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Perinatal high fat diet and early life methyl donor supplementation alter one carbon metabolism and DNA methylation in the brain

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Abstract

One carbon metabolism is regulated by the availability of nutrients known as methyl donors, and disruption of this pathway can affect multiple physiological systems. DNA methylation, critical for the regulation of gene expression, is linked to one carbon metabolism, and can be altered by perinatal diet. In the current study, dams (n=12/group) were fed HF or standard control (SC) diet through pregnancy and lactation, and male and female offspring were then fed either SC or methyl donor supplemented diet (MDS) between 3 and 6 weeks of age (n=20–26/group). Concentration of one carbon intermediates and other related metabolites were assessed within brain tissue (prefrontal cortex, PFC) through the use of mass spectrometry at 6 weeks of age. Additionally, expression of target genes and enzymes that participate in DNA methylation or are relevant to one carbon metabolism were measured. We found that MDS increases the concentration of folate intermediates in the PFC, and that this increase is blunted in male offspring from dams fed a HF diet. Additionally, perinatal HF diet increased the concentration of cysteine in the PFC of both male and female offspring, consistent with oxidative stress. Further, both maternal HF diet and postnatal MDS altered global DNA methylation in the PFC in males but not females. Collectively, these data demonstrate sex differences in changes in one carbon metabolites in the prefrontal cortex in response to early life high fat diet and methyl donor supplementation.

Graphical Abstract

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Disclosure

The authors have no financial interests or conflicts to disclose.

To determine how dietary changes affect 1-carbon metabolites in brain tissue, pregnant dams were fed high fat (HF) or standard control (SC) diet through pregnancy and lactation, and male and female offspring were then fed either SC or methyl donor supplemented diet (MDS) between 3 and 6 weeks of age. Concentration of one carbon intermediates and other related metabolites were altered by perinatal diet within the prefrontal cortex in a sex-dependent manner. This is a potential mechanism whereby early life dietary alterations affect DNA methylation within the brain, a process that is dependent on 1-carbon metabolism.

Keywords

High Fat Diet; Methyl Donors; One Carbon Metabolism; DNA methylation; Developmental programming

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 the 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) (Robles *et al.* 2015; Van Lieshout *et al.* 2011; Krakowiak *et al.* 2012; Rivera *et al.* 2015). Interestingly, consumption of excess calories during pregnancy is related to lower concentrations of many micronutrients in plasma including methyl donors (i.e. folate, vitamin B12, methionine, zinc, betaine and choline) (Sen *et al.* 2014; Kim *et al.* 2012), and when maternal micronutrient consumption is low, this deficiency is mirrored in the child (Allen 2005; Visentin *et al.* 2015; Plumptre *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 in adulthood, offspring from these dams have prefrontal cortex (PFC)-mediated cognitive and executive function deficits (16 weeks of age) (Grissom *et al.* 2015), as well as altered sucrose and fat preference, RNA gene expression and DNA hypomethylation in reward-related circuitry, including the PFC (18–24 weeks of age) (Vucetic *et al.* 2010). Furthermore, pre- and post-natal dietary methyl donor supplementation (MDS) can normalize some of 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.

One carbon metabolism is at the intersection of folate metabolism and methionine metabolism (Figure 1), and provides building blocks for purine and pyrimidine synthesis, methyl groups for methylation reactions, precursors for phospholipid synthesis and substrates for reactive oxygen species removal (Fox and Stover 2008; Anderson *et al.* 2012). Methyl donor nutrients are crucial to one carbon metabolism, as they act as both intermediates and cofactors within the metabolism pathway. Multiple methyl donor nutrients and cofactors (highlighted in gray, Figure 1) are essential nutrients provided solely by the diet. Studies have shown that deficiency, imbalance or excess of one or more of these nutrients throughout various periods of life can alter development, cognition and disease risk (Pannia *et al.* 2016; Veena *et al.* 2016; Morris 2012; Reynolds 2006; O'Neill *et al.* 2014). We previously reported that MDS given concurrently with a 60% HF diet during pregnancy and lactation normalizes palatable food preference, increases locomotor activity, and normalizes gene expression and both gene specific and global DNA hypomethylation in male and female offspring (Carlin *et al.* 2013). Changes in DNA methylation can alter gene expression, and represents an important mechanism through which an organism responds to environmental challenges. Others have found that maternal MDS 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, MDS has been shown to alter cognition and motivation (McKee *et al.* 2017), reduce fatty liver and alter DNA methylation within the liver (Dahlhoff *et al.* 2014; Cordero *et al.* 2013a; Cordero *et al.* 2013b). However, it remains unknown whether MDS 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. Given our previous findings that early life MDS can alter long term behavior driven by the PFC and RNA gene expression in the PFC (McKee *et al.* 2017), we were interested in understanding the immediate changes that occur in brain after supplementation. Additionally, because sex differences in the prevalence rates of many neurological disorders are well documented, one primary goal of the present work 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 brain tissue as previous research has been limited to measurements in cerebrospinal fluid and plasma. Therefore, to determine whether maternal HF diet and postnatal early life MDS alter one carbon metabolism directly in the brain, we analyzed the concentration of one carbon metabolism intermediates, RNA expression of genes involved in one carbon metabolism and DNA methylation in the PFC of the offspring. All experiments were performed when animals were 6 weeks of age at the conclusion of a 3 week supplementation with MDS.

Methods

Animals and experimental model:

All animals were cared for according to the guidelines of the University of Cincinnati Institutional Animal Care and Use Committee (#15-09-02-01). An experimental timeline is seen in Figure 2. C57BL/6J (RRID:IMSR_JAX:000664) virgin females were bred with DBA/2J (RRID:IMSR_JAX:000671) males (Jackson Laboratories, Bar Harbor, ME). F1 hybrid mice are used, as a 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) (Vucetic *et al.* 2010; Grissom and Reyes 2013). Specific diet formulations are shown in Table 1. Litter size was normalized 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 arbitrarily assigned to one of two diets; (1) control or (2) control diet plus methyl donor supplementation (MDS) (Test Diet, Richmond, IN), and were group housed with 4 animals per cage (McKee *et al.* 2017). After weaning, dams and offspring not included in the study were returned to the colony. The MDS supplementation involved addition of the following nutrients per kg/diet; 15g choline chloride, 15g betaine, 15g folic acid, 1.5 mg vitamin B12, 7.5g L-methionine, and 150 mg zinc. At 6 weeks, all offspring were sacrificed for experimentation (rapid decapitation using DecapiCones (BrainTree Scientific)). Animals were fed *ad libitum* throughout the entire study period. The HF offspring had increased body weight at weaning (3 weeks), and at 6 weeks of age, consistent with our previous reports (Vucetic *et al.* 2010; Grissom and Reyes 2013) (Supplemental Fig 1).

Metabolite Analysis:

At sacrifice, animals underwent rapid decapitation using DecapiCones (BrainTree Scientific), the brain was removed and the prefrontal cortex was immediately dissected from a 2mm coronal slice from bregma +2.3 to 0.3 and flash frozen on dry ice (~3 minutes after sacrifice). Frozen tissue was ground into a powder (Retsch Cryomill). Folate metabolites were extracted and analyzed by LC-MS as previously described (Ducker *et al.* 2016). Measured folate metabolites included tetrahydrofolate (THF) (which is in abiotic exchange with methylene-tetrahydrofolate), 5-formyl-tetrahydrofolate (5-formyl-THF), methenyl-tetrahydrofolate (methenyl-THF) (which is in abiotic exchange with 10-formyl-tetrahydrofolate) and 5-methyl-tetrahydrofolate (5-methyl-THF). Methionine cycle metabolites were extracted and measured as previously described (Shlomi *et al.* 2014). Metabolites measured included cystathionine, cysteine, S-adenosyl-homocysteine (SAH), and S-adenosyl-methionine (SAM). All metabolites are reported in ion counts and are compared to male controls. n=5/group.

Gene expression in Prefrontal Cortex:

Whole brain was collected and 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). 220µg/10µl 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. 2µl of the pooled assay mix was combined with 2µl cDNA and 4µl 2X Taqman Preamp Master Mix (Life technologies). The preamplification reaction was cycled following the manufacturer's protocol in a 7500 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 HF 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 HF+MDS 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 manufacturer's 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-mC% or 5-hmC% = ((Sample OD/Negative Control OD)/(Slope of Standard Curve x 100))*100.

Data analysis:

Two and three-way ANOVA (maternal diet x supplementation; maternal diet x supplementation x sex) were used to evaluate results and normality was confirmed using D'Agostino & Pearson normality test using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). Minimum group numbers were based on historical data (Vucetic *et al.* 2010; Grissom *et al.* 2015; McKee *et al.* 2017; Carlin *et al.* 2013). 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<0.05 was considered significant. Experimental personnel were not blinded to the experimental conditions. The study was not pre-registered.

Results

To determine whether perinatal HF diet and early life supplementation of methyl donors alter global DNA methylation and brain specific one carbon metabolism, we supplemented offspring from both maternal SC and HF diets with dietary methyl donors from 3–6 weeks of age and compared them to SC fed offspring (Figure 2; dams: n=12/group). At six weeks of age, male offspring fed a maternal diet of 60% fat had decreased global DNA methylation within the PFC (Figure 3A). Early life methyl donor supplementation reversed this decrease (interaction of maternal diet and MDS: F(1,39)=9.19, p=0.004; SC vs HF: $t_{39}=2.69$, p=0.021; HF vs HF+MDS: $t_{39}=4.25$, p<0.001). No changes in global DNA methylation were

seen in the females (Figure 3B). Global hydroxymethylation was not changed in either sex (Supplemental Figure 1)).

Because DNA methylation is driven in part by the availability of methyl groups provided by one carbon metabolism, we examined how metabolites and enzymes within one carbon metabolism were altered due to perinatal HF and short-term dietary MDS (Figure 4A). We determined that MDS increased the expression, only in male offspring, of three enzymes, the dehydrogenase MTHFD1 (main effect of MDS: $F(1,40)=5.4$, $p=0.025$) and the reductases MTHFR (main effect of MDS: $F(1,40)=6.93$, $p=0.012$) and MTRR (main effect of MDS: $F(1,40)=5.09$, $p=0.003$), by about 6, 9 and 7% each, respectively (Figure 4B, Table 3). DHFR and MTR, were not changed by either dietary manipulation, and no gene expression changes of these enzymes were observed in female offspring. Using mass spectrometry, we found that MDS increased the concentration of THF, 5-formyl-THF, methenyl-THF and 5-methyl-THF in both male and female offspring (Figure 4C-F)(THF: main effect of MDS: $F(1,1)=22.17$, $p<0.001$; 5-formyl-THF: main effect of MDS: $F(1,1)=24.39$, $p<0.001$; Male – main effect of maternal diet: $F(1,16)=9.82$, $p=0.006$; SC+MDS vs HF+MDS: $t_{16}=3.62$, $p=0.005$; methenyl-THF: main effect of MDS: $F(1,1)=34.95$, $p<0.001$; 5-methyl-THF: main effect of MDS: $F(1,1)=20.08$, $p<0.001$). Interestingly, in 5-formyl-THF, MDS-driven increases were attenuated in the HF male offspring (a similar trend was observed for methenyl-THF ($p=0.053$)). In male offspring, MDS increased free methionine levels in the PFC, while perinatal HF exposure attenuated this increase (Figure 4C) (interaction of maternal diet and MDS: $F(1,16)=4.58$, $p=0.048$; $p=0.002$; SC+MDS vs HF+MDS: $t_{16}=2.63$, $p=0.037$). Interestingly, even though methionine is one of the supplemented nutrients, levels of methionine remained unchanged in the female offspring PFC in any diet group. Further, 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 reaction with ATP to form S-adenosyl methionine (SAM). A variety of methyl transferases then utilize the methyl group from SAM, generating S-adenosyl-homocysteine (SAH) as the byproduct (Figure 5A). We found that in males, expression of two genes within this portion of the pathway were increased due to MDS (DNMT3a, 6% and SETD7, 11%), while in females these genes remained unchanged (Figure 5B, Table 3). In females, PNMT expression was decreased by 25% due to MDS, but was unchanged in males. Interestingly, in male offspring, perinatal HF diet decreased the expression of PEMT (5%), but the expression was unchanged in female offspring. Perinatal HF diet increased the concentration of SAM in both sexes (Figure 5C) (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 5D) (main effect of maternal diet: $F(1)=4.18$, $p=0.049$). Analysis of the SAM/SAH ratio revealed a significant 3-way ANOVA ($p=0.013$, $F(1,32)=6.91$, Fig 5E), with no difference between groups in the male offspring. However, in female offspring, HF offspring had an increase in SAM/SAH (main effect of maternal diet: $p=0.038$, $F(1,16)=5.137$; posthoc: SC vs HF: $p=0.046$, $t=2.507$, $df=16$), a difference that was attenuated in supplemented offspring.

SAH is cleared by hydrolysis into homocysteine. Homocysteine can then be either re-methylated to methionine or shuttled through the transsulfuration pathway to generate cystathionine and ultimately cysteine, which is required for glutathione synthesis and thus antioxidant defense (Figure 6A). In males, MDS increased the expression of SAHH (8%) and CBS (7%), but maternal HF diet decreased the expression of CTH (8%) (Figure 6B, Table 3). In females, maternal HF diet decreased expression of EAAT3 (4%). We found that maternal HF diet increased the concentration of cysteine in the PFC (Figure 6C) (main effect of maternal diet: $F(1,1)=9.05$, $p=0.005$), while cystathionine remained unchanged (Supplemental Figure 2), in both sexes.

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 (Vucetic *et al.* 2010; Grissom *et al.* 2015), which can be in part normalized by maternal and early life MDS (Carlin *et al.* 2013; McKee *et al.* 2017). To determine whether diet can 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. Prior to this study, 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 have completed an analysis of the one carbon metabolism cycle from brain tissue from a specific region, measuring intermediates using mass spectrometry, and enzyme expression using RT-qPCR. Additionally, prior studies were completed only in male offspring, and our studies included both male and female offspring to directly examine possible sex differences. Overall, we see a distinct difference between how folate metabolism intermediates and how methionine metabolism intermediates (SAM, SAH and cysteine) respond to maternal HF diet and postnatal MDS within the PFC. Generally, folates and methionine concentration were increased due to MDS, however, male HF offspring were partially resistant to these increases. SAM, SAH and cysteine were unaffected by MDS, but were increased in response maternal HF diet.

We have previously reported global DNA hypomethylation at 52 weeks of age in HF diet offspring. We wanted to determine whether this pattern could be seen as early as 6 weeks of age. At 6 weeks of age, we observed global DNA hypomethylation in male offspring, but not female, in the PFC, and this male-specific DNA hypomethylation was normalized by the postnatal MDS. As organisms age, a progressive pattern of hypomethylation is observed in many tissues, including the brain (Christensen *et al.* 2009). However, even within the context of global DNA hypomethylation, select loci can still exhibit hypermethylation, highlighting a complex relationship between levels of DNA methylation and expression of any one target gene of interest. Additionally, in the human PFC, it has been shown that about 86% of CpG loci show a sexually dimorphic pattern of DNA methylation, with 73% of these loci showing higher methylation in females (Numata *et al.* 2012). It is possible that the HF diet induced

hypomethylation occurs more readily in male offspring than female, and examination at a later time-point may reveal a similar response in the female HF offspring.

Dietary consumption of methyl vitamins is known to increase plasma concentrations of one carbon metabolites (Gargari *et al.* 2011; Veenema *et al.* 2008; Jacques *et al.* 1999), and here we demonstrate that adolescent MDS increased folates (THF, 5-formyl-THF, methenyl-THF and 5-methyl-THF) and methionine concentrations directly in the PFC. Interestingly, these MDS-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 methenyl-THF ($p=0.053$). The collective consequences of this blunted response in males is unknown. It is also worth noting that this blunted response was observed even in the presence of high, likely saturating, levels of folic acid in the supplementation, suggesting that the reduced levels of 5-formyl-THF in the HF+MDS offspring were not a result of inadequate folic acid supplementation, but rather something fundamentally altered in the metabolic pathway. For example, 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. In fact, MDS increased the expression of a number of enzymes important for one carbon metabolism and methylation in male offspring, including MTHD1, MTHFR, MTRR, DNMT3a, SETD7, SAHH and CBS. Therefore, this could indicate that the presence of higher methyl donor concentrations in the brain in turn upregulates expression of enzymes involved in their metabolism. 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 regard to changes in the one carbon metabolism pathway in the PFC. It should be noted that the fold change increase in the enzyme transcripts was approximately 5–10%. While statistically significant, the biological relevance of these small fold changes in gene expression is not immediately clear, however, these data were obtained in a heterogeneous cell population (containing neurons, astrocytes, and glia). Further, it is possible that while the expression changes were small, changes in activity of the enzymes could have a significant effect on one carbon metabolism. Future studies designed to examine enzymatic activity would be important in this context. Determining whether these changes are biologically important or driven by one specific cell type will be important for future work.

At the point where folate and methionine cycles meet, methionine concentrations were not increased in the MDS females as was observed in MDS males. Because the precursors were all increased in a similar way it was 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, although 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 MDS, 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 MDS specifically in females to shuttle methionine through the folate cycle. Identifying the relationship between MDS and enzymatic activity may help shed light onto this sex difference.

Unlike the folate intermediates, MDS surprisingly did not alter the concentrations of SAM, SAH or cysteine. 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 HF diet leads to global and gene promoter-specific DNA hypomethylation in the brain, specifically within the PFC (Carlin *et al.* 2013; Vucetic *et al.* 2010). The hypomethylation in the male offspring (and lack of hypomethylation in the female offspring) observed here does not seem to be driven by the relative changes in SAM and SAH concentrations, or by the methylation potential (SAM:SAH ratio). This suggests that other factors, such as the activity of DNA methyltransferases, are more likely to explain changes in global methylation in this experimental context.

SAH is next hydroxylized to homocysteine, which can either be re-cycled through methionine metabolism or shuttled toward the transsulfuration pathway. We observed an increased concentration of cysteine within the PFC of both sexes due to perinatal HF diet, indicative of increased flux through the transsulfuration pathway. Transsulfuration flux has been reported to increase in response to oxidative stress (Vitvitsky *et al.* 2006), perhaps to meet the need for cysteine to support GSH synthesis and defense against reactive oxygen species (ROS). ROS are chemically reactive moieties that contain oxygen and are known to oxidize proteins, lipids and DNA. HF diet has been reported to increase oxidative stress in the brain. Various neurological diseases (Alzheimer's and Parkinson's) are also associated with increased oxidative stress (Mattson and Shea 2003; Clarke *et al.* 1998). The increased concentration of cysteine in both sexes due to maternal HF diet are consistent with increased oxidative stress and homocysteine concentrations noted in various neurological disorders (Kruman II *et al.* 2002; Kruman *et al.* 2000; Mattson and Shea 2003).

Further, 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. We saw that maternal HF diet altered the expression level of EAAT3, excitatory amino acid transporter 3, specifically in the females. Much 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 this would be an important 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, neuroinflammatory signals, neonatal hormone exposure and cellular differences in genetic sex (sex chromosomes acting within cells to drive sex differences) have all been shown to associate with sex-specific differences in DNA methylation (McCarthy *et al.* 2009; McCarthy and Arnold 2011), and may contribute to the observed differences in one carbon metabolism as well. The sex

differences seen here in DNA methylation, HF diet-induced folate and methionine concentration blunting and various differences in RNA gene expression may contribute to the observed sex differences in prevalence of many neurological disorders including autism, ADHD, schizophrenia and mood/anxiety disorders.

The present data provide initial tissue level resolution of the effects of maternal HF diet and early life MDS 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. The supplementation approach used in the present experiments involved multiple methyl donors, rather than a single nutrient, as methyl donors are known to work in concert and because these nutrients tend to co-exist in natural food sources. Understanding the relative impact of any one methyl donor in isolation would be another area of future research. When looking in a heterogeneous cell population within the PFC, we see that early life MDS increases folate concentrations and that maternal HF exposure increases SAM, SAH and cysteine. In males, MDS increased expression of enzymes important to one carbon metabolism and DNA methylation. These findings indicate that further investigation of local brain specific one carbon metabolism is warranted and disruption of this metabolism could contribute to the link between maternal malnutrition and offspring neurological disease risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations:

SC	Standard control diet
HF	High fat
MDS	Methyl donor and cofactor supplementation
PFC	Prefrontal cortex
DNMT	DNA methyltransferase
RRID	Research Resource Identifier
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
THF	Tetrahydrofolate

THF	5-formyl-tetrahydrofolate, 5-formyl
THF	methenyl-tetrahydrofolate, methenyl
THF	5-methyl-tetrahydrofolate, 5-methyl

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Figure 1.

One carbon metabolism. Simplified schematic of the intermediates, enzymes and nutrients involved in one carbon metabolism. Essential nutrients are highlighted in gray. 5-formyl-THF, 5-formyl-tetrahydrofolate; 5-methyl-THF, 5-methyl-tetrahydrofolate; 10-formyl-THF, 10-formyl-tetrahydrofolate; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine-beta-synthase; CH₃, methyl; CTH, cystathionine gamma-lyase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, N,N-dimethylglycine; DNMT, DNA methyltransferase; MAT1, methionine adenosyltransferase 1; methenyl-THF, methenyl-tetrahydrofolate; methylene-THF, methylene-tetrahydrofolate; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methylenetetrahydrofolate-homocysteine methyltransferase reductase; PEMT, phosphatidylethanolamine N-methyltransferase; SAH, S-adenosylhomocysteine; SAHH, adenosylhomocysteinase; SAM, S-adenosylmethionine, THF, tetrahydrofolate

Figure 2.

Experimental design. Mouse dams were fed either a 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 (MDS) diets, creating 4 experimental groups (control, SC; high fat, HF, methyl supplemented control, SC+MDS; methyl supplemented high fat, HF+MDS). All animals were killed for tissue collection at 6 weeks of age.

Figure 3.

Maternal and postnatal diet alter global DNA methylation in the male PFC. (a) Male maternal HF diet offspring have global hypomethylation in the PFC. Three week supplementation with methyl donor diet reversed his hypomethylation to the level of control offspring. (b) Neither maternal HF diet nor postnatal MDS altered global DNA methylation in female offspring. * $p < 0.05$ SC vs HF, ^ $p < 0.05$ HF vs HF+MDS. N (number of mice): SC=10, HF=11, SC+MDS=10, HF+MDS=12 in (a), n: SC=10, HF=10, SC+MDS=10, HF+MDS=10 in (b)

Figure 4.

Methyl donor supplementation increases folate metabolite concentrations in the PFC. (a) diagram of folate metabolism. **Bolded** intermediates were measured by mass spectrometry and *italicized* genes measured by qPCR. (b) MDS diet increases expression of multiple folate metabolism enzymes in male offspring. (c-g) MDS increased concentrations of folate metabolism intermediates in the PFC (THF (c), 5-formyl-THF (d), methenyl-THF (e) and 5-methyl-THF (f)). (g) Free methionine concentration is increased in MDS supplemented male, but not female PFC. * $p < 0.05$ main effect of maternal diet, ^ $p < 0.05$ main effect of MDS, \$ $p < 0.05$ main effect of sex, @ $p < 0.05$ SC+MDS vs HF+MDS; male n: SC=10, HF=12, SC+MDS=10, HF+MDS=12, female n: SC=10, HF=10, SC+MDS=10, HF+MDS=9 in (b), (c-g) both sexes N (number of mice): SC=5, HF=5, SC+MDS=5, HF+MDS=5. SC=black, HF=white, SC+MDS= dark gray, HF+MDS=light gray.

Figure 5.

Maternal HF diet increased 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-e) Maternal HF diet increased the concentrations of SAM and SAH in the PFC, while SAM:SAH ratio was increased only in female offspring. * $p < 0.05$ SC vs SC+MDS, ^ $p < 0.05$ main effect of MDS, # $p < 0.05$ HF vs. HF+MDS, % $p < 0.05$ main effect of maternal diet, \$ $p < 0.05$ main effect of sex, @ $p < 0.05$ SC+MDS vs HF+MDS; male n: SC=10, HF=12, SC+MDS=10, HF+MDS=12, female N (number of mice): SC=10, HF=10, SC+MDS=10, HF+MDS=9 in (b), (c-e) both sexes n: SC=5, HF=5, SC+MDS=5, HF+MDS=5. SC=black, HF=white, SC+MDS= dark gray, HF+MDS=light gray.

Figure 6.

Maternal HF diet increased 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 MDS. (c) Maternal HF diet increased the concentration of cysteine. * $p < 0.05$ SC vs SC+MDS; male N (number of mice): SC=10, HF=12, SC+MDS=10, HF+MDS=12, female n: SC=10, HF=10, SC+MDS=10, HF+MDS=9 in (b), (c) both sexes n: SC=5, HF=5, SC+MDS=5, HF+MDS=5. SC=black, HF=white, SC+MDS=dark gray, HF+MDS=light gray.

Table 1.

Specific diet information

	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%	51.7%
Protein	18.3%	18.6%	19.7%
Energy (kcal/gram)	4.07	5.21	3.87
<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.

Details of the statistical results presented in the figures.

Prefrontal cortex	3-way	Male 2-way	Female 2-way
THF	MDS: $p<0.001$ $F(1,1)=22.17$		
5-formyl-THF	MDS: $p<0.001$ $F(1,1)=24.39$	Maternal: $p=0.006$ $F(1,16)=9.82$	
methenyl-THF	MDS: $p<0.001$ $F(1,1)=34.95$		
5-methyl-THF	MDS: $p<0.001$ $F(1,1)=20.08$		
Methionine	Sex: $p=0.006$ $F(1,1)=8.7$	Interaction: $p=0.048$ $F(1,16)=4.583$	
	MDS: $p=0.002$ $F(1,1)=11.92$		
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 for gene expression results

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

Table 4.

Gene Primer 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	methyltetrahydrofolate 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