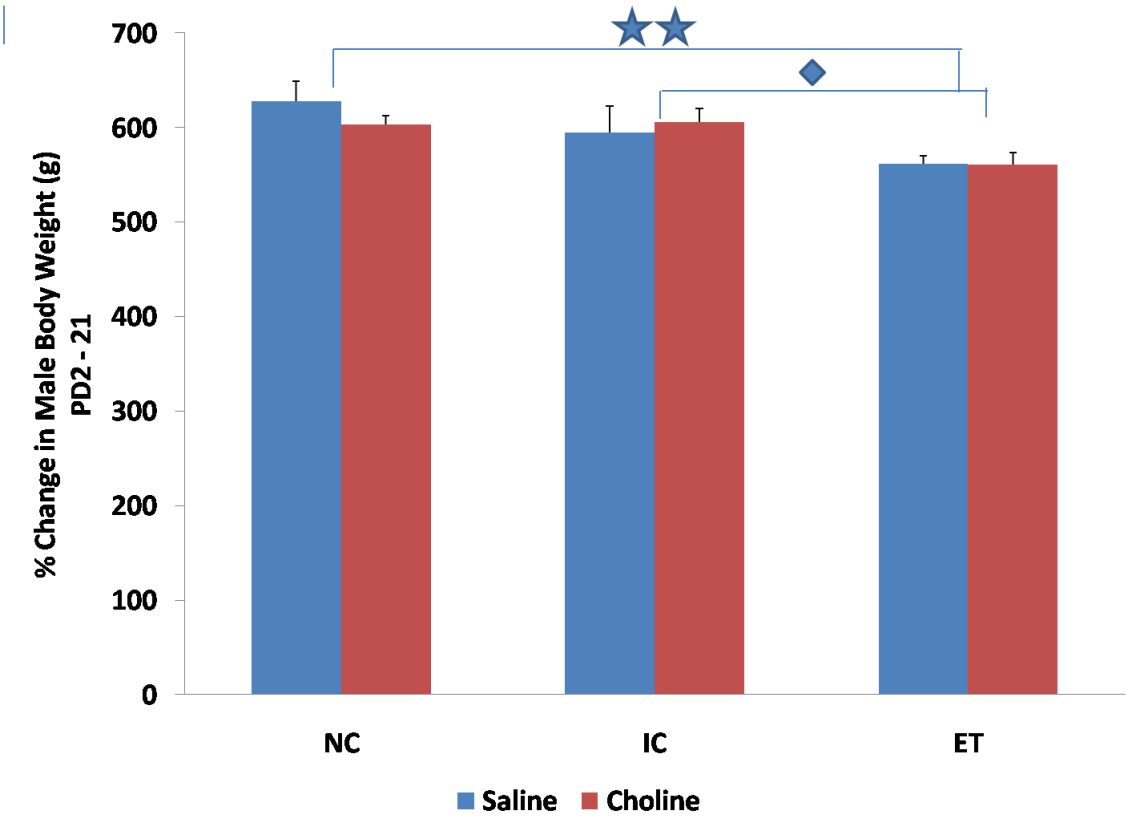


Figure 3: Average percent change in body weight for male subjects from PD 2 to 21 across treatment and supplement groups. There was a significant main effect of treatment, where the ET groups gained weight at a significantly slower rate than the NC groups ($p < .01$), indicated by ★★ and marginally slower than the IC group, indicated by ◆. Error bars represent the SEM.

Table 3. Mean body weight (g) and SEMs in female subjects across treatments and supplement. The * and ** indicate significant differences from the NC group with p's < .05 and .01 respectively; the ° and °° indicate significant differences from the IC group with p's < .05 and .01 respectively

CHOLINE											
	PD 2	**PD 3**	**PD 4**	**PD 5**	**PD 6**	**PD 7**	**PD 8**	**PD 9**	**PD 10**	**PD 11**	**PD 12**
ET	6.9 ± 0.1	7.7 ± 0.2	8.7 ±0.2*°	9.8 ±0.3*°	11.3 ±0.4*°	12.8 ±0.4*°	14.8 ±0.5*°	16.7 ±0.5*°	18.6 ±0.6*°	20.9 ±0.6*°	23.0 ±0.5*°
IC	6.8 ± 0.2	7.9 ± 0.2	9.3 ± 0.2	10.8 ± 0.3	12.6 ± 0.4	14.4 ± 0.5	16.3 ± 0.5	18.6 ± 0.6	20.7 ± 0.6	23.1 ± 0.7	24.9 ± 0.7
NC	6.8 ± 0.2	7.8 ± 0.3	9.1 ± 0.3	10.5 ± 0.4	12.2 ± 0.5	14.1 ± 0.5	16.1 ± 0.5	18.3 ± 0.5	20.4 ± 0.5	23.1 ± 0.5	25.1 ± 0.5
	PD 13	**PD 14**	**PD 15**	**PD 16**	**PD 17**	**PD 18**	**PD 19**	**PD 20**	**PD 21**		**% Change PD 2 - 21**
ET	25.1 ±0.6*°	27.5 ±0.6*°	29.6 ± 0.6*	31.7 ±0.8*°	33.7 ± 0.8*	35.3 ± 0.9*	37.5 ± 1.0*	41.7 ±1.0*°	45.8 ± 1.0*		569.2 ± 11.6**°°
IC	27.0 ± 0.7	29.4 ± 0.8	31.4 ± 0.9	33.8 ± 1.0	35.8 ± 1.1	37.4 ± 1.1	39.8 ± 1.2	44.8 ± 1.3	49.1 ± 1.4		628.0 ± 15.2
NC	27.2 ± 0.6	29.6 ± 0.5	31.9 ± 0.6	33.9 ± 0.7	35.9 ± 0.8	37.6 ± 0.8	40.3 ± 1.0	44.7 ± 1.1	48.3 ± 1.4		611.4 ± 8.5
SALINE											
	PD 2	**PD 3**	**PD 4**	**PD 5**	**PD 6**	**PD 7**	**PD 8**	**PD 9**	**PD 10**	**PD 11**	**PD 12**
ET	6.7 ± 0.1	7.5 ± 0.2	8.6 ±0.3*°	9.6 ±0.3*°	11.1 ±0.4*°	12.8 ±0.4*°	14.6 ±0.4*°	16.4 ±0.5*°	18.3 ±0.5*°	20.6 ±0.6*°	22.6 ±0.5*°
IC	6.9 ± 0.3	8.0 ± 0.3	9.3 ± 0.4	10.9 ± 0.6	12.7 ± 0.6	14.4 ± 0.7	16.4 ± 0.8	18.6 ± 0.9	20.6 ± 0.9	23.1 ± 1.0	24.9 ± 1.0
NC	6.9 ± 0.2	8.2 ± 0.2	9.6 ± 0.3	11.1 ± 0.5	12.8 ± 0.6	14.5 ± 0.6	16.6 ± 0.7	18.9 ± 0.8	21.1 ± 0.8	23.5 ± 0.8	25.8 ± 0.8
	PD 13	**PD 14**	**PD 15**	**PD 16**	**PD 17**	**PD 18**	**PD 19**	**PD 20**	**PD 21**		**% Change PD 2 - 21**
ET	24.9 ±0.6*°	27.0 ±0.6*°	29.3 ± 0.8*	31.1 ±0.7*°	33.2 ± 0.8*	34.9 ± 0.8*	37.5 ± 0.9*	41.3 ±1.0*°	46.3 ± 1.2*		589.5 ± 13.4**°°
IC	26.9 ± 1.0	29.1 ± 1.0	31.3 ± 1.0	33.7 ± 1.0	35.5 ± 1.1	37.0 ± 1.1	40.0 ± 1.2	44.4 ± 1.3	48.8 ± 1.4		614.3 ± 12.0
NC	27.8 ± 0.9	30.1 ± 0.9	32.5 ± 1.0	34.6 ± 1.0	36.7 ± 1.1	38.5 ± 1.2	41.5 ± 1.4	45.8 ± 1.6	50.4 ± 1.6		628.0 ± 13.5

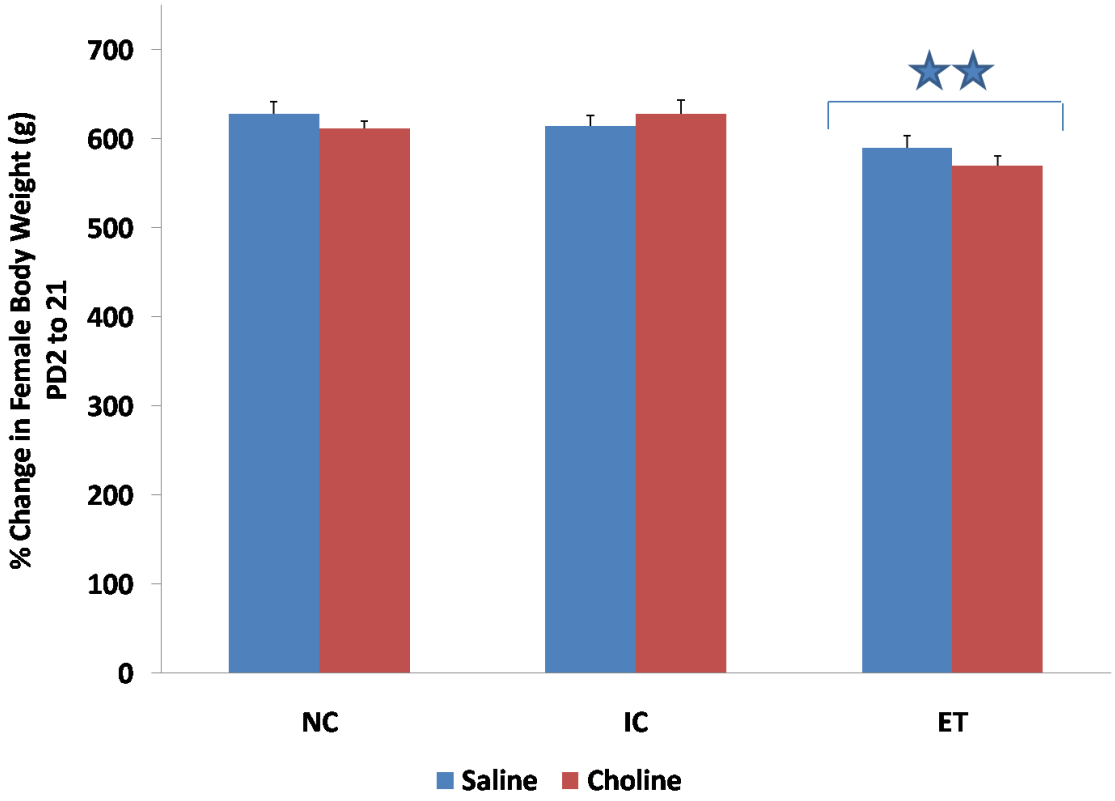
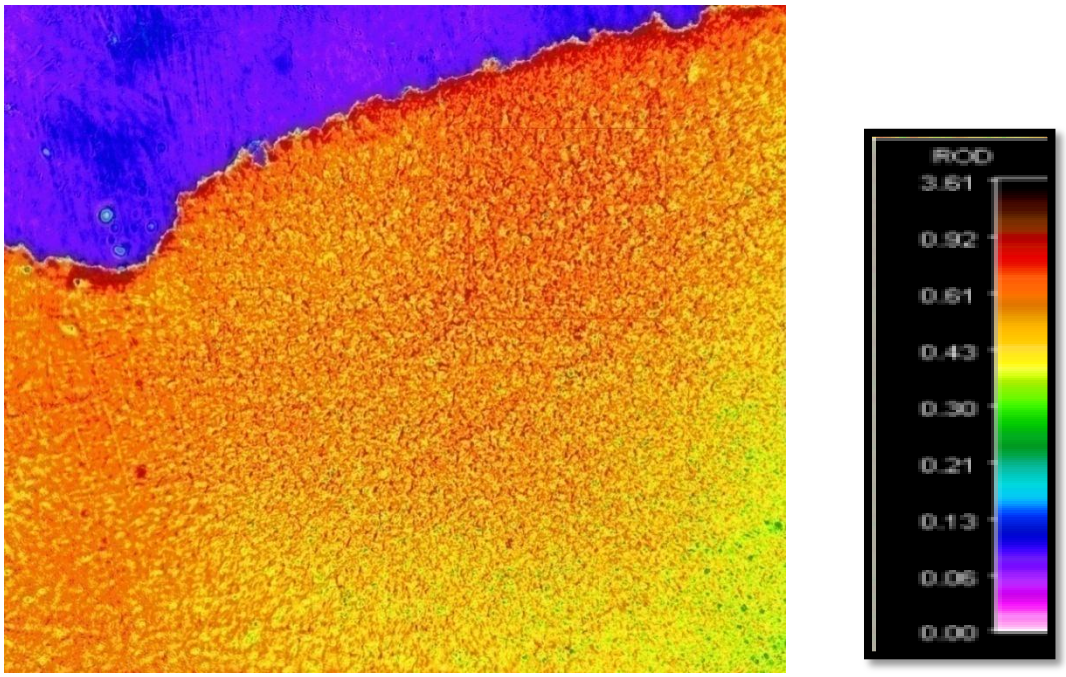


Figure 4: Average percent change in body weight for female subjects from PD 2 to 21 across treatment and supplement groups. There was a significant main effect of treatment, where the ET groups gained weight at a significantly slower rate than both control groups ($p < .01$), indicated by ★★. Error bars represent the SEM.

a)



b)

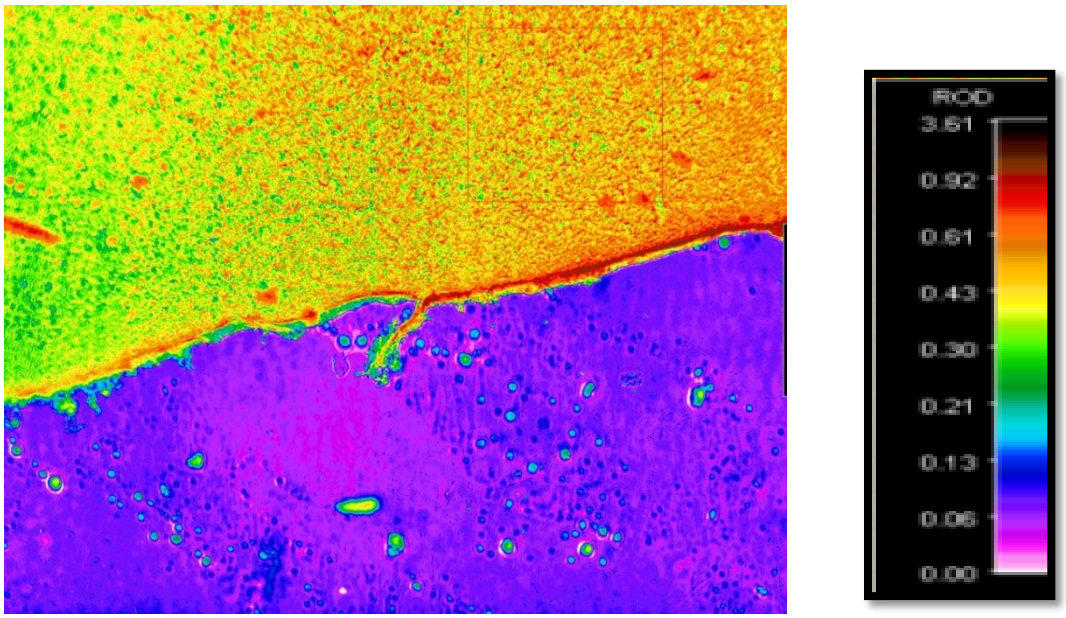
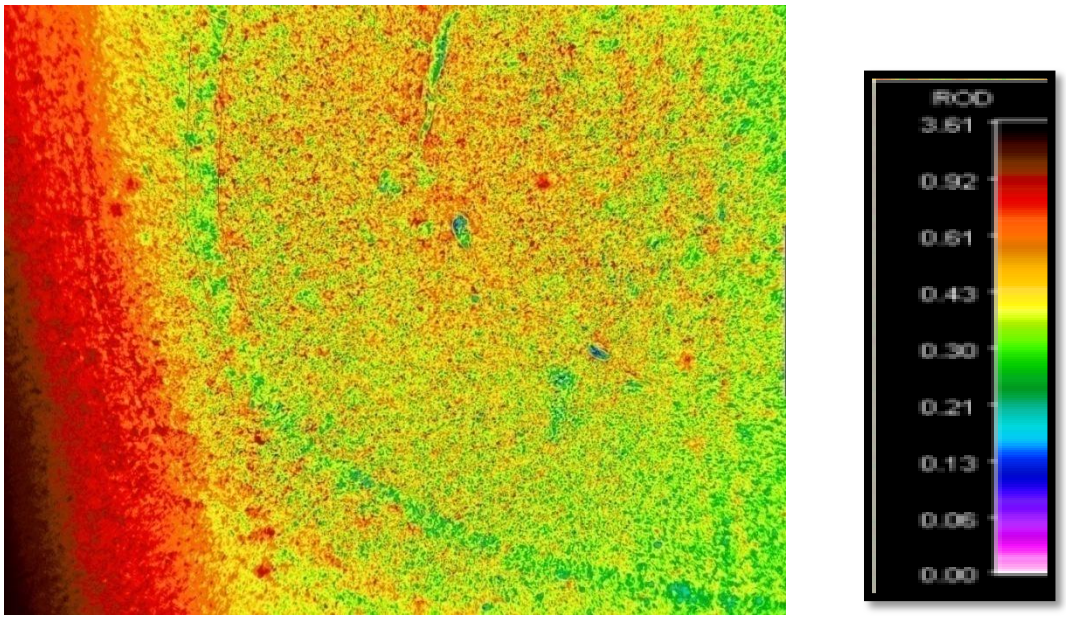


Figure 5: (a) ROD levels after NSE staining in the mPFC in an ET male subject supplemented with choline. The legend to the right shows the optical density by color. (b) ROD levels in the mPFC of an IC male subject supplemented with saline.

(a)



(b)

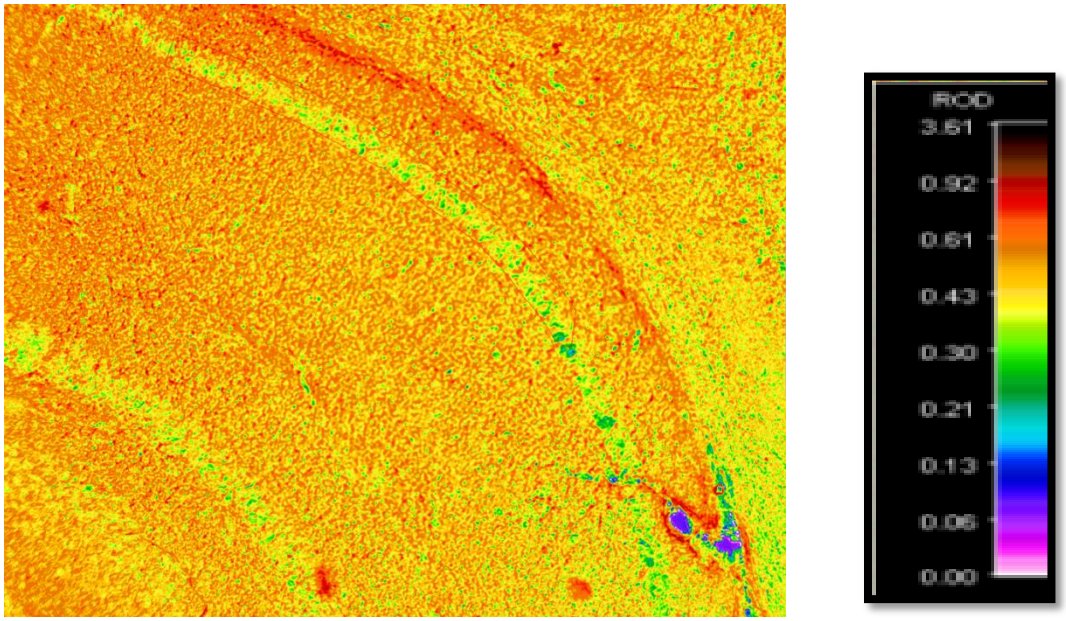


Figure 6: (a) ROD levels after NSE staining in the CA1 brain region of an NC male subject supplemented with choline. The legend to the right shows the optical density by color. (b) ROD levels in the CA1 region of an ET male subject supplemented with choline.

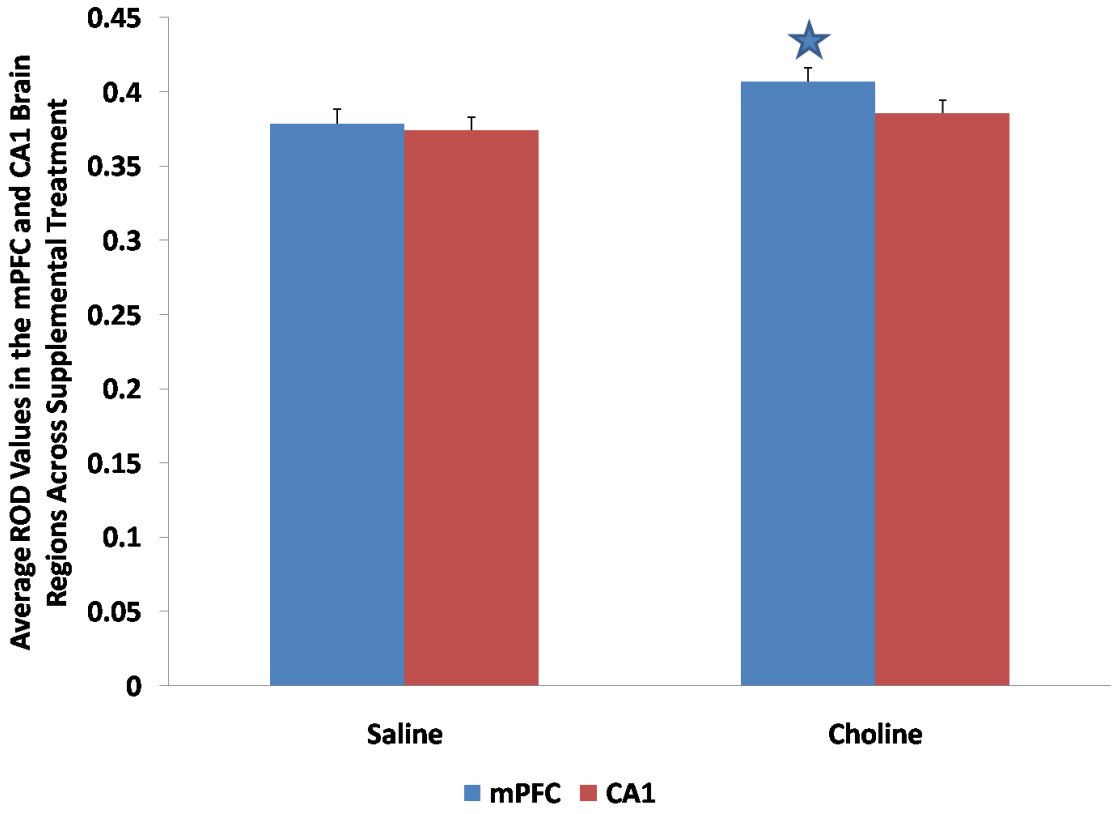


Figure 7: Average ROD values in the mPFC and CA1 brain regions across supplemental groups. There was a significant main effect of supplement in the mPFC, where the choline groups showed significantly higher density values than the saline groups ($p < .05$) indicated by ★. Data are collapsed across treatment and sex. Error bars represent the SEM.

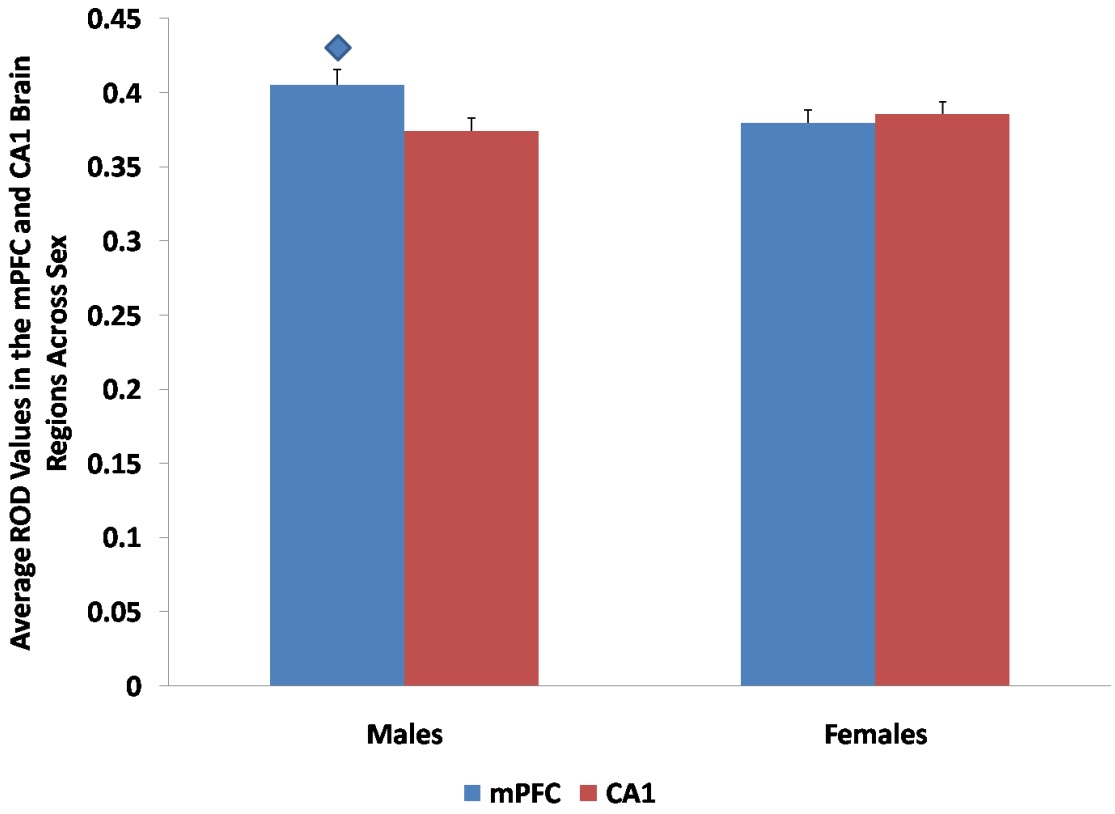


Figure 8: Average ROD values in the mPFC and CA1 brain regions across sex. There was a trend toward a main effect of sex in the mPFC, where males showed marginally higher density values than females ($p = .06$), indicated by ◆. Data are collapsed across supplement and treatment. Error bars represent the SEM.

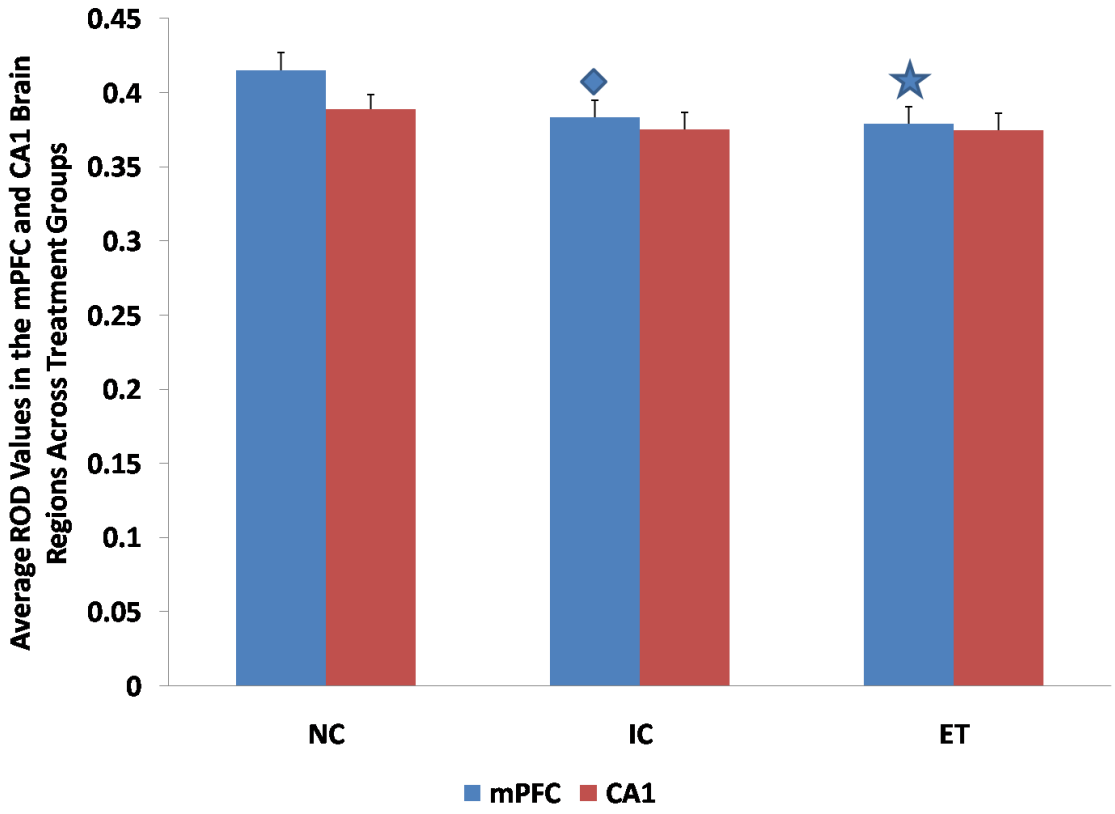


Figure 9: Average ROD values in the mPFC and CA1 brain regions across treatment. There was a trend towards a main effect of treatment in the mPFC and LSD *post hoc* tests showed that subjects in the ET group showed significantly lower density values than those in the NC group ($p < .05$), indicated by ★ and subjects in the IC group showed marginally lower density than those in the NC group, indicated by ◆. There were no effects in the hippocampus. Data are collapsed across supplement and sex. Error bars represent the SEM.

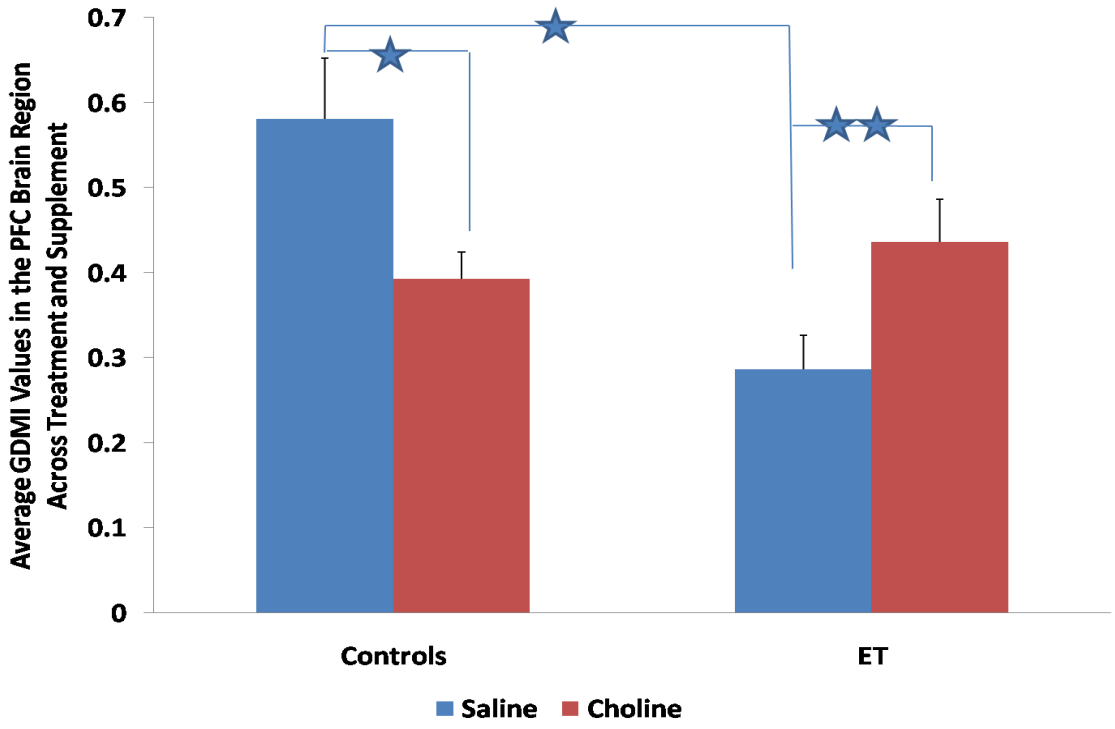


Figure 10a: Average GDMI values in the PFC brain region across treatment and supplement condition. There was a significant main effect of treatment, where subjects in the ET group showed significantly lower GDMI values than those in the combined control group ($p < .05$), indicated by ★. There was also a significant treatment x supplement interaction ($p < .01$), indicated by ★★ where choline led to a significant decrease in methylation in the ET group, but a significant increase in methylation in the combined control group. Data are collapsed across sex. Error bars represent the SEM.

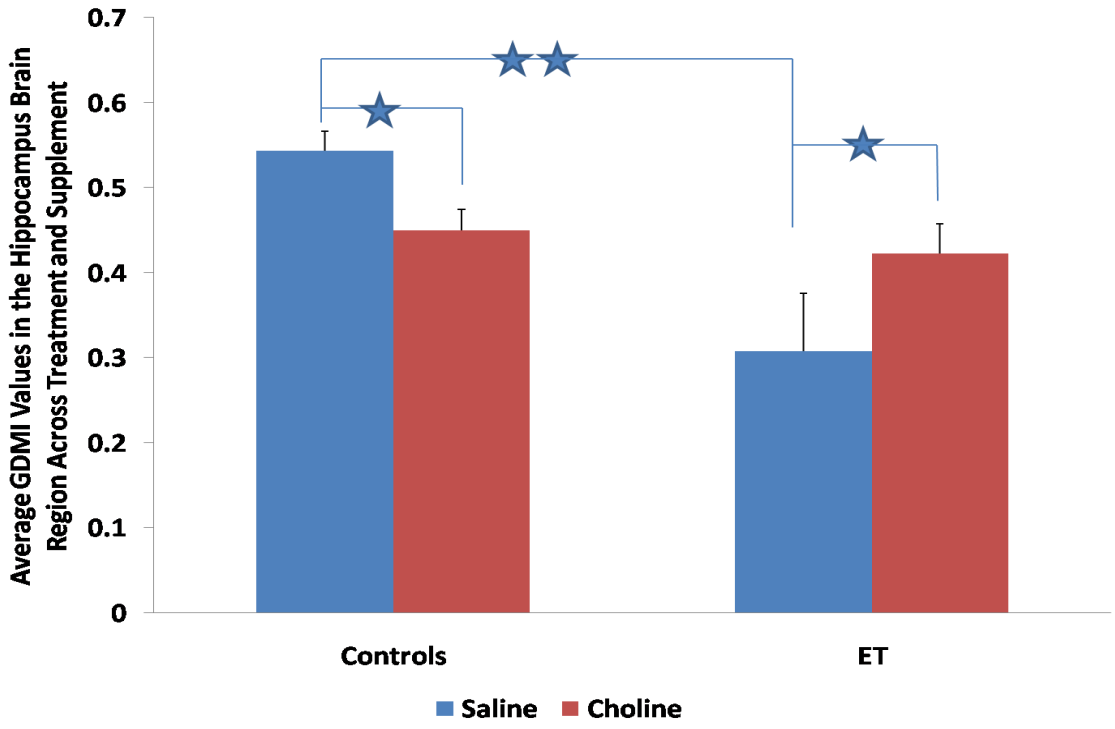


Figure 10b: Average GDMI values in the hippocampus brain region across treatment and supplement condition. There was a significant main effect of treatment, where subjects in the ET group showed significantly lower GDMI values than those in the combined control group ($p < .01$), indicated by ★★. There was also a significant treatment x supplement interaction ($p < .05$), indicated by ★ where choline led to a significant decrease in methylation in the ET group, but a significant increase in methylation in the combined control group. Data are collapsed across sex. Error bars represent the SEM.

VI. DISCUSSION

6.1 Summary of Results

This study is the first to show in a rat model of FASD that alcohol exposure during a period equivalent to the third trimester of human pregnancy causes changes in global DNA methylation in the PFC and hippocampus, and these changes are evident in the early adolescent stage. Developmental alcohol exposure led to a state of increased methylation (hypermethylation) in both these brain areas, compared to control subjects. This study is also the first to show significant changes in global DNA methylation in ethanol-exposed animals after supplementation with the nutrient choline. Choline given to ethanol-exposed subjects from PD 2 to 20 led to a decrease in methylation, while control animals showed a state of increased methylation after choline supplementation in both brain regions. The effects of choline were opposite in the two groups of animals.

The neuroanatomical findings did not mirror the findings on DNA methylation. Supplementation with choline led to an increase in the concentration of cells (as measured by density of immunohistochemical staining for NSE) only in the mPFC, regardless of sex and treatment condition. There was also a trend towards main effect of sex and a main effect of treatment in the mPFC, where males showed a marginally higher concentration of cells than the females, and the ET group showed a marginally lower cell concentration than the NC group. No differences in NSE staining were found in the CA1 brain region of the hippocampus, and no differences were found due to ethanol exposure in either brain area.

6.2 Ethanol- induced changes in methylation states in the literature

The finding that developmental alcohol causes increased methylation in the DNA of affected subjects did not agree with the first hypothesis. Given the initial literature (Garro et al., 1991) that reported a state of hypomethylation after exposure to gestational alcohol, and that decreases in methylation have been implicated in reductions in learning and memory abilities, it was hypothesized that developmental alcohol during the third trimester equivalent would lead to decreases in global DNA methylation. However, the present findings corroborate a more recent report demonstrating that developmental alcohol exposure increases the probability of transcriptional silencing (hypermethylation) of the allele, *Agouti viable yellow* (*Avy*), in the mouse (Kaminen-Ahola, Ahola, Maga, Mallitt, Fahey et al., 2010). Pregnant *a/a* (black coat color) female mice, were bred with *Avy/a* (yellow coat color) males and given an *ad libitum* diet containing 10% (v/v) ethanol from GD 0.5 to 8.5 (equivalent to the first trimester of human pregnancy). While ethanol did not affect Mendelian inheritance such that both alcohol-treated and control pregnant mice produced the same numbers of offspring with the *Avy/a* gene, ethanol exposure led to the production of a significantly higher percentage of pseudoagouti *Avy/a* offspring, compared to controls. In other words, gestational alcohol caused increased methylation, or transcriptional silencing, at the *Avy* allele, resulting in more offspring carrying the *Avy/a* gene to show an agouti-colored (black) phenotype.

On the other hand, the alcohol-induced increase in methylation is not compatible with the Garro et al. (1991) study, which is the earliest known study to examine methylation changes due to early prenatal alcohol exposure. However, the timing of both alcohol administration and DNA methylation analysis are different. Whereas the present study looked at alcohol exposure during the third trimester equivalent and methylation states during the adolescent period, Garro et al.

(1991) administered alcohol from GD 9 to 11 and measured DNA methylation states on GD 11 fetuses. Another difference is that the earlier study measured the overall methylation state of the entire fetus (or at least the specific region of interest was not mentioned). In the present study, methylation states were measured specifically in the hippocampus and mPFC brain regions.

Similar to Garro et al. (1991), another study examining developmental alcohol on changes in DNA methylation states also examined whole embryo tissue (Liu et al., 2009b). The researchers examined methylation changes based on genes associated with GC content including high (HCP), intermediate (ICP) and low CpG density (LCP), as well as whether the alcohol-exposed embryo had the neural tube opened (NTO) or closed (NTC). Alcohol exposure led to significantly more genes with an increase in methylation levels in HCP and significantly more genes in a state of hypomethylation in LCP. In NTO alcohol-exposed embryos, significantly more genes showed demethylation and, in NTC alcohol-exposed embryos, significantly more genes showed a state of hypermethylation compared to control embryos. Moreover, there were significantly more alterations in methylation status in the NTO group compared to the NTC group. In the NTO group, a greater number of genes in the HCP region and LCP region showed significantly more genes displaying hypermethylation and hypomethylation, respectively, compared to the same regions in the NTC group. These findings suggest that differential findings in methylation states in the literature may result from differences of the state of the embryos and suggest that future research should focus on understanding ethanol-induced methylation changes as a function of CpG density profiles of a given region of interest.

Given that both hypo- and hypermethylation can lead to deficits in brain function and behavior, it is possible that developmental alcohol has differential effects on DNA methylation patterns based on the timing, length and route of administration, and the developmental stage at

which methylation patterns are examined. As can be extrapolated from the Liu et al. (2009b) study, factors may also include whether there is an alcohol-related neural tube defect and the CpG density of a given region of interest. These assumptions are supported by the fact that the teratogenic effects of alcohol are based on the relationship between dosage and timing of exposure, where higher concentrations at critical periods of development lead to specific and more severe deficits in humans (O'Leary, Nassar, Kurinczuk & Bower, 2009a; O'Leary, Bower, Zubrick, Geelhoed, Kurinczuk et al., 2009b; O'Leary, Nassar, Zubrick, Kurinczuk, Stanley et al., 2010) and in animal models (Miller, 1995; Gohlke, Griffith & Faustman, 2005; O'Leary-Moore, McMechan, Mathison, Berman & Hannigan, 2006; Hunt, Jacobsen & Torok, 2009). Even though our understanding of the methylation patterns throughout gestation and early development is not complete, studies have reliably shown that DNA methylation states not only show drastic changes under normal conditions during early development (Reik, Dean & Walker, 2001; Santos, Hendrich, Reik & Dean, 2002), but that the same teratogen can have contrasting effects on DNA methylation patterns depending on the area of interest (Pogribny, Ross, Wise, Pogribna, Jones et al., 2006; Pogribny et al., 2008). This latter group showed that while dietary methyl deficiency in a rat model causes hypomethylation in the liver, this same diet leads to hypermethylation in brain cortical tissue. The present study is among the first to show evidence at the molecular level of alterations in DNA methylation patterns due to developmental alcohol exposure. It is expected that these findings will ultimately lead to understanding the basis through which alcohol teratogenicity leads to physical, cognitive and behavioral anomalies in affected offspring.

6.3 Comparable deficits of DNA hypermethylation and developmental alcohol

In the current experiment, alcohol exposure during the period equivalent to the third trimester in human pregnancy was enough to cause an increase in global DNA methylation in both the hippocampus and mPFC in juvenile rats. Studies have shown that while hypomethylated regions of the DNA show increases in gene expression, hypermethylation is associated with the transcriptional silencing of a gene. Both forms of the methylation states have been observed to be a molecular abnormality that is evident in many disease states (Bird, 2002; Holliday & Pugh, 1975; Jenuwein, 2006). For instance, in cancer studies, hypermethylation leads to the silencing of tumor suppressor gene promoters (Jones & Baylin, 2002) while hypomethylation leads to continued expression of the gene responsible for aberrant cell growth (Feinberg & Tycko, 2004).

Individuals who are born with mental retardation, a defect of the fragile X mental retardation 1 (FMR1) gene, also show hypermethylation of the gene (Crawford, Acuna & Sherman, 2001; Jiang et al., 2004). It is important to note that this gene has been shown to be a folate-sensitive fragile site that is strongly affected by a diet deficient in folic acid and thymidine (Robertson, 2005). As will be discussed later on, it is postulated that prenatal alcohol exposure may be linked to effects on the folate pathway to DNA methylation, and may be a molecular mechanism through which this teratogen is causing deficits in affected offspring, such as mental retardation and deficits in memory performance.

Some studies have shown an association between DNA hypermethylation and increases in the levels and expression of certain genes and proteins. For instance, Pogribny et al. (2008) have found a strong link between DNA hypermethylation and a significant increase in the methyl-CpG-binding 2 (MeCP2) protein. Studies examining effects of hypermethylation and those examining effects due to overexpression of the MeCP2 protein have linked these two

molecular mutations to disorders such as Rett syndrome (Collins, Levenson, Vilaythong, Richman, Armstrong et al., 2004; Jiang et al., 2004; Muhle et al., 2004), X-linked mental retardation (Couvert, Bienvenu, Aquaviva, Poirier, Moraine et al., 2001; Meloni, Bruttini, Longo, Mari, Rizzolio et al., 2000; Crawford et al., 2001; Jiang et al., 2004), autism (Carney, Wolpert, Ravan, Shahbazian, Ashley-Koch et al., 2003; Muhle et al., 2004), schizophrenia (Cohen, Lazar, Couvert, Desportes, Lippe et al., 2002), and, most important to the present study, impairments in specific memory abilities including hippocampal-dependent spatial memory, contextual fear memory, and social memory (Moretti, Levenson, Battaglia, Atkinson, Teague et al., 2006). In this last study, mice with a mutated MeCP2 allele (*Mecp2*308/Y) (a mouse model of Rett syndrome) showed impaired learning in the MWM task, an electric shock fear conditioning task, and social investigation of a juvenile counterpart. These impairments were associated with a reduction in the number of larger post-synaptic densities (PSDs) and an increased number of smaller PSDs, as well as deficits in paired-pulse facilitation, early phase LTP and induction of paired-pulse long-term depression in the hippocampus – all indicative of impairment in synaptic plasticity.

Similar to these observed memory impairments caused by mutations in the MeCP2 protein, the alcohol literature has reliably shown deficits in memory abilities caused by developmental alcohol exposure and it has been well established that such effects are associated with the vulnerability of the hippocampus to this teratogen (see introduction). For instance, from the earliest studies examining the effects of developmental alcohol in a rodent model, deficits in the MWM has been a hallmark of FAE on spatial memory performance (see introduction for references). So too, studies have shown prenatal alcohol-induced effects on avoidance tasks indicative of contextual fear memory performance (Abel, 1979; Bond & DiGiusto, 1978). Kelly,

Leggett and Cronise (2009) also showed deficits in recognition memory among adult rats exposed to ethanol throughout gestation, and these effects were sexually dimorphic in nature. Subjects were required to investigate same-sex juveniles in a period of time that involved a first session of investigation that lasted for either 2, 3 or 5 minutes; a delay of 30, 60, 120 or 180 minutes with no juvenile, and a second session of investigation that lasted 5 minutes. In ethanol-exposed males, deficits in memory occurred when the the first session lasted for 2 or 3 minutes and the delays between sessions were long. In contrast, ethanol-exposed females showed deficits in social recognition memory when the first session lasted for 2 minutes and the delays between sessions were short. According to the authors, these findings suggest that while alcohol-exposed males may show a deficit in how long the memory of the juvenile lasts, the alcohol-exposed females have a deficit in the actual encoding of the memory.

Extensive clinical and experimental research on FASD have reliably shown effects of prenatal alcohol on the memory abilities of affected offspring comparable to that found in MeCP2 dysfunction, including impaired spatial memory performance. This suggests that developmental alcohol exposure during the period equivalent to the third trimester in humans may be causing an increase in the MeCP2 gene expression, leading to the hypermethylation of DNA that is currently observed. This concept is further explored below.

6.4 Epigenetic effects of choline supplementation and deprivation

The methylation changes observed after choline supplementation in ET-subjects led to a significant decrease in the alcohol-induced hypermethylation state of the DNA. This decrease in methylation levels after choline supplementation was not significantly different from the methylation levels of control subjects that were also given choline. This finding supports the

second hypothesis, which stated that choline supplementation given to ethanol-exposed subjects will lead to levels of global DNA methylation in both hippocampus and PFC similar to those of control animals. The choline-induced changes found in the both ET-exposed and control subjects in the present study corroborate past research showing that methylation changes do occur in the brains of animals treated with varying levels of choline availability. However, results of such studies seem to vary. While some studies examining methylation changes after choline supplementation have found hypomethylation of the DNA (Kovacheva et al., 2007), others have shown DNA hypomethylated states after a choline-deficient diet (Niculescu et al., 2006). So too, while some have found increased methylation states after choline deprivation (Mehedint et al., 2010), others failed to find any effect on DNA methylation when subjects were fed a choline-deficient diet (Kovacheva et al., 2007). These differences are generally due to methylation analysis in different brain regions and cells, as well as in the specific genes of interest. For instance, a recent study examined epigenetic changes in both the liver and frontal cortex of GD17 rat fetuses due to choline supplementation and deprivation (Kovacheva et al., 2007). This study observed hypomethylated DNA in the frontal cortex of fetuses exposed to a choline supplemented diet *in utero* (GD 11 to 17). On the other hand, there was no significant difference in the neural methylation state between choline-deprived subjects and controls. However, when examining the methylation changes in the *Igf2* gene, this past study showed hypermethylation on positions 7 and 19 CpG sites of the differentially methylated region 2 (DMR2) of the gene due to choline deficiency, and a general hypomethylation of all CpG sites in subjects treated with a diet rich in choline.

In another study, a deficiency in choline availability resulted in increased methylation within the CpG site 3 of the Calb1 promotor in neural progenitor cells (Mehedint et al., 2010).

There have been reports of decreases in global DNA methylation due to choline deficiency, rather than to choline supplementation. For instance, choline deficiency has led to global DNA hypomethylation in IMR-32 human neuroblastoma cells and a cell cycle regulator known as the cyclin-dependent kinase inhibitor 3 gene of the Ammon's horn ventricular and sub-ventricular areas of the hippocampus (Niculescu et al., 2006). This decrease in methylation was associated with an increase in the protein levels of the cell cycle inhibitor called kinase-associated phosphatase (Kap) (Niculescu et al., 2006) and an increase in gene expression that led to decreased cell proliferation of IMR-32 human neuroblastoma cells in culture (Niculescu et al., 2004). The tissue from which these cells derived is not well stated in this past study, but given the literature review it is assumed that they were taken from the hippocampus area. A related study by this group examined changes in gene expression due to a deficit in choline availability among GD14 mouse neural precursor cells (Niculescu, Craciunescu & Zeisel, 2005). Results showed significant overexpression of 846 genes, and significant reduced expression of 157 genes. Of these, 331 were related to cell proliferation, differentiation, apoptosis, calcium-binding proteins, methyl group metabolism and gene methylation. With the majority of the genes revealing a state of increased expression due to choline deficiency, this finding coincides with the group's observation of global DNA hypomethylation after dietary restriction of choline.

Even though both developmental alcohol and choline deficiency leads to cognitive, specifically memory, deficits and changes in brain areas such as the hippocampus and frontal cortex (see Introduction), the present study shows increased DNA methylation after developmental alcohol exposure. In contrast, the majority of past studies show decreases in DNA methylation states due to choline deficiency. What is more, choline supplementation in the present study also led to contrasting states of DNA methylation between ethanol-exposed

subjects and their control counterparts. While the ET animals showed decreased DNA methylation when supplemented with choline, control animals showed a level of DNA hypermethylation with extra choline administration when compared to subjects in the placebo condition. This finding may be explained by observations that DNA methylation is regulated by a number of factors such as *DNMT* enzymes and binding proteins (Robertson, 2005), shows co-activity with other epigenetic processes such as histone methylation (Pogribny et al., 2008), which in turn are regulated by multiple factors (Davison et al., 2009). Recent studies have shown that ethanol results in increased homocysteine levels (Bonsch, Lenz, Reulbach, Kornhuber & Bleich, 2004), changes in chromatin modulation (Mahadev & Vemuri, 1998) and increased histone H4 acetylation (Wang, Krishnan, Ghezzi, Yin & Atkinson, 2007). It can thus be speculated that, compared to otherwise healthy controls, developmental ethanol may differentially impact how the availability of choline in the diet affects DNA methylation changes.

However, the fact that choline availability shows significant epigenetic changes in ET-exposed subjects in the present study suggests that developmental alcohol may be causing alterations in neural DNA methylation somewhere along the path from choline availability to *DNMT* functioning. Effects may be via a direct impact on the *DNMT* genes or via an indirect impact on one or more of the factors or processes involved along the path to DNA methylation. Since the choline path is considered an 'alternate pathway' from that of the well-known folate pathway in being a methyl donor for DNA methylation (Finkelstein, 2000) (see Figure 11a), developmental alcohol may be affecting factors or processes on the folate path to methylation. In support of this, one study showed strikingly similar results to the present study in changes in the methylation pattern after folate/choline deficiency (Pogribny et al., 2008). As was found in the present study with ET-exposed subjects, this past study found significant global DNA

hypermethylation in subjects exposed to a combined lack of folic acid and choline in the diet compared to subject on a diet deficient in choline alone. Thus, developmental alcohol may be causing changes in DNA methylation somewhere along the folate path. With an obstruction of activity in this pathway, extra choline availability in ET-exposed subjects may be attenuating the ET-induced effects, not by 'fixing' the effects in the folate path, but by compensating for deficits in the folate path via its own alternate path to DNA methylation. The potential mechanisms will be addressed below.

Based on the results of these past studies examining the molecular bases for choline's actions in the brain, it is suggested that choline availability leads to varying patterns of DNA methylation depending on the specific gene and region being analyzed, as well as the developmental period during which choline is given. These factors may be important in later studies that seek to determine specific methylation changes due to developmental alcohol exposure. Choline supplementation helps to attenuate some of the deficits caused by developmental alcohol (Thomas et al., 2000; 2004; 2007; Thomas, Abou & Dominguez, 2009; Wagner & Hunt, 2006; Ryan et al., 2008), and this present study has not only found epigenetic changes in ET-exposed subjects, but also in ET-exposed subjects treated with choline. In particular, choline supplementation from the third trimester equivalent to the onset of adolescence in the rat model leads to reduction in ET-induced hypermethylation in the hippocampus and PFC regions.

6.5 Developmental alcohol effects on the folate/choline path to DNA methylation

The prevailing effect of choline deficiency is that of decreased global DNA methylation (Niculescu et al., 2005; 2006), while the ET exposure in the present study caused increases in

global DNA methylation. However, a combined lack of choline and folic acid leads to a similar hypermethylated state of global DNA (Pogribny et al., 2008) as that of ethanol exposure during the third trimester equivalent in the present study. This suggests that a possible decrease in choline levels is not enough to explain the molecular effects of developmental ethanol exposure, at least during the third trimester equivalent. Even though studies have yet to look at brain choline levels in offspring exposed to gestational ethanol, one study has examined possible changes in the level of choline and its substrates betaine aldehyde and betaine in the rat liver after 4 weeks of ethanol consumption (Chern, Gage & Pietrusko, 2000). In this past study, while ET consumption led to a significant decrease in betaine levels, ethanol did not have any effects on the levels of choline or betaine aldehyde. In addition to the decrease in betaine levels, ethanol has been shown to cause increases in betaine-homocysteine methlytransferase activity, which is responsible for transferring a methyl group from betaine to homocysteine to form methionine (see Barak, Beckenhauer & Tuma, 1996). Even though these findings were observed in the liver, it may be that developmental ET also may not show significant effects on choline or betaine aldehyde levels in the brain (see Figure 11b). This may explain the differences in DNA methylation states in the brain due to choline deprivation versus developmental alcohol exposure, even though both factors show similar effects on the hippocampus, frontal cortex and memory performance. Also, the observation that ethanol consumption leads to depleted betaine, but no effect on betaine's precursors suggests that ethanol may be affecting betaine levels via effects on another point in the path to DNA methylation.

As was mentioned earlier, in Pogribny et al. (2008), folate/choline deficiency led to DNA hypermethylation which is associated with an increase in the amino acid, homocysteine, but not in SAM, the physiological methyl group donor for protein, RNA and DNA methylation (Jeltsch,

2002), S-adenosylhomocysteine (SAH), a product of homocysteine which acts as primary predictor of tissue-specific methylation status (James, Melnyk, Pogribna, Pogribny & Caudill, 2002; Chen, Yang, Capecci, Gu, Achafer et al., 2010) or the SAM/SAH ratio. In this same study, the folate/methyl deficient diet also led to a significant decrease in the expression of the *DNMT1* gene (responsible for maintenance of methylation states) and a significant increase in the expression of the *DNMT3a* gene (responsible for *de novo* methylation) and the MeCP2 protein - an important transcriptional repressor (Meehan, Lewis & Bird, 1992). In this respect, it is important to note that another role of the *DNMT3a* gene is the compensation of an inadequacy in the maintenance of methylated regions by the *DNMT1* gene (Liang, Chan, Tomigahara, Tsai, Gonzales et al., 2002), which may have led to the increased methylation status found in the Pogribny et al (2008) study.

If developmental alcohol is causing similar deficits on the folate/methyl pathways to DNA methylation as does the folate/methyl deficiency diet, then it is possible that developmental ethanol exposure may be causing similar changes in the levels of the amino acid homocysteine, methyltranferases and/or MeCP2 protein, ultimately leading to the observation of a significant increase in hypermethylation in our present study. One particular study in the alcohol literature that helps to support this proposal examined changes in DNA methylation in alcoholic patients (Bonsch et al., 2004). In this study, blood samples from patients with a medical history of alcoholism showed a significant increase in global DNA methylation, which was also significantly associated with an increase in homocysteine levels. From this, it can be suggested that in the present study that an increase in the levels of homocysteine may be one mechanism, or part of a mechanism, through which developmental alcohol may be causing global DNA hypermethylation (see Figure 11b).

As was already reviewed by Mason and Choi (2005), other studies have also examined alcohol's effect on the folate pathway, giving clues as to the molecular bases of alcohol's effects. For instance, several weeks of exposure to alcohol has led to impaired activity of methionine synthase (MTR) (Barak, Beckenhauer, Tuma & Badakhsh, 1987; Halsted, Villanueva, Devlin, Niemela, Parkkila et al., 2002). One possibility of how this impairment occurs may be through acetaldehyde which is produced during alcohol metabolism and produces 'adducts' that bind to the MTR protein, resulting in partial inactivation of MTR activity (Barak et al., 1987). As a consequence of this impaired activity, these two aforementioned studies have also observed decreased concentrations of two products of the MTR reaction, methionine and SAM, along with a rise in two of the reaction's precursors, homocysteine and SAH (see Fig 11a & b). The Bonsch et al. (2004) study mentioned earlier also supports the findings of a significant increase in homocysteine levels among alcoholic patients. In addition, studies have observed decreased concentrations of intracellular folate (Cravo, Gloria, Selhub, Nadeau, Camilo et al., 1996) and hepatic vitamin B-12 (Kanazawa & Herbert, 1985). However, while it is more or less accepted that the reduction in folate concentrations is an indirect effect of alcohol, the mechanism of this indirect effect is still up for debate (Cravo et al., 1996).

It is important to mention here that these studies looking at effects of alcohol in the folate pathway include animal models of alcoholism or clinical studies examining effects in alcoholics, and not studies looking at developmental alcohol in the offspring. Also, these studies have examined alcohol's effects in the liver and not in the brain. Thus, precaution should be taken in generalizing the findings of these studies to those using animal models of FASD and the present study. Nevertheless, while there is the need for future studies to examine perinatal alcohol exposure on the folate pathway in affected offspring, particularly in the brain, the literature on

effects of alcohol in adult organisms may provide clues as to the molecular basis of alcohol's teratogenic effects.

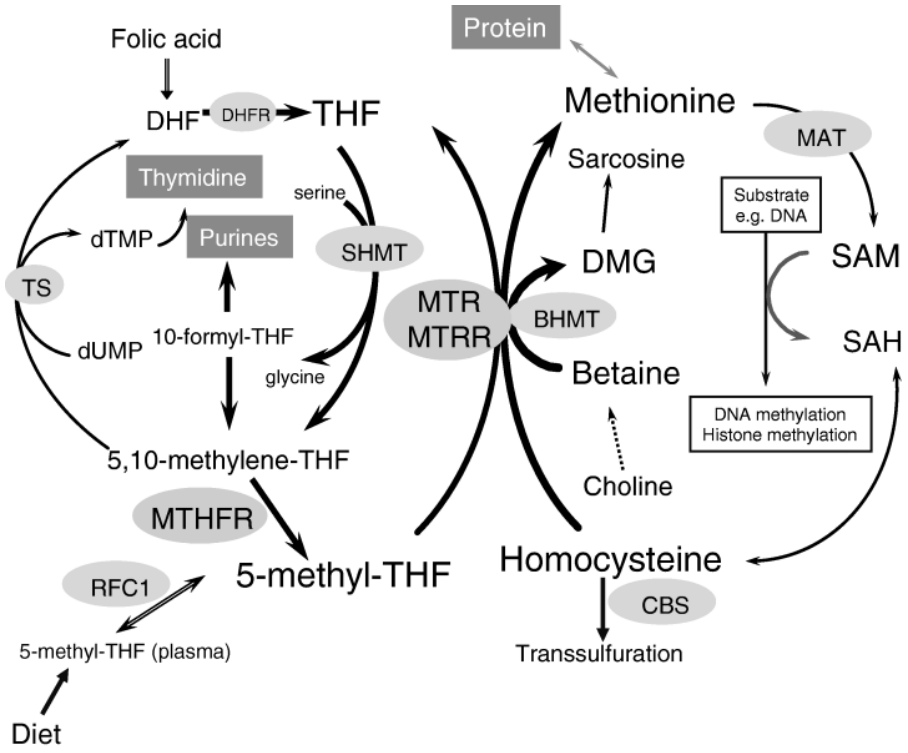


Figure 11a. The folate and choline pathways leading to DNA methylation (Kelly & Trasler, 2004).

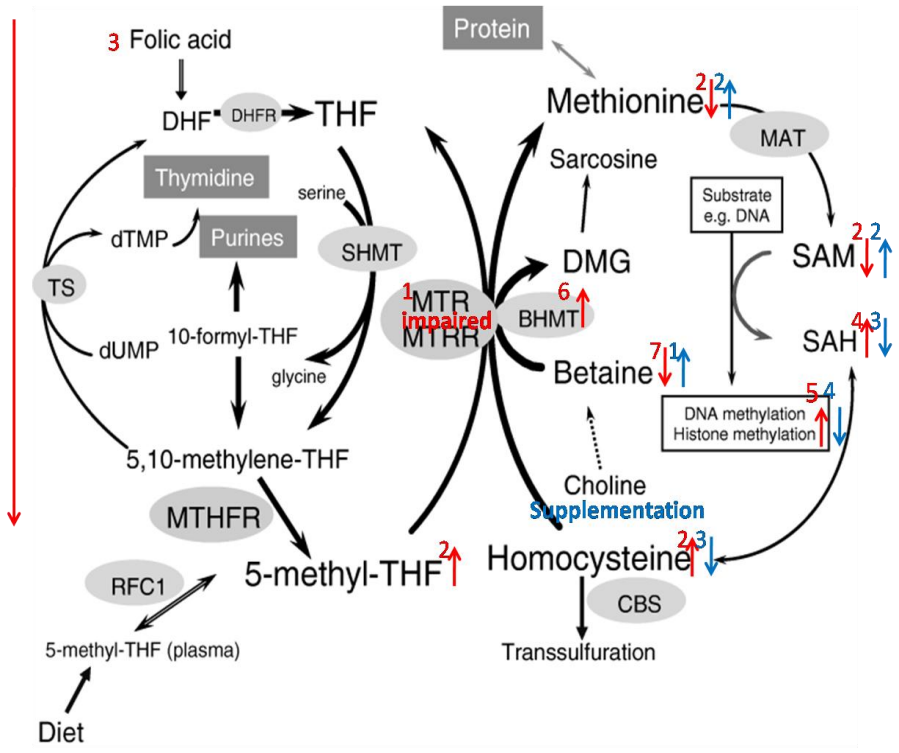


Figure 11b. The folate and choline pathways showing proposed ET-induced effects in red and choline's attenuating effects in blue.

6.6 Proposed molecular mechanisms of developmental alcohol effects

Determining a definite mechanism(s) through which developmental alcohol causes effects characteristic of FASD at the molecular level is still a long way off. However, with new evidence pointing towards a molecular basis along with the pulling together of alcohol-related abnormalities from the literature, this endeavor may be successful. As was mentioned earlier, given the fact that both choline supplementation in alcohol-exposed subjects and choline availability in otherwise healthy subjects result in DNA methylation changes, it is quite possible that alcohol may be interfering with DNA methylation patterns via insults somewhere along the path from folate/choline availability and *DNMT* activity. As is shown in Figure 11b, a similar figure is also found in Mason and Choi (2005). The major differences between the figures include the role of the choline pathway, changes up to folate levels, and how these changes lead to hypermethylation rather than hypomethylation of the DNA in the present figure.

Following from Figure 11b, it is postulated here that developmental alcohol interferes with the folate/choline path to DNA methylation initially at the site of MTR activity[1] (Barak et al., 1987; Halsted et al., 2002), and this impairment in activity may be due to the binding of factors provided by acetaldehyde to MTR causing inactivity, as was proposed by Barak et al. (1987). MTR activity is responsible for transferring N5-methyl tetrahydrofolate (5-methyl-THF) to vitamin B-12 to form a methyl cobalamine, which in turn provides a methyl group to homocysteine to form methionine (see Barak et al. 1996). Given this, it is postulated that the impaired functioning of the MTR results in decreases in vitamin B-12 concentration at the site of MTR activity, as it is unable to transfer the 5-methyl-THF to vitamin B-12 and provide the methyl group to homocysteine to form methionine. This is supported and further highlighted by Kanazawa & Herbert (1995) who observed a decrease in hepatic vitamin B-12 levels in the liver.

Consequently, with impaired MTR activity, there are increases in the levels of both 5-methyl-THF[2] (Horne, Briggs & Wagner, 1978) and homocysteine[2] (Bonsch et al., 2004), with a simultaneous reduction in methionine[2] and SAM[2] (Barak et al., 1987; Halsted et al., 2002). With an increase in the levels of 5-methyl-THF, there may be a negative feedback loop affecting folate availability, such that there is a decrease in levels of intracellular folate[3] (Cravo et al., 1996). With an increase in homocysteine levels, there is a consequential increase in SAH levels[4] (Barak et al., 1987; Halsted et al., 2002), leading to an imbalance in the SAM:SAH ratio, which has been shown to play a pivotal role in the methylation status of the DNA. Increases in SAH has been associated with an apparent increase in hypomethylation states (Yi, Melnyk, Pogribna, Pogribny & Hine, 2000), which may lead to a decrease in *DNMT1* activity after alcohol exposure. However, as a consequence of a decrease in *DNMT1* activity, overcompensation by the *DNMT*3 enzymes may occur (Liang et al., 2002), leading to enhanced *de novo* methylation[5]. This mechanism was also postulated in Pogribny et al. (2008) when examining effects of folate/choline deficiency. In the present study, these enhanced *de novo* methylation would explain the hypermethylation states observed in the hippocampus and PFC areas after ethanol exposure during development.

As was mentioned earlier, it is also postulated that choline supplementation may be a way of attenuating some of alcohol's teratogenic effects on the folate path to DNA methylation. The impact of choline would be via an indirect mechanism and not via a direct impact on the folate path postulated here to mediate the effects of alcohol exposure during development of the brain. With impairment of MTR activity, the folate pathway may be compensating for decreased methionine levels by increasing its demand on the betaine-homocysteine methyltransferase (BHMT) activity[6], hence the increase in BHMT activity due to alcohol exposure in some studies

(Barak et al., 1996). Because of increased BHMT activity, the supply of betaine is more quickly depleted[7], which would be consistent with the significant decrease in betaine levels resulting from alcohol consumption (Chern et al., 2000). There are no studies examining the effects of alcohol on the levels of choline or the enzymes that catalyze this nutrient into betaine. However, giving a choline supplement, and thus enhancing choline supply in the pathway, may be a way of enhancing betaine supply[1], enough to meet the increased demand on BHMT activity. If this is the case, increased choline availability may help to increase both methionine[2] and SAM[2] levels. So too, with the compensating increase in BHMT activity utilizing the ethanol-induced increases in homocysteine, the level of both homocysteine[3] and SAH[3] also drops closer to normal levels, and further stabilizes the SAM:SAH ratio. With a SAM:SAH ratio nearer to normal levels, there is an increase in *DNMT1* activity, reducing the need for overcompensation by the *DNMT3* group of enzymes, and thus leading to a decrease in the *DNMT3*-mediated hypermethylation state of the DNA[4].

This postulated mechanism for both developmental alcohol and choline effects on DNA methylation is plausible. However, further studies are needed to test this hypothesis at each step in the process of folate/choline availability to DNA methylation changes. Given the variability of DNA methylation among different tissue regions, the variability of and regional specificity of alcohol's teratogenic effects, and the variability of the effects of choline availability on changes in the DNA methylation, this mechanism may be limited to the methodology of the present study where the time periods of developmental alcohol exposure and choline availability lead to such effects on the folate/choline path. Nevertheless, this proposed mechanism can provide a general pattern of how developmental alcohol may be working to not only induce changes in methylation

states, but cause phenotypical characteristics of FASD and how methyl donors, such as choline, may be used to compensate for these changes.

It is also important to note that literature show that the impact of choline availability on changes in DNA methylation states is highly variable. This variability is dependent on the time period and the length of choline supplementation, the organ or brain regions of interest, and the original state of the individual or organism. For instance, Pogribny and colleagues (2006; 2008) found that while a diet deficient in choline led to decreased methylation in the liver, the same diet caused hypermethylation in the brain. Thus, while the current proposal shows a compensating effect of choline supplementation on the ET-induced hypermethylation state of the hippocampus and PFC, the mechanisms through which choline supplementation may be working after ET-induced damage to the liver may be strikingly different.

Given the myriad of effects of choline at the level of the brain, choline may also have indirect effects on DNA methylation other than the proposed mechanisms provided above. For instance, the hippocampus is rich in cholinergic neurons, which rely on choline for the synthesis of the neurotransmitter acetylcholine (Blusztajn et al., 1998). Indeed, studies have shown that choline supplementation leads to increases in acetylcholine release (Cermak et al., 1998). Choline also has effects on growth factors and NMDA-receptor mediated transmission (Montoya & Swartzwelder, 2000), which is involved in long-term potentiation. The impact of choline on the NMDA receptor is likely followed by a host of cellular events, including changes in gene expression (Bliss & Collindgridge, 1993). Choline has also been shown to be a complete and selective agonist at the alpha 7 subunit nAChRs (Alkondon et al., 1997; Albuquerque et al., 1998; Alkondon et al., 1999; Uteshev et al., 2003), which are suggested to mediate the memory-enhancing effects of choline (Alkondon & Albuquerque, 2006). Thus, this enhancement in neural

functioning may provide another path through which changes in DNA methylation and changes in behavior are observed after choline supplementation.

6.7 Possible genes vulnerable to alcohol/choline-induced epigenetic changes in present study

It is important to further study and understand the processes driving the ET-induced hypermethylation in the hippocampus and PFC and the choline-induced methylation changes that were observed in the present study. However, it is also equally important to further study and understand which genes are involved and highly vulnerable to such changes in these two brain regions and whether the genes are related to learning and memory abilities. In a study by Hard, Abdolell, Robinson and Koren (2005), ET-induced alterations in gene expression were observed in the fetal brains of mice exposed to the 2.9 g/kg ET on GD 7 and 9. According to the results, six genes with known biological functions were significantly down-regulated due to the alcohol exposure. Even though DNA methylation changes were not observed in the study, reduced gene expression has been reliably linked to hypermethylation of the DNA (see above).

One of the ET-induced down-regulated genes included *Timp-4* (tissue inhibitor of metalloproteinase), which is one of 4 *Timp* (*Timp 1-4*) isotopes that controls matrix metalloproteinase (MMP) activity (Brew, Dinakarpandian & Nagase, 2000; Higuchi, Yasuda, Kawamoto, Yotsui, Baba et al., 2007). MMPs themselves have been observed to be an important regulator of synaptic activity in the hippocampus, affecting learning and memory performance (Ethell & Ethell, 2007). While levels of *Timp-4* has been implicated in cardiac and other peripheral processes (Mishra & Metreveli, 2010; Tummalapalli, Heath & Tyagi, 2001), another study (Baba, Yasudo, Takemura, Ishikawa, Ohishi et al., 2009) has observed that *Timp-3* knock-out mice have deficits in memory performance in the water maze task and reduced habituation in

the open-field test. Even though Hard et al. (2005) only found reduced expression in the *Timp-4* isotope after early ethanol exposure, it may be that the length (GD 7 and 9), dose (2.9 g/kg) or administration (*i.p.* injection) of ET exposure in that study was not sufficient to affect expression of the other *Timp* members, specifically that of *Timp-3*.

One particular study examined the expression of several genes in the PFC of alcoholic patients with or without comorbidity to smoking (Flatscher-Bader & Wilce, 2006). Alcoholism was related to an increase in the expression of the *Timp-3* gene. Over-expession of this inhibitory gene has been linked to dose-dependent inhibition of cell proliferation and disruption of intracellular cell cycle regulatory mechanisms (Baker, Zaltsman, George & Newby, 1998). What is more, a choline deficient and a low methionine (1.7g/Kg) and amino acid-defined diet (CDAA diet, #518753) from Dyets Inc. (Philadelphia, PA, USA) has been shown to significantly reduce the expression of *Timp-3* (Wang, Hsu, Majumder, Kutay, Huang et al., 2010). Even though this past study focused on changes in the liver, it provides a hint as to possible changes in *Timp-3* expression in both PFC and hippocampus-dependent cognition after choline/amino acid deficient diet. It has also been demonstrated in the literature that the expression of *Timp-3* is regulated by methylation (Van der Velden, Zuidervaart, Hurks, Pavey, Ksander et al., 2003) such that *Timp-3* hypermethylation is associated with lower *Timp-3* protein levels (Ninomiya, Kawakami, Fushida, Fujimura, Funaki et al., 2008).

Thus, future studies should examine levels of expression, as well as DNA methylation changes in the *Timp* group of genes after developmental alcohol. This will add to the understanding of which genes are most vulnerable to epigenetic and molecular changes due to early alcohol, and how these changes are associated with deficits in PFC and hippocampus-dependent cognitive performance. Given the results of our present study, it is expected that

alcohol exposure during the third trimester equivalent will lead to reduced expression of *Timp-3* via DNA hypermethylation, and that the reduction in expression may be dose- and time-dependent. In addition, since choline availability seems to influence expression levels of this gene (Baba et al., 2009), the choline supplementation administered in the present study may have attenuated the alcohol-induced reduction in *Timp-3* expression, via an attenuation of the alcohol-induced hypermethylation of the DNA.

Another gene vulnerable to early ethanol-induced reduction in expression is the bone morphogenetic protein (*Bmp15*) (Hard et al., 2005), which is a member of the transforming growth factor beta (TGF-β) super-family (Kingsley, 1994). Even though there is no evidence of a role of expression of this gene in either the hippocampus or PFC, the observation of a prenatal ethanol effect on its expression hints at possible epigenetic changes that may be occurring within the TGF-β signaling pathway. Other members of this super-family may be affected. For instance, in the hippocampus, another member of this signaling pathway – TGF-β2 – has been shown to be involved in the processes underlying long-term synaptic facilitation (Fukushima, Liu & Byrne, 2007), Alzheimer's disease (Tashiro, Dohura & Iwaki, 1998) and retention of spatial memory ability (Harris-White, Balverde, Lim, Kim, Miller et al., 2004).

Moreover, intrauterine ET exposure has been shown to disrupt proper functioning of the TGF-β pathway during early development. Miller and Luo (2002) showed that in-vitro ethanol exposure to cortical neurons obtained from GD16 rat fetuses inhibits TGF-β mediated cell growth and cell proliferation. Lombard, Tiffin, Hofmann, Bajic, Hide et al. (2007) also makes a link between the TGF-β pathway's role in neuronal apoptosis and alcohol-induced widespread apoptosis in the developing rat forebrain during a period of vulnerability equivalent to the third trimester and several years postnatal in humans (Ikonomidou, Bittigau, Ishimaru, Wozniak, Koch

et al., 2000). The mechanism through which the alcohol exposure leads to apoptosis is not clear. However, while Lombard et al. (2007) proposes a mechanism via the dysfunction of the TGF-β pathway, the present study furthers this hypothesis via a possible alcohol-induced hypermethylation, or transcriptional silencing of genes associated with the TGF-β pathway, thus leading to functional inhibition of this pathway and causing neuronal apoptosis. This may be especially evident in the PFC and hippocampus areas, both of which show alcohol-related hypermethylation during the 3rd trimester equivalent in the present study, and show vulnerability to alcohol-related neurodegenerative apoptosis in the Ikonomidou et al. (2000) study.

The TGF-β pathway also shows vulnerability to choline availability in the hippocampus, especially during early development (Albright, Tsai, Mar & Zeisel, 1998). In this past study, effects on the TGF-β1 protein levels in fetal hippocampus were examined in rats exposed to a choline-supplemented, choline-deficient or conrol diet. At GD18, animals supplemented with choline showed less intense immunohistochemical staining for the protein in all areas of the hippocampus compared to controls. In contrast, choline deficient subjects showed more intense staining in the CA1 and CA3 regions of the hippocampus compared to controls,. Increased expression of TGF-β1 has been shown in a previous study to be indicative of neuronal cell death in the CA1 region of the hippocampus, and occurred simultaneously with decreased expression of the other two TGF-β isoforms, -β2 and β3 (Knuckey, Finch, Palm, Primiano, Johanson et al., 1996). This suggests that the increase in TGF-β1 protein levels in choline-deficient fetal hippocampus may be indicative of an increase in apoptosis caused by this diet deficiency, thus making this protein a likely marker for hippocampal damage caused by choline deficiency and, maybe also, fetal alcohol exposure.

6.8 Neuroanatomical Findings

As was mentioned earlier, the present study found an increase in the concentration of cells (as measured by density of immunohistochemical staining for NSE) only in the mPFC due to supplementation with choline, regardless of sex and treatment condition. On the other hand, no differences in NSE staining were found in the CA1 brain region of the hippocampus, and no differences were found due to ethanol exposure in either brain area. These findings only partially support the original hypothesis. As was expected, the results showed an increase in NSE staining density due to choline supplementation. However, while it was hypothesized that choline supplementation will lead to an increase in density values in both brain areas of interest, this effect of choline was only observed in the mPFC region and not in the CA1. It was also hypothesized that ET exposure would lead to a marked decrease in density staining compared to controls. However, the results showed no main effect of treatment on either brain area, although there was a trend towards a decrease in density due to developmental alcohol in the mPFC region.

There are several possible reasons for the lack of effect of ET on the neuroanatomical measures even though there was an impact on global methylation. Firstly, while global DNA methylation was determined in the entire PFC and the entire hippocampus, optical density of NSE staining was measured in only one part of the PFC (the mPFC) and of the hippocampus (the CA1). Thus, limiting the area in which optical density was measured may have led to a failure to detect adequate differences among groups.

Another possible reason for the failure to detect any differences in the CA1 area in particular is that this measure may not have been sensitive enough to show alcohol-induced deficits or choline-induced changes relative to the experimental procedure that was used in the

present study. ROD values of NSE staining are a measure of the concentration of the staining with the antibody and not cell number. Thus, more sensitive measures might include neuronal structure, neuronal connectivity, and actual cell number, as was observed in past studies (Abel et al., 1983; Barnes & Walker, 1981; West et al., 1981). In the Abel et al. (1983) study, alcohol-exposed offspring showed significantly decreased spine number in the CA1 region of the hippocampus, as well as a shift in the type of spines that were more prominent compared to controls. Barnes and Walker (1981) found that prenatal alcohol exposure caused significant reductions in the total number of pyramidal cells in the CA1, while West et al. (1981) found changes in the topography of the mossy fibers in rats exposed to 35% EDC from GD 1 to 20.

From another perspective, failure to find significant differences in ROD of NSE staining in the CA1 region may also be due to the timing of the alcohol exposure in the present study. Exposure to alcohol occurred postnatally, during the time equivalent to the third trimester of human pregnancy. However, proliferation of pyramidal cells in the CA1 is completed prenatally in the rat (see discussion in Barnes and Walker, 1981 or Bayer, 1980). This suggests that the early postnatal exposure to alcohol did not lead to deficits in the concentration of the cells of CA1 per se. Instead, the deficits may be more evident at this stage in the structure or proper functioning of these cells. Moreover, Miller (1995) examined differences in neuronal generation in the hippocampus after pre- or early postnatal alcohol exposure, and observed no effect on CA1 neuronal numbers after early postnatal alcohol treatment. In Barnes and Walker (1981) study, prenatal alcohol exposure in the rat did not affect the number of dentate granule cells, which reach their peak time of proliferation after the second postnatal week (Schlessinger, Cowan & Gottlieb, 1975), when no alcohol was administered to the subjects. On the other hand, in a rat model, Tran and Kelly (2003) observed the effects of intragastric intubations of ethanol during

critical periods of development on hippocampal cell numbers. Results showed significant reductions in the CA1 regions for adult offspring exposed to developmental alcohol during the third trimester and all three trimesters equivalent to human pregnancy.

Despite a lack of effect in the CA1, the present results showed significant effects in the mPFC region. Subjects supplemented with choline showed a significantly higher density of cells in the mPFC compared to subjects in the placebo condition, regardless of treatment or sex. To date, no other study has examined the effect of choline availability in the mPFC region. However, studies have observed the role of choline's derivatives in functioning in the frontal region. It has been shown that the neurotransmitter, Ach is involved in cognitive tasks such as cue-detection in a rat model (Parikh, Kozak, Martinez & Sarter, 2007). Cholinergic projections to the frontal cortex are believed to be involved in visual attentional performance, with further evidence suggesting that, in animal models, learning deficits such as reversal learning and conditional discrimination may be due to cholinergic depletions in the PFC region (Everitt & Robbins, 1997). In addition, McGaughy, Koene, Eichenbaum and Hasselmo (2005) observed impaired performance in a delayed non-matching to sample task in cholinergic-lesioned rats, which was associated with significantly lower AchE positive fiber count in the entorhinal cortex, all compared to sham controls. Given this, it can be speculated that choline supplementation may have enhancing effects not only on the levels of the neurotransmitter Ach, but also on the functioning of cholinergic activity and morphology of its projections to the mPFC. The particular NSE antibody used in this study (polyclonal rabbit anti-NSE - AB951; Chemicon) is used to stain many neuronal cell bodies and processes within the CNS. Thus, the augmentation of neuronal processes due to choline-induced functioning of cholinergic activity in the mPFC may have resulted in the increased density of NSE found in the mPFC of subjects supplemented with

choline in the present study. Future work should explore this further by examining the effects of choline supplementation at critical periods of development using several techniques such as unbiased stereological cell counting and morphological changes in spine density.

Also in the present study, ethanol-exposed subjects showed a significantly lower density of NSE staining in the mPFC when compared to non-treated controls, independent of supplementation or sex. This finding corroborates a previous report demonstrating that *in utero* alcohol exposure from PD 4 to 9 in rats led to decreased dendritic complexity, shorter dendritic length, as well as significant differences in spine phenotype in Layer II/III neurons of the mPFC, all compared to suckle control subjects (Hamilton, Whitcher & Klintsova, 2010). Thus, the decrease in NSE staining found in the present study may be indicative of deficits in the dendritic processes of the mPFC neurons.

A difference in mPFC density was also sexually dimorphic in nature. Males showed a marginally higher density of NSE staining than females, regardless of treatment or supplement. Sex differences in neuroanatomical measures in the mPFC have also been observed in other studies. For instance, Markham, Morris and Juraska (2007) found that from adolescence to adulthood, female rats showed a more pronounced decrease in neuron number in the ventral mPFC compared to male rats, revealing sexual differentiation in adulthood but not during adolescence. Another study showed that from adulthood to old-age, male rats, but not females, began showing neuronal loss in the ventral mPFC (Yates, Markham, Anderson, Morris & Juraska, 2008). Kolb, Pellis and Robinson (2004) observed sex differences in the dendritc density of cells in the mPFC where male rats show greater dendritic arborization than female counterparts. Such differences are said to be hormone-dependent since this neuroanatomical differentiation between the sexes is removed after castration or ovariectomy during the neonatal

period (Kolb & Stewart, 1991). Thus, the higher optical density of NSE staining found in our male subjects compared to the females may be indicative of greater dendritic densities in the cells of the mPFC, a feature that the present NSE antibody is capable of staining, according to the manufacturer.

Thus, differences in ROD values for NSE staining that are found in the mPFC region show the vulnerability of this region not only to early neonatal alcohol exposure, but also to early postnatal choline availability. Investigation of how these changes in mPFC morphology and function are related to this area's connections to other brain areas, such as the hippocampus (also highly vulnerable to developmental alcohol and choline availability) is vital in furthering our understanding of mechanisms underlying alcohol's teratogenic effects and choline's attenuation of these effects on the brain and behavior.

6.9 Physical Data

With regards to weight gain, both male and female subjects were similarly affected whereby exposure to alcohol during the third trimester equivalent to human pregnancy caused growth retardation all the way to the adolescent stage (PD 21). This finding suggests that alcohol-related growth defects is not transient, as was found in studies such as Abel (1979, 1982), especially when the level of the alcohol exposure is high (see Table 1 for BAC levels). However, other studies have found that such effects on weight gain are transient. For instance, Barnes and Walker (1981) found that rat offspring prenatally exposed to 35% EDC weighed significantly less than controls only at birth, but not at PDs 5, 10, 15 or 60. Thus, another possible reason for the continued weight effect in the present study is the mixed litter design in which siblings of the same litter belonged to one of all three treatment conditions. This latter

suggestion corroborates a previous study in which control pups elicited significantly more maternal responsiveness than their alcohol-treated counterparts from non-drug treated dams (Abel, 1982). Thus, as regards the present study, pups belonging to the control group may have had an advantage over their alcohol-treated siblings of the same litter in maternal responsiveness. This latter interpretation is also supported by another study that examined critical periods during early development for alcohol's teratogenic effects in a rat model (Tran, Cronise, Marino, Jenkins & Kelly, 2000). Subjects were exposed to alcohol from GD 1 to 10, GD 11 to 20, PD 2 to 10 or all three periods combined. According to the results, only the subjects given alcohol from GD 11 to 20 and those given alcohol for all three periods combined showed significantly lower body weights compared to controls. Subjects administered alcohol during the third trimester equivalent (PD 2 to 10), similar to those in the present study, showed no significant effect on weight. This further supports the analysis that the reduction in weight found among the alcohol subjects in the present study is more likely due to maternal responsiveness and not a direct effect of the alcohol exposure.

Also in the present study, ET animals did not weigh significantly less than their IC counterparts, suggesting that stress due to the experimental procedure may account for some of effects of growth retardation that are observed. Despite this disadvantage to the study, alcohol administration via gavauge has its important advantages. As was pointed out by Abel (1979), this procedure allows for the same dose of alcohol to be administered to each animal at the same time, thus resulting in high BAC levels as well as BAC levels that are similar per treatment group.

6.10 Clinical Implications

The global DNA methylation changes after developmental alcohol exposure that were observed in the present study have some implications regarding children born with FASD and impaired learning and memory abilities. In the clinical literature, learning and memory impairment is one of the most commonly reported deficits of FASD and has been associated with ET-induced damage to the hippocampus and PFC brain regions (see Introduction). By examining global DNA methylation levels in these two aforementioned brain areas, this study provides evidence that hypermethylation of the hippocampus and PFC due to third trimester alcohol exposure may be a mechanism through which developmental alcohol causes deficits in learning and memory abilities among affected children. Also, given the literature showing that DNA methylation is a reversible process (e.g. Weaver et al., 2004), the current methylation changes observed after developmental alcohol exposure suggests that deficits in learning and memory observed in FASD children can be reversed, or at least ameliorated, using appropriate treatment.

The current study also shows that choline supplementation reversed the direction of the ET-induced increase in global DNA methylation, leading to a decrease in methylation state that was not significantly different than that observed in control subjects that were also supplemented with choline. Given the recent line of studies by Thomas and colleagues (Thomas et al., 2000; 2004; 2007; 2009; Ryan et al., 2008) showing that choline supplementation in an animal model of FASD attenuates some of the learning and memory deficits of ET-exposed subjects at the behavioral level, the current data suggest that choline supplementation is an appropriate and effective treatment for FASD children suffering with learning and memory impairments. Since choline availability is shown to reverse the ET-induced methylation changes at the level of the

DNA, the present findings also suggest that choline treatment to affected children will provide long-term amelioration of the learning and memory impairments.

6.11 Limitations to present study and interpretation of results

One limitation to the interpretation of the alcohol-induced methylation changes in the present study is that, to date, no study has examined normal methylation patterns throughout the entire gestation (all three trimesters equivalent) and during the early postnatal period. Thus far, there is some understanding of the methylation reprogramming and methylation patterns occurring up to the time of implantation (Kelly & Trasler, 2004). However, normal methylation changes that occur throughout the entire gestation period are yet to be examined and understood. More so is the examination and comparison of this process in different species, such as humans, rats, mice, and non-human primates. Until then, determining how environmental teratogens, such as alcohol, modify methylation states is limited to comparisons with control counterparts in a given study.

Being able to compare the methylation process throughout the entire gestation among the species highlights another limitation to the present study, which is the ability to sufficiently infer the findings to human cases. As is highlighted by Lombard et al. (2007), given the high variability of findings in the alcohol literature, including evidence that not all children exposed to developmental alcohol are born with FAS (Chaudhuri, 2000) or with the same alcohol-related deficits, it is expected that there is an association between the alcohol exposure and the variable genetic background of each individual. Even animal models examining the effects of developmental alcohol on different strains of mice have found differences in alcohol-induced deficits, including differences in behavior (Thomas et al., 2000; Ogawa, Kuwagata, Ruiz &

Zhou, 2005; Boehm, Lundahl, Caldwell & Gilliam, 1997; Gilliam, Mantle, Barkhausen & Tweden, 1997). Thus, the severity of alcohol-related epigenetic reprogramming and the effectiveness of choline supplementation may be different for a given individual. Furthermore, by the time the severity and extent of a developmental alcohol effect is established in a child born with a disorder, it may be too late to begin therapeutic intervention.

Also, not only is there the challenge of variability in the teratogenic effects of alcohol among affected individuals, but also high variation in methylation states across organs and regions, both within and outside of CpG islands. For instance, De Bustos, Ramos, Young, Tran, Menzel et al. (2009) observed differences in methylation profiles from different organ tissues of the same individual, including the lobes of the brain, cerebellum, medulla oblongata, pons, heart, liver, lung, testis and ovary. These variations in methylation states between tissues are also highlighted by a series of studies by Pogribny et al. (2006; 2008) (mentioned above) where exposure to the same dietary methyl deficiency at the same developmental time point in a rat model caused contrasting methylation states in the liver compared to brain cortical tissue. Differences in methylation states are also evident among promoters of varying levels of GC content where both high and low CpG densities show significantly lower numbers of hypermethylated genes than promoters with intermediate CpG content (Liu et al., 2009b). In addition, this same past study not only found significant differences among these three CpG densities in methylation states during the early embryonic period, but also that early alcohol exposure show varied modifications of DNA methylation dependent on CpG density and neural tube phenotype (Liu et al., 2009b) (see above). Given this, global treatment of alcohol's teratogenic effects may lead to attenuation of effects in some areas, but also undesirable results in other areas or no effect at all. This may hold true when examining the differential findings in

studies examining environmental factors such as environmental enrichment, vitamin E, exercise, choline and other agents used to determine their ability to ameliorate perinatal alcohol effects (see introduction).

6.12 Future Work

Further exploration and understanding of epigenetic changes due to developmental alcohol may be of value in screening individuals born with FASD. As is highlighted by Bernstein, Meissner and Lander (2007), a more complete understanding of the roles played by the different epigenetic mechanisms, such as DNA methylation and histone modification, in normal development is still needed. As was pointed out by Holliday (2006), with the sequencing of the human genome complete, the next necessary step is to determine DNA methylation's role in specialized gene functions (the 'epigenome project'). It is this knowledge that will clarify whether gene expression is primarily controlled at the level of the DNA or the chromatin. In another review, Holliday (2005) highlights the need to use modern techniques, such as Nothern blots, Western blots, immunofluorescence, microchip arrays, or proteome analysis, as a way of determining whether DNA methylation is necessary and sufficient to cause gene silencing. Holliday goes on to propose that if cytosine methylation is not the central mechanism for the silencing of the genes, then chromatin configurations may also be involved.

Moreover, how these different mechanisms function and interact with each other throughout the entire developmental stage – from conception to the early postnatal stages – is imperative in understanding how outside agents, such as developmental alcohol, lead to epigenetic changes that ultimately are expressed in both physical, CNS and behavioral deficits.

More recently, studies have begun to show evidence of the importance of both DNA and histone methylation in proper development and functioning. For instance, Davison et al. (2009) examined changes in histone methylation and expression of its methyltransferases induced by gestational choline availability. This study aimed to compare the results to those observed in an earlier study looking at the effects of choline availability on changes in DNA methylation (Kovacheva et al., 2007). Concluding data showed that choline deficiency led to over-expression of certain *DNMT*s with a resulting hypermethylation of the DNA (Kovacheva et al., 2007). This increase in methylation state was associated with reduced expression of two histone methyltransferases (*G9a* and *Suv39h1*) and low levels of lysines that are methylated by these enzymes (Davison et al., 2009) . On the other hand, a high supply of choline *in utero* results in reduced *DNMT* expression and DNA hypomethylation (Kovacheva et al., 2007), which paralleled increased expression of the histone methyltransferases and high levels of the associated lysines (Davison et al., 2009). Even though their main findings were in the liver, it highlights the fact that both epigenetic mechanisms play a concerted role in the proper regulation and expression of DNA.

With the need for future studies to compare multiple epigonomic activities specifically in the brain, Pogribny et al. (2008) compared changes in global DNA methylation with changes in histone lysine methylation after a diet deficient in both folic acid and choline. Unlike the Davison and Kovacheva studies mentioned above, this study focused on epigenetic changes in the brain. A folate/methyl-deficient diet induced global DNA hypermethylation, which was associated with significant changes in the trimethylation states of histone H3 lysine 9 (H3K9), H3K27 and H4K40. While there was a significant decrease in the levels of both H3K9 and H3K27, there was a significant increase in the levels of H3K40. Moreover, a study done by

Gupta, Kim, Artis, Molfese, Schumacher et al. (2010) found that trimethylation of the H3K4 and dimethylation of H3K9 in the hippocampus area are involved in contextual fear conditioning whereby mice deficient in H3K4 show deficits in this type of learning. Thus, histone methylation also seems to be involved in hippocampus-associated types of learning and memory. With more studies examining the co-occurrence of changes in both DNA and histone methylation, a better understanding of the workings of these epigenetic processes will help with future studies aimed at examining and determining the molecular mechanisms driving developmental alcohol effects, specifically on cognitive functioning such as learning and memory.

What is more, the regulation of gene expression via epigenetic processes is heritable from parent to offspring (Anway & Skinner, 2006). For instance, not only are the effects of irradiation exposure heritable from the exposed parent to the unexposed offspring in a mice model (Mohr, Dasenbrock, Tillmann, Kohler, Kamino et al., 1999), but also, the mutagenesis and tumor formation caused by such exposure are inherited in the germ line at least up to the second generation in mice (Barber, Plumb, Boulton, Roux & Dubrova, 2002). Animal models of developmental alcohol have also begun to explore effects on second generation offspring that are not directly exposed to the teratogen. Lam, Homewood, Taylor and Mazurski (2000) found that in five-day-old offspring of dams exposed to fetal alcohol showed deficits in the righting reflex. Even though more studies are needed to explore multigenerational effects of teratogenic alcohol on cognition and behavior, these initial findings by Lam et al. (2000) peak future interest in examining whether epigenetic modifications caused by developmental alcohol are also inherited by second generation offspring. Further studies can also examine whether the attenuation of alcohol effects by choline supplementation is also passed down from one generation to the other,

as well as the number of future generations in which these teratogenic alterations in DNA imprinting occur.

VII. CONCLUSION

Along with the Garro et al. (1991), Kaminen-Ahola et al. (2010) and Liu et al. (2009b) studies, the present finding of an alcohol-induced change in DNA methylation (especially in two areas of the brain well-known for their susceptibility to developmental alcohol insults) points towards a central role of epigenetic mechanisms in the etiology of FASD and a first step in understanding alcohol's teratology at the molecular level. As is echoed by Haycock (2009), examining the effects of developmental alcohol from an epigenetic viewpoint will help to explain the several behavioral and physical deficits definitive of FASD. This implication is further supported by the observation of a significant change in the alcohol-induced methylation state after supplementation with choline, a nutrient that has been shown, behaviorally, to attenuate some of the learning and memory deficits caused by developmental alcohol. This 'epigenetic reprogramming' by choline also shows that, at least up to the adolescent stage, some of the deficits due to developmental alcohol in the PFC and hippocampus are reversible, and points to a possible effective therapeutic strategy to treat affected individuals.

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