

**OF MOUSE AND METHYL:
AN INVESTIGATION OF METHYL DONORS, METHYLATION AND
METHYLTRANSFERASES IN THE PFC**

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DEDICATION

This work is dedicated to Dr. John W. McKee.

I hope to impact as many people, maintain my passion for science and carry the Dr. title just as well as you have.

I hope this work makes you proud.

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ABSTRACT

OF MOUSE AND METHYL:

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IN THE PFC

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Gaining excessive weight during pregnancy occurs in over 50% of pregnancies in the United States. This excessive weight gain can lead to development of Large for Gestational Age (LGA) babies, or babies born in the top 10% for weight class at birth. LGA babies have increased risk for neurological disorders, including autism, schizophrenia and ADHD. Our lab models excessive gestational weight gain and LGA in a mouse model by feeding dams a 60% high fat (HF) diet throughout gestation and lactation. At weaning all offspring are fed control diet for the remainder of life. HF offspring have increased preference for palatable foods, increased learning and motivation deficits, disrupted gene expression in reward system neurocircuitry and both global- and promoter-specific DNA hypomethylation within the prefrontal cortex (PFC). Dietary methyl donor nutrient supplementation (MS) during pregnancy has been shown to ameliorate some of these phenotypes in HF offspring as well as alter DNA methylation patterns. Because the PFC continues developing postnatally, we were interested in understanding whether MS given during early postnatal life (3-6 weeks) would also ameliorate behavioral and molecular phenotypes. Also, because HF offspring exhibit DNA hypomethylation throughout the brain, we were interested in whether DNA methyltransferase (DNMT) function could mediate these changes. Utilizing operant behavior training, gene and protein expression analysis and mass spectrometry we were able to answer these questions. We determined that early life MS can counteract deficits in motivation and learning in female offspring. Also that MS alters the concentrations of folate and methionine

intermediates and gene expression directly after supplementation in the PFC tissue of male and female offspring. Finally, we determined that perinatal HF diet alters overall DNMT activity within the PFC without altering DNMT1 or DNMT3a expression in adult male and female offspring. This work adds to our understanding that long-term behavior and disease risk can be influenced by both maternal and early life diet. Therefore, it is important to continue study the mechanistic links between excessive gestational weight gain, perinatal HF diet exposure and MS to make better dietary recommendations that may combat disease risk.

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List of Abbreviations

5-CSRTT, 5-choice serial reaction timed task
5-formyl-THF, 5-formyl-tetrahydrofolate
5,10-methylenyl-THF, 5,10-methylenyl-tetrahydrofolate
5-methyl-THF, 5-methyl-tetrahydrofolate
BHMT, betaine-homocysteine methyltransferase
BMI, body mass index
CNS, central nervous system
DNMT, DNA methyltransferase
dTMP, thymidine monophosphate
dUMP, deoxyuridine monophosphate
FR1, fixed ratio 1 task
GWG, gestational weight gain
HF, high fat
KO, knock out
LGA, large for gestational age
MS, methyl donor supplementation
MTR, methionine synthase
PFC, prefrontal cortex
PR, progressive ratio
SAH, S-adenosyl-homocysteine
SAM, S-adenosyl-methionine
SC, standard control
THF, tetrahydrofolate

Chapter One

General Introduction

The choice of what food to fuel the body with is an important one. Due to the advancement of technology and the domestication of animals, our current food landscape is vastly different than the previous generations. The last 200 years have seen an increase in the variety of dairy products, cereal grains, refined sugars, refined oils and alcohols in our diet (Cordain et al., 2005). The USDA recommends a balanced diet of whole fruits, vegetables, whole grains, lean protein foods and non-fat dairy (ChooseMyPlate.gov). However, less than 20% of Americans meet the USDA guidelines for a healthy diet (Krebs-Smith et al., 2010).

The Western diet that dominates the US and other developed countries is characterized by the consumption of high-energy foods that are low in essential nutrients. These calorie (energy) dense foods are high in fats and high in added sugars. According to the CDC, current total food energy from the 3 major macronutrient categories are 49.5% from carbohydrate (which includes sugar), 15.7% from protein and 32.9% from fat (National Center for Health Statistics, 2016). For men, 12.7% of total calories come from added sugar, this is slightly increased to 13.2% for women (Ervin and Ogden, 2013). Sugar consumption has increased from 55.5 kg per capita in 1970 to 69.1 kg per capita in 2000 (Cordain et al., 2005). Refined sugars lack any vitamin or mineral, therefore as consumption of energy-dense refined sugar has increased, the micronutrient density of the diet has decreased (Cordain et al., 2005), creating a population that is at the same time being over-fed while being under-nourished with calorie-dense, nutrient deficient foods. As the population has become more deficient in micronutrients, there is a growing reliance on dietary supplements and food fortification to add micronutrients to the diet (Gahche et al., 2011). One study showed that if enrichment and fortification were not present, inadequate

concentrations of vitamin A, vitamin C, vitamin D, vitamin E, thiamine, folate, calcium, magnesium and iron would be found in a large portion of the US population (Weaver et al., 2014).

Pregnancy is a unique time period when an offspring does not get the choice as to what is fueling their development. It has also been recognized that nutrition during pregnancy can have long term implications for the offspring, specifically with regard to brain development (Georgieff, 2007; Grissom et al., 2014). The objective of this work is to add to the understanding of how specific nutrient excesses and deficiencies during pregnancy alter development of the offspring brain, building upon earlier work done in the laboratory (Carlin et al., 2013; Grissom et al., 2015; Vucetic et al., 2010). A specific focus was made to expand the understanding as to whether *postnatal* nutrient supplementation can be given during early life to alter or ameliorate molecular patterns or physical behaviors set-up by excessive maternal fat intake during pregnancy. This first chapter is used to create an understanding of excessive gestational weight gain (GWG), how it can be modeled in the lab, how it affects the offspring brain development and behavior. It also serves to introduce DNA methylation, a mechanism by which the perinatal environment can alter long-term offspring health, and to introduce methyl donor nutrients and one carbon metabolism and their role in epigenetics and cognition.

Prevalence of obesity and excessive gestational gain

Just over 36% of adults and 17% of youths are considered obese, having a Body Mass Index (BMI) over 30 (Ogden et al., 2015, 2014). An additional 33% of the adult population is considered overweight, having a BMI between 25 and 30 (Flegal et al., 2012). That means 2 in 3 adults in the United States is considered either overweight or obese. Rates of overweight and obesity have steadily risen over the past 30-40 years, it is expected that these numbers will continue to rise. One group estimates that by 2030 86.3% of adults will be overweight or obese, of these 51.1%, obese (Wang et al., 2008).

The Institute of Medicine recommends that women who are normal weight (BMI 18.5-24.9) gain between 25-35 pounds during a pregnancy (Institute of Medicine and National Research Council, 2009). Over 50% of all women exceed these guidelines, and of women who were considered normal weight prior to pregnancy 43.5% overgained during the gestational period (Simas et al., 2011). Women classified as overweight or obese prior to pregnancy overgained at rates of 68.9 and 59.8%, respectively. Gestational weight gain (GWG) is known to affect the growth and health of the fetus. Excessive GWG is a known risk for Large for Gestational Age (LGA) babies, or babies born in the top 10% of weight for their gestational age (Ludwig and Currie, 2010; Sridhar et al., 2016). Greater GWG is associated with increased BMI in offspring at all ages (Schack-Nielsen et al., 2010). In addition to risk for obesity, LGA offspring are at increased risk for diabetes, autism, attention deficit/hyperactivity disorder (ADHD), cognitive deficits, depression, schizophrenia, anxiety and mortality (Alfaradhi and Ozanne, 2011; Colman et al., 2012; Herva et al., 2008; Krakowiak et al., 2012; Lubchenco et al., 1972; Robles et al., 2015; Van Lieshout et al., 2011). Understanding the link between excessive GWG and short and long-term offspring health risks is important to creating recommendations and providing education and interventions to decrease adverse health risks for offspring.

Excessive GWG and LGA can be modeled in the laboratory by feeding mouse dams a high fat (HF) diet (60% calories from fat versus 22% calories from fat in controls) during conception, gestation and lactation (Vucetic et al., 2010). At birth, HF dams and offspring weigh significantly more than the control dams and offspring. At 3 weeks of age, all offspring are weaned onto control diet and body weights begin to normalize. It has been reported that adult offspring from this model have increased insulin resistance, increased fatty liver and adiposity, increased density of neurons producing orexigenic peptides, increased orexigenic signaling, impaired spatial learning, worsened memory deficits and heightened anxiety-like behaviors (Ashino et al., 2012; Chang et al., 2008; Hatanaka et al., 2016; Martin et al., 2014; Muhlhausler et al., 2006; Stachowiak et al., 2013; Sullivan et al., 2014; Tozuka et al., 2010). Our lab specifically has reported that HF offspring have increased preference for sucrose and fat, altered dopamine and

opioid regulation in the brain regions important to reward-seeking behaviors (hypothalamus (HYP), nucleus accumbens (NAC), ventral tegmental area (VTA) and prefrontal cortex (PFC)), global and promoter-specific DNA hypomethylation in these brain regions and learning and motivation deficits in PFC-mediated operant behaviors (Grissom et al., 2015; Vucetic et al., 2010).

DNA methylation

DNA methylation is one proposed epigenetic mechanism by which maternal GWG and nutrition can influence offspring development throughout gestation and adulthood. Epigenetics, meaning “on” or “above” the genome, is the study of both heritable and reversible molecular modifications to the underlying DNA sequence, without altering the base pattern, that in part regulate gene expression (Li, 2002). DNA methylation, the addition of a methyl group to the 5-carbon position on a cytosine base, is a well-studied epigenetic modification. At the beginning of development, the genome of the primordial germ cells is highly methylated (Reik et al., 2001). Upon fertilization, a rapid demethylation throughout the genome occurs, completed by embryonic day (E) 13 to 14. Remethylation of the genome then takes place, beginning around E16 for male gametes and a bit later in female (Reik et al., 2001). Once thought to be a stable epigenetic mark, mounting evidence shows that the genome can be modified throughout pre- and post-natal life via external stimuli such as chemical exposure, diet and age (Anderson et al., 2012; Xin et al., 2015; Zampieri et al., 2015). Additionally, incomplete erasure of DNA methylation from various DNA regions can also alter development (Kawasaki et al., 2014; Miyoshi et al., 2016; Trerotola et al., 2015).

DNA methylation throughout the genome is generally symmetrical, when DNA is methylated on one DNA strand, it should be methylated on the opposing strand. A class of enzymes known as DNA methyltransferases (DNMTs) mitigate this symmetry. DNMTs bind to regions of the DNA and covalently bind methyl moieties to cytosine bases. DNMT1, 3a and 3b are the main DNMTs (Li, 2002). DNMT3a and 3b, the de novo methyltransferases, work to proofread the DNA adding methyl groups to appropriate bases where no methyl group was previously, creating hemi-

methyated DNA. DNMT1, preferential to hemi-methyated DNA and therefore known as the maintenance methyltransferase, works to establish DNA methylation on newly transcribed strands of DNA, either by matching the methylation pattern from the parent strand or filling in hemi-methyated DNA. DNA methylation present within a promoter region of a gene is canonically associated with a tightly closed chromatin state and RNA gene silencing (Robertson, 2002; Robertson and Wolffe, 2000). While the absence of DNA methylation within the same region is associated with RNA gene expression. However, studies continue to reveal the complexity of this relationship (Suzuki and Bird, 2008; Weber et al., 2007). Methylation at specific DNA sites varies significantly across neuroanatomical regions (Ladd-Acosta et al., 2007) and is dynamically regulated across the lifespan (Siegmund et al., 2007). DNMTs are also differentially expressed across cell type within the brain (Veldic et al., 2005, 2004).

Unlike typical somatic cells, most neurons are non-dividing cells, therefore, neuronal DNA generally is not replicated. However, the necessity of both maintenance and de novo methyltransferases for survival has been proven through the use of genetically modified whole-body and brain specific DNMT knock-out (KO) mouse models. Whole body KO of *DNMT1* and *DNMT3b* are embryonic lethal, while *DNMT3a* KO animals develop neural tube defects during late gestation and die prematurely (Li et al., 1992; Okano et al., 1999). Mice lacking functional DNMT3a in the CNS are born healthy, but survive with neuromuscular and motor coordination deficits, and die prematurely (Nguyen et al., 2007). A forebrain-specific *DNMT1* KO mouse model showed global DNA hypomethylation, neurodegeneration, changes in RNA gene expression and deficits in learning and memory (Hutnick et al., 2009). Deletion of *DNMT1* in post-mitotic neurons did not change DNA methylation levels, but deletion in mitotic CNS precursor cells, resulted in DNA hypomethylation in daughter cells (Fan et al., 2001). Another group developed a conditional *DNMT1* mutant mouse with ~90% of cortical and hippocampal cells lacking *DNMT1* in the dorsal forebrain at E13.5 (Hutnick et al., 2009). About 25% of the cortical neurons lacking DNMT1 survived into adulthood, and the mice had defects in learning and memory. Mice with a double KO of *DNMT1* and *DNMT3a* in the forebrain excitatory neurons have deficits in hippocampal CA1

plasticity, smaller hippocampal neurons and show deficits in learning and memory (Feng et al., 2010). Mature neurons showed CpG hypomethylation and altered gene expression. These deficits were not seen when only *Dnmt1* or *Dnmt3a* were knocked out independently. These results indicate that the DNMTs possibly play overlapping roles in post-mitotic neurons. In vitro, neural stem cells derived from embryonic stem cells lacking *Dnmt3a* were globally hypomethylated and had increased proliferation rates, possibly indicating that *Dnmt3a* could play a role the timing of neural cell differentiation (Z. Wu et al., 2012). On the opposite end, when DNMT1 is overexpressed in mouse embryonic stem cells, neurons show increased dendritic branching and arborization (D'Aiuto et al., 2011). In sum, these studies indicate a complex role for DNMTs throughout brain development.

In humans, DNA methylation and proper DNMT function is not only integral to normal CNS function, but the dysregulation of DNA methylation also underlies multiple neurological diseases (Gräff et al., 2011). Disrupted DNA methylation and methyltransferase function has been implicated in autism, Parkinson's disease, schizophrenia and ADHD (Loke et al., 2015; Masliah et al., 2013; Schuch et al., 2015; Weng et al., 2013). Mutations in DNMT1 are linked to cognitive function decline, hearing loss, sensory neuropathy and result in imbalanced DNMT1 protein homeostasis (Baets et al., 2015). Disrupted localization of DNMT1 has been implicated in Alzheimer's and Parkinson's disease (Desplats et al., 2011; Mastroeni et al., 2013).

One carbon metabolism and methyl donor nutrients

DNMTs receive methyl groups through the conversion of S-adenosyl-methionine (SAM) to S-adenosyl-homocysteine (SAH). This reaction is one in a cyclical series of biochemical reactions called one carbon metabolism (Figure 1). One carbon metabolism (Figure 1) refers to the intersection of folate and methionine metabolism cycles that donate and regenerate one-carbon units for various reactions in the cell (Ducker and Rabinowitz, 2016; Fox and Stover, 2008; Tibbetts and Appling, 2010). Through these reactions, our cells synthesize purines, pyrimidines, thymidylate, creatine, phosphatidylcholine and multiple hormones. One carbon metabolism also

provides methyl groups for at least 50 different methylation reactions that occur within the cell, including methylation of RNA, DNA, histones, neurotransmitters and other small proteins. One carbon metabolism is also involved in the catabolism of choline and histidine and the interconversion of serine and glycine. One carbon metabolism is involved in almost every cellular process and thus it is important that these reactions occur efficiently.

One carbon metabolism is highly dependent on nutritional status. In Figure 1, highlighted in gray are essential nutrients for one carbon metabolism known as methyl donors. An essential nutrient is a nutrient that cannot be synthesized by the body and therefore must be consumed via the diet. Vitamin B12, vitamin B6, vitamin B9 (folate), methionine, betaine, choline and zinc are necessary for one carbon metabolism. These methyl donor nutrients are critical intermediates or cofactors for enzymes involved in one carbon metabolism. Imbalances in methyl donors can negatively impact enzyme function, SAM regeneration and DNA methylation (Anderson et al., 2012; Fernández-Roig et al., 2012; Ly et al., 2012; Niculescu et al., 2006; Niculescu and Zeisel, 2002; Sable et al., 2014). Additionally, deficiencies and excesses of these nutrients also can alter cognition, behavior and risk for disease (Araújo et al., 2015; Boeke et al., 2013; Bottiglieri, 2005; Breimer and Nilsson, 2012; Grayson and Guidotti, 2012; Tomizawa et al., 2015; Zhang et al., 2016).

Folate metabolism serves to create and transfer one carbon units for the biosynthetic processes listed above. Natural folates in the body and diet are typically in reduced form, mainly 5-methyl-tetrahydrofolate (THF) in humans (Suh et al., 2001). The THF backbone, the active form of folate, is used to carry and transfer one carbon units. One carbon units, also known as methyl groups, are covalently bound to the 5- and 10-position on the pteridine ring of THF. THF can be maintained in three oxidative states, each which play specific biosynthesis roles. These roles were reviewed thoroughly by Ducker and Rabinowitz (Ducker and Rabinowitz, 2016). Briefly, 5,10-methylene-THF produces thymidine and serine, 5-methyl-THF produces methionine and 10-formyl-THF produce purines, formate and CO₂.

The most abundant form of folate, 5-methyl-THF, can be converted to methionine via a vitamin B12 dependent methyltransferase in which the methyl group from 5-methyl-THF is transferred to homocysteine. Resulting in the formation of methionine and unsubstituted THF. THF can then begin the folate cycle again, and methionine can continue through its unique metabolism. Methionine is the precursor of SAM, the universal methyl donor. Donation of a methyl group from SAM creates SAH, which is reversibly metabolized into homocysteine. Homocysteine can be remethylated to reform methionine via betaine-homocysteine methyltransferase (BHMT, not found in the brain) or methionine synthase (MTR), a vitamin B12 dependent methyltransferase, to re-cycle through methionine metabolism. Alternatively, homocysteine can enter the transsulfuration pathway, which is required to support glutathione synthesis and remove reactive oxygen species from the brain (Vitvitsky et al., 2006).

Methyl donor nutrients are necessary for normal CNS growth and development. Both deficiency and imbalanced methyl donor concentration has been linked with abnormal CNS development as well as neurological diseases. For example, choline deficiency during gestation alters global and gene-specific DNA methylation in the developing mouse hippocampus, a region important to learning and memory (Niculescu et al., 2006). Increased homocysteine is a potential risk factor for cognitive impairment and other neurological diseases (Jiang et al., 2017; Setién-Suero et al., 2016). Additionally, low vitamin B12 status has also been associated with poorer memory performance and reduced microstructural integrity of the hippocampus (Köbe et al., 2016). Higher folate intake has been associated with lower risk for dementia (Lefèvre-Arbogast et al., 2016). It has been suggested that using B vitamins to help remethylate homocysteine to methionine could be one mechanism to improve cognition or slow cognitive decline.

The liver is thought to mediate about 80% of the one carbon reactions in the body (Stead et al., 2006), thus all of the pathway information has been generalized to liver one carbon metabolism. There is evidence that shows altered one carbon cycles in various cell types and diseases (Ducker and Rabinowitz, 2016). Unfortunately for the present review, there have been

no studies elucidating the specific one carbon cycle present in the CNS. Citing the associations of one carbon metabolism reactions and efficiency in neural tube closure (Blom, 2009; Zeisel, 2009), neurological diseases (Kennedy, 2016; McGarel et al., 2015), cognition (Morris, 2012) and the current studies of this dissertation (McKee et al., 2017), more studies are needed to form a more complete picture of CNS specific, even cell type specific, one carbon metabolism. Understanding whether neurons, astrocytes, oligodendrocytes and microglia have the same patterns of one carbon metabolism under various metabolic conditions could be important to understanding how specific neurological diseases develop. All together one carbon metabolism is a series of biochemical reactions necessary for the viability of the cell. The efficiency is highly dependent on nutritional status and its dysregulation is correlated to many disease states. Further elucidation of the connection between nutritional status, one carbon metabolism dysregulation and disease states will help us understand whether nutritional interventions can be used for therapeutic benefit.

Methyl donors and neurodevelopment and cognition

Beginning at conception methyl donors are important for the development of the CNS, specifically closure of the neural tube. Emerging evidence suggests that maternal folate status throughout pregnancy affects offspring neurodevelopment and postnatal behavior and cognition (McGarel et al., 2015). During pregnancy concentrations of folate decrease by about 50% (Milman et al., 2006). There is also an increased energy and nutrient demand required for continuous fetal development. Fortification of grain products with folic acid, a stable oxidized form of folate, was introduced in the United States in the 1990's to help increase the folate concentration consumed by women of childbearing age to prevent the risk of neural tube closer defects and other midline defects. Since the introduction of folic acid fortification, incidence of neural tube closer defects has decreased by about 25% (Blom, 2009). The use of folic acid supplementation as well as other methyl donors (mainly vitamin B12, choline and methionine) as therapeutic interventions to enhance CNS development, prevent neurological disease and slow

down cognitive decline has burgeoned in the last 20 years. This section will review a few of the studies and highlight the questions that remain.

Maternal (perinatal) supplementation

Maternal choline availability influences offspring brain development and function (reviewed by Zeisel and Niculescu) (Zeisel and Niculescu, 2006). In animal models the positive relationship between maternal choline supplementation and neurodevelopment and cognition is well developed. Maternal choline supplementation enhances cognition (fewer working memory errors, responses to reach criterion, reference memory errors, faster behavior acquisition and shorter escape latency in the Morris water maze) prevents age-related memory decline, enhances hippocampal function (McCann and Ames, 2007; Zhu et al., 2016), reduces the effects of fetal alcohol exposure in the offspring (Thomas et al., 2010, 2009), can increase nerve growth factor levels in the hippocampus and frontal cortex (Sandstrom et al., 2002) and normalize choline-deficiency induced DNA hypermethylation (Davison et al., 2009). Choline supplementation can also reverse the effect of folate deficiency on neurogenesis and apoptosis at E17 in the fetal forebrain (Craciunescu et al., 2010) as well as iron deficiency-induced reprogramming within the hippocampus (Tran et al., 2016). In a mouse model of Down syndrome, maternal supplementation of choline attenuated degeneration of the basal forebrain cholinergic neuron system (Kelley et al., 2014). In a mouse model of autism, high maternal choline consumption improves anxiety-like behaviors and increases social interaction (Langley et al., 2015). (For further review see Jiang et al. 2014 (Jiang et al., 2014)) Because of the beneficial findings in rodent models, effects in humans are being investigated. The current results are mixed. Early second trimester maternal plasma choline levels are positively correlated with early cognitive development (B. T. F. Wu et al., 2012) and at 7 years (Boeke et al., 2013). Neither supplementing with phosphatidylcholine (Cheatham et al., 2012) or maternal cord blood choline concentrations (Signore et al., 2008) correlated with enhanced infant cognition.

Opposite to choline, the positive link between maternal folic acid supplementation and neurodevelopment and cognition is stronger in humans than it is in animal models. In humans, maternal folic acid supplementation is associated with improved verbal, motor, verbal-executive scores in 4 year olds (Julvez et al., 2009). Maternal use of folic acid supplements is associated with reduced risk of severe language delay at 3 years of age (Roth et al., 2012). Higher plasma folate during the 30th week of pregnancy is associated with increased learning, long-term storage and memory retrieval and decreased inattention at 10 years old (Veena et al., 2010). Folic acid intake during the first trimester is associated with reduced language delay, increased communication and verbal skills, and increased cognitive performance (Chatzi et al., 2012; Roth et al., 2012; Villamor et al., 2012). Supplementation with folic acid during the second and third trimester decreased the levels of homocysteine in maternal and cord blood, but offspring outcomes were not studied (McNulty et al., 2013). In animal models, offspring from rodents deficient in methyl donors during pregnancy, but supplemented with folic acid during late gestation, had reduced the risk for structural and functional defects in the CNS during the prenatal period (Geoffroy et al., 2016). Late gestational folic acid supplementation restored expression of specific microRNAs in the brain when compared to control and methyl donor deficient animals (Geoffroy et al., 2016). Additionally, reduced folate intake in pregnant mice impaired short-term memory in offspring as well as increased apoptosis in the hippocampus (Jadavji et al., 2015). Expression of a variety of gene are altered in offspring brain after folic acid supplementation (Barua et al., 2014).

In natural food sources, a combination of multiple methyl donor nutrients are often found. Additionally, methyl donor nutrients work in concert with each other within one carbon metabolism. As a result, supplementing with a variety of methyl donor nutrient mixtures can alter cognitive and neurobiological outcomes. Children of mothers given a mixed micronutrient supplementation which included multiple methyl donors had increased procedural memory and higher general intellectual ability at 10 years of age (Prado et al., 2017). A study of maternal methyl donor consumption found positive associations between folic acid intake and cognition at

3 years, but no associations were found for choline, betaine or methionine (Villamor et al., 2012). In rodents, methyl donor supplementation during pregnancy and lactation normalized preference for palatable rewards and altered global DNA methylation and RNA gene expression in brain regions involved in reward function (Carlin et al., 2013; Cooney et al., 2002; O'Neill et al., 2014). Additionally, maternal micronutrient imbalance alters DNA methylation and expression of neurotropic factors in the offspring cortex (Sable et al., 2015, 2014).

Early postnatal supplementation

Early life nutritional supplementation with methyl donors and cognition is a relatively unstudied field when compared to maternal or adult supplementation time periods. Evidence shows that infants supplemented with choline had fewer attention problems and less social withdrawal at 40 months (Ross et al., 2016), but that 6 weeks of choline supplementation in school-aged children did not improve cognition (T. T. Nguyen et al., 2016). Betaine, but not choline status, in 5 year olds is positively associated with total language scores (Strain et al., 2013). 3 months of a mixed B vitamin supplementation in kindergarten children improved folate and homocysteine status, but did not affect cognitive performance (Rauh-Pfeiffer et al., 2014). In rodent models, early life postnatal methyl donor supplementation in female mice increased motivation and learning in an operant chamber task (McKee et al., 2017). Postnatal supplementation of multiple methyl donors between P2-9 restored methionine levels and early life stress induced adult cognitive impairments (Naninck et al., 2016). 3 weeks of folic acid prevented anxiogenic and memory impairment in an open field test, possibly by partial recovery of the Na^+, K^+ -ATPase in the frontal cortex (Carletti et al., 2012). 18 weeks of dietary methyl donor supplementation normalized depression-like behaviors induced by maternal separation during lactation (Paternain et al., 2016). 3 weeks of methyl donor supplementation increased DNA methylation in the amygdala and reduced depression-like behaviors in forced swim test, and that 3 weeks of 90% methyl donor depletion increased depression-like behaviors (McCoy et al.,

2017). Rat pups deficient in folate from birth to 3 weeks of age exhibited long-term memory, spatial learning and set-shifting deficits (Berrocal-Zaragoza et al., 2014).

Adult supplementation

Memory loss, increased neurological disease risk and increased oxidative stress in the brain is characteristic of aging adults. In rodents, 8 weeks of folic acid supplementation in aged rats showed improved memory and decreased lipid peroxidation, a marker of aging, in multiple brain regions (Singh et al., 2011). 7 days of folic acid treatment in rats showed decreased locomotor activity and decreased lipid peroxidation in the hippocampus (Brocardo et al., 2010). One week of methyl donor supplementation increased motivation in mice when either exposed to a maternal high fat diet or previously exposed to methyl donors early in life (McKee et al., 2017).

In animal models, the benefits of methyl donor supplementation in the aged population have not been well-examined. However, the use of methyl donors in clinical trials is extensive, and the results are mixed. A meta-analysis reviewed whether 6 months of folic acid supplementation in healthy individuals, with or without other B vitamins, altered cognitive function (i.e. memory speed, language and executive function), and found no changes to cognition (Wald et al., 2010). But 3 years of folic acid supplementation in healthy individuals reported higher memory, information processing speed and sensorimotor speed scores (Durga et al., 2007). Trials supplementing with folic acid and B12 for years, measured cognitive status at both 12 and 24 months found that only at 2 years did supplementation promote improved cognitive function including immediate and delay memory performance (Walker et al., 2012). But, two years of B12 and folic acid supplementation in older adults did not reduce depressive symptoms (de Koning et al., 2016). A recent meta-analysis revealed that 77 human trials have been performed attempting to improve cognition in elderly patients with Alzheimer disease or dementia by providing B vitamin supplements to reduce homocysteine levels. A study published earlier in 2017, Using the Mini-mental state examination score as a measure of cognition, found that B vitamins do reduce homocysteine levels, but that there were no overall changes in cognition in these patients (Zhang

et al., 2017). Following this population further will elucidate whether these B vitamins could perhaps slow progression of cognitive decline. Additionally, a study showed that lower B6 and riboflavin (B2) status were associated with an accelerated rate of cognitive decline when using the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS), but B2 did not have the same association when using the MMSE to measure cognition (Porter et al., 2017). Indicating that current measures for cognition are not uniform, so it is difficult to compare across studies that use different methods of detection.

Adverse effects of supplementation

Too much of a good thing is no longer healthy. Because of supplementation and dietary recommendations, 5% of the US adult population and between 30-66% of children 1-13 have plasma concentrations higher than the recommended daily dose of methyl vitamins (Regan L Bailey et al., 2010; Regan L. Bailey et al., 2010). As this subset of the population emerged, studies began showing the detrimental effects of too much methyl donor supplementation. The current recommendations for daily folic acid intake are 400 µg/day for adult males and non-pregnant females. The recommendation increases to 600 µg/day for pregnant and 500 µg/day for lactating females. Offspring from women who consumed higher than recommended folic acid concentrations during pregnancy (5000 µg/day, almost 10 times the recommended daily allowance) had lower psychomotor scores at 1 year of age (Valera-Gran et al., 2014). In mice, offspring from dams fed high levels of folic acid (10 times the control, 20mg/kg of diet) during gestation displayed altered development of the cortical layers at E17.5 and short-term memory impairment and decreased hippocampal size at 3 weeks (Bahous et al., 2017). High vitamin diets during pregnancy have been shown to increase metabolic syndrome risk in offspring by altering the neural pathways involved in energy homeostasis and feeding (Clara E Cho et al., 2013; Pannia et al., 2014), but this could be prevented by providing a post-weaning high multivitamin or high folate diet (C E Cho et al., 2013). Excess folate (4 times the control, 8.0mg/kg of diet) during adolescence has been correlated with deficits in motivation and spatial memory tasks (Sittig et

al., 2012). Another factor in methyl donor supplementation are genetic variations in the enzymes that metabolize the nutrients. It is well known that for individuals with certain mutations in MTHFR, supplementing with folic acid is detrimental as they cannot break down the folic acid into active folate (Mattson and Shea, 2003). In dams with a MTHFR deficiency, methyl donor supplementation was associated with embryonic delay and growth retardation at E10.5 in their offspring, but longer-term outcomes were not studied (Pickell et al., 2011). It now is evident that there is a genetic and environmental interaction in which concentrations of methyl donor nutrients are no longer healthful, but harmful, and studying these interactions are very important (further reviewed Shorter et al. 2015 (Shorter et al., 2015)).

Conclusions about supplementation

We now understand that both too much and too little methyl donor is detrimental to offspring and adult neurodevelopment and cognition. Understanding which nutrients to supplement with, concentrations of each nutrient, how long should a supplement be taken and what populations should be taking supplementation still need to be answered and standardized when deciding whether methyl donor supplementation can alter CNS development and long-term cognition. Many of the basic research studies point to methyl donors as a way to modulate cognition, but the current clinical literature is inconclusive (Veena et al., 2016). Earlier diagnosis of homocysteine levels, understanding critical periods for supplementation and which populations would most benefit from supplementation could all help to increase efficacy. For studies that show detrimental outcomes, elucidating whether it is the effect of taking a bolus amount of one nutrient (instead of a mixed cocktail) or the actual increased concentration that is detrimental would help to make consumption recommendations. Going forward, controlled experimental designs, consistent methods and definitions when performing studies (i.e. What is cognition?, How do we measure cognition?, What are “high” or “low” concentrations?, Do we use natural folates or folic acid? (Colapinto et al., 2016)) and mechanistic studies will help reveal these answers.

The prefrontal cortex

The prefrontal cortex (PFC) is a highly developed region of the forebrain known to mediate higher cognitive function. The PFC lies within the frontal lobes. The size of the PFC is the main anatomical difference between humans and other primates. The increased size and highly developed nature of the PFC led scientists to believe that this region is critical for the higher brain functioning specific to *Homo sapiens*, including working memory and planning of behavior. Studies involving humans with lesions to the PFC provided insight as to the PFC's role (Cato et al., 2004; Harlow, 1868; Tranel et al., 2007). Phineas Gage is the most famous case. In 1848, while Gage was working on the railroad an iron bar flew straight through the left side of his face through the frontal lobe of his brain. There was no indication that Gage lost consciousness and could function well within an hour. Characteristic of patients with damage to the PFC, Gage did not exhibit any changes in IQ, long-term memory function or motor and language skills. In the years following his accident, Gage was described as "no longer Gage", his personality and behavior had changed, but his intelligence level had not. Once thought to be a well-balanced businessman, hard-working and responsible, Gage became impatient, fitful and irreverent (Harlow, 1868).

Since the days of Gage, the PFC has been shown to be an important driver of higher-level cognition (executive function, working memory and goal orientation) (Funahashi and Andreau, 2013). This includes supporting the ability to determine between good and bad, organize behavior in time and in context, regulates impulse control and learn and execute complex behaviors (Kolb et al., 2012). Disorders of the prefrontal cortex include Attention-Deficit/Hyperactivity Disorder, Antisocial Personality Disorder, Parkinson's disease, Schizophrenia, Obsessive-Compulsive Disorder and Autism.

Development of the PFC is altered by a variety of pre- and post-natal factors (reviewed by Kolb et al. 2012) (Kolb et al., 2012). Nervous system development begins during the early embryonic period. Neural stem cells arise during gastrulation, and then migrate to the appropriate brain structure, mature and form synapses. In humans peak synaptic density throughout the brain

is reached between 1 and 5 years old, studies show that within the PFC this peak could be even later (Gogtay et al., 2004). After this peak, the real work begins as the glia begin to prune synapses that are unused. Pruning of the PFC occurs at the slowest rate within the brain (Kolb et al., 2012). This long period of pruning indicates why environmental exposures and experiences could have greater influence on the synaptic density and functioning of the PFC than other brain regions. Early-life social interactions, chemical exposure and nutrition can all alter PFC development and function (Kolb et al., 2012).

In humans, scientists utilize a battery of behavior assessments to study cognitive processes driven by the prefrontal cortex. Similarly, by utilizing operant behavior chambers and various other behavior paradigms, we can use animal models to shed light on mechanisms by which the PFC, or other brain regions, drive higher cognitive processes. In this dissertation, a 9-hole operant chamber is used to understand learning, attention and motivational behaviors in mice, all mediated by the PFC. These chambers have 9-recessed holes at the back of the chamber, 5 of which have LED lights that can be turned on and off. At the front of the chamber there is a magazine which dispenses palatable reinforcer. Animals learn the association between nose-poking a lit recessed hole at the back of the chamber and the following dispensation of palatable reinforcer to the front of the chamber. We can use various schedules of the work followed by reward association to measure learning, motivation, inattention and impulsivity.

Conclusion

Due to the high prevalence of excessive GWG in the United States, it is important for the continued investigation of how this affects the offspring, specifically in regards to brain development and risk for neurological disorders and cognition. By continuing to investigate the differences in brain development, we can identify risks and possible therapeutic targets to ameliorate adult disease. Specifically in this thesis, investigating how excessive GWG alters DNMT function within the PFC. Additionally, examining whether dietary methyl donor supplementation in early life, while the brain is still developing, can alter long-term brain

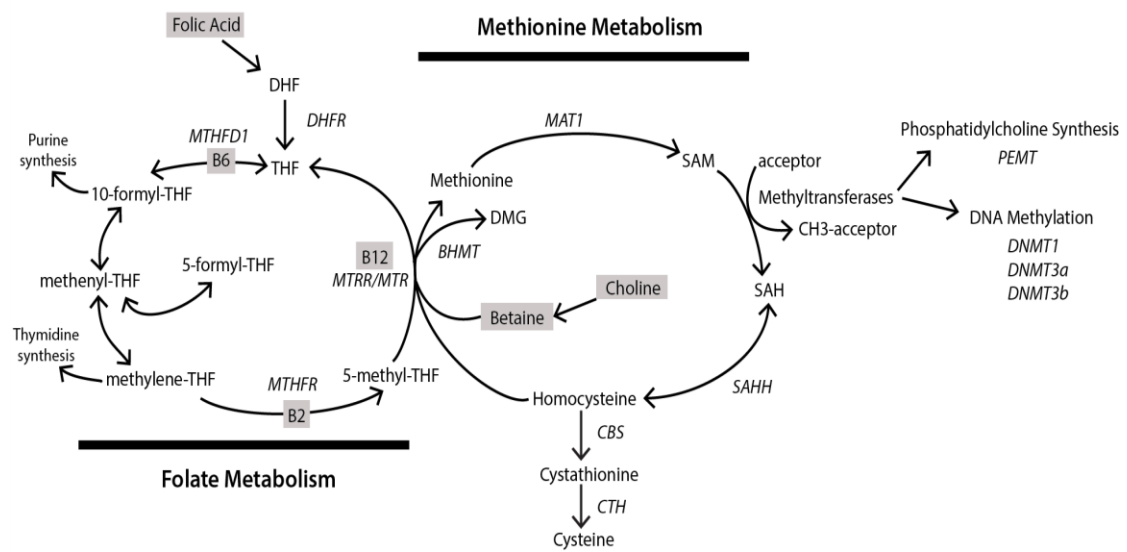
development and cognition. This will provide insights as to sensitive time periods of brain development that can be targeted by nutritional interventions.

Figure Legends

Figure 1. Simplified schematic of the intermediates, enzymes and nutrients involved in one carbon metabolism. DHF, dihydrofolate; THF, tetrahydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DHFR, dihydrofolate reductase; MTHFD, methyltetrahydrofolate dehydrogenase; MTHFR, methyltetrahydrofolate reductase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; BHMT, betaine homocysteine S-methyltransferase; MAT1, methionine adenosyltransferase; SAHH, S-adenosylhomocysteine hydrolase; CBS, cystathionine- β -synthase; TYMS, thymidylate synthetase; DNMT, DNA methyltransferase; PEMT, phosphatidylethanolamine N-methyltransferase; GNMT, glycine N-methyltransferase

Figures

Figure 1.



Chapter Two

Methyl donor supplementation alters cognitive performance and motivation in female offspring from high fat diet fed dams

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Introduction

Over the past three decades overweight and obesity rates have more than doubled (Ogden et al., 2014, 2012). Pregnancy is a particularly vulnerable time when women are susceptible to excessive weight gain, as over 75 percent of pregnant women will gain excessive weight during pregnancy (Schack-Nielsen et al., 2010). This maternal obesity can lead to an increased risk for neurological disorders in offspring, such as attention deficit/hyperactivity disorder (ADHD), autism and schizophrenia (Krakowiak et al., 2012; Rivera et al., 2015; Robles et al., 2015; Van Lieshout et al., 2011). Excessive gestational weight gain can be modeled in mice by feeding dams a 60% high fat diet (HFD) from the onset of breeding through pregnancy and lactation (Grissom et al., 2014; Vucetic et al., 2010). And previously, we have shown that offspring from dams fed a HFD have cognitive and executive function deficits (e.g., impulsivity) in an operant 5-choice serial reaction timed task (5-CSRTT) (Grissom et al., 2015).

DNA methylation, the addition of a methyl group to a cytosine base within the DNA sequence, is an epigenetic mark, that in part, regulates gene expression (Jones, 2012). DNA methylation is hypothesized to link gestational environment with adult disease (Waterland and Jirtle, 2003) through persistent changes in gene expression. In autism, schizophrenia and ADHD, aberrant DNA methylation and methyltransferase function have been implicated in development of these diseases and cognitive dysfunction (Loke et al., 2015; Schuch et al., 2015; Weng et al., 2013). In our mouse model, we have shown that offspring exposed to maternal HFD have whole-

genome DNA hypomethylation in multiple brain regions, including the PFC, along with promoter-specific hypomethylation and dysregulated gene expression (Vucetic et al., 2010). Additionally, expression of two methyltransferase enzymes, DNA methyltransferase 1 (DNMT1) and catechol-o-methyltransferase, in the PFC correlate with overall poor performance in operant behavioral tasks (Grissom et al., 2015), suggesting that maternal HFD exposure disrupts methyltransferase function which could lead to altered gene expression and behavioral deficits.

Methylation reactions, including DNA methylation, require dietary consumption of a group of nutrients known as methyl donors (folate, vitamin B12, methionine, zinc, betaine and choline) (Anderson et al., 2012; Fox and Stover, 2008). Pregnant obese women have lower plasma concentrations of methyl donors and this is correlated with levels found in the fetus (Sen et al., 2014). The explanation for these lower levels is unclear, but may include differences in bioavailability or metabolism of the methyl donors in the context of higher adiposity (da Silva et al., 2013) or decreased intake of the recommended multivitamins (Masho et al., 2016). Lowered concentrations could be one mechanism by which aberrant DNA methylation is induced, resulting in increased disease risk for the offspring. Conversely, methyl donor supplementation can alter DNA methylation, RNA gene expression and behavior in mice (Cooney et al., 2002; O'Neill et al., 2014; Waterland and Jirtle, 2003), and supplementation of maternal HFD with methyl donors during pregnancy has been shown to ameliorate palatable food preference, increase locomotor activity and gene expression (Carlin et al., 2013), alter promoter-specific methylation and gene expression (Paul Cordero et al., 2013a) and prevent metabolic disease (Goodspeed et al., 2015; Seferovic et al., 2015). Beyond the early life window, methyl donor supplementation in adults has been shown to affect cognitive endpoints. Adult methyl donor supplementation can improve memory function (Yu et al., 2016), has been used to target synaptic dysfunction in Alzheimer's disease (Van Wijk et al., 2014) and slow memory decline and improve processing speed in some populations (Morris, 2012).

Given that methyl donor supplementation during pregnancy or in adulthood alters gene expression as well as cognitive and behavioral endpoints, we wanted to determine whether early

life supplementation (e.g., in adolescence, when the prefrontal cortex is still developing) could alter performance in a cognitive task. The human brain continues to develop extensively after birth (Jiang and Nardelli, 2015), and the PFC is the last brain region to mature (Gogtay et al., 2004; Sowell et al., 2001), not reaching functional capacity until late adolescence (Spear, 2000). Behavioral tasks, such as the 5-CSRTT, can be used to assess PFC-mediated executive function. Deficits in PFC-mediated executive function are central to ADHD, autism and schizophrenia (Bari and Robbins, 2013; Duncan, 2013; Floresco and Jentsch, 2011). Therefore, in the present studies, we utilized the 5-CSRTT behavioral task to determine whether early life methyl donor supplementation could block or attenuate PFC-mediated behavioral deficits associated with maternal HFD exposure. Additionally, targeted gene expression analysis of the PFC was used to identify potential molecular mechanisms underlying behavioral changes, with a focus on genes related to neurotransmission, neuroinflammation and epigenetics.

Methods and Procedures

Animals and experimental model: All animals were cared for according to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. An experimental timeline is seen in **Figure 1A**. C57BL/6J virgin females were bred with DBA/2J males. The B6D2F1/J hybrid mice are used in our work as the hybrid background is more similar to heterogeneity observed in humans, as opposed to a pure inbred strain. All animals were fed control diet prior to breeding (Table 1). From the onset of breeding, through pregnancy and lactation, dams (n=10/group) were fed one of two purified diets (Test Diet, Richmond, IN); (1) standard control (SC) and (2) 60% high fat (HF) (Grissom and Reyes, 2013; Vucetic et al., 2010). Specific diet formulations are shown in **Table 1**. Litters were culled to 8-9 pups as necessary, to equalize access to nutrition throughout lactation. At weaning, 1-2 offspring of each sex from each litter were randomly assigned to one of two diets (to control for litter or maternal effects); (1) control or (2) control diet plus methyl donor supplementation (MS) (Test Diet, Richmond, IN), and were group housed with 5 animals per cage. At 6 weeks, all offspring were returned to control diet

for the remainder of the experiment. (Specific litter information: Litter numbers; SC: n=8, HF: n=10; pups reared; SC: n=67, HF: n=86; initial experimental group size; Male: SC: n=15, HF: n=15, SC+MS: n=15, HF+MS: n=15; Female: SC: n=15, HF: n=13, SC+MS: n=15, HF+MS: n=18)

Behavior: Operant behavior testing occurred as previously described (Grissom et al., 2015).

Briefly, male and female animals were group housed (5 mice/cage) in a reverse light cycle 12h:12h (0900 lights off) room so testing could be done during the active period (lights off).

Animals were food restricted to 90% of free feeding weight. Prior to training, animals were pre-exposed to the palatable reinforcer (Yoo-hoo, Mott's, Plano, TX, USA) for 24 hours to decrease neophobic responses and ensure that all animals consumed the reinforcer.

Operant behavior training: Simultaneously, male and female animals were trained in a 9-hole operant chamber (Lafayette Instruments, Lafayette, IN). The back of the chamber contains 9 holes with recessed lights visible to the animal. The chambers detect nose pokes to any of the 9 holes by infrared beam breaks. A magazine, where reinforcer is deposited, is located at the front of the chamber. Infrared beam breaks upon magazine entry were also recorded. Magazine training (Pavlovian Conditioned Approach, PCA) was given for the first 14 days. In magazine training, the back center light is turned on for 8 seconds and then terminated. Upon termination a droplet of reinforcer is delivered to the magazine at the front of the chamber (30 trials/30 minute training session).

Following magazine training, Fixed Ratio 1 (FR1) training began. The back center hole (hole 5) remained lit until one nosepoke was made to this hole. The light was then terminated and reinforcer was dispensed into the magazine. When the animal completed a magazine entry, the cycle repeated. Responses to the other 8 holes did not initiate reinforcer. Each animal completed one 30 minute session each day and responses were recorded each day. Criterion for task acquisition was 50 nosepoke responses within the 30 minute session. Animals which failed to acquire the nosepoke response were eliminated from FR1 and progressive ratio (PR) analyses (number of animals that failed to learn: male: 1 HF, 3 HF+MS; female: 1 HF, 1 SC+MS).

Progressive ratio testing occurred after 2.5 weeks of FR1 training. The number of nosepokes to dispense reinforcer now increased arithmetically every third trial (i.e. 1, 1, 1, 2, 2, 2, 4, 4, 4, 7, 7, 7...). Sessions continued for 60 minutes or until the animal made no nosepoke response for 5 minutes. Breakpoint was measured at the trial in which the animal would no longer nosepoke for the reinforcer.

Animals that met FR1 criterion continued onto 5 choice serial reaction (5-CSRTT) schedule A training, previously described (Grissom et al., 2015; Young et al., 2009). Briefly, animals initiated the training by completion of a magazine entry. This triggered an inter-trial interval (ITI) through which the animal waited for the illumination of one of the five odd-numbered holes at the back of the chamber. The animal then was required to nosepoke the illuminated hole for completion of a correct trial. A premature trial was recorded if an animal made a response during the ITI. If an animal responded to an un-illuminated nokepoke hole then an incorrect trial was recorded. If no response was made an omitted trial was recorded. Only a response at the correct hole during the 8-second stimulus or a 2-second limited hold after stimulus termination was reinforced. Performance measures are reported for first day where criterion (>20 responses, with 50% correct) was met.

Once criterion was met for schedule A training, animals were transitioned to 5-CSRTT titration schedule, a more challenging version of the task, as described in Martin et al., 2015 (Martin et al., 2015). Briefly, in contrast to schedule A training in which the stimulus duration and ITI remained constant throughout the session, within a single session of the titration schedule, stimulus duration is titrated based on an animals' individual performance. Specifically, after a successful magazine entry to initiate training for the session, the first light stimulus is 10 seconds (stimulus duration). Upon each correct nosepoke, reinforcer is received, and the stimulus duration for the subsequent trial is shortened one step down in the series (10, 8, 6, 4, 2, 1, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 seconds respectively). Incorrect and omitted responses elicit a lengthened stimulus duration one step up the series for the subsequent trial. While premature responses maintain the same stimulus duration. The ITI remained constant for this training. Median stimulus

duration (median of stimulus lengths for all trials) and shortest cue duration (shortest stimulus duration reached within a single training session) were calculated for each animal. Performance measures are reported for day 1 of titration training.

Adult methyl donor supplementation: Upon completion of 5-CSRTT titration training, PR was run to evaluate baseline motivation prior to acute methyl donor supplementation. All animals were then fed the methyl supplemented diet for 7 days (each week day running 5-CSRTT training to maintain operant responding) and then tested in PR after acute supplementation. Each animals' breakpoint was compared before and after supplementation to assess differences in motivation due to acute methyl supplementation. Only animals that successfully performed schedule A training were included in the analysis (animals not included: HF: 2, SC+MS=1, HF+MS=1; 1 additional SC+MS animal not included because chamber malfunctioned).

Gene expression in Prefrontal Cortex: After completion of operant training, female animals were sacrificed. Brains were placed in RNAlater and stored at -20C. The medial prefrontal cortex was dissected from a 2mm coronal slice from bregma +2.3 to 0.3 and DNA and RNA were extracted using AllPrep DNA/RNA Mini Kit (Qiagen). 220ug/10ul cDNA was synthesized using High Capacity Reverse Transcriptase kit (Applied Biosystems). Concentrated cDNA was used in a specific target preamplification following the manufacturer's recommendations (Fluidigm Corp. South San Francisco, CA). Briefly, Taqman assays for 32 genes were pooled to a final concentration of 0.2X. 2ul of the pooled assay mix was combined with 2ul cDNA and 4ul 2X Taqman Preamp Master Mix (Life technologies). The preamplification reaction was cycled following the manufactures protocol in a 750 fast qPCR machine. Samples were then diluted 1:50 using 1X TE. Samples were then run on a 96.96 Dynamic Array IFC on the Biomark HD machine (Fluidigm Corp). We were interested in the expression of genes involved in DNA methylation, inflammation, reward and learning behaviors, as these genes are known to be altered after HFD exposure (**Table 2**). Gene expression values were correlated with median stimulus duration in the

5-CSRTT titration schedule, an endpoint increased by maternal HFD and normalized by MS. Expression of targets was normalized to the mean of the housekeeping gene ACTB (expression was not changed across conditions), expressed as RQ values, and analyzed by two-way ANOVA. Nonparametric Spearman correlations were calculated between each animal's performance for all behaviors and the expression of each gene as $2^{-\Delta Ct}$.

Data analysis: Two-way ANOVA (maternal diet x supplementation) was used to evaluate results using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). Repeated measures by time were conducted for Fixed Ratio 1 training. Bonferroni post hoc planned comparisons were conducted when appropriate. The comparisons of interest were; (1) control versus high fat offspring (to determine the effect of maternal HF diet) and (2) unsupplemented versus supplemented (to determine the effect of supplementation). Interactions and main effects are described in the results and significant post hoc comparisons are indicated in the figures, with $p \leq 0.05$ considered significant.

Results

To determine whether early life methyl donor supplementation can alter operant behavior in offspring from dams fed a 60% HFD throughout pregnancy, we supplemented offspring with dietary methyl donors from 3-6 weeks of age and tested them in a series of operant behavior tasks (Fig. 1A). Male mice from all dietary conditions reached criterion around day 5 of FR1 training (Fig. 1B), while all females met the criterion around day 3 (Fig. 1C) with no significant difference between diet groups in either sex. After all animals had learned the FR1 task, a progressive ratio task was used to assess motivation. In male offspring, motivation was unchanged by maternal ($F(1,51)=2.396$, $p=0.128$) or postnatal ($F(1,51)=1.319$, $p=0.256$) diet (Fig 1D). However, female HF offspring showed decreased motivation (lower breakpoint) compared to offspring from SC dams (main effect of maternal diet, $F(1,54)=5.91$, $p=0.019$) (Fig 1E). Interestingly, we found that female offspring provided with MS during early life had increased

motivation to work for reinforcer as compared to the unsupplemented females (main effect of MS, $F(1,54)=9.32$, $p=0.004$) (Fig. 1E).

To evaluate executive function, animals were tested in the 5-choice serial reaction time test (5-CSRTT) task. Due to an insufficient number of control males acquiring the 5-CSRTT schedule A task, we were unable to move forward with the male analysis, so from this point forward all studies were completed only in female offspring. In female offspring, we found that between days 9-13 fewer of the high fat offspring had learned the task, but over the entire course of the learning period, this difference only resulted in a trend toward a differential learning rate between the groups (Mantel-Cox Log-rank test: $\chi^2(3)=6.209$, $p=0.1$) (Fig. 2A). Notably, MS female offspring reached criterion in fewer training sessions than unsupplemented females (main effect of MS, $F(1,51)=7.16$, $p=0.01$) (Fig. 2B). All female offspring performed approximately 75% correct responses (Fig. 2C) and there were no differences in error type (data not shown). MS significantly increased the total number of trials performed during the session (main effect of MS, $F(1,51)=10.64$, $p=0.002$; Bonferroni post hoc SC vs SC+MS $t_{51}=2.77$, $p<0.05$) (Fig2D) and decreased the time required to complete a correct response (main effect of MS, $F(1,51)=4.52$, $p=0.038$) (Fig2E). MS also decreased the amount of time to perform a premature trial error when this error type was performed (main effect of MS, $F(1,50)=9.75$, $p=0.003$; Bonferroni post hoc HF vs. HF+MS, $p=0.006$) (Fig 2F), while the time to perform an incorrect responses was not affected.

After 3 consecutive days of criterion performance on 5-CSRTT schedule A, females were moved onto the more difficult 5-CSRTT titration schedule. On this schedule, the response performed by the animal directly affected the stimulus length for the subsequent trial (Fig 3A), which is in contrast to Schedule A in which the stimulus length remained constant throughout the 30 minute training session. This titration schedule identifies each animal's individual performance limit (shortest stimulus duration) by titrating between a stimulus length at which a correct response was possible and a stimulus length below the performance limit (Martin et al., 2015). Interestingly, we found that HF females were unable to reach the shortest stimulus length reached by the SC females (Fig 3B), and this was ameliorated by MS (interaction of maternal diet

and MS, $F(1,46)=4.793$, $p=0.034$; Bonferroni post hoc SC vs HF, $t_{46}=3.16$, $p=0.014$; HF vs HF+MS, $t_{46}=3.26$, $p=0.011$). We also found that MS decreased the median stimulus needed to perform a correct response (main effect of MS, $F(1,46)=6.58$, $p=0.014$; Bonferroni post hoc HF vs HF+MS, $t_{46}=2.52$, $p=0.031$) (Fig 3C). Additionally, similar to the observation in Schedule A, MS females completed more total trials than unsupplemented females (main effect of MS, $F(1,46)=6.61$, $p=0.013$) (Fig 3D), and the time to complete successful trials for the MS females was decreased compared to the unsupplemented females (main effect of MS, $F(1,46)=6.62$, $p=0.017$) (Fig 3E). Unlike in schedule A, the titration schedule increased the percentage of premature errors performed by the MS females (main effect of MS, $F(1,46)=4.36$, $p=0.042$), but did not significantly change the incorrect or omission errors (Fig 3F). The titration task is designed to detect the performance limit of each animal (e.g. an equal number of success and failures), therefore, as expected, all animals performed about 50% successful trials within this titration schedule (Fig 3G).

Following completion of operant training, we wanted to determine if acute *adult* exposure to MS would alter motivation apart from cognitive performance (e.g. memory or learning). After 5-CSRTT was completed, females were tested in PR while fed the control diet to establish a baseline. This baseline PR performance did not differ from the initial PR conducted prior to the 5-CSRTT testing (data not shown). All animals were then fed the MS diet for 7 days, during which time animals continued daily 5-CSRTT testing to maintain operant responding, before testing PR for a second time (Fig. 1A). We found that 7 days of acute MS increased the number of trials performed across all the groups except for the controls when compared to their own baseline (SC, $t_{13}=1.137$, $p=0.27$; HF, $t_{13}=3.636$, $p=0.009$; SC+MS, $t_{12}=5.025$, $p<0.001$; HF+MS, $t_{15}=2.132$, $p<0.05$) (Fig. 4).

After completion of all operant training we screened for genes (Table 2) with expression changes altered by either maternal or postnatal diet and correlated with median stimulus duration. We identified two chemokines, CXCL10 (Fig 5A) and CCL2 (also known as MCP-1 (Fig 5B)), whose expression levels correlated with median stimulus duration on the 5-CSRTT titration

schedule. The expression of both chemokines was positively correlated with stimulus length (CXCL10: $r=0.477$, $p<0.001$; CCL2: $r=0.291$, $p=0.047$). We also found that overall expression levels of CCL2 was increased in HFD offspring and this increase was attenuated by methyl donor supplementation (Fig 5C) (interaction of maternal diet and MS, $F(1,48)=11.15$, $p=0.002$; Bonferroni post hoc SC vs HF, $t_{48}=2.75$, $p<0.05$, HF vs HF+MS, $t_{48}=3.44$, $p=0.007$). There were no group differences in CXCL10 expression (data not shown). No other genes correlated with stimulus duration, and only one other gene was affected by HFD or MS, with TNF- α levels increased by MS ($F(1,48)=4.093$, $p=0.049$, data not shown).

Discussion

Methyl donor supplementation has been implicated in altering behavior, gene expression and DNA methylation when given either during pregnancy or in late adulthood. We hypothesized that because the prefrontal cortex continues to develop after birth, exposure to methyl donors in the postnatal period would alter PFC-dependent cognitive performance. We found that in mice, methyl donor supplementation during 3-6 weeks of age can both reverse certain HFD-induced deficits, as well as improve other cognitive endpoints which were not affected by HFD. By utilizing, for the first time in mice, a 5-CSRTT titration schedule we were able to determine individual limits of performance for each animal. Incorporation of this more difficult version of the 5-CSRTT proved to be valuable in identifying parameters affected by both maternal HFD, as well as MS.

Consumption of a HFD during pregnancy has been shown to affect offspring cognition, for example it can impair spatial learning (Lu et al., 2011; Tozuka et al., 2010) and worsen memory deficits (Martin et al., 2014). Here, using a more difficult version of the 5-CSRTT, the titration protocol, we demonstrate that maternal HFD impairs cognitive performance in female offspring such that the stimulus length required for a correct response was significantly longer in the HFD offspring. Importantly, MS was able to reverse this deficit. In HFD offspring, the shortest stimulus length obtained matched the time required to perform a correct response, potentially indicating

that an illuminated cue was necessary to complete a correct response. In all other groups, the shortest stimulus reached was shorter than the time required to perform a correct response, signifying that a correct response could be performed after the light cue was terminated. These data indicate that HFD offspring could have either a deficit in working memory or a slower reaction time to the task. Neurons in the PFC have been implicated in working memory, specifically in the encoding and retention of temporal information (Sakurai et al., 2004). This type of working memory is critical to the 5-CSRTT titration schedule. As the stimulus duration shortens, animals must remember where the light cue was previously seen. Synaptic plasticity also contributes to working memory (Mayford et al., 2012), and synapse number and signaling across synapses are two ways to measure synaptic function. Maternal HFD has been shown to adversely affect both synaptic stability (Hatanaka et al., 2016), as well as dendritic complexity in new neurons (Tozuka et al., 2010). Conversely, it is known that increased plasma concentration of specific methyl donor nutrients, which are precursors for synapses, can promote synthesis of new brain synapses (Cansev et al., 2008; Wurtman et al., 2010), stimulate neurite outgrowth (Fujii et al., 1996) and neurogenesis (Craciunescu et al., 2003). Because the methyl donor supplementation contains multiple precursors for brain synapse development, one possible mechanism through which MS improved the HFD phenotype is through an increase in synapse number and signaling, however further studies will be required to support this hypothesis.

Interestingly, we found that gene expression of two chemokines, CCL2 and CXCL10, was positively correlated with median stimulus length in the 5-CSRTT titration task, with higher chemokine levels associated with poorer performance. Additionally, overall expression of CCL2 was increased in HFD offspring, and this increase was normalized with early life exposure to MS. We hypothesize that increased expression of chemokines, particularly CCL2, negatively affect cognition, an idea with additional support in the literature. CCL2 overexpression in a mouse model of Alzheimer's disease was found to accelerated deficits in spatial and working memory (Kiyota et al., 2009). Additionally, in humans, increased expression of these chemokines has been associated with neurocognitive deficits in Alzheimer's disease (Westin et al., 2012) and

cognitive status in Parkinson's disease patients (Rocha et al., 2014). Beyond their classically defined function in the immune system, to attract and activate immune cells, there is an increasing appreciation for the importance of chemokine action within the CNS (Mélis-Parsadaniantz and Rostène, 2008). CCL2 specifically is known to modulate neuronal function (Gosselin et al., 2005; Alice Guyon et al., 2009), and can alter membrane resistance in dopaminergic neurons (A. Guyon et al., 2009). Behavioral effects of CCL2 have also been noted, as chronic infusion of CCL2 was shown to increase sweetened ethanol consumption in an operant self-administration task (Valenta and Gonzales, 2016). Importantly, it has been shown that prenatal fat exposure disrupts proper functioning of the CCL2 chemokine system in embryonic neurons in the hypothalamus (Poon et al., 2013). Collectively, these findings support the conclusion that early life exposure to HFD or MS can affect the CCL2 chemokine system, representing a potential mechanistic link between these dietary manipulations and effects on cognition in adulthood.

In addition to improving HFD-driven phenotypes, early life methyl donor supplementation also increased motivation and performance (independent of HFD). Methyl donor supplementation increased progressive ratio breakpoint and increased the number of trials performed within a session on both 5-CSRTT training schedules, as well as decreasing the days required to learn the 5-CSRTT, and decreasing 5-CSRTT reaction time. These data indicate increased overall motivation and cognitive performance in methyl donor supplemented animals. However, these increases in performance were not without some cost. Methyl donor supplementation also increased the number of impulsive premature errors in the titration experiment, and decreased the time to make an impulsive error in the standard 5-CSRTT. The mechanism whereby MS affects cognitive performance remains unknown. It is possible that methyl donor supplementation increases acetylcholine concentration during development (by providing the precursor choline) (Cohen and Wurtman, 1976) and could in turn enhance cholinergic signaling pathways that are known to in part modulate learning and memory (Klinkenberg et al., 2011; Savelkoul et al., 2012). Further, MS has the potential to broadly affect gene expression through alteration of the

methylation status of target genes or regulatory regions. We previously reported the maternal HFD leads to global hypomethylation within the PFC, which is reversed by MS (Carlin et al., 2013) and that high gene expression within the PFC, canonically associated with decreased promoter methylation, was related to poor executive function performance (Grissom et al., 2015). Therefore, it is possible that methyl donor supplementation leads to increased methylation of specific gene promoter regions within the PFC, leading to reduced gene expression, and improvements in motivation and performance. However, it is important to note that assessments of global hypomethylation include DNA methylation in many genomic regions outside of the promoter regions (i.e. intergenic and intronic regions in which methylation affects overall stability of the genome). Therefore, at the level of individual gene promoters both hypo- and hyper-methylation will exist. Identification of the target genes affected by MS in the prefrontal cortex is an important next step.

It has been shown previously that adult methyl donor supplementation, either with single- or multiple-nutrients, increased memory retention and decreased/slowed progression of dementia (Morris, 2012; Yu et al., 2016). Our data add to this literature by demonstrating an acute positive effect on motivation. While the effects of adolescent supplementation on both behavior and gene expression persisted well into adulthood, it is unclear how long the effects of *acute* supplementation on motivation may last. Interestingly, motivation increased in all experimental groups, but not the controls, suggesting that prior exposure to either HFD or MS is required for the beneficial effects of adult supplementation. While the precise mechanism of acute MS action on cognition remains unclear, the current data demonstrate that early life exposure to either HFD or MS increases responsiveness to later life methyl supplementation.

The addition of folic acid to many grain products was mandated by the U.S. Food and Drug Administration in 1996 to decrease risk for spina bifida and other neural tube closure defects. More recently, a critical role for folate is increasingly being appreciated with regard to adverse neurodevelopmental outcomes. Folate in the brain prevents accumulation of homocysteine by remethylation of homocysteine to methionine. Epidemiological studies have linked folate

deficiency with homocysteine accumulation in stroke, Alzheimer's disease and Parkinson's disease patients (Mattson and Shea, 2003), while experimentally in rats, folate deficiency led to both increased homocysteine as well as learning and memory deficits (Berrocal-Zaragoza et al., 2014). Folate insufficiency can be caused by decreased dietary folate consumption, genetic defects or blockage of folate transport to the brain. Folate receptor alpha (FR α) autoantibodies block the transport of folate into the brain and have been associated with cerebral folate-deficiency-related developmental disorders, autism and schizophrenia (Desai et al., 2016; Frye et al., 2016; Ramaekers et al., 2014). Exposure to FR α autoantibodies during gestation have been associated with increased anxiety-like behaviors in mice (Sequeira et al., 2016), and folinic acid supplementation during gestation can alter adult cognition, specifically ameliorating adult communication and sociability deficits in mice exposed to FR α autoantibodies (Desai et al., 2016). The use of various other methyl donors postnatally has generally been limited to preventing or slowing cognitive decline (Morris, 2012). Our findings indicate that early life postnatal consumption of a cocktail of methyl vitamins, possibly through a vitamin or supplementation protocol, could affect learning and cognition into adulthood, and may be particularly beneficial to those children exposed to HFD and/or obesity during gestation. These data also provide support for the hypothesis that adult methyl donor supplementation remains a potential mechanism through which cognition could be altered in the adult or aging population. While folic acid is the most broadly used supplement clinically, the present studies provided a cocktail of methyl vitamins, as this is important for proper functioning of the entire one carbon metabolism cycle, helping to catalyze enzymes reliant on folate, vitamin B12 and choline (Selhub and Paul, 2011). Whether the same beneficial effects would be found with the use of single nutrient supplements (e.g., only folic acid or choline) remains to be determined.

We have previously reported that offspring of a maternal high-fat diet show deficits acquiring motivated behavior (Grissom et al., 2015). Here we saw no differences in time to acquire motivated behavior in either sex. This could be due to differences in the study design, including; (1) age of animals during training, (2) housing conditions or (3) dietary differences. In the previous

study, restriction began at 12 weeks, while in the current study, restriction began at 20 weeks. Age of testing has been shown to be a factor in locomotor activity, social behavior, depression-related behavior and spatial and cued fear memory (Shoji et al., 2016), while the age of food restriction has been shown to alter overall goal- and sign-tracking in an operant task, with adolescence being a more vulnerable time-period than adulthood (Anderson et al., 2013). It is possible that cortical circuits underlying learning and motivation are more vulnerable to food restriction occurring at an earlier age. Housing conditions were another key difference between our previous and current study. In the previously reported study, male and female experiments were performed sequentially, while in the current study, male and female experiments were performed simultaneously, in the same room. It is possible that housing both sexes in the same room instead of in separate rooms within an experiment could affect behavioral results (Beatty, 1979). The presence of female odors could distract male mice enough to displace their motivation from performing the operant task to acquisition of a mate (Kavaliers et al., 2001; Kavaliers and Choleris, 2013). This could also explain the inability of control male offspring to successfully learn the 5-CSRTT task, which precluded their further behavioral testing. As such, it is important to note that these findings apply only to females, and it will be important to confirm these findings in males. Finally, differences in the diets could account for differential behavioral findings. Previously we used defined diets during pregnancy and lactation and then animals were weaned onto the house chow. In the current study, defined diets were used throughout the entire study. Lab Diet 5001 (previous weaning diet) contains 13% fat, 57% carbohydrate and 30% protein with over double the amounts of all methyl donor nutrients, while Test Diet 5755 (current weaning diet) contains 22% fat, 59% carbohydrate and 18% protein. Nutrient composition differences between regular chow and defined diet are significant and is a confounding factor overlooked by about 75% of diet studies (Warden and Fisler, 2008). Further, it is also important to note that the HFD used here also has lower carbohydrate content than the control diet, so any observed effects of the HFD may be related to increased fat content or decreased carbohydrate content. There are also various increases in micronutrient concentration per gram of diet in the HFD. However, mice

consume less total volume of the HFD to compensate for the excess calories, so intake of the micronutrients is likely to be similar.

In sum, using a novel variation of the 5-CSRTT schedule, we have shown that female HFD offspring fail to reach the same performance standard (stimulus length duration) as control offspring, and early life methyl donor supplementation ameliorated this deficit possibly through modulation of the chemokines CCL2 and CXCL10. Further, MS improved a number of cognitive and motivational endpoints, leading the MS female offspring to learn more rapidly, perform more trials, and display a reduced reaction time. Therefore, methyl donor supplementation in early life has the potential to both reverse negative outcomes associated with HFD during pregnancy, as well as promote an overall increase in cognitive performance. Future studies will be directed at identifying the molecular mechanisms by which methyl donor supplementation alters these cognitive endpoints. Deciphering these mechanisms could point toward novel therapeutic targets to improve human cognition.

Table and Figure Legends

Table 1. Diet Information

Table 2. qPCR Gene Primer List

Figure 1. Maternal and postnatal diet alter cognition. (a) Mouse dams are fed either standard control (SC) or high fat (HF) breeder diet throughout gestation and lactation. Offspring were weaned either onto standard control or methyl donor supplemented diets, creating 4 experimental groups (control, SC; high fat, HF; methyl supplemented control, SC+MS; methyl supplemented high fat, HF+MS). Male and female offspring were then tested in a series of operant tasks. (b-c) Neither maternal nor postnatal diet impacted animals' ability to learn the fixed ratio (FR1) task (male: left; female: right). (d) Male offspring motivation is not altered by diet. (e) Female HF offspring are less motivated to work for reinforcer on a progressive ratio (PR) task. Female offspring supplemented with methyl donors are more motivated to work for reinforcer. * $p < 0.05$ main effect of maternal diet, $p < 0.05$ main effect of MS. n: male: SC=15, HF=15, SC+MD=14, HF+MS=15, female: SC=14, HF=13, SC+MS=15, HF+MS=18 in all figures. PCA, Pavlovian-conditioned approach. 5-CSRTT, 5-choice serial reaction time test.

Figure 2. Postnatal methyl donor supplementation enhances ability to learn operant behavioral task. (a) Rates at which the criterion was reached. Criterion was reached when animals performed 20 trials with 50% correct in the 30 minute session. (b) Postnatal MS decreased the number of training sessions for the animals to reach criterion. (c) All offspring performed around 70% correct once the criterion was met. (d) MS significantly increased the number of trials performed during the session. (e) MS significantly decreased the amount of time required to perform a correct response. (f) MS significantly decreased the amount of time to perform a

premature error. $p < 0.05$ main effect of MS, $p < 0.05$ SC vs SC+MS, $p < 0.05$ HF vs. HF+MS. n: SC=14, HF=11, SC+MS=14, HF+MS=16 in all figures.

Figure 3. High fat diet offspring require a longer stimulus to perform correct responses. (a) As shown in the schematic, on the 5-CSRTT titration training, the response performed within each trial directly impacted the stimulus length of the subsequent trial. This allowed each animal to reach an individual set point for the task. (b) High fat diet offspring required the longest stimulus to perform a correct trial, this was ameliorated by MS. (c) MS significantly decreased the median stimulus needed to perform a correct response. (d) As designed, animals performed about 50% successful trials within this titration schedule. (e) MS increased the number of trials performed during the 30 minute session. (f) MS decreased the time to complete a successful trial within this schedule. (g) MS significantly increased the number of premature errors, but did not alter incorrect or omission errors. $p < 0.05$ main effect of MS, $p < 0.05$ SC vs HF, $p < 0.05$ HF vs HF+MS. n: SC=11, HF=10, SC+MS=13, HF+MS=16 in all figures.

Figure 4. Acute adult methyl donor supplementation alters motivation. At the conclusion of cognitive testing, motivation was again tested with the PR task. Following this testing, animals were given acute access to MS for 7 days (during which maintenance 5-CSRTT was performed) before motivation was again assessed with PR. Acute MS increased the motivation of the high fat diet offspring and offspring that had previous supplementation of methyl donors early in life. Motivation remained unchanged in control offspring. $p < 0.05$ when compared to 0. n: SC=14, HF=11, SC+MS=13, HF+MS=16.

Figure 5. High fat diet offspring have increased expression of CCL2 in the prefrontal cortex which is attenuated by methyl donor supplementation. At the conclusion of operant testing RNA gene expression was measured in the prefrontal cortex. (a) High fat diet offspring have increased CCL2 gene expression, MS attenuated this increase. (b-c) Expression of two chemokines, CCL2 (b) and

CxCl10 (c), correlated with median stimulus duration on the 5-CSRTT titration schedule. $*p < 0.05$
SC vs HF, $^{\wedge}p < 0.05$ HF vs HF+MS. n: SC=13, HF=11, SC+MS=13, HF+MS=15 (1 HF+MS
expression lost in processing) in (a), animals that did not reach the titration schedule were left out
of correlation analysis (b-c) n: SC=11, HF=10, SC+MS=13, HF+MS=15.

Figures and Tables

Table 1.

	Control Diet	High Fat Diet	Methyl Supplemented Diet
Test Diet No.	5755	58G9	5AAX
Fat	22.1%	59.9%	23.2%
Carbohydrate	59.6%	21.4%	57.1%
Protein	18.3%	18.6%	19.7%
<i>Choline Chloride</i>	1,400 ppm	1,784 ppm	11,900 ppm
<i>Betaine</i>	not detected	not detected	1.50%
<i>Folic Acid</i>	4.2 ppm	5.1 ppm	15,004 ppm
<i>Vitamin B12</i>	24 mcg/kg	25 mcg/kg	72 mcg/kg
<i>L-methionine</i>	0.15%	0.19%	0.90%
<i>Zinc</i>	27 ppm	35 ppm	178 ppm

Table 2.

Gene	Assay ID
ACTB	Mm00607939_s1
ADORA2a	Mm00802075_m1
C1Qa	Mm00432142_m1
CCL2	Mm00441242_m1
CD86	Mm00444543_m1
COMT	Mm00514377_m1
CXCL10	Mm00445235_m1
CXCL5	Mm00436451_g1
DDARP-32	Mm00454892_m1
DNMT1	Mm00599763_m1
DNMT3A	Mm00432881_m1
DRD1A	Mm01353211_m1
DRD2	Mm00438545_m1
GADD45B1	Mm00435123_m1
GAPDH	Mm99999915_g1
GRM5	Mm00690332_m1
HDAC2	Mm00515108_m1
HDAC5	Mm01246076_m1
IL-10	Mm01288386_m1
IL-1 β	Mm01336189_m1
IL-6	Mm00446190_m1
MECP2	Mm01193537_g1
MTR	Mm01340053_m1
NGP	Mm01250218_m1
OPRD1	Mm00443063_m1
OPRK1	Mm01230885_m1
PNMT	Mm00476993_m1
SET7	Mm00499823_m1
SLC6A3	Mm00438388_m1
TH	Mm00447557_m1
TLR4	Mm00445273_m1
TNF α	Mm00443258_m1

Figure 1.

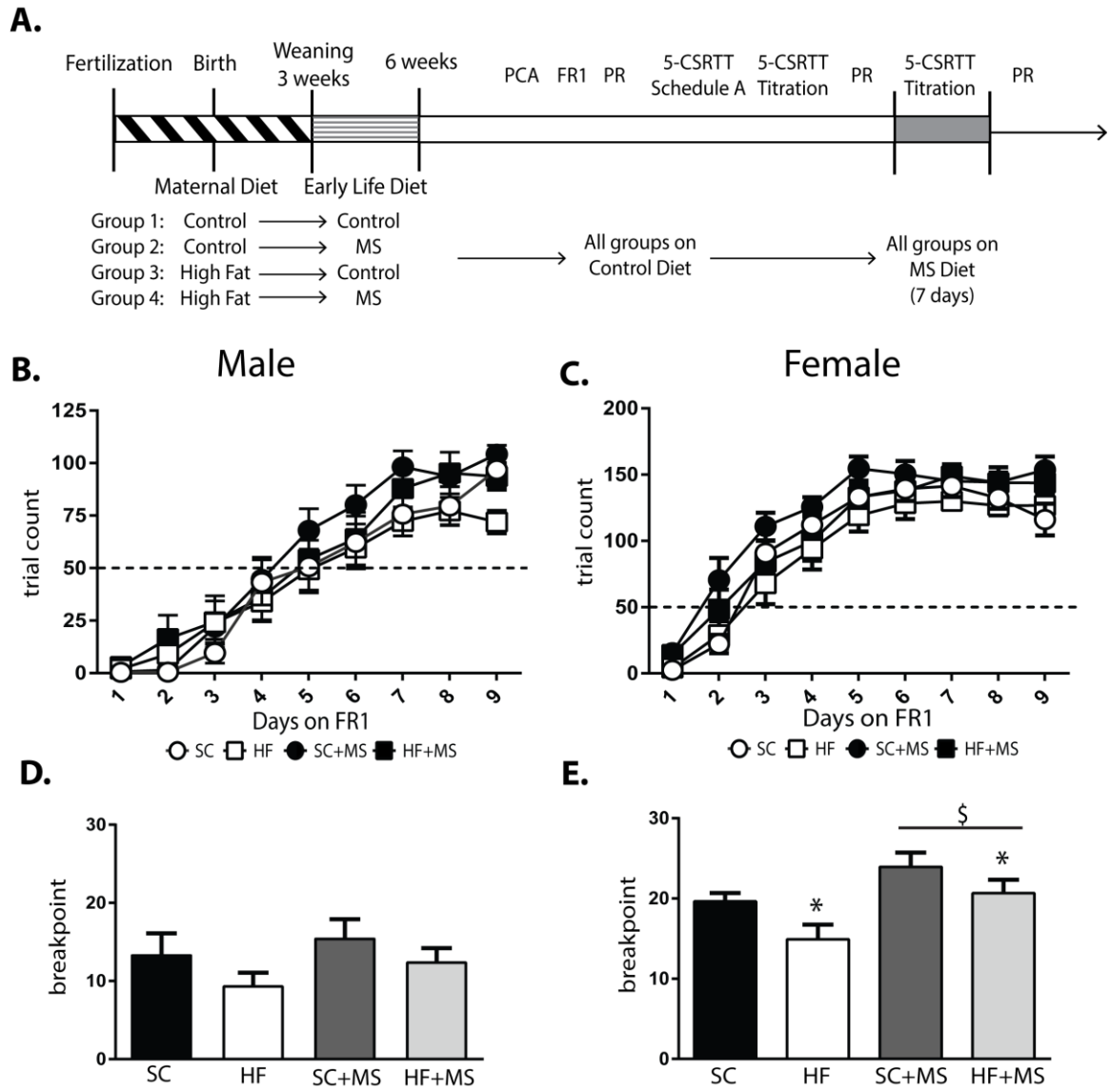


Figure 2.

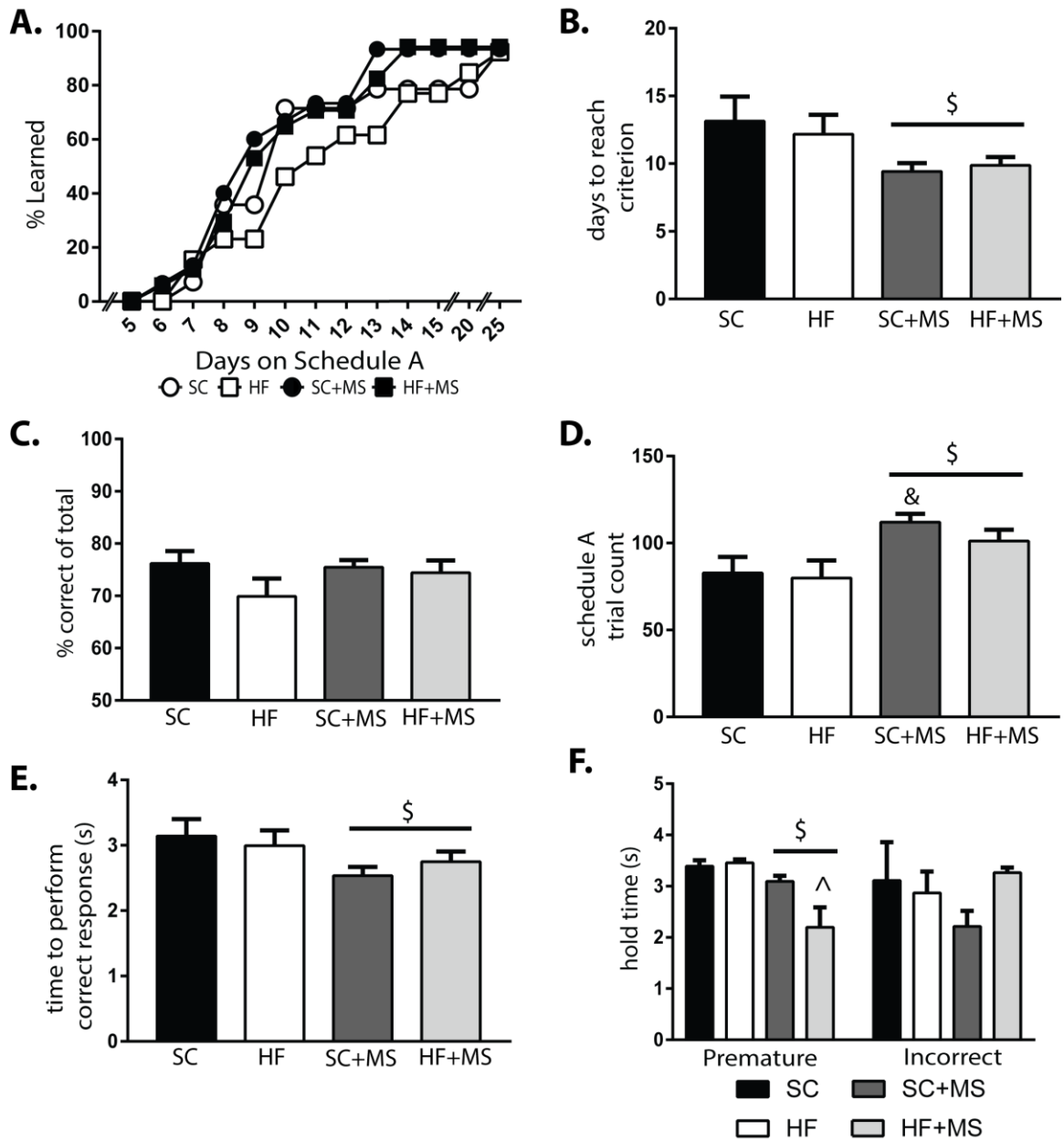


Figure 3.

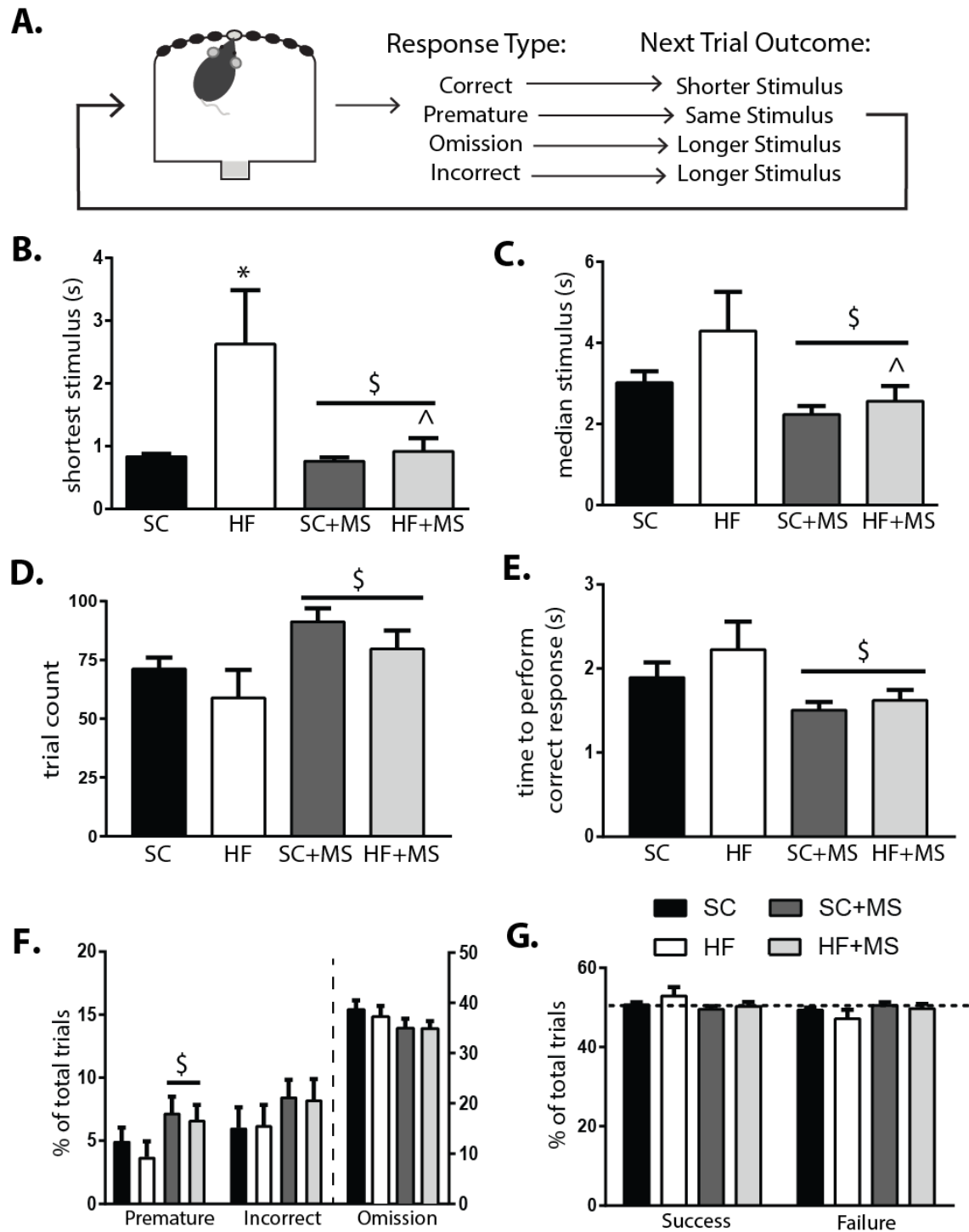


Figure 4.

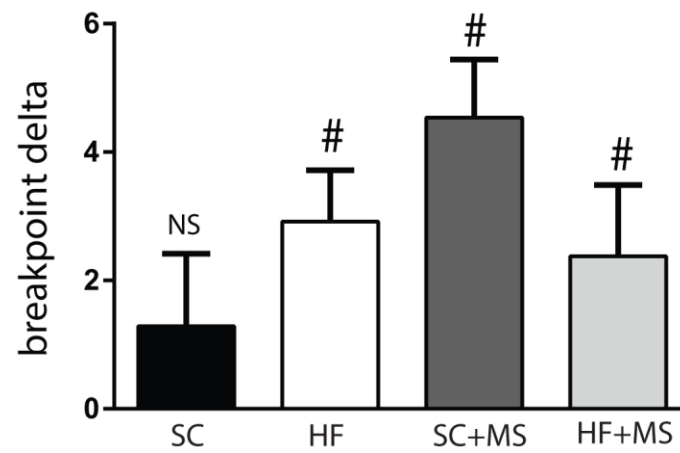
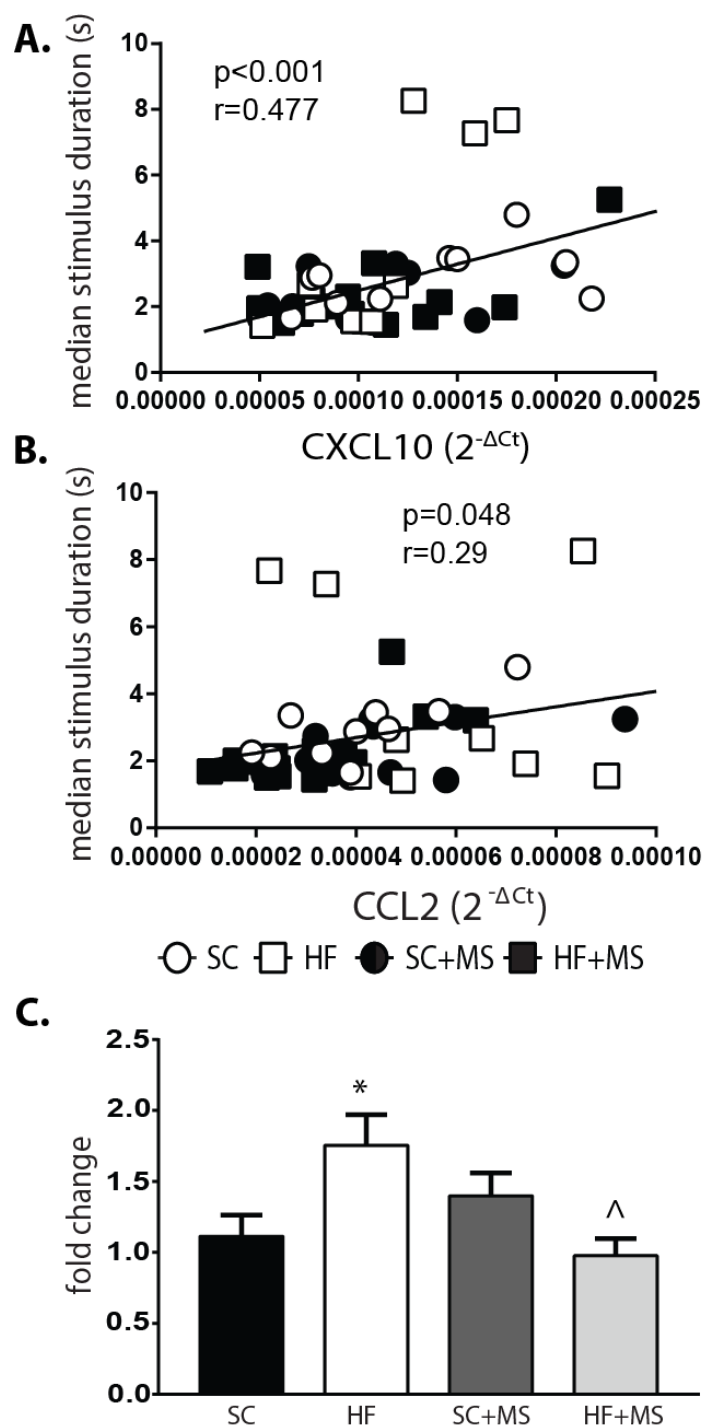


Figure 5.



Chapter Three

Short term effects of early life methyl donor supplementation on the prefrontal cortex of offspring exposed to maternal high fat diet

Sarah E. McKee, Sisi Zhang, Li Chen, Joshua D. Rabinowitz, Teresa M. Reyes

Introduction

In the United States, recommended weight gain during pregnancy is exceeded in 75% of pregnancies (Schack-Nielsen et al., 2010). Overnutrition during pregnancy increases offspring's risk for metabolic (i.e. obesity and type 2 diabetes) (Alfaradhi and Ozanne, 2011) and neurologic disorders (i.e. Alzheimer's, schizophrenia, ADHD and autism) (Krakowiak et al., 2012; Rivera et al., 2015; Robles et al., 2015; Van Lieshout et al., 2011). Interestingly, consumption of excess calories during pregnancy is actually shown to lower concentrations of many micronutrients in plasma including methyl donors (i.e. folate, vitamin B12, methionine, zinc, betaine and choline) (Kim et al., 2012; Sen et al., 2014), and when maternal micronutrient consumption is low, this deficiency is mirrored in the fetus or child (Allen, 2005; Plumptre et al., 2015; Visentin et al., 2015). Whether this micronutrient deficiency is responsible for offspring health risk remains to be determined.

Maternal overnutrition can be modeled in mice by feeding dams a 60% high fat (HF) diet from the onset of breeding throughout pregnancy and lactation (Vucetic et al., 2010). We have previously shown that offspring from these dams have cognitive and executive function deficits (Grissom et al., 2015), altered sucrose and fat preference, RNA gene expression and DNA methylation in reward-related circuitry (Vucetic et al., 2010). Furthermore, pre- and post-natal dietary methyl donor supplementation (MS) can normalize these changes (Carlin et al., 2013; McKee et al., 2017), possibly through increasing the access of methyl donor nutrients to the fetus or offspring during early development.

The intersection of folate metabolism and methionine metabolism (two methyl donor nutrients) constitute a series of one-carbon transfers known as one carbon metabolism (Figure 1). One carbon metabolism is critical to the vitality of every cell in the body, as it provides the building blocks for purine and pyrimidine synthesis, methyl groups for methylation reactions, precursors for phospholipid synthesis and substrates for reactive oxygen species removal (Anderson et al., 2012; Fox and Stover, 2008). Methyl donor nutrients are crucial to one carbon metabolism as they are both intermediates and cofactors within the pathway.

Methyl donor nutrients (highlighted in gray, Figure 1) are essential nutrients provided solely by the diet. Studies have shown that deficiency, imbalance or excess of one of more of these nutrients throughout various periods of life can alter development, cognition and disease risk (Morris, 2012; O'Neill et al., 2014; Pannia et al., 2016; Reynolds, 2006; Veena et al., 2016). We previously reported that MS given concurrently with a 60% HF diet during pregnancy and lactation normalizes palatable food preference, increases locomotor activity and alters gene expression and DNA methylation in offspring (Carlin et al., 2013). Others have found that maternal MS prevents transgenerational amplification of obesity (Waterland et al., 2008), metabolic disease (Goodspeed et al., 2015; Seferovic et al., 2015) and rescues early-life stress induced cognitive impairments (Naninck et al., 2016). When given postnatally, MS has been shown to alter cognition and motivation (McKee et al., 2017), reduce fatty liver and alter DNA methylation within the liver (P Cordero et al., 2013; Paul Cordero et al., 2013a; Dahlhoff et al., 2014). However, it remains unknown whether MS can directly affect one carbon metabolism in the brain and whether there are downstream consequences for methylation reactions.

Obesity has been shown to disrupt methionine metabolism (Nathanielsz et al., 2015) and affect short-term folate pharmacokinetics (da Silva et al., 2013). It is possible that offspring from dams fed a HF diet receive smaller concentrations of methyl donor nutrients throughout gestation. Because we have seen that early life methyl donor supplementation can alter long term behavior driven by the prefrontal cortex (PFC) and RNA gene expression in the PFC (McKee et al., 2017), we were interested in understanding the immediate changes that occur after supplementation.

Additionally, because sex differences in the prevalence rates of many neurological disorders are well documented, one main goal was to compare male and female offspring to determine whether sex differences at the level of one carbon intermediates were present. It was also important to measure these metabolites and enzymes directly within the brain tissue as previous research has been limited to measurements in the cerebral spinal fluid and plasma. Therefore, to determine whether maternal HF diet and postnatal early life MS alter one carbon metabolism directly in the brain, we analyzed the concentration of one carbon metabolism intermediates, RNA gene expression of genes involved in one carbon metabolism and DNA methylation in the PFC tissue. All experiments were performed when animals were 6 weeks of age at the conclusion of a 3 week supplementation with MS.

Methods and Procedures

Animals and experimental model: All animals were cared for according to the guidelines of the University of Cincinnati Institutional Animal Care and Use Committee. An experimental timeline is seen in **Figure 2**. C57BL/6J virgin females were bred with DBA/2J males. The B6D2F1/J hybrid mice are used in our work as the hybrid background is more similar to heterogeneity observed in humans, as opposed to a pure inbred strain. All animals were fed control diet prior to breeding. From the onset of breeding, through pregnancy and lactation, dams (n=12/group) were fed one of two purified diets (Test Diet, Richmond, IN); (1) standard control (SC) and (2) 60% high fat (HF) (Grissom and Reyes, 2013; Vucetic et al., 2010). Specific diet formulations are shown in **Table 1**. Litters were culled to 8-9 pups as necessary, to equalize access to nutrition throughout lactation. At weaning, 1-2 offspring of each sex from each litter were randomly assigned one of two diets; (1) control or (2) control diet plus methyl donor supplementation (MS) (Test Diet, Richmond, IN), and were group housed with 4 animals per cage (McKee et al., 2017). At 6 weeks, all offspring were sacrificed for experimentation.

Metabolite Analysis: At sacrifice, the prefrontal cortex was dissected from a 2mm coronal slice from bregma +2.3 to 0.3 and flash frozen on dry ice. Folate metabolites were extracted as previously described using the LC-MS (Ducker et al., 2016), with the exception of the initial step, where the frozen tissue was ground into powder (Retsch Cryomill). Folate metabolites measured include tetrahydrofolate (THF), 5-formyl-tetrahydrofolate (5-formyl-THF), 5,10-methylenyl-tetrahydrofolate (5,10-methylenyl-THF) and 5-methyl-tetrahydrofolate (5-methyl-THF). Methionine cycle metabolites were extracted and measured as previously described (Shlomi et al., 2014). Metabolites measured include cystathionine, cysteine, S-adenosyl-homocystine (SAH), S-adenosyl-methionine (SAM), homocysteine, Thymidine monophosphate (dTMP) and deoxyuridine monophosphate (dUMP). All metabolites are reported in ion counts and are normalized to control males. n=5/groups.

Gene expression in Prefrontal Cortex: Whole brain was collected and was placed in RNAlater and stored at -20C. The medial prefrontal cortex was dissected and DNA and RNA was extracted using AllPrep DNA/RNA Mini Kit (Qiagen). 220ug/10ul cDNA was synthesized using High Capacity Reverse Transcriptase kit (Applied Biosystems). Concentrated cDNA was used in a specific target preamplification following the manufacturer's recommendations (Fluidigm Corp. South San Francisco, CA). Briefly, Taqman assays for all assays were pooled to a final concentration of 0.2X. 2ul of the pooled assay mix was combined with 2ul cDNA and 4ul 2X Taqman Preamp Master Mix (Life technologies). The preamplification reaction was cycled following the manufactures protocol in a 750 fast qPCR machine. Samples were then diluted 1:50 using 1X TE. Samples were then delivered to the Molecular Profiling Core at the University of Pennsylvania, where they were run on a 96.96 Dynamic Array IFC on the Biomark HD machine (Fluidigm Corp). We were interested in the expression of genes involved in DNA methylation and one carbon metabolism as these genes are known to be altered after high fat diet exposure (**Table 4**). Expression of targets was normalized to the geometric mean of the housekeeping

genes (ACTB and GAPDH), expressed as RQ values, and analyzed by two-way ANOVA. n=10/group except female HFMS n=9.

Global DNA methylation and hydroxymethylation: Measurements were completed using the MethylFlash Global DNA Methylation and Hydroxymethylation ELISA Easy Kits (Epigentek, P-1030 and P-1032). According to manufacturers instructions, 100 ng of DNA was run. Absorbance was read using a microplate reader (Epoch BioTek Instruments Inc., USA). The % methylation or hydroxymethylation was calculated $5\text{-mC}\%$ or $5\text{-hmC}\% = ((\text{Sample OD}/\text{Negative Control OD})/(\text{Slope of Standard Curve} \times 100)) \times 100$.

DNMT activity: At sacrifice, brains were collected and prefrontal cortex was immediately dissected and flash frozen on dry ice. Tissue was then homogenized and nuclear protein was extracted with an EpiQuik Kit (Epigentek, USA) according to manufacturer's instructions. After protein quantification, DNMT activity was measured in nuclear protein with the EpiQuik DNMT Activity/Inhibition Assay Ultra Kit (Epigentek) according to manufacturer's instructions. Absorbance was read at 450 and 655 nm on a microplate reader (Epoch, BioTek Instruments, Inc., USA). The DNMT activity was calculated using $\text{DNMT activity} = (\text{sample OD} - \text{blank OD})/[\text{protein amount } (\mu\text{g}) \times \text{h}] \times 1000$. All activity was normalized to SC controls.

Data analysis: Two and Three-way ANOVA (maternal diet x supplementation; maternal diet x supplementation x sex) were used to evaluate results using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). Bonferroni post hoc planned comparisons were conducted when the omnibus F test was significant. The planned comparisons of interest were; (1) control versus high fat offspring, (2) unsupplemented versus supplemented offspring (3) supplemented controls versus supplemented high fat diet offspring and (4) male versus female. $P \leq 0.05$ was considered significant.

Results

To determine how early life supplementation of methyl donors could alter methylation and brain specific one carbon metabolism, we supplemented offspring from both maternal control and HF diet with dietary methyl donors from 3-6 weeks of age and compared them to control fed offspring (Figure 2). At six weeks of age, male offspring fed a maternal diet of 60% fat had decreased global DNA methylation within the PFC (Figure 3A). Importantly, early life methyl donor supplementation reversed this decrease (interaction of maternal diet and MS: $F(1,39)=9.19$, $p=0.004$; SC vs HF: $t_{39}=2.69$, $p=0.021$; HF vs HF+MS: $t_{39}=4.25$, $p<0.001$). No changes in global DNA methylation were seen in the females (Figure 3B). Global hydroxymethylation was not changed in either sex (data not shown). Analysis of DNA methyltransferase activity revealed a significant interaction in male offspring (maternal diet x MS: $F(1,26)=4.57$, $p=0.042$, Figure 3C), such that MS increased activity in offspring from control fed dams, but not those from HF dams (SC vs SC+MS: $t_{26}=2.38$, $p<0.05$; SC+MS vs HF+MS: $t_{26}=2.68$, $p=0.025$). In female offspring, a significant interaction was again observed (maternal diet x MS: $F(1,37)=17.01$, $p<0.001$, Figure 3D), however the pattern was different. Female offspring from high fat fed dams had decreased DNMT activity which was rescued by early life methyl donor supplementation (SC vs HF: $t_{37}=3.81$, $p<0.001$; HF vs HF+MS: $t_{37}=3.92$, $p<0.001$).

Because we determined that MS could alter both global methylation and nuclear DNMT enzymatic activity in the PFC we were interested in characterizing how intermediates and enzymes within one carbon metabolism were altered due to short-term dietary MS. Our first step was to analyze enzymes and intermediates within folate metabolism (Figure 4A). We determined that MS increased the expression, only in male offspring, of two enzymes, the dehydrogenase MTHFD1 (main effect of MS: $F(1,40)=5.4$, $p=0.025$) and a reductase MTHFR (main effect of MS: $F(1,40)=6.93$, $p=0.012$), by about 6 and 9% each, respectively (Figure 4B, Table 3). Using mass spectrometry, we found that MS increased the concentration of THF, 5-formyl-THF and 5,10-methenyl-THF in both male and female offspring (Figure 4C-E)(THF: main effect of MS: $F(1,1)=22.17$, $p<0.001$; 5-formyl-THF: main effect of MS: $F(1,1)=24.39$, $p<0.001$; Male – main

effect of maternal diet: $F(1,16)=9.82$, $p=0.006$; SC+MS vs HF+MS: $t_{16}=3.62$, $p=0.005$; 5,10-methenyl-THF: main effect of MS: $F(1,1)=34.95$, $p<0.001$). Interestingly, for 5-formyl-THF, this increase was absent in the HF male offspring (a similar trend was observed for 5,10-methenyl-THF ($p=0.053$)).

In addition to feeding into methionine metabolism, folate metabolites feed directly into the pyrimidine synthesis pathway (Figure 4A). We measured dUMP and dTMP levels in the PFC, and found that in males the levels of dTMP were increased by maternal HF diet, and unaffected by MS (Figure 4F) (Male – main effect of maternal diet: $F(1,16)=12.38$, $p=0.003$). Neither dUMP concentration (data not shown) nor the enzyme that converts dUMP to dTMP (TYMS) concentrations were changed by maternal diet or MS in either sex (Figure 4B, Table 3).

The intersection of folate and methionine metabolism is where the exchange of a one carbon methyl group from 5-methyl-THF is added to homocysteine to re-methylate it to methionine (Figure 5A). We found that in male offspring MS increased the expression of MTRR by about 7%, but not MTR (Figure 5B, Table 3)(main effect of MS: $F(1,40)=5.09$, $p=0.003$). Neither gene was altered in female offspring. We again found that MS increased the concentration of the folate intermediate, 5-methyl-THF in both males and females (Figure 5C)(main effect of MS: $F(1,1)=20.08$, $p<0.001$). For methionine, there was an interaction of maternal diet and MS in the males, such that MS increased methionine levels only in control offspring (Figure 5D)(interaction of maternal diet and MS: $F(1,16)=4.58$, $p=0.048$; $p=0.002$; SC+MS vs HF+MS: $t_{16}=2.63$, $p=0.037$). Interestingly, no changes were seen in female offspring for any diet group, and the levels of methionine were significantly lower in females overall (main effect of sex: $F(1,1)=8.7$, $p=0.006$).

Following the re-methylation of homocysteine to methionine, methionine is activated by the addition of adenosine to form S-adenosyl methionine (SAM). A variety of methyl transferases then utilize the methyl group from SAM in various reactions throughout the cell. Once the methyl group is donated from SAM, S-adenosyl-homocysteine remains (SAH) (Figure 6A). We found that in males, expression of two genes within this portion of the pathway were increased due to MS

(DNMT3a, 6% and SETD7, 11%), while in females these genes were unchanged (Figure 6B, Table 3). In females, PNMT expression was decreased by 25% due to MS, but was unchanged in males. Interestingly, in male offspring maternal HF diet decreased the expression of PEMT (5%), but the expression was unchanged in female offspring. Maternal HF diet increased the concentration of SAM (Figure 6C) (main effect of maternal diet: $F(1,1)=9.77$, $p=0.004$), with overall lower levels in females (main effect of sex: $F(1,1)=6.55$, $p=0.016$), as well as increased the levels of SAH (Figure 6D) (main effect of maternal diet: $F(1)=4.18$, $p=0.049$).

After the donation of a methyl group to various methyltransferase reactions, SAH is hydrolyzed into homocysteine. Homocysteine can then be either re-methylated to methionine and return through methionine metabolism or it can be shuttled through the transsulfuration pathway to help mitigate reactive oxygen species elimination (Figure 7A). We found that males were particularly susceptible to increases in shuttling to the transsulfuration pathway. In males, MS increased the expression of SAHH (8%) and CBS (7%), but maternal HF diet decreased the expression of CTH (8%) (Figure 7B, Table 3). In females, maternal HF diet decreased expression of EAAT3 (4%). We found that maternal HF diet increased the concentration of homocysteine (Figure 7C) (Male – main effect of maternal diet: $F(1,16)=6.37$, $p=0.023$; HF vs HF+MS: $t_{16}=2.71$, $p=0.031$) in male offspring and cysteine (Figure 7D) (main effect of maternal diet: $F(1,1)=9.05$, $p=0.005$) in both sexes. Strikingly, HF diet male offspring with MS had particularly high concentrations of homocysteine when compared to controls and unsupplemented HF diet offspring.

Discussion

We previously reported that both male and female offspring fed a maternal HF diet had global DNA hypomethylation, altered cognition and RNA gene expression within the PFC (Grissom et al., 2015; Vucetic et al., 2010), which can be in part normalized by maternal and early life MS (Carlin et al., 2013; McKee et al., 2017). To determine whether the specific nutrients in the diet alter one carbon metabolism directly in brain tissue and possibly mediate these molecular and

behavioral changes, we fed offspring from SC and HF maternal diets a methyl donor cocktail from 3-6 weeks of age and then measured the levels of one carbon intermediates and RNA gene expression of the enzymes within the pathway at 6 weeks of age in male and female mice. Most notably, prior to our studies, only one recent study had measured one carbon intermediates in the brain, and these measurements were completed in whole brain at postnatal day 9 (with many intermediates below the level of detection) (Naninck et al., 2016). For the first time, we completed a brain region specific analysis of the one carbon metabolism cycle, measuring intermediates using LC-MS, and enzyme expression using RT-qPCR. Additionally, prior studies were only completed in male offspring, and our studies included both male and female offspring to directly examine possible sex differences. Overall, we see a distinct line between how folate metabolism (including methionine) and how methionine metabolism (including SAM, SAH and homocysteine) respond to maternal HF diet and postnatal MS within the PFC. Generally, folates and methionine concentration were increased due to MS, however, male HF offspring were partially resistant to these increases. While SAM, SAH and transsulfuration pathway intermediates were unaffected by MS, but were increased in response maternal HF diet.

Given our hypothesis that MS has the potential to reverse adverse effects of prenatal HF diet exposure, we were particularly interested in effects of HF diet which were then rescued by MS. This pattern was seen in global DNA methylation in male offspring. We found that at 6 weeks, global DNA hypomethylation in male offspring was normalized by the postnatal MS. A similar pattern was observed for DNMT activity in the females. HF offspring had decreased DNMT activity, which was normalized by MS. We had previously seen that behavior deficits induced by maternal HF diet could be normalized by early life MS in females (McKee et al., 2017), it was key to determine if the MS was inducing early molecular changes which could underlie these long-term behavioral changes. These findings indicate that nutritional interventions that alter DNMT activity within the brain could underlie changes to long term cognition.

Dietary consumption of methyl vitamins is known to increase plasma concentrations of one carbon metabolites (Gargari et al., 2011; Jacques et al., 1999; Veenema et al., 2008), and we

here we demonstrate that adolescent MS increased folates (THF, 5-formyl-THF, 5,10-methenyl-THF and 5-methyl-THF) and methionine concentrations directly in the PFC. Interestingly, these MS-induced increases of folate and methionine were blunted in male offspring exposed to HF diet *in utero*. This blunted pattern was seen in 5-formyl-THF and methionine, with a similar trend in 5,10-methenyl-THF ($p=0.053$). The collective consequences of this blunted response in males is unknown, however, we also demonstrate a functional outcome consistent with this blunted response pattern, namely, DNMT activity in the male offspring. Control offspring supplemented with MS had increased overall DNMT activity while HF offspring supplemented with MS did not. Males exposed to maternal HF diet may metabolize methyl vitamins differently than controls when fed higher concentrations of the vitamins, possibly through altered activity of enzymes important to folate metabolism. And in fact, MS increased the expression of a number of enzymes important for one carbon metabolism and methylation, including MTHD1, MTHFR, MTRR, DNMT3a, SETD7, SAHH and CBS. In female HF offspring, we did not observe this blunted response nor were the expression levels of these enzymes changed, which may indicate that female offspring (at 6 weeks of age) are relatively protected from the effects of maternal HF diet exposure with regards to changes to one carbon metabolism in the PFC. The fold change increase in the enzyme expression was approximately 5-10%, and while the biological relevance of small fold changes in gene expression is not immediately clear, it is important to remember that these data were obtained in a heterogeneous cell population. Determining whether these changes are biologically important or driven by one specific cell type (i.e. neuron, astrocytes, glia) will be important for future work.

At the point where folate and methionine cycles meet, we see that in females MS does not increase the concentration of methionine as it does in males. Because the precursors were all increased in a similar way it is unexpected that methionine concentration was not also increased in both males and females. The enzyme levels for MTRR and MTR were not different between males and females, and we did not measure the expression of methionine adenosyltransferase 1a (MAT1A), a key enzyme in the conversion of methionine to SAM. It is possible that in females

MAT1A expression is increased due to MS, a pattern seen previously in the liver (Jacometo et al., 2017), or that enzymatic activities for either MTRR, MTR or MAT1A are increased due to MS specifically in females to shuttle methionine through the folate cycle. Identifying the relationship between MS and enzymatic activity will help shed light onto this sex difference.

Unlike the folate intermediates, MS surprisingly did not alter the concentrations of SAM, SAH or homocysteine. Instead maternal HF diet increased the concentrations of these three metabolites in the PFC. The conversion of SAM to SAH is integral in the donation of methyl groups to methylation reactions in the cell. We have previously reported that maternal high fat diet correlates with global and gene promoter-specific DNA hypomethylation in the brain, specifically within the PFC (Carlin et al., 2013; Vucetic et al., 2010). It is possible that increased SAH concentration within the brain could be the molecular link which mediates these changes as SAH is known to have high binding affinity for methyltransferases, inhibiting their function.

SAH is next hydroxylized to homocysteine, which can either be re-cycled through methionine metabolism or shuttled toward the transsulfuration pathway. Flux toward the transsulfuration pathway is known to increase when oxidative stress is more prevalent (Vitvitsky et al., 2006), and the synthesis of GSH through the transsulfuration pathway is the most important mechanism by which cells eliminate reactive oxygen species (ROS), a marker of increased oxidative stress. ROS are chemically reactive moieties that contain oxygen and are known to oxidize proteins, lipids and DNA. HF diet is known to increase oxidative stress in the brain and various neurological diseases (Alzheimer's and Parkinson's) are also associated with increased oxidative stress (Clarke et al., 1998; Mattson and Shea, 2003). The increased concentrations of homocysteine (males only) and cysteine (both sexes) due to maternal HF diet are consistent with this literature (Kruman et al., 2000; Kruman II et al., 2002; Mattson and Shea, 2003).

Oxidative damage to DNA is also known to increase dNTP concentration (Håkansson et al., 2006), a mechanism generally thought to increase the pool of DNA building blocks for the creation of new DNA or DNA repair. We found increased dTMP concentration, the building blocks for pyrimidine bases, in HF male offspring. We did not measure directly whether HF males have

increased oxidative damage, but the increase in homocysteine and cysteine seen in male offspring from HF fed dams is consistent with increased DNA damage. Possibly indicating that male HF offspring have increased shuttling of folate intermediates to dTMP synthesis, which increases the pool of DNA building blocks used to repair broken DNA. Further investigation into the relationship between HF diet, oxidative damage and DNA synthesis is needed. Additionally, if increases in oxidative damage are more prevalent in males, as the increased dTMP concentration suggests, this could explain why DNA hypomethylation is seen specifically in male offspring, as oxidative damage to methyl-CpG sites is thought to interfere with the ability of binding proteins and methyltransferases to interact and work on DNA (Valinluck et al., 2004), thus resulting in overall DNA hypomethylation.

Cysteine concentration is the rate limiting substrate for GSH synthesis and the removal of ROS (Hodgson et al., 2013). Unlike homocysteine, we observed increased concentrations of cysteine in both male and female offspring. Cysteine concentration within an individual cell can be regulated in two ways, first through increased flux through the transsulfuration pathway and second through increased flux of extracellular cysteine through cysteine transporters into the cell. Despite not seeing an increase in homocysteine in female offspring, we did see that HF diet altered the expression level of EAAT3, excitatory amino acid transporter 3, specifically in the females. 90% of extracellular cysteine uptake is mediated via EAAT3 (Hodgson et al., 2013), which is found both within the cytoplasm and at the cell membrane (Bjørn-Yoshimoto and Underhill, 2016). The cellular localization of EAAT3 within our samples is currently unknown, and would be a valuable area for future research. Further, astrocytes are the main synthesizer of GSH (Aoyama et al., 2008), and it would be interesting to determine the specific cell type responsible for increased cysteine concentration.

Our findings indicate that there are sex differences in the way maternal diet and early life diet interact to alter brain one carbon metabolism and DNA methylation in the brain. Evidence suggests that epigenetic regulation in the brain is often sex-specific (McCarthy et al., 2009). Maternal care, estrogen receptor (ER) expression and neonatal hormone exposure all have been

shown to correlate with sex-specific differences in DNA methylation (McCarthy et al., 2009). The sex differences seen in DNA methylation, DNMT activity, HF diet-induced folate and methionine concentration blunting, dTMP concentration and various differences in RNA gene expression may contribute to the observed sex specific differences in prevalence of many neurological disorders including autism, ADHD, schizophrenia and mood/anxiety disorders.

For the first time, the present data provide tissue level resolution of the effects of maternal HF diet and early life MS within the PFC. Further understanding of how one carbon metabolism changes across neurons, astrocytes and glia when compared to other dividing cell types is warranted. When looking in a heterogeneous cell population within the PFC, we see that early life MS increases folate concentrations and that perinatal HF exposure increases SAM, SAH and the transsulfuration pathway. In males, MS increased expression of enzymes important to one carbon metabolism and DNA methylation. Further investigation is necessary to understand the specific mechanisms by which these changes occur, why these changes occur, how these changes alter enzymatic activities of one carbon metabolism and whether a specific cell type mediates these changes. These findings do create many more questions, but they also highlight the need for brain and sex specific investigation into how nutrients can alter brain biochemistry.

Table and Figure Legends

Table 1. Specific diet information

Table 2. Details of the statistical main effects presented in the figures for protein

Table 3. Details of the statistical main effects and fold changes for gene expression

Table 4. qPCR Gene Primer List

Figure 1. One carbon metabolism. Simplified schematic of the intermediates, enzymes and nutrients involved in one carbon metabolism.

Figure 2. Experimental design. Mouse dams are fed either a standard control (SC) or high fat (HF) breeder diet throughout gestation and lactation. Male and female offspring were weaned either onto SC or methyl donor supplemented (MS) diets, creating 4 experimental groups (control, SC; high fat, HF, methyl supplemented control, SC+MS; methyl supplemented high fat, HF+MS). All animals were sacrificed and tissues appropriately collected at 6 weeks of age.

Figure 3. Maternal and postnatal diet alter global DNA methylation and DNA methyltransferase activity in the PFC. (a) Male maternal high fat diet offspring have global hypomethylation in the PFC. Interestingly, when supplemented with 3 weeks of MS these offspring this hypomethylation is reversed to the level of control offspring. (b) Maternal HF diet nor postnatal MS alter global DNA methylation in female offspring. (c) Nuclear DNMT activity was increased in control males supplemented with MS. (d) Nuclear DNMT activity in HF females was decreased, but this activity level was normalized by MS. * $p < 0.05$ SC vs HF, ^ $p < 0.05$ HF vs HF+MS, \$ $p < 0.05$ SC vs SC+MS, @ $p < 0.05$ SC+MS vs HF+MS. n: SC=10, HF=11, SC+MS=10, HF+MS=12 in (a), n: SC=10,

HF=10, SC+MS=10, HF+MS=10 in (b), n: SC=6, HF=7, SC+MS=10, HF+MS=7 in (c), n: SC=10, HF=12, SC+MS=8, HF+MS=11 in (d)

Figure 4. Methyl donor supplementation increases folate metabolite concentrations in the PFC.

(a) diagram of the first few steps of folate metabolism. **Bolded** intermediates were measured by mass spectrometry and *italicized* genes measured by high throughput PCR. (b) RNA gene expression changes to enzymes within folate metabolism and pyrimidine synthesis due to maternal HF diet exposure or postnatal MS. (c-e) MS increased concentrations of folate metabolism intermediates in the PFC (THF (c), 5-formyl-THF (d), and 5,10-methenyl-THF). (f) Maternal high fat diet increased the concentration of dTMP in male offspring PFC. *p<0.05 SC vs SC+MS, ^p<0.05 main effect of MS, *p<0.05 main effect of maternal diet, @p<0.05 SC+MS vs HF+MS; male n: SC=10, HF=12, SC+MS=10, HF+MS=12, female n: SC=10, HF=10, SC+MS=10, HF+MS=9 in (b), (c-f) both sexes n: SC=5, HF=5, SC+MS=5, HF+MS=5.

Figure 5. In male offspring, maternal high fat diet exposure limits the extent to which methyl donor supplementation increases methionine in the PFC. (a) diagram of the intersection of folate and methionine metabolism. **Bolded** intermediates were measured by mass spectrometry and *italicized* genes measured by high throughput PCR. (b) MS increased expression of MTRR, but not MTR specifically in male offspring. (c) MS increased the concentration of 5-methyl-THF in male and female offspring. (d) Sex-specific concentrations of methionine were observed. Diet did not influence the concentration of methionine within the female PFC, while MS increased the concentration in males. HF diet supplemented males had a blunted increase in concentration compared to the MS controls. ^p<0.05 main effect of MS, \$p<0.05 main effect of sex, @p<0.05 SC+MS vs HF+MS; male n: SC=10, HF=12, SC+MS=10, HF+MS=12, female n: SC=10, HF=10, SC+MS=10, HF+MS=9 in (b), (c-d) both sexes n: SC=5, HF=5, SC+MS=5, HF+MS=5.

Figure 6. Maternal high fat diet increases the concentrations of SAM and SAH in the PFC. (a) diagram of the conversion of SAM to SAH and donation of methyl groups to methylation reactions. **Bolded** intermediates were measured by mass spectrometry and *italicized* genes measured by high throughput PCR. (b) Sex-specific changes in methyltransferase expression due to maternal and postnatal diet. (c-d) Maternal HF diet increased the concentrations of SAM and SAH in the PFC. * $p < 0.05$ main effect of maternal diet, \$ $p < 0.05$ main effect of sex; male n: SC=10, HF=12, SC+MS=10, HF+MS=12, female n: SC=10, HF=10, SC+MS=10, HF+MS=9 in (b), (c-d) both sexes n: SC=5, HF=5, SC+MS=5, HF+MS=5.

Figure 7. Maternal high fat diet increases expression of the transsulfuration pathway in male offspring. (a) diagram of the transsulfuration pathway. **Bolded** intermediates were measured by mass spectrometry and *italicized* genes measured by high throughput PCR. (b) Gene expression of enzymes involved in homocysteine metabolism and the transsulfuration pathway is altered by maternal HF diet and postnatal MS. (c-d) Maternal high fat diet increased the concentration of transsulfuration pathway intermediates (c) homocysteine and (d) cysteine. # $p < 0.05$ HF vs. HF+MS, * $p < 0.05$ main effect of maternal diet; male n: SC=10, HF=12, SC+MS=10, HF+MS=12, female n: SC=10, HF=10, SC+MS=10, HF+MS=9 in (b), (c-d) both sexes n: SC=5, HF=5, SC+MS=5, HF+MS=5.

Figures and Tables

Table 1.

Gene	Full name	Primer ID
DNMT1	DNA methyltransferase (cytosine-5) 1	Mm00599763_m1
DNMT3a	DNA methyltransferase 3a	Mm00432881_m1
DNMT3b	DNA methyltransferase 3b	Mm01240113_m1
MECP2	methyl CpG binding protein 2	Mm01193537_g1
TET1	Ten-eleven translocation methylcytosine dioxygenase 1	Mm01169087_m1
TET2	Ten-eleven translocation methylcytosine dioxygenase 2	Mm00460654_m1
ACTB	actin, beta	Mm00607939_s1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1

Table 2. Details of the statistical main effects presented in the figures

Prefrontal cortex	3-way	Male 2-way	Female 2-way
THF	MS: $p < 0.001$ $F(1,1)=22.17$		
5-formyl-THF	MS: $p < 0.001$ $F(1,1)=24.39$	Maternal: $p=0.006$ $F(1,16)=9.82$	
5,10-methenyl-THF	MS: $p < 0.001$ $F(1,1)=34.95$		
5-methyl-THF	MS: $p < 0.001$ $F(1,1)=20.08$		
Methionine	Sex: $p=0.006$ $F(1,1)=8.7$	Interaction: $p=0.048$ $F(1,16)=4.583$	
	MS: $p=0.002$ $F(1,1)=11.92$		
SAM	Maternal: $p=0.004$ $F(1,1)=9.77$		Maternal: $p=0.004$ $F(1,16)=11.1$
SAH	Maternal: $p=0.049$ $F(1,1)=4.18$		
Hcy		Maternal: $p=0.023$ $F(1,16)=6.37$	
Cysteine	Maternal: $p=0.005$ $F(1,1)=9.05$	Maternal: $p=0.028$ $F(1,16)=5.873$	
dTMP		Maternal: $p=0.003$ $F(1,16)=12.38$	

Table 3. Details of the statistical main effects of gene expression

	Male	Female
CBS	MS: $p=0.003$ $F(1,40)=5.08$	--
CTH	Maternal: $p=0.03$ $F(1,40)=5.04$	--
DNMT3a	MS: $p=0.045$ $F(1,40)=4.29$	--
EAAT3	--	Maternal: $p=0.046$ $F(1,35)=4.28$
MTHFD1	MS: $p=0.025$ $F(1,40)=5.4$	--
MTHFR	MS: $p=0.012$ $F(1,40)=6.926$	--
MTRR	MS: $p=0.003$ $F(1,40)=5.09$	--
PEMT	Maternal: $p=0.002$ $F(1,40)=10.81$	--
PNMT	--	MS: $p=0.022$ $F(1,35)=5.8$
SAHH	MS: $p=0.002$ $F(1,40)=11.44$	--
SETD7	MS: $p=0.005$ $F(1,40)=9.023$	--

Table 4. Gene List

Gene	Full name	Primer ID
CBS	cystathionine-Beta-synthase	Mm00460654_m1
CGL	Cystathionine Gamma-Lyase	Mm01317283_m1
COMT	catechol-O-methyltransferase 1	Mm00514377_m1
DHFR	dihydrofolate reductase	Mm00515662_m1
DNMT1	DNA methyltransferase (cytosine-5) 1	Mm00599763_m1
DNMT3a	DNA methyltransferase 3a	Mm00432881_m1
EAAT3	Solute Carrier Family 1 Member 1	Mm00436590_m1
EHMT2	Euchromatic Histone-Lysine N-Methyltransferase 2	Mm01132261_m1
GADD45β	growth arrest and DNA-damage-inducible 45 beta	Mm00435123_m1
HDAC2	histone deacetylase 2	Mm00515108_m1
HDAC5	histone deacetylase 5	Mm01246076_m1
MECP2	methyl CpG binding protein 2	Mm01193537_g1
MTHFD1	methylenetetrahydrofolate dehydrogenase	Mm00507092_m1
MTHFR	methylenetetrahydrofolate reductase	Mm00487787_m1
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	Mm01340053_m1
MTRR	5-methyltetrahydrofolate-homocysteine methyltransferase reductase	Mm00549977_m1
PEMT	phosphatidylethanolamine N-methyltransferase	Mm00839436_m1
PNMT	Phenylethanolamine N-Methyltransferase	Mm00476993_m1
SAHH	S-adenosylhomocysteine hydrolase-like 1	Mm00461101_m1
SETD7	SET domain containing (lysine methyltransferase) 7	Mm00499823_m1
TET1	Ten-eleven translocation methylcytosine dioxygenase 1	Mm01169087_m1
TET2	Ten-eleven translocation methylcytosine dioxygenase 2	Mm00460654_m1
TYMS	thymidylate synthase	Mm01702970_m1
ACTB	actin, beta	Mm00607939_s1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1

Figure 1.

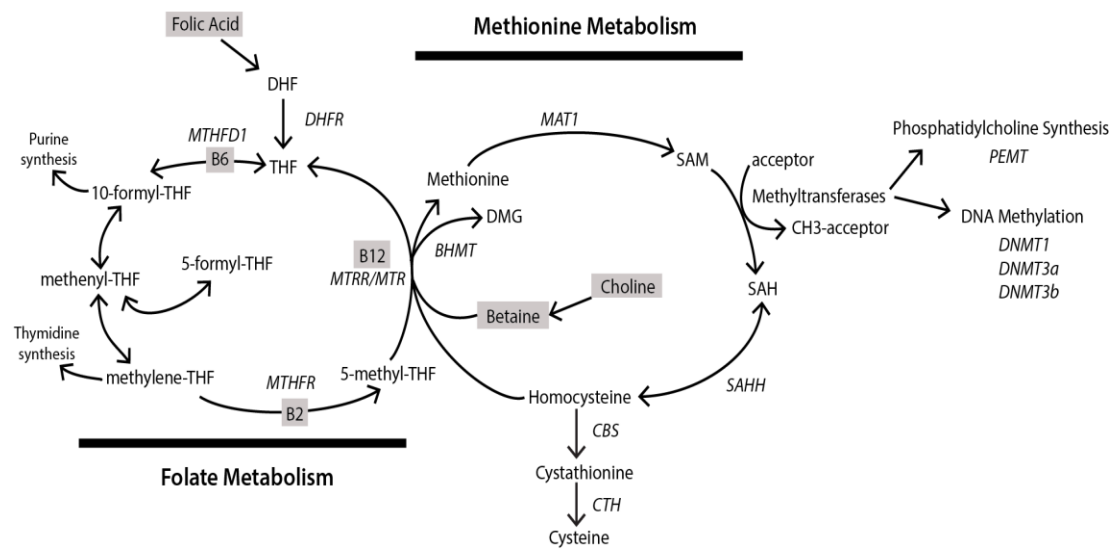


Figure 2.

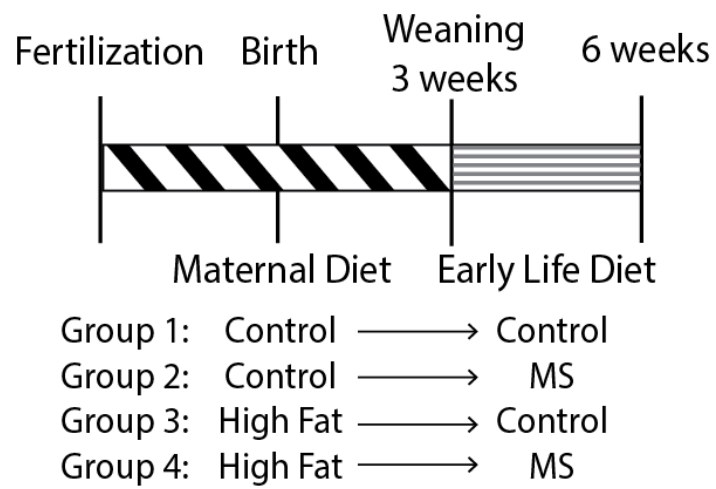
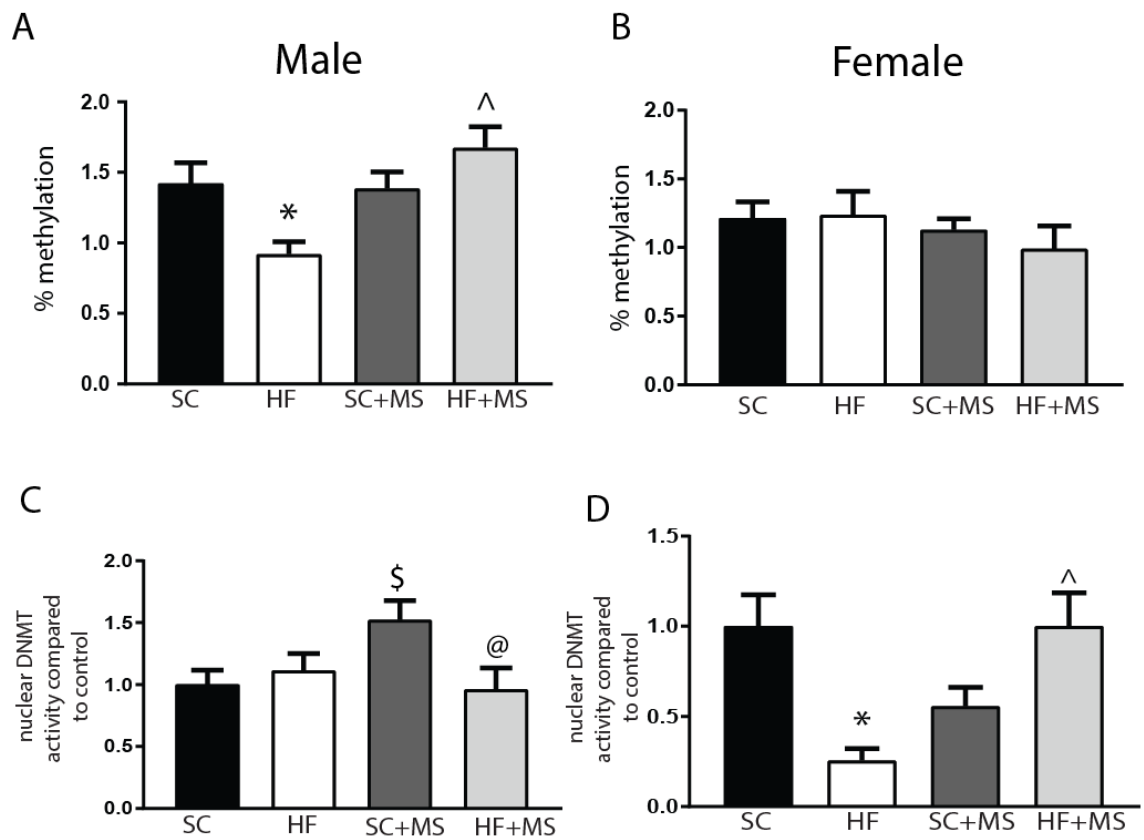
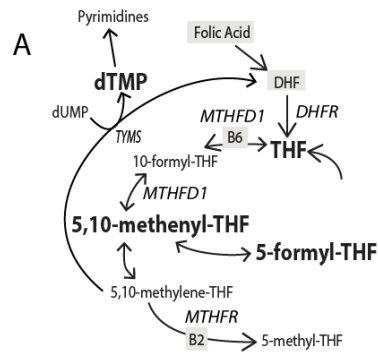


Figure 3.

* SC vs HF
 ^ HF vs HF+MS
 \$ SC vs. SC+MS
 @ SC+MS vs HF+MS



■ SC ^ main effect of MS
 □ HF * main effect of Maternal Diet
 ■ SC+MS @ SC+MS vs HF+MS
 ■ HF+MS



Gene	Male	Female
DHFR	--	--
MTHFD1	MS ↑ 6%	--
MTHFR	MS ↑ 9%	--
TYMS	--	--

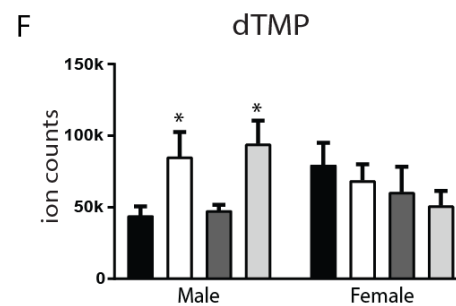
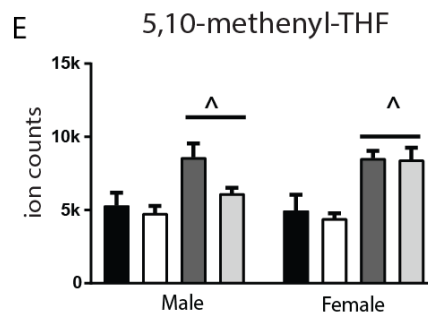
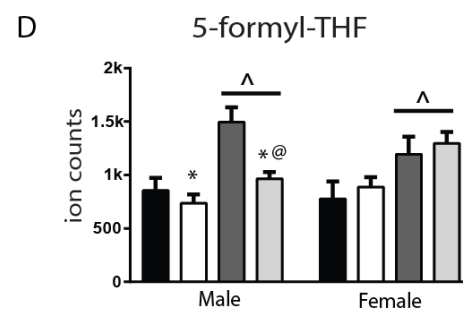
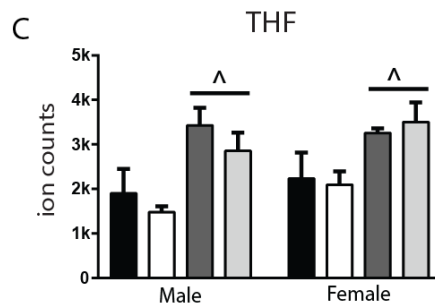
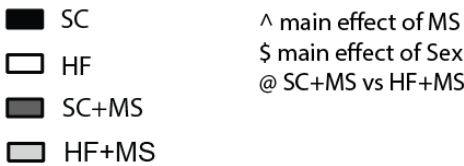
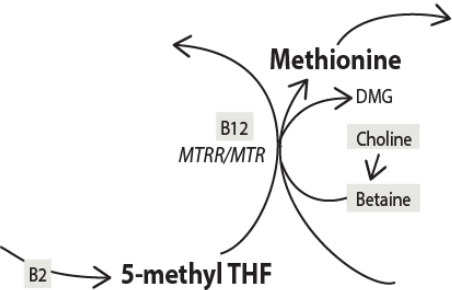


Figure 5.



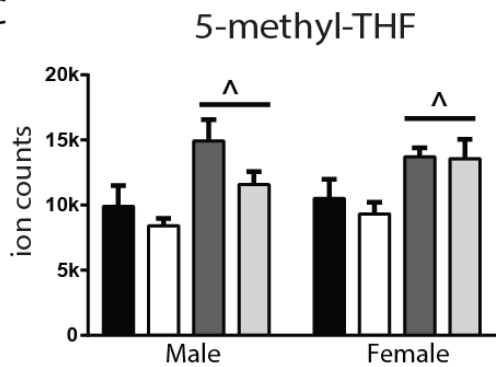
A



B

Gene	Male	Female
MTRR	MS ↑ 7%	--
MTRR	--	--

C



D

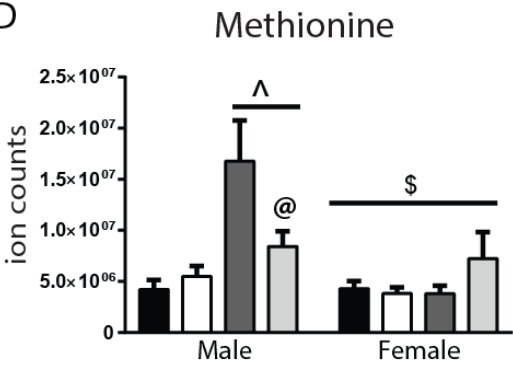
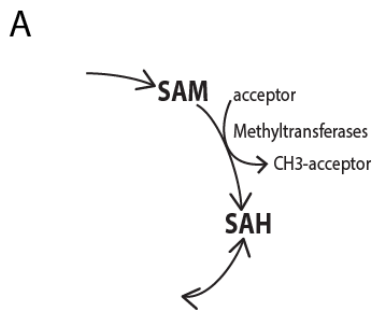
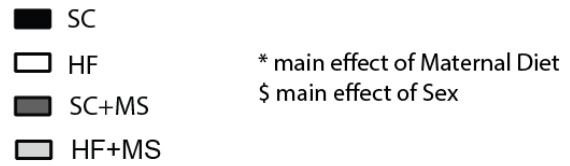


Figure 6.



B

Gene	Male	Female
DNMT1	--	--
DNMT3a	MS ↑ 6%	--
SETD7	MS ↑ 11%	--
PEMT	High Fat ↓ 5%	--
PNMT	--	MS ↓ 25%

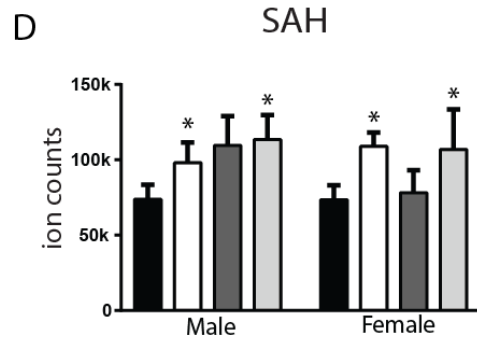
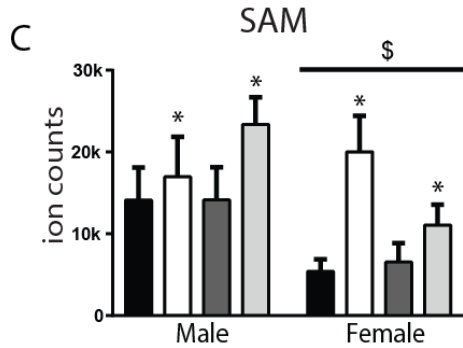
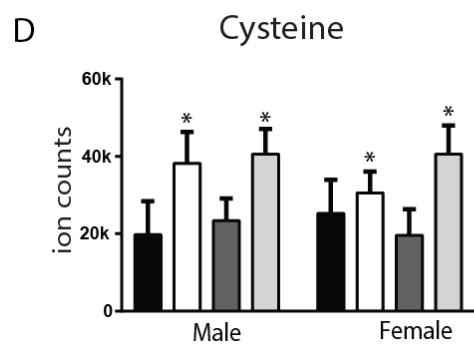
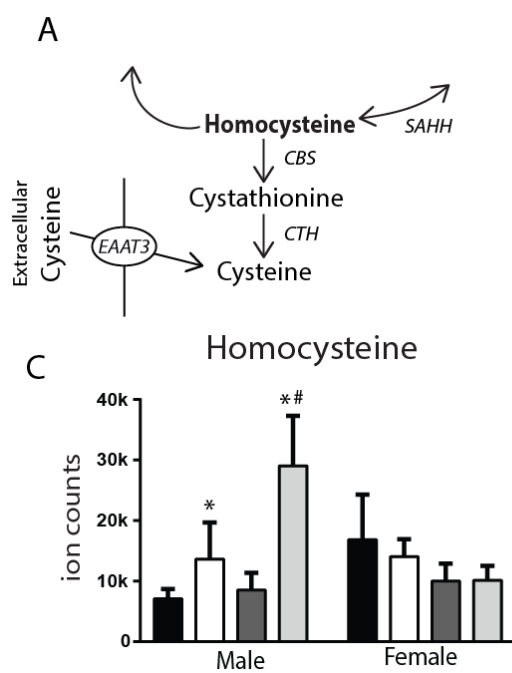
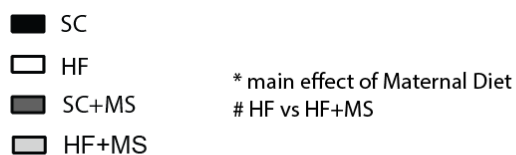


Figure 7.



Chapter Four

Perinatal high fat diet alters DNA methyltransferase activity in offspring prefrontal cortex without altering expression

Sarah E. McKee, Kelsey R. Lloyd, Nicola M. Grissom, Brittany L. Smith, Teresa M. Reyes

Introduction

Over 50% of women exceed recommended weight gain during pregnancy, also known as excessive gestational weight gain (GWG) (Simas et al., 2011). This weight gain is often driven by over consumption of highly palatable, energy dense foods (i.e. foods high in fats and/or sugars). Poor maternal nutrition during pregnancy is known to predispose offspring to increased risk for metabolic and neurological disorders, including obesity, diabetes, schizophrenia, autism and attention deficit and hyperactivity disorder (ADHD) (Alfaradhi and Ozanne, 2011; Colman et al., 2012; Herva et al., 2008; Krakowiak et al., 2012; Lubchenco et al., 1972; Robles et al., 2015; Van Lieshout et al., 2011). Elucidating the link between GWG and long-term offspring health outcomes is of the utmost importance.

Using a mouse model of excessive GWG, in which dams are fed a 60% high fat (HF) diet throughout gestation and lactation, we have reported that offspring have altered brain development, including increases preference to palatable foods (i.e. sugars and fats) and increased risk for cognitive and motivation deficits mediated primarily by the prefrontal cortex (PFC) (Carlin et al., 2013; Vucetic et al., 2010). We have also seen changes in RNA gene expression within reward circuitry and global and promoter-specific DNA hypomethylation within the PFC. DNA methylation is a well-studied epigenetic mark that is known to in part regulate gene expression. It is also a proposed mechanistic link between gestational environment and changes in offspring brain development and long-term cognition and health outcomes. DNA methylation patterns are known to be altered by diet, chemical exposure and behavioral environment (Anderson et al., 2012; Xin et al., 2015; Zampieri et al., 2015). Found on the 5-carbon position of

cytosine bases, when present DNA methylation is canonically known to repress gene expression by associating with a tightly closed chromatin state (Robertson, 2002; Robertson and Wolffe, 2000). While gene expression is associated with the absence of DNA methylation within the same region. Studies are continuing to reveal the complex nature of this relationship (Suzuki and Bird, 2008; Weber et al., 2007). It is known that methylation patterns vary across neuroanatomical regions (Ladd-Acosta et al., 2007) and are dynamically regulated across the lifespan (Siegmund et al., 2007).

DNMT1, DNMT3a and DNMT3b methyltransferases (collectively known as DNMTs) are large multi-domain proteins that introduce DNA methylation. DNMT1 is known for its preferential binding to hemi-methylated DNA, categorizing it as the maintenance methyltransferase, methylating newly synthesized daughter DNA strands. DNMT3a and DNMT3b, do not show preference between hemi- or un-methylated DNA, designating them as de novo methyltransferases. Typically characterized within somatic dividing cell types, evidence reveals that all three DNMTs are necessary for maintenance of neuronal DNA methylation. Specifically, a forebrain-specific DNMT1 knockout (KO) mouse showed global DNA hypomethylation, changes in RNA gene expression, neurodegeneration and deficits in learning and memory (Hutnick et al., 2009). Deletion of DNMT1 in post-mitotic neurons resulted in DNA hypomethylation in daughter cells (Fan et al., 2001). Mice lacking functional DNMT3a in the CNS survive with neuromuscular and motor coordination deficits and die prematurely (Nguyen et al., 2007). Mice with a double KO of DNMT1 and DNMT3a in forebrain excitatory neurons have smaller hippocampal neurons, CpG hypomethylation, altered gene expression and show deficits in learning memory (Feng et al., 2010). In humans, disrupted DNA methylation and methyltransferase function has been implicated in autism, Parkinson's disease, schizophrenia and ADHD (Loke et al., 2015; Masliah et al., 2013; Schuch et al., 2015; Weng et al., 2013). DNMT1 mutations are linked to cognitive function decline, hearing loss, sensory neuropathy and result in imbalanced DNMT1 protein homeostasis (Baets et al., 2015). Disrupted localization of DNMT1 has been also implicated in Alzheimer's and Parkinson's disease (Desplats et al., 2011; Mastroeni et al., 2013). Taken

together, within non-dividing neurons, functional DNMT1, DNMT3a and DNMT3b are necessary for proper neural development and function.

Given that perinatal HF diet exposure alters both gene expression and global and promoter-specific hypomethylation in the PFC and the importance of DNMT function in DNA methylation, we wanted to determine whether DNMT activity or abundance could mediate the DNA hypomethylation reported. Utilizing offspring from our mouse model of excessive GWG, we measured total neuronal DNMT activity, RNA gene expression of the DNMTs, whole cell DNMT protein concentration, cytoplasmic and nuclear DNMT protein concentration, quantified cellular localization of DNMT1 using immunohistochemistry, and measured association with known kinases and binding partners using co-immunoprecipitation. We hoped to elucidate a mechanism by which perinatal HF diet can alter DNA methylation patterns in offspring.

Methods and Procedures

Animals and experimental model: All animals were cared for according to the guidelines of the University of Cincinnati Institutional Animal Care and Use Committee. An experimental timeline is seen in **Figure 2**. C57BL/6J virgin females were bred with DBA/2J males. All animals were fed control diet prior to breeding. From the onset of breeding, through pregnancy and lactation, dams (n=12/group) were fed one of two purified diets (Test Diet, Richmond, IN); (1) standard control (SC) and (2) 60% high fat (HF) (Grissom and Reyes, 2013; McKee et al., 2017; Vucetic et al., 2010). Specific diet formulations are shown in **Table 1**. Litters were culled to 8-9 pups as necessary, to equalize access to nutrition throughout lactation. Animals were weaned at 3 weeks of age. Animals were sacrificed at 10-12 weeks of age for experimentation. At sacrifice, brains were collected and prefrontal cortex was immediately dissected and flash frozen on dry ice for protein, placed in RNeasy lysis buffer and stored at -20C for RNA or perfused for immunohistochemistry.

Nuclear/Cytoplasmic fractionation: Prefrontal cortex tissue was homogenized with a Dounce homogenizer (50 strokes) and cytoplasmic and nuclear protein was extracted with the EpiQuik

Nuclear Extraction Kit (Epigentek, USA) according to manufacturer's instructions with two minor additions. 1:1000 PIC and DTT solutions were added to the NE1 buffer to keep the cytoplasmic proteins from breaking down and an ice-cold PBS wash (400µl) step was added between the pulling off the cytoplasmic fraction and before the nuclear proteins were extracted, to ensure cleaner separation of the nuclear and cytoplasmic proteins. To ensure that protein was concentrated enough for western blot analysis, 3 PFC (from the same litter) were combined into one sample. 360 µl of NE1 and 100 µl of NE2 was used. Protein concentration was quantified using Pierce BCA Protein Assay (Pierce Biotechnology, Rockford, IL).

Western blotting: Proteins were run on 4-20% polyacrylamide gel (BioRad, USA) for 45 minutes at 200 V at room temperature. Then were transferred on to an Immobilon-FL PVDF (Millipore, Cork, IRL) for 60 minutes at 100 V at 4°C. Membranes were incubated overnight at 4°C in 1:1 Odyssey Blocking Buffer (TBS) (LI-COR, USA) and Tris-buffered saline (TBS) containing 0.1% Tween 20, and then incubated with either DNMT1 (1:250; ab13537), DNMT3a (1:500; ab23565), GAPDH (1:1000; sc25778 or ab8245), or LAMINb (1:1000; ab16048) overnight at 4°C. Membranes were then washed with TBST 4 times 5 minutes and incubated with appropriate secondary antibodies (1:20k) donkey anti-mouse (Li-COR 926-68021) or goat anti-rabbit (Li-COR 926-32212) for one hour at room temperature in a black incubation box. Membranes were then scanned using an Odyssey LI-COR scanner. Proteins were then quantified with Image Studio Lite Software (version 3.1) (LI-COR) and normalized to either GAPDH or LAMINb.

Co-immunoprecipitation: Whole-cell proteins were extracted using Whole-cell extraction buffer from the Universal Magnetic Co-IP Kit (Active Motif, California, USA) following manufacturer's instructions. Protein concentration was quantified as described above. Co-immunoprecipitation was completed using 500 µg of whole-cell protein extract and 5 µg of DNMT1 (39204) antibody. Final bead pellet was resuspended in 20 µl of 2x Laemmli buffer. Samples were run on a western blot as described above with the addition of an input control lane for each sample which

contained 5 µg of the final protein extract. Primary antibodies DNMT1 (1:250; ab13537), AKT (1:500; CS9272S), MECP2 (1:500; Upstate 07013), and CK (1:500; ab136052) were analyzed. Secondary LI-COR antibodies anti-rabbit CW800 (1:20k) and anti-mouse LT 680 (1:20k) were used. % IP enrichment for each protein was calculated by normalizing the protein concentration to DNMT1 concentration. DNMT1 concentration did not differ between groups.

DNA methyltransferase Activity: DNMT activity was measured in 2 µg (male) or 10 µg (female) of nuclear protein and 10 µg of cytoplasmic protein with the EpiQuik DNMT Activity/Inhibition Assay Ultra Kit (Epigentek) according to manufacturer's instructions. Absorbance was read at 450 and 655 nm on a microplate reader (Epoch, BioTek Instruments, Inc., USA). The DNMT activity was calculated using $\text{DNMT activity} = (\text{sample OD} - \text{blank OD}) / [\text{protein amount } (\mu\text{g}) \times \text{h}] \times 1000$. All activity was normalized to SC controls.

Tissue processing and histology: Animals were injected with an overdose of sodium pentobarbital and transcardially perfused with saline followed by ice-cold 4% paraformaldehyde in 0.1% borate buffer at pH 7.5. Brains were postfixed for 4 hr and cryoprotected overnight in 30% sucrose at 4°C. Brains were sectioned (30 µm) using a sliding microtome, collected in cold ethylene glycol-based cryoprotectant, and stored at -20 °C until histochemical processing.

Immunohistochemistry: Immunohistochemistry (IHC) for DNMT1 (ab188453, 1:1000), DNMT3a (MA5-16171, 1:1000), NeuN (ab104520, 1:2000 or MABN140, 1:1000) and DAPI (300nM) (was performed on a 1-in-4 series of brain sections using standard IHC procedures. Briefly, after being washed in 50mM potassium phosphate buffered saline (KPBS), sections were incubated in blocking solution (containing 0.1% bovine serum albumin and 0.2% Triton-X) for 45 minutes, incubated in primary antibody in blocking solution overnight at 4°C. Sections were then incubated with fluorescent secondary antibodies (a10520 and a11001, 1:2500) for one hour. The sections were then mounted, dried, and stained with DAPI, dried, and coverslipped with polyvinyl alcohol

mounting medium (Sigma-Aldrich). Immunolabeling in the PFC (Bregma +2.10mm, Paxanos and Weston atlas) was visualized and photographed at 40x magnification using fluorescence microscopy (Zeiss Imager Z1 with Apotome, AxioCam camera, AxioVision version 4.8.2 software, Carl Zeiss Microscopy, Jena, Germany). Relative abundance of DNMT1 and 3a was measured in the region of interest using ImageJ software (NIH). Neurons and nuclei were traced in 5 images in each PFC section. Fluorescence was averaged across cells in each image, then averaged for each animal. Fluorescence within the nuclei was subtracted from total neuronal fluorescence to achieve a relative fluorescence in the cytoplasm. All analyses were performed by an observer unaware of group assignment.

Gene expression in Prefrontal Cortex: At sacrifice the medial prefrontal cortex was dissected from a 2mm coronal slice from bregma +2.3 to 0.3 and DNA and RNA were extracted using AllPrep DNA/RNA Mini Kit (Qiagen). cDNA was synthesized using High Capacity Reverse Transcriptase kit (Applied Biosystems). Expression of target genes was determined by qRT-PCR using gene-specific TaqMan probes (Applied Biosystems) on the ABI7900Fast real-time PCR cycler. Probes used for RT-PCR are listed in **Table 2**. The relative amount of each transcript was determined using the delta cycle threshold (ΔC_t) values as previously described (Pfaffl, 2001). Changes in gene expression were calculated using relative quantitation of target genes against the geometric mean of two housekeeping genes, ACTB and GAPDH.

Data analysis: Data were analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). Student's *t* was used to analyze differences between offspring from HF-fed and control dams. A *P* value ≤ 0.05 considered significant.

Results

Because we have repeatedly reported that perinatal high fat diet leads to offspring global and promoter-specific DNA hypomethylation in PFC we were interested in determining whether

differences in DNMT could underlie these changes. We fed dams either a SC or HF diet from breeding through lactation and weaned both male and female offspring onto standard vivarium chow and examined the PFC for DNMT activity, abundance and localization between 8 and 12 weeks of age (Fig. 1). We first examined nuclear DNMT activity and found that perinatal HF decreased total DNMT activity in the male PFC ($p=0.03$, $t=2.454$, $df=12$) (Fig. 2A). In contrast, in female offspring, perinatal HF diet increased total DNMT activity in the PFC ($p=0.042$, $t=2.198$, $df=17$) (Fig. 2B).

We were then interested in evaluating gene expression of the different DNMTs to determine whether one particular DNMT could mediate these changes in DNMT activity. We ran standard rt-qPCR on DNMT1, 3a and 3b as well as methyl CpG binding protein, MECP2, and tet methylcytosine dioxygenase 1 and 2, TET1 and 2. All genes known to mediate RNA gene expression. We found that overall, for both male and female offspring, there were no changes in gene expression for any of these genes (Fig. 3). In females, DNMT1 expression had a trending decrease ($p=0.059$, $t=2.134$, $df=10$), but this difference did not reach significance.

It is well known that changes in gene expression, or lack thereof, do not always correlate with protein expression, so we examined whole cell homogenate protein expression of DNMT1 and DNMT3a. We again found that neither DNMT1 nor DNMT3a have altered protein expression in male or female PFC (Fig. 4A-D). We next wanted to separate cytoplasmic versus nuclear fractions within the protein homogenate, as DNMTs function generally within the nucleus on the DNA. In male offspring, perinatal HF diet did not alter DNMT1 or 3A levels within the PFC in either the nuclear or cytoplasmic fractions (Fig. 5A-D). In female offspring, perinatal HF diet increased the concentration of DNMT1 in the cytoplasm (Fig. 6A) ($p=0.0227$, $t=2.505$, $df=17$), but not in the nucleus (Fig. 6B). DNMT3a expression was not different in either fraction (Fig. 6C-D).

We next used immunohistochemistry to determine cellular localization of DNMT expression to determine whether there was active cytosolic sequestration of DNMT as seen in other neurological disorders (Desplats et al., 2011; Mastroeni et al., 2013). We stained brains with DNMT1, NeuN (a neuronal marker) and Dapi (a nuclear marker) and measured total

fluorescence, and fluorescence inside and outside of the nucleus. Interestingly, opposite that of the protein fractionization findings, we found no changes in DNMT1 fluorescence in female offspring, neither total expression nor changes in cellular localization (data not shown). In male offspring, we did not see any differences in total DNMT1 signal over the neuronal area (Fig. 7A). We did see an increase in the percent of DNMT1 expression outside of the nucleus in HF diet offspring (Fig. 7B) ($p=0.003$, $t=3.861$, $df=10$). Surprisingly, we also found that perinatal HF diet decreases the average nuclei size (Fig. 7C) ($p=0.019$, $t=2.8$, $df=10$) and neuronal area measured (Fig. 7D) ($p=0.042$, $t=2.34$, $df=10$), a pattern not seen in the female offspring.

Finally, it is known that DNMT1 is regulated by various kinases and binding proteins which regulate localization into the nucleus and activation of the protein. Therefore, we performed co-immunoprecipitation experiments to determine whether there were changes in association to these proteins that could explain the hypomethylation phenotypes reported. We first immobilized the bound proteins and precipitated DNMT1. We then measured the abundance of kinases AKT and CK and binding protein MECP2. We found that perinatal HF diet did not change the associations of DNMT1 with these proteins in either male (Fig. 8A) or female (Fig. 8B) offspring.

Discussion

Perinatal HF diet exposure increases offspring risk for development of both metabolic and neurological disorders. Utilizing a mouse model of excessive GWG, we previously reported altered RNA gene expression and global and promoter- specific DNA hypomethylation within the offspring PFC. The current studies identified that between 8-12 weeks of age perinatal HF diet exposure alters total nuclear DNMT activity in both male and female offspring in a sex-dependent manner. These activity changes do not seem to be mediated by changes to overall RNA expression, whole-cell or cytoplasmic/nuclear fractioned protein concentration. Activity changes were also not dependent on association with kinases CK and AKT or methyl binding protein MECP2. There is some evidence possibly indicating that DNMT1 expression in male offspring is

sequestered to the cytoplasm. Further exploration for mechanisms controlling DNMT1 expression is necessary.

In the current study, we found that perinatal HF diet can alter nuclear DNMT activity at 8 weeks of age. It should be recognized that the current assay utilized measured activity of all present DNMTs. Methods to separate the activities of specific DNMTs have not been developed. Therefore, we cannot determine which, if any, particular DNMT is mediating these changes in activity. However, because we do not see any changes in DNMT1 or 3a protein concentration in the western blot within the nuclear fraction, we can be confident that changes in overall DNMT concentration utilized in the assay did not alter activity levels, but that there are actually functional differences in the DNMT proteins. Additionally, for the purpose of this discussion, DNMT3b will not be addressed as it is no longer active at this stage of development (Feng et al., 2005).

DNMT1 and DNMT3a are multi-domain proteins that contain both a N-terminal regulatory domain and a C-terminal catalytic domain. The catalytic C-terminal domain is structurally very similar in each DNMT. It is involved with S-adenosyl methionine (SAM) binding, DNA recognition and binding and target base flipping and catalysis (Jeltsch and Jurkowska, 2016). The N-terminal regulatory domain in DNMT1 is much larger than that in the DNMT3 family (Hermann et al., 2004). Within DNMT1's N-terminus there are 7 different functional domains, while only 2 functional domains comprise DNMT3a's N-terminus. The functions of each of these domains is reviewed thoroughly by Jeltsch and Jurkowska (2016) (Jeltsch and Jurkowska, 2016). The regulatory domain is highly targeted by post-translational modifications (PTMs), which can alter nuclear localization and interactions with other proteins, nucleic acids and chromatin. Further investigation of how perinatal HF diet alters this regulatory domain could shed light onto why DNMT activity is altered in these offspring.

Presently we determined that DNMT activity changes were not mediated by association with kinases known to phosphorylate DNMT1 casein kinase, CK, known to decrease DNA binding affinity to DNMT1, and protein kinase B, AKT, known to increase DNMT1 stability. We did not however quantify levels of phosphorylation within these offspring. Therefore, it remains unknown

whether the actual phosphorylation levels are altered in these animals, possibly leading to altered DNMT activity. We also found no changes in association with binding protein methyl CpG binding protein 2, MECP2, known to bind to methylated DNA and canonically repress gene expression.

Because PTMs are highly involved in regulating DNMT activity (Peng et al., 2011a; Turk et al., 1995; Valinluck et al., 2004), altered phosphorylation, methylation, ubiquitination, acetylation and sumoylation not examined in our studies could mediate the changes seen in DNMT activity. Over 100 PTM sites have been identified on human and mouse DNMT1 (Jeltsch and Jurkowska, 2016). One example is deubiquitination of DNMT1 by Ubiquitin-specific protease 7, USP7. USP7 is a protease known to bind to DNMT1 and increase the stability through deubiquitination of the protein. In vitro, binding of USP7 with DNMT1 increased overall activity (Felle et al., 2011). Individuals with mutations in USP7 are characterized with intellectual disability, autism and seizures (Fountain and Schaaf, 2016). Therefore, if USP7 association or levels of ubiquitin are altered in the current model it could explain why perinatal HF diet also correlates with increased cognitive deficits. A second possibility is the presence of microRNAs (miRNAs) which are also known to bind to DNMT1 and inhibit activity, canonically resulting in increased gene expression of various target genes (Di Ruscio et al., 2013; Peschansky and Wahlestedt, 2014). miR-155-5p has been specifically implicated in DNMT1 activity inhibition. A recent study showed that deletion of miR-155-5p prevented diet-induced obesity by upregulating genes involved in thermogenic potential, brown adipose differentiation and insulin sensitivity (Gaudet et al., 2016). These data indicate that diet can change miRNAs content within the body and could mediate gene changes through inhibition of DNMT1 activity. There have been no studies identifying whether perinatal HF diet alters expression of miRNA in the brain. However, it is known that perinatal HF diet alters miRNA expression in the liver (Benatti et al., 2014; Zhang et al., 2009), so we would hypothesize similar changes within the brain. An additional possibility is the mediation through changes in expression or activity of sirtuin 1, SIRT1. SIRT1 is a deacetylase known to alter DNMT function (Peng et al., 2011b). SIRT1 expression and activity is known to be altered by HF diet (Chalkiadaki and Guarente, 2012; Heyward et al., 2012; Pfluger et al., 2008; Scheibye-Knudsen et al., 2014),

implicated in multiple neurological disease models (Ng et al., 2015; Tulino et al., 2016), and have sex specific expression in the brain (Lafontaine-Lacasse et al., 2010). Additional studies are needed to link SIRT1 and the DNMT activity changes due to perinatal HF diet. Taken together, there are multiple possibilities that have not been examined in the current experiments that could explain the changes in nuclear DNMT activity.

We also observed a sex-dependent pattern to changes in nuclear DNMT activity. Perinatal HF diet decreased DNMT activity in males, while it increased DNMT activity in females. Previous research shows that epigenetic regulation in the brain is often sex-specific (McCarthy et al., 2009). Additionally, between 8-12 weeks of age sex hormones are fully present and active. The presence of sex hormones in the brain is known to regulate neurodevelopment, offer protection to neural injury better in one sex, in part drive sex differences in mental health vulnerability, alter gene expression and most importantly to this research induce epigenetic changes (T.-V. Nguyen et al., 2016). There could be an interaction with DNMT and one of the sex hormones that could regulate the activity changes observed. In a previous study, it was found that 24 hours after birth DNMT activity in male offspring was significantly greater than in females, and, interestingly, exogenous estradiol treatment in females increased DNMT activity. Importantly, this study also found no differences in protein levels of DNMT1 or DNMT3a, similar to our findings, therefore it seems that DNMT activity is specifically regulated by sex hormones (McCarthy et al., 2009). Sex hormones are also known to regulate many of the above mentioned activity modifiers; acetylases, deacetylases, histone modifications, DNA binding proteins, methyl binding proteins to name a few (reviewed thoroughly by McCarthy et al., 2009) (McCarthy et al., 2009).

Although total DNMT1 expression was not changed in the male offspring, cytosolic sequestration of DNMT1 was increased. Cytosolic sequestration is also seen in human brains of patients with Alzheimer's and Parkinson's disease (Desplats et al., 2011; Mastroeni et al., 2013). Onset of mild cognitive impairment (MCI), which can precede Alzheimer's onset, and Parkinson's disease is earlier than in men than women (Gillies et al., 2014; Mielke et al., 2014). This possibly indicates that if we were to look at DNMT1 localization later in life in female offspring we would

see this same pattern. It is also known that DNMT1 cellular localization changes throughout the cell cycle in replicating cells (Jeltsch and Jurkowska, 2016). Because neurons are non-replicating cellular localization is not well characterized in the brain.

Immunohistochemistry revealed that perinatal HF diet may interact with the size of the neuronal nucleus in male offspring. We observed significantly smaller nuclei area in the HF diet male offspring. We also observed decreased neuronal area within the measured markers. It will be important to follow this finding up with a tubulin stain to determine the entire neuronal area to see if the pattern holds. There seems to be a small bit of evidence that nuclear area decreases with age, but it varies by region and is controversial (Flood and Coleman, 1988). The nucleus is the storage site of an individual's genetic information. Lamins make up a large portion of the nuclear membrane, a structure thought to protect the genome from mechanical stress (Scaffidi et al., 2005). Lamins are now known to interact with chromatin and potentially play a role in regulating gene expression (Gruenbaum et al., 2005). The sex specific nature of these findings are interesting as males are predisposed to develop certain neurological disorders. Determining whether perinatal HF diet alters neuron cellular architecture could provide new insights into mechanisms by which offspring risk for disease is increased.

Overall, we have determined that perinatal HF diet alters DNMT activity in male and female offspring in a sex-dependent manner. We did not however discover the mechanism by which the DNMT activity is changed and whether the same mechanism underlies the sex-specific changes. There were no changes in gene expression, protein expression or associations with CK, AKT or MECP2. It will be important to investigate additional known PTMs and protein associations to shed light on this mechanism. Understanding how perinatal HF diet alters DNMT activity could provide additional therapeutic targets to mediating offspring risk for metabolic and neurologic disease.

Table and Figure Legends

Table 1. Gene primer list.

Figure 1. Experimental design. Mouse dams were fed either a standard control (SC) or high fat (HF) breeder diet throughout gestation and lactation. Male and female offspring were weaned onto standard vivarium diet, creating 2 experimental groups (control, SC; high fat, HF). All animals were sacrificed and tissues appropriately collected between 8-12 weeks of age.

Figure 2. Perinatal high fat diet alters DNA methyltransferase (DNMT) activity in the PFC. (a) Male HF offspring have decreased nuclear DNMT activity. (b) Female HF offspring have increased nuclear DNMT activity. * $p < 0.05$ SC vs HF. N: SC=7, HF=7 in (a), n: SC=10, HF=9 in (b)

Figure 3. Perinatal high fat diet does not change gene expression of DNA methylation machinery in the PFC (a) male offspring. (b) female offspring. n=6 for all groups (a-b).

Figure 4. Perinatal high fat diet does not change whole cell DNMT protein levels in the PFC. (a) DNMT1 whole cell protein expression in male offspring. (b) DNMT1 whole cell protein expression in female offspring. (c) DNMT3a whole cell protein expression in male offspring. (d) DNMT3a whole cell protein expression in female offspring. n=8 for all groups (a-d).

Figure 5. Perinatal high fat diet does not change DNMT protein expression in nuclear or cytoplasmic cellular fractions in male offspring PFC. Cytoplasmic fraction protein expression of DNMT1 (a) and DNMT3a (c). Nuclear fraction protein expression of DNMT1 (b) and DNMT3a (d). n: SC=8, HF=11 in all figures.

Figure 6. Perinatal high fat diet increases cytoplasmic DNMT1 protein in female offspring. (a) DNMT1 is increased in female offspring exposed to perinatal HF. (b) DNMT1 protein concentration does not change in the nucleus. DNMT3a protein concentration does not change in the cytoplasm (c) or nucleus (d) in female offspring due to perinatal HF. * $p < 0.05$ SC vs HF. n: SC=10, HF=9 in all figures.

Figure 7. Perinatal high fat decreases the size of the nucleus specifically in male offspring neurons, while increasing the concentration of DNMT1 outside of the nucleus. (a) Total DNMT1 expression was not changed by perinatal HF diet. (b) DNMT1 is localized more to the cytoplasm in male offspring PFC. (c) Perinatal HF decreases the average area of nuclei in male offspring PFC. (d) Total surface area is decreased due to perinatal HF diet in male offspring. * $p < 0.05$ SC vs HF. n: SC=6, HF=6 in all groups.

Figure 8. Perinatal high fat diet does not alter DNMT1 binding to known proteins that regulate nuclear import and activation. Immunoprecipitation of DNMT1 with kinases AKT and CK and binding partner MECP2 in male (a) and female (b) offspring. n: SC=8, HF=8 in (a), n: SC=8, HF=7 in (b).

Figures and Tables

Table 1.

Gene	Full name	Primer ID
DNMT1	DNA methyltransferase (cytosine-5) 1	Mm00599763_m1
DNMT3a	DNA methyltransferase 3a	Mm00432881_m1
DNMT3b	DNA methyltransferase 3b	Mm01240113_m1
MECP2	methyl CpG binding protein 2	Mm01193537_g1
TET1	Ten-eleven translocation methylcytosine dioxygenase 1	Mm01169087_m1
TET2	Ten-eleven translocation methylcytosine dioxygenase 2	Mm00460654_m1
ACTB	actin, beta	Mm00607939_s1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1

Figure 1.

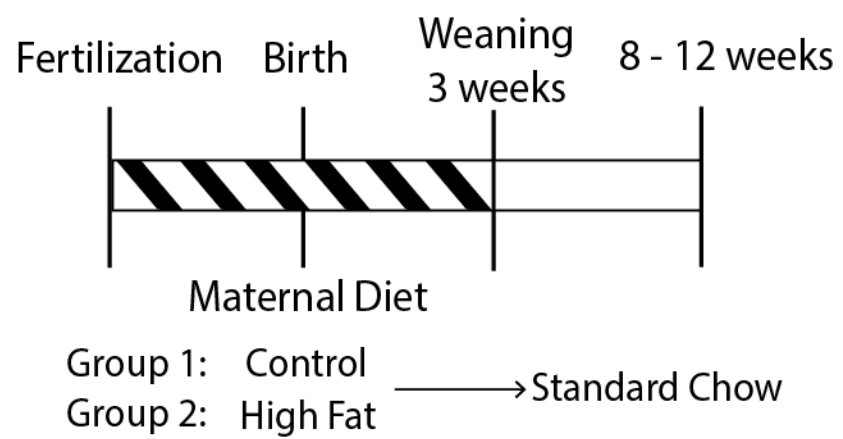


Figure 2.

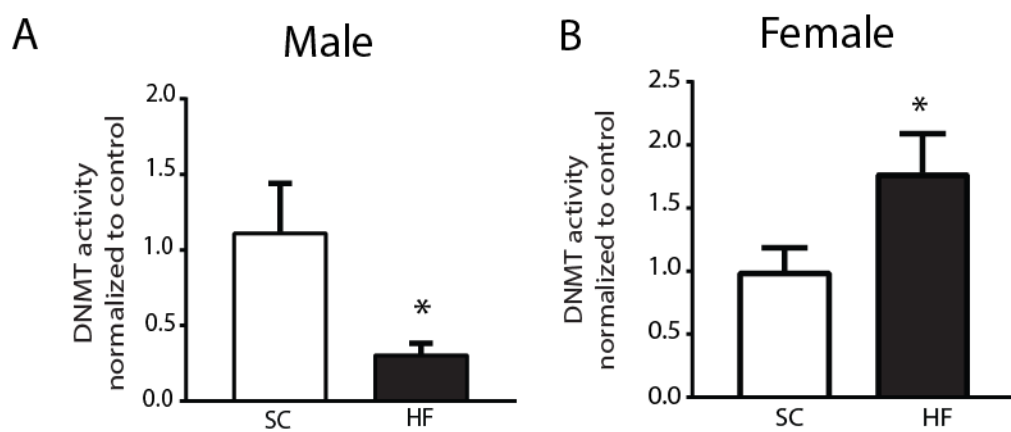


Figure 3.

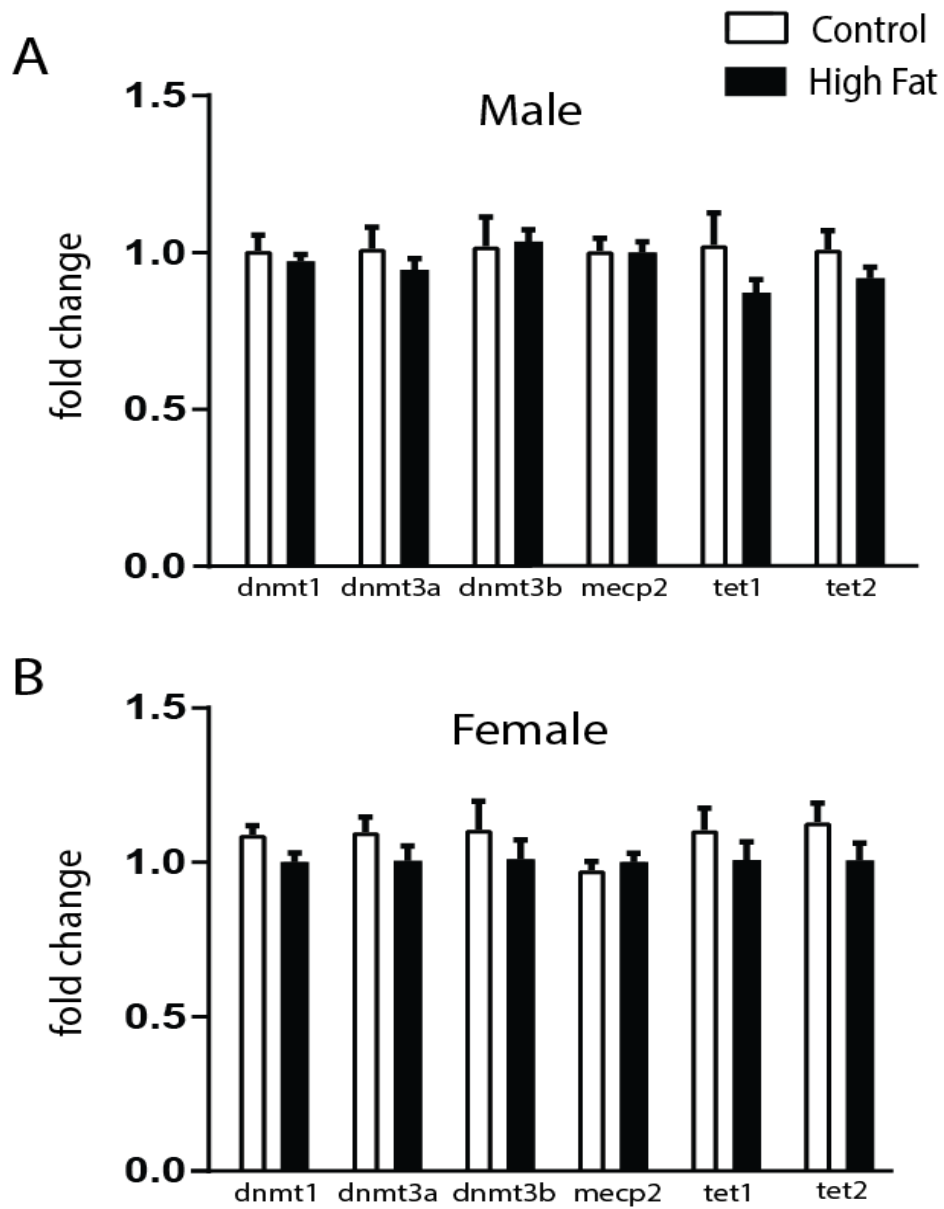


Figure 4.

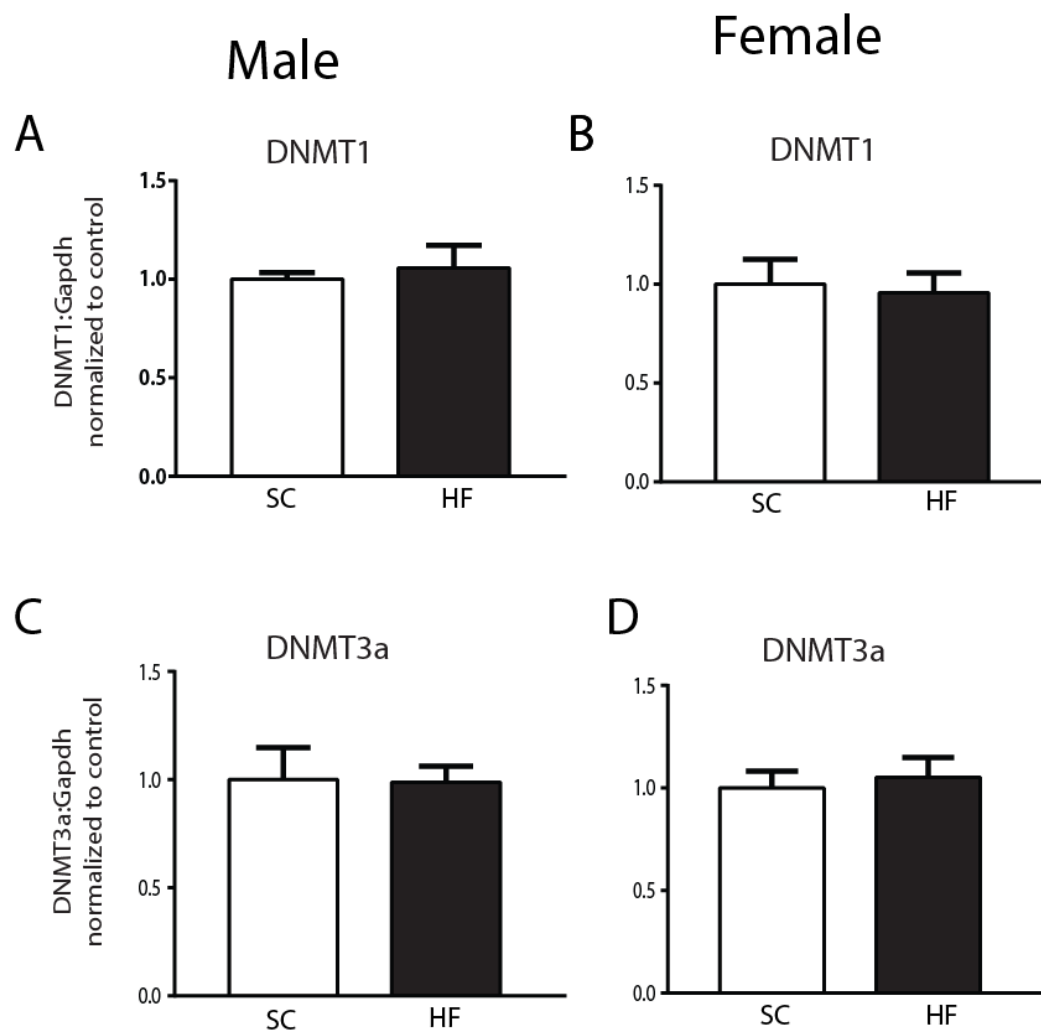


Figure 5.

Male

Cytoplasm

Nucleus

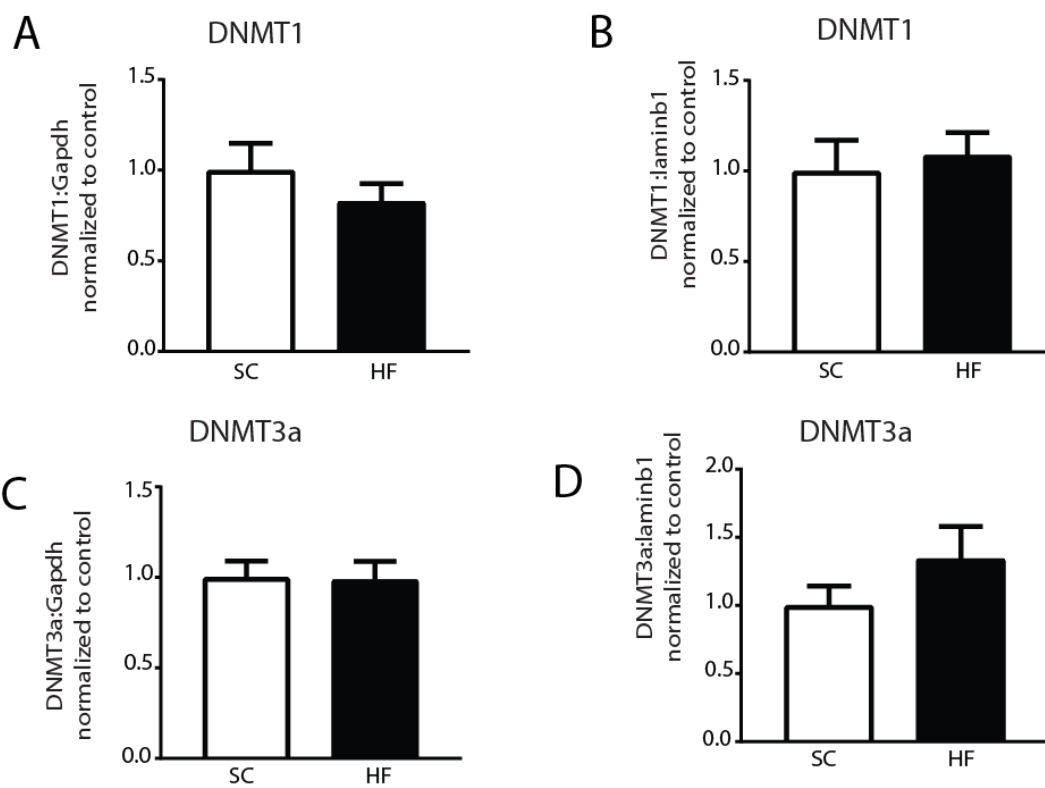


Figure 6.

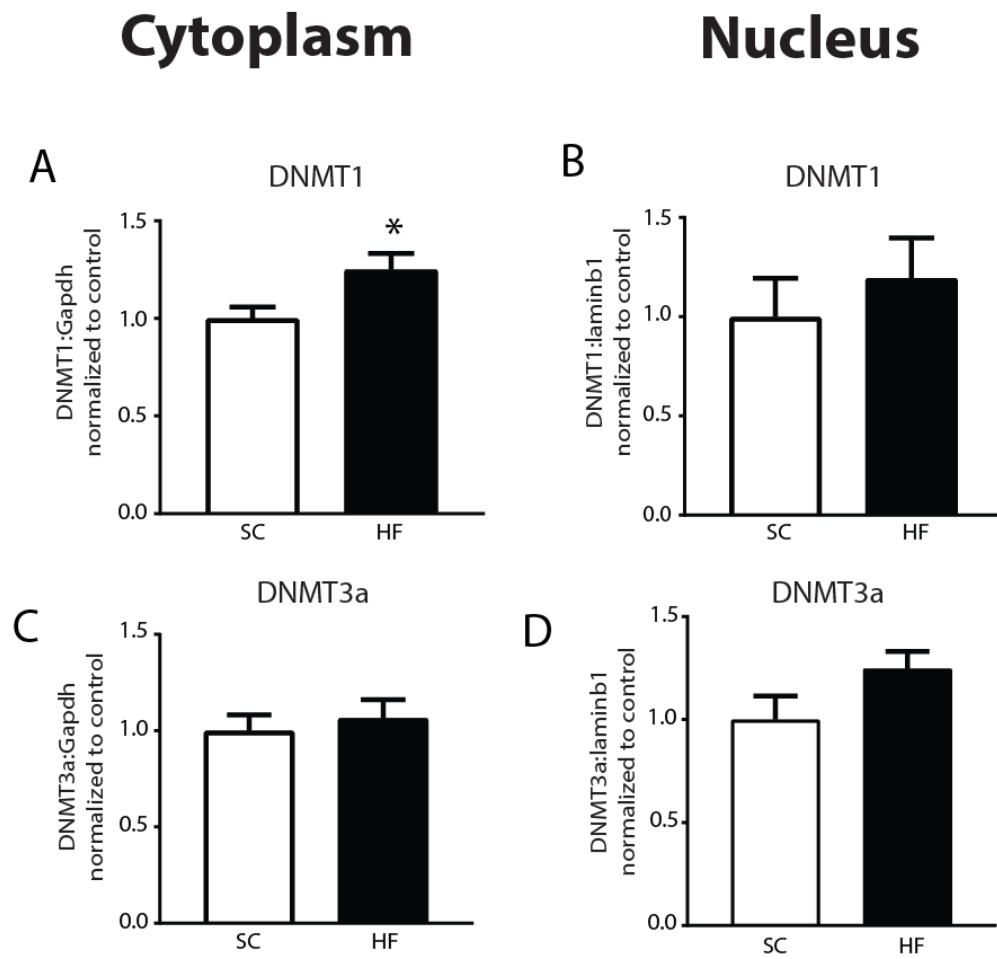


Figure 7.

Male

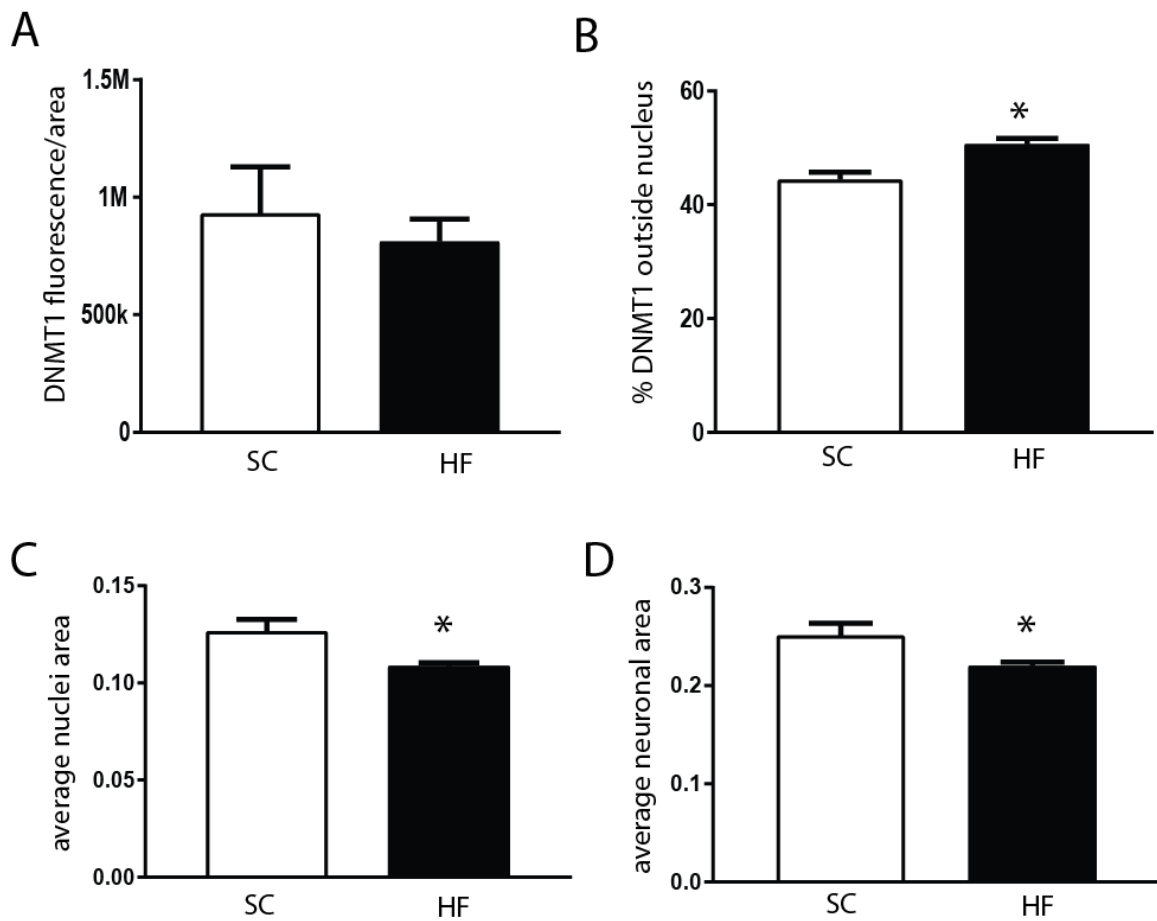
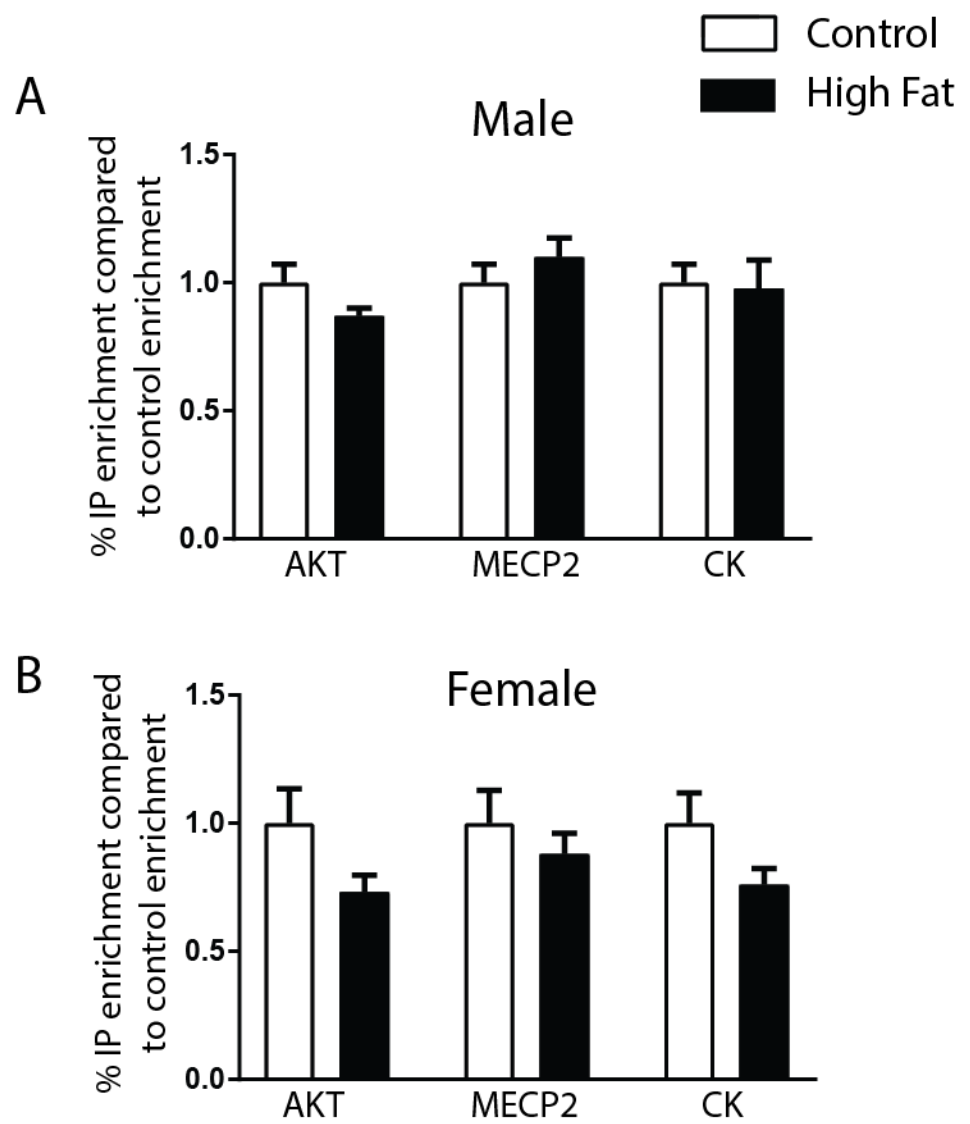


Figure 8.



Chapter Five

General Discussion

Obesity rates have continued to rise over the last 30-40 years, a trend that is expected to continue (Wang et al., 2008). Currently, two-thirds of the adult population is considered overweight or obese (Flegal et al., 2012; Ogden et al., 2015, 2014). The rise in obesity has coincided with advancement in food technologies increasing the variety of processed foods high in refined grains, fats, sugars and oils in our diet (Cordain et al., 2005). During pregnancy women are told to gain between 25-35 pounds to promote healthy fetal development. However, in over 50% of pregnancies weight gained exceeds the recommended weight gain (Institute of Medicine and National Research Council, 2009). The Developmental Origins of Health and Disease (DOHaD) hypothesis, created by David Barker about 25 years ago, led to the understanding that the gestational environment (i.e. gestational nutrition, weight gain, chemical exposure etc.) can shape offspring development and adult health (Barker et al., 1989; Dover, 2009). Excessive weight gain during pregnancy is correlated with increased birth weight of the offspring (Ludwig and Currie, 2010), also known as Large for Gestational Age (LGA). LGA offspring are at increased risk for developing many neurological disorders including autism, attention deficit/hyperactivity (ADHD), cognitive deficits, depression, schizophrenia and anxiety (Alfaradhi and Ozanne, 2011; Colman et al., 2012; Herva et al., 2008; Krakowiak et al., 2012; Robles et al., 2015; Van Lieshout et al., 2011). Elucidating the mechanistic link between excessive gestational weight gain (GWG) and offspring risk for neurological disorders is an important research question. However, investigation of possible interventions or therapies to reduce risk is also important.

Because of the increased consumption of processed energy-dense foods high in fats and sugars while being low in nutrients, the current population, including pregnant women, is both over-fed while being under-nourished (Cordain et al., 2005). Therefore, nutrient fortification of food has become necessary (Weaver et al., 2014). Currently, many dietary recommendations and

nutritional interventions are recommended to modify the gestational environment to prevent excessive GWG and offspring risk for disease. However, when women become pregnant, studies show that they will reduce smoking and alcohol consumption, but that they will not change their fruit and vegetable intake (Crozier et al., 2009a). Additionally, younger age at pregnancy and lower education levels are also predictors of non-compliance with health recommendations (Crozier et al., 2009b). Therefore, evidence shows that the addition of healthful behaviors during pregnancy is challenging.

Herein lies the motivation for this current research. We were interested in determining whether we could utilize a short-term *postnatal* nutritional intervention in LGA offspring to ameliorate adult disease risk. We wanted to take advantage of the fact that the brain, specifically the prefrontal cortex (PFC), continues to develop extensively after birth (Gogtay et al., 2004; Kolb et al., 2012). Utilizing a mouse model of excessive GWG, we discovered that methyl donor supplementation given between 3 and 6 weeks of age can alter learning and motivation into adulthood (Chapter 2). Specifically, methyl donor supplementation increased motivation and decrease response time in female control and HF offspring. For the first time, we characterized the concentrations of one carbon intermediates and expression of DNA methylation machinery genes within the PFC immediately after early life methyl donor supplementation (Chapter 3). We determined that methyl donor supplementation increases the concentration of folate intermediates, while perinatal HF increases the concentration of SAM, SAH and transsulfuration pathway intermediates. Gene expression of enzymes within the pathway were also changed in a sex dependent manner. Finally, it was determined that perinatal high fat (HF) diet can alter DNA methyltransferase (DNMT) activity, decreases in males and increases in females, without altering the gene expression or protein concentration present in the offspring PFC (Chapter 4). These changes in DNMT activity were also not due to changes in binding or association with kinases, CK and AKT, or binding protein, MECP2.

Diet composition

Because we were interested in manipulating the maternal and postnatal diet, it was important for us to use matched purified research diets. It is hypothesized that in about 75% of diet studies the composition differences between diets have been overlooked (Warden and Fisler, 2008). Matching dietary composition between experimental groups is important to consider when planning and executing diet studies. Throughout the majority of these studies we used purified diets that matched the levels of both macro- and micro- nutrients as much as possible between diets. Only in Chapter 4 did we use a standard vivarium chow diet, but that was only after weaning and all animals were fed the chow diet. A short correspondence piece, written by Warden and Fisler (Warden and Fisler, 2008), outlined a few of the ways purified diets are the gold standard for research diets. Unpurified standard vivarium chow is palatable to animals and inexpensive to produce. There are no regulations on chow manufacturers to report if there is a change in dietary source or composition. Additionally, phytoestrogen, a dietary compound derived from plants with similar structure to the primary female sex hormone, 17- β -oestradiol, content is known to be variable in chow diets (Warden and Fisler, 2008). It is important for phytoestrogen content to remain constant because it is known to influence cardiovascular disease risk, obesity, brain function and cancer risk (Rietjens et al., 2016). On the other hand, each manufactured lot of purified diet is tested for dietary composition. This allows for consistency throughout manufacturing and experimentation, reducing the number of unknown confounding variables introduced by diet. In addition, in order to increase the fat content of the experimental HF diet, there was a marked reduction in carbohydrate concentration. Therefore, the different experimental groups are exposed to different fat/carbohydrate and carbohydrate/protein ratios. Studies show that altering these ratios can alter cognition and differentially activate reward neurocircuitry (Fischer et al., 2002; Herrick et al., 2003; Hoch et al., 2015).

Recognition of the type of fat consumed is also important because it can influence cognition. The 60% HF diet is composed primarily of lard fat. The HF diet is higher in cholesterol (5x),

saturated (4x) and monounsaturated fatty acids (4x) than the control diet. Many studies indicate the specific type of fat to alter outcomes. It has been shown that higher intakes of saturated fat and trans-unsaturated fats, but not total fats consumed are associated with greater cognitive decline in older individuals (Morris et al., 2004). Higher intake of n-3 polyunsaturated fatty acids and docosahexaenoic acid is associated with reduced risk of Alzheimer disease, while intake of eicosapentaenoic acid is not (Morris et al., 2003). Trans-fatty acids are known to promote pro-inflammatory signaling (Hirata et al., 2017). Higher intake of saturated fatty acids are associated with cognitive impairment (i.e. impaired prospective memory, memory speed and flexibility and increased age related deficits) (Beilharz et al., 2015). While, increased intake of polyunsaturated fatty acids and high ratios of polyunsaturated to saturated fatty acids are associated with better memory function (Beilharz et al., 2015). A recent study identified a specific dietary cholesterol, 27-hydroxycholesterol, to mitigate the negative effects of cholesterol on memory impairment in mice (Heverin et al., 2015). Specifics of these studies aside, they show that understanding the specific fat composition of a studied diet could lead to very different behavioral and molecular results. Going forward it will be increasingly important to understand the nutrient composition of research diets to be able to identify the biochemical mechanisms that underlie the effects.

Single versus mixed nutrient supplementation

Chapters 2 and 3 investigated the long and short-term effects of early life methyl donor supplementation on the prefrontal cortex. Generally, we found that early life methyl donor supplementation could alter long-term cognition and motivation, long- and short-term gene expression, concentrations of one carbon metabolism intermediates and activity of DNA methyltransferases. The methyl donor supplementation cocktail, which included folic acid, methionine, vitamin B12, zinc, choline and betaine, was chosen because it has been used repeatedly throughout the literature and has been shown to induce physical appearance, behavior, molecular and epigenetic changes (Carlin et al., 2013; Paul Cordero et al., 2013a,

2013b; McKee et al., 2017; Waterland et al., 2008; Waterland and Jirtle, 2003). This supplement provides the nutrients at physiologically relevant concentrations, provides a cocktail of nutrients that work in concert with each other and is more consistent with what would be found in a natural food source.

In reviewing the current supplements used to enhance brain health, we found that many studies promote single nutrient supplementation while other tout a mixed cocktail approach. We believe that the mixed cocktail is the best approach because it is more relevant to what is found in naturally occurring sources. Additionally, there is the unique relationship between folate and vitamin B12 deficiencies, where one surplus may mask the other's deficiency (i.e. excess folate/folic acid may mask a vitamin B12 deficiency). Generally, cellular folate is present in low concentrations, therefore, regenerating THF from 5-methyl-THF is extremely important for one carbon metabolism and regulating cellular homocysteine levels. This regeneration can occur in two ways. One is dependent on the availability of vitamin B12 to act as a co-enzyme for methionine synthase. When vitamin B12 is not present, the cell has increased concentrations of both 5-methyl-THF, which has been shown to impair DNA and RNA synthesis (Herbert and Zalusky, 1962), and homocysteine, which has been observed in many neurological disorders (Bottiglieri, 2005; Ducker and Rabinowitz, 2016; Kennedy, 2016; Mattson and Shea, 2003). Folic acid supplementation has been shown to decrease levels of homocysteine, the biomarker routinely tested for proper cellular levels of vitamin B12 and folic acid. However, when low homocysteine levels are paired with low vitamin B12 levels, individuals present with pernicious anemia, macrocytosis and cognitive impairment (Morris et al., 2010, 2007; Selhub and Paul, 2011). Therefore, a cocktail approach to methyl donor supplementation may in part mitigate these masking effects.

Adverse effects of supplementation also need to be considered. We did find that methyl donor supplementation increased premature responses in our behavior training. Revealing that supplementation can have its downsides as well. 5% of adults and 30-66% of children have

higher plasma concentrations of methyl vitamins than recommended (Regan L Bailey et al., 2010; Regan L. Bailey et al., 2010). A human study showed that offspring from women who consume higher than recommended concentrations of folic acid during pregnancy have lower psychomotor scores at age 1 (Valera-Gran et al., 2014). In animal models, high methyl donor diets alter neural reward pathways, perturb cortical development, decrease hippocampal size, and impair short-term memory and motivation (Bahous et al., 2017; C E Cho et al., 2013; Clara E Cho et al., 2013; Pannia et al., 2014; Sittig et al., 2012). The Swedish physician Paracelsus, said, “The dose makes the poison”, which has become the most basic principle of toxicology. It is important to remember this principle when making dietary recommendations.

One carbon metabolism and the brain

Methyl donor nutrients are important to one carbon metabolism primarily because of their necessity as critical intermediates and cofactors in donation of methyl groups to methylation reactions. The liver is the main metabolism organ in the body, therefore, one carbon metabolism has generally been functionally characterized within the liver. It is thought by many that metabolism of these nutrients is completed by the liver and then shuttled through the blood to be distributed throughout the body, passing through the blood brain barrier to reach the brain. However, the presence of the metabolism enzymes within the brain tissue would suggest that local one carbon metabolism can also occur.

Even though liver one carbon metabolism is well studied and we have an understanding of how perturbations such as diet or disease alter this metabolism, brain one carbon metabolism has not been well characterized. Few functional differences have been found between brain and liver one carbon metabolism. As recently as 2006, there remained a controversy surrounding whether the brain contained a functional transsulfuration pathway, important for the elimination of reactive oxygen species (ROS) (Vitvitsky et al., 2006). Because the brain proportionally synthesizes the most oxygen of any organ, generating high ROS concentrations, effective

elimination of ROS is necessary. Both Alzheimer and Parkinson disease patients show possible impairments to the brain transsulfuration pathway (Vitvitsky et al., 2006). Levels of cystathionine are increased in the brain compared to other organs, while γ -cystathionase activity in the brain is 100-fold lower compared to the liver. We now understand that the brain contains a functional transsulfuration pathway, but these two functional differences provide evidence that this pathway may function differently than the liver.

Additionally, the brain lacks the enzyme BMHT, betaine-homocysteine methyltransferase (Bottiglieri, 2005). Therefore, the brain lacks the ability to utilize betaine to resynthesize methionine from homocysteine, a pathway present in the liver. This may indicate that the brain has increased reliance on the presence of vitamin B12 to aid in the maintenance of cellular methionine levels than the liver. There is evidence that the brain is extremely resilient when nutrient deficiencies are present. One study found that when feeding a folate-free diet to rats the total folate concentrations in the liver and kidney were decreased by 60%, but the brain maintained the same levels of folate throughout the study (Selhub, 1991). Additionally, a recent paper showed that when folate deficiency is induced, the brain, when compared to plasma, erythrocytes, liver, kidney, and spleen, can maintain folate metabolite levels better and the other organs or biomarkers (Kopp et al., 2017). These studies indicate a possible maintenance system or protective mechanism present in the brain, but not the other organs. However, there is also a study that showed decreased brain vitamin B12 when a dietary folate deficiency is present (Birn et al., 2003). In chapters 2 and 3, we learned that levels of one carbon intermediates can be changed by both maternal diet and offspring diet, and that they changes may in part alter the molecular and behavioral changes observed. These data indicate that the brain one carbon metabolism may be reactive to dietary consumption of methyl donor vitamins. Because we know that there are functional differences in the brain transsulfuration pathway, no conclusive evidence of how the brain reacts to changes to methyl donor supply, and no determination of whether brain nutrient concentration changes are mitigated through local or peripheral metabolism, it is important that characterization of local brain one carbon metabolism occur.

It is also well known that the brain is comprised of multiple cell types that help the brain to function. Evidence reveals that cell type could alter one carbon metabolism (Ducker and Rabinowitz, 2016). One could imagine that neurons, glia and astrocytes would utilize the same nutrients in different ways because of their functional differences. One possibility is the increased reliance on astrocytes and oligodendrocytes to mediate transsulfuration pathway reactions. The brain consumes a disproportionate amount of the body's oxygen and therefore must eliminate copious amounts of reactive oxygen species. Synthesis of glutathione is critical to this elimination. In turn glutathione synthesis is dependent on cysteine concentration. EAAT3 is the main transporter of extracellular cysteine. EAAT3 has been found in greater densities on astrocytes and oligodendrocytes (Bjørn-Yoshimoto and Underhill, 2016), possibly indicating increased participation of these two cell types in transsulfuration pathway-mediated reactive oxygen species elimination. Further characterization of the roles each cell type plays in brain one carbon metabolism is warranted.

Sex differences influence outcomes

The differences between male and female offspring were evident throughout these studies. In chapter 2, only in female offspring did early life methyl donor supplementation increase motivation in the progressive ratio task. In chapter 3, we saw that in male supplemented HF offspring the folate intermediate concentration increase was blunted when compared to supplemented controls. While both supplemented control and HF female offspring had similar increases. Finally, in chapter 4, we saw that perinatal high fat diet altered DNMT activity in opposite directions in male and female offspring. We also showed that there are sex difference in the prevalence of many neurological disorders, including Parkinson's, Alzheimer's, cognitive decline, autism spectrum disorders, mood and anxiety disorders, major depression, trauma-related disorders, depressive disorders, autoimmune disease affecting the nervous system, ADHD and neurodegenerative disorders (Zagni et al., 2016). The pervasiveness of sex

differences in the brain and neurological disorders warrants the topic of sex differences be addressed.

In mammals, the presence of SRY on the male Y chromosome drives the development of the testis. Gonadal sex hormones (androgens, estrogens and progestins) then drive the sexual differentiation of the tissues. During development, the male brain is exposed to a testosterone surge which gets converted to estradiol. At the same time, in females, the estradiol levels remain low (Lenz et al., 2012). Within the brain these sex hormones are known to influence neuroanatomy, neurochemistry and neuron structure (Zagni et al., 2016). One interesting difference is the growth speed of the cortex. During development it is known that cortical volume within male brains grows faster than within female brains (Raznahan et al., 2011). This growth difference is hypothesized to in part drive the predisposition in males for autism spectrum disorders (Raznahan et al., 2010). Our methyl donor supplementation period is given during this critical time period of differing growth speeds, therefore there may be a complex interaction between cortical growth speed and nutrient availability that in part drives the molecular and behavioral differences we observed. Additionally, during development, maternal care, estrogen receptor (ER) expression and neonatal hormone exposure all have been shown to correlate with sex-specific differences in DNA methylation which in turn can influence development and behavior (McCarthy et al., 2009).

These sex hormones can also exhibit effects during adulthood, when females are exposed to high levels of estradiol and progesterone while males are exposed to high testosterone levels. To add complexity, the female estrus cycle alters the levels of the female sex hormones which is known to change behavior (Conrad et al., 2004; Frye, 1995; Kolb et al., 2012; Korol et al., 2004; Stackman et al., 1997). In all of the current studies, we measured effects after 6 weeks of age, when the sex hormones are present and active within the mice and can create sex-specific behaviors. Steroid hormone receptors, which can be found in different densities throughout the brain, are known to act as transcription factors and influence epigenetic machinery (co-activators and co-repressors) (Lenz et al., 2012; McCarthy et al., 2009). Interestingly, there is even a sex-

specific difference in these co-activators expression (Auger et al., 2000). These epigenetic differences could in part explain the differences in expression and DNMT activity we observed. A limitation to this research is that we did not measure the level of sex hormone present in our animals throughout these studies. Nor did we measure the estrus cycle in the females. Both measures could have played a role in the sex differences seen throughout our studies. All animals were the same age throughout each study and measurement and tissue collection occurred at the same time each day to minimize the effect of circadian variation. In the future, it would be important to monitor both estrogen and testosterone levels throughout the study, specifically when taking specific measurements or collecting tissue. Also monitoring female estrus cycle would decrease variances and confounds that can occur throughout the cycle. This would allow us to collect samples at the same point in estrus for each animal. It would also inform us to whether the observed sex differences remain throughout the entire estrus cycle, or are stronger at one point versus another.

The relationship between sex and neurological disease predisposition and development is very complex. Overall, we observed interesting sex differences in DNMT activity, one carbon metabolism intermediate and enzyme expression levels and effects on motivation. These sex differences could in part mediate the sex differences seen in neurological disease prevalence. Further studies are needed to understand both the downstream consequences of these changes, but also the mechanisms that underlie their development.

Future Directions

The current studies made a crucial first step into identifying a postnatal time-period in which a nutritional intervention can alter brain development. Overall, it is most important to verify that the one carbon metabolism pathway functions similarly in the brain as it does in the liver, where it is currently characterized. Understanding the differences between how somatic dividing cells and non-dividing cells utilize nutrients in metabolic processes will provide insights as to why diet composition plays a role in brain development and health. This could be completed using isotope

tracing and mass spectrometry. Using labeled nutrients and tracing them throughout the body, and specifically the brain, will help us link mechanistically nutrition, one carbon metabolism efficiency and biological processes such as DNA methylation to disease outcomes. This would also allow us to begin to parse apart how the one carbon metabolism pathways in the different cell types within the brain work in response to changes in nutritional status. Determining which nutrients are important for each cell type would not only further our understanding of proper dietary recommendations for methyl donor vitamins, but it will help us determine the best balance for all nutrients for total brain health and possibly provide new insights for disease prevention. Once characterized different nutritional or disease challenges could be performed to then observe how the metabolism system responds.

The current studies measured the concentrations of metabolized intermediates within one carbon metabolism, but not nutrient concentration consumed or delivered to the fetus. Because mice self-regulate calorie consumption, increasing the fat content of the diet decreases the total volume of diet consumed by the mice. Therefore, it is hypothesized that the concentration of the methyl donors consumed is decreased in the HF dams. That in turn would mean less concentration available to the developing fetus. Decreased methyl donor availability in the fetus is one possible mechanism by which DNA hypomethylation in the brain could occur. It would be important to verify that the methyl donor nutrient concentrations (i.e. folate, vitamin B12, choline and betaine levels) are decreased in both dam and offspring tissue, the blood (dam) and brain (offspring) specifically. This could be accomplished using HPLC or mass spectrometry and would help to connect mechanistically to the decrease in nutrients and molecular endpoints.

Genetic interactions between diet and outcome is increasingly of interest. Although we did not study gene differences specifically in these studies, it is easy to understand how they could play a role in the outcome. Single polymorphisms (SNP) within different genes can alter the function. The most studied within one carbon metabolism are SNP in the MTHFR gene. MTHFR is the enzyme that catalyzes the reaction of 5,10-methylene-THF to 5-methyl-THF (the circulating and most stable form of folate). SNPs within MTHFR alter catalytic domain of the enzyme and

change the enzymatic activity. This leads to slower rates of folate conversion. Individuals with polymorphisms that reduce enzymatic activities must be cognizant of the sources of folate and folic acid consumed and avoid medications that adversely affect homocysteine levels. MTHFR is not the only gene within one carbon metabolism where genetic changes can alter protein abundance or enzymatic activity. Further research can be completed to determine how perinatal high fat diet impacts individuals with these genetic differences. Additionally, determining whether supplementation is helpful or harmful when different polymorphisms are present would lead to more individualized nutritional recommendations which is important for preventing and treating disease.

Further investigation into different critical periods is also important. In the current studies, 3-6 weeks was chosen because the PFC is largely still developing during this time. However, studies continue to report maturation of the brain well into the mid-20's in humans (Gogtay et al., 2004). Therefore, it would be interesting to determine if waiting longer to intervene would yield the same result. We began to answer this in Chapter 2 by the addition of a 7-day supplementation period later in life and we found the outcomes dependent on the diet exposure in early life. Simple manipulation of the supplementation period to 6-10 weeks or 16-20 weeks of age would reveal whether nutritional interventions can be used throughout life to improve brain health.

Finally, the next important steps would be to investigate specific questions that remain in the current studies. In chapter 2, we were unable to examine how early life methyl donor supplementation alters more difficult PFC-mediated behaviors in male offspring due to an insufficient number of males learning the behavior paradigms in the allotted time frame. Repeating the experiment to further understand how supplementation alters male offspring learning and motivation is critical. In chapter 3, determining how the enzymatic activities are altered for the enzymes within one carbon metabolism and methylation machinery because of perinatal high fat diet or methyl donor supplementation still need to be completed. In chapter 4, we were unable to determine specific mechanisms by which perinatal high fat diet alters DNMT activity. Further investigation of other post-translational modifications could elucidate these

mechanisms. Additionally, in chapter 4, determining whether all changes in DNMT activity are driven by one specific DNMT remains. Filling in these holes will help reveal more complete mechanism for each of these projects.

Conclusions

The mechanistic link between excessive gestational weight gain and offspring disease risk remains unclear. Whether it is the additional weight gain during gestation, a change in maternal metabolism, differences in fat consumption, possible nutrient deficiencies or a completely novel mechanism that induces the disease predisposition in LGA offspring. We have moved the field forward firstly, by adding to the knowledge that perinatal exposure to high fat diet can modify adult behavior, DNA methylation machinery and protein concentration of one carbon metabolism intermediates and methylation in both male and female offspring. Secondly, by characterizing a developmental window in which postnatal methyl donor supplementation may attenuate these changes. Continued experiments investigating this mechanistic link will reveal new therapeutic opportunities and increased understanding of how the brain functions.

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