Special Article

Effect of supplementation with methyl-donor nutrients on neurodevelopment and cognition: considerations for future research

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Pregnancy represents a critical period in fetal development, such that the prenatal environment can, in part, establish a lifelong trajectory of health or disease for the offspring. Poor nutrition (macro- or micronutrient deficiencies) can adversely affect brain development and significantly increase offspring risk for metabolic and neurological disease development. The concentration of dietary methyl-donor nutrients is known to alter DNA methylation in the brain, and alterations in DNA methylation can have long-lasting effects on gene expression and neuronal function. The decreased availability of methyl-donor nutrients to the developing fetus in models of poor maternal nutrition is one mechanism hypothesized to link maternal malnutrition and disease risk in offspring. Animal studies indicate that supplementation of both maternal and postnatal (early- and later-life) diets with methyl-donor nutrients can attenuate disease risk in offspring; however, clinical research is more equivocal. The objective of this review is to summarize how specific methyl-donor nutrient deficiencies and excesses during pre- and postnatal life alter neurodevelopment and cognition. Emphasis is placed on reviewing the current literature, highlighting challenges within nutrient supplementation research, and considering potential strategies to ensure robust findings in future studies.

INTRODUCTION

Pregnancy and early life are critical periods of development, particularly with regard to neurocognitive endpoints, because significant brain maturation continues throughout the postnatal period. Beginning in pregnancy but continuing well into adolescence, neural systems, particularly those in the prefrontal cortex that are essential for cognition and executive function processes such as attention and cognitive flexibility, are established and refined. This significant postnatal development means that the neural systems underlying cognition remain vulnerable to adverse environmental influences, such as poor diet, alcohol or drugs of abuse, stressors, or inflammation, for a prolonged period of time. Early exposure to these environmental challenges can therefore increase the risk for a wide range of adverse neurodevelopmental outcomes, such as cognitive deficits, executive function deficits, and other social and emotional behavioral problems.[1,2]

Epigenetic modifications and their resultant changes in gene expression have been proposed as one mechanism linking poor early-life environment with long-term changes in physiology and increased disease risk. Epigenetics, meaning "on" or "above" the genome, is the study of modifications to DNA or histones that can regulate gene expression, some of which can be heritable and/or reversible.[3] In the context of poor early-life nutrition, the decreased availability of methyl-donor nutrients to the developing offspring is one mechanism

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hypothesized to link maternal malnutrition and disease risk in offspring. Animal studies indicate that supplementation of both maternal and postnatal (early- and later-life) diets with methyl-donor nutrients can attenuate disease risk in offspring. This review will first examine 1-carbon metabolism and methyl-donor nutrients and will then summarize the literature pertaining to effects of methyl-donor supplementation on cognitive endpoints in both animals and humans. It will conclude with a discussion of some of the caveats and limitations within the field and will consider opportunities for future studies to fill gaps in the current knowledge.

ONE-CARBON METABOLISM AND METHYL-DONOR NUTRIENTS

Methyltransferases receive methyl groups to perform methylation reactions through the conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine. This reaction is one in a cyclical series of biochemical reactions called 1-carbon metabolism (Figure 1). One-carbon metabolism refers to the intersection of folate and methionine metabolism cycles that donate and regenerate 1-carbon units for various reactions in the cell.[4–6] Through these reactions, cells synthesize purines, thymidylate, creatine, phosphatidylcholine, and multiple hormones. One-carbon metabolism also provides methyl groups for at least 50 different methylation reactions that occur within the cell, including methylation of RNA, DNA, histones, neurotransmitters, and other proteins. It is also involved in the catabolism of choline and histidine, the interconversion of serine and glycine, and in almost every cellular process; thus, it is important that these reactions occur efficiently.

One-carbon metabolism is highly dependent on nutritional status. In Figure 1, highlighted in gray are nutrients essential for 1-carbon metabolism, known as methyl donors. Vitamin B_{12}, vitamin B_6, vitamin B_9 (folate), methionine, betaine, choline, and zinc are all necessary for 1-carbon metabolism. These methyl-donor nutrients are critical intermediates or cofactors for enzymes involved in 1-carbon metabolism. Specifically within the brain, methyl-donor nutrients are known to be involved in multiple neurotransmitter systems, epigenetic modifications, and cellular membrane structures. For example, choline is a precursor for acetylcholine synthesis, a major neurotransmitter system involved in attention, memory, and motivation.[7–9] It is also a precursor for phosphatidylcholine, a class of phospholipids integral to the formation of biological membranes.[10] Folate is integral to the donation of methyl groups that are utilized by multiple methyltransferases.[11] DNA methyltransferases mediate the addition of methyl groups to DNA, a well-studied epigenetic

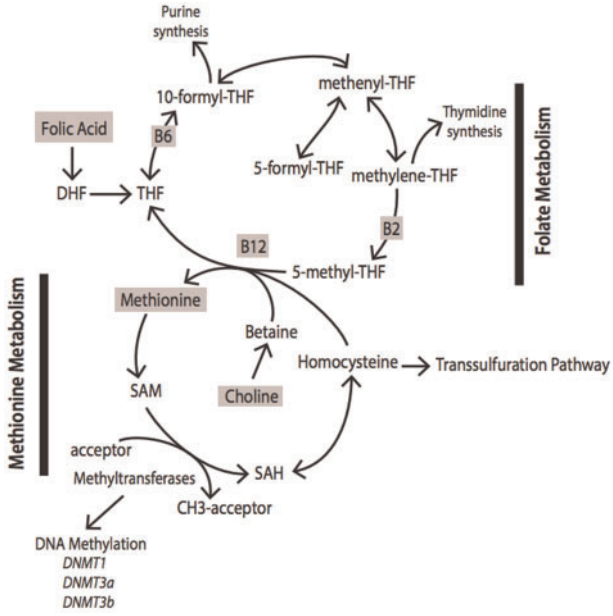

Figure 1 **Simplified schematic diagram of the intermediates, enzymes, and nutrients involved in 1-carbon metabolism**. *Abbreviations*: DHF, dihydrofolate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

modification known to mediate gene expression changes and neuronal function. Catechol-*O*-methyltransferase, another methyltransferase, utilizes methyl groups to aid in the degradation of catecholamines (dopamine, epinephrine, norepinephrine). Imbalances in methyl donors can also negatively affect methyltransferase function, *S*-adenosylmethionine regeneration, and DNA methylation.[12–17] Additionally, deficiencies and excesses of these nutrients also can alter cognition, behavior, and risk for disease.[18–24]

Folate metabolism serves to create and transfer 1-carbon units for the biosynthetic processes listed above. Natural folates in the body and diet are typically found in reduced form, mainly 5-methyltetrahydrofolate in humans.[25] The tetrahydrofolate backbone, which is the active form of folate, is used to carry and transfer 1-carbon units. One-carbon units, also known as methyl groups, are covalently bound to the 5- and 10-positions on the pteridine ring of tetrahydrofolate. Tetrahydrofolate can be maintained in 3 oxidative states, each of which plays a specific role in biosynthesis (recently reviewed by Ducker and Rabinowitz[5]). Briefly, 5,10-methylenetetrahydrofolate produces thymidine and serine, 5-methyltetrahydrofolate produces methionine, and 10-formyltetrahydrofolate produce purines, formate, and carbon dioxide.

The most abundant form of folate, 5-methyltetrahydrofolate, can be converted to methionine via a vitamin B_{12}–dependent methyltransferase in which the methyl group from 5-methyltetrahydrofolate is transferred

to homocysteine, resulting in the formation of methionine and unsubstituted tetrahydrofolate. Tetrahydrofolate can then begin the folate cycle again, and methionine can continue through its unique metabolism. Methionine is the precursor of *S*-adenosylmethionine, the universal methyl donor, as it is the final carrier of methyl groups before they are donated to the methyltransferases. Donation of a methyl group from *S*-adenosylmethionine creates *S*-adenosylhomocysteine, which is reversibly metabolized into homocysteine. Homocysteine can be remethylated to reform methionine via betaine–homocysteine methyltransferase (expressed in liver and kidney, but not found in the brain or other tissues) or methionine synthase, a vitamin B_{12}–dependent methyltransferase, to recycle through methionine metabolism. Alternatively, homocysteine can enter the transsulfuration pathway, which is required to support glutathione synthesis and remove reactive oxygen species from the brain.[26]

Methyl-donor nutrients are necessary for normal growth and development of the central nervous system (CNS). Deficiencies in methyl donors and imbalanced concentrations of methyl donors have both been linked to abnormal CNS development as well as to neurological diseases. For example, choline deficiency during gestation alters global and gene-specific DNA methylation in the developing mouse hippocampus, a region important for learning and memory.[17] Increased homocysteine is a potential risk factor for cognitive impairment and other neurological diseases.[27,28] Additionally, low vitamin B_{12} status has also been associated with poorer memory performance and reduced microstructural integrity of the hippocampus.[29] Conversely, higher folate intake has been associated with lower risk for dementia,[30] and it has been suggested that using B vitamins to help remethylate homocysteine to methionine could be one mechanism to improve cognition or to slow cognitive decline.[31]

The efficiency and reactions of 1-carbon metabolism have been shown to affect a number of CNS outcomes, including neural tube closure,[32,33] neurological diseases,[34,35] and cognition.[36] The efficiency of this cycle is highly dependent on nutritional status, and studies in both animals and humans suggest that nutritional interventions can be used for therapeutic benefit. The subsequent sections will summarize animal studies (Table 1)[37–50] and human clinical trials (Table 2)[23,30,31,51–72] linking methyl-donor supplementation with neurodevelopment and cognition.

METHYL DONORS, NEURODEVELOPMENT, AND COGNITION

Beginning at conception, methyl-donor nutrients are important for the development of the CNS, specifically for closure of the neural tube. Beyond this, emerging evidence suggests that maternal folate status throughout pregnancy affects offspring neurodevelopment, postnatal behavior, and cognition.[34] Folate concentrations may decline during pregnancy because of increased demand, larger blood volume, decreased intestinal absorption, or inadequate intake.[73] The demand for energy and nutrients required for continuous fetal development also increases during pregnancy. Fortification of grain products with folic acid, a stable oxidized form of folate, was introduced in the United States in the 1990s to help increase the amount of folate consumed by women of childbearing age to prevent the risk of neural tube and other midline defects. Since the introduction of folic acid fortification, the incidence of neural tube defects has decreased by about 25%.[32] The use of supplementation with folic acid and other methyl-donor nutrients (mainly vitamin B_{12}, choline, and methionine) as therapeutic interventions to enhance CNS development, prevent neurological disease, and slow cognitive decline has burgeoned in the last 20 years.

Maternal supplementation during pregnancy or lactation

Maternal choline availability influences offspring brain development and function (reviewed by Zeisel and Niculescu[10]). In animal models, the positive relationship between maternal choline supplementation and neurodevelopment and cognition is well established. Maternal choline supplementation enhances offspring cognition (fewer working memory errors, fewer responses to reach criterion, fewer reference memory errors, faster behavior acquisition, and shorter escape latency in the Morris water maze), prevents age-related memory decline, enhances hippocampal function,[40,74] reduces the effects of fetal alcohol exposure in the offspring,[37,38] increases levels of nerve growth factor in the hippocampus and frontal cortex,[75] and normalizes choline deficiency–induced DNA hypermethylation.[76] Choline supplementation can also reverse both the effect of folate deficiency on neurogenesis and apoptosis at E17 in the fetal forebrain[77] and iron deficiency–induced reprogramming within the hippocampus.[78] In a mouse model of Down syndrome, maternal supplementation with choline attenuated degeneration of the basal forebrain cholinergic neuron system,[79] while in a mouse model of autism, high maternal choline consumption improved anxiety-like behaviors and increased social interaction[41] (for review, see Jiang et al[80]). Because of the beneficial findings in rodent models, effects in humans are being investigated. The current results are mixed. Choline levels in early second-trimester maternal plasma are positively correlated with early cognitive development[51] as well as with cognitive

Table 1 **Animal studies linking methyl-donor supplementation with neurodevelopment and cognition**

Reference	Behavior	Supplementation period	Nutrient(s) supplemented	Outcomes
Thomas et al. (2009)[37]	Motor coordination and balance, open field test, spatial learning, working memory	Pregnancy	Choline	Choline supplementation reduced effects of prenatal ethanol exposure; increased brain weight; and normalized righting reflex, motor coordination, and negative geotaxis
Thomas et al. (2010)[38]	Spontaneous alternation, parallel bar motor coordination, Morris water maze, spatial working memory	Pregnancy	Choline	Choline supplementation may reduce severity of fetal alcohol effects. It increased rates of spontaneous alternation; increased memory retention
Naninck et al. (2016)[39]	Elevated plus maze, object recognition task, object location task, Morris water maze, and T maze	Pregnancy	Folic acid, vitamin B_{12}, vitamin B_6, choline, betaine, zinc, and methionine	Supplementation normalized object recognition but did not change object location or performance on Morris water maze
Zhu et al. (2016)[40]	Barnes maze test	Pregnancy	Choline	Choline supplementation decreased spatial learning deficits
Langley et al. (2015)[41]	Open field test, elevated plus maze, marble burying test	Pregnancy + lactation	Choline	Choline supplementation lowered anxiety levels
Carlin et al. (2013)[42]	Fat and sucrose preference	Pregnancy + lactation	Folic acid, vitamin B_{12}, choline, betaine, zinc, and methionine	Supplementation decreased palatable food preference
Bahous et al. (2017)[43]	Open field test, ladder beam test, novel object recognition test, Y-maze	Pregnancy + lactation	Folic acid	Excessive folic acid supplementation impaired short-term memory; no difference observed in movement score, anxiety, or spatial memory
McKee et al. (2017)[44]	5-choice serial reaction time task	Early life (3–6 wk)	Folic acid, vitamin B_{12}, choline, betaine, zinc, and methionine	Methyl-donor supplementation increased motivation, enhanced operant learning, and shortened reaction time needed to perform correct responses
Carletti et al. (2012)[45]	Open field test and inhibitory avoidance task	Early life (PD7–30)	Folic acid	Folic acid supplementation reversed neonatal hypoxia-ischemia–induced anxiogenic effects and impaired aversive memory
Sittig et al. (2012)[46]	Morris water maze	Early life (PD30–60)	Folic acid	Excessive folic acid led to deficits in motivation and spatial memory
Paternain et al. (2016)[47]	Open field test, forced swim test, novel object recognition task	Adult (8–26 wk)	Folic acid, vitamin B_{12}, betaine, and choline	Methyl-donor supplementation reversed early-life–induced depression in rats
Singh et al. (2011)[48]	Active avoidance, passive avoidance, and elevated plus maze	Adult (6, 11, 16 mo)	Folic acid	Folic acid supplementation for 8 wk improved memory in behavioral tasks

(continued)

Table 1 **Continued**

Reference	Behavior	Supplementation period	Nutrient(s) supplemented	Outcomes
Brocardo et al. (2010)[49]	Open field test	Adult (3–4 mo)	Folic acid	Folic acid supplementation decreased hyperlocomotion in a rodent model of bipolar disorder
McKee et al. (2017)[44]	Progressive ratio task	Adult (32 wk)	Folic acid, vitamin B_{12}, choline, betaine, zinc, and methionine	Methyl-donor supplementation increased motivation
Barua et al. (2014)[50]	Ultrasonic vocalization (P2, P4, P6), social approach task, trace fear conditioning, open field test (2–8 mo)	Pregnancy, lactation, and postweaning	Folic acid	Folic acid supplementation during both gestational development and postweaning period increased anxiety-like behavior, ultrasonic vocalization, and hyperactivity

Abbreviation: PD, postnatal day.

development at 7 years.[23] However, neither supplementing with phosphatidylcholine[59] nor maternal cord blood choline concentrations[53] correlated with enhanced infant cognition (for review, see McCann and Ames[74] and Jiang et al[80]).

In contrast to the link between maternal choline supplementation and neurodevelopment, the positive link between maternal folic acid supplementation and neurodevelopment and cognition is stronger in humans than it is in animal models. In humans, maternal folic acid supplementation is associated with improved verbal, motor, and verbal-executive scores in 4-year-olds.[54] Maternal use of folic acid supplements is associated with reduced risk of severe language delay in children at 3 years of age.[55] Higher plasma folate during week 30 of pregnancy is associated with increases in learning, long-term storage, and memory retrieval and a decrease in inattention at 10 years of age.[56] Folic acid intake during the first trimester is associated with reduced language delay, increased communication and verbal skills, and increased cognitive performance.[55,57,58] Furthermore, supplementation with folic acid during the second and third trimesters decreased the levels of homocysteine in maternal and cord blood, although outcomes in offspring were not studied.[81] In animal models, offspring from rodents deficient in methyl donors during pregnancy but supplemented with folic acid during late gestation had reduced risk for structural and functional defects in the CNS during the prenatal period.[82] Additionally, reduced intake of folate in pregnant mice impaired short-term memory in offspring and increased apoptosis in the hippocampus.[83] Folic acid supplementation in late gestation restored expression of specific microRNAs in the brain in methyl donor–deficient animals.[82] Expression of a number of genes related to neuronal function is altered in the brain of offspring after folic acid supplementation.[50]

In naturally occurring food sources, a combination of multiple methyl-donor nutrients is typically found within a single food. Additionally, methyl-donor nutrients work in concert with each other within 1-carbon metabolism. Therefore, supplementation with mixtures of methyl-donor nutrients, as opposed to supplementation with single nutrients, has been examined as well. Children of mothers supplemented with a mixture of micronutrients that included multiple methyl-donor nutrients had increased procedural memory and higher general intellectual ability at 10 years of age.[60] A study of maternal methyl-donor nutrient consumption found positive associations between folic acid intake and cognition in children at 3 years, but no associations were found for choline, betaine, or methionine consumption.[58] In rodents, methyl-donor supplementation during pregnancy and lactation increased DNA methylation in the brain[42,84] and normalized the increased preference for palatable foods observed in offspring from high-fat diet-fed dams.[42] Postnatal supplementation with multiple methyl-donor nutrients during lactation (postnatal day 2–9) restored methionine levels and reduced early-life-stress–induced cognitive impairments in adult animals.[39] Additionally, maternal micronutrient imbalance (excessive folic acid and/or vitamin B_{12} deficiency) leads to global DNA hypomethylation and reduced expression of neurotrophins in the brain of offspring.[14,85]

Early postnatal supplementation

Supplementation with methyl-donor nutrients during early life (infancy, childhood, or adolescence) is

Table 2 **Clinical studies in humans linking methyl-donor supplementation with neurodevelopment and cognition**

Reference	Behavioral test	Supplementation period	Nutrient(s) supplemented or analyzed	Outcomes
Boeke et al. (2013)[23]	Offspring visual memory and intelligence	Pregnancy (1st and 2nd trimesters)	Analyzed: choline, vitamin B_{12}, betaine, and folate (maternal intake questionnaire)	Increased gestational choline intake was associated with better visual memory at age 7; no associations for other methyl donors
Wu et al. (2012)[51]	Bayley Scales of Infant Development, Third Edition	Pregnancy (2nd trimester)	Analyzed: choline, betaine, dimethylglycine, methionine, homocysteine, cysteine, vitamin B_{12}, holotranscobalamin, and folate (maternal plasma)	Maternal plasma free choline and betaine in the first half of pregnancy associated with increased infant cognitive test scores
Valera-Gran et al. (2014)[52]	Bayley Scales of Infant Development (1 y)	Pregnancy	Analyzed: folic acid (maternal intake questionnaire)	Children from mothers who consumed over 5000 μg of folic acid daily had lower psychomotor scores and increased risk for delayed psychomotor development
Signore et al. (2008)[53]	Intelligence quotient (5 y)	Pregnancy	Analyzed: choline (maternal cord blood)	No correlations found
Julvez et al. (2009)[54]	McCarthy Scale of Children's Abilities (4 y)	Pregnancy (1st trimester)	Analyzed: folic acid supplementation (maternal intake questionnaire)	Reported folic acid supplement use during pregnancy was associated with improved neurodevelopment and decreased inattention symptoms
Roth et al. (2011)[55]	Children's language competency (3 y)	Pregnancy (4 wk before conception to 8 wk after conception)	Analyzed: folic acid supplementation (maternal intake questionnaire)	Folic acid supplementation during early pregnancy was associated with reduced risk of severe language delay
Veena et al. (2010)[56]	Cognitive function assessment—memory, attention and fluid reasoning (9–10 y)	Pregnancy (30 wk)	Analyzed: folic acid, vitamin B_{12} and homocysteine (maternal plasma)	Increased folic acid concentrations associated with increased cognitive test scores; no associations were seen with vitamin B_{12} or homocysteine
Chatzi et al. (2012)[57]	Bayley Scales of Infant Development, Third Edition (18 mo)	Pregnancy (14–18 wk)	Analyzed: folic acid supplementation (maternal intake questionnaire and RBC concentration)	Folic acid supplementation (5 mg/d) was associated with enhanced vocabulary development, communication skills, and verbal comprehension
Villamor et al. (2012)[58]	Peabody Picture Vocabulary Test III and Wide Range Assessment of Visual Motor Abilities (3 y)	Pregnancy (1st and 2nd trimesters)	Analyzed: folic acid, vitamin B_{12}, choline, betaine, and methionine (maternal intake questionnaire)	Higher folate intake in 1st trimester was associated with higher receptive language; weak inverse association between vitamin B_{12} intake during 2nd trimester and receptive language; no association with choline, betaine, or methionine
Cheatham et al. (2012)[59]	Short-term visuospatial memory, long-term episodic memory, language development, and global development	Pregnancy + lactation (18 wk gestation to 90 d post partum)	Supplemented: phosphatidylcholine	No effects seen

(continued)

Table 2 **Continued**

Reference	Behavioral test	Supplementation period	Nutrient(s) supplemented or analyzed	Outcomes
Prado et al. (2017)[60]	General intellectual ability, declarative memory, procedural memory, executive function, academic achievement, fine motor dexterity, and socioemotional health (9–12 y)	Pregnancy + lactation (pregnancy + 3 mo post partum)	Supplemented: multiple micronutrients (iron, folic acid, retinol, vitamins D, E, C, B_1, B_2, B_6, B_{12}, zinc, copper, selenium, and iodine) or iron and folic acid	Supplementation with multiple micronutrients increased child cognitive development more than that with iron and folic acid alone
Ross et al. (2016)[61]	Child Behavior Checklist (40 mo)	Pregnancy + lactation (2nd trimester to 3 mo)	Supplemented: phosphatidylcholine	Phosphatidylcholine supplementation decreased attention problems and social withdrawal
Nguyen et al. (2016)[62]	Memory, executive function, attention, and hyperactivity	Early life (5–10 y)	Supplemented: choline (6 wk)	No effects seen
Strain et al. (2013)[63]	Finger tapping test, Preschool Language Scale–Revised, Woodcock-Johnson Test of Scholastic Achievement, Child Behavior Checklist, and Kaufman Brief Intelligence Test	Early life (5 y)	Analyzed: choline, betaine, dimethylglycine, methionine, and homocysteine (plasma)	No associations found
Rauh-Pfeiffer et al. (2014)[64]	HAWIVA-III, WPPSI-III, Kaufman Assessment Battery for Children	Early life (4–6 y)	Supplemented: folic acid, riboflavin, pyridoxine, cobalamin, and calcium lactate pentahydrate (3 mo)	Supplementation decreased plasma homocysteine concentration, but no changes were seen in cognitive performance
Lefèvra-Arbogast et al. (2016)[30]	Dementia	Adult	Analyzed: folic acid, vitamins B6 and B_{12} (24-h recall)	Higher folate intakes associated with decreased risk of dementia; no associations found for vitamin B6 or B_{12}
Morris et al. (2010)[65]	Wechsler Adult Intelligence Scale III	Adult ($\geq$ 60 y)	Analyzed: folic acid, vitamin B_{12}, and 5-MeTHF (serum)	Normal vitamin B_{12} status together with higher serum 5MeTHF associated with higher cognitive test scores
Morris et al. (2007)[66]	Wechsler Adult Intelligence Scale III	Adult ($\geq$ 60 y)	Analyzed: folic acid and vitamin B_{12} (serum)	Low vitamin B_{12} together with high serum folate associated with cognitive impairment; normal vitamin B_{12} status together with high serum folate associated with protection against cognitive impairment
Wald et al. (2010)[67]	Cognitive function test scores (3 y from start of treatment)	Adult ($\geq$45 y)	Analyzed: meta-analysis of 9 placebo-controlled randomized trials, supplementing folic acid with or without other B vitamins	No associations found
Durga et al. (2007)[68]	Performance for memory, sensorimotor speed, complex speed, information processing speed, and word fluency	Adult (50–70 y)	Supplemented: folic acid (3 y)	3-y changes in memory, information processing speed, and sensorimotor speed were significantly better in the folic acid–supplemented group

(continued)

Table 2 **Continued**

Reference	Behavioral test	Supplementation period	Nutrient(s) supplemented or analyzed	Outcomes
Walker et al. (2012)[69]	Telephone Interview for Cognitive Status–Modified, the Brief Test of Adult Cognition by Telephone, and the Informant Questionnaire on Cognitive Decline in the Elderly	Adult (60–74 y)	Supplemented: folic acid and vitamin B_{12} (2 y)	Long-term supplementation of daily oral folic acid and vitamin B_{12} improved immediate and delayed memory performance; no differences seen in orientation, attention, semantic memory, processing speed, or informant reports
de Koning et al. (2016)[70]	Geriatric Depression Scale	Adult ($\geq$ 65 y)	Supplemented: folic acid and vitamin B_{12} (2 y)	No changes in depressive symptoms observed
Zhang et al. (2017)[31]	MMSE	Adult ($\approx$ 75 y)	Analyzed: meta-analysis of 77 studies, supplementing folic acid, and vitamin B_{12} and/or vitamin B_6 (supplementation length varied from 6 to 18 mo)	No associations found
Clarke et al. (2014)[71]	Specific cognitive domains and MMSE	Adult ($\approx \geq$60 y)	Analyzed: meta-analysis of 11 studies, supplementing B vitamins (supplementation length varied from 0.3 to 7 y)	No associations found
Porter et al. (2017)[72]	MMSE, RBANS, and the Frontal Assessment Battery	Adult ($\geq$ 60 y)	Analyzed: folate, vitamins B_{12}, B_6, B_2, and homocysteine (at 5-y follow-up)	Lower vitamin B_6 status was associated with accelerated rate of cognitive decline as assessed by RBANS and MMSE; lower vitamin B_2 status was associated with accelerated rate of cognitive decline as assessed by RBANS, but not MMSE

Abbreviations: HAWIVA-III, Hannover-Wechsler-Intelligenztest für das Vorschulalter; MMSE, Mini Mental State Examination; RBC, red blood cell; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; WPPSI-III, Wechsler Preschool and Primary Scale of Intelligence, 3rd edition; 5-MeTHF, 5-methyltetrahydrofolate.

relatively understudied, as the majority of supplementation studies have focused on supplementation during pregnancy or later life. Infants supplemented with choline were shown to have fewer attention problems and less social withdrawal at 40 months.[61] However, if administered at a later time point (to school-aged children), 6 weeks of choline supplementation did not improve cognition.[62] Similarly, 3 months of mixed B vitamin supplementation in kindergarten children improved folate and homocysteine status but did not affect cognitive performance.[64] However, betaine status, but not choline status, in 5-year-olds was positively associated with total language scores.[63] In rodent models, rat pups deficient in folate from birth to 3 weeks of age exhibited deficits in long-term memory, spatial learning, and set-shifting.[86] Three weeks of 90% methyl-donor depletion increased depression-like behaviors in the forced swim test, while 3 weeks of methyl-donor supplementation increased DNA methylation in the amygdala and reduced depression-like behaviors.[87] Similarly, 3 weeks of folic acid supplementation prevented hypoxia-ischemia–induced anxiogenic and memory impairments, possibly by partial recovery of Na^+, K^+-ATPase in the frontal cortex.[45] Finally, early-life postnatal supplementation with methyl-donor nutrients during weeks 3 to 6 of life in female mice increased motivation and learning in an operant chamber task.[44] Collectively, these studies demonstrate that methyl-donor supplementation can affect neurodevelopment and cognition beyond the well-established critical period of pregnancy; however, studies in humans indicate the window of opportunity for intervention may be reduced by the time children have reached school age.

Supplementation in adults

Memory loss, increased neurological disease risk, and increased oxidative stress in the brain are characteristic

of aging adults. In aged rats, 8 weeks of folic acid supplementation improved memory and decreased lipid peroxidation, a marker of aging, in multiple brain regions.[48] In adult rats, 7 days of folic acid treatment decreased locomotor activity and lipid peroxidation in the hippocampus in a pharmacological model of manic behavior.[49] Furthermore, 1 week of methyl-donor supplementation in mice increased motivation to respond in an operant task, although only if animals had been exposed to a high-fat diet during pregnancy/lactation or had been previously exposed to methyl-donor nutrients early in life.[44] Lastly, 18 weeks of dietary methyl-donor supplementation normalized depression-like behaviors induced by maternal separation during lactation.[47]

While studies of methyl-donor supplementation in aged animals are limited, the use of methyl-donor nutrients in clinical trials is extensive. The results, however, are mixed. A meta-analysis examined whether 6 months of folic acid supplementation, with or without other B vitamins, altered cognitive function (ie, memory speed, language, and executive function) in healthy individuals and found no changes in cognition.[67] However, a study of 3 years of folic acid supplementation in healthy individuals reported higher scores for memory, information processing speed, and sensorimotor speed.[68] In a trial of 2-year supplementation with folic acid and B_{12}, improved cognitive functioning, including improved immediate and delayed memory performance, was observed.[69] However, in 2 separate trials, 2 years of B_{12} and folic acid supplementation in older adults did not reduce depressive symptoms.[70,88] A recent meta-analysis identified 77 human trials designed to examine whether B vitamin supplementation reduced homocysteine levels and improved cognition in elderly patients with Alzheimer disease or dementia.[31] After filtering and selection, 4 randomized controlled trials were included in the analysis. With the Mini-Mental State Examination score used as a measure of cognition, the meta-analysis found that B vitamins do reduce homocysteine levels; however, there was no overall improvement in cognition in these patients.[31] Similarly, another meta-analysis of 11 studies involving 22 000 study participants failed to find an effect of B vitamin supplementation on cognition, even though verified reductions in homocysteine and increases in folate were observed.[71] Future longitudinal studies may focus on whether these B vitamins could perhaps slow the progression of cognitive decline or be efficacious in select clinical populations.[89] Additionally, a study showed that lower concentrations of vitamin B_6 and riboflavin (B_2) were associated with an accelerated rate of cognitive decline as assessed by the Repeatable Battery for the Assessment of Neuropsychological Status, but the same association with vitamin B_2 was not found when the Mini-Mental State Examination was used to measure cognition.[72]

Adverse effects of supplementation

The Swedish physician Paracelsus said, "The dose makes the poison," which is a basic principle of toxicology and applies equally to the study of dietary supplementation. Owing to both supplementation of processed food products and adherence to dietary recommendations for nutrient consumption, 5% of the US adult population and between 30% and 66% of children aged 1 to 13 years consume more than the recommended daily dose of methyl vitamins.[90,91] As this subset of the population emerged, studies began showing the detrimental effects of excessive methyl-donor supplementation. The current recommendation for folic acid intake is 400 µg/d for adult males and nonpregnant females. The recommendation increases to 600 µg/d for pregnant females and 500 µg/d for lactating females. Offspring of women who consumed higher-than-recommended amounts of folic acid during pregnancy (5000 µg/d, almost 10 times the recommended daily allowance) had lower psychomotor scores at 1 year of age.[52] In mice, offspring of dams fed high levels of folic acid (10 times the control, 20 mg/kg of diet) during gestation displayed altered development of the cortical layers at E17.5 and short-term memory impairment and decreased hippocampal size at 3 weeks.[43] High-vitamin diets during pregnancy have been shown to increase the risk of metabolic syndrome in offspring by altering the neural pathways involved in energy homeostasis and feeding,[92,93] but this could be prevented by providing a postweaning diet high in multivitamins or folic acid.[94] Excessive folic acid intake (4 times the control, 8.0 mg/kg of diet) during adolescence has been correlated with deficits in motivation and spatial memory tasks.[46] Another factor that affects response to methyl-donor supplementation is genetic variations in the enzymes that metabolize the nutrients, such as methylenetetrahydrofolate reductase (MTHFR). In dams with a MTHFR deficiency, methyl-donor supplementation was associated with embryonic delay and growth retardation at E10.5 in their offspring, but longer-term outcomes were not studied.[95] It is evident that a genetic and environmental interaction exists in which concentrations of methyl-donor nutrients are no longer healthful, but are harmful. Studying these interactions remains critical (reviewed by Shorter et al[96]).

CONSIDERATIONS FOR FUTURE RESEARCH

It is clear that both excessive and inadequate levels of methyl levels are detrimental to neurodevelopment and

cognition. Further research is needed to understand and standardize which nutrients to provide supplementally, which concentrations of each nutrient are appropriate, how long a supplement should be taken, and which populations should receive supplementation as well as to determine whether methyl-donor supplementation can alter CNS development and long-term cognition. Many of the basic research studies point to methyl-donor supplementation as a way to modulate cognition, but the current clinical literature is inconclusive.[97] A number of experimental variables need to be thoughtfully considered to ensure the robustness of research in this field. The focus here is on animal research, but similar considerations are applicable to human clinical research as well.

Single-nutrient vs mixed-nutrient supplementation

In reviewing the current supplements used to enhance brain health, many studies promote single-nutrient supplementation while others tout a mixed-nutrient approach. The mixed-nutrient approach may be more relevant to what is found in naturally occurring food sources (which contain multiple methyl donors). Additionally, there is the unique relationship between folate deficiency and vitamin B_{12} deficiency, wherein the surplus of one may mask a deficiency of the other (ie, excess folate/folic acid may mask a vitamin B_{12} deficiency). Generally, cellular folate is present in low concentrations; therefore, regenerating tetrahydrofolate from 5-methyltetrahydrofolate is extremely important for 1-carbon metabolism and regulation of cellular homocysteine levels. This regeneration can occur in 2 ways. One is dependent on the availability of vitamin B_{12} to act as a coenzyme for methionine synthase. When vitamin B_{12} is not present, the cell has increased concentrations of both 5-methyltetrahydrofolate, which has been shown to impair DNA and RNA synthesis,[98] and homocysteine, high levels of which have been observed in many neurological disorders.[5,24,35,99] Folic acid supplementation has been shown to decrease the homocysteine concentration, the biomarker routinely used to determine cellular levels of vitamin B_{12} and folic acid. However, when high folate levels exist together with low vitamin B_{12} levels, individuals present with anemia, macrocytosis, and cognitive impairment.[65,66,100] Therefore, a mixed-nutrient approach to methyl-donor supplementation may mitigate these masking effects.

One-carbon metabolism and the brain

The liver is responsible for the vast majority of metabolic processes in the body. Therefore, 1-carbon metabolism has largely been functionally characterized within the liver. It is widely thought that these methyl-donor nutrients are metabolized by the liver, and then metabolites are transported by the blood to be distributed throughout the body, passing through the blood–brain barrier to reach the brain. However, because enzymes that mediate 1-carbon metabolism are present within the brain, this would suggest that local 1-carbon metabolism can also occur within the brain.

Even though 1-carbon metabolism in the liver is well studied and research to date has provided an understanding of how perturbations such as diet or disease alter this metabolism, 1-carbon metabolism in the brain has not been well characterized. It is thought, on the basis of increased homocysteine concentrations in plasma and cerebrospinal fluid, that 1-carbon metabolism in the brain is disrupted in various neurological diseases such as Alzheimer disease, depression, and schizophrenia.[101–104] This relies, however, on the assumption that peripheral metabolite levels are reliable biomarkers of brain levels, which has not yet been demonstrated. Importantly, a few functional differences between 1-carbon metabolism in the brain and 1-carbon metabolism in the liver have been found. As recently as 2006, there remained controversy about whether the brain contained a functional transsulfuration pathway, important for the elimination of reactive oxygen species.[26] Because the brain consumes a relatively high level of oxygen, effective strategies for the elimination of high levels of reactive oxygen species are necessary. Possible impairments to the brain transsulfuration pathway are found both in patients with Alzheimer disease and in those with Parkinson disease.[26] Furthermore, levels of cystathionine are higher in the brain than in other organs, while γ-cystathionase activity in the brain is 100-fold lower than that in the liver. It is now known that the brain contains a functional transsulfuration pathway, but these 2 functional differences provide evidence that this pathway may function differently in the brain than in the liver.

Additionally, the brain lacks the enzyme betaine-homocysteine methyltransferase.[24] Therefore, it lacks the ability to utilize betaine to resynthesize methionine from homocysteine, a pathway present in the liver. This may indicate that the brain, compared with the liver, has increased reliance on the presence of vitamin B_{12} to aid in the maintenance of cellular methionine levels. There is evidence that the brain is extremely resilient when nutrient deficiencies are present. One study found that, when rats were fed a folate-free diet, total folate concentrations in the liver and kidney decreased by 60%, but levels of folate in the brain remained the same throughout the study.[105] Additionally, a recent study showed that, when folate deficiency was induced, the

brain—as compared with plasma, erythrocytes, liver, kidney, and spleen—maintained folate metabolite levels better than the other organs.[106] These studies indicate a possible maintenance system or protective mechanism present in the brain but not in other organs. One potential mechanism could involve folate-binding proteins, which are expressed at high levels within the choroid plexus[107,108] and may contribute to higher folate levels in the brain at the expense of levels in other tissues. This same phenomenon may also occur in other organs with high expression of folate-binding proteins, such as the placenta,[109,110] which would result in folate being provided to the offspring at the expense of the mother. However, it has also been shown that vitamin B_{12} levels in the brain decreased during dietary folate deficiency.[111] Current gaps in the literature include a lack of studies evaluating brain levels of 1-carbon metabolites in response to dietary changes. There is also a need to determine whether plasma levels of metabolites reflect changes in brain levels, since findings of an association could support the potential use of plasma levels as a biomarker for brain dysfunction.

It is well known that the brain is composed of multiple cell types, including neurons, astrocytes, microglia, and endothelial cells, that work coordinately. Functional differences in various cell types could alter 1-carbon metabolism.[5] For example, the reliance on glucose to provide serine for 1-carbon metabolism is higher in the brain than in other organs, since serine does not easily pass through the blood–brain barrier.[5] Additionally, the lack of betaine-homocysteine methyltransferase in the brain alters 1-carbon metabolism by eliminating the ability to utilize betaine directly to remethylate homocysteine. Neurons, glia, and astrocytes could also conceivably utilize the same nutrients in different ways because of their functional differences. One possible example is the increased reliance of the brain on astrocytes and oligodendrocytes to mediate transsulfuration pathway reactions. The brain consumes a disproportionate amount of the body's oxygen and therefore must eliminate copious amounts of reactive oxygen species. Synthesis of glutathione is critical to this elimination. In turn, glutathione synthesis is dependent on the cysteine concentration. Excitatory amino acid transporter 3, the main transporter of extracellular cysteine, has been found in greater densities on astrocytes and oligodendrocytes,[112] possibly indicating increased participation of these 2 cell types in transsulfuration pathway–mediated elimination of reactive oxygen species. Further characterization of the roles each cell type plays in brain 1-carbon metabolism will be important in developing a full appreciation of the role of 1-carbon metabolism in the brain.

Influence of sex on outcomes

The pervasiveness of sex differences in the brain and neurological disorders warrants a discussion in this context. While investigations of sex differences are increasingly included in preclinical research and are typically included in the human clinical literature, analysis of both sexes remains underrepresented in the current animal literature. Only 4 of the 14 studies shown in Table 1 included a comparison between sexes, while 8 focused on a single sex, 1 studied combined sexes without analysis, and another did not report the sex of the animals. Sex differences within 1-carbon metabolism have not been extensively examined, although some reports suggest that sex is an important variable with regard to the study of 1-carbon metabolism and brain function. Sex was found to influence vitamin B_{12} and 1-carbon–related enzymes as well as plasma homocysteine levels in the fetal liver, and sex moderated the effect of maternal smoking on these outcomes.[113] Plasma levels of homocysteine were found to correlate with some brain metabolite levels (as determined through imaging) in elderly females, but not males.[114] Furthermore, in an animal study involving supplementation of high-dose folic acid to pregnant dams, the expression of transcription factors related to neuronal function in whole brain samples was oppositely affected in males (decreased) compared with females (increased).[115] In addition, in a mouse model with a deletion of the *Mthfr* gene, early-life manipulations, either neonatal stress[116] or administration of a γ-aminobutyric acid–potentiating drug,[117] were found to have sex-specific effects on behavior.

Sex differences in DNA methylation patterns in the brain have also been noted. At postnatal day 1, female rats were found to have significantly higher activity of DNA methyltransferase 1 as well as an increase in DNA methylation[118] in the preoptic area of the hypothalamus. Sex differences in the expression of genes that either increase DNA methyltransferases[119] or decrease (Gadd45b) DNA methylation[120] have been demonstrated in the amygdala, and sex differences in promoter-specific DNA methylation of the *bdnf* gene have been observed within the prefrontal cortex.[121] It has been well characterized that sex alters the prevalence of many neurological disorders, including Parkinson disease, Alzheimer disease, cognitive decline, autism spectrum disorders, mood and anxiety disorders, major depression, trauma-related disorders, depressive disorders, autoimmune disease affecting the nervous system, attention-deficit/hyperactivity disorder, and neurodegenerative disorders.[122] Collectively, these findings suggest that understanding sex differences in 1-carbon metabolism in the brain, both at baseline and

in response to methyl-donor supplementation, may increase the current understanding of sex differences in neurodevelopmental outcomes.

In mammals, the presence of *SRY* on the male Y chromosome drives the development of the testis. Gonadal sex hormones (androgens, estrogens, and progestins) then drive the sexual differentiation. During development, the male brain is exposed to a testosterone surge, which is converted to estradiol. At the same time, in females, the estradiol levels remain low.[123] Within the brain, these sex hormones are known to influence neuroanatomy, neurochemistry, and neuron structure.[122] One interesting difference between the sexes is the growth speed of the cortex. During development, cortical volume increases faster in males than in females.[124] This growth difference is hypothesized to drive, in part, the predisposition in males for autism spectrum disorders.[125] Additionally, during development, maternal care, estrogen receptor expression, and neonatal hormone exposure all have been shown to correlate with sex-specific differences in DNA methylation, which in turn can influence development and behavior.[126]

Sex hormones can also have effects during adulthood, when females are exposed to high levels of estradiol and progesterone and males are exposed to high levels of testosterone. To add complexity, the female estrus cycle alters the levels of the female sex hormones, which is known to change behavior.[127–131] Additionally, steroid hormone receptors, which can be found in different densities throughout the brain, are known to act as transcription factors and influence epigenetic machinery (coactivators and corepressors).[123,126] Interestingly, there is even a sex-specific difference in the expression of these coactivators.[132] Monitoring the estrus cycle would inform whether observed sex differences remain throughout the entire estrus cycle or are stronger at one point versus another, while testing at a standard stage of the estrus cycle may decrease the variance that could be observed in female study participants. Lastly, the presence of sex chromosomes, or genetic sex (XX vs XY), can also influence neurodevelopment and sexual differentiation in the brain (reviewed by McCarthy and Arnold[133]). Thus, genetic sex and the presence of sex hormones during development and throughout adulthood both have the potential to influence the physiological endpoints of interest. The inclusion and analysis of both sexes will prove essential to understanding how supplementation and dietary interventions alter neurodevelopment and cognition.

CONCLUSION

The current literature suggests that dietary methyl-donor supplementation is a promising therapeutic strategy with the potential to improve or protect cognition and neurodevelopment in vulnerable populations. Earlier diagnosis of nutrient deficiencies and excesses, understanding critical periods for intervention, and identification of which populations would most benefit from supplementation could all help to increase the efficacy of supplementation. Ensuring robustness in animal studies through the careful consideration of diet composition, tissue and cellular specificity, and sex differences will significantly advance the understanding of how methyl-donor supplementation affects neurodevelopment on a mechanistic level as well as increase the potential for translation to at-risk human populations.

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