Maternal Folic Acid Supplementation During Pregnancy Promotes Neurogenesis and Synaptogenesis in Neonatal Rat Offspring

Xinyan Wang¹, Wen Li¹, Zhenshu Li¹, Yue Ma¹, Jing Yan², John X. Wilson³ and Guowei Huang¹

¹Department of Nutrition and Food Science, School of Public Health, Tianjin Medical University, Tianjin 300070, China, ²Department of Social Medicine and Health Administration, School of Public Health, Tianjin Medical University, Tianjin 300070, China and ³Department of Exercise and Nutrition Sciences, School of Public Health and Health Professions, University at Buffalo, Buffalo, NY 14214-8028, USA

Address correspondence to Dr Guowei Huang, Department of Nutrition and Food Science, School of Public Health, Tianjin Medical University, 22 Qixiangtai Road, Heping District, Tianjin 300070, China. E-mail: huangguowei@tmu.edu.cn orcid.org/0000-0002-0346-6303

Abstract

Maternal folic acid supplementation during pregnancy is associated with improved cognitive performances in offspring. However, the effect of supplementation on offspring’s neurogenesis and synaptogenesis is unknown, and whether supplementation should be continued throughout pregnancy is controversial. In present study, 3 groups of female rats were fed a folate-normal diet, folate-deficient diet, or folate-supplemented diet from 1 week before mating until the end of pregnancy. A fourth group fed folate-normal diet from 1 week before mating until mating, then fed folate-supplemented diet for 10 consecutive days, then fed folate-normal diet until the end of pregnancy. Offspring were sacrificed on postnatal day 0 for measurement of neurogenesis and synaptogenesis by immunofluorescence and western blot. Additionally neural stem cells (NSCs) were cultured from offspring’s hippocampus for immunocytochemical measurement of their rates of proliferation and neuronal differentiation. The results demonstrated that maternal folic acid supplementation stimulated hippocampal neurogenesis by increasing proliferation and neuronal differentiation of NSCs, and also enhanced synaptogenesis in cerebral cortex of neonatal offspring. Hippocampal neurogenesis was stimulated more when supplementation was continued throughout pregnancy instead of being limited to the periconceptional period. In conclusion, maternal folic acid supplementation, especially if continued throughout pregnancy, improves neurogenesis and synaptogenesis in neonatal offspring.

Key words: folate, hippocampus, neural stem cell, neuron, periconceptional period

Introduction

Folate-mediated one-carbon metabolism is essential for nucleic acid synthesis and methylation reactions (Stover 2009; Craciunescu et al. 2010; Chen et al. 2012; McGarel et al. 2015). Mammals require folate from food or supplements to support cell proliferation and tissue growth, especially in pregnant women and developing embryos (Chen et al. 2012; Bailey et al. 2015; Jadavji et al. 2015). Low folate status during pregnancy is associated with neural tube defects, congenital heart defects and fetal growth retardation in offspring (Bailey et al. 2015).
Supplementation with folic acid during the periconceptional period (from 4 to 12 weeks preconception to the end of first trimester of pregnancy) decreases neural tube defects incidence (Chen et al. 2012; Gomes et al. 2016) and improves cognitive performance in offspring (McGarel et al. 2015). In vitro studies have shown that folic acid stimulates cultured neural stem cells (NSCs) to proliferate (Zhang et al. 2009; Li et al. 2013; Yu et al. 2014) and differentiate into neurons (Luo et al. 2013). However, these studies in vitro may not fully represent central nervous system development in vivo, and we are not aware of any detailed in vivo experiments giving direct evidence of the effect of maternal folic acid supplementation during pregnancy on neuronal development in offspring.

Recommendations by public health authorities concerning folic acid intake for women of childbearing age differ between countries (Gomes et al. 2016). In particular, there is a lack of consensus about whether maternal folic acid supplementation should be limited to the periconceptional period or continued throughout pregnancy. Our previous study found that continuation of folic acid supplementation throughout pregnancy kept serum folate concentration high in rat mothers and improved neurobehavioral development from infancy to adulthood in their offspring (Wang et al. 2018).

We hypothesized in the present study that maternal folic acid supplementation stimulates neurogenesis and synaptogenesis in neonatal offspring and that these effects are increased when supplementation is continued throughout pregnancy. Neonatal offspring within groups were sacrificed on postnatal day 0 (PND0), neurogenesis was assessed by immunofluorescence assay of neurons and proliferating NSCs, synaptogenesis was assessed by western blot assay of brain-derived neurotrophic factor (BDNF) and synaptophysin (SYP). BDNF is crucial for neurogenesis and synaptic plasticity (Griva et al. 2017), such as enhancing long-term potentiation in the hippocampus and modulating dendritic growth and complexity in the cerebral cortex (Yamada and Nabeshima 2003). SYP is a synaptic vesicles-associated protein that is a reliable marker for synaptogenesis and synaptic density (Hami et al. 2016; Griva et al. 2017). Postnatal NSC represent only a small portion of the total hippocampal cell population, and it is not technically feasible to inject 5-bromo-2-deoxyuridine (BrdU) into PND0 rats, therefore, in vivo analysis alone cannot identify long-term effects on NSC proliferation and neuronal differentiation. To circumvent this technical obstacle, we measured proliferation and neuronal differentiation in NSC cultures derived from the hippocampus of PND0 neonatal offspring.

**Materials and Methods**

**Rats and Dietary Treatment**

All animal procedures were approved by the Tianjin Medical University Animal Ethics Committee (TMUaeEC2015001). Three-month-old female Sprague-Dawley rats (Charles River Laboratories, Beijing, China) were assigned randomly into 4 groups (12 rats/group): 1) folate-deficient diet (DD) group fed the folate-deficient diet from 1 week before mating until the end of pregnancy; 2) folate-normal diet (ND) group fed the folate-normal diet from 1 week before mating until the end of pregnancy; 3) folate-supplemented diet short-period (FD-S) group fed the folate-normal diet from 1 week before mating until 10 days after mating, and then fed the folate-supplemented diet until the end of pregnancy; and 4) folate-supplemented diet long-period (FD-L) group fed the folate-supplemented diet from 1 week before mating until the end of pregnancy.

![Figure 1. Flow chart for the experimental design with maternal diet. Three-month-old female Sprague-Dawley rats were assigned randomly into 4 groups (12 rats/group): (1) folate-deficient diet (DD) group fed the folate-deficient diet from 1 week before mating until the end of pregnancy; (2) folate-normal diet (ND) group fed the folate-normal diet from 1 week before mating until the end of pregnancy; (3) folate-supplemented diet short-period (FD-S) group fed the folate-normal diet from 1 week before mating until mating, then fed the folate-supplemented diet until 10 days after mating, and then fed the folate-normal diet until the end of pregnancy; and (4) folate-supplemented diet long-period (FD-L) group fed the folate-supplemented diet from 1 week before mating until the end of pregnancy.](https://academic.oup.com/cercor/advance-article-abstract/doi/10.1093/cercor/bhy207/5078224)
matured form of hippocampus was not developed until weaning (Gobinath et al. 2017). Thus, the hippocampus and cerebral cortex were used to detect neurogenesis and synaptogenesis in neonatal offspring, respectively. In present study, the neonatal offspring were sacrificed on PND0. For in vivo study, the brain tissue (cerebellum was removed) was either fixed with 4% paraformaldehyde for immunofluorescence staining to detect neurogenesis, or stored at −80 °C after liquid nitrogen flash-freezing for western blot assay to detect synaptogenesis. For in vitro study, NSCs were cultured from hippocampus and their proliferation and neuronal differentiation were measured as described below.

**Immunofluorescence Analysis**

Hippocampal neurogenesis was quantified by the number of neurons and proliferating NSCs. Neurons were identified as neuron-specific nuclear protein (NeuN)-positive cells. Proliferating NSCs were identified as proliferating cell nuclear antigen (PCNA)/sex determining region Y box 2 (SOX2)-double-positive cells. SOX2 is essential to maintain self-renewal of undifferentiated stem cells, and it is commonly used to identify NSCs (Suh et al. 2007; Li et al. 2013). The brain tissue was fixed with 4% paraformaldehyde for 24 h at 4 °C, then dehydrated with gradient alcohol, embedded in paraffin, and cut coronally into 4 μm-thick sections. After deparaffinization with xylene, rehydration with gradient ethanol, permeabilization with 0.2% Triton X-100 and heat mediated antigen retrieval, the sections were blocked with 10% goat serum for 1 h at 37 °C and then incubated with primary antibodies (mouse anti-NeuN [1:200, Abcam, Cambridge, UK]; mouse anti-PCNA [1:400, Abcam]; rabbit anti-SOX2 [1:100, Abcam]) overnight at 4 °C. After washing with phosphate-buffered saline (PBS), sections were incubated with appropriate secondary antibodies (tetramethyl rhodamine isothiocyanate [TRITC]-conjugated antirabbit antibody, 1:100; fluorescein isothiocyanate-conjugated antirabbit antibody, 1:100, Jackson, USA) for 1 h at room temperature. Finally, sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) contained Vectashield (H1200, Vector, CA, USA). Immunofluorescence signals were captured using Olympus IX81 microscope (Olympus, Tokyo, Japan) and analyzed by Image-Pro Plus 6.0 software.

**Western Blot Analysis**

Synaptogenesis in cerebral cortex was assessed by western blot analysis of BDNF and SYP (Hami et al. 2016; Griva et al. 2017). Brain tissue extracts were prepared in TNE-NP40 buffer with a motor-driven tissue homogenizer (PT1200E, Kinematica, Luzern, Switzerland). Protein was loaded on 4–20% PAGE Gels (GenScript, USA), separated in an electric field, then transferred onto PVDF membranes. The membranes were blocked with 5% nonfat milk for 1 h at room temperature, incubated with primary antibody (rabbit anti-BDNF [1:1000, Abcam]; rabbit anti-SYP [1:10000, Abcam]; horseradish peroxidase-conjugated anti-β-actin [1:2000; Cell Signaling Technology, USA]) overnight at 4 °C, then followed with secondary antibody (horseradish peroxidase-conjugated antirabbit antibody, 1:2000, Proteintech, China) for 1 h at room temperature. The bands were diluted with 5% nonfat milk contained tris-buffered saline and Tween-20. Proteins bands were visualized using chemiluminescence reagent (Millipore, MA, USA) and exposed to ChemiDoc™ XR+ Imaging System (Bio-Rad, CA, USA), then quantified by Image J software. The intensity of each protein band was normalized to the respective β-actin band.

**NSC Culture**

Primary NSC culture protocols have been described previously (Li et al. 2013). Briefly, hippocampus was dissected from PND0 offspring and plated at 1 × 10⁶ cells/mL in T25 culture flasks (Corning, NY, USA), then cultured in a 5% CO₂ contained humidified atmosphere at 37 °C. NSCs were cultured in serum-free Dulbecco’s modified Eagle’s medium (DMEM) and nutrient mixture F-12 Ham (F12) (1:1) (Corning), supplemented with 2% B27 supplement (Gibco, USA), 20 ng/mL epidermal growth factor (EGF; PeproTech, USA), 20 ng/mL basic fibroblast growth factor (bFGF; PeProTech), 100 U/mL penicillin and phytoypcin (Gibco) and 2 mmol/L L-glutamine (Sigma, USA) for 7 days. The identification of cultured NSC was performed by immunocytochemistry as previously described (Li et al. 2013).

**Cell Viability Assay**

NSC viability was measured by Alamar Blue (resazurin) assay. The cells were incubated with 0.1 mg/mL Alamar blue reagent (Gibco) in proliferative medium for 3 h and then the optical density was read using a microplate reader (ELX800uv, Bio-Tek Instrument Inc., VT, USA) at 575 nm (Li et al. 2015).

**Cell Proliferation Assay**

NSC proliferation was measured by BrdU incorporation (Liu et al. 2013). NSC neurospheres were gently mechanically dissociated and plated on laminin (100 μg/mL) coated coverslips, incubated with 10 μM BrdU (Sigma, USA) in proliferative medium for 24 h, then fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were washed with PBS, permeabilized with 0.1% Triton X-100 for 15 min at room temperature, blocked with 10% goat serum for 1 h at 37 °C, and incubated with primary mouse anti-BrdU antibody (1:100, Sigma) overnight at 4 °C. After another washing with PBS, the coverslips were incubated with secondary TRITC-conjugated antimouse antibody (1:100, Jackson) for 1 h at room temperature and counterstained with DAPI contained Vectashield (H1200, Vector). Immunofluorescence signals were captured using Olympus IX81 microscope (Olympus) and analyzed by Image-Pro Plus 6.0 software.

**Neuronal Differentiation Assay**

The potential capacity for neuronal differentiation of cultured NSC was assessed by immunocytochemistry. NSC neurospheres were gently mechanically dissociated and the resulting cells were plated on laminin (100 μg/mL) coated coverslips at a density of 2 × 10⁴ cells/mL in DMEM/F12 medium supplemented with 5% fetal bovine serum (FBS; Gibco), 2% N₂ (Gibco) and 100 U/mL penicillin and phytoypcin (Gibco), but without B27, EGF, or bFGF (Luo et al. 2013). After 8 days of differentiation, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, the cells were blocked with 10% goat serum for 1 h at 37 °C, and incubated with primary mouse anti-β-III-tubulin antibody (1:1000, Abcam) overnight at 4 °C. After another washing with PBS, the coverslips were incubated with secondary TRITC-conjugated antimouse antibody (1:100, Jackson) for 1 h at room temperature and counterstained with DAPI contained Vectashield (H1200, Vector). Immunofluorescence signals were captured using Olympus IX81 microscope (Olympus) and analyzed by Image-Pro Plus 6.0 software.
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**Statistical Analysis**

The data were expressed as mean ± standard deviation (SD). Comparisons between different groups were performed by one-way ANOVA and followed by Student–Newman–Keuls tests for multiple comparisons. The statistical software package SPSS 19.0 was used to evaluate the differences within groups, which was considered statistically significant at P < 0.05.

**Results**

**Maternal Folic Acid Supplementation Stimulated Hippocampal Neurogenesis in Neonatal Offspring**

The numbers of neurons and proliferating NSCs were quantified to determine if maternal folic acid supplementation could improve brain development by promoting hippocampal neurogenesis in neonatal offspring. Immunofluorescence analysis of hippocampal sections showed that the DD group had the fewest NeuN-positive and PCNA/SOX2-double-positive cells, whereas folic acid supplementation throughout pregnancy increased the abundance of both cell types significantly (P < 0.05; Fig. 2a–d). Comparison of the 2 folate-supplemented groups showed that the FD-L group had larger numbers of both cells types than did the FD-S group. These results indicate that maternal folic acid supplementation during pregnancy stimulated neuron generation and NSC proliferation in hippocampus in neonatal offspring. The results also show that supplementation is most effective when continued throughout pregnancy.

**Identification of NSC in Cell Culture**

Hippocampus was dissected from PND0 offspring for use as donors to create cell cultures in B27-, bFGF-, and EGF-supplemented, serum-free DMEM/F12 medium. NSCs proliferated and aggregated into neurospheres during the 7 days of culture (Fig. 3a). Almost all the cells in neurospheres were SOX2-positive and still had potential to proliferate as indicated by BrdU incorporation assay results (Fig. 3b). Then the neurospheres were mechanically dissociated and the resulting cells were cultured in DMEM/F12 medium supplemented with 5% FBS, 2% N2, but without B27, EGF, or bFGF. After 8 days in this differentiation medium, most of the NSCs had differentiated into neurons or astrocytes, which were identified as β-III-tubulin-positive and glial fibrillary acidic protein (GFAP)-positive cells, respectively (Fig. 3c). Taken together, these results demonstrate that the cultured neurospheres were comprised of NSCs with the capacity for self-renewal as well as for neuronal and astrocytic differentiation.

![Figure 2](https://academic.oup.com/cercor/advance-article-abstract/doi/10.1093/cercor/bhy207/5078224)

**Figure 2.** Maternal folic acid supplementation increased the abundance of neurons and proliferating neural stem cells (NSCs) in the hippocampus of neonatal offspring. Dams were fed as described in Figure 1. (a) Representative micrographs of immunofluorescence in hippocampus, in which neurons are stained with NeuN (red) and DAPI (blue). Each below merge column depicts a magnified image of the rectangular region of its corresponding image in the upper merge column. (b) Quantitative analysis of NeuN-positive cells in hippocampus. (c) Representative micrographs of immunofluorescence in hippocampus, in which proliferative NSCs are stained with PCNA (red), SOX2 (green), and DAPI (blue). Each below merge column depicts a magnified image of the rectangular region of its corresponding image in the upper merge column. (d) Quantitative analysis of PCNA/SOX2-double-positive cells in hippocampus. Scale bar = 50 μm. Data are expressed as mean ± SD (n = 6 rat offspring for each group). *P < 0.05 compared with DD group. †P < 0.05 compared with ND group. ‡P < 0.05 compared with FD-S group.
Maternal Folic Acid Supplementation Increased Cell Viability and Proliferative Capacity in Hippocampus-Derived NSCs in Vitro

Further to confirm whether maternal folic acid supplementation could promote hippocampal NSC proliferation as a long-term effect, NSCs derived from hippocampus of PND0 offspring within groups were cultured in proliferative medium for 7 days. Cell viability and proliferative capacity were measured by Alamar Blue and BrdU incorporation assay, respectively. The results showed that the DD group had the lowest numbers of viable cells and BrdU-positive cells, whereas folic acid supplementation throughout pregnancy increased the abundance of both significantly \((P < 0.05); \text{Fig. 4}\) – (c). Comparison of the 2 folate-supplemented groups showed that the FD-L group had higher cell viability and proliferative capacity than did the FD-S group. That maternal folic acid supplementation during pregnancy can increase cell viability and stimulate proliferation in hippocampus-derived NSCs in vitro suggests that the supplementation may have a long-term effect on hippocampal neurogenesis by improving the capacity of NSCs for neuronal differentiation after birth.

Maternal Folic Acid Supplementation Promoted Neuronal Differentiation Capacity in Hippocampus-Derived NSCs in Vitro

We used immunocytochemical assay of the neuron marker \(\beta\)-III-tubulin to determine if the effect of maternal folic acid supplementation on hippocampal neuron abundance was also associated with stimulation of NS differentiation into neuron. When hippocampus-derived neurospheres were mechanically dissociated and the resulting NSCs were subcultured in differentiation medium for 8 days, the DD group had the lowest rate of \(\beta\)-III-tubulin-positive cells, whereas folic acid supplementation increased the abundance of this cell type significantly \((P < 0.05, \text{Fig. 5}\) – a,b). However, there was no significant difference between the FD-S and FD-L groups. That maternal folic acid supplementation during pregnancy can promote the differentiation of hippocampus-derived NSCs into neurons in vitro suggests that the supplementation may have a long-term effect on hippocampal neurogenesis by improving the capacity of NSCs for neuronal differentiation after birth.

Maternal Folic Acid Supplementation Promoted Synaptogenesis in Cerebral Cortex of Neonatal Offspring

Immunofluorescence assay of NeuN-positive cells showed that maternal folic acid supplementation stimulated neuron generation in the cerebral cortex of neonatal offspring \((P < 0.05, \text{Fig. 6}\) – a,b). Maternal folic acid supplementation also increased BDNF and SYP protein levels \((P < 0.05, \text{Fig. 6c–f}), \text{indicating that the}\) supplementation had stimulated synaptogenesis in cerebral cortex. However, there was no significant difference between the FD-S and FD-L groups. Taken together, the results indicate maternal folic acid supplementation can promote neuron generation and enhance synaptogenesis in cerebral cortex of neonatal offspring.

Discussion

In present study, for the first time, we provide direct in vivo evidence that maternal folic acid supplementation during pregnancy improves neurogenesis and synaptogenesis in neonatal offspring. Compared with the folate-deficient group, the folate-supplemented groups showed significant improvements in
neurogenesis and synaptogenesis, as evidenced by increased number of PCNA/SOX2-double-positive cells in hippocampus, increased number of NeuN-positive cells in hippocampus and cerebral cortex, and increased protein expression of BDNF and SYP in cerebral cortex of neonatal offspring. Furthermore, we found in hippocampus-derived cell cultures that the supplementation exerts a long-term stimulatory effect on the capacities of NSC for proliferation and neuronal differentiation, which could improve neurogenesis in the hippocampus after birth.

The layers of the cerebral cortex start to form around 7 weeks postfertilization and E15, respectively, in human and rats (Fletcher et al. 2017). Newly generated neurons migrate from the ventricular surface to cortical layers during this period and subsequently continue dendritic maturation and synaptogenesis throughout gestation (Fletcher et al. 2017). In rodents, the hippocampus undergoes continued neurogenesis to develop into its mature form at approximately PND21 (Gobinath et al. 2017) and the majority of dentate gyrus granule neurons are generated during this postnatal period (Gobinath et al. 2017; Sourial and Doering 2017). Proliferating NSCs become neural precursor cell at approximately PND6 (Ortega-Martinez and Trejo 2015). The proliferation of neural precursor cell in hippocampus peaks in the first postnatal week and these cells may greatly influence neonatal and adult neurogenesis, learning, and memory (Sourial and Doering 2017). Thus, the neonatal offspring’s neurogenesis and synaptogenesis were detected, respectively, in hippocampus and cerebral cortex.

The folate-mediated one-carbon cycle is required for the synthesis of purines and thymidylate essential for cell proliferation (Stover 2009; Craciuencu et al. 2010; Chen et al. 2012; McGarel et al. 2015). Folate metabolism also supports reactions that methylate DNA and histones, and it therefore influences epigenetic regulation of gene expression (Stover 2009; Gueant et al. 2013; Barua et al. 2014; McGarel et al. 2015). DNA reprogramming is vital to early embryonic development, and the de novo DNA methylation initiated around the time of implantation is essential for completing embryonic development (Barua et al. 2016). Maternal plasma folate status during pregnancy may impact neurodevelopmental and behavioral outcomes by modulating DNA methylation patterns in offspring (Barua et al. 2014, 2016; Joubert et al. 2016). In vitro studies have indicated

Figure 4. Maternal folic acid supplementation increased cell viability and proliferative capacity in hippocampus-derived NSCs in vitro. Dams were fed as described in Figure 1, and NSC cultures were created as described in Figure 3. (a) Cell viability was assessed by Alamar Blue. (b) Representative micrographs of the BrdU incorporation assay, in which proliferative cells are stained with BrdU (red) and DAPI (blue). Scale bar = 50 μm. (c) Quantitative analysis of BrdU-positive cells. Data are expressed as mean ± SD. (Overall, 18 offspring per group were used to collect the results of cell viability assay, and 18 offspring per group were used to collect the results of BrdU incorporation assay, respectively. Specifically, hippocampus dissected from 6 rat offspring for each group was used to create one single NSC culture, and the assay was repeated in 3 cell cultures.) *P < 0.05 compared with DD group. #P < 0.05 compared with ND group. &P < 0.05 compared with FD-S group.

Figure 5. Maternal folic acid supplementation improved neuronal differentiation capacity in hippocampus-derived NSCs in vitro. Dams were fed as described in Figure 1, NSC cultures were created as described in Figure 3, then the NSC neurospheres were mechanically dissociated into cell suspension and cultured in differentiation medium for 8 days. (a) Representative micrographs of immunocytochemical assay, in which neurons are stained with β-III-tubulin (red) and DAPI (blue). Scale bar = 50 μm. (b) Quantitative analysis of β-III-tubulin-positive cells. Data are expressed as mean ± SD. (Overall, 18 offspring were used to collect the results for each group. Specifically, hippocampus dissected from 6 rat offspring for each group was used to create one single NSC culture, and the assay was repeated in 3 cell cultures.) *P < 0.05 compared with DD group.
that folate acts through a DNA methyltransferase-mediated mechanism to stimulate cultured NSCs to proliferate and differentiate into neurons (Li et al. 2013; Luo et al. 2013). The present study’s folate-supplemented diet has been shown to increase serum folate concentration in rat mothers and improve their offspring’s neurobehavioral development from infancy to adulthood, compared with the folate-deficient diet (Wang et al. 2018). Our new data elucidate plausible mechanisms for those neurobehavioral effects.

Maternal folic acid supplementation during pregnancy plays important roles in preventing neural tube defects and improving cognitive performances in offspring (McGarel et al. 2015). However, there is no consensus about whether the supplementation should be continued throughout pregnancy (Gomes et al. 2016). In present study, comparison of the FD-S and FD-L groups indicated that prolonged supplementation throughout pregnancy had greater effect on hippocampal neurogenesis, as evidenced by increased number of NeuN-positive and PCNA/ SOX2-double-positive cells in hippocampus and increased proliferative capacity in hippocampus-derived NSC cultures.

Neural tube closure begins around E8.5 and completes around E10.5 with the posterior neuropore finally closing in mice embryos (Mao et al. 2010), and this process begins at approximately 21 days postfertilization and completes by 26–28 days postfertilization in human fetuses (Greene and Copp 2014). Thus maternal folic acid supplementation that was limited to the periconceptional period could be effective for preventing neural tube defects (Chen et al. 2012). However, the development of central nervous system continues after neural tube closure has finished, and areas such as hippocampus, cerebral cortex, and striatum undergo rapid morphogenesis and synaptogenesis to gain their functions throughout the late fetal and early postnatal periods (McGarel et al. 2015; Fletcher et al. 2017). Experiments with pregnant rats have shown that maternal serum folate concentration decreases soon after folic acid supplementation is stopped (Wang et al. 2018). Therefore, it is plausible that stopping supplementation prematurely may lower folate status to a level that is inadequate for the developing brain’s increasing need for DNA synthesis and methylation reactions. Indeed, maternal folic acid deficiency from E11 through E17 altered neural progenitor cell proliferation and apoptosis in fetal mouse brain (Craciunescu et al. 2010). Similarly the results of the present study indicate that maternal folic acid intake impacts hippocampal neurogenesis by altering NSCs proliferation late in gestation, after neural tube closure. Taken together, these data provide support for the opinion that maternal folic acid supplementation should be prolonged throughout pregnancy.

In conclusion, the present study shows that maternal folic acid supplementation exerts a long-term effect on hippocampal neurogenesis in offspring by stimulating the capacities of NSC for proliferation and neuronal differentiation. The supplementation
also enhances synaptogenesis in the cerebral cortex of neonatal offspring. Maternal folic acid supplementation was more effective when continued throughout pregnancy instead of being limited to the periconceptional period. These results provide evidence that folic acid supplementation in women of childbearing age may be most beneficial if begun prior to conception and continued throughout pregnancy.

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**Notes**
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