MATERNAL THIRD-TRIMESTER CHOLINE SUPPLEMENTATION, FETAL
NR3C1 METHYLATION, AND BEHAVIOR PROBLEMS AT 7 YEARS OF AGE

A Thesis
Presented to the Faculty of the Graduate School
of Cornell University
In Partial Fulfillment of the Requirements for the Degree of
Master of Science

by
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ABSTRACT

Less than 10% of pregnant women consume the AI of choline, a nutrient important for development. In animals, maternal choline supplementation (MCS) improves offspring behavior, but few studies have tested this in humans. In the present RCT, MCS caused higher placental and lower cord NR3C1 methylation, which is opposite the pattern associated with early adversity and offspring behavior problems in prior studies. We tested the independent associations between MCS and NR3C1 methylation and child behavior (BASC-III; n=21) at age 7, using adjusted linear regression. Mothers consumed 480 or 930 mg choline/d during the 3rd trimester. Cord blood and placentas were collected at delivery for methylation analysis, which focused on average and site-specific NR3C1 methylation. The 930 mg group reported fewer emotional self-control problems. Cord CpG 30-32 methylation was associated with fewer internalizing and anxiety problems. Results suggest that choline-induced changes in NR3C1 methylation may have functional consequences for child behavior.
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POSITION TITLE: Master’s Student in Human Nutrition

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)

A. Personal Statement

My long-term research interest has been in the nutritional mechanisms (gene-nutrient interactions, energy availability) underlying the development and maintenance of healthy hypothalamic-pituitary-adrenal (HPA) axis functioning. My academic training and prior research experience have given me background in several key disciplines required for the success of this project: nutrition, biochemistry, molecular biology, and epigenetics. As an undergraduate at the College of Saint Benedict (CSB), I designed and conducted a human research study to examine the prevalence and predictors of hypothalamic amenorrhea in female collegiate cross country runners. This study was conducted under the direction of Dr. Amy Olson, a professor in the Nutritional Sciences department. Through this experience, I gained experience working with human subjects and gained familiarity in the topic of HPA-axis functioning. The
following summer, I worked as a researcher in the Chemistry department, under the
direction of Dr. Rachel Hutcheson. The goal of our lab was to identify novel S-
adenosyl methionine (SAM) enzymes within the BL21(DE3) E. Coli genome. During
this time, I obtained specialized training in a broad range of biochemistry and
molecular biology based approaches such as primer design, quantitative PCR, and
DNA isolation. This project also sparked my interested in SAM-mediated DNA
methylation pathways. Since beginning my graduate studies at Cornell, I have built
upon this background by obtaining training in experimental approaches relevant to my
thesis topic, including: child cognitive testing, salivary cortisol ELISA, and DNA
methylation analysis (global and site-specific). My advisor, Dr. Barbara Strupp is an
internationally-recognized researcher with experience studying the effects of various
exposures (e.g., nutritional and toxicological) on human cognitive dysfunction. My
epidemiology minor advisor, Dr. Pat Cassano is the Chair of Epidemiology at Cornell
and has extensive experience in genetic epidemiology. My thesis work and my
committee members have collectively allowed me to achieve my goal of studying the
roles of nutrition in the development of HPA-axis functioning.

B. Positions and Honors

Positions and Employment

2013-2014  Undergraduate Teaching Assistant, Nutrition Department, College of
Saint Benedict, St. Joseph, MN
2013-2014  Research Assistant and Project Coordinator, Amy Olson Lab,
College of Saint Benedict, St.
Joseph, MN
2014 summer  Undergraduate Research Assistant, Rachel Hutcheson Lab,
Chemistry Department, College
of Saint Benedict, Saint Joseph, MN
2015-present Graduate Teaching Assistant, Division of Nutritional Sciences,
Cornell University, Ithaca, NY
2015-present Graduate Student and Research Assistant, Barbara Strupp Lab,
Division of Nutritional
Sciences, Cornell University, Ithaca, NY

Professional Memberships

2016-present Member, American Academy for the Advancement of Science
(AAAS)

Honors and Awards

2011 Recognition Scholarship, College of Saint Benedict, St. Joseph, MN
2011-2014 Trustee’s Award, College of Saint Benedict, St. Joseph, MN
2011-2014 Academic All-Regional Cross-Country, NCAA Division III,
Midwest Region
2012-2014 Academic All-Conference Track and Field, Minnesota
Intercollegiate Athletic Conference
2014 Dean’s List, College of Saint Benedict, St. Joseph, MN
2014 Runner-up, Poster Competition, Nutritional Sciences Department, College of Saint Benedict, St. Joseph, MN
2015 Phi Beta Kappa Honor Society Inductee, College of Saint Benedict, St. Joseph, MN
2017 Outstanding Teaching Assistant Award, Division of Nutritional Sciences, Cornell University, Ithaca, NY

C. Contribution to Science

**Undergraduate Chemistry Research:** As a member of Dr. Rachel Hutcheson’s laboratory at the College of Saint Benedict (CSB), I helped characterize potential radical S-adenosyl methionine (SAM) enzymes encoded within the BL21(DE3) *E. Coli* genome. Many previously identified radical SAM genes are highly conserved across species, but whether or not these genes encode proteins with radical SAM activity remains to be determined. By evaluating the structure and function of these putative gene products, our lab contributed to the growing database of known radical SAM enzymes.

**Undergraduate Nutrition Research:** Under the direction of Dr. Amy Olson at CSB, I conducted a 3-month observational study to investigate the relationship between energy balance, menstrual function, resting metabolic rate, and serum osteoprotegerin (inhibits bone resorption) in female collegiate cross-country runners. This body of work has important implications for providing dietary recommendations to young female athletes to promote maintenance of healthy reproductive functioning and bone growth.

**Related Written and Oral Work**


**Graduate Research:** As a member of Dr. Barbara Strupp’s laboratory at Cornell University I will be studying the effects of prenatal and early post-partum choline supplementation on offspring cognition, temperament, and HPA-axis reactivity and the epigenetic mechanisms underlying these effects. My current research activities include finalizing saliva collection and cognitive testing protocols, as well as creating a health history questionnaire that will be used to collect information about potential confounding variables in our subject population. Additionally, I work with a second graduate student researcher to oversee 10 undergraduate research assistants. The results of our study will have important implications regarding dietary recommendations for choline during pregnancy which may eventually lead to a shift
towards greater cognitive functioning and affective regulation, in addition to decreases in HPA axis reactivity and stress related diseases in the US population.

D. Additional Information: Research Support and Scholastic Performance

Research Support

Expired
Undergraduate Research Conference Travel Grant: A grant to prepare and present the findings of my undergraduate thesis project at the Northland Chapter of the American College of Sports Medicine 2014, Saint Cloud, MN. Award: $100

Scholastic Performance

Graduate Courses:

Statistical Methods I  
(Fall 2015, Cornell University)  A
Micronutrients  
(Fall 2015, Cornell University)  A
International Seminar in Nutrition  
(Fall 2015, Cornell University)  Satisfactory
Seminar in Nutritional Sciences  
(Fall 2015-present, Cornell University)  Satisfactory
Graduate Fellowship Review  
(Fall 2015, Cornell University)  Satisfactory
Statistical Methods II  
(Spring 2016, Cornell University)  A-
What does the Hippocampus do?  
(Spring 2016, Cornell University)  A
Foundations of Epidemiology  
(Fall 2016, Cornell University)  A
Grant Writing  
(Fall 2016, Cornell University)  Satisfactory
Epigenetics  
(Fall 2016, Cornell University)  B-
Survival Analysis  
(Fall 2017, Cornell University)  In Progress
ACKNOWLEDGMENTS

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Introduction

Dietary choline requirements increase during pregnancy to support fetal and placental growth, but there is growing concern that many pregnant women do not consume enough choline to keep up with these demands. Choline is the precursor to cell membrane components sphingomyelin and phosphatidylcholine and to the neurotransmitter acetylcholine. These three compounds are required in abundance during early development to support the rapid growth, differentiation, and myelination of neurons (Lauder and Schambra, 1999). Choline also supplies methyl groups for epigenetic modifications (i.e., DNA and histone methylation) in the fetus and placenta, two tissues that exhibit remarkable epigenomic plasticity. Animal studies have shown that methyl donor supplementation during pregnancy can induce stable epigenetic changes in the offspring (Davison et al., 2009; Lillycrop et al., 2005; Mehedint et al., 2010) and lead to sustainable functional alterations across the lifespan (Kovacheva et al., 2009; Waterland et al., 2008). The choline utilized for these critical developmental functions is supplied almost entirely by the maternal diet (Yan et al., 2012). Pregnancy depletes maternal choline pools in individuals consuming the recommended amount (Adequate Intake or AI) (Yan et al., 2012; Zeisel et al., 1995), and even consuming double the AI does not prevent a reduction in choline stores and choline metabolites relative to non-pregnant women (Yan et al., 2012). Less than 9% of pregnant women even consume the AI (Wallace and Fulgoni, 2017), and it is not known whether such low intake puts the children at increased risk of cognitive or psychosocial impairment throughout the lifespan.
Animal studies have repeatedly demonstrated that increasing maternal choline intake (above levels provided by standard chow) improves several domains of offspring cognition. Providing dams with a choline-deficient diet during pregnancy has been shown to impair memory and attention in the adult offspring (Meck and Williams, 1999, 2003). Conversely, providing pregnant dams with additional choline (maternal choline supplementation; MCS) has been shown to exert lasting beneficial effects on offspring memory (Meck, 1988; Meck and Williams, 1999) and attention (Meck et al., 2007; Mohler et al., 2001; Moon et al., 2010). Few studies have examined this important question in humans. Observational studies have shown that higher maternal choline intake (Boeke et al., 2013) and higher maternal plasma free choline (Wu et al., 2012) are associated with improved offspring cognition. A recent randomized controlled trial (RCT) demonstrated that MCS (930 vs 480 mg choline/d) significantly improved infant visual processing speed, and markedly improved infant explicit memory (Caudill et al., in press), two early predictors of child IQ (Dougherty and Haith, 1997). To the best of our knowledge, only one other study has examined the effects of MCS (750 mg PtdCh/d, equivalent to 100 mg choline/d, versus placebo) on infant cognition (Cheatham et al., 2012). This study found no main effect for choline, but evidence of moderation by genotype led the authors to conclude that genetic differences may have masked the effects of the intervention. The results of these studies, although mixed, provide suggestive evidence that the effects of increased maternal choline in animals may translate to humans.

The observed benefits of MCS also extend to offspring behavioral and psychosocial functioning, based on the rodent literature. MCS increases object-
exploration and diminishes age-related decline in open-field exploration (both indicative of lower fearfulness) in the offspring (Glenn et al., 2008). Maternal choline supplementation improves offspring emotion regulation (Cheng et al., 2008), and ameliorates deficits in behavioral and emotional regulation in rodent models of autism (Langley et al., 2015), Rett syndrome (Nag and Berger-Sweeney, 2007; Nag et al., 2008; Ward et al., 2009), and Down syndrome (Moon et al., 2010). In a murine model of Down syndrome, MCS partially attenuated the disruptive effect of committing an error on performance in a vigilance task (Moon et al., 2010). Finally, other studies have shown that MCS mitigates the effects of adverse *in utero* exposures including alcohol (Thomas et al., 2000, 2004, 2007, 2009), iron deficiency (Kennedy et al., 2014; Tran et al., 2016), and maternal stress (Schulz et al., 2014a) on offspring behavioral development.

Few studies have evaluated the effects of varying maternal choline intake on offspring behavior and psychosocial functioning in humans. One trial reported significantly improved sensory gating (indicative of reduced risk of later psychiatric illness) in infants whose mothers were randomized to receive 5,300 mg PtdCh/d during pregnancy relative to placebo controls (Ross et al., 2013). A 40-month follow-up study of these children revealed fewer attention problems and less social withdrawal in the supplemented group relative to the controls, as measured by the Child Behavior Checklist (CBCL), a parent-report of child behavior. The supplemented group also had lower scores (indicating fewer problems) on the five other behavior subscales and the externalizing and internalizing composite scales, but none of these differences were statistically significant (Ross et al., 2016). A second
RCT also revealed substantially lower parent-reported infant fearfulness at 13 months of age among infants whose mothers consumed the higher dose of choline (930 vs 480 mg choline/d) (Caudill et al, in press). Although limited, the results of these human studies align well with the rodent literature in terms of behavioral domains affected by maternal choline intake (i.e., internalizing, fearfulness, emotional regulation).

Increased maternal choline intake may also improve offspring hypothalamic-pituitary-adrenal (HPA) axis functioning. In our prior RCT, higher maternal choline intake resulted in lower cord blood cortisol at delivery and altered methylation of the HPA-axis regulating genes, CRH and NR3C1, in cord blood and placenta (Jiang et al., 2012). The HPA axis is the primary neuroendocrine system responsible for generating the stress response and maintaining circulating stress hormone (e.g., cortisol) levels (Smith and Vale, 2006). Dysregulation of the HPA-axis has been associated with internalizing behaviors in the offspring (Goodyer et al., 2001; Lopez et al., 2004). Among adults, dysregulation of the HPA-axis is associated with both anxiety and depression, in addition to other psychiatric illnesses including schizophrenia, bipolar disorder, and PTSD (Benjet et al., 2010; Green et al., 2010; Slopen et al., 2014). The role of in utero exposures in the development of mental disorders has been increasingly recognized. Prenatal programming of the HPA axis by epigenetic modification (i.e., DNA methylation) is one mechanism by which in utero exposures modulate risk of these disorders (Heim and Binder, 2012; Palma-Gudiel et al., 2015a; Parade et al., 2016). Large cohort studies have shown that even small exposure-associated methylation differences (i.e. 2-10%) can be statistically significant and
clinically meaningful, due to low natural variation in DNA methylation across individuals (Breton et al., 2017).

The glucocorticoid receptor gene, *NR3C1* (Benjet et al., 2010), is perhaps the most widely-studied gene in the context of epigenetic programming of human behavior. In a landmark study by Weaver et al., maternal neglect resulted in higher *NR3C1* methylation in offspring hippocampal DNA (Weaver et al., 2004). Altered *NR3C1* methylation has been observed in infants exposed to early life adversity including maternal neglect (Francis et al., 1999; Liu et al., 1997; Weaver et al., 2004) and maternal smoking (Stroud et al., 2016a), psychosocial stress (Mulligan et al., 2012; Palma-Gudiel et al., 2015a), and depression during pregnancy (Oberlander et al., 2008; Palma-Gudiel et al., 2015b; Stroud et al., 2016b). Variation in *NR3C1* methylation in fetal tissues (i.e., cord leukocytes and placenta) also predicts infant neurobehavior (Bromer et al., 2013; Conradt et al., 2013) and infant cortisol stress response (Oberlander et al., 2008). Finally, *NR3C1* methylation may mediate the effects of *in utero* exposures on offspring behavior. Two recent studies reported independent additive effects of maternal depression (Conradt et al., 2013) and maternal smoking (Stroud et al., 2016a) with *NR3C1* methylation on infant neurobehavior. A third study revealed that *NR3C1* methylation in child saliva significantly mediated the effects of prenatal stress on childhood internalizing behaviors (Parade et al., 2016).

One prior RCT reported marked effects of maternal choline intake on methylation of the cortisol-regulating genes *NR3C1* and *CRH* (corticotropin releasing hormone) in cord blood and placenta. Furthermore, infants of supplemented moms
exhibited a reduced cortisol response to the stress of labor and delivery, suggesting a functional effect of these choline-induced epigenetic modifications (Jiang et al., 2012). Given prior associations between fetal NR3C1 methylation and childhood behavior, and given the effects of MCS on fetal NR3C1 methylation, we hypothesized that NR3C1 methylation may mediate the hypothesized effects of MCS on child behavioral and psychosocial outcomes. If this is the case, then examining the association between NR3C1 methylation and child behavior may provide a much stronger analysis than testing the effects of MCS on child behavior directly.

The present study is a 7-year follow-up of the mother-child pairs from a prior randomized controlled maternal choline feeding study during the third trimester of pregnancy. At 7 years of age, a more complete behavioral assessment is possible, compared to the assessment conducted during infancy. The present study has three aims: First, we aim to characterize the effects of MCS on child behavior (primary analysis). We hypothesize that higher maternal choline intake will be associated with fewer problems in domains shown to be sensitive to MCS in previous animal and human studies (i.e., internalizing, anxiety, withdrawal, and emotional self-control). Second, we aim to replicate the findings of Jiang et al. with regard to MCS and NR3C1 methylation in our smaller follow-up sample (confirmatory analysis). Third, we will evaluate whether cord and placental NR3C1 methylation differences within our sample predict child behavior outcomes (plausibility analysis). Given that MCS strongly influenced NR3C1 methylation in this sample (Jiang et al., 2012), evidence for a relationship between methylation and behavior would support our hypothesis that
MCS influences child behavior via downstream changes in cord and placental NR3C1 methylation (Figure 1).

**FIGURE 1**
Overall analysis plan. In the primary analysis, we examined the relationship between MCS and child behavior. In a second, confirmatory analysis, we aimed to replicate the findings of Jiang et al. regarding the effects of MCS on offspring NR3C1 methylation in our reduced sample size (n=18/19 for cord/placenta vs n=23/24 in Jiang et al.). In our third, plausibility analysis, we examined the association between NR3C1 methylation and child behavior. Methylation served as a proxy for MCS in this analysis, given our hypothesized mechanism whereby MCS is involved in fetal programming of child behavior via altered NR3C1 methylation.
Methods

Study Participants

This study was a follow-up to a randomized controlled maternal choline feeding study conducted at Cornell University. The detailed methods of the original trial, which was designed to assess biomarkers of choline metabolism in pregnant versus nonpregnant women (ClinicalTrials.gov as NCT101127022) have been described elsewhere (Yan et al., 2012). Briefly, between January 2009 and October 2008, third-trimester pregnant (gestational week 27) women were randomized (n=29) to either 480 (approximately the AI for choline; n=12) or 930 (n=12) mg choline/d for 12-weeks. Choline was supplied from dietary sources (380 mg/d; all participants) and choline chloride supplements (100 or 550 mg/d for the 480 and 930 mg choline/d groups, respectively). All food and beverages were provided by study investigators, as detailed in Yan et al. (Yan et al., 2012). Participants consumed 1 meal/d on-site and received the remaining meals, snacks, and beverages as take-aways. Participants also received daily prenatal multivitamins (Pregnancy Plus; Fairhaven Health, Bellingham, WA, USA) and DHA supplements (200 mg; Neuromins; Nature’s Way Products, Lehi, UT, USA) as well as a potassium and magnesium supplement (General Nutrition Corp, Pittsburgh, PA, USA) 3X/wk. The daily choline supplement was consumed during the onsite meal under the supervision of study investigators. Participants continued on their choline supplements after completing the 12-week feeding study until the delivery of their babies. This resulted in slight variation in the total number of days on the supplement based on differences in gestational age at study entry and delivery.
Twenty-six of the 29 women who started the feeding study completed it.

Women who completed the choline feeding study were re-contacted for participation in the follow-up study between July 2016 to May 2017. A total of 21 mother-child pairs consented to participate (Figure 2).

Parents brought their children to Cornell University for two cognitive testing sessions on consecutive days within 7 months after the child’s 7th birthday. These sessions were part of a larger study to evaluate the effects of maternal choline intake on child behavior, cognition, and hypothalamic-pituitary-adrenal (HPA) axis reactivity. Children completed a variety of cognitive tasks while the accompanying
parent completed behavior and health history surveys. For the purpose of this study, we investigated the behavior survey data to assess the association of choline dose with child internalizing behaviors, anxiety, withdrawal, and emotional self-control.

Sample Collection

Cord venous blood and placentas were obtained from 18 and 19 of the women in this study, respectively (n=9-10 per choline intake group). Cord venous blood was collected in 10 mL EDTA tubes (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ, USA) immediately after delivery (Yan et al., 2012). Placenta samples were processed within 60-90 minutes of delivery, and a majority were processed within 10-30 minutes. Placentas were processed as follows: The amniotic sac was removed and the placenta was visually divided into 4 quadrants. Full thickness biopsies (0.5 cm x 0.5 cm x placenta depth) were obtained from the center of each quadrant, rinsed with PBS, and flash frozen in liquid nitrogen. Biopsies from each of the 4 quadrants were pooled and ground in liquid nitrogen for DNA extraction (Jiang et al., 2012). The placenta is a heterogeneous tissue, so DNA methylation measurements in this study represent average methylation patterns across a subset of placental cells.

Measurements

NR3C1 Methylation

Genomic DNA was extracted via the DNeasy blood and tissue kit (Qiagen), and methylation was measured by base-specific cleavage and mass spectrometry (Hartmer et al., 2003). Briefly, 1µg extracted DNA was bisulfite treated using the EZ
96-DNA methylation kit (Zymo, Irvine, CA, USA) and the sequence of interest, the 5’ untranslated exon 1F of *NR3C1*, was PCR-amplified using the bisulfite-treated DNA as the template and primers that incorporate the T7 tag. Sequences for both the primers and the NR3C1 target region have previously been published by Jiang et al., (Jiang et al., 2012). Amplification products were analyzed using the MassArray EpiTyper system (Sequenom, San Diego, CA, USA) at the Cornell Life Sciences Core Laboratories Center (Cornell University). Results are reported as percent cytosine methylation across the entire region (average *NR3C1* methylation) and at the level of specific cytosine-phosphate-guanine (CpG) dinucleotides. The EpiTyper system cannot distinguish adjacent CpG sites, so in some cases site-specific results are reported as an average across 2-3 adjacent CpG dinucleotides (Jiang et al., 2012).

The *NR3C1* exon 1F region contains 47 CpG sites. We analyzed average methylation across the 47 sites and methylation at specific *a priori* selected CpG units in relationship to child behavior outcomes. These units, which include CpGs 30-32, and CpGs 37-38, correspond to canonical NGFI-A (a transcription factor) binding regions. Methylation of these units is influenced by maternal care (Weaver et al., 2004) and early life stress (Hompes et al., 2013; van der Knaap et al., 2014; Oberlander et al., 2008; Radtke et al., 2011; Romens et al., 2015; Stroud et al., 2016; Tyrka et al., 2015), and has been shown to modulate *NR3C1* expression (McGowan et al., 2009) and stress reactivity across the lifespan (Edelman et al., 2012; Stroud et al., 2014, 2016b).
Child behavior was assessed using the Behavior Assessment System for Children, Third Edition, Parent Rating Scale, Child Form (BASC-III; Reynolds and Kamphaus, 2015) administered to caregivers using an online survey platform (Qualtrics Survey Platform, Provo, UT, USA) in the presence of a study investigator who was trained to answer questions relating to the instrument. The BASC-III is a reliable and valid measure with strong test-retest reliability (Cecil R. Reynolds, PhD and Randy W. Kamphaus, PhD, 2015). In most instances (90.5%), assessments were completed by the child’s mother; the father completed the remaining assessments (9.5%).

The BASC-III provides a comprehensive measure of adaptive and problematic child behaviors in home and community settings, and is suitable for children ages 6-11 years old. Primary outcomes of interest were the Internalizing Behaviors composite scale (comprised of three clinical subscales: Depression, Somatization, and Anxiety), the Anxiety clinical scale, the Emotional Self-Control scale, and the Withdrawal content scale. Parents assessed their children on 175 behaviors using a 4-point scale ranging from “Never” to “Almost Always”. Standardized age and sex-specific t-scores were calculated relative to the BASC-III normative sample, which included children from different ethnicities and designed to “resemble the U.S. population with respect to sex, socio-economic status, race/ethnicity, geographic region, and special-education classification”. Possible t-scores range from 0-100, with a mean of 50 and standard deviation of 10 in the normative sample. Scores between 60-69 are considered “At-Risk”, and scores greater than 70 are considered “clinically significant” (Cecil R.
Emotional Self Control Scores were not available for the normative sample, so raw scores were used for all analyses involving this scale. For all of the scales analyzed, higher scores indicate more problems.

Covariates

Maternal and newborn information were obtained from a questionnaire administered in the screening phase of the initial feeding study and from medical charts at the time of delivery. Maternal information included age, gestational age at study entry, education, parity, height, weight, and complications during pregnancy or delivery. Newborn information included birth weight, head circumference, gestational age, method of delivery, and sex (Yan et al., 2012). Child and caregiver information at the 7-year follow-up was obtained from a questionnaire administered using an online survey platform (Qualtrics Survey Platform, Provo, UT, USA). Child information included height, weight, race primary language, duration of breastfeeding, family history of depression or anxiety, and ”Recent Stressor Index”. The ”Recent Stressor Index” score was calculated by summing the number of potentially stressful events (from a pre-selected 12-item checklist, Appendix A) experienced by the child in the past 12 months.

Ethics

The protocols for the feeding trial and for the follow-up behavioral and cognitive study were both approved by the Institutional Review Board for Human
Participant Use at Cornell University (Ithaca, NY, USA). The feeding trial was also approved by the Institutional Review Board at Cayuga Medical Center (Ithaca, NY, USA). Written informed consent was obtained from all participants before the feeding study, and again before participation in the present study. Child assent was also obtained before participation in the present study.

Statistical Analysis

The sample size for the feeding study was based on a power calculation that predicted differences of 20% in biomarkers of choline metabolism (choline and betaine) with a power of 80% at an alpha of 0.05 between choline intake groups. The sample size for the follow-up study had 80% power at an alpha of 0.05 to detect a 13-point difference in behavior t-scores between the two intake groups, assuming a standard deviation of 10 (G*Power 3.1, Universitaat Dusseldorf, Dusseldorf, Germany). This was an intent-to-treat analysis, so all participants with available data were included in the analyses. Statistical analyses were conducted using general linear models (glm's) in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and all tests were two-tailed.

Primary Analysis: Maternal Choline Intake and Child Behavior

We examined the effects of maternal choline supplementation on four indices of child behavior outcomes (Internalizing Problems, Anxiety, Withdrawal, and Emotional Self Control) using unadjusted models. Sex was included as a covariate in the unadjusted model for Emotional Self-Control, because raw scores are not
comparable across sexes (Cecil R. Reynolds, PhD and Randy W. Kamphaus, PhD, 2015). For adjusted analyses (Models 2 and 3), we pre-specified inclusion of covariates known to be prognostic of child internalizing behaviors. In Model 2, we included maternal education at randomization, child sex, and pregnancy and delivery complications. In Model 3 we added a “Recent Stressor Index” variable (See Appendix A) to remove variation in the behavior outcomes that was not likely to be due to the exposure.

**Confirmatory Analysis: Maternal Choline Intake and Infant DNA Methylation**

We also aimed to recapitulate the findings of Jiang et. al., which showed that maternal choline intake alters offspring DNA methylation (Jiang et al., 2012), in the subset of subjects who participated in our follow-up study (n=19 for cord, n=18 for placenta). For this supplemental analysis, we conducted unadjusted individual contrasts assessing the effect of choline on each \( NR3C1 \) methylation site (i.e., average, CpG30-32, and CpG37-38) in cord leukocytes and placenta.

**Plausibility Analysis: Infant DNA Methylation and Childhood Behavior Patterns**

We hypothesized that maternal choline supplementation would influence child behavior by altering cord and placental \( NR3C1 \) methylation, which has been shown to predict child behavior (Appleton et al., 2015; Bromer et al., 2013; Conradt et al., 2013; Parade et al., 2016; Stroud et al., 2016a) and stress-reactivity (Oberlander et al., 2008; Stroud et al., 2016b). We had measurements of \( NR3C1 \) methylation in cord blood and placenta. In this analysis, \( NR3C1 \) methylation is an indicator of response to treatment
(Jiang et al., 2012). To explore this, we evaluated the effects of offspring cord and placental average \textit{NR3C1} exon 1F methylation and methylation at sites CpG 30-32 and CpG37-38 on child behavior using adjusted models (Model 3 above). We applied a Bonferroni correction to adjust for multiple testing (three methylation sites in two tissues), and the statistical significance threshold was conservatively set at $P=0.008$. 
Results

Behavior data was collected for 21 of 26 eligible children (Figure 2). Table 1 shows demographic characteristics of mothers and children that comprised the statistical models for the 480 and 930 mg choline/d groups. Maternal education at randomization was significantly higher in the 480 mg choline/d group. This imbalance justified the inclusion of maternal education as a covariate in our a priori adjusted models (Models 2 and 3). Five mother-child pairs were lost to follow-up. These mothers were not systematically different than the 21 follow-up participants in any of the measured covariates. Among those lost to follow-up, 2 women were Caucasian, 3 were employed full- or part-time, and 2 held graduate degrees.

Maternal Choline Supplementation

In the regression model adjusted only for sex (Model 1), there were lower Emotional Self-Control scores (indicating fewer problems) in the 930 mg choline/day group compared to the 480 mg choline/d group ($\beta = -2.28$, $p=0.10$, Figure 3). Lower scores were also reported on the Internalizing Problems, Anxiety, and Withdrawal scales in the 930 mg choline/d group compared to the 480 mg choline/d group, however none of these results were statistically significant.

In the a priori adjusted model (Model 2) of the association between MCS and Emotional Self-Control was attenuated. There was little to no association of MCS with Internalizing Problems, Anxiety, or Withdrawal scores in the adjusted analyses, and none of the covariates were significantly associated with the behavior outcomes.
TABLE 1  Selected characteristics of 21 children who were followed up at age 7 y by treatment

<table>
<thead>
<tr>
<th></th>
<th>480 mg (n = 9)</th>
<th>930 mg (n = 12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal characteristics at random assignment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>28.4 ± 3.0 ²</td>
<td>27.3 ± 3.8</td>
<td>0.45</td>
</tr>
<tr>
<td>Gestational age (wks)</td>
<td>26.6 ± 1.9</td>
<td>26.9 ± 1.2</td>
<td>0.59</td>
</tr>
<tr>
<td>Education</td>
<td>2.8 ± 0.4</td>
<td>1.5 ± 1.1</td>
<td>0.0004</td>
</tr>
<tr>
<td>Parity (primiparous/multiparous)</td>
<td>5/4</td>
<td>4/8</td>
<td>0.31</td>
</tr>
<tr>
<td>Pre-pregnancy weight (kg)</td>
<td>61.5 ± 5.6</td>
<td>65.5 ± 11.0</td>
<td>0.33</td>
</tr>
<tr>
<td>Pre-Pregnancy height (cm)</td>
<td>165.7 ± 5.2</td>
<td>164.8 ± 8.3</td>
<td>0.78</td>
</tr>
<tr>
<td>Pre-pregnancy BMI (kg/m²)</td>
<td>22.4 ± 1.2</td>
<td>24.0 ± 3.0</td>
<td>0.13</td>
</tr>
<tr>
<td>Offspring characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>3/6</td>
<td>4/8</td>
<td>1</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3468.4 ± 549.3</td>
<td>3457.8 ± 314.7</td>
<td>0.96</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>50.2 ± 2.2</td>
<td>50.8 ± 2.4</td>
<td>0.57</td>
</tr>
<tr>
<td>Head circumference at birth (cm)</td>
<td>34.4 ± 1.3</td>
<td>34.1 ± 1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>40.2 ± 1.5</td>
<td>39.9 ± 0.7</td>
<td>0.57</td>
</tr>
<tr>
<td>Delivery (cesarean/vaginal)</td>
<td>3/6</td>
<td>2/10</td>
<td>0.37</td>
</tr>
<tr>
<td>Pregnancy and/or delivery complications (y/n)</td>
<td>5/4</td>
<td>4/12</td>
<td>0.31</td>
</tr>
<tr>
<td>Duration of breastfeeding (mo)</td>
<td>16.6 ± 8.3</td>
<td>16.9 ± 13.1</td>
<td>0.95</td>
</tr>
<tr>
<td>Height at age 7 y (cm)</td>
<td>128.2 ± 5.7</td>
<td>125.1 ± 5.8</td>
<td>0.23</td>
</tr>
<tr>
<td>Weight at age 7 y (cm)</td>
<td>26.3 ± 4.2</td>
<td>25.6 ± 5.4</td>
<td>0.77</td>
</tr>
<tr>
<td>BMI at age 7 y</td>
<td>15.9 ± 1.3</td>
<td>16.3 ± 2.2</td>
<td>0.68</td>
</tr>
<tr>
<td>Race (Caucasian/African American/Asian/ Native American)</td>
<td>8/0/0/1</td>
<td>7/2/2/1</td>
<td>NA</td>
</tr>
<tr>
<td>Primary Language (English/other)</td>
<td>8/1</td>
<td>9/3</td>
<td>0.42</td>
</tr>
<tr>
<td>Family hx depression and/or anxiety (y/n)</td>
<td>2/7</td>
<td>2/10</td>
<td>0.75</td>
</tr>
<tr>
<td>Recent Stress Index Score</td>
<td>1.3 ± 1.0</td>
<td>1.7 ± 0.9</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Child Behavior Scores

<table>
<thead>
<tr>
<th></th>
<th>480 mg (n = 9)</th>
<th>930 mg (n = 12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internalizing Problems (t-score)</td>
<td>51.44 ± 6.86</td>
<td>50.33 ± 8.61</td>
<td>0.75</td>
</tr>
<tr>
<td>Anxiety (t-score)</td>
<td>50.78 ± 5.76</td>
<td>50.17 ± 8.97</td>
<td>0.86</td>
</tr>
<tr>
<td>Withdrawal (t-score)</td>
<td>52.89 ± 10.30</td>
<td>50.83 ± 7.53</td>
<td>0.60</td>
</tr>
<tr>
<td>Emotional Self-Control (raw score)</td>
<td>9.44 ± 3.47</td>
<td>7.17 ± 2.44</td>
<td>0.09</td>
</tr>
</tbody>
</table>

¹ P values were determined with the use of the t test for comparisons of means and chi-square test for comparisons of proportions.

² Mean ± SD (all such values)

³ Maternal education was measured as a categorical variable (indicator variables were: 0= H.S. Diploma, 1=Associates or Vocational Degree, 2= Bachelor's Degree, 3=Master’s Degree, 4=Doctorate or Professional degree) and analyzed as a continuous variable

⁴ See Appendix A for a description of how this was calculated

⁵ Behavior Assessment System for Children (BASC-III) Internalizing Problems is a composite score which includes the Somatization, Depression, and Anxiety Subscales.

⁶ BASC-III Anxiety measures “the tendency to be nervous, fearful, or worried about real or imagined problems”

⁷ BASC-III Withdrawal measures “the tendency to evade others or to avoid social contact”

⁸ BASC-III Emotional Self Control measures “the ability to regulate one’s affect and emotions in response to environmental changes
To refine our assessment of child behavior to focus on trait-level behaviors (i.e., not situationally induced), we explored the addition of a “Recent Stressor Index” score to our *a priori* adjusted model. In this model, Model 3, the magnitude of the estimate for MCS on mean Emotional Self-Control scores was greater than in the unadjusted model, but was not statistically significant ($\beta = -3.09, p=0.12$). No associations of MCS with Internalizing Problems, Anxiety, or Withdrawal were observed in these refined adjusted analyses and none of the covariates were statistically significant (*Table 2*).
### TABLE 2
Associations of MCS with child behavior outcomes at age 7 y, as measured by the BASC-III Parent Report

<table>
<thead>
<tr>
<th>Behavior Outcome</th>
<th>Model 1</th>
<th></th>
<th></th>
<th>Model 2</th>
<th></th>
<th></th>
<th>Model 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>S.E.</td>
<td>P</td>
<td>β</td>
<td>S.E.</td>
<td>P</td>
<td>β</td>
<td>S.E.</td>
<td>P</td>
</tr>
<tr>
<td><strong>Internalizing Problems</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline (930 mg/day)</td>
<td>-1.11</td>
<td>3.49</td>
<td>0.75</td>
<td>1.78</td>
<td>4.50</td>
<td>0.70</td>
<td>0.20</td>
<td>4.87</td>
<td>0.97</td>
</tr>
<tr>
<td>Sex (M)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-1.63</td>
<td>4.00</td>
<td>0.69</td>
<td>3.85</td>
<td>4.75</td>
<td>0.43</td>
</tr>
<tr>
<td>Maternal Education</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.58</td>
<td>2.38</td>
<td>0.52</td>
<td>0.69</td>
<td>2.60</td>
<td>0.79</td>
</tr>
<tr>
<td>Pregnancy and/or delivery complications</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.92</td>
<td>4.11</td>
<td>0.35</td>
<td>5.08</td>
<td>4.35</td>
<td>0.26</td>
</tr>
<tr>
<td>Recent Stress Index Score</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.10</td>
<td>2.39</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>Anxiety</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline (930 mg/day)</td>
<td>-0.61</td>
<td>3.43</td>
<td>0.86</td>
<td>1.25</td>
<td>4.43</td>
<td>0.78</td>
<td>0.41</td>
<td>4.89</td>
<td>0.93</td>
</tr>
<tr>
<td>Sex (M)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.90</td>
<td>3.94</td>
<td>0.82</td>
<td>0.29</td>
<td>4.76</td>
<td>0.95</td>
</tr>
<tr>
<td>Maternal Education</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.62</td>
<td>2.35</td>
<td>0.79</td>
<td>0.14</td>
<td>2.61</td>
<td>0.96</td>
</tr>
<tr>
<td>Pregnancy and/or delivery complications</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.83</td>
<td>4.05</td>
<td>0.25</td>
<td>5.45</td>
<td>4.36</td>
<td>0.23</td>
</tr>
<tr>
<td>Recent Stress Index Score</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.13</td>
<td>2.39</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>Withdrawal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline (930 mg/day)</td>
<td>-2.06</td>
<td>3.88</td>
<td>0.60</td>
<td>1.72</td>
<td>5.06</td>
<td>0.74</td>
<td>-0.29</td>
<td>5.44</td>
<td>0.96</td>
</tr>
<tr>
<td>Sex (M)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-2.08</td>
<td>4.49</td>
<td>0.65</td>
<td>4.90</td>
<td>5.30</td>
<td>0.37</td>
</tr>
<tr>
<td>Maternal Education</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.87</td>
<td>2.68</td>
<td>0.30</td>
<td>1.74</td>
<td>2.90</td>
<td>0.56</td>
</tr>
<tr>
<td>Pregnancy and/or delivery complications</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.48</td>
<td>4.62</td>
<td>0.92</td>
<td>1.96</td>
<td>4.85</td>
<td>0.69</td>
</tr>
<tr>
<td>Recent Stress Index Score</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.68</td>
<td>2.66</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Emotional Self-Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline (930 mg/day)</td>
<td>-2.28</td>
<td>1.32</td>
<td>0.10</td>
<td>-2.26</td>
<td>1.77</td>
<td>0.22</td>
<td>-3.09</td>
<td>1.88</td>
<td>0.12</td>
</tr>
<tr>
<td>Sex (M)</td>
<td>-0.21</td>
<td>1.39</td>
<td>0.88</td>
<td>-0.02</td>
<td>1.57</td>
<td>0.99</td>
<td>1.20</td>
<td>1.83</td>
<td>0.52</td>
</tr>
<tr>
<td>Maternal Education</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.13</td>
<td>0.94</td>
<td>0.89</td>
<td>-0.34</td>
<td>1.00</td>
<td>0.74</td>
</tr>
<tr>
<td>Pregnancy and/or delivery complications</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.67</td>
<td>1.62</td>
<td>0.68</td>
<td>-0.05</td>
<td>1.67</td>
<td>0.97</td>
</tr>
<tr>
<td>Recent Stress Index Score</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.11</td>
<td>0.92</td>
<td>0.24</td>
</tr>
</tbody>
</table>

**Note:** Model 1 includes maternal choline supplementation dose only. Model 2 includes variables causally associated with child behavior outcomes. Model 3 includes a “Recent Stress Index Score” to adjust for uncharacteristic child behavior problems. β’s represent values when all variables in each model are entered simultaneously.

Maternal education was measured as a categorical variable (indicator variables were: 0= H.S. Diploma, 1= Associates or Vocational Degree, 2= Bachelor's Degree, 3= Master's Degree, 4= Doctorate or Professional degree) and analyzed as a continuous variable.

1See Appendix A for a description of how this was calculated.
Placental NR3C1 Methylation

Placental methylation data was available for 19 of 21 children with behavior data (Figure 1). Unadjusted regression analyses revealed 0.9% higher NR3C1 exon 1F average methylation ($p<0.001$) and 2% higher CpG 30-32 methylation ($p=0.02$) in the 930 mg choline/d group compared to the 480 mg choline/d group (Table 3). These results are of a similar magnitude and direction as the results obtained by Jiang et. al. in their adjusted analyses of all women who completed the feeding study and from whom placentas were collected (n=24)(Jiang et al., 2012). Higher CpG 37-38 methylation was also observed in the 930 mg choline/day group, but the effect was not statistically significant.

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>Choline (930 mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cord Leukocyte NR3C1</strong></td>
<td>β</td>
</tr>
<tr>
<td>CpG 30-32</td>
<td>-0.49</td>
</tr>
<tr>
<td>CpG 37-38</td>
<td>-0.63</td>
</tr>
<tr>
<td>Exon 1F, Average</td>
<td>-0.46</td>
</tr>
<tr>
<td><strong>Placental NR3C1</strong></td>
<td>β</td>
</tr>
<tr>
<td>CpG 30-32</td>
<td>2.00</td>
</tr>
<tr>
<td>CpG 37-38</td>
<td>1.00</td>
</tr>
<tr>
<td>Exon 1F, Average</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Adjusted analyses (Model 3) indicated that placental NR3C1 methylation (average exon 1F, CpG 30-32, and CpG 37-38) was not a significant predictor of any of assessed child behavior outcomes (Table 4).
Cord Leukocyte NR3C1 Methylation

Cord leukocyte methylation data was available for 18 of 21 children with behavior data (Figure 1). Unadjusted regression analyses revealed lower average NR3C1, CpG 30-32, and CpG 37-38 methylation in the 930 mg choline/day group relative to the 480 mg choline/day group, however none of these effects were statistically significant (Table 2).

Adjusted analyses (Model 3) revealed higher cord CpG 30-32 methylation among children with higher Internalizing Problems and Anxiety scores. Each 1% higher CpG 30-32 methylation was associated with a 2.8-point higher Internalizing Problems score (p=0.02) and a 3.2-point greater Anxiety score (p=0.01, Figure 4).

Similarly, there was a trend for higher Internalizing Problems (p=0.11) and Emotional...
Self-Control scores (p=0.11) among children with greater average exon 1F methylation (Table 4).

**FIGURE 4**
BASC-III Anxiety t-scores as a function of cord leukocyte NR3C1 CpG 30-32 methylation. In adjusted regression analyses, each 1% higher methylation was associated a 3.2% greater Anxiety score.
**Discussion**

Two major findings were observed in the present study. First, parents reported fewer emotional self-control problems in the 930 mg choline/day group compared to the 480 mg choline/day group. Second, higher cord leukocyte NR3CI Cpg 30-32 methylation was associated with more anxiety and internalizing behaviors in the full sample.

**MCS and Child Behavior**

Higher maternal choline intake (930 vs 480 mg choline/d) was associated with a 3-point decrease in emotional self-control problems. The mean Emotional Self-Control score in the full sample was 8.14± 3.07, so this association corresponds to a full standard deviation increase in emotional self-control problems. The association persisted across all 3 models, suggesting that the choline effect was not highly sensitive to adjustment. The observed benefits of maternal choline supplementation (MCS) on child emotional self-control are consistent with the results of prior rodent studies. A study by Cheng et al. reported less “burst responding” (an indicator of frustration) among MCS rats versus controls during an operant conditioning task, suggesting a superior ability to regulate emotional reaction among the MCS rats (Cheng et al., 2008). In a second study, MCS attenuated the heightened “emotional disruption” typically observed in a mouse model of Down syndrome after committing an error during testing on an attention task (Moon et al., 2010). The present study is the first to demonstrate that the observed benefits of MCS on offspring emotional regulation may translate to humans.
We did not observe any meaningful associations of maternal choline intake with withdrawal, anxiety, or internalizing problems. We had predicted effects in these domains, based on prior rodent studies, which have shown fewer anxiety-related behaviors in offspring of MCS dams (Schulz et al., 2014b; Thomas et al., 2004) and one prior human study that showed less social withdrawal among 40-month-old MCS offspring relative to unsupplemented controls, based on parent report (Ross et al., 2016).

Additional findings by Ross et al. regarding child CHRNA7 genotype may explain why they found effects of MCS on child withdrawal while we did not (Ross et al., 2016). Ross et al. reported that MCS attenuated withdrawal, but only in children with particular CHRNA7 genotypes at rs3087454, specifically those with the AC and CC genotypes (Ross et al., 2016). Frequency of the CHRNA7 rs3087454 minor C allele predicts attention problems (Hyde et al., 2016) and lifetime risk of various mental disorders including attention deficit hyperactivity disorder (ADHD; Williams et al., 2012), autism spectrum disorder (ASD; Allen-Brady et al., 2010), and schizophrenia (Freedman et al., 1997). These findings suggest that the potential-to-benefit from MCS may be greatest in children with at least one CHRNA7 rs3087454 minor allele. Failure to adjust for CHRNA7 genotype in the present study may prevent us from detecting effects of MCS on child outcomes that depend on an upregulation of this pathway.

Alternatively, it is also possible that emotion regulation is more strongly affected by increased maternal choline intake than the other indices of affect (anxiety, internalizing). Emotional self-control scale may have been the most sensitive to MCS
because it combines effects on both cognition and emotion, domains which both seem to be affected by MCS, based on the animal studies. Emotional self-control measures the responsivity of various cognitive functions (i.e., attention and executive function) to a variety of emotions (i.e., sadness, frustration, and fear)(Cecil R. Reynolds, PhD and Randy W. Kamphaus, PhD, 2015). In contrast, anxiety and internalizing are more specific to the affective domain (Figure 5).

**FIGURE 5**
The effects of choline on child behavior via three independent developmental pathways. Capitalized/bold letters indicate behaviors for which there is some evidence of moderation by maternal choline intake from animal or human studies. The various contributions of each pathway in programming child behavior is not fully understood. Ptd = Phosphatidylcholine.
Fetal Programming of Child Behavior by NR3C1 Methylation

We focused our subsequent analyses on DNA methylation as a potential mechanism by which MCS may modulate child behavior. Specifically, we examined the relationship between NR3C1 methylation and child behavior. A prior study showed that MCS altered NR3C1 methylation in cord leukocytes and the placenta (Jiang et al., 2012), so the goal of this analysis was to directly evaluate the functional consequences of the methylation changes. The NR3C1 gene, which encodes the glucocorticoid receptor (GR), has been implicated in epigenetic programming of the offspring HPA axis (Benjet et al., 2010; Oberlander et al., 2008), and methylation of this gene is associated with infant neurobehavior (Appleton et al., 2015; Bromer et al., 2013; Stroud et al., 2016a). Prior work with our sample showed increased placental NR3C1 methylation and decreased cord leukocyte NR3C1 methylation resulting from higher maternal choline intake (930 mg choline/day vs 480 mg choline/day; Jiang et al., 2012). Therefore, methylation is a proxy for response to the choline treatment in the present study. The relationship between NR3C1 methylation and child behavior provides the strongest analysis of the effects of choline on behavior because it eliminates the variance introduced by subjects who did not respond to the choline treatment with changes in NR3C1 methylation (Figure 6).

The extent to which increased choline availability increases methylation of specific genes, such as NR3C1, may vary across individuals, based on their ability to convert choline to betaine, which is modulated by several genetic polymorphisms. For example, genetic polymorphisms of the human BHMT gene have been shown to
impair methyl-group metabolism (Li et al., 2008) and influence DNA methylation (Lupu et al., 2017). If present in our sample, these polymorphisms may have attenuated the effects of MCS on cord and placental DNA methylation. Consequently, if some of the effects of MCS on child behavior are mediated by effects on *NR3C1* methylation, we would expect *NR3C1* methylation to be a better predictor of child behavior than maternal choline intake because it eliminates the variance introduced by those who did not respond to the choline treatment with increased methylation.

**FIGURE 6**
A proposed mechanism by which higher maternal choline intake results in fewer child behavior problems via altered methylation of cord and placental *NR3C1* methylation. Given the associations between MCS and *NR3C1* methylation in our sample (Pathways 1 and 2), *NR3C1* is a proxy for response to the choline treatment. The effect of MCS on *NR3C1* methylation is sensitive to various effect modifiers. Therefore, the associations between *NR3C1* methylation and child behavior problems (Pathways 3 and 4) provide a stronger analysis of the possible effects of MCS on child behavior. Pathway 5 suggests a possible mechanism by which placental NR3C1 methylation may influence fetal NR3C1 methylation.
In this plausibility analysis, NR3C1 methylation is viewed as an indicator of response to the choline treatment. In our sample, MCS caused higher average placental NR3C1 promoter methylation and higher placental NR3C1 CpG 30-32 methylation. In contrast, in cord leukocytes, MCS caused lower average NR3C1 promoter methylation (Jiang et al., 2012). Notably the pattern of effects in both placenta and cord leucocytes is opposite to that produced by early stress and other environmental adversities. Specifically, decreased placental NR3C1 methylation is associated with maternal smoking (Stroud et al., 2016a), chronic war stress (Kertes et al., 2016), and depression (Conradt et al., 2013) during pregnancy. In contrast, increased cord leukocyte NR3C1 methylation is associated with maternal chronic war stress (Kertes et al., 2016), depression (Oberlander et al., 2008), and anxiety (Hompes et al., 2013; see Palma-Gudiel et al., 2015b for review). Taken together, these results suggest that maternal choline intake may offer some protection against some of the effects of in utero stress adversity on epigenetic programming of the NR3C1 gene.

The pattern of changes in NR3C1 methylation produced by MCS are in the direction of improved behavioral and cognitive outcomes, based on prior studies evaluating methylation of this gene in relation to early life stressors. For example, higher placental NR3C1 methylation has been associated with better infant attention, self-regulation, habituation, and quality of movement (Appleton et al., 2015; Bromer et al., 2013; Stroud et al., 2016a). Second, higher placental (Stroud et al., 2016b) and lower cord (Oberlander et al., 2008) NR3C1 methylation have been associated with better infant stress-regulation. Moreover, several studies have shown that cord and
placental *NR3C1* methylation mediate the effects of in utero adversity on offspring behavior (Appleton et al., 2015; Conradt et al., 2013) and stress-regulation (Oberlander et al., 2008; Stroud et al., 2016a). Based on these results, we anticipated that higher placental *NR3C1* methylation and lower cord *NR3C1* methylation would be associated with fewer behavior problems.

Some observations in the present study are consistent with this prediction: We observed associations between *NR3C1* methylation and behavior in the anticipated directions, but only cord *NR3C1* CPG 30-32 methylation was a significant predictor of child behavior. Each 1% increase in CpG 30-32 methylation was associated with a 2.8-point increase in internalizing problems and a 3.2-point increase in anxiety. We were particularly interested in methylation at this site (CpG 30-32) because of its demonstrated ability to block *NR3C1* expression in rodent (Weaver et al., 2004) and human (McGowan et al., 2009) cell lines. *NR3C1* encodes the glucocorticoid receptor (GR). The control of fetal exposure to glucocorticoids is critical to the normal programming of the developing infant’s neural circuitry. The placenta modulates fetal exposure to maternal cortisol, and this function is linked, in part, to the activities of *CRH* and *NR3C1*. (Myatt, 2006; Paquette et al., 2016). The effects of MCS on methylation of these two genes would have the effect of reducing fetal exposure to maternal glucocorticoids, and possibly lowering HPA stress reactivity in the child.
Conclusions

The study findings suggest that increased maternal choline intake during the third trimester of pregnancy may improve offspring emotional self-control at age 7. A prior study showed that higher maternal choline intake was associated with higher placental and lower cord NR3C1 methylation, which is opposite the pattern of effects produced by prenatal adversity. In this study, we showed that these NR3C1 methylation patterns, modulated by maternal choline intake, may have functional consequences for child internalizing behaviors. NR3C1 is the most widely-studied gene in the context of epigenetic programming of the HPA-axis. To the best of our knowledge, this is the first study to assess matched cord and placental NR3C1 methylation in relationship to child behavior outcomes. In light of these findings, maternal choline supplementation may have the potential to mitigate the effects of in utero stress exposure on child behavior. Future studies are needed to replicate these results in larger cohorts and deepen our understanding of the pathways whereby maternal choline intake modulates child behavior.
REFERENCES


46. Breton, C. V. *et al.* Small-Magnitude Effect Sizes in Epigenetic End Points are Important in Children’s Environmental Health Studies: The Children’s


APPENDIX A

Recent Stressor Index*

This checklist includes events that children may find bad or upsetting. If an event DID happen to your child in the LAST 12 MONTHS, tick the box under the word ‘YES’. If the event did not happen to your child, tick the box under ‘NO’.

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Someone special to my child moved away (non-family)</td>
</tr>
<tr>
<td></td>
<td>Someone in our family was really sick or injured</td>
</tr>
<tr>
<td></td>
<td>My child was teased or bullied</td>
</tr>
<tr>
<td></td>
<td>My child’s pet died, got sick, lost or injured</td>
</tr>
<tr>
<td></td>
<td>My child was really sick or injured</td>
</tr>
<tr>
<td></td>
<td>My partner and I split up</td>
</tr>
<tr>
<td></td>
<td>My child saw something bad happen (e.g., car accident, someone being robbed)</td>
</tr>
<tr>
<td></td>
<td>Someone in our family died</td>
</tr>
<tr>
<td></td>
<td>Someone broke into our house</td>
</tr>
<tr>
<td></td>
<td>My child was in a fight (not with people in our family)</td>
</tr>
<tr>
<td></td>
<td>Someone special to my child was really sick or injured (non-family)</td>
</tr>
<tr>
<td></td>
<td>Someone special to my child (who is not in our family) died</td>
</tr>
</tbody>
</table>

*This checklist has been adapted from the “Child and Adolescent Survey of Experiences: Parent Version” developed by the Center of Emotional Health at Macquarie University. Items were selected based on: 1) Propensity to elicit internalizing behaviors in a child, and 2) outside of the child’s control (i.e., not a result of a child’s intrinsic behavior patterns). The recent stressor index score was calculated by summing the number of events for which a parent selected ‘YES’.