Dietary folic acid increases neurogenesis in the ventral hippocampus of adult female rats

Wansu Qiu[1], Aarthi R. Gobinath[2], Stephanie E. Lieblich[2], Jehannine Austin[3], Liisa A.M. Galea[1,2,4*]

[1]Graduate Program in Neuroscience, [2]Department of Psychology, and [3]BC Children's Hospital Research Institute, [4]Djavad Mowafaghian Centre for Brain Health

University of British Columbia

*Corresponding Author:

2215 Wesbrook Mall

Vancouver, BC, Canada

V6T 1Z3

liisa.galea@ubc.ca

ABSTRACT

Folate is an important regulator of hippocampal neurogenesis, and in utero spinal cord development. Due to the fortification of folic acid in our food, women may be receiving higher doses of synthetic folic acid than recommended. In addition, in patients with major depressive disorder, folate deficiencies, lower levels of hippocampal neurogenesis, elevated levels of homocysteine, and/or the stress hormone, cortisol, are all associated with depression. Here, we are interested in whether different doses of natural folate or synthetic folic acid diets can influence neurogenesis in the hippocampus, levels of plasma homocysteine, and serum corticosterone in adult female rats. Adult female Sprague-Dawley rats underwent dietary interventions for 29 days. Animals were randomly assigned two six different dietary groups: folate deficient + SST, low 5-MTHF, low 5-MTHF + Succinylsulfathiazole (SST), high 5-MTHF + SST, low folic acid, and high folic acid. (SST) was added to a subset of the 5-methyltetrahydrofolate (5-MTHF) diets to eliminate folic acid production in the gut. Before and after dietary treatment, blood samples were collected for corticosterone and homocysteine analysis, and brain tissue was collected for neurogenesis analysis. High folic acid increased the number of immature neurons within the ventral hippocampus compared to folate deficient controls. Low 5-MTHF without SST significantly increased the number of immature neurons compared to low 5-MTHF + SST, indicating that SST interfered with elevations in neurogenesis. Low folic acid reduced plasma homocysteine levels compared to controls, but there was no significant effect of diet on serum corticosterone levels. Overall, folic acid, rather than 5-MTHF dietary treatment, influenced neurogenesis and homocysteine clearance. Furthermore, the addition of SST negated the effects of 5-MTHF to increase neurogenesis.

Keywords: corticosterone, folic acid, folate, neurogenesis, homocysteine

1. INTRODUCTION

Folate is one of the natural B-complex vitamins, is responsible for one-carbon metabolism[1], and converts homocysteine to methionine[2]. This process of reducing homocysteine levels is crucial for DNA synthesis and overall health. The human body is incapable of making folate endogenously. Thus, folate must be obtained through diet, such as by eating leafy greens, legumes, and citrus fruits[3]. Folic acid is a synthetic folate analog and is added to several breads and cereal products to fortify folate levels within the general population. There are however, microorganisms within the human gut microbiome that are capable of synthesizing folic acid[4]. The antibacterial agent, succinylsulfathiazole (SST) is commonly added to folate diets in research studies to prevent folic acid synthesis through the gut microbiome[5].

Increased folic acid supplements are added to food products in part to help prevent fetal neural tube development defects during embryonic development[6]. Overall, folic acid is more bioavailable than natural folate[7]. It is important to distinguish the differences between natural folate such as 5-methyltetrahydrofolate (L-methylfolate; 5-MTHF) and synthetic folic acid. Overconsumption of folic acid can lead to excessive levels of unmetabolized folic acid accumulating in blood[8]. Increased and prolonged exposure to unmetabolized folic acid may confer some toxic effect of high folic acid intake in older female populations[9]. While there are numerous studies supporting folate supplementation in women for the benefit of child development, studies rarely examine whether 5-MTHF or folic acid can affect women's health. Here, we investigate two doses of both 5-MTHF and folic acid in diet to determine whether the effects of natural folate and synthetic folic acid are distinct on neurogenesis in the hippocampus of adult female rats.

Folate is an important regulator of neuroplasticity, including neurogenesis, during development[10,11], and during adulthood[12,13]. Particular attention has been paid to folate deficiencies during pregnancy as it is detrimental for fetal nervous system development in utero[14]. In adult mammals, the hippocampus retains the ability to generate new neurons throughout life[15,16] Although less well studied, folate levels also influence hippocampal neurogenesis in adult or aging populations[12,13]. However, to our knowledge no studies have been conducted examining neurogenesis in adult female rodents after folate manipulations and this

72 study sought to rectify this deficiency in the literature. Metabolites of the 5-MTHF and folic acid
73 metabolic cycle contribute to the formation of purine rings and the conversion of uracil to
74 thymidine for DNA synthesis[17]. Elevated homocysteine levels due to folate deficiencies are
75 associated with cell death and DNA damage [18–20] that may lead to reduced neurogenesis. In this
76 study we used the endogenous marker, doublecortin (DCX), as it is expressed in proliferating
77 progenitors up until 21-30 days in rats[21], we chose this endogenous protein to examine the effects
78 of 5-MTHF or folic acid over a wide range of immature neuron maturation (during earlier stages
79 of cell proliferation as a proxy for levels of DNA synthesis, or when cells are more mature).

80 Most studies have examined the effect of folate deficiencies on neurogenesis, rather than
81 folate supplementation. Previously, Kronenberg and colleagues[12] showed that in aged mice (sex
82 not specified), a chronic folic acid-deficient diet for 3 months reduced the number of immature
83 neurons in the dentate gyrus (DG) of the hippocampus compared to animals given a folic acid
84 enriched diet. In a separate study, one-month-old (juvenile) male mice maintained on a folate-
85 deficient standard rodent chow for 3.5 months showed reduced adult hippocampal neurogenesis
86 compared to a control diet[13]. While these reports show how folate and folic acid deficiencies can
87 reduce neurogenesis, no research to our knowledge has specifically investigated the effects of
88 folate dietary interventions on adult hippocampal neurogenesis in healthy female rodents.

89 Finally, folate and folic acid may have antidepressant properties as assessed in both clinical
90 and preclinical studies[22]. For example, women are less likely to present with perinatal depression
91 when not folate deficient[23]. In addition, female rodents express less depressive-like behaviour
92 with higher doses of folic acid[24] and, folic acid can eliminate depressive-like endophenotypes in
93 female mice that had undergone a corticosterone-induced model of depression[25], which was
94 comparable to pharmacological antidepressant treatment. Depression is associated with reduced
95 hippocampal neurogenesis[26], elevated cortisol levels[27], and lower levels of folic acid and
96 elevated homocysteine[28,29]. One mechanism by which folic acid may contribute to mood
97 elevation is via its effects on stress hormones via homocysteine metabolism[30]. For example,
98 acute restraint stress increased homocysteine levels in female rats[31], and 7 days of folic acid
99 supplementation (30mg/kg) slightly, but not significantly, reduced corticosterone levels in
100 female mice after three weeks corticosterone treatment[25]. This finding suggests that folic acid
101 may elicit antidepressant effects by reducing homocysteine and modulating the corticosterone

102 system. Thus, in the present study we examined homocysteine and corticosterone levels with
103 folate and folic acid diets.

104 Few studies have studied the differential effects of folic acid versus folate dietary
105 supplementation and thus this studied was designed to examine differences between the two diets
106 at different doses on hippocampal neurogenesis, plasma homocysteine and serum corticosterone
107 levels in adult female rats. As folic acid can be synthesized in the gut and not elsewhere in the
108 body, here we used 5-MTHF diets with or without SST to determine whether this would impact
109 our findings. We expected that there would be differential effects of 5-MTHF versus folic acid to
110 increase neurogenesis in the hippocampus of adult female rats that may be associated with
111 changes in plasma homocysteine and corticosterone and the use of SST.

Name of the Diet	Folic acid	L-methylfolate	1% SST	n
FD+ (Folate Deficient)	0 mg/kg	0 mg/kg	✓	5
LF+ (Low 5-methyltetrahydrofolate)	0 mg/kg	5 mg/kg	✓	5
HF+ (High 5-methyltetrahydrofolate)	0 mg/kg	15 mg/kg	✓	5
LF (Low 5-methyltetrahydrofolate	0 mg/kg	5 mg/kg	-	5
LFA (Low Folic Acid)	5 mg/kg	0 mg/kg	-	5
HFA (High Folic Acid)	15 mg/kg	0 mg/kg	-	5

Table 1. Description of dietary treatments.

2. MATERIALS AND METHODS

2.1 Animals

Thirty adult female Sprague-Dawley (Charles River, Quebec) rats (7 months old) were individually housed in transparent polyurethane bines (24 x 16 x 46 cm) with aspen chip bedding, in order to ensure proper monitoring of food consumption. Rats were maintained in a 12 h: 12 h light/dark cycle (lights on at 7:00 am) and provided rat chow in different groups as described below (folic acid and L-methylfolate was purchased from Vitcost.com, Boca Raton, FL, USA, and formulated with by Harlan Laboratories, Inc., Madison WI, US) and tap water *ad libitum*. All protocols were in accordance with ethical guidelines set by Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. For an overview of experimental procedures, refer to Figure 1.

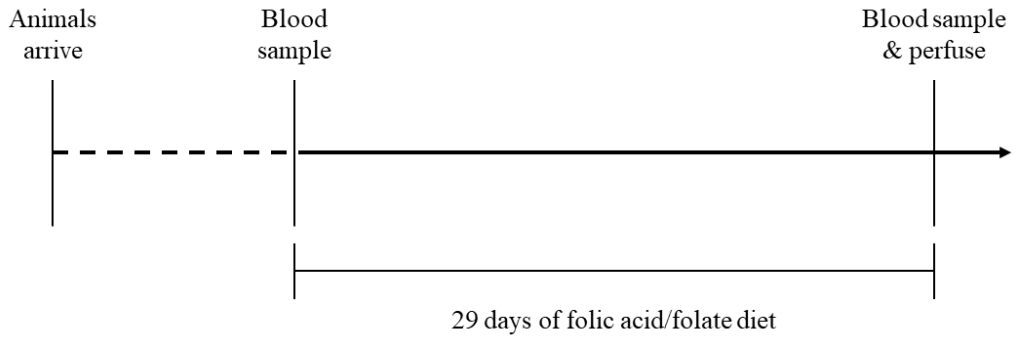


Figure 1. Timeline of Experiment.

135 *2.2 Dietary Interventions*

136 Animals were randomly assigned to one of the following diets (n=5/group; total n=30): 1.
137 Folate deficient + 1% SST (FD+) that will serve as the control group, 2. Low 5MTHF + 1% SST
138 (LF+), 3. Low 5-MTHF (LF), 4. High 5-MTHF + 1% SST (HF+), 5. Low folic acid (LFA), 6.
139 High folic acid (HFA). The antibacterial agent, succinylsulfathiazole (SST) was added to folate
140 diets to prevent folate synthesis through the gut microbiome[5]. In animals with SST treatment, it
141 was assumed that there would not be any *de novo* folic acid synthesis by gut bacteria. Doses
142 were chosen based on studies in which similar doses showed efficacy in increasing neurogenesis
143 in male rats in a model of cerebral ischemia[32]. Food consumption was monitored for weekly
144 intake of folic acid or folate diets. Body mass was also monitored every 7 d. All other vitamins
145 as well as caloric value remained the same among all 6 diets. For an overview of details
146 regarding the diets, refer to Table 1. Diets were given for 29 days.

147 *2.3 Blood collection, corticosterone, and homocysteine analysis*

148 Twenty-four hours prior to dietary interventions and again after 29 days of dietary
149 intervention, blood samples were collected via tail vein. All blood samples were collected within
150 3 min of touching the cage.

151 Plasma samples were collected with the anticoagulant EDTA. Plasma blood was then
152 centrifuged at 10,000 g for 15 min immediately. Serum blood samples were stored overnight at
153 4ºC to allow blood to clot completely, and then centrifuged at 10,000 g for 15 min. The serum
154 and plasma were collected and stored at -20ºC until radioimmunoassay.

155 Total CORT (bound and free) was measured on the serum samples using the ImmuChem
156 Double Antibody 125I radioimmunoassay Kit (MP Biomedicals, Solon, OH, USA). The
157 antiserum cross-reacts 100% with CORT, 0.34% with deoxycorticosterone, 0.05% with cortisol,
158 and does not cross-react with dexamethasone (<0.01%). All reagents were halved, and samples
159 run in duplicates. Percent change in serum CORT was calculated following the formula ((CORT
160 levels after treatment – CORT levels prior to treatment)/CORT levels prior to treatment) * 100.

161 Plasma homocysteine levels were measured by liquid chromatography-tandem mass
162 spectrometry in the Analytical Core for Metabolomics and Nutrition laboratories located at BC

163 Children's Hospital Research Institute at the University of British Columbia as described by
164 Dominguez-Salas et al.[33].

165 *2.4 Tissue Collection*

166 On Day 29, rats were then weighed and given an overdose of Euthanyl. Adrenal glands
167 and ovarian tissue were collected and weighted. Rats were then transcardially perfused with 60
168 ml cold saline followed by 120 ml cold 4% paraformaldehyde. Brains were then extracted and
169 placed in a vial containing 4% paraformaldehyde overnight at 4ºC. Brains were then transferred
170 to 30% sucrose in phosphate buffer at 4ºC. Hippocampus was rapidly frozen and sectioned using
171 a freezing microtome (Leica, Richmond Hill, ON, Canada) at 30 µm in a series of 10. Sections
172 were stored in antifreeze (30 % ethylene glycol/ 20% glycerol; Sigma) and stored at -20ºC until
173 processing.

174 *2.5 DCX Immunohistochemistry*

175 Immunohistochemistry for DCX was conducted as previously described [34,35]. Briefly,
176 brain slices were rinsed with 0.1*M* phosphate-buffered saline, then treated with 0.3% hydrogen
177 peroxide (30 min). Tissue was rinsed then incubated in primary DCX antibody solution (1:1000,
178 goat anti-DCX, Santa Crux Biotechnology, Santa Cruz, CA, USA) at 4˚C for approximately
179 24hrs. Rinsed tissue section were then incubated in secondary antibody solution (1:500, rabbit
180 anti-goat, Vector Laboratories, Burlington, ON, Canada) at 4˚C for approximately 24 h. Rinsed
181 tissues were then incubated in Avidin Biotin complex (ABC elite kit, 1:1000, Vector) for 4 h.
182 Sections were developed using 3,3'-diaminobenzidine + nickel (DAB Peroxidase substrate kit,
183 Vector). Sections were mounted on superfrost slides, dehydrated with increasing concentrations
184 of ethanol, cleared with Xylene, and coverslipped with Permount (Fisher Scientific) for
185 microscopy.

186 *2.6 Microscopy*

187 Hippocampal sections (1/10th) were exhaustively counted for DCX-expressing cells using
188 the 40x objective of an Olympus C×22LED brightfield microscope. Sixty cells (n = 30 within the
189 dorsal regions; n = 30 within the ventral region) were randomly selected and were classified by
190 type into 1 out of 3 developmental stages of DCX-expressing neurons (proliferative,

191 intermediate, and postmitotic) based on morphological attributes for cell maturity analysis[34–36].
192 Total DCX-expressing cell counts were then averaged by the number of slices per region (dorsal
193 or ventral) to obtain an averaged DCX-expressing cell count data. Areas of these sections were
194 quantified using ImageJ (NIH, Bethesda, MD, USA) and used for relative $1/10^{th}$ volume
195 calculations (areas × 30 μm). Experimenters were blind to the treatment conditions when
196 conducting microscopy analyses.

197 *2.7 Data Analyses*

198 All data were analyzed using one-way analysis of variance (ANOVA) unless otherwise
199 specified with diet (FD+, LF+, LF, HF+, LFA, and HFA) as between-subject factors. Adrenal
200 mass, and ovarian mass were analyzed using one-way analysis of covariance (ANCOVA) using
201 body mass as a covariate. DCX-expressing cells and relative $1/10^{th}$ hippocampal volume was
202 analyzed using repeated measures ANOVA with diet as between-subject factors, and region
203 (dorsal, ventral) as the within-subjects factor, and an additional within-subject factor of
204 developmental stages (proliferative, intermediate, and post-mitotic) for cell maturity analysis.
205 Effect sizes are reported for significant effects. Post hoc comparisons used Newman-Keuls. *A*
206 *priori* comparisons were subjected to Bonferroni correction. Pearson product-moment
207 correlations were conducted between body mass, relative organ mass, and serum CORT, dorsal
208 and ventral DCX-expressing cells, and homocysteine levels. All data were analyzed using
209 Statistica software (v. 9, StatSoft, Inc., Tulsa, OK, USA). All effects were considered statistically
210 significant if $p \leq 0.05$, trends are discussed if $p \leq 0.10$.

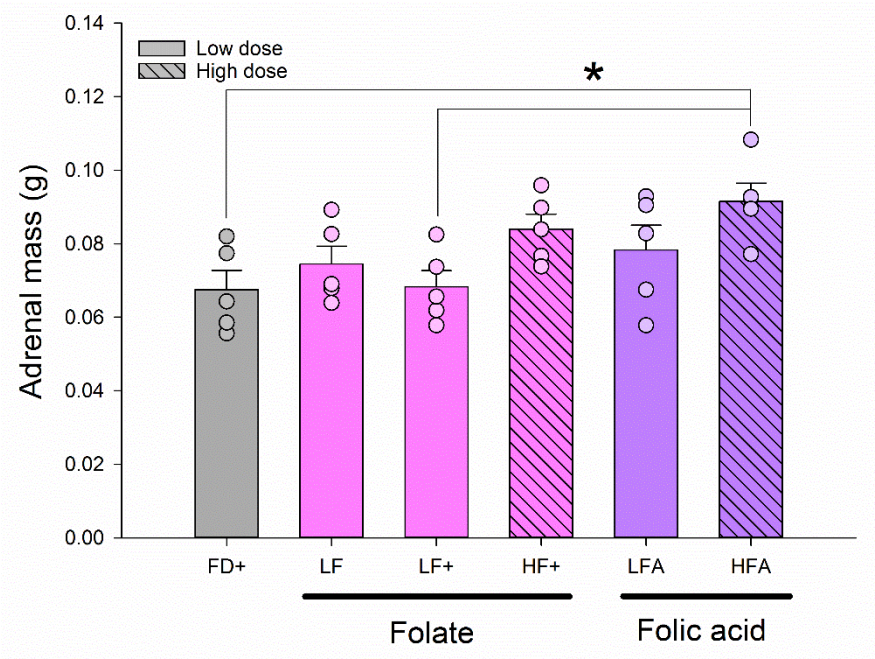


Figure 2. Mean and + SEM of adrenal mass, and individual data points of adrenal mass dependent on dietary interventions. HFA animals showed increased adrenal mass when controlling for final body mass compared to FD+ and LF+ animals, $\alpha = 0.05$.

3. RESULTS

3.1 High folic acid increased adrenal mass, and low folic acid diets reduced homocysteine levels diets did not influence body mass, ovarian mass, or percent change in serum CORT levels

The HFA group had a significant increase in adrenal mass compared to FD+ ($p = 0.037$, Cohen's $d = 2.900$) and LF+ groups ($p = 0.033$, Cohen's $d = 3.307$; main effect of diet: $p = 0.025$, $\eta_p^2 = 0.409$), but there were no other significant effects on adrenal mass (all p's ≥ 0.126; Figure 2). Diet did not statistically influence body mass or ovarian mass (all p's ≥ 0.480). Diet did not statistically influence percent change in serum CORT levels ($p = 0.169$). Diet did not statistically influence the relative $1/10^{th}$ volume of the hippocampus ($p = 0.811$).

LFA animals showed a significantly lower homocysteine levels compared to FD+ controls ($p = 0.049$, Cohen's $d = 1.501$; main effect of diet $p = 0.025$, $\eta_p^2 = 0.408$.), and a slight trend towards significance compared to LF animals ($p = 0.073$). HF+ animals also showed a trend towards significance of reduced homocysteine levels compared to FD+ ($p = 0.060$) and LF ($p = 0.081$) animals but no other significant differences were evident (Figure 3).

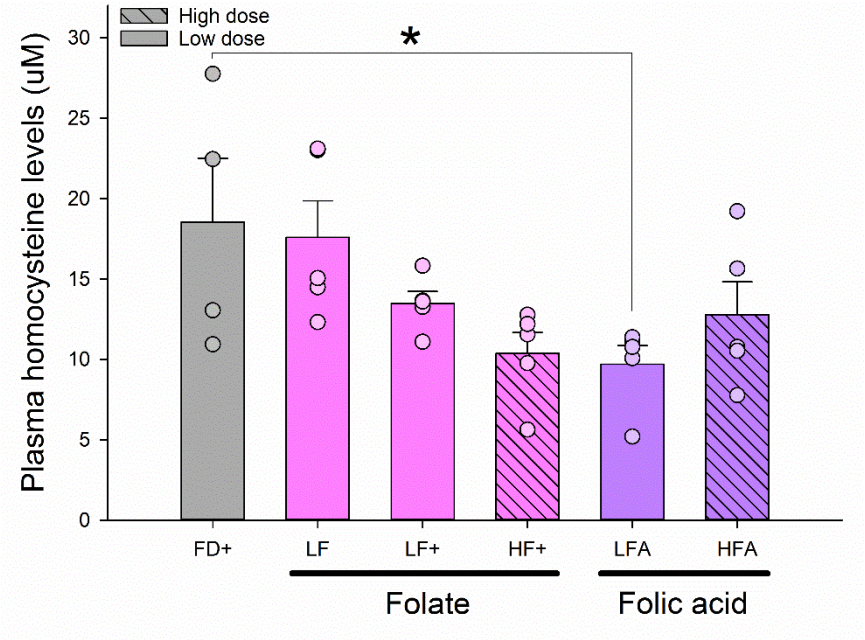
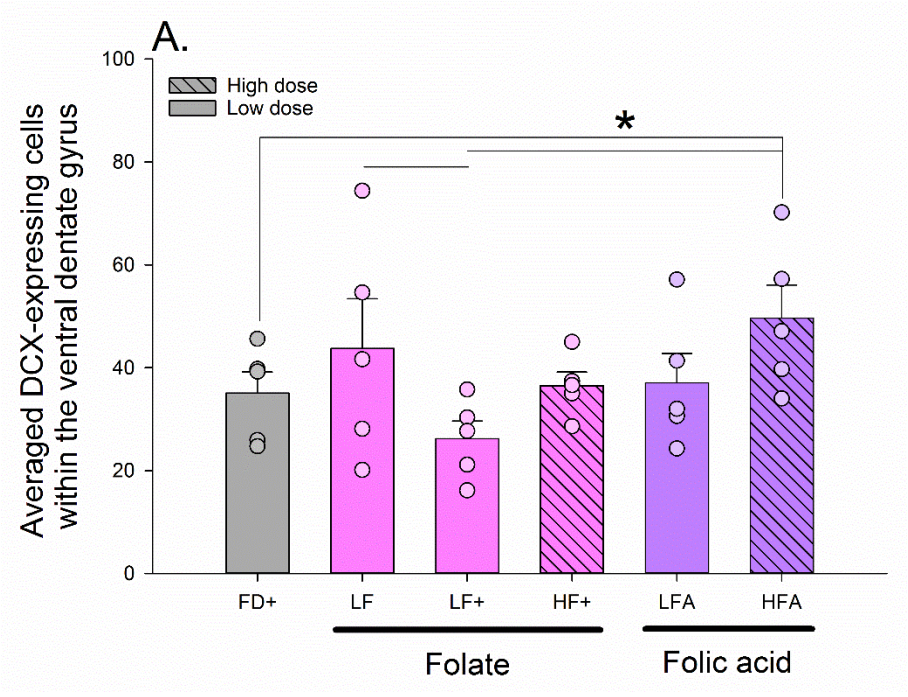


Figure 3. Mean and +SEM of plasma homocysteine levels (uM) and individual data points of homocysteine levels. LFA animals showed a significant decrease in homocysteine levels compared to FD+ controls. LFA animals also showed a trend towards significance compared to LF animals. HF+ animals showed a trend towards significance compared to both LF and FD+ animals, $\alpha = 0.05$.

3.2 High folic acid and low folate (without SST) increased DCX expression in the ventral dentate gyrus

The HFA diet significantly increased the number of ventral hippocampal DCX-expressing cells compared to LF+ group ($p < 0.001$, Cohen's $d = 1.384$), LFA group ($p = 0.009$, Cohen's $d = 1.094$), FD+ group ($p = 0.008$, Cohen's $d = 0.894$), and the HF+ group ($p = 0.012$, Cohen's $d = 0.909$; with a significant interaction between diet and dentate gyrus region, $p = 0.018$, $\eta_p^2 = 0.416$). Furthermore, the LF diet significantly increased the number of DCX-expressing cells compared to the LF+ group ($p = 0.003$, Cohen's $d = 1.164$); a trend towards significance emerged when LF diet was compared to the LFA group ($p = 0.097$, Cohen's $d = 0.888$). There was a main effect of region ($p < 0.001$, $\eta_p^2 = 0.683$ but no significant main effect of diet $p = 0.392$). No other significant pairwise comparisons were observed (all p's ≥ 0.116; Figure 4).



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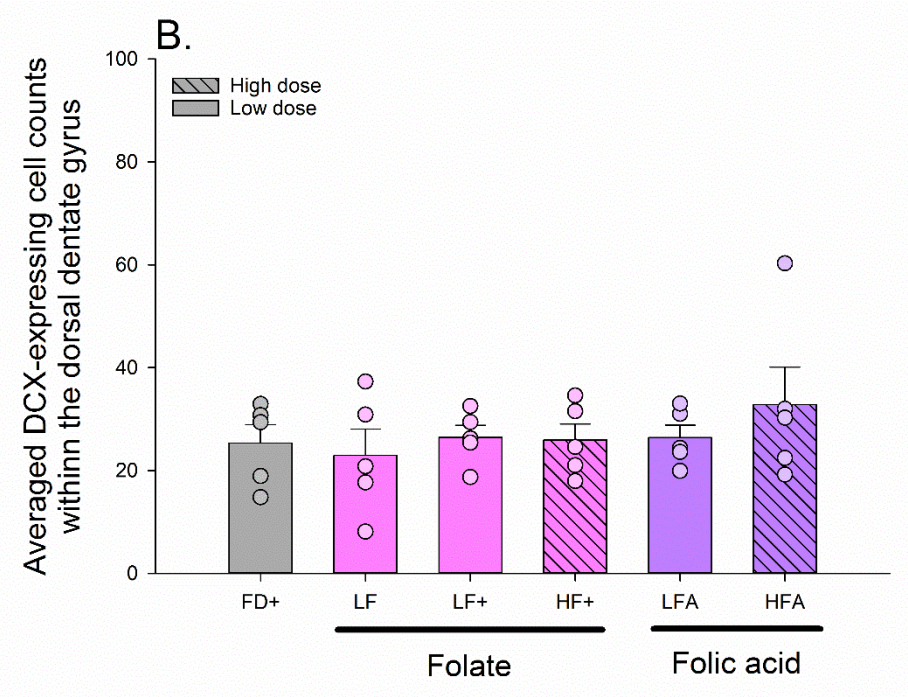

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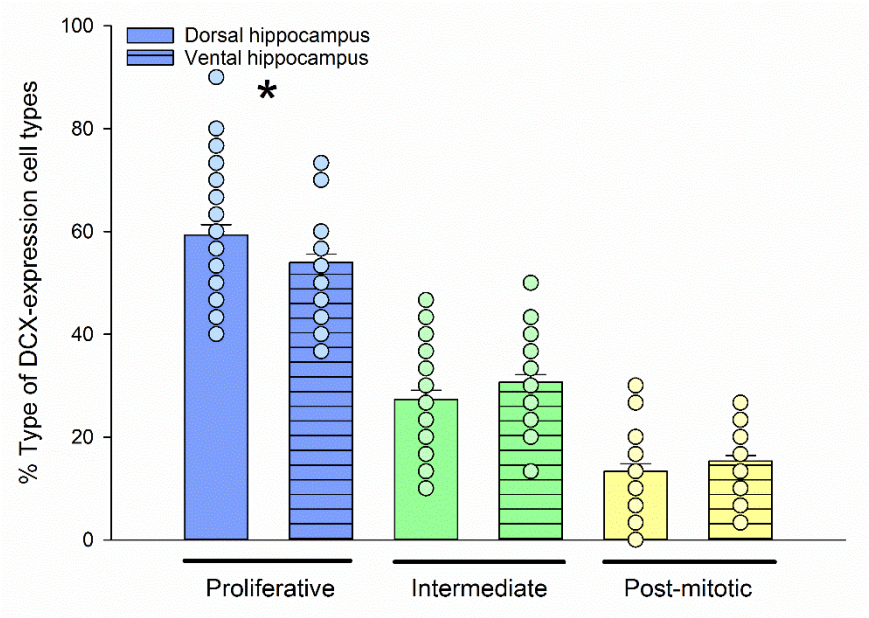
250 **Figure 4. A.** Mean and + SEM of DCX-expressing cell counts, and individual data points of
251 DCX-expressing cell counts dependent on dietary interventions within the ventral dentate gyrus
252 region of the hippocampus. HFA group showed significantly more DCX-expressing immature
253 neurons within the ventral region compared to all other groups except the LF group. LF group
254 showed higher DCX-expressing immature neurons only compared to the LF+ group, $\alpha = 0.05$. **B.**

255 Mean and + SEM of DCX-expressing cell counts, and individual data points of DCX-expressing
256 cell counts dependent on dietary interventions within the dorsal dentate gyrus region of the
257 hippocampus. No statistically significant differences were found within the dorsal region, α =
258 0.05.

259 *3.3 Animals showed significantly higher numbers of proliferative doublecortin cells overall*

260 Post-hoc analysis indicated significantly more DCX-expressing cells in the proliferative
261 stage than in the other two stages: intermediate ($p > 0.001$, Cohen's d = 4.007) and post-mitotic
262 stages ($p > 0.001$, Cohen's d = 6.473; significant main effect of cell maturity stages, $p < 0.001$,
263 $\eta_p^2 = 0.909$). No significant main effect of diet ($p = 0.676$). A trend emerged towards
264 significance between the interaction of dentate gyrus region and cell maturity, $p = 0.065$, this was
265 only driven by the differences in cell maturity within regions (all p's < 0.001), and not between
266 regions (all p's ≥ 0.058; Figure 5).



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268 **Figure 5.** Mean and + SEM of % type of DCX-expressing cells, and individual data points of %
269 type of DCX-expressing cells. All animals showed higher proliferative type cells within both the
270 dorsal and ventral dentate gyrus, regardless of dietary interventions, α = 0.05.

271 *3.4 Within low folate without SST animals, relative adrenal mass negatively correlated with*
272 *serum CORT levels. Ventral DCX cell counts weakly negatively correlated with serum CORT*
273 *levels after dietary interventions*

Within the LF group, relative adrenal mass was negatively correlated with serum CORT levels after dietary treatment both before and after removing the outlier, r = -0.997, $p > 0.001$. No other significant differences were found ($p \geq 0.282$; Figure 6).

When looking at all dietary groups together, for total DCX-expressing cells (dorsal + ventral regions) there was a trend for a negative correlation with serum CORT levels after dietary intervention, r = -0.337, $p = 0.074$, as well as the dorsal region, r = -0.341, $p = 0.071$. DCX-expressing cells in the ventral region negatively correlated with serum CORT levels r = -0.359, $p = 0.060$. When correlating between specific dietary groups to serum CORT levels after dietary interventions after removing the outlier, no statistically significant correlations emerged after correcting for multiple comparisons. (all p's ≥ 0.026). However, after an outlier was removed, both the trends towards significance were eliminated, but a trend towards significance emerged.

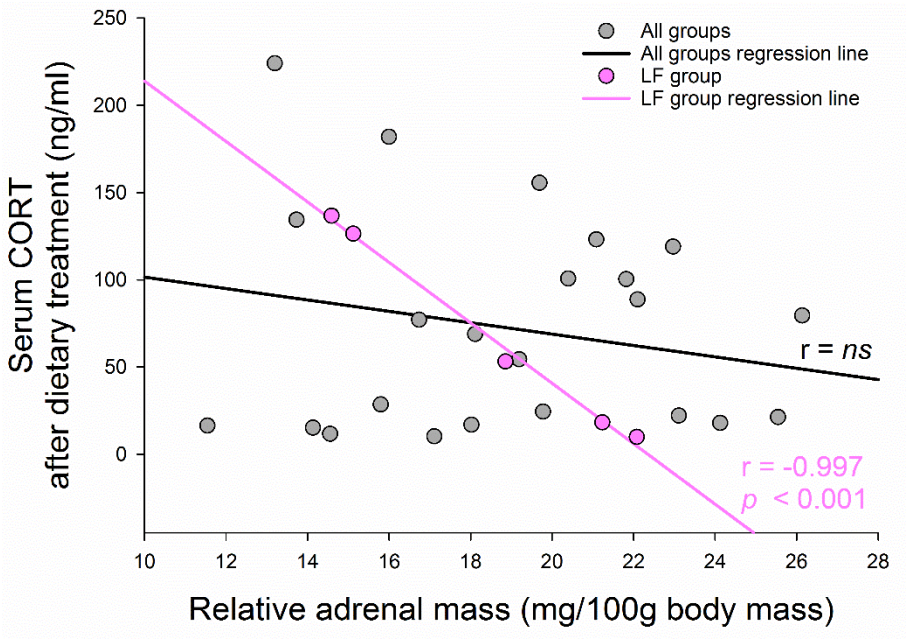

Figure 6. Correlation matrix between serum CORT after dietary treatment and relative adrenal mass, where no statistically significant correlation was observed when all dietary animals were group together, $\alpha = 0.05$. When separating animals by dietary interventions, only within the LF group, a significant positive correlation emerged after Bonferroni's correction, $\alpha = 0.0083$.

292 ## 4. DISCUSSION

293 In the present study, we find that high folic acid increased the number of DCX-expressing
294 cells in the ventral hippocampus compared to the folate deficient diet. Furthermore, the addition
295 of SST inhibited neurogenesis in the low 5-MTHF group as the low 5-MTHF diet group without
296 SST had higher number of DCX-expressing cells in the ventral hippocampus compared to the
297 low 5-MTHF group given SST. The diet-dependent changes in DCX-expressing cells did not
298 affect the maturation of immature neurons, as all dietary groups showed elevated levels of type I
299 (proliferative) immature neurons. High folic acid increased adrenal mass compared to folate
300 deficient animals and low folic acid decreased plasma homocysteine levels compared folate
301 deficient animals. Diet did not influence the percent change in serum CORT levels across
302 treatment but there was a suggestion that the ventral DCX-expressive cells were negatively
303 correlated with CORT levels. Collectively, these data suggest that there may be beneficial, dose-
304 dependent effects of 5-MTHF and/or folic acid on ventral hippocampal neurogenesis in the adult
305 female rat, and that SST may interfere with these neurogenic effects.

306 *4.1 High folic acid increased the number of immature neurons within the ventral hippocampus,*

307 Here, we show that high folic acid increased neurogenesis in the ventral dentate gyrus
308 compared to folate deficient diet. Our findings are consistent with other reports indicating that
309 folate deficiency induced by a folate deficient diet + SST treatment decreases hippocampal
310 neurogenesis in male mice[13]. Our findings were specific to the ventral dentate gyrus, and this is
311 intriguing because the ventral hippocampus is more involved in the stress response via its
312 interaction to provide negative feedback to the hypothalamic-pituitary-adrenal (HPA) axis.
313 Because regulating negative feedback of the HPA axis and antidepressant efficacy are closely
314 linked in patients with major depressive disorder (Ising et al., 2007), increased ventral
315 hippocampal neurogenesis as a result of increased folic acid intake may be a possible mechanism
316 by which folic acid exerts its antidepressant effects. Given past evidence of the influence of folic
317 acid to prevent depressive-like endophenotypes in female mice, increased neurogenesis in the
318 ventral hippocampus may be one mechanism by which folic acid could exert its antidepressant
319 effects[22]. Furthermore, low 5-MTHF without SST, significantly increased the number of ventral
320 DCX-expressing cells compared to low 5-MTHF with SST. Because, SST prevents folic acid
321 synthesis via the gut microbiota, this suggests that SST interfered with the ability of folate to

322 increase neurogenesis in the low 5-MTHF + SST group. Thus, we can infer that the addition of
323 SST reduced neurogenesis, which may have been due to its effects on the microbiota or the
324 reduction in folic acid. Interestingly, another study has shown that antibiotic use reduced
325 hippocampal neurogenesis in adult female C57BL/6 mice[37], consistent with our findings.
326 Overall, our results suggest that the introduction of SST (and consequently suppressing gut folic
327 acid synthesis) can interfere with any potential effects of 5-MTHF to promote neurogenesis in
328 the adult female rat.

329 *4.2 The effects of folic acid and folate on neurogenesis, adrenal glands and CORT: Implications*
330 *for depression*

331 In the present study, we found low folic acid diet reduced plasma homocysteine levels,
332 with a trend for high 5-MTHF to do the same. It is important to note that we utilized a folate
333 deficient diet as a comparative control group. The purpose of this study was to compare and
334 contrast the effects of different doses of 5-MTHF and folic acid to a folate deficient group.
335 Furthermore, we saw that high folic acid increased adrenal mass (but not serum CORT), and a
336 potential relationship between CORT following 5-MTHF/folic acid manipulations to
337 neurogenesis in the ventral dentate gyrus. As mentioned earlier, increased folate and folic acid
338 levels have been associated with antidepressant-like properties. Given that depression is
339 associated with higher levels of homocysteine[38] and elevated levels of cortisol[27,39] it may be that
340 these two measures are related via in part their effects following dietary folate. Other reports
341 show in a population of healthy middle-aged men and women, high cortisol levels were
342 negatively associated with serum folate levels[40], and positively associated with serum
343 homocysteine levels. In our study, we see suggestions of associations between reduced
344 homocysteine, increased adrenal mass and increased neurogenesis in the ventral hippocampus
345 and it is possible that with more prolonged exposure of the diets we would see stronger
346 associations between these variables.

347 We also found that high folic acid enhanced neurogenesis, specifically in the ventral
348 hippocampus, an area of the hippocampus that is more responsible for regulating stress and
349 mood[41]. While in this study, we did not assess depressive-like behaviour after dietary treatment,
350 the ability of folic acid and 5-MTHF to modulate neurogenesis in the ventral hippocampus
351 suggests a potential mechanism by which dietary folate could modify mood. Although, high folic

352 acid did increase neurogenesis, neurogenesis was not correlated to the change in serum CORT
353 levels, suggesting an independent effect of folic acid to increase neurogenesis than via the HPA
354 axis. Furthermore, as suggested above, the current study indicates that the introduction of the
355 antibacterial SST to eliminate gut folic acid synthesis negates the effects of natural 5-MTHF on
356 neurogenesis and should not be adopted in studies wishing to increase neurogenesis.

357 The antidepressant-like properties of folate or folic acid are still under debate. Folic acid
358 can either act as an antidepressant alone without co-current pharmacological agent[42] or as an
359 adjuvant with traditional antidepressants[43]. This effect may be more efficacious for women[43], and
360 only effective when dietary interventions are prolonged[44]. Elevated levels homocysteine and low
361 folic acid are associated with depressive symptoms in middle-aged cohorts of both men and
362 women[28,29]. The current study does show that within the folate deficient group, there are higher
363 homocysteine levels and lower ventral neurogenesis levels suggesting potential endophenotypes
364 of depression. In the present study, our dietary treatment was only for 29 days and it is possible
365 we would see a stronger influence on both blood corticosterone and hippocampal neurogenesis if
366 the dietary treatments were prolonged and future studies should investigate longer timelines.
367 Here, we showed that there are differences between synthetic folic acid and natural 5-MTHF,
368 different doses of folic acid, the addition of the antibacterial SST, and how they influenced
369 neurogenesis, homocysteine and CORT with just 29 days of dietary intervention.

370 *4.3 Folic acid treatment can have different outcomes than 5-MTHF treatment*

371 In the present study, we show some distinct differences in the effects of folic acid vs 5-
372 MTHF on homocysteine clearance and adult hippocampal neurogenesis. In the literature, there
373 are more studies utilizing folic acid compared to 5-MTHF, likely due to the fact that folic acid is
374 more bioavailable than other forms of folate and more stable[7,45], making it a clear benefit for diet
375 fortification, and more relevant for human studies and animal models. It is then important to note
376 that folic acid and other forms of folate such as 5-MTHF act through distinct pathways of
377 metabolism[17] and that the mode of delivery and amount consumed can change the efficacy of
378 both folic acid and folate[46]. In the present study, only animals with low folic acid or high folic
379 acid diets showed significantly reduced levels of plasma homocysteine levels compared to the
380 folate deficient group, with no significant change with 5-MTHF diets. Although high 5-MTHF
381 did reduce homocysteine levels this effect was not significant. Furthermore, only animals fed the

382 high folic acid diet, but not the 5-MTHF diets, showed increased neurogenesis compared to
383 controls. Thus, collectively these results suggest that folic acid diets have greater efficacy to
384 increase neurogenesis and reduce homocysteine levels than do 5-MTHF diets.

385 The differential effects of natural forms of folate such as 5-MTHF and folic acid may be
386 the result of their underlying modes of metabolism, differences in structure, and ultimately, their
387 functional pathways. While in general, elevated homocysteine levels are associated with cell
388 death and DNA damage that can lead to reduced neurogenesis, both in vitro[18–20], and in vivo[20]. In
389 older male rats and mice, folate supplementation lasting for at least 8-20 weeks is effective at
390 inducing genomic changes[47,48]. In the present study, supplement duration for 29 days may not be
391 sufficient to significantly reduce homocysteine levels and to have significant effects on
392 neurogenesis. It is also crucial to note that here, we utilized healthy female rats to determine
393 whether folate and folic acid could affect biomarkers of depression such as reduced
394 neurogenesis, high levels of CORT or homocysteine. This suggests that it is worthwhile to
395 investigate the effects of dietary folate and folic acid diet in an animal model of depression.

396 **5. CONCLUSIONS**

397 In summary, high folic acid increased the number of immature neurons in the ventral
398 hippocampus after 29 days of dietary treatment in adult female rats. Low 5-MTHF without the
399 antibacterial SST also increased neurogenesis compared to low 5-MTHF treated with the SST,
400 indicating that SST impaired the ability of 5-MTHF to enhance neurogenesis, perhaps via its
401 effects on the microbiome. High folic acid was the only dietary intervention that increased
402 adrenal mass compared to the folate deficient group but surprisingly higher adrenal mass did not
403 correspond to higher serum CORT levels, which may indicate some disturbances in the HPA
404 axes functioning. Low folic acid significantly reduced plasma homocysteine levels at the end of
405 dietary treatment but did not significantly affect neurogenesis levels. Overall, our findings
406 indicate that natural 5-MTHF and synthetic folic acid may act on different pathways to influence
407 hippocampal neurogenesis and homocysteine metabolism. The lack of effects of 5-MTHF diets
408 with SST compared to folate deficient animals suggests that modifications to the gut microbiota
409 (or reductions in folic acid) significantly alter the functionality and efficacy of 5-MTHF to
410 modulate neurogenesis in the hippocampus. The current study suggests that folic acid and 5-

411 MTHF have similar but differential effects on neurogenesis, homocysteine metabolism, adrenal
412 mass and serum CORT release. Thus, we suggest that folic acid and 5-MTHF are not
413 interchangeable. Furthermore, future research should be cautioned that the use of SST in folate
414 studies may interfere with neuroplasticity.

415 **CONFLICTS OF INTEREST**

416 The authors have nothing to declare.

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