Dietary folic acid deficiency impacts hippocampal morphology and cortical acetylcholine metabolism in adult male and female mice

Calli Bennett BS[1,2], Jacalyn Green PhD[3], Mae Ciancio PhD[4], Joanna Goral PhD[5], Lenore Pitstick[3] BS, Matthew Pytynia BA[4], Alice Meyer MBS[5], Neha Kwatra BSc[1,6], Nafisa M. Jadavji PhD[1,7,8]

Affiliations
[1] Biomedical Sciences Program, College of Graduate Studies, Midwestern University, Glendale, US
[2] College of Osteopathic Medicine, Midwestern University, Glendale, US
[3] Biochemistry and Molecular Genetics, College of Graduate Studies, Midwestern University, Downers Grove, US
[4] Biomedical Sciences Program, College of Graduate Studies, Midwestern University, Downers Grove, US
[5] Anatomy, College of Graduate Studies, Midwestern University, Downers Grove, US
[6] College of Dental Medicine, Midwestern University, Glendale, US
[7] College of Veterinary Medicine, Midwestern University, Glendale, US
[8] Department of Neuroscience, Carleton University, Ottawa, Canada

Corresponding Author
Nafisa M. Jadavji, PhD
Department of Biomedical Sciences
College of Graduate Studies
Midwestern University
19555 N 59th Ave
Glendale, AZ 85308, USA
Phone: 623-572-3665
Fax : 623-572-3647
E-mail: njadav@midwestern.edu

Abstract

Objective

One-carbon metabolism is a metabolic network that integrates nutritional signals with biosynthesis, redox homeostasis, and epigenetics. There are sex differences in hepatic one-carbon metabolism. However, it is unclear whether there are sex differences in dietary deficiencies of one-carbon metabolism in the brain. The aim of this study was to investigate if sex modulates the effects of dietary folic acid deficiency in brain tissue using a mouse model.

Methods

Male and female C57Bl/6J mice were placed on a folic acid deficient (FD) or control diet (CD) at six weeks of age. Mice were maintained on these diets for six months, after which animals were euthanized and brain tissue and serum were collected for analysis. Serum folate levels were measured. In brain tissue, hippocampal volume and morphology including Cornu Ammonis 1 and 3 (CA1; CA3), and dentate gyrus thickness were measured. Apoptosis within the hippocampus was assessed using active caspase-3 immunofluorescence staining. Additionally, cortical acetylcholine metabolism was measured in brain tissue using immunofluorescence staining of acetylcholinesterase (AChE), or choline acetyltransferase (ChAT), and neuronal nuclei (NeuN).

Results

Male and female FD mice had reduced serum levels of folate. Both males and females maintained on a FD showed a decrease in the thickness of the hippocampal CA1-CA3 region. Interestingly, there was a sex difference in the levels of active caspase-3 within the CA3 region of the hippocampus. In cortical tissue, there were increased levels of neuronal ChAT and reduced levels of AChE in FD females and male mice.

Conclusions

The results indicated that FD impacts hippocampal morphology and cortical neuronal acetylcholine metabolism. The data from our study indicate that there was only one sex difference and that was in hippocampal apoptosis. Our study provides little evidence that sex modulates the effects of dietary folate deficiency on hippocampal morphology and cortical neuronal acetylcholine metabolism.

Keywords

One- carbon metabolism; folic acid; hippocampus; apoptosis; acetylcholine; sex differences

1. Introduction

One-carbon (1C) metabolism is a key metabolic network that integrates nutrition signals with biosynthesis, redox homeostasis, and epigenetics. It plays an essential role in the regulation of cell proliferation, stress resistance, and embryonic development. Folate, also known as vitamin B9, is the main component of 1C metabolism. Folates act as 1C donors in the regeneration of methionine from homocysteine, nucleotide synthesis and repair, and DNA methylation (1).

While gastrointestinal bacteria produce small amounts of folate, the majority are obtained in our diets. However, the bioavailability and stability from these dietary sources are much lower than synthetic, folic acid (2). Many countries fortify foods with folic acid in order to reduce deficiencies and related negative health outcomes (3). However, even in countries in which fortifying foods is routine, deficiencies remain fairly common. Deficiencies are seen in individuals that have a high rate of tissue turnover (e.g., pregnant women), have an alcohol use disorder (4), or in those using folate antagonist drugs (e.g., methotrexate) (5). Additionally, deficiencies are common in the elderly population, which is especially vulnerable to malnutrition and folate absorption impairments (6) .

Folate's role in the regeneration of methionine from homocysteine proves especially significant when considering vascular and brain health. Elevated levels of homocysteine, that can be a result of folic acid deficiency, have been linked to several negative health outcomes, including increased risk for Alzheimer's Disease (7), and cerebrovascular diseases including stroke (8). Additionally, hyperhomocysteinemia is an important indicator of post-stroke recovery (9), mortality (10), and risk of recurrence (11). Homocysteine leads to downstream oxidative damage (12), indicating a potential mechanism behind its role in neuronal degeneration and atherosclerosis (13).

Choline, another component of 1C metabolism, is an essential nutrient. Folate and choline metabolism are tightly linked. In model systems that study the impact of reduced levels of dietary folates, choline metabolism is also affected (14–17). Choline can act as a 1C donor, especially in situations of diminished folate. In the brain, choline is also involved in the production of acetylcholine, the main neurotransmitter of the parasympathetic nervous system, and lipid membrane synthesis. Acetylcholine is synthesized by choline acetyltransferase (ChAT) and hydrolyzed by acetylcholinesterase (AChE). Reduced levels of choline have been reported to result in more apoptosis in cultured cells (18) and were observed in the hippocampus of offspring of choline deficient females (19). *In vivo* rodent studies have also shown cognitive impairment, because of a dietary choline deficiency. The hippocampus is particularly vulnerable to both choline and folate dietary deficiencies (14,16,20,21).

There is growing evidence that there are significant sex differences with regard to 1C metabolism (22–24), including quantifiable differences in homocysteine levels (25). Males homozygotic for the polymorphism in methylenetetrahydrofolate reductase are more vulnerable to factors (e.g., smoking) that increase levels of homocysteine (25). Estrogen may serve as a protective factor because it is linked to reduced homocysteine levels in blood. Decreased homocysteine levels lead to declined risk for vascular diseases (25). Furthermore, using a model, five key hepatic 1C metabolism enzymes are differentially expressed in males and females(23). Estrogen and other female hormones impact expression of these enzymes, leading to increased levels of homocysteine in males, and increased levels of choline and betaine in females(23). Further, folate intake and expression of genes in 1C metabolism regulate steatosis in a sex specific manner (26). Despite the significant evidence of hepatic 1C differences between sexes, limited evidence exists on the brain. A recent study, including both male and female mice, demonstrated

that there are significant sex differences in behavior observed caused by a methylenetetrahydrofolate reductase deficiency (22,24). The aim of this study was to investigate if sex modulates the effects of dietary folic acid deficiency in brain tissue using a mouse model. We hypothesized dietary folic acid deficiency would result in reduced levels dietary folate in males and females and that sex would impact on hippocampal morphology and cortical acetylcholine metabolism in mice maintained on a folic acid deficient diet.

2. Methods

2.1 Experimental design

All experiments were done in accordance with IACUC animal welfare protocols and were approved by the Midwestern University Downers Grove IACUC Committee. Male and female C57Bl/6J mice were put on a folic acid deficient (FD) or control (CD) diets (Envigo) at 6 weeks of age (Table 1). Animals had access to food and water *ad libitum*. Each diet group had 5 males and 5 females; a total of 20 animals were used.

The CD and FD contained the same concentrations of macro-micro-nutrients, except for folic acid (Table 1). The CD contained 2.0 mg/kg of folic acid (TD.04194), while FD contained 0.2 mg/kg of folic acid (TD.95247) (27,28). Mice were maintained on these diets for 6 months. Animals were weighed weekly throughout the experiment. At 8 months of age mice were euthanized with CO_2 inhalation and cervical dislocation. This corresponds to mature adults in humans (29).

132 **Table 1**. List of micro- and macro-nutrient contents in Envigo control (TD.04194) and folic acid
133 deficient diet (TD.95247). The Envigo folic acid deficient (TD.95247) was identical to the control
134 diet (TD.04194) except it contained 0.2 mg/kg folic acid.

Category	Concentration	Category	Concentration
Amino acids	**(g/kg)**	**Lipids**	**(g/kg)**
Lysine	14	Total fat	60.0
Methionine	4.7	Saturated fat	9.0
Cystine	3.6	Monounsaturated fat	13.8
Arginine	6.6	Polyunsaturated fat	36.6
Phenylalanine	8.8	4:0 Butyric acid	0.0
Tyrosine	9.2	6:0 Caproic Acid	0.0
Histidine	5.1	8:0 Caprylic Acid	0.0
Isoleucine	10.1	10:0 Capric acid	0.0
Leucine	16	12:0 Lauric acid	0.0
Threonine	7.6	14:0 Myristic Acid	0.0
Tryptophan	2.1	16:0 Palmitic acid	6.6
Valine	11.7	16:1 Palmitoleic acid	0.0
Aspartic Acid	12.1	18:0 Stearic acid	2.4
Glutamic acid	36.6	18:1 Oleic acid	13.8
Alanine	5.2	18:2 Linoleic acid	31.8
Glycine	3.2	18:3 Linolenic acid	4.8
Proline	18.1	Cholesterol	8.8 mg/kg
Serine	10		
Fat-soluble Vitamins	**(IU/kg)**	**Minerals**	**(mg/kg)**
Vitamin A	4000	Calcium	5100
Vitamin D	1000	Phosphorus	3000
Vitamin E	75	Potassium	3600
Vitamin K	0.8	Sodium	1000
Water-soluble Vitamins	**(mg/kg)**	Chlorine	1600
Biotin	0.20	Magnesium	515
Choline	1147.5	Copper	6.0
Folic acid	2.0	Iron	36.5
Niacin	30.0	Zinc	35.6
Pantothenate	14.7	Manganese	10.5
Riboflavin	6.0	Iodine	0.21
Thiamin	4.9	Selenium	0.15
Vitamin B_6	5.8	Molybdenum	0.15
Vitamin B_{12}	0.03	Chromium	1.00
Vitamin C	0.0		

135

2.2 Tissue collection

At time of euthanization body and brain weights were recorded. Tissue including brain and blood were collected for analysis. Serum was isolated from blood and stored at -80°C until analysis. Brain tissues were fixed in 4% paraformaldehyde overnight and then switched over to a 70% ethanol solution. Tissue was processed and embedded in paraffin in a coronal orientation. Brain tissue was sectioned using a microtome (Leica) at 5-µm thickness. Sections were slide mounted and stored at room temperature for staining.

2.3 Microbiological assay for serum folate

Serum folate was measured using a microbiological assay (30). *Lactobacillus rhamnosus* (ATCC) was grown overnight in Folic Acid Casei Medium (Difco) supplemented with 0.025% sodium ascorbate (A7631, Sigma-Aldrich,) and 1.2 ng/ml of (6S)-5-formyltetrahydrofolate (Schirks Laboratories). An equal volume of 80% sterile glycerol was added to the overnight culture and 1 ml aliquots were frozen and stored at -80°C. The day of the assay, an aliquot of *L. rhamnosus* was thawed and 100ul was added to 50ml of Folic Acid Casei Medium supplemented with 0.025% sodium ascorbate. The approximately 20 mg/liter folic acid stock (Sigma-Aldrich) was 0.22 µm filter sterilized, verified by absorbance spectra at 282 (molar absorptivity coefficient= 27,000 M^{-1}, MW= 441.4), and stored at -20°C. Dilutions of the folic acid stock and test sera were made in freshly prepared 0.22 µm filter sterilized 0.5% sodium ascorbate. Through a series of dilutions, a folic acid standard curve with 100µl/well in duplicate was generated from 0 to 166 pg/ml final concentration. Test sera was diluted with sterile 0.5% sodium ascorbate before the assay. Several dilutions of sera, 1:600-1:2400 final concentration, at 100µl/well in duplicate were tested to ensure that an A_{600} fell within the standard curve. The inoculated media was added at 200µl/well to the

158 10 x10 Bioscreen honeycomb plate (Fisher Scientific). The plate was loaded into the Bioscreen C
159 accompanied with EZExperiment 1.26 software (OY Growth Curves Ab Ltd), incubated at 37°C
160 with shaking for 24 hours, and A_{600} read at 24 hours (30).

161 ***2.4 Brain tissue morphological analysis***

162 Series of brain tissue samples were stained with 1% cresyl violet (Sigma) to assess
163 morphological changes. Images were taken using Nikon Ni-U Compound Light microscope.
164 Analysis of morphological changes focused on the hippocampal formation as previous studies
165 demonstrate that this area is particularly vulnerable to deficiencies in 1C metabolism (14,16,20).
166 Thickness measurements of the granular cell layer were taken using ImageJ (NIH) (31,32) within
167 the dentate gyrus, Cornu Ammonis 1 (CA1) and 3 (CA3) regions of the hippocampus. The total
168 volume of the hippocampus was measured using ImageJ (NIH) (31,32) by tracing the perimeter of
169 the structure. For each measurement a minimum of 3 brain tissue sections per animals were used
170 and each hemisphere was measured. An average of 6 measurements was used per animal.

171 ***2.5 Immunofluorescence***

172 Antigen retrieval on paraffin embedded brain tissue sections was performed prior to
173 staining as previously described (33). A series of brain tissue sections were stained with the
174 following antibodies: anti-choline acetyltransferase (ChAT) (Millipore, AB144P) at 1:100; anti-
175 acetylcholinesterase (AChE) (Sigma, SAB2500018) at 1:100; or anti-active caspase-3 (Cell
176 Signaling, 9662) at 1:100. All samples were co-stained with anti-neuronal nuclei antibody (NeuN,
177 ab104224) (AbCam) at 1:200 and (4',6-diamidino-2-phenylindole) DAPI (Fisher Scientific) at
178 1:10,000.

Imaging of ChAT and AChE was conducted using a Zeiss Apotome microscope equipped with a camera. Two brain tissue sections with three subfields within each cortex were imaged. Imaging of active caspase-3 was performed using an Olympus inverted microscope equipped with a camera. The hippocampus was analyzed using two images of CA1, CA3, or three dentate gyrus for each animal. Only cells demonstrating colocalization of ChAT, AChE, or active caspase-3 along with NeuN and DAPI were considered positive and counted.

2.6 Data and statistical analysis

All data analysis was conducted by 2 individuals blinded to experimental groups. Data were analyzed using GraphPad Prism 9.0 and IBM SPSS Statistics 25. Weekly body weights were analyzed using repeated measures ANOVA using IBM SPSS Statistics 25. Two-way analysis of variance (ANOVA) was used to determine interactions and main effects between diet groups (folic acid deficient or control) and sex (male or female). Analyses included brain and body weight, morphological measurements, immunofluorescence of choline metabolism (AChE and ChAT), and active caspase-3. Pairwise post-hoc testing was performed using the Tukey's multiple comparison test. In all analyses, $p<0.05$ was considered statistically significant.

3. Results

3.1 Body and brain weight

Body weights were collected on a weekly basis; a difference between male and females (Figure 1A, $F(_{1,14}) = 50.80$, $p<0.001$). However, there was no difference by diet ($F(_{1,14}) = 0.048$, $p = 0.830$). Brain and body weights were collected at time of euthanization and the data was analyzed. There was a sex difference in body weight (Figure 1B, $F\ (_{1,\ 14}) = 48.48$, $p < 0.001$), but

there was no effect of diet ($F_{(1, 14)} = 1.97$, $p = 0.18$). There was no difference in brain weight observed (Figure 1C, Sex: $F_{(1,14)} = 2.58$, $p = 0.13$, Diet: $F_{(1,14)} = 1.13$, $p = 0.31$). When analyzing brain weight as a percent of body weight, females had a higher percent brain weight than males (Figure 1D, $F_{(1,14)} = 73.99$, $p < 0.0001$). Female FD mice had smaller brain weight as percent of body when compared to female CD mice ($F_{(1,14)} = 6.217$, $p = 0.026$).

3.2 Serum folate measurements

Folate levels were measured in serum collected from all animals to confirm dietary deficiency. There was no sex difference ($F_{(1,14)} = 2.40$, $p = 0.14$). Compared to CD mice, animals maintained on the FD had reduced levels of folate in their serum (Figure 1A; $F_{(1,14)} = 10.52$, $p = 0.0059$).

3.3 Hippocampal volume and morphology

Previous work has shown that the hippocampus is affected by a 1C deficiency (14,19,21). The hippocampus volume was analyzed using ImageJ, and thickness of the dentate gyrus, CA1, and CA3 regions. There was no sex difference (Figure 2B; $F_{(1,12)} = 0.37$, $p = 0.55$) in the total hippocampal volume. However, there was a trend for lower hippocampal volume of mice maintained on FD ($F_{(1,12)} = 4.37$, $p = 0.059$). There was no sex (Figure 2C; $F_{(1,12)} = 0.86$, $p = 0.37$) or diet ($F_{(1,12)} = 1.15$, $p = 0.31$) differences in the thickness of the dentate gyrus. There was no sex difference in the thickness of CA1 and 3 regions ($F_{(1,13)} = 0.94$, $p = 0.35$). However, there was a decrease in the thickness of the CA1 and 3 regions of mice maintained on a FD diet (Figure 2D; $F_{(1,13)} = 37.3$, $p < 0.0001$).

3.4 Hippocampal apoptosis

Hippocampal apoptosis was assessed by counting active caspase-3 positive neuronal cells, such cells were counted within the dentate gyrus, CA1, and CA3 regions of the hippocampus. There was no difference in neuronal apoptosis between groups within the dentate gyrus (sex: $F_{(1,12)} = 0.035$, $p = 0.86$, diet: $F_{(1,13)} = 0.033$, $p = 0.86$). There was a trend for a sex difference of apoptotic neurons within the CA1 region ($F_{(1,12)} = 3.502$, $p = 0.084$), but there was no difference between dietary groups ($F_{(1,12)} = 0.00083$, $p = 0.99$). Representative images of active caspase-3 staining within the CA3 are shown in Figures 3A to D. There was a difference in the number of positive apoptotic neurons between females and males (Figure 3E; ($F_{(1,12)} = 7.36$, $p = 0.019$)), but no dietary difference ($F_{(1,12)} = 1.44$, $p = 0.25$).

3.5 Cortical choline metabolism

Neuronal cortical choline metabolism was characterized by counting the number choline acetyltransferase (ChAT) positive cells. This enzyme is involved in the synthesis of acetylcholine at the synapse. Representative images are shown in Figure 4A to D. Quantification revealed no difference between sexes ($F_{(1,12)} = 1.79$, $p = 0.21$), however, there were increases in ChAT positive neurons in the FD groups when compared to CD mice (Figure 4E; $F_{(1,12)} = 4.58$, $p = 0.050$).

To confirm changes in choline metabolism in brain tissue, we also measured acetylcholine esterase (AchE) levels in neurons within the cortex. Representative images are shown in Figure 5A to D. Quantification revealed no difference between sexes ($F_{(1,12)} = 0.49$, $p = 0.49$), but did reveal decreases in FD mice compared to CD animals (Figure 5E; $F_{(1,12)} = 7.29$, $p = 0.019$).

4. Discussion

One-carbon metabolism involves multiple complex factors that coordinate gene expression and metabolism in response to nutritional signals that impact cellular anabolism, redox

246 homeostasis, and epigenetics. There is growing evidence that sex plays a significant role in one-
247 carbon metabolism and in regulating levels of blood homocysteine (22–25). Despite this, there is
248 limited evidence for sex differences in brain tissue. Using a mouse model, our study investigated
249 the impact of sex and dietary folic acid deficiency on hippocampal morphology and brain choline
250 metabolism. As expected, the FD mice had lower serum folates than CD mice. Notably, our results
251 indicate a significant decrease in the thickness of the granular layer within the CA1-CA3 region in
252 both males and females maintained on a FD. In cortical tissue we demonstrate changes in
253 acetylcholine metabolism between diet groups. The only sex difference we report in our study is
254 the decreased apoptosis observed in the CA3 region of the hippocampus in males compared to
255 females.

256 Consistent with prior research, our results demonstrate an intrinsic link between choline
257 and folate metabolism (14–17). Specifically, our results show the novel finding of increased levels
258 of cortical neuronal ChAT, the enzyme that catalyzes the synthesis of acetylcholine from choline
259 at the synapse and decreased levels of AChE, the enzyme that catalyzes the destruction of
260 acetylcholine within cortical neurons. Choline can act as a 1C donor in the liver and kidney
261 via betaine homocysteine methyltransferase, especially when folate levels are low (33,34). This
262 compensatory function of choline shunts choline away from the production of acetylcholine,
263 potentially leading to lower levels of the neurotransmitter (14,15,17). Therefore, we suggest a
264 potential mechanism for the demonstrated changes in cortical choline metabolism is a
265 compensatory upregulation of ChAT and downregulation of AChE in response to decreased levels
266 of acetylcholine. Low levels of acetylcholine have been associated with cognitive impairments,
267 including mild cognitive impairment and dementia (35). An interesting follow-up to this study

268 would be to assess cognitive function in adult females and males maintained on folic acid deficient
269 and control diets.

270 The hippocampus is impacted by a genetic or dietary deficiency in 1C metabolism, as
271 shown by others; our results demonstrate this effect as well (14,16,21). The different areas of the
272 hippocampus and their functions are topics of ongoing research. In humans, the CA3 region has
273 been specifically linked to spatial representations and episodic memory. Additionally, the CA3
274 region is susceptible to neurodegeneration, possibly accounting for our results (36). Both *in vivo*
275 and *in vitro* studies have demonstrated that reduced levels of folate result in an increase in apoptotic
276 markers (19,37–39), possibly due to increases in oxidative stress (40), DNA damage (38), or
277 neuroexcitatory mechanisms (41). Recent *in vivo* studies have indicated similar results, with
278 hyperhomocysteinemia, a direct consequence of 1C deficiency, leading to increased autophagy
279 and apoptosis of cortical neurons(42).

280 While our results did not indicate a diet difference in apoptosis, there was a significant sex
281 difference in apoptosis in the CA3 region of the hippocampus. The CA3 region is the most
282 interconnected region of the hippocampus and has been implicated in memory functions and
283 neurodegeneration (36). In order to measure levels of apoptosis, we stained for active caspase-3,
284 which, as an executioner caspase leading to cell death, acts as an indirect measure of apoptosis
285 (43). Previous research demonstrates a distinct sex difference in the activation of caspases,
286 specifically following brain injury (44,45). These differences could be accounted for by sex
287 hormones, potentially via the pro-apoptotic protein Bax (46). Model studies have indicated a
288 significant sex difference in gross hippocampal morphology within the hippocampus including the
289 dentate gyrus, CA1 and CA3 regions (47). A recent human study indicates that after adjusting for
290 total hippocampal volume, regional sex differences exist, however, they are not present in the

CA2/CA3 regions or the dentate gyrus (48). We are not observing a diet impact on apoptosis, which has been previously reported (19). We think that the duration and time point of dietary deficiency may be an important factor, since there is minimal apoptosis that occurs in the normal adult brain (49). Apoptosis in the brain is associated with key time points, such as after birth (49) and damage (50,51).

In conclusion, our study demonstrates stronger evidence that folate availability modulates changes in brain tissue more than sex in adult mice. Future research should aim to include behavioral analysis measuring cognitive function of male and females maintained on a 1C deficient diets.

Acknowledgments

The authors would like to the College of Graduate Studies for the generous support of this collaboration. CB was funded by the Kenneth A. Suarez Research Fellowship.

Disclosure statement

None

Notes on contribution

Calli Bennett BSc is a Doctor of Osteopathic Medicine student at Midwestern University. Calli graduated with an Honors Bachelor of Science degree in Anthropology from the University of Utah.

Jacalyn Green PhD is a Professor in the Department of Biochemistry and Molecular Genetics at Midwestern University Downers Grove campus.

Mae Ciancio PhD is an Associate Professor in the Biomedical Sciences Program at Midwestern University Downers Grove campus.

Joanna Goral PhD is an Associate Professor in the Department of Anatomy at Midwestern University Downers Grove campus.

Lenore Pitstick BS is a Department Research Coordinator in the Department of Biochemistry and Molecular Genetics at Midwestern University Downers Grove campus.

Matt Pytynia BA is a Senior Research Specialist in the Biomedical Sciences Program at Midwestern University Downers Grove campus.

Alice Meyer MBS is a Senior Research Associate in the Department of Anatomy at Midwestern University Downers Grove campus.

Neha Kwatra BSc is a Doctor of Dental Medicine student at Midwestern University. Neha graduated with a BSc in Physiology from the University of Arizona.

Nafisa M. Jadavji PhD is an Assistant Professor in Biomedical Sciences at Midwestern University (US) and Research Assistant Professor in Neuroscience at Carleton University (Canada).

Figure Captions

Figure 1. The impact of dietary folic acid deficiency and sex on body and brain weight. Weekly body weights (A) for duration of 6-month experimental period. At time of euthanization body weight (B), brain weight (C) and brain weight as a percent of body weight (D). Line graph and scatter plot with mean ± SEM of 3 to 5 mice per group. * $p<0.05$ indicate sex difference after repeated measures 2-way ANOVA. *** $p<0.001$ indicate Tukey's pairwise comparison between groups.

Figure 2. The impact of dietary folic acid deficiency and sex on serum folate levels (A) and hippocampal volume (B), and thickness of dentate gyrus granular cell layer (C) and Cornu Ammonis (CA) 1 and 3 region (D). Scatter plot with mean ± SEM of 3 to 5 mice per group. * $p<0.05$ and ** $p<0.01$ indicate Tukey's pairwise comparison between groups.

Figure 3. The impact of dietary folic acid deficiency and sex on active caspase-3 levels within the hippocampal Cornu Ammonis 3 (CA3) region of brain tissue. Representative images of active caspase-3, neuronal nuceli (NeuN), and 4'6-diamidino-2-phenyloindole (DAPI) from all experimental groups (A -D). Blinded quantification of active caspase-3, NeuN, and DAPI positive cells (E). Scatter plot with mean ± SEM of 3 to 5 mice per group. * $p<0.05$ indicates sex main effect between males and females.

Figure 4. The impact of dietary folic acid deficiency and sex on neuronal choline acetyltransferase (ChAT) levels within the cortex of adult mice. Representative images from all experimental groups (A to D) of ChAT, neuronal nuclei (NeuN), and 4′,6-diamidino-2-phenylindole (DAPI).

364 Quantification of ChAT, NeuN, and DAPI positive cells (E). Scatter plot with mean ± SEM of 3
365 to 5 mice per group. * $p<0.05$ indicate Tukey's pairwise comparison between groups.
366
367 **Figure 5.** The effect of dietary folic acid deficiency and sex on neuronal acetylcholine transferase
368 (AChE) levels within the cortex of adult mice. Representative images from all experimental groups
369 (A to D) of AChE, neuronal nuclei (NeuN), and 4′,6-diamidino-2-phenylindole (DAPI).
370 Quantification of AChE, NeuN, and DAPI positive cells (E). Scatter plot with mean ± SEM of 3
371 to 5 mice per group. * $p<0.05$ indicate Tukey's pairwise comparison between indicated groups.
372

References

1. Folate in Health and Disease [Internet]. Routledge & CRC Press. [cited 2021 Apr 24]. Available from: https://www.routledge.com/Folate-in-Health-and-Disease/Bailey/p/book/9781138111882

2. Caudill MA. Folate bioavailability: Implications for establishing dietary recommendations and optimizing status. Am J Clin Nutr. 2010 May;91(5):1455S-1455S.

3. Crider KS, Bailey LB, Berry RJ. Folic acid food fortification-its history, effect, concerns, and future directions. Nutrients. 2011 Mar;3(3):370–84.

4. Halsted CH, Villanueva JA, Devlin AM. Folate deficiency, methionine metabolism, and alcoholic liver disease. In: Alcohol. 2002. p. 169–72.

5. Morgan SL, Baggott JE, Vaughn WH, Austin JS, Veitch TA, Lee JY, et al. Supplementation with folic acid during methotrexate therapy for rheumatoid arthritis: A double-blind, placebo-controlled trial. Ann Intern Med. 1994 Dec;121(11):833–41.

6. Enderami A, Zarghami M, Darvishi-Khezri H. The effects and potential mechanisms of folic acid on cognitive function: a comprehensive review. Neurol Sci. 2018 Oct;39(10):1667–75.

7. Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med. 2002;346(7):476–83.

8. Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: Evidence on a causal link from mendelian randomisation. Lancet. 2005;365(9455):224–32.

9. Kwon H-M, Lee Y-S, Bae H-J, Kang D-W. Homocysteine as a predictor of early neurological deterioration in acute ischemic stroke. Stroke. 2014 Mar;45(3):871–3.

10. Shi Z, Guan Y, Huo YR, Liu S, Zhang M, Lu H, et al. Elevated Total Homocysteine Levels in Acute Ischemic Stroke Are Associated With Long-Term Mortality. Stroke. 2015;46(9):2419–25.

11. Kumral E, Saruhan G, Aktert D, Orman M. Association of Hyperhomocysteinemia with Stroke Recurrence after Initial Stroke. J Stroke Cerebrovasc Dis. 2016;1–8.

12. Ho PI, Ashline D, Dhitavat S, Ortiz D, Collins SC, Shea TB, et al. Folate deprivation induces neurodegeneration: roles of oxidative stress and increased homocysteine. Neurobiol Dis. 2003;14:32–42.

13. Castro R, Rivera I, Blom HJ, Jakobs C, de Almeida IT. Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: An overview. J Inherit Metab Dis. 2006 Feb;29(1):3–20.

14. Jadavji NM, Deng L, Leclerc D, Malysheva O, Bedell BJ, Caudill MA, et al. Severe methylenetetrahydrofolate reductase deficiency in mice results in behavioral anomalies with morphological and biochemical changes in hippocampus. Mol Genet Metab. 2012 Jun;106(2):149–59.

15. Chan A, Tchantchou F, Graves V, Rozen R, Shea TB. Dietary and genetic compromise in folate availability reduces acetylcholine, cognitive performance and increases aggression: Critical role of S-adenosyl methionine. J Nutr Health Aging. 2008 Apr;12(4):252–61.

16. Bahous RH, Jadavji NM, Deng L, Cosín-Tomás M, Lu J, Malysheva O, et al. High dietary folate in pregnant mice leads to pseudo-MTHFR deficiency and altered methyl metabolism, with embryonic growth delay and short-term memory impairment in offspring. Hum Mol Genet. 2017;26(5):888–900.

17. Dam K, Füchtemeier M, Farr T, Boehm-Strum P, Foddis M, Dirnagal U, et al. Deficiencies in methylenetetrahydrofolate reductase and dietary folic acid alter choline metabolism during chronic hypoperfusion. Behav Brain Res. 2017;321:201–8.

18. Yen C-LE, Mar M-H, Craciunescu CN, Edwards LJ, Zeisel SH. Deficiency in methionine, tryptophan, isoleucine, or choline induces apoptosis in cultured cells. J Nutr. 2002 Jul;132(7):1840–7.

19. Jadavji NM, Deng L, Malysheva OV, Caudill MA, Rozen R. MTHFR deficiency or reduced intake of folate or choline in pregnant mice results in impaired short-term memory and increased apoptosis in the hippocampus of wild-type offspring. Neuroscience. 2015;300:1–9.

20. Troen AM, Chao W-H, Crivello NA, D'Anci KE, Shukitt-Hale B, Smith DE, et al. Cognitive impairment in folate-deficient rats corresponds to depleted brain phosphatidylcholine and is prevented by dietary methionine without lowering plasma homocysteine. J Nutr. 2008 Dec;138(12):2502–9.

21. Chen Z, Schwahn BC, Wu Q, He X, Rozen R. Postnatal cerebellar defects in mice deficient in methylenetetrahydrofolate reductase. Int J Dev Neurosci Off J Int Soc Dev Neurosci. 2005 Aug;23(5):465–74.

22. Levav-Rabkin T, Blumkin E, Galron D, Golan HM. Sex-dependent behavioral effects of Mthfr deficiency and neonatal GABA potentiation in mice. Behav Brain Res. 2011;216(2):505–13.

23. Sadre-Marandi F, Dahdoul T, Reed MC, Nijhout HF. Sex differences in hepatic one-carbon metabolism. BMC Syst Biol [Internet]. 2018 Oct;12(1). Available from: https://pubmed.ncbi.nlm.nih.gov/30355281/

24. Blumkin E, Levav-Rabkin T, Melamed O, Galron D, Golan HM. Gender-Specific Effect of Mthfr Genotype and Neonatal Vigabatrin Interaction on Synaptic Proteins in Mouse Cortex. Neuropsychopharmacology. 2011 Jul;36(8):1714–28.

25. Stanisławska-Sachadyn A, Woodside JV, Brown KS, Young IS, Murray L, McNulty H, et al. Evidence for sex differences in the determinants of homocysteine concentrations. Mol Genet Metab. 2008 Apr;93(4):355–62.

26. Christensen KE, Wu Q, Wang X, Deng L, Caudill MA, Rozen R. Steatosis in mice is associated with gender, folate intake, and expression of genes of one-carbon metabolism. J Nutr. 2010;140(10):1736–41.

27. Kim MH, Kim WH, Kim CG, Kim DW. Synthesis and Evaluation of 99mTc-Labeled Folate-Tripeptide Conjugate as a Folate Receptor-Targeted Imaging Agent in a Tumor-Bearing Mouse Model. Nucl Med Mol Imaging. 2015 Sep;49(3):200–7.

28. Martínez-Vega R, Murillo-Cuesta S, Partearroyo T, Varela-Moreiras G, Varela-Nieto I, Pajares MA. Long-term dietary folate deficiency accelerates progressive hearing loss on CBA/Ca mice. Front Aging Neurosci. 2016;8(AUG):1–8.

29. Flurkey K, Currer JM, Harrison DE. Mouse Models in Aging Research. In: Fox J, Barthold S, Davisson M, Newcommer C, Quimby F, Smith A, editors. The-Mouse-in-Biomedical-Research, Normative Biology, Husbandry, and Models [Internet]. 2nd ed. Elsevier.; 2007. p. 637–72. Available from: https://mouseion.jax.org/stfb2000_2009/1685

30. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol. 1997;281:43–53.

31. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: An open-source platform for biological-image analysis. Nat Methods. 2012 Jul;9(7):676–82.

32. Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, et al. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics. 2017 Dec;18(1):529–529.

33. Prieur EAK, Pjetri E, Zeisel SH, Jadavji NM. Reduced brain volume and impaired memory in betaine homocysteine S-methyltransferase knockout mice. Appl Physiol Nutr Metab. 2017;42(11).

34. Niculescu MD, Zeisel SH. Diet, Methyl Donors and DNA Methylation: Interactions between Dietary Folate, Methionine and Choline. J Nutr. 2002 Aug;132(8):2333S-2335S.

35. Sabri O, Meyer PM, Gräf S, Hesse S, Wilke S, Becker GA, et al. Cognitive correlates of α4β2 nicotinic acetylcholine receptors in mild Alzheimer's dementia. Brain. 2018 Jun;141(6):1840–54.

36. Cherubini E, Miles R. The CA3 region of the hippocampus: How is it? What is it for? How does it do it? Front Cell Neurosci [Internet]. 2015 Feb;9(FEB). Available from: https://pubmed.ncbi.nlm.nih.gov/25698930/

37. Kasture VV, Sundrani DP, Joshi SR. Maternal one carbon metabolism through increased oxidative stress and disturbed angiogenesis can influence placental apoptosis in preeclampsia. Life Sci. 2018;206(May):61–9.

38. Kruman II, Culmsee C, Chan SL, Kruman Y, Guo Z, Penix L, et al. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J Neurosci Off J Soc Neurosci. 2000 Sep 15;20(18):6920–6.

39. Kruman II, Kumaravel TS, Lohani A, Pedersen WA, Cutler RG, Kruman Y, et al. Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease. J Neurosci Off J Soc Neurosci. 2002 Mar 1;22(5):1752–62.

40. Hoffman M. Hypothesis: Hyperhomocysteinemia is an indicator of oxidant stress. Med Hypotheses. 2011 Dec;77(6):1088–93.

41. Lipton SA, Kim W-K, Choi Y-B, Kumar S, D'Emilia DM, Rayudu PV, et al. Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. Proc Natl Acad Sci. 1997 May 27;94(11):5923–8.

42. Zhang JW, Yan R, Tang YS, Guo YZ, Chang Y, Jing L, et al. Hyperhomocysteinemia-induced autophagy and apoptosis with downregulation of hairy enhancer of split 1/5 in cortical neurons in mice. Int J Immunopathol Pharmacol. 2017;30(4):371–82.

43. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol. 2013 Apr;5(4):1–28.

44. Liu F, Li Z, Li J, Siegel C, Yuan R, Mccullough LD. Sex differences in caspase activation after stroke. Stroke. 2009 May;40(5):1842–8.

45. Siegel C, Li J, Liu F, Benashski SE, McCullough LD. miR-23a regulation of X-linked inhibitor of apoptosis (XIAP) contributes to sex differences in the response to cerebral ischemia. Proc Natl Acad Sci U S A. 2011 Jul;108(28):11662–7.

46. Forger NG, Rosen GJ, Waters EM, Jacob D, Simerly RB, De Vries GJ. Deletion of Bax eliminates sex differences in the mouse forebrain. Proc Natl Acad Sci U S A. 2004 Sep;101(37):13666–71.

47. Yagi S, Chow C, Lieblich SE, Galea LAM. Sex and strategy use matters for pattern separation, adult neurogenesis, and immediate early gene expression in the hippocampus. Hippocampus. 2016 Jan;26(1):87–101.

48. van Eijk L, Hansell NK, Strike LT, Couvy-Duchesne B, de Zubicaray GI, Thompson PM, et al. Region-specific sex differences in the hippocampus. NeuroImage. 2020 Jul;215:116781–116781.

49. Kolb B, Whishaw IQ, Teskey GC. An Introduction to Brain and Behavior. Fifth. New York: Worth Publishers; 2016.

514 50. Jadavji N, Emmerson JT, Shanmugalingam U, Willmore WG, Macfarlane AJ, Smith PD. A
515 genetic deficiency in folic acid metabolism impairs recovery after ischemic stroke. Exp
516 Neurol. 2018;309:14–22.

517 51. Jadavji NM, Mosnier H, Kelly E, Lawrence K, Cruickshank S, Stacey S, et al. One-carbon
518 metabolism supplementation improves outcome after stroke in aged male MTHFR-deficient
519 mice. Neurobiol Dis. 2019;132.

520

Figure 1

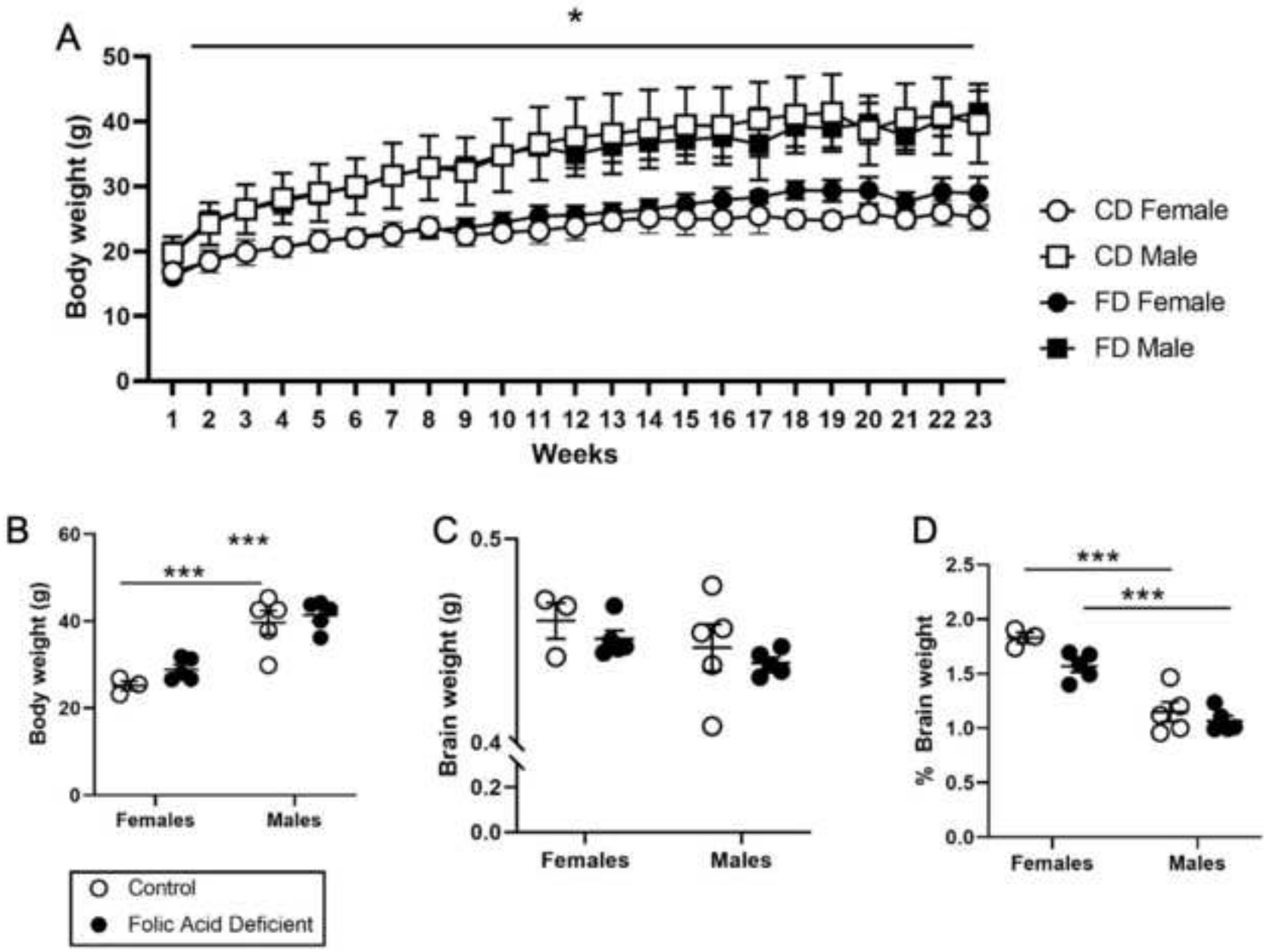

Figure 2