Eur J Nutr (2013) 52:1157–1167
DOI 10.1007/s00394-012-0426-1

ORIGINAL CONTRIBUTION

Dietary folic acid intake differentially affects methionine metabolism markers and hippoccampus morphology in aged rats

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Received: 3 January 2012 / Accepted: 16 July 2012 / Published online: 26 July 2012
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Abstract
Purpose Folic acid (FA) is an emerging nutritional factor in the pathogenesis of diverse neurodegenerative disorders by still unknown mechanisms. The hippocampus is altered during the loss of cognitive abilities in humans and selectively affected when homocysteine increases. The aim was to evaluate the potential protective role of folic acid in the maintenance of biochemical markers related to the methionine cycle, as well as the integrity of the hippocampus as part of the brain in aged rats.
Methods Male Sprague–Dawley rats (18 months old) were assigned to four different folic acid groups (0 mg FA/kg diet, deficient; 2 mg FA/kg diet, control; 8 mg FA/kg diet, *moderate* supplementation; 40 mg FA/kg diet, extra supplementation) for 30 days. We evaluated several parameters related to the methionine cycle. In addition, hippocampus areas were immunostained for specific neuronal markers and astrocytes.
Results Serum folate levels increased according to FA dietary level ($p < 0.01$). There was a significant increase in the serum homocysteine concentrations in the folic acid-deficient diet group ($p < 0.01$). However, brain S-adenosylmethionine and S-adenosylhomocysteine did not differ significantly between the folic acid groups. Consequently, the methylation ratio was also unchanged. The morphometric analysis did not show any differences in the number of neurons and astrocytes between groups, except when comparing the folic acid-deficient diet versus folic acid-supplemented diet in the *striatum* of the hippocampus.
Conclusions Clearly, the dietary FA deficiency negatively affects the methionine metabolism biomarkers, while excessive supplementation seems to be unnecessary for optimal maintenance of the methylation cycle and hippocampus integrity.

Keywords Folic acid · Supplementation · Homocysteine · S-adenosylmethionine · S-adenosylhomocysteine · DNA methylation · Brain · Hippocampus · Aging

Introduction

The hippocampus is a structure intimately involved in several aspects of learning and memory and is especially vulnerable to the course of aging [1]. The loss of neurons has been reported as a natural event in the hilus of the dentate gyrus of the hippocampal formation in elderly people [2] and aged rats [3].

Folate one-carbon units are essentials for purine and thymidine biosynthesis, and folate deficiency has been shown to impair embryonic neuroepithelial cell proliferation [4], as well as to increase homocysteine (Hcy) levels, a highly neurotoxic compound formed during the metabolism of methionine. Severe hyperhomocysteinemia (HHcy) may be caused by genetic factors, although it has been also associated with a low folate status and other metabolically related B-vitamins, mainly vitamin B_{12} and vitamin B_6 [5–7]. Kruman et al. [8] reported that cultured hippocampal neurons seem to be much more vulnerable to homocysteine than cultured vascular endothelial cells or astrocytes. Recently, HHcy has been closely linked to memory loss and

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the development of dementia in the elderly. HHcy and low dietary folate are being considered as potential independent risk factors for the development of Alzheimer's disease and other neurodegenerative dementias [9]. Likewise, HHcy is an independent risk factor for memory deficit and the development of cognitive impairment without dementia [10]. Attempts to understand the molecular mechanism implied in memory loss have focused mainly on the toxic effects of Hcy on mature neurons [11–13].

It has been hypothesised that the association between Hcy and cognitive performance in aging may be attributed to the following: exposure to the neurotoxic effects of Hcy [8, 14–16]; folate and vitamin B_6 and B_{12} deficiencies [17–19]; and/or microvascular rarefaction.

However, there is a lack of information on the influence of different dietary folic acid conditions on hippocampus functioning and its association to methionine metabolism. The aim of this work was to evaluate several methionine-cycle-related biochemical markers, and the hippocampus integrity (as reflected in the number of neurons and astrocytes) in 18-month-old rats fed with different dietary levels of FA.

Methods and materials

Animals and diets

A total of thirty-four male OFA (Sprague–Dawley) 18-month-old rats (Charles River SA, Spain) were randomly divided into four groups on the basis of the experimental diet administered. All diets were adjusted to rat requirements and were based on the pure amino acid diet (Dyets, Bethlehem, PA, USA) [20] modifying only the FA content as follows: FA-deficient diet (Group Def; 0 mg FA/kg diet, $n = 9$), FA-supplemented diet (Group Sup; 8 mg FA/kg diet, $n = 8$), FA extra-supplemented diet (Group extra-sup; 40 mg FA/kg diet, $n = 9$) and control diet (Group Con; 2 mg FA/kg diet, $n = 8$). Animals were maintained on a 12:12 h dark/light cycle, under controlled temperature and humidity conditions at the individual animal care unit of the Universidad CEU San Pablo (Madrid, Spain). Rats were fed their respective diets ad libitum for 30 days. Manipulation of the animals was performed following the European Union Normative (2003/65/CE). These diets have been already successfully employed in previous studies by our research group [21–28].

Tissue collection

The anesthetised animals were killed by decapitation after an overnight fasting. The encephalon was quickly removed, meninges were detached and the brain was divided through the mid-sagittal fissure in two pieces: the right hemisphere was used for biochemical processing, and the left hemisphere for histological assessment. The blood was collected from all rats, and the serum and plasma were separated by centrifugation and kept at −80 °C until analyzed.

Folate, total homocysteine (tHcy) and vitamin B_{12} serum determinations

Serum folate, tHcy and vitamin B_{12} levels were determined by an IMx System (Abbot Laboratories, Abbot Park, IL, USA). The IMx Hcy assay, IMx folate assay and IMx B_{12} assay are based on Microparticle Enzyme Immunoassay (MEIA) technology.

Vitamin B_6

Plasma vitamin B_6 levels were determined by High-Performance Liquid Chromatography (HPLC) using a vitamin B_6 (piridoxal-5 phosphate and piridoxal) plasma kit (Chromsystems Instruments & Chemicals GmbH, Munich, Germany) followed by fluorescent detection.

S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy)

Brain AdoMet and AdoHcy levels were determined by HPLC according to the method proposed by Fell et al. [29] with some modifications. Portions of frozen brain (about 100 mg) were homogenised in three volumes of 0.4 M of $HClO_4$ and then centrifuged at 10.000 g at 4 °C for 10 min. The clear supernatant fractions were removed, filtered, and appropriate samples were analyzed for AdoMet and AdoHcy. Brain protein levels were determined by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA), to express AdoMet and AdoHcy per gram of protein.

DNA methylation

DNA was extracted using the Qiagen Kit (Hilden, Germany). The capacity of brain DNA preparations to serve as methyl group acceptor was determined by the method proposed by Christman et al. [30] which was modified by replacing the DNA methylase from Friend erythroleukemia cells with SssI methylase from *E.Coli* (New England Biolabs, Beverly, MA, USA). Briefly, DNA (2 µg), SssI (2 U), and [^{3}H-methyl] AdoMet (5 µCi) in 20 µl buffer containing 50 mM NaCl, 10 mM Tris/HCl, and 10 mM EDTA pH = 8 were mixed and incubated for 3 h at 37 °C. The reaction was stopped by heating the mixture for 20 min at 65 °C. The mixture was then applied in a disk of

Whatman DE-81 paper (Whatman International Ltd, Maidstone, Kent, UK), and the disks were washed with 20 ml buffer of 5 % NaH_2PO_4 for 45 min. The radioactivity retained on the disk was determined by scintillation counting using a non-aqueous scintillation fluor. The amount of radiolabel bound to a filter from the incubation mixture without DNA was used as background and was subtracted from the values obtained with mixtures containing DNA. Since this is an inverse assay, a higher incorporation of [^{3}H]-methyl groups into DNA in the in vitro assay indicates a diminished in vivo methylation of DNA.

Immunostaining analysis

The hemiencephalons were immersed for 3 days in two successive 4 % formaldehyde solutions, cleared in water and maintained in 70 % ethanol until processed and embedded in paraffin. Coronal sections (12 μm) were made using a Leica JUNG RM 2035 microtome and applied to glass slides. The paraffin sections were reviewed, and one out of fifteen slides was Nissl stained with cresyl violet for morphological analysis.

To identify the cell types, a set of sections from 5 to 6 animals was immunostained for specific neuronal marker (NeuN, a soluble nuclear protein that is local to the cell nucleus and in the neuronal cytoplasm of postmitotic neuron). Within the hippocampus, NeuN can be used as a marker of postmitotic cells and labels both "normal" postmitotic neurons and newly generated postmitotic neurons, or glial fibrillary acidic protein (GFAP, widely known as a marker for mature astrocytes in the adult brain) for astrocytes [31]. Sections were first incubated in 2 % hydrogen peroxide in methanol for 10 min in the dark at room temperature, to quench endogenous peroxidase activity. For NeuN staining, sections were pre-treated by heating in 0.01 M citrate, pH: 6–6.2 for 40 min in the oven for antigen retrieval. Unspecific binding sites were blocked with a mixture of 0.1 % FCS (Fetal Calf Serum, Gibco, Grand Island, NY), 0.1 % BSA (Bovine Serum Albumine, Sigma, Spain) in PBT (PBS + 0.4 % Triton-X 100) for 30 min. Sections were incubated with a mouse NeuN monoclonal antibody diluted 1:100 (Chemicon International, Tameluca, CA) diluted in PBT for 1 h at 37 °C, or with rabbit anti-GFAP polyclonal antibody (1:500, Chemicon International) overnight at 4 °C. Sections were rinsed in PBT and incubated for 45 min at room temperature in biotinylated anti-mouse/anti-rabbit BA-1400 (Vector Laboratories, Burlingame, CA) diluted 1:50. Immunostaining was amplified with the avidin–biotin system (ImmunoPure ABC Peroxidase Staining Kit, Pierce, Rockford, IL) for 30 min, and peroxidase was detected using liquid diaminobenzidine (DAB, Dako Cytomation) following the instructions of manufacturer. Finally, sections were dehydrated and coverslip mounted with Eukit (Sigma, Spain).

Negative control sections for each antigen were processed in the same manner except that the primary antibody incubation step was replaced by continued incubation in the normal serum. Neither immunostaining nor any recognisable background staining was observed under these conditions in negative control sections.

Morphometric analysis

Sections were analyzed with a Leica DMR Microscope equipped with a Leica DFC 320 video camera connected to a computer, using an Adobe Photoshop CS 8.0 image capture card for digital image capture of standard images. The public domain NIH ImageJ program [32] was used for morphometry.

The number of neurons (positive NeuN staining, $NeuN^+$) and astrocytes (positive GFAP staining, $GFAP^+$) was estimated following the approach of West et al. [33]. The anterior third of each hippocampal formation was delimited following Witter and Amaral (2004) criteria. From this region, two comparable sections of each hippocampal formation, 100 μm apart, were divided into four parts (DG, *cornu ammonis 4* (CA4) + hilus (H), CA3 and *strata: stratum lucidum, stratum radiatum* and *stratum lacunosum-moleculare*) and photographed at different magnifications depending on the cellular density (Fig. 1a, b and c), ten photos in total for NeuN. For $GFAP^+$, only two files were considered, CA4 + H and *strata*, where two photos at 10× magnification were necessary (Fig. 1d).

For each photograph, counting was done manually, and only complete cells were considered, excluding border cut cells and cells without nucleus for $NeuN^+$. For GFAP, a positive cell was counted when cell body or three to four thick $GFAP^+$ processes joined. The ImageJ program allowed us to visualise enlarged images to distinguish background staining or DAB deposits and did not take them into account. Results are expressed as numerical density, calculated by dividing the number of $NeuN^+$ or $GFAP^+$ by the reference space (cells/mm^2).

Statistical analyses

The number of animals studied in each experiment is indicated in the table legends. Values are expressed as mean and confidence interval per group, when the data are parametric, and as median and interquartile range when the data are nonparametric. The parametric data were statistically analyzed by one way ANOVA. When ANOVA resulted in differences, multiple comparisons between means were studied by the Tukey's test. The nonparametric data were statistically analyzed by the Kruskal–Wallis test.

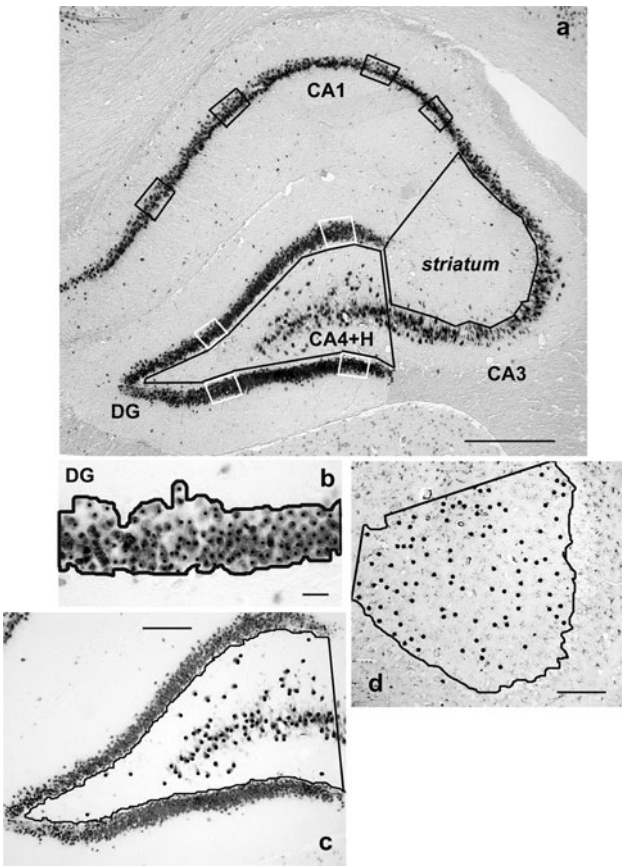

Fig. 1 **a** Morphometric analysis of the hippocampus for NeuN$^+$. Each hippocampal formation was divided into four files: dentate gyrus (DG), CA4 + hilus (CA4 + H), CA3 and *striatum: stratum lucidum, stratum radiatum* and *stratum lacunosum-moleculare*) and photographed at different magnifications depending on the cellular density as follows: **b** 4 photos at ×40 magnification for the granular layer of the DG, **c** 1 photo at ×10 magnification for the pyramidal layer in the CA4 + H, 4 photos at ×40 magnification for the pyramidal layer of the CA3, **d** 1 photo at ×10 magnification for the *striatum,* ten sectors in total. *Scale bar*: 1 mm

Differences were considered significant at $p < 0.05$. To establish whether the samples were parametric or non-parametric, the Kolmogorov–Smirnov test was used (SPSS 12.0).

Results

Nutritional parameters

Dietary intake throughout the whole period of the study was similar in all groups, with no significant differences (group Def: 22.7 (SEM 6.5) g/d; group Con: 20.1 (SEM 4.1) g/d; group Sup: 20.7 (SEM 3.2) g/d; group Extra-sup: 23.3 (SEM 3.3) g/d). In addition, there were no significant differences in body weight (group Def: 691.9 (SEM 79.2) g; group Con: 662.8 (SEM 62.7) g; group Sup: 658.8 (SEM 25.7) g; group Extra-sup: 718.7 (SEM 58.2) g). In consequence, the administration of neither a folate-deficient diet nor a folate-supplemented diet modified these critical parameters during the aging process under study.

Biochemical parameters

As expected, we observed significant differences in folate serum concentration in accordance with dietary folate level: much lower in the deficient group and higher in the groups 8 and 40 mg FA/kg diet respective to the control group ($p < 0.01$) (Table 1). A critical biomarker involved in the functioning of the methionine/methylation cycle, serum Hcy concentration, was significantly increased in group 0 mg FA/kg diet ($p < 0.01$) when compared with control animals (Table 1). By contrast, serum B_{12} vitamin and plasma B_6 levels, both also involved in the nutritional regulation of the methylation cycle, were unmodified by the dietary FA level (Table 1). Brain AdoMet and AdoHcy concentrations (Table 2) did not show significant inter-group differences. In consequence, the AdoMet/AdoHcy concentration ratio remained unchanged regardless of the dietary FA level. However, it is important to consider that the so-called "methylation ratio" reached a 30 % lower value for the extra-supplemented FA group when compared to the control. In fact, this effect is more pronounced than that observed for the deficient group. Nevertheless, Table 2 showed that the in vitro incorporation of [^{3}H]-methyl groups from radioactive AdoMet (an in vivo indicator of DNA methylation) was lower in FA deficiency group when compared to the control group ($p < 0.001$).

Histological staining and morphometry

The Nissl stain showed no remarkable differences in hippocampus morphology between the different dietary groups. In the same way, NeuN$^+$ immunostaining showed a generally comparable morphology (Fig. 2) for hippocampus in neuronal distribution between the four FA dietary groups. Morphometric analysis (Table 3) revealed that the only significant difference ($p < 0.01$) was observed for the strata when the FA-deficient group and FA supplementation group are compared.

The GFAP$^+$ immunostaining did not revealed significant differences in the astrocyte distribution through the hippocampus (Table 4 and Fig. 3).

Discussion

The present study investigated the possibility of weather dietary FA deficiency, or moderate and high supplementation may modify some biochemical markers related to the

Table 1 Serum folate, vitamin B_{12} and vitamin B_6 plasma (pyridoxal-5-phosphate and pyridoxal) and serum total homocysteine (Hcy) concentrations

Group	Folate (ng/ml)	Vitamin B_{12} (pg/ml)	Pyridoxal-5-phosphate (μg/l)	Pyridoxal (μg/l)	Hcy (μmol/l)
Def	2.4**	1160.7	89.6	58.3	12.2**
$n = 7$	(2.3–4.3)	(948.8–1372.6)	(69.8–109.3)	(34.1–82.5)	(7.4–17.1)
Con	11.2	1218.8	123.0	54.6	2.9
$n = 7$	(9.5–13.3)	(981.6–1456.1)	(90.7–155.3)	(37.6–71.7)	(1.3–4.4)
Sup	15.5**	1099.4	150.5	75.6	3.4
$n = 8$	(15.3–16.3)	(986.2–1212.6)	(117.3–183.7)	(66.5–84.7)	(2.5–4.4)
Extra-sup	18.7**	1167.5	148.7	80.1	4.0
$n = 9$	(17.5–123.5)	(992.1–1342.9)	(116.7–181.4)	(57.8–102.4)	(1.1–6.9)

Serum folate, vitamin B_{12}, plasma vitamin B_6 (Pyridoxal-5-phosphate and Pyridoxal) and total Hcy concentrations in male rats fed FA deficiency, control, FA-supplemented and FA extra-supplemented diets. Values are means (confidence interval per group) or median (interquartile range per group)

A significant (** $p < 0.01$) difference in serum folate concentration is observed in groups Def, Sup and Extra-sup versus group Con

A significant (** $p < 0.01$) difference in serum tHcy concentration was observed in group Def versus group Con

Table 2 Brain S-adenosylmethionine (AdoMet), S-adenosylhomocysteine (AdoHcy) concentrations, methylation ratio (AdoMet/AdoHcy) and brain DNA methylation

Group	AdoMet (μg/g protein)	AdoHcy (μg/g protein)	AdoMet/AdoHcy	Brain DNA methylation
Def	339.3	414.5	0.9	299.4***
$n = 7$	(214.2–464.3)	(170.9–658.0)	(0.7–1.1)	(92.2–650.3
Con	519.4	568.0	1.0	1336.8
$n = 7$	(288.4–750.4)	(291.0–845.1)	(0.5–1.4)	(984.0–1715.1)
Sup	433.3	526.8	0.8	2033.5
$n = 8$	(264.8–601.8)	(376.3–677.2)	(0.6–1.0)	(901.0–4442.3)
Extra-sup	394.9	531.44	0.7	1739.1
$n = 9$	(155.7–634.0)	(367.6–695.3)	(0.3–1.1)	(1381.3–2067.3)

Brain AdoMet, AdoHcy concentrations and AdoMet/AdoHcy ratio in male rats fed FA-deficient, control, FA-supplemented and FA extra-supplemented diets. Values are means (confidence interval per group). In global brain methylation, the results are expressed as ^{3}H-methyl group incorporation (cpm) into DNA isolated from rat brain. Values are means (confidence interval per group) or median (interquartile range per group)

A significant (*** $p < 0.001$) difference in global methylation in brain was observed in group Def versus group Con

methionine cycle and hippocampus integrity in 18-month-old rats.

FA dietary deficiency for 30 days significantly increases serum Hcy concentrations when compared with control animals. Both FA-supplemented diets (groups Sup and Extra-sup) showed an expected increase in serum folate concentrations, as previously also shown in other animal models [21, 34–37], which did not result, however, in a further reduction of Hcy concentration when compared to the control group [21, 23]. Nonetheless, reduced serum Hcy levels due to FA supplementation were previously observed under other physiological situations related to growth such as pregnancy and weaning [24, 38], in accordance with a nonlinear inverse correlation model between folate and Hcy [39].

In our experimental animal model, under FA deficiency and supplementation conditions, vitamins B_{12} and B_6 remained unchanged. In previous studies under different experimental conditions, these vitamins also remained unaffected by varying the dietary folate [21, 23, 24].

AdoMet and AdoHcy are considered key biomarkers in the methionine cycle, due to their critical regulation of the transmethylation reactions. FA supplementation may potentially induce a higher availability of methyl groups, and the opposite may occur for a deficiency state. When AdoMet and AdoHcy were determined in aged rats, both were not significantly modified by the effect of dietary FA (Table 2). These results are in accordance with previous studies when feeding rats with folate-enriched diets [21, 23, 24, 38]. Nevertheless, some studies have previously described that FA-deficient diets may decrease hepatic AdoMet concentration and increase AdoHcy concentrations [37, 40]. The brain, as part of the central nervous system, seems to be more resistant than the liver to modifications when methyl-donor nutrient (folate, choline or methionine)–deficient diets are fed [21, 26, 27, 41].

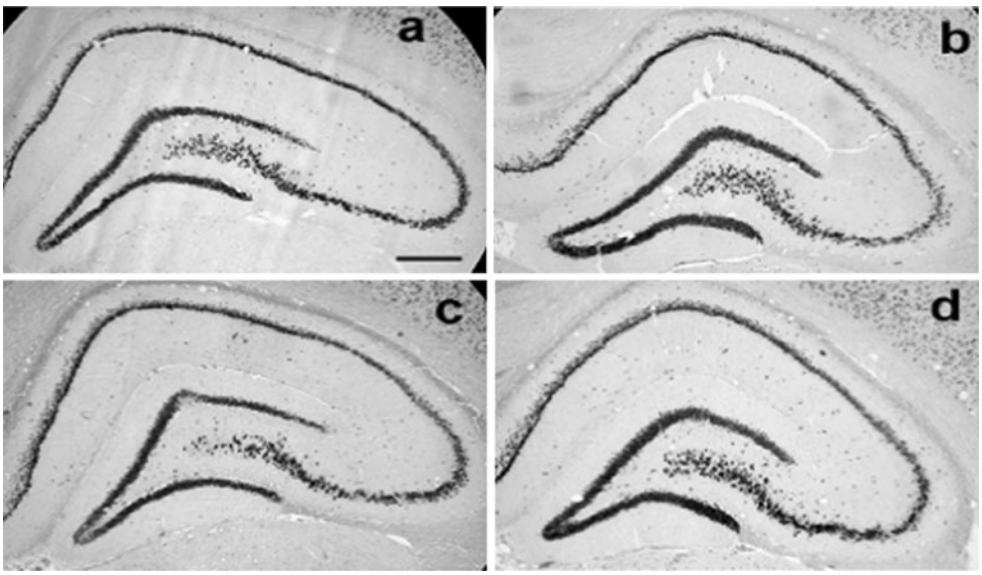

Fig. 2 NeuN$^+$ hippocampus of the four diet groups. **a** Control group. **b** Supplement group. **c** Deficient group. **d** Extra-supplement group. *Scale bar*: 1 mm

Table 3 Number of NeuN$^+$ neurons/mm^2 in the four hippocampus sectors

Group	DG	CA3	CA4 + H	*Strata*
Def	9648.1	5133.3	371.4	84.9**
$n = 6$	(8872.1–10424.1)	(4007.1–6259.7)	(271.5–471.3)	(66.2–102.5)
Con	10519.8	5695.7	409.8	108.5
$n = 5$	(9412.8–11626.7)	(5135.2–6256.2)	(284.4–535.2)	(84.1–132.9)
Sup	9567.0	5489.5	439.9	129.0
$n = 6$	(7285.0–11848.7)	(4830.3–6148.7)	(220.2–659.6)	(110.4–147.7)
Extra-sup	9769.5	5424.7	461.6	116.2
$n = 6$	(9028.3–10510.6)	(4263.0–6586.3)	(438.0–485.2)	(87.5–144.9)

DG granular layer of the dentate gyrus, *CA3* pyramidal layer of CA3, *CA4 + H* pyramidal layer of the CA4$^+$ hilus

Strata: stratum lucidum, stratum radiatum and stratum lacunosum-moleculare

Values are means (confidence interval) per group. Significant difference (** $p < 0.01$) between groups 0 and 8 mg FA/kg diet in NeuN$^+$ cells/mm^2 in *strata*

A similar effect occurred in the present study. The higher stability of the brain may explain the maintenance of neuron and astrocyte density shown in the hippocampus regardless of the dietary treatment. Future studies may be necessary to confirm or refute the present observations.

DNA methylation is a crucial process associated with basic biological processes, that is, gene expression, maintenance of genome integrity, cellular differentiation and carcinogenesis [42–44]. DNA methylation may be modified by the availability of dietary methyl groups, namely FA, since it participates directly in AdoMet synthesis. The decreased AdoMet and increased AdoHcy result in a lowered AdoMet/AdoHcy ratio, that is, the so-called "methylation ratio". In the present study, FA supplementation did not cause any remarkable change in brain DNA methylation (Table 2). Therefore, an FA extra-supplemented diet did not seem to induce a higher potential activity of the transmethylation reactions. On the other hand, FA deficiency caused the only significant decrease in the incorporation of methyl group ("brain hypermethylation"), whereas in the liver, it has been observed that folate/methyl deficiency caused an increase in methyl group incorporation, what may be considered as "hepatic DNA hypomethylation" [45]. This observation is in agreement with other studies, where methyl group–deficient diets (folate and choline) were fed to animals [46–48]. Mulder et al. [49] reported no statistical differences for the transmethylation activity in the cerebrospinal fluid from Alzheimer disease patients *versus* controls. Siegmund et al. [50] predict that approximately one half of genes encoded

Table 4 Number of $GFAP^+$ glia cells/mm^2 in the two hippocampus sectors

Group (mg FA/kg diet)	CA4 + H	*Strata*
Def	279.0	209.2
$n = 5$	(201.2–357.0)	(161.3–257.2)
Con	284.5	170.0
$n = 5$	(148.3–420.6)	(114.2–225.8)
Sup	282.6	186.6
$n = 6$	(225.8–339.4)	(156.6–216.7)
Extra-sup	270.4	186.9
$n = 6$	(232.1–308.7)	(141.0–232.8)

*CA4 + H:*CA4 + hilus

Strata: stratum lucidum, stratum radiatum and stratum lacunosum-moleculare

Values are means (confidence interval) per group. No significant differences were seen among the experimental groups

in the genome will show age-related DNA methylation changes (increases) in the human brain, which affect neuronal gene expression and thus potential cognition and behavior functions. According to our results, FA deficiency seems to enhance the methylation of genes and therefore the potential acceleration of processes associated with aging. At present, there is much interest in investigating the effect of FA and vitamins B_{12} and B_6 supplementation on cognitive function or neurodegenerative disorders. Some studies show that the data do not yet provide adequate evidence [51–53]; however, the FACIT trial showed a statistically significant improvement in memory, information processing speed and sensorimotor speed in the FA group than in the placebo group after 3 years of vitamin supplementation [54].

It is well established that the hippocampus plays a critical role in learning and memory capabilities, being susceptible to dysfunction and degeneration in aging and, specifically, Alzheimer's disease [55]. Similarly, the dentate gyrus (DG) of the hippocampal formation is one of the two privileged areas of the adult mammalian brain, in conjunction with the subventricular zone (SVZ)/olfactory bulb [56]. The neural stem cell niches in the adult brain contain various cell types and tissue components, including stem/progenitor cells and their progeny, astrocytes, oligodendroglia, microglia, immune cells, and the vasculature [57]. Proliferative "hot spots" are often located close to the vasculature, and consequently, important regulation for neurogenesis has been suspected to come from blood vessels [58]. Neurogenesis could be altered under various physiological and pathophysiological conditions [59–61]. Moreover, behavior and the environment have a striking influence on the rate of adult neurogenesis within the DG [61–66].

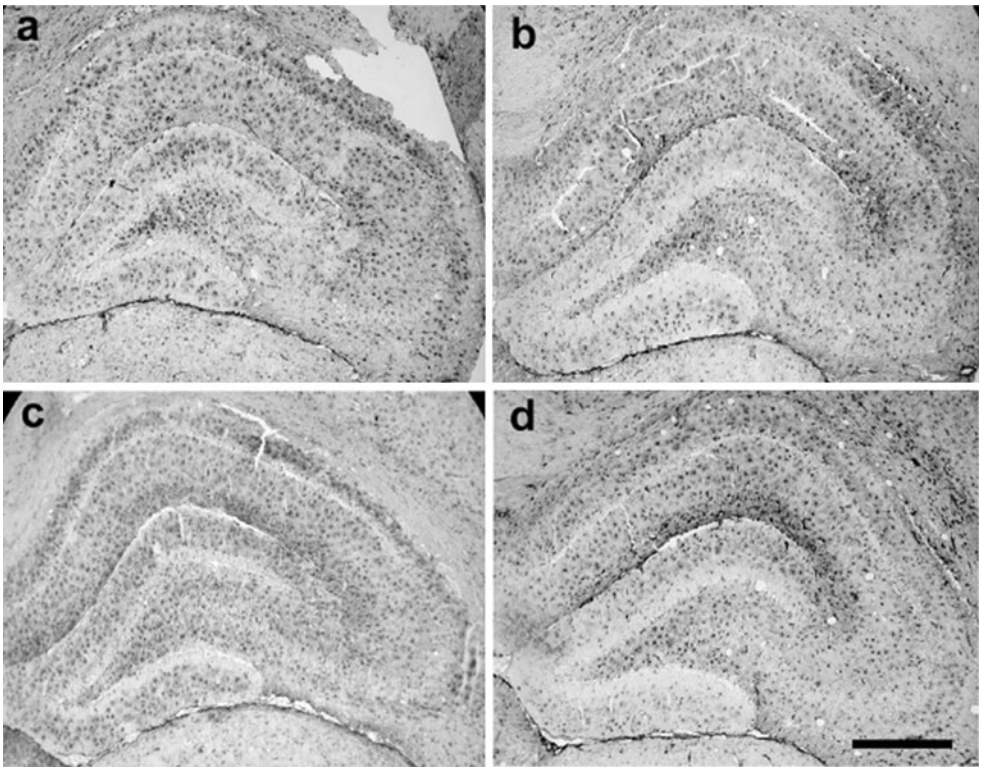

Fig. 3 $GFAP^+$ hippocampus of the four diet groups. **a** Control group. **b** Supplement group. **c** Deficient group. **d** Extra-supplement group. *Scale bar*: 1 mm

Folate deficiency adverse effects on the developing nervous system suggested the possibility that folate deficiency and elevated homocysteine levels may also have adverse effects on the adult nervous system. Although this developmental role for folate may not appear of direct relevance to aging or the disease of aging, the underlying cellular and molecular factors at the end of life may be fundamentally the same, involving alterations in cell proliferation, differentiation and survival. Besides other metabolic sequelae already described, low folate-induced HHcy compromises neurogenesis in the DG of mice that fed a folate-deficient diet [13]. Also, a reduced neurogenesis in the cerebellum of postnatal methylenetetrahydrofolate reductase knockout mice with hyperhomocysteinemia was observed [67], as well as in the caudal hippocampus, but not in the rostral region [68].

Although the antiproliferative effect of HHcy appears evident, these studies [67, 68] have failed to detect neuronal cell death in the hippocampus. Indeed, even the direct injection of a large dose of homocysteine into the hippocampus of wild type mice did not result in neuron death [8]. Dietary hyperhomocysteinemia has only been found to enhance neuronal cell death in mice that are predisposed to neurodegeneration, such as the transgenic mutant amyloid precursor protein model of Alzheimer's disease [13] or in mice rendered pharmacologically susceptible to Parkinson's disease [35], or lacking the uracil DNA glycosylase [69]. Similarly, feeding apolipoprotein-E-deficient mice with vitamin-B-deficient diets induces both hyperhomocysteinemia and cognitive impairment without neurodegeneration, astrogliosis or demyelination [70]. Such studies prove the capacity of homocysteine to contribute to cognitive dysfunction by fortifying a primary neurodegenerative insult, but not by direct neurotoxicity as the primary mechanism leading to homocysteine-related cognitive dysfunction.

Rabaneda et al. [68] divide the DG into two regions, rostral and caudal, stating that both impaired differently the generation of neuronal progenitors after an animal model of hyperhomocysteinemia treatment. These authors also showed that unexpectedly, no Hcy-induced differences in hippocampal volume were found, whereas the proliferation was reduced in the caudal area and the cell survival remained unaffected. These same authors hypothesise that the differences in the proliferation rate during the 2 weeks' treatment were not large enough to induce detectable changes in hippocampal volume, and probably, the four-week model used in the present study was not enough to achieve this either. In the present study, dietary FA only modifies the neuronal density of the *strata* for FA-deficient animals, with no neurodegeneration and astrocyte maintenance in the other sectors and groups, from which it may be concluded that density is not statistically affected. Thus, in absence of neurodegeneration, alternative mechanisms such as cerebrovascular dysfunction may be more important for homocysteine-related cognitive impairment [71]. On the other hand, the reduction in number of neurons in the strata layer does not initiate inflammatory processes including regional changes in the appearance of glial fibrillary acid protein (GFAP)-positive astrocytes which usually follows transient neuronal damage, at least under our experimental tools.

Low FA levels are accompanied by hyperhomocysteinemia and may produce alterations in the methylation process of neuronal circuits and vessels, resulting in a cognitive dysfunction in the elderly. The results obtained in the present study allow us to conclude that FA deficiency seems to negatively affect several markers of the methionine/methylation cycle, increase homocysteine and DNA methylation but also induce less neurons in the *strata*. However, an extra FA supplementation does not appear to have an additional benefit over control. In fact, once a better folate status is reached, additional folate seems to be ineffective in improving the different functioning markers. On the other hand, the hippocampus is a region that undergoes changes in humans during the loss of cognitive skills and is affected selectively when homocysteine, in whose regulation folic acid is involved, increases. In the hippocampal anterior third, our study showed that both the number of neurons and the number of astrocytes remained unchanged under different dietary FA concentrations, except in the area of the *strata*. In this area, under folic acid deficiency, we observed a statistically significant decrease in the number of neurons but no significant differences in astrocyte density, suggesting that these cells may be impervious to the potential FA diet effect in these two zones. It seems that the quantity of neurons could be preserved more efficiently with a physiological supplementation rather than with a supranormal one. It is important to note that the number of $NeuN^+$ or $GFAP^+$ was not intended to reflect an exact number of neuron and astrocyte cells in the hippocampus; rather, these measures were taken to indicate an overall difference in brain damage under the different experimental conditions applying in this study.

In conclusion, recommended doses of folic acid may be sufficient to avoid hyperhomocysteinemia and changes in the methylation cycle, extra contributions of this vitamin being unnecessary. Moreover, when the reported adverse effect of folate on masking vitamin B_{12} deficiency in an elderly population is evident, more caution is needed, mainly by exposing a potential worsening of neurological and neuropsychiatry disorders. Whether these findings in rodents apply equally humans is not yet known and further studies are needed to confirm or refute these observations on the role of methionine/methylation capacity in aging.

Acknowledgments Authors wish to thank A. Cerro and D. Arroyo for technical assistance in histology, C. Martinez-Alvarez and A. Merchan-Perez for help in morphometry. This work was supported by grants UCM-CAM, ref: PR45/05-14174, from the Comunidad Autónoma de Madrid and by "Plan Nacional de I + D + I 2000-2003", ref: BFI2003-09538 (Ministry of Science and Innovation, MICINN, Spain).

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