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Early Manifestations of Brain Aging in Mice Due to Low Dietary Folate and Mild MTHFR Deficiency

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Abstract

Folate is an important B vitamin required for methylation reactions, nucleotide and neurotransmitter synthesis, and maintenance of homocysteine at nontoxic levels. Its metabolism is tightly linked to that of choline, a precursor to acetylcholine and membrane phospholipids. Low folate intake and genetic variants in folate metabolism, such as the methylenetetrahydrofolate reductase (*MTHFR*) 677 C>T polymorphism, have been suggested to impact brain function and increase the risk for cognitive decline and late-onset Alzheimer's disease. Our study aimed to assess the impact of genetic and nutritional disturbances in folate metabolism, and their potential interaction, on features of cognitive decline and brain biochemistry in a mouse model. Wild-type and *Mthfr*$^{+/-}$ mice, a model for the *MTHFR* 677 C>T polymorphism, were fed control or folate-deficient diets from weaning until 8 and 10 months of age. We observed short-term memory impairment measured by the novel object paradigm, altered transcriptional levels of synaptic markers and epigenetic enzymes, as well as impaired choline metabolism due to the *Mthfr*$^{+/-}$ genotype in cortex or hippocampus. We also detected changes in mRNA levels of *Presenillin-1*, neurotrophic factors, one-carbon metabolic and epigenetic enzymes, as well as reduced levels of *S*-adenosylmethionine and acetylcholine, due to the folate-deficient diet. These findings shed further insights into the mechanisms by which genetic and dietary folate metabolic disturbances increase the risk for cognitive decline and suggest that these mechanisms are distinct.

Keywords Aging · Brain · Choline · Epigenetics · Folate · MTHFR

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Introduction

In the last decades, the proportion of older individuals has been dramatically increasing, and the number of people over 60 years of age is expected to double by 2050, reaching approximately 2.1 billion individuals. It is estimated that 150 million of these individuals will be affected by cognitive decline and neurodegenerative disorders such as Alzheimer's disease (AD). Aging is considered a major risk fcactor for these chronic diseases [1]. The cells of the nervous system are particularly affected by aging, which changes the brain structurally and functionally. These changes include but are not limited to the following: loss of synaptic function and plasticity, decreased expression of neurotrophic factors, global downregulation of DNA methylation, dysregulation of histone acetylation, and breakdown of myelin, which ultimately facilitate and accelerate cognitive impairment and susceptibility to degenerative disorders [2, 3]. Characteristic pathological changes of the natural aging process are also present in AD but with a different severity. Thus, some investigators support the

idea of a continuum from brain aging to AD, which is susceptible to genetic, epigenetic, and environmental influences [4].

AD is an age-related neurodegenerative disorder characterized by progressive memory impairment, loss of cognitive function, and deposition of intracellular neurofibrillary tangles (phosphorylated Tau protein filaments) and extracellular senile plaques (amyloid beta (Aβ)). Late-onset Alzheimer's disease (LOAD) is the most common form of AD, occurring sporadically in individuals over 65 years of age [5]. Converging evidence suggests that the pathophysiological process of LOAD starts decades before the first clinical symptoms, and there is no known causative factor [6]. However, several genetic and environmental factors can increase the risk of the disease [7, 8], including low folate levels and the genetic variant in methylenetetrahydrofolate reductase (*MTHFR*), *MTHFR* 677C>T (A222V) [9–12].

Folate is an essential B vitamin involved in many biochemical processes. It is critical for optimal brain function since it is required for nucleotide and neurotransmitter synthesis, DNA and histone methylation reactions, myelin synthesis, and maintenance of homocysteine at non-toxic levels [13, 14]. 5-Methyltetrahydrofolate (methylTHF), the main circulatory form of folate, is used to remethylate homocysteine to methionine, which is the precursor of *S*-adenosylmethionine (SAM), the primary methyl donor in most mammalian reactions. *MTHFR* is an important gene in folate metabolism; it is ubiquitously expressed and generates methylTHF for methionine and SAM synthesis. The *MTHFR* 677C>T polymorphism is present at 10–15% homozygosity in many North American and European populations and is associated with mild hyperhomocysteinemia (HHcy) and low methylTHF [15, 16].

The main mechanisms by which a folate-deficient (FD) diet or mild MTHFR deficiency increases the risk of developing LOAD remain unknown. Several authors have suggested that the increased risk is mediated by the adverse effects of HHcy which include negative cardiovascular effects, oxidative stress, and chromosome instability [17, 18]. Other hypotheses point to the dysregulation of one-carbon and choline metabolism. Folate and MTHFR deficiency can lead to decreased SAM and increased *S*-adenosylhomocysteine (SAH) [19, 20], which could eventually alter methylation of DNA and histones [21, 22]. In addition, disturbances in folate metabolism often disrupt choline metabolism. Choline is an alternate methyl donor for remethylation of homocysteine [23], mainly in the liver. It can be oxidized to betaine, which then donates its methyl group to homocysteine. When folate metabolism is impaired, choline-dependent methionine/SAM synthesis is upregulated, with potential consequences on choline pools [24–26]. Choline is the precursor for acetylcholine (Ach) and for many important lipids such as phosphatidylcholine and sphingomyelin, which are critical for optimal brain function. Decreased levels of these choline-derived metabolites are associated with cognitive impairment and AD [27, 28].

Murine studies have been used to identify the mechanisms underlying the associations of AD with folate dysmetabolism. However, most of the research has been performed in AD transgenic mouse models which overexpress mutations in the amyloid precursor protein (APP) and therefore resemble familial AD rather than sporadic LOAD [21, 29], or in mice fed folate-deficient diets combined with other nutritional changes, which lead to a more severe nutritional deficiency [30]. Although these studies have been informative, they do not recapitulate LOAD and many questions remain unanswered.

In order to better explore the mechanisms by which genetic or dietary folate deficiency, or their interaction, contribute to risk for LOAD and the early associated changes in aging, we investigated the effects of a folate-deficient diet (FD) or a control diet (CD), initiated at weaning, on brain function of 8- and 10-month-old wild-type and *Mthfr*$^{+/-}$ mice, a model for the *MTHFR* 677 C>T polymorphism [20]. We chose these time points to uncover early effects of folate dysmetabolism that could increase the risk for cognitive impairment and neurodegeneration in later stages**.** In particular, we were interested in alterations in pathways known to be affected in aging and neurodegeneration including epigenetic changes, synaptic function and plasticity, expression of neurotrophic factors and amyloid processing genes. We therefore assessed changes in (1) behavioral tests (novel object recognition, Y-maze and open field); (2) cortical and hippocampal mRNA expression of epigenetic genes, synaptic and neurotrophic factors, APP processing enzymes, and genes involved in methylation homeostasis and cholinergic signaling; (3) cortical levels of SAM, SAH, methionine, and choline-derived metabolites; and (4) hepatic expression of folate and one-carbon metabolic enzymes.

Results

Ten-Month-Old *Mthfr*$^{+/-}$ Mice Have Short-term Memory Impairment and Increased Anxiety

Male *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice were placed on a control diet (CD) or a folate-deficient diet (FD) at weaning and studied at 8 or 10 months of age. This FD diet has previously been shown to result in folate deficiency and hyperhomocysteinemia in these mice [31].There were no body weight differences between groups at 10 months of age (CD$^{+/+}$ 31.29 ± 0.57, FD$^{+/+}$ 31.95 ± 0.35, CD$^{+/-}$ 31.16 ± 0.71, FD$^{+/-}$ 31.93 ± 0.65). They were subjected to a series of behavioral tests in the following order: the novel object recognition test (NOR) to assess short-term memory, the Y-maze test to assess working memory, and

the open-field test to assess general locomotor activity levels and anxiety.

Ten-month-old *Mthfr*$^{+/-}$ mice showed short-term memory impairment in the NOR (Fig. 1a, two-way ANOVA, $F_{(1,22)} = 7.71$, $p = 0.01$); there was no dietary effect. For the Y-maze test, there were no differences in the number of visits or % alternation, suggesting similar working memory between groups (Fig. 1b). In the open-field test, there was no difference in distance traveled or speed between groups; however, *Mthfr*$^{+/-}$ mice spent significantly less time in the center areas (i.e., the exposed areas) compared to *Mthfr*$^{+/+}$ mice, suggesting they were more anxious (Fig. 1c, two-way ANOVA, $F_{(1,29)} = 4.45$, $p = 0.04$). No differences due to diet or genotype were seen in 8-month-old mice (data not shown).

Cortex of FD Mice Shows Disturbances in Methyl Metabolism

SAM levels in the cortex were decreased although they did not reach statistical significance by two-way ANOVA (Fig. 2a, $F_{(1,16)} = 3.26$, $p = 0.08$). No differences were seen in SAH levels (the product of SAM-dependent methylation reactions) (Fig. 2b) or in the SAM/SAH ratio. We observed increased *Mat2a* mRNA, which synthesizes SAM from methionine in the brain, due to FD (Fig. 2c, two-way ANOVA, diet: $F_{(1,14)} = 10.67$, $p = 0.005$; DxG: $F_{(1,14)} = 8.67$, $p = 0.01$). This upregulation could constitute a compensatory mechanism to increase SAM levels. PEMT is a major consumer of SAM for the synthesis of phosphatidylcholine and we observed a significant diet and genotype interaction (Fig. 2d, two-way ANOVA, $F_{(1,13)} = 4.64$, $p = 0.05$). For these results, the dietary differences appeared to be greater in the wild-type group, as compared to the mutant group. To further examine this trend, we evaluated the effect of diet in the *Mthfr*$^{+/+}$ group, by *t* test. In this analysis, SAM was lower in the FD *Mthfr*$^{+/+}$ group (*t* test, $p = 0.052$). *Mat2a* was significantly upregulated in FD *Mthfr*$^{+/+}$ mice (*t* test, $p < 0.005$), and there was an increase in *Pemt* mRNA in this group which did not quite reach significance (*t* test, $p = 0.06$). By performing a *t* test analysis, we also observed a significant increase in methionine levels in the cortex in the FD *Mthfr*$^{+/+}$ group (Table 1, *t* test, $p < 0.05$). Taken together, these data suggest that wild-type mice may be trying to compensate for reduced SAM levels. In contrast, in the *Mthfr*$^{+/-}$ group, *t* test analyses did not show any differences due to diet.

An increasing number of studies in age-related disorders have reported changes in key epigenetic enzymes [3]. Given the changes in SAM, we measured the expression levels of the DNA methyltransferases *Dnmt1*, *Dnm3b*, and *Dnmt3a* in the cortex of 10-month-old mice. We observed a significant increase in *Dnmt1* (Fig. 3a, two-way ANOVA, $F_{(1,31)} = 9.29$, $p = 0.004$) and *Dnmt3b* mRNA (Fig. 3b, two-way ANOVA, $F_{(1,27)} = 4.87$, $p = 0.04$) due to FD. We did not observe differences in *Dnmt3a* expression (Fig. 3c). Since the differences in SAM were more pronounced in the *Mthfr*$^{+/+}$ group, we also assessed the effect of diet in the *Mthfr*$^{+/+}$ group on *Dnmt* expression by *t* test. Both *Dnmt1* and *Dnmt3b* were significantly upregulated due to FD in *Mthfr*$^{+/+}$ mice (*t* test, $p < 0.05$). No differences were seen in the *Mthfr*$^{+/-}$ group due to diet by *t* test analysis.

We also measured gene expression of the histone deacetylase *Hdac3*, the main class I histone deacetylase in the brain; increased levels of this enzyme have been negatively associated with memory [32]. We saw a significant increase of *Hdac3* mRNA due to diet and genotype (Fig. 3d, two-way ANOVA, diet: $F_{(1,31)} = 9.91$, $p = 0.003$; genotype: $F_{(1,31)} = 4.43$, $p = 0.04$; DxG: $F_{(1,31)} = 14.2$, $p = 0.0007$). Tukey's post hoc analysis showed that for all three groups *Hdac3* levels were significantly higher than those in the CD *Mthfr*$^{+/+}$ group (CD *Mthfr*$^{+/-}$, FD *Mthfr*$^{+/+}$, and FD *Mthfr*$^{+/-}$, $p < 0.005$). No

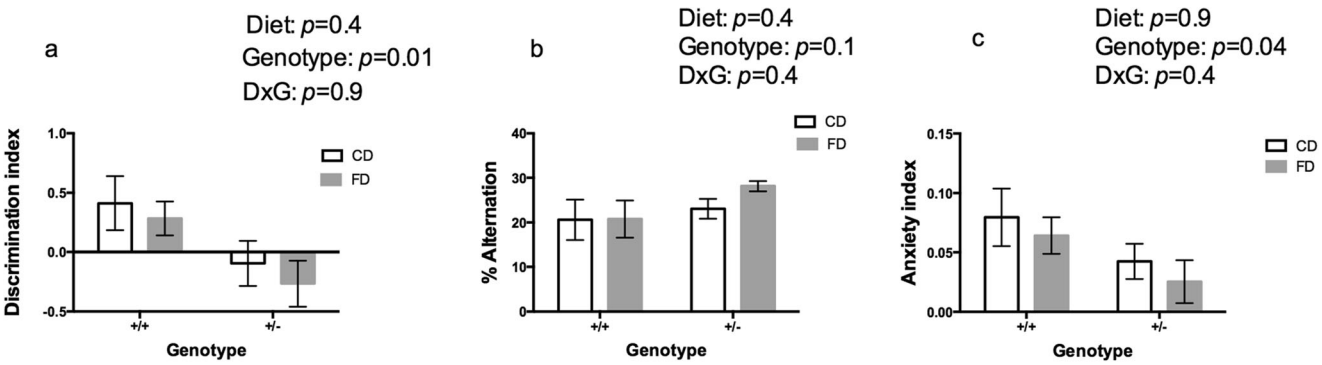


Fig. 1 Ten-month-old *Mthfr*$^{+/-}$ mice had short-term memory impairment. Behavioral testing was performed on 10-month-old male mice. **a** Short-term memory impairment was seen due to the *Mthfr*$^{+/-}$ genotype on the novel object recognition test. A negative discrimination index indicates that less time was spent with the novel object versus the familiar object. **b** No differences were seen in working memory as measured by the Y-maze test. **c** *Mthfr*$^{+/-}$ mice had lower values, which is indicative of increased anxiety, as measured by the open-field test. $N = 5$–10 mice/group. Values are means ± SEM. *p* values from two-way ANOVA indicated at the top of each graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

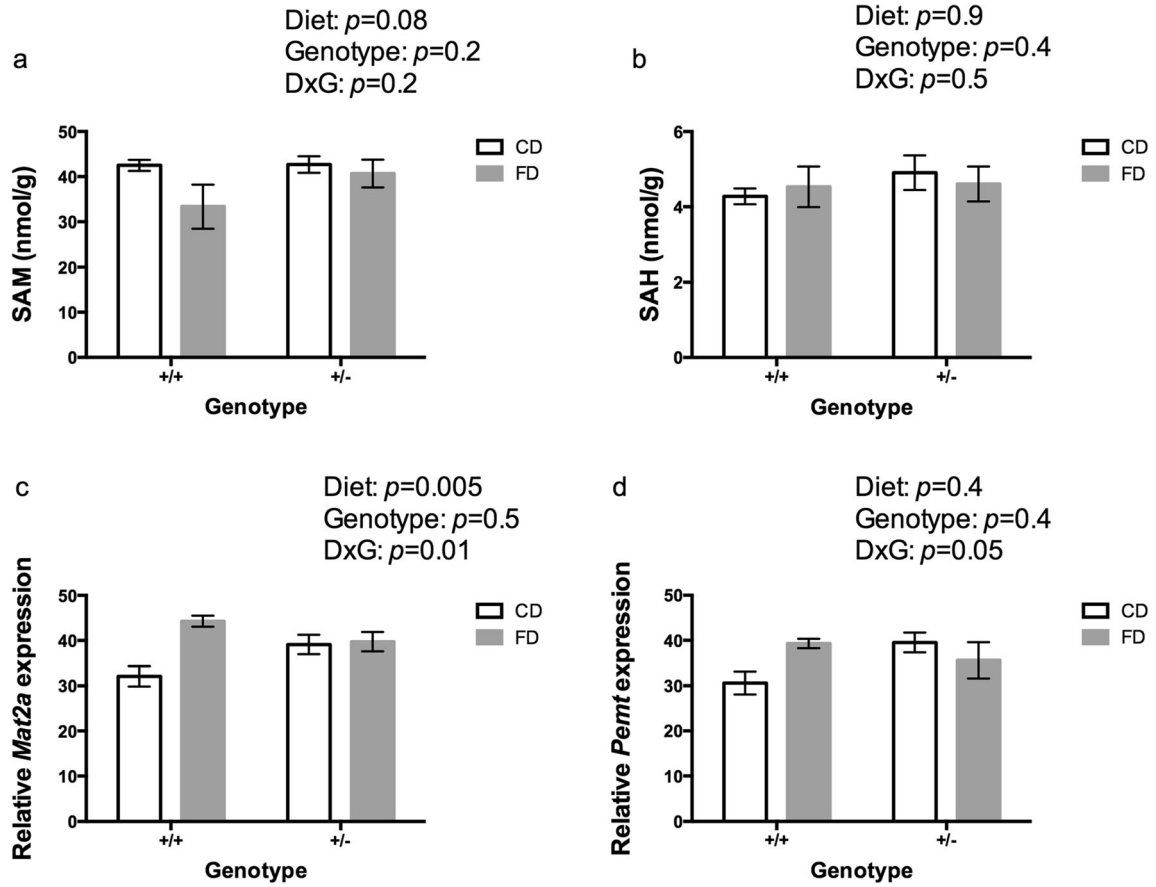


Fig. 2 Altered SAM metabolism in cortex due to low folate. Two-way ANOVA did not show statistically significant differences for SAM (**a**) or SAH (**b**) levels. **c** mRNA levels of *Mat2a* were significantly increased due to FD. **d** There was a significant diet and genotype interaction in mRNA expression of *Pemt*. $N = 4–5$ mice/group. Values are means ± SEM. p values for two-way ANOVA are indicated at the top of each graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

changes in expression were seen for members of two other HDAC families, *Hdac5* (class IIa) and *Sirt1* (class III) (data not shown).

Since epigenetic changes such as DNA methylation and histone acetylation can precede cognitive changes and Alzheimer's pathology [33], we measured the expression of the aforementioned enzymes in the cortex of 8-month-old mice, which did not show short-term memory impairment. Interestingly, we observed an increase in *Dnmt1* expression due to diet that did not reach statistical significance (Fig. 3e, two-way ANOVA, $F_{(1,16)} = 3.57$, $p = 0.07$). This finding was similar to that seen in 10-month-

Table 1 Choline metabolites in cortices of 10-month-old mice. *Mthfr*$^{+/-}$ genotype and/or folate deficiency did not alter the concentration (nmol/g) of choline metabolites in the cortex of 10-month-old mice, when assessed by a two-way ANOVA

Metabolite	CD +/+	FD +/+	CD +/−	FD +/−
Methionine[a]	79.09 ± 1.36	94.10 ± 3.95	76.12 ± 1.96	77.14 ± 12.99
Betaine	10.84 ± 1.01	13.94 ± 1.48	12.1 ± 1.00	10.63 ± 1.04
Choline	157.58 ± 13.33	186.90 ± 12.05	177.74 ± 17.29	167.13 ± 18.64
GPC	735.05 ± 18.35	817.8 ± 50.46	794.71 ± 17.35	712.1 ± 39.61
PCho	372.06 ± 18.08	403.6 ± 29.25	385.14 ± 24.80	371.2 ± 35.49

$N = 4–5$ mice/group

Values are means ± SEM (analyzed by two-way ANOVA)

[a] t test analysis for the effect of FD on *Mthfr*$^{+/+}$ mice showed increased methionine ($p < 0.05$)

CD control diet

FD folate-deficient diet

GPC glycerophosphocholine

Pcho phosphocholine

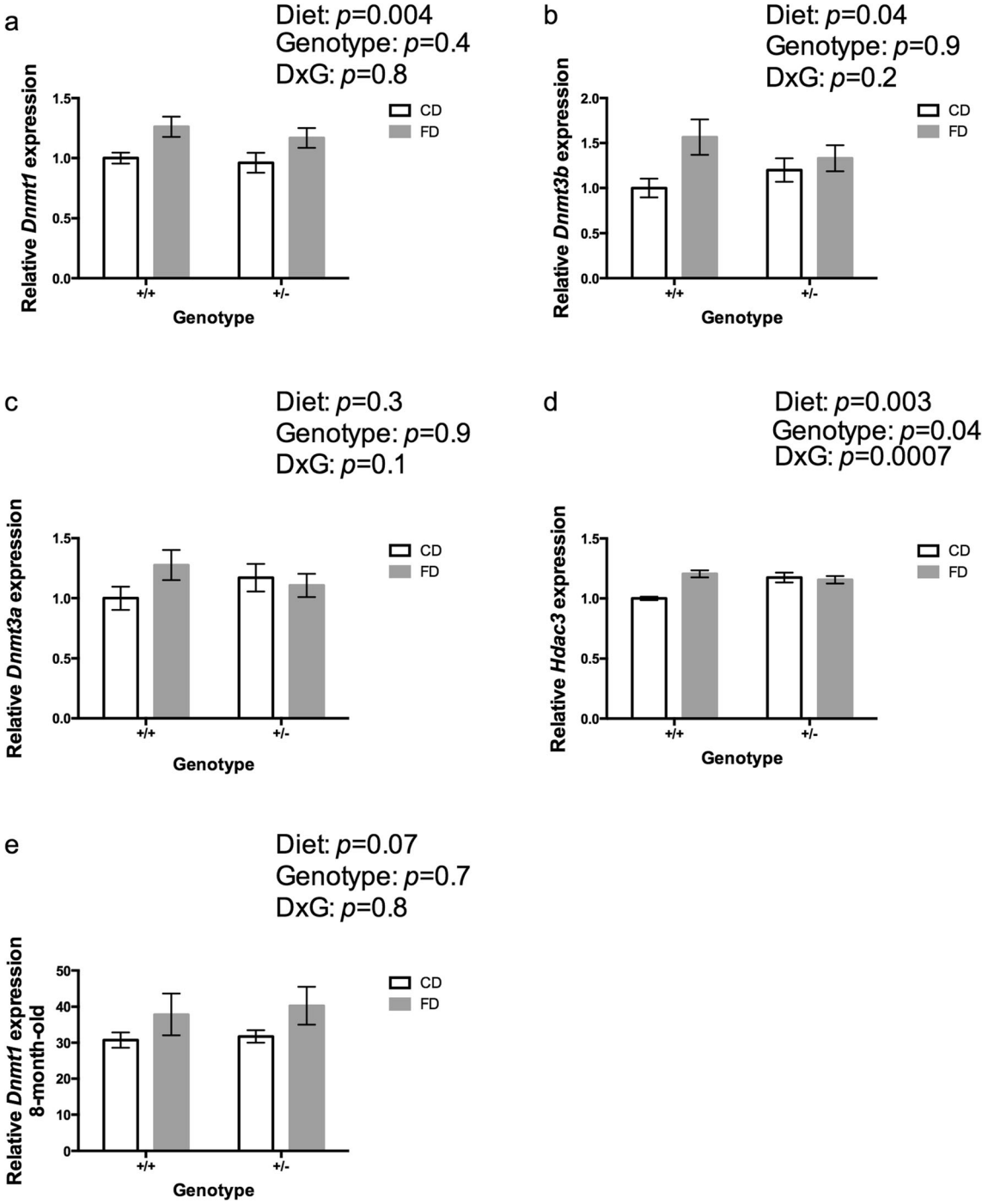


Fig. 3 Low folate resulted in epigenetic alterations in the cortex. **a** mRNA levels of *Dnmt1* were significantly increased due to diet (data from two independent experiments, $n = 9–10$ mice/group in total). **b** There was a significant increase of *Dnmt3b* mRNA due to diet (data from two independent experiments, $n = 9–10$ mice/group in total). **c** No changes were seen in *Dnmt3a* mRNA. **d** There was a significant increase of *Hdac3* mRNA due to diet and genotype (data from two independent experiments, $n = 9–10$ mice/group in total). **e** The increase in *Dnmt1* mRNA in the cortex of 8-month-old mice due to diet did not reach statistical significance ($n = 5$ mice/group). Values are means ± SEM. *p* values for two-way ANOVA indicated at the top of the graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

old mice, which had a significant marked increase in *Dnmt1* expression due to diet. No changes in expression were seen for the other enzymes.

To determine if these changes in *Dnmt* expression affected global DNA methylation, we measured DNA methylation in cortices of 8- and 10-month-old mice with the LUMA assay.

Differences were not observed between groups (Supplementary Fig. 1a,b).

Disturbances in Folate Metabolism Lead to Cholinergic and Glutamatergic Changes in Cortex

We observed a significant decrease of Ach levels in the cortex due to FD (Fig. 4a, two-way ANOVA, $F_{(1,15)} = 5.63$, $p = 0.03$). Although there were no significant differences due to diet or genotype by two-way ANOVA for expression of cortical acetylcholinesterase (*AchE*) (Fig. 4b), the enzyme responsible for Ach breakdown, Tukey post hoc analysis showed a non-significant increase in FD $Mthfr^{+/+}$ relative to CD $Mthfr^{+/+}$ mice, with the same p value as the D × G interaction by ANOVA ($p = 0.07$). We did not see any changes in protein levels of choline acetyltransferase (ChAT), the enzyme responsible for Ach synthesis, by immunoblotting (Fig. 4c). When we stained for cholinergic neurons, we did not observe a change in ChAT intensity or in number of cholinergic neurons (data not shown).

Homocysteine is a known agonist of NMDA receptors and HHcy has been shown to lead to excitotoxicity of these receptors [34]. The basic functional structure of NMDA receptors is formed by NR1 subunits, and these receptors are highly expressed in the human brain throughout adulthood [35]. We measured mRNA levels of the *Nmda nr1* receptor and observed an increase due to folate deficiency (Fig. 4c, two-way ANOVA, $F_{(1,31)} = 11.05$, $p = 0.02$).

We measured other choline-derived metabolites in cortices of 10-month-old mice (Table 1). The values for methionine were discussed above. There were no significant differences in betaine, choline, glycerophosphocholine, or phosphocholine. However, we observed a decrease in phosphatidylcholine (PtdCho) due to the $Mthfr^{+/-}$ genotype (Fig. 5a, two-way ANOVA, $F_{(1,16)} = 12.52$, $p = 0.002$). We also observed a significant diet and genotype interaction for sphingomyelin levels (Fig. 5b, two-way ANOVA, $F_{(1,15)} = 12.03$, $p = 0.003$). Sphingomyelin was significantly decreased due to $Mthfr^{+/-}$ genotype in mice fed FD (Tukey's post hoc, $p = 0.02$ FD $Mthfr^{+/+}$ vs FD $Mthfr^{+/-}$). These data suggest that

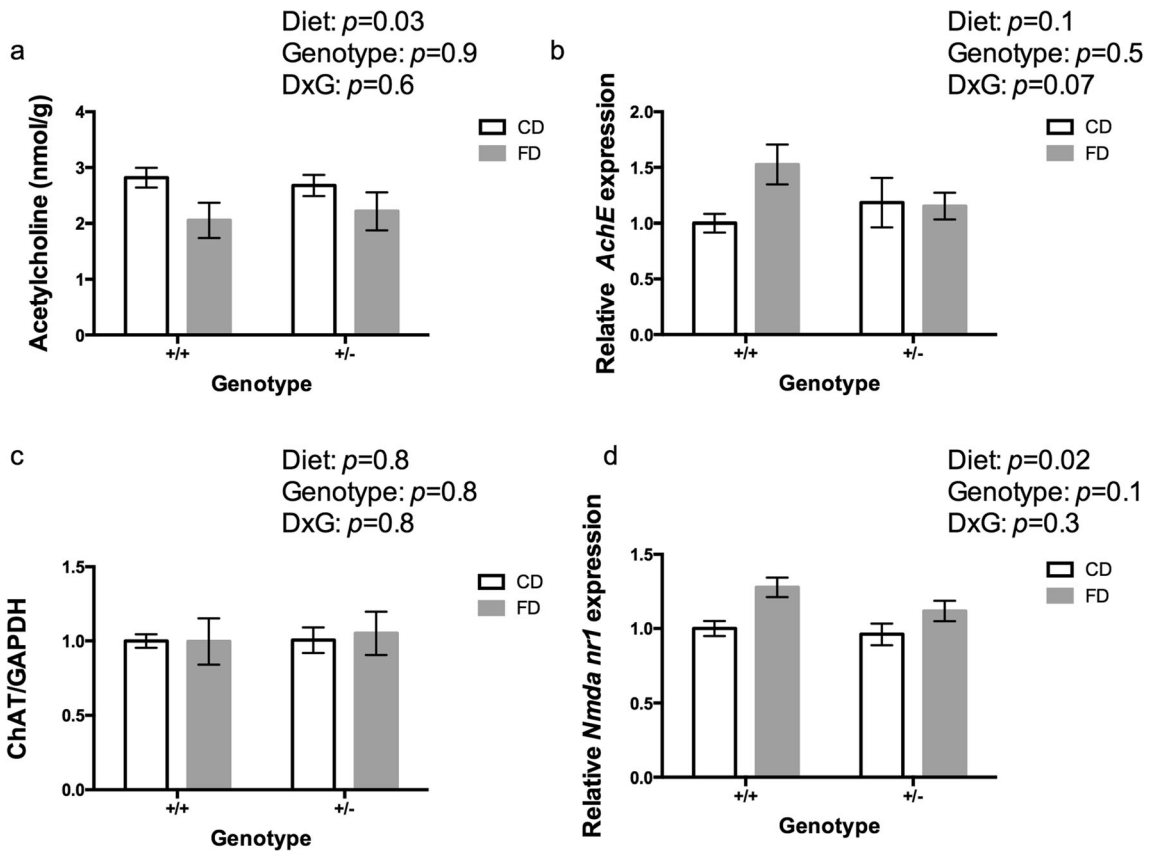


Fig. 4 Cholinergic and glutamatergic changes in cortex of 10-month-old mice. **a** Ach levels were decreased due to FD and **b** the interaction of diet and genotype for mRNA levels of acetylcholinesterase (*AchE*) was borderline significant (combined data from two independent experiments, $n = 9$–10 mice/group in total). **c** There were no differences in choline acetyltransferase (ChAT) levels by immunoblotting ($n = 5$ mice/group). **d** mRNA levels of the glutamate receptor *NMDA* were significantly increased due to FD (combined data from two independent experiments, $n = 9$–10 mice/group in total). Values are means ± SEM. p values for two-way ANOVA indicated at the top of the graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

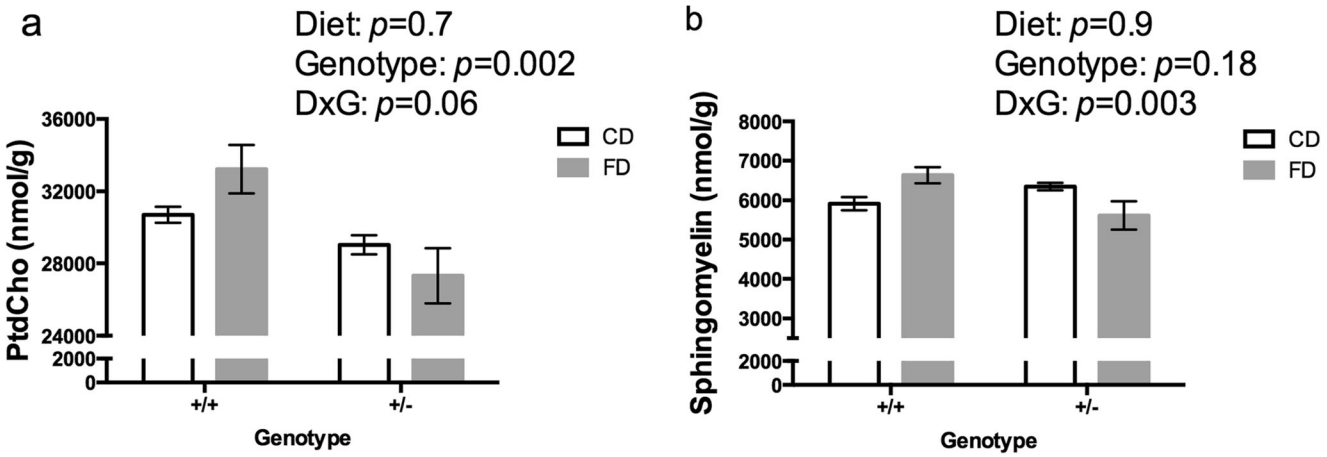


Fig. 5 Choline-derived phospholipids in cortex of 10-month-old mice. **a** Phosphatidylcholine (PtdCho) concentration was decreased due to MTHFR genotype and **b** there was a significant diet and genotype interaction for concentration of sphingomyelin. $N = 4$–5 mice/group. Values are means ± SEM. p values for two-way ANOVA indicated at the top of the graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

dietary and genetic folate disturbances can alter choline metabolism in the cortex.

Folate and MTHFR Deficiency Alter Cortical Expression of Genes Involved in Neuronal Survival and Synaptic Transmission

Decline in cognitive function in the aging brain is often accompanied by loss or diminished synaptic function. We measured the expression of some neurotrophic factors that are key players in these processes. We did not observe a significant effect of diet or genotype on *Ngf* mRNA (data not shown). *Bdnf* mRNA levels were decreased due to genotype but this did not reach statistical significance (Fig. 6a, two-way ANOVA, $F_{(1,28)} = 3.66$, $p = 0.06$). However, since the p value was 0.06, we pursued possible involvement of BDNF by measuring DNA methylation in the promoter and by assessing expression of some downstream targets. We observed increased *Bdnf* methylation at CpG5 in the promoter region (Fig. 6b, Table 2 and Supplementary Fig. 2, two-way ANOVA, $F_{(1,14)} = 4.48$, $p = 0.05$) due to $Mthfr^{+/-}$ genotype. We measured gene expression of *Synapsin 1a* and *1b* (combined), *Synapsin 1a*, *Synapsin 2a*, and *Synapsin 2b*, which encode the phosphoproteins that associate with synaptic vesicles but there was no significant effect of diet or genotype (data not shown). However, *synaptophysin*, a downstream target of *Bdnf*, which encodes an integral protein of synaptic vesicles, showed increased expression due to folate deficiency (Fig. 6c, two-way ANOVA, $F_{(1,31)} = 6.8$, $p = 0.01$). *Bdnf* and synaptophysin expression showed a significant positive correlation ($r = 0.4$, $p = 0.018$) (Fig. 6d), suggesting that the decrease in neurotrophin expression may result in decreased synaptic transmission and activity. We also measured mRNA expression of *Neuritin*, another downstream target of *Bdnf* involved in plasticity, and did not observe any changes due to diet or genotype but again found a significant positive correlation with *Bdnf* ($r = 0.44$, $p = 0.009$).

mRNA Level of Aβ Processing Enzyme Is Altered in Cortex Due to Folate Deficiency with no Changes in Aβ Protein Levels

To determine if our model showed any signs of AD pathology, we examined some aspects of amyloid plaque processing. The amyloidogenic APP processing pathway involves cleavages by β- and γ-secretases resulting in the generation of Aβ fragments which oligomerize and fibrillize contributing to AD pathology, whereas α-secretases are involved in the non-amyloidogenic APP processing pathway [36].

There were no statistically significant changes in mRNA levels of β-secretase (*Bace1*) (Fig. 7a). There was a significant increase of mRNA for *Presenillin-1* (*Psen1*), the catalytic subunit of the γ-secretase, due to FD (Fig. 7b, two-way ANOVA, $F_{(1,30)} = 5.53$, $p = 0.02$). We also observed decreased methylation at CpGs −421 and −470 in the promoter of *Psen1*, suggesting that the decreased methylation could have led to the increased gene expression (Fig. 7c, d, Supplementary Fig. 2). These CpGs have been previously reported to be hypomethylated due to a vitamin B-deficient diet [21].

To determine if these changes had any consequences on Aβ processing, we evaluated by immunoblotting the levels of the different Aβ oligomers in the cortex. We did not observe any differences in oligomer expression (at any molecular weight; data not shown), suggesting that at this stage (10 months of age), there were no consequences on amyloid processing.

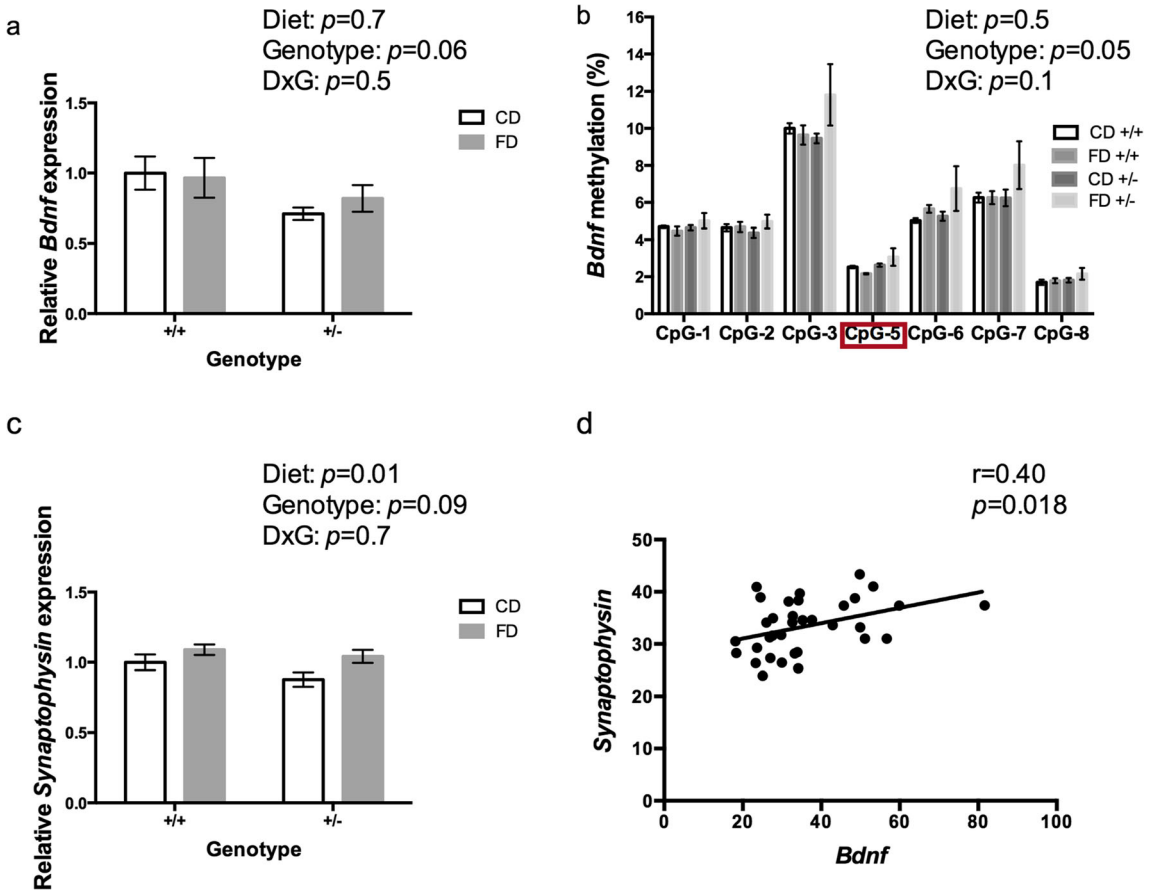


Fig. 6 Altered expression of genes involved in synaptic transmission in cortex of 10-month-old mice. **a** There was a trend towards decreased mRNA levels of brain-derived neurotrophic factor (*Bdnf*) due to genotype and **b** increased methylation at CpG5 due to genotype ($n = 5$ mice/group). **c** mRNA levels of synaptophysin were significantly increased due to FD and **d** synaptophysin expression positively correlated with *Bdnf* expression ($r = 0.4$, $p = 0.018$). Data from two independent experiments, $n = 9$–10 mice/group. Values are means ± SEM. p values for two-way ANOVA indicated at the top of the graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

Hippocampal Changes in Gene Expression of Neurotrophic Factors and Epigenetic Enzymes

Given the role of the hippocampus in memory and learning, we examined all the aforementioned genes in the hippocampi of our 10-month-old mice. We identified changes in two epigenetic enzymes that were not altered in the cortex. mRNA levels of *Dnmt3a* were decreased due to genotype (Fig. 8a, two-way ANOVA, $F_{(1,15)} = 7.72$, $p = 0.01$) and *Hdac5* mRNA was decreased due to FD (Fig. 8b, two-way ANOVA, $F_{(1,14)} = 7.4$, $p = 0.02$). *Hdac5* positively correlated with NOR behavioral results ($r = 0.59$, $p < 0.01$), such that mice with worse scores also showed lower *Hdac5* levels.

The expression of neurotrophic factors *Bdnf* and *Ngf* was downregulated due to FD in hippocampus (Fig. 8c, two-way ANOVA, $F_{(1,15)} = 5.3$, $p = 0.03$ and Fig. 8d, two-way ANOVA, $F_{(1,15)} = 10.24$, $p = 0.006$). Consistent with the decreased expression, we observed increased methylation of the *Bdnf* gene in the hippocampus in CpG 2 due to FD. There was a diet-genotype interaction for CpGs 5 and 7 (Table 2, Supplementary Fig.2) which demonstrated borderline increases due to FD in the $Mthfr^{+/+}$ group when analyzed by t test. In the aforementioned studies in cortex, *Bdnf* had shown decreased expression due to genotype and CpG 5 had also shown increased methylation.

Altered Hepatic One-Carbon Metabolism

Liver is the key organ for folate and choline metabolism. We measured MTHFR immunoreactive protein in liver and observed significant upregulation due to FD (Fig. 9a, two-way ANOVA, $F_{(1,16)} = 31.28$, $p < 0.0001$). Interestingly, we did not observe changes in MTHFR protein levels in cortex (data not shown). Several genes in choline and folate metabolism did not show hepatic mRNA changes (Supplementary Table 1). However, we did see increased expression due to genotype of choline dehydrogenase (*Chdh*), which converts choline to betaine for the alternative methylation pathway (Fig. 9b, two-way ANOVA, $F_{(1,16)} = 8.65$, $p = 0.009$).

Table 2 Increased methylation in CpGs of the *Bdnf* gene in 10-month-old mice. Table summarizes *p* values from two-way ANOVA for methylation levels at different CpGs in the *Bdnf* gene in cortex and hippocampus of 10-month-old mice

			Diet			Genotype			Diet × Genotype		
			df	*F*	*p* value	df	*F*	*p* value	df	*F*	*p* value
Bdnf promoter	CpG − 1	Hippocampus	1, 16	0.427	0.523	1, 16	2.412	0.140	1, 16	0.440	0.517
		Cortex	1, 16	0.098	0.758	1, 16	1.038	0.323	1, 16	1.353	0.262
	CpG − 2	Hippocampus	1, 16	6.248	**0.024**	1, 16	0.203	0.658	1, 16	1.927	0.184
		Cortex	1, 16	1.284	0.274	1, 16	0.001	0.992	1, 16	1.066	0.317
	CpG − 3	Hippocampus	1, 15	0.017	0.898	1, 15	1.468	0.244	1, 15	0.053	0.820
		Cortex	1, 16	1.197	0.290	1, 16	0.784	0.389	1, 16	2.449	0.137
	CpG − 5	Hippocampus	1, 16	0.413	0.529	1, 16	0.562	0.464	1, 16	3.359	*0.086*[a]
		Cortex	1, 14	0.487	0.497	1, 14	4.483	**0.053**	1, 14	2.601	0.129
	CpG − 6	Hippocampus	1, 16	1.844	0.193	1, 16	1.562	0.229	1, 16	2.073	0.169
		Cortex	1, 15	2.336	0.147	1, 15	0.358	0.558	1, 15	0.013	0.911
	CpG − 7	Hippocampus	1, 16	0.228	0.640	1, 16	0.006	0.938	1, 16	5.476	**0.033**[b]
		Cortex	1, 15	0.917	0.353	1, 15	0.679	0.423	1, 15	1.076	0.316
	CpG − 8	Hippocampus	1, 16	0.302	0.509	1, 16	0.582	0.457	1, 16	2.233	0.155
		Cortex	1, 15	0.901	0.357	1, 15	1.189	0.293	1, 15	0.220	0.646

df degrees of freedom. Bold values indicate $p \leq 0.05$

Italicized value indicates a trend for significance

[a] *t* test CD *Mthfr*$^{+/+}$ vs FD *Mthfr*$^{+/+}$; $p = 0.09$

[b] *t* test CD *Mthfr*$^{+/+}$ vs FD *Mthfr*$^{+/+}$; $p = 0.07$

Discussion

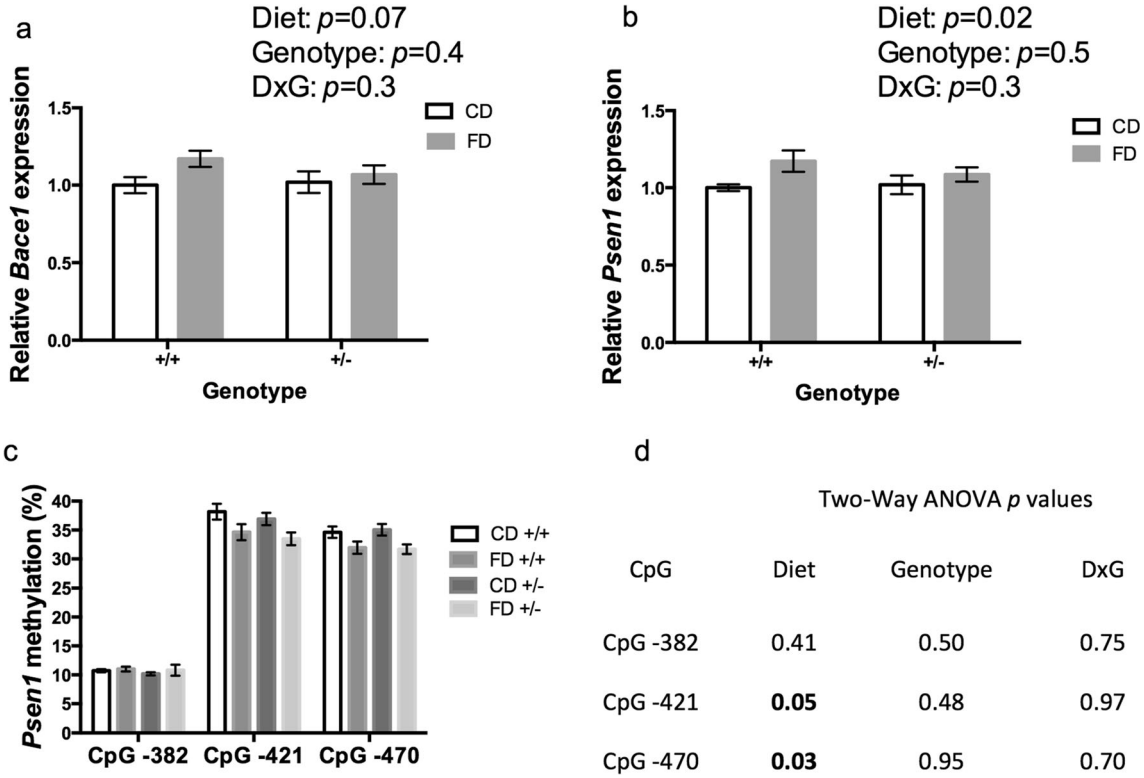
Our study was designed to investigate the early impact of genetic and/or dietary disturbances in folate metabolism on cognitive decline and brain metabolism that could increase the risk for cognitive impairment and neurodegeneration in later stages. In contrast to other studies, we used a non-transgenic model of AD, a diet deficient in folate rather than in multiple B vitamins, and examined the liver, a key organ of folate metabolism. Towards that end, we fed *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice with control or folate-deficient diets as of weaning until 8 or 10 months of age. One limitation of our work was studying exclusively male mice. Subsequent studies should include female mice, particularly since AD is more prevalent in women [37].

At 10 months of age, we observed short-term memory impairment and increased anxiety due to MTHFR deficiency. The memory impairment is consistent with clinical studies that have reported increased risk of mild cognitive impairment in individuals with the *MTHFR* 677 C>T polymorphism [12, 38]. We did not see cognitive changes due to folate deficiency. It is possible that behavioral changes due to FD might be manifest at later time points.

Changes in the epigenetic landscape, mainly global DNA hypomethylation and decreased levels of histone acetylation, are common in aging and age-related disorders such as AD [3]. In our study, we observed increased cortical *Dnmt1* and *Dnmt3b* expression in 10-month-old mice due to folate deficiency, particularly in the *Mthfr*$^{+/+}$ group. Increased *Dnmt* gene expression with low folate or methyl-deficient diets has been reported in different models and tissues [39–41]. Disturbances in folate metabolism, either dietary or genetic, have been shown to reduce levels of SAM, the primary methyl donor, in a variety of tissues. Previous studies have reported lower SAM levels in the cerebrospinal fluid [42] and in brains of patients with cognitive decline [43] and AD [44], as well as in animals fed folate-deficient diets [45, 46]. We observed decreased cortical SAM levels due to FD, mainly in *Mthfr*$^{+/+}$ mice. Since DNMTs are very sensitive to SAM levels [47], it is possible that the increase in DNMT expression is a compensatory mechanism to prevent methylation disturbances. The significant increase of *Mat2a* mRNA and the increased methionine levels in cortex, as well as the increase in hepatic MTHFR protein, may also be compensatory mechanisms to replenish SAM.

Our results are distinct from a study in which transgenic AD mice, fed a vitamin B-deficient diet, showed a difference in SAM/SAH ratios and a downregulation of DNMT3a/b protein levels [48]. This difference may be due to the fact that our mice do not overexpress a mutant APP gene and our diet is deficient only in folate not 3 vitamins (B_{12}, folate, and B_6) [49].

We did not observe differences between groups in global DNA methylation by LUMA assay, but there could be



CpG	Diet	Genotype	DxG
CpG -382	0.41	0.50	0.75
CpG -421	**0.05**	0.48	0.97
CpG -470	**0.03**	0.95	0.70

Fig. 7 Low folate diet can alter mRNA levels of APP processing genes in cortex of 10-month-old mice. **a** Significant differences were not observed for mRNA levels of β-secretase (*Bace1*) (data from two independent experiments, $n = 9$–10 mice/group in total). **b** mRNA levels of *Psen1* were increased due to FD diet (data from two independent experiments, $n = 9$–10 mice/group in total). **c**–**d** Methylation levels were decreased at CpG −421 and −470 in the promoter of *Psen1* due to FD ($n = 5$ mice/group). Significant p values in **d** are in bold. Values are means ± SEM. p values for two-way ANOVA indicated at the top of the graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

changes at specific genes involved in AD or age-related cognitive decline without changing global methylation, as we observed for *Psen1* and *Bdnf*. Identification of additional genes would be useful since early changes in DNA methylation are being considered as preclinical markers of AD [33].

In the hippocampus, the decrease of *Dnmt3a* due to genotype could be contributing to the poor performance of *Mthfr*$^{+/-}$ mice in the NOR test. *Dnmt3a* is necessary for normal memory formation and its downregulation has been previously associated with memory impairment and variable cognitive disability in rodents and humans respectively [50–52]. Importantly, *Dnmt3a* levels are significantly decreased in the hippocampus of AD patients [53].

We observed an increase in cortical *Hdac3* expression, the most highly expressed class I HDAC in the brain, due to FD diet and *Mthfr*$^{+/-}$ genotype. HDAC3 has been previously described as a negative regulator of memory formation and its inhibition can enhance long-term object recognition memory in mice [32, 54]. A study in the rat H19-7 hippocampal cell line found that folate deficiency led to an increase in the expression of other HDACs such as HDAC 4, 6, and 7 [55]. A loss of HDAC5 has been reported to damage memory function in a mouse model for amyloid pathology [56] and we observed downregulation of *Hdac5* due to FD diet in the hippocampus. Moreover, *Hdac5* positively correlated with NOR behavioral results, with lower *Hdac5* levels in mice with poor NOR scores. To our knowledge, this is the first report showing modulation of *Hdac3* and *Hdac5* by folate deficiency. Since there is an interplay between histone acetylation and DNA methylation, the changes in DNA methylation in folate disturbances could be underlying the modulation of *Hdacs* [57, 58].

Cholinergic and Ach deficiencies are very common in cognitive decline and AD [59]. We observed decreased Ach levels due to FD in cortices of 10-month-old mice, as well as a minor increase in *AchE* expression in the *Mthfr*$^{+/+}$ group fed FD. This finding is consistent with a previous study where rats with hyperhomocysteinemia, a feature of MTHFR deficiency and FD in our mouse model [20, 31], showed increased *AchE* expression [60]. Increased AchE activity is often reported in AD patients and the use of AchE inhibitors is a common therapy to increase Ach levels in the synaptic space [61, 62]. We also observed decreased levels of cortical PtdCho in *Mthfr*$^{+/-}$ mice. PtdCho is essential for membrane integrity, axonal elongation and growth. Genes in PtdCho metabolism have been reported to be involved in AD [63, 64], and a study

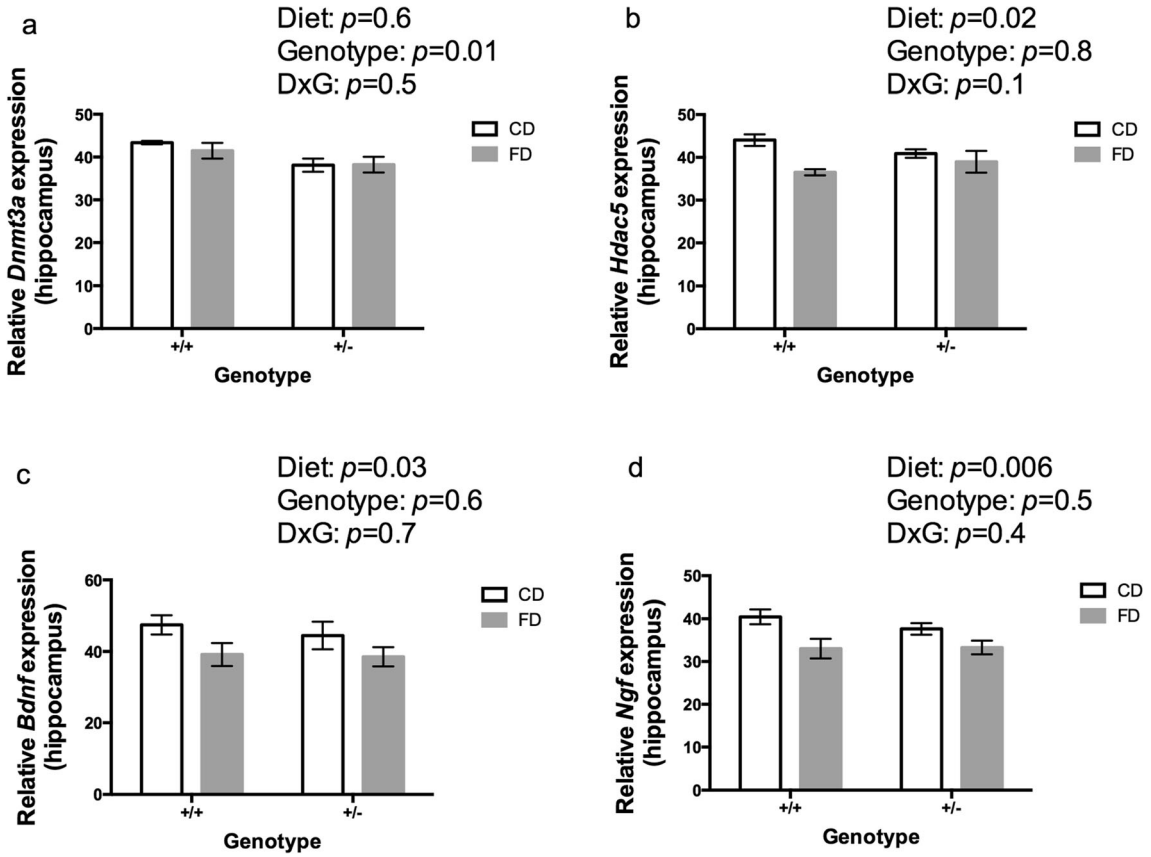


Fig. 8 Decreased expression of epigenetic enzymes and neurotrophic factors in hippocampus of 10-month-old mice due to MTHFR deficiency or folate deficiency. **a** mRNA levels of *Dnmt3a* were decreased due to *Mthfr*$^{+/-}$ genotype. mRNA levels of **b** *Hdac5*, **c** *Bdnf*, and **d** *Ngf* were decreased due to FD ($N = 5$ mice/group). Values are means ± SEM. p values for two-way ANOVA indicated at the top of the graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

in folate-deficient rats reported depletion of PtdCho in the brain [27]. Our results here and in other work [65, 66] suggest that folate disturbances trigger changes in choline metabolism. Since choline is an alternate methyl donor for methionine/SAM synthesis, choline pools may be depleted in *Mthfr*$^{+/-}$ mice in an attempt to maintain SAM synthesis [24, 26], with impairment of PtdCho and Ach synthesis and potential adverse consequences in the brain.

We observed an increase in *Nmda nr1* gene expression in the cortex due to folate deficiency. Glutamate excitotoxicity has been reported in the context of folate and MTHFR deficiency, mainly due to increased homocysteine levels [67].

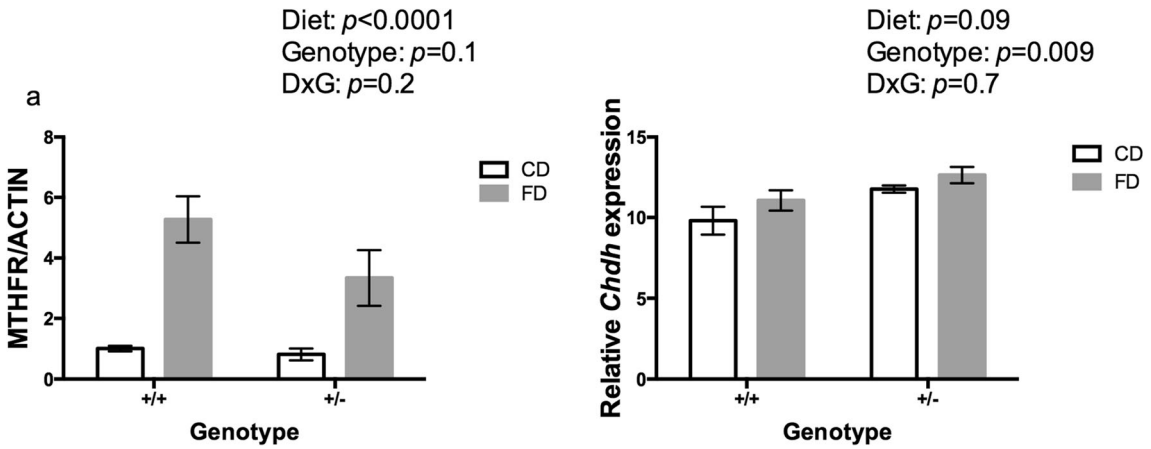


Fig. 9 One-carbon metabolism is altered in liver of 10-month-old mice. **a** Total MTHFR protein expression was increased due to FD. **b** mRNA levels of choline dehydrogenase (*Chdh*) were significantly increased due to genotype. $N = 5$ mice/group. Values are means ± SEM. p values for two-way ANOVA indicated at the top of the graph. CD control diet, FD folate-deficient diet, DxG interaction of diet and genotype

Glutamate excitotoxicity is associated with neurodegeneration and an NMDA receptor channel blocker, memantine, has been approved for the treatment of AD [68].

In studies of one-carbon metabolism in brain, the liver is not always examined despite its critical role in supplying methyl metabolites to the circulation. In this study, we observed increased expression of MTHFR due to folate deficiency, as we previously reported [65], and a significant increase of *Chdh* mRNA due to genotype. The liver is the main site of folate and choline metabolism, and these changes may constitute a mechanism to replenish methyl donors into the circulation for supplying the brain and other tissues.

Low folate has been associated with decreased methylation of *Psen1* in human and animal studies [21, 69]. This finding is consistent with our results, i.e., decreased *Psen1* methylation and increased mRNA expression in FD mice but we did not observe changes in protein levels of APP and APP oligomers at this time point. Hoffman et al. recently reported an increase in APP phosphorylation, which could promote Aβ production, due to folate deficiency and $Mthfr^{+/-}$ genotype in an age-specific and brain region-specific manner [70]. However, since PSEN1 has many different substrates in addition to APP, further research is needed to address the potential impact of *Psen1* mRNA changes on other pathways altered in AD such as the Notch and Wnt signaling pathways [36]. Moreover, alterations in non-CpG methylation levels of *Psen1* have been reported [71]. It would be interesting to measure these sites, since the primers used in this study were not designed for this assessment.

The decreased expression of *Bdnf* in the cortex of $Mthfr^{+/-}$ mice and in the hippocampus of FD mice and the corresponding increase in methylation in the promoter region are alterations that have been reported in the context of aging and AD [72]. We measured DNA methylation in the promoter upstream of exon IV as it has been shown to be more responsive to neuronal activity [73]. This region of exon IV has also been shown to be responsive to environmental factors and hypermethylated in aging [74, 75]. Although statistically significant, the percent increase of methylation in our study was minor. Similar studies exploring DNA methylation in brain found that small changes could be explained by the presence of some heavily methylated cells amongst fully unmethylated ones in the same tissue [76]. Thus, additional work should explore particular areas in the cortex and hippocampus to determine whether this regulation is more important in certain groups of neurons.

Expression of *Ngf*, another neurotrophic factor shown to be affected by folate [77], was decreased by FD in the hippocampus. The synaptic marker synaptophysin exhibited a significant positive correlation with *Bdnf* expression. *Bdnf* is known to modulate expression of synaptic markers, and decreased synaptophysin has been reported in aging and AD [78]. *Neuritin* expression, another synaptic marker [79], also showed a significant positive correlation with *Bdnf* expression. The correlation between *Bdnf* expression in hippocampus and NOR results is also consistent with its involvement in the memory impairment.

Our work suggests that MTHFR deficiency or low folate may contribute to brain dysfunction, possibly through different mechanisms. These mechanisms may not be acting alone. Other mechanisms that have previously been reported in folate-deficient rodents include changes in the vasculature [80], nuclear receptor dysregulation [81], increased apoptosis [82], ER stress [83], and inflammation [84]. In most cases, the modulation by diet in our study seemed to be manifested more in $Mthfr^{+/+}$ mice, whereas in $Mthfr^{+/-}$ animals the differences between CD and FD were not significant. Importantly, $Mthfr^{+/-}$ mice have a genetic disturbance prenatally and postnatally; therefore, it is possible that altered development or the long time frame could have contributed to the memory impairment seen at 10 months of age. Moreover, complex regulatory or compensatory mechanisms may have come into play, which could explain why mutant mice are less sensitive to FD.

In summary, MTHFR-deficient mice exhibited cognitive impairment, evidence for synaptic dysfunction (decreased *Bdnf*), and altered choline metabolism. The folate-deficient diet resulted in reduced SAM and acetylcholine levels and changes in mRNA levels of *Psen1*, *Ngf*, *Bdnf*, *Hdacs*, *Dnmts*, and *Nmda nr1* and one-carbon metabolism enzymes (*Mat2a*, *Pemt*, *Chdh*). Future studies could assess changes in protein levels of some of the critical aforementioned genes and examine later time points to determine whether the alterations identified in this study contribute to the more deleterious phenotype associated with folate dysmetabolism in human epidemiological studies. Although some of the observed changes appeared to be minor, they are in keeping with clinical studies suggesting that the alterations leading to AD consist of multiple changes in multiple pathways that eventually shift the process from normal aging to disease [85, 86]. Overall, our work has identified early changes in pathways/markers that are sensitive to folate status, thereby adding further evidence that low dietary folate and mild MTHFR deficiency, such as that seen with the 677C>T polymorphism, may be risk factors for brain biochemical disturbances and cognitive decline. Adequate folate status, which can be achieved by modest folate supplementation, overcomes the effect of both dietary and genetic deficiencies and may prevent or reverse the changes we observed.

Materials and Methods

Animal Experimentation and Diets

Experiments were performed according to the guidelines of the Canadian Council on Animal Care and approved by

the Animal Care Committee of the Research Institute of the McGill University Health Centre. The animals were kept in clean pathogen-free facilities in a controlled environment (12-h light/dark cycle) with food and water ad libitum. At weaning, BALB/c *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ male mice (total $n = 40$) were randomly placed on an amino acid-defined control diet (CD, 2 mg folate/kg diet, the recommended amount for rodents, TD.09704, Harlan Laboratories, Inc., Madison, WI, USA) or folate-deficient diet which was identical to CD except for folate content (FD; 0.3 mg folate/kg diet TD.09705, Harlan Laboratories, Inc., Madison, WI, USA), such that each of the four diet/genotype groups had $n = 10$. Mice were maintained on diets until 8 or 10 months of age. Both diets contained 1% succinylsulfathiazole, an antibiotic that inhibits folate synthesis by intestinal flora. We have previously shown that this diet leads to folate deficiency and hyperhomocysteinemia as indicated by the plasma homocysteine levels measured for the same background strain and the same diet for 1 year: *Mthfr*$^{+/+}$ CD, 8.55 ± 1.19 μmol/L; *Mthfr*$^{+/-}$ CD, 13.97 ± 3.29 μmol/L; *Mthfr*$^{+/+}$ FD, 18.93 ± 3.58 μmol/L; and *Mthfr*$^{+/-}$ FD, 38.87 ± 5.42 μmol/L [31]. Mice were sacrificed after completion of behavioral testing in random order by CO_2 asphyxiation and body weights were recorded. Cortex, hippocampus, and liver were collected and weighed. Tissues were snap frozen on dry ice and stored at − 80 °C.

Behavioral Testing

Mice were subjected to three behavioral tests: the novel object recognition test (NOR); the Y-maze; and the open-field test. Animals were able to rest for 48 h between each test. The tests have been previously described [26] and are briefly summarized below.

Novel Object Recognition Test

Twenty-four hours prior to the test, mice were habituated to the apparatus (open field) for 10 min. Animals explored two identical copies of object one in the apparatus for 8 min (trial phase). One hour later, the test phase began and animals had to explore the familiar object (object 1) and a novel object (object 2) for 5 min. Both phases were recorded and the time spent exploring the objects was measured by two independent researchers. The discrimination index was then calculated according to the following formula: (time novel − time familiar) / (time (novel + familiar)). Given the curious nature of mice, they should spend more time exploring the novel object. A negative value indicates that the animal spent less time with the novel object and is indicative of short-term memory impairment.

Y-Maze

The Y-maze test was used to evaluate working memory. At the beginning of the test, mice naive to the maze were placed at the end of the longest arm of the maze and allowed to move freely for a period of 8 min. The session was recorded and the entries into each arm were noted. The number of alternations, i.e., successive entries into three arms on overlapping triplet sets, was measured using Y-maze software.

Open-Field Test

The open-field test is used to assess anxiety, general locomotor activity levels, and willingness to explore. The mouse is placed in the middle of an open-field box (opaque gray Plexiglas) and its movement is recorded with a video camera for 5 min. All sessions were analyzed using the Any-Maze software (Stoelting Co., IL, USA) to measure time spent in the center or edges of the box, movement pattern, and distance [87]. The anxiety index is calculated as the ratio of distance traveled in the center areas relative to total distance traveled.

Quantitative Real-time PCR

Experiments were performed as previously described [66]. Briefly, the total RNA extraction from frozen hippocampus (~ 20 mg) and cortex (~ 30 mg) was performed using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Toronto, Canada) and RNA from liver was extracted using RNeasy Mini Kit (Qiagen, Toronto, Canada). cDNA synthesis was achieved using iScript™ cDNA Synthesis Kit (Bio-Rad, Quebec, Canada). RT-PCR was carried out with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Quebec, Canada) on a Lightcycler LC480 (Roche Diagnostics). The following reference genes were evaluated: β-actin (*Actb*), β-2-microglobulin (*B2m*), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The two most stably expressed reference genes were used to calculate one normalization factor for target gene expression by geNorm v.3.4 (Ghent University Hospital Center for Medical Genetics) [88]. Relative gene expression was measured using values calculated relative to the standard curve (eight serial dilutions (1:2) from a pool of all samples) and then normalizing by the normalization factor. Primer sequences and reaction conditions are summarized in Supplementary Table 2.

DNA Methylation Analysis

Pyrosequencing

Bisulfite pyrosequencing was used to determine the percent methylation at single CpG sites, as we previously described

[89]. Briefly, genomic DNA extraction from frozen hippocampus (~ 20 mg) and cortex (~ 30 mg) was done using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Toronto, Canada). Five hundred nanograms was used for bisulfite conversion (EpiTect Bisulfite Kit; Qiagen, Toronto, Canada). To amplify regions of interest, 1 μL of bisulfite converted DNA was run in a PCR using the PyroMark PCR kit (Qiagen, Toronto, Canada) and specific primers, one of which was biotinylated at the 5′ end. Pyrosequencing was performed on a PyroMark Q24 pyrosequencer using PyroMark Gold Q24 Reagents (Qiagen, Toronto, Canada) and a specific sequencing primer. PCR amplification and pyrosequencing primer sequences were designed by PyroMark Assay Design 2.0 software (Qiagen, Toronto, Canada). The average methylation levels of CpG sites were quantified using PyroMark Q24 2.0.4 software (Qiagen, Toronto, Canada). Primers are shown in Supplementary Table 3.

Global DNA Methylation

Global methylation was measured using a modification of the luminometric methylation assay (LUMA) [90]. Briefly, 500 ng of genomic DNA was incubated at 37 °C for 4 h with the *Eco*RI restriction enzyme mix and either *Hpa*II or *Msp*I. The cut sites were then tested on a Pyromark Q24 Pyrosequencer (Qiagen). Dispensation order is TAAACCCT TTGGGCCC; analyzed sequence is TA/CTGC. "A" (dATPαS) and "T" (dTTP) nucleotides were undiluted. For the "C" dispensation, a mixture of dCTP and dGTP was used, whereas for the "G" dispensation, the undiluted dCTP nucleotide was provided as a negative control. For normalization, the peak height of the first "C" (from *Hpa*II or *Msp*I digestion) was divided by the peak height of A (from EcoRI digestion), and peak height of C (from MspI digestion) was divided by the peak height of the first "A" (from *Eco*RI digestion). The percent methylation was calculated from the obtained ratio using the following formula: $100 \times [1 - [(\text{HpaII} \quad \text{EcoRI}) / (\text{MspI} \quad \text{EcoRI})]]$.

Western Blotting

Protein extracts from hippocampus, cortex, and liver were prepared and immunoblotting was performed as previously described [91]. The primary antibodies were MTHFR, ChAT, and APP (Millipore, Billerica, USA), GAPDH (Cell Signaling Technology, Boston, USA), and β-actin (Sigma-Aldrich). Secondary antibodies were horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (GE Healthcare, Mississauga, Canada) and HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, USA), as appropriate. Detection was achieved using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Mississauga, Canada). Bands were quantified by densitometry using Image J software and normalized to GAPDH, β-actin, or total protein (measured by amido black staining) as appropriate.

Measurement of Choline Metabolites

Choline, betaine, glycerophosphocholine, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine, phosphocholine, and acetylcholine were measured in cortex according to protocols previously published with modifications [92–94]. SAM and SAH concentrations were determined by liquid chromatography-electrospray ionization tandem mass spectrometry with modifications based on our instrumentation [95].

Immunohistochemistry

At sacrifice, brains were cut sagitally and the entire right hemisphere of the cortex was fixed in 4% paraformaldehyde for 24 h and stored at 4 °C in 70% ethanol until embedded in paraffin. Five-micrometer thick sections were mounted on slides for immunohistochemical analysis. Cholinergic staining was done as previously described using a ChAT polyclonal goat antibody (Millipore, Billerica, USA) [96]. Slides were scanned using the Zeiss Axio Scan.Z1 (Oberkochen, Germany).

Statistical Analysis

Statistical analysis was performed using SPSS (version 19.0) and GraphPad software package 5.01. Two-way ANOVA [two genotypes ($Mthfr^{+/+}$, $Mthfr^{+/-}$) × 2 diets (CD, FD)] was conducted to assess genotype and diet effects. Comparisons between groups were performed by Tukey's post hoc test. All data are presented as means ± S.E.M and p values (p) below 0.05 were considered statistically significant. Statistical outliers (≥ two standard deviations from the mean) and a mouse with a tumor were removed from the analyses. Partial correlations (controlling for genotype and/or diet effect) were utilized to analyze the possible association between expression of two genes or between gene and behavioral testing variables. For some cortical mRNA experiments, as indicated, results were obtained from a first experimental set of 10-month-old mice ($n = 5$/group) and then validated in a second set of 10-month-old mice ($n = 5$/group). The combined results of the two experiments are shown and expressed as a fold change relative to the control mice (Control diet, $Mthfr^{+/+}$) and then analyzed by two-way ANOVA. Unpaired t test analysis was occasionally performed using GraphPad software, as indicated.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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