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

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**Abstract**

***Background***

Recent evidence suggests that sex plays a role when there are deficiencies in one-carbon metabolism, however, the impact on brain tissue remains unknown.

***Objective***

The aim of the study was to examine the impact of sex differences and dietary folic acid deficiency on brain tissue in adult mice.

***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) and choline acetyltransferase (ChAT).

***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 CA3 region. Interestingly, there was a sex difference in the levels of apoptosis within the CA1 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 through increased apoptosis and changes acetylcholine metabolism within the cortex. The data from our study indicate a sex difference in apoptosis and differences in hippocampal morphology and choline metabolism as a result of dietary folic acid deficiency.

## 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 (1). 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)(2)

While gastrointestinal bacteria produce small amounts of folate, the majority of folates are obtained in our diets. However, the bioavailability and stability from these dietary sources are much lower than synthetic, folic acid (3). Many countries fortify foods with folic acid in order to reduce deficiencies (4). 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, or in individuals using folate antagonist drugs (e.g., methotrexate) (5)(6). Additionally, deficiencies are common in the elderly population, which is especially vulnerable to malnutrition and folate absorption impairments (7).

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 (8), and cerebrovascular diseases including stroke (9). Additionally, hyperhomocysteinemia is an important indicator of post-stroke recovery (10), mortality (11), and risk of recurrence (12). Homocysteine leads to downstream oxidative damage, 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 studying the impact of reduced levels of dietary folates, choline metabolism is also affected (14–16). 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 (17) and were observed in the hippocampus of offspring of choline deficient females (18). *In vivo* rodent studies have also shown cognitive impairment, as a result of a dietary choline deficiency (19). The hippocampus is particularly vulnerable to both choline and folate dietary deficiencies (20–22).

There is growing evidence that there are significant sex differences with regard to 1C metabolism (23–26), including quantifiable differences in homocysteine levels have (24). Males homozygotic for the polymorphism in methylenetetrahydrofolate reductase are more vulnerable to factors (e.g., smoking) that increase levels of homocysteine(24). Estrogen may serve as a protective factor, limiting hyperhomocysteinemia that in turn leads to an increased risk for vascular diseases (24). Furthermore, five key hepatic 1C metabolism enzymes are differentially expressed in males and females. 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 (24). Further, folate intake and expression of genes in 1C metabolism regulate steatosis in a sex specific manner (23). Despite the significant evidence of hepatic 1C differences between sexes, limited evidence exists on sex related 1C metabolism differences in the brain. A recent study, including both male and female mice, demonstrates that there are significant sex differences in behavior observed caused by a methylenetetrhydratefolate reductase deficiency (25). The aim of this study was to investigate if sex modulates the effects of folate deficiency in brain tissue

using a mouse model system. We hypothesize that in addition to dietary folate levels, sex will have a significant effect on hippocampal morphology and cortical 1C metabolism.

## Methods

### *Experimental design*

All experiments were done in accordance with IACUC animal welfare protocols and were approved by the Midwestern University Downers Grove IACUC Committee (protocol #2501). Male and female C57Bl/6J mice were put on a folic acid deficient (FD) or control (CD) diets (Envigo) at 6 weeks of age. Each diet group had 5 males and 5 females; a total of 20 animals were used. During experiments, two females prior to the planned end-point were euthanized from the CD group because of health concerns.

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 folic acid (TD.04194), while FD contained 0.2 mg/kg folic acid (TD.95247) (27,28)Mice were maintained on these diets for 6 months and at 8 months of age mice were euthanized with $CO_2$ inhalation and cervical dislocation. This corresponds to mature adults in humans (29).

**Table 1**. List of micro- and macro-nutrient contents in Envigo control diet (TD.04194). The folic acid deficient diet (TD.95247) was identical 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 | | |

***Tissue collection***

At time of euthanization brain and blood were collected. 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. Serum was isolated from blood and stored at -80°C until analysis.

***Microbiological assay for serum folate***

*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 10 x10 Bioscreen honeycomb plate (Fisher Scientific). The plate was loaded into the Bioscreen C accompanied with EZExperiment 1.26

software (OY Growth Curves Ab Ltd), incubated at 37°C with shaking for 24 hours, and $A_{600}$ read at 24 hours (30).

***Brain tissue morphological analysis***

Series of brain tissue samples were stained with 1% cresyl violet (Sigma) to assess morphological changes. Images were taken using Nikkon Ni-U Compound Light microscope. Analysis of morphological changes focused on the hippocampal formation as previous studies demonstrate that this area is particularly vulnerable to 1C metabolism (15,31). Thickness measurements of the granular cell layer were taken using Fiji (NIH) (32) within the dentate gyrus, Cornu Ammonis 1 (CA1) and 3 (CA3) regions of the hippocampus as well as the total volume of

***Immunofluorescence***

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

### *Data and statistical analysis*

All data analysis was conducted by 2 individuals blinded to experimental groups. Data were analyzed using GraphPad Prism 9.0. Two-way analysis of variance (ANOVA) was used to compare treatment groups (folic acid deficient or control diets) 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.

## Results

### *Body and brain weight*

Brain and body weight were collected at time of euthanization. There was a sex difference in body weight ($F_{(1, 14)} = 48.48$, $p < 0.001$), but there was no effect of diet ($F_{(1, 14)} = 1.97$, $p = 0.18$). There was no difference in brain weight observed (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 ($F_{(1,14)} = 73.99$, $p < 0.0001$). Female FD mice had smaller brain weight as percent of body ($F_{(1,14)} = 6.217$, $p = 0.026$).

### *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; $F_{(1,14)} = 10.52$, $p = 0.0059$).

***Hippocampal volume and morphology***

Previous work has shown that the hippocampus is affected by a 1C deficiency (15,34–36). The hippocampus volume was analyzed using FIJI, and thickness of the dentate gyrus, CA1, and CA3 regions. There was no sex difference ($F_{(1,12)} = 0.37$, $p = 0.55$) in the total hippocampal volume. However, there was a trend for lower hippocampal volume of mice maintained on FD (Figure 1B; $F_{(1,12)} = 4.37$, $p = 0.059$). There were no sex ($F_{(1,12)} = 0.86$, $p = 0.37$) or diet ($F_{(1,12)} = 1.15$, $p = 0.31$) differences in the thickness of the dentate gyrus (Figure 1C). Representative images of cresyl violet staining from all groups are shown in Figure 2A. There was no sex ($F_{(1,13)} = 0.07$, $p = 0.88$) or diet ($F_{(1,13)} = 4.28$ $p = 0.06$) difference in CA1 thickness. There was no sex difference in the thickness

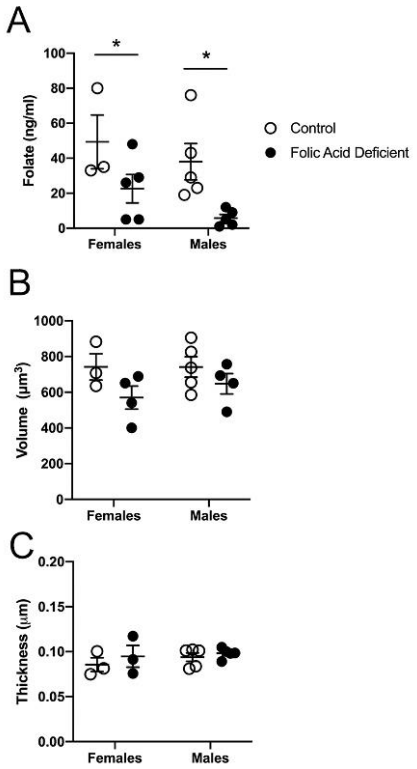


**Figure 1.** The impact of dietary folic acid deficiency and sex on serum folate levels (A) and hippocampal volume (B), and thickness of dentate gyrus granular cell layer (C). Scatter plot with mean ± SEM of 3 to 5 mice per group. *p<0.05 indicate Tukey's pairwise comparison between indicated groups.

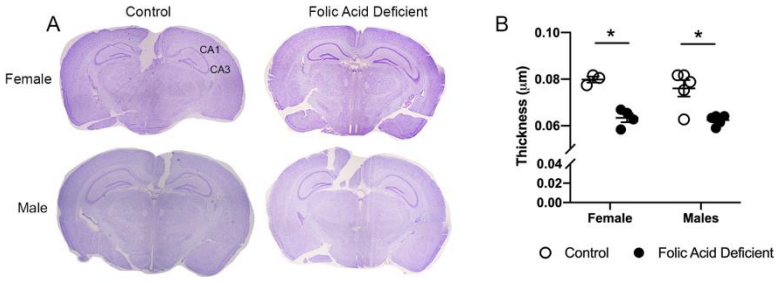


**Figure 2**. The impact of dietary folic acid deficiency on hippocampal Cornu Ammonis (CA) 1 and 3 region thickness. Representative images from all experimental groups with CA1 and CA3 regions highlighted (A). Quantification of thickness of the CA3 region (B). Scatter plot with mean ± SEM of 3 to 5 mice per group. *p<0.05 and **p<0.01 indicate Tukey's pairwise comparison between indicated groups.

of CA3 regions ($F_{(1,13)} = 0.94$, $p = 0.35$). However, there was a decrease in the thickness of the CA3 region of mice maintained on a FD diet (Figure 2B; $F_{(1,13)} = 37.3$, $p < 0.0001$).

***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 ($F_{(1,12)} = 1.44$, $p = 0.25$).

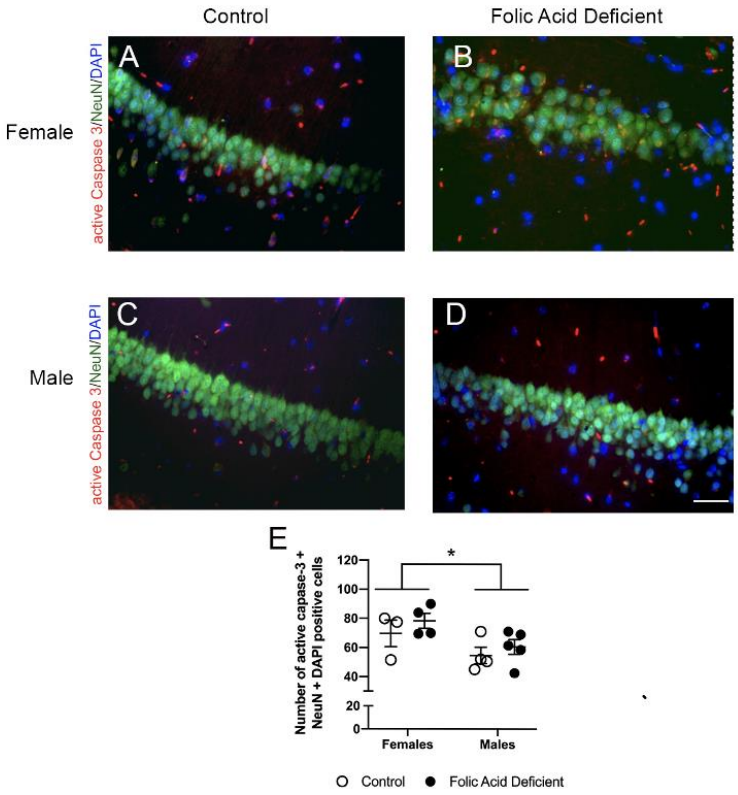


**Figure 3**. The impact of dietary folic acid deficiency and sex on active caspase-3 levels within the hippocampal Cornu Ammonis 3 (CA3). Representative images from experimental groups (A -D). Quantification of active caspase-3, neuronal nuclei (NeuN), and 4′,6-diamidino-2-phenylindole (DAPI) positive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

**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; $F_{(1,12)} = 4.58$, $p = 0.050$).

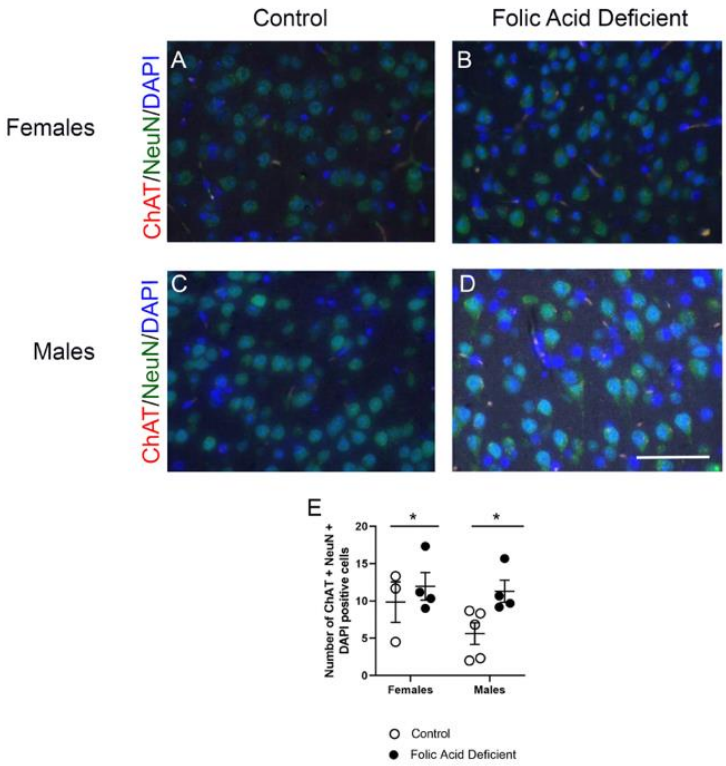


**Figure 4.** The impact of dietary folic acid deficiency and sex on choline acetyltransferase (ChAT) levels within the cortex of adult mice. Representative images from all experimental groups (A to D). Quantification of ChAT, neuronal nuclei (NeuN), and 4′,6-diamidino-2-phenylindole (DAPI) positive cells. Scatter plot with mean ± SEM of 3 to 5 mice per group. * $p<0.05$ indicate Tukey's pairwise comparison between indicated groups.

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; $F_{(1,12)} = 7.29$, $p = 0.019$).

**Discussion**

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

homeostasis, and epigenetics. There is growing evidence that sex plays a significant role in the metabolism and levels of homocysteine (23–26). Despite this, there is limited evidence for sex differences in the brain tissue. Using a mouse model, our study investigated the impact of sex and dietary folic acid deficiency on hippocampal morphology and brain choline metabolism. As expected, the FD mice had lower serum folates than CD mice. Notably, our results indicate a significant decrease in the thickness of the granular layer within the CA3 region in both males and females maintained on a FD. In cortical tissue we demonstrate changes in cholinergic metabolism between diet groups. The only sex difference we report in our study is the decreased apoptosis observed in the CA3 region of the hippocampus.

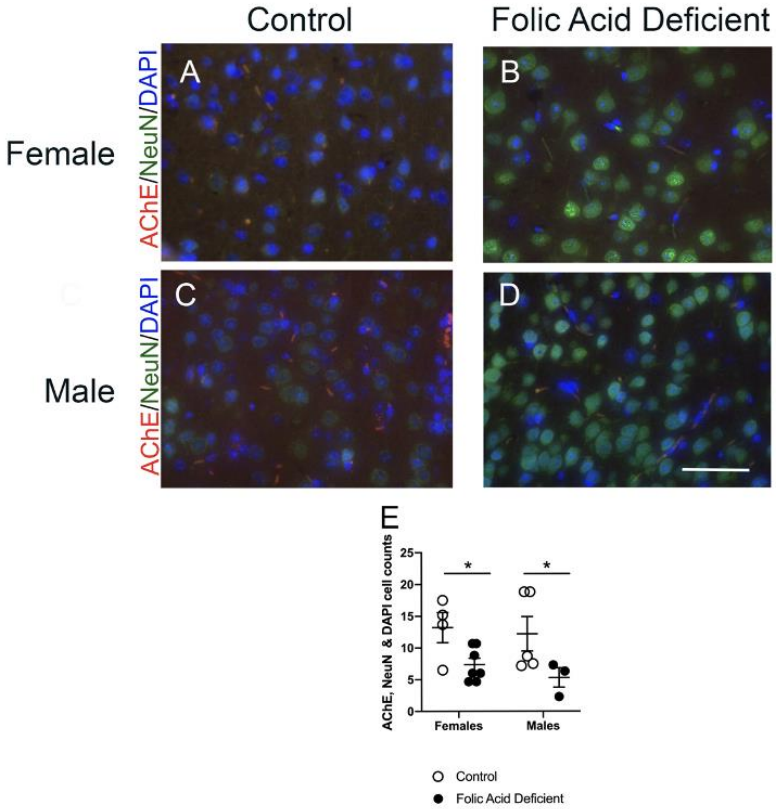


**Figure 5.** The effect of dietary folic acid deficiency and sex on acetylcholine transferase (AChE) levels within the cortex of adult mice. Representative images from all experimental groups (A to D). Quantification of AChE, neuronal nuclei (NeuN), and 4′,6-diamidino-2-phenylindole (DAPI) positive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

Consistent with prior research, our results demonstrate an intrinsic link between choline and folate metabolism (15,16,37,38). A folic acid deficient diet resulted in increased levels of

ChAT, the enzyme that catalyzes the synthesis of acetylcholine from choline, and decreased levels of AChE, the enzyme that catalyzes the destruction of acetylcholine. Choline can act as a 1C donor in the liver and kidney via betaine homocysteine methyltransferase, especially when folate levels are low (33,39). This compensatory function of choline would shunt choline away from the production of acetylcholine, potentially leading to lower levels of the neurotransmitter (15,16). Therefore, we suggest that a potential mechanism for these demonstrated changes in cortical AChE and ChAT is a compensatory upregulation of ChAT and downregulation of AChE in response to decreased levels of acetylcholine. Low levels of acetylcholine have been associated with cognitive impairments, including mild cognitive impairment and dementia (40).

The hippocampus is impacted by a genetic or dietary deficiency in 1C metabolism (15,34). Our results demonstrate this effect as well, with a significant diet effect in the thickness of the CA3 granular layer. The different areas of the hippocampus and their functions are topics of ongoing research. In humans, the CA3 region has been specifically linked to spatial representations and episodic memory. Additionally, this region is susceptible to neurodegeneration, possibly accounting for our results (41). However, previous research also demonstrates that alterations in 1C metabolism leads to decreased thickness in the CA1 region and the dentate gyrus(15). Our results did not support this observation, possibly because our study investigated a dietary deficiency, whereas the reported study investigated a genetic deficiency in 1C. We hypothesized that the differences in the CA3 region were due to increased levels of apoptosis following a folic acid deficient diet, however, our results did not demonstrate diet effects when measuring for apoptosis.

While our results did not indicate a diet difference in apoptosis, there was a significant sex difference in apoptosis in the CA3 region of the hippocampus. The CA3 region of the hippocampus

is the most interconnected region of the hippocampus and has been implicated in memory functions and susceptible to neurodegeneration (41). In order to measure levels of apoptosis, we stained for active caspase-3, which is as an executioner caspase leading to cell death, thus acting as an indirect measure of apoptosis (42). Previous research demonstrated a distinct sex difference in the activation of caspases, specifically following ischemic stroke (43,44). These differences could be accounted for by sex hormones, potentially via the pro-apoptotic protein Bax (45). Model studies have indicated a significant sex difference in gross hippocampal morphology within the hippocampus including the dentate gyrus, CA1 and CA3 regions (46). A recent human study indicates that after adjusting for total hippocampal volume, regional sex differences exist, however, they are not present in the CA2/CA3 regions or the dentate gyrus (47).

In conclusion, our study demonstrates stronger diet effects than sex differences in brain tissue of mice maintained on a folic acid deficient diet. Further research should in the area could aim to include functional measurements, such as behavioral analysis.

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**Statement of authors' contributions to manuscript**
N.M.J, J.G., M.C., J.G. designed research; L.P., M.P., A.M., C.B., N.M.J. conducted research; C.B. and N.M.J analyzed data; and C.B., L.P., J.G., N.M. J. wrote the paper. N.M.J. had primary responsibility for final content. All authors read and approved the ifnal manuscript.

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