

# Prenatal choline availability modulates hippocampal neurogenesis and neurogenic responses to enriching experiences in adult female rats

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## Abstract

Increased dietary intake of choline early in life improves performance of adult rats on memory tasks and prevents their age-related memory decline. Because neurogenesis in the adult hippocampus also declines with age, we investigated whether prenatal choline availability affects hippocampal neurogenesis in adult Sprague–Dawley rats and modifies their neurogenic response to environmental stimulation. On embryonic days (ED) 12–17, pregnant rats ate a choline-supplemented (SUP-5 g/kg), choline sufficient (SFF-1.1 g/kg), or choline-free (DEF) semisynthetic diet. Adult offspring either remained in standard housing or were given 21 daily visits to explore a maze. On the last ten exploration days, all rats received daily injections of 5-bromo-2-deoxyuridine (BrdU, 100 mg/kg). The number of BrdU+ cells was significantly greater in the dentate gyrus in SUP rats compared to SFF or DEF rats. While maze experience increased the number of BrdU+ cells in SFF rats to the level seen in the SUP rats, this enriching experience did not alter cell proliferation in DEF rats. Similar patterns of cell proliferation were obtained with immunohistochemical staining for neuronal marker doublecortin, confirming that diet and exploration affected hippocampal neurogenesis. Moreover, hippocampal levels of the brain-derived neurotrophic factor (BDNF) were increased in SUP rats as compared to SFF and DEF animals. We conclude that prenatal choline intake has enduring effects on adult hippocampal neurogenesis, possibly via up-regulation of BDNF levels, and suggest that these alterations of neurogenesis may contribute to the mechanism of life-long changes in cognitive function governed by the availability of choline during gestation.

## Introduction

Choline is a vital nutrient with several biological functions. As a constituent of phosphatidylcholine, it contributes to the construction of cellular membranes and lipid transport; upon conversion to betaine it becomes a methyl group donor; and as a component of platelet aggregating factors, lysosphosphatidylcholine and sphingosylphosphorylcholine, it participates in cell signalling (Blusztajn, 1998; Zeisel, 2004, 2006). Choline is also a precursor to the neurotransmitter, acetylcholine.

The supply of choline during gestation is critical for brain development. Compared to offspring of mothers that ate a choline sufficient or deficient diet during pregnancy, prenatal choline supplemented rats have enhanced spatial and temporal memory, specifically showing augmented memory capacity and precision (Meck *et al.*, 1988, 1989; Meck & Williams, 1997a, 1999; pre- and postnatal choline, Ricceri & Berger-Sweeney, 1998; Tees & Mohammadi, 1999; Tees, 1999a, 1999b), that resists even normal, age-related decrements (prenatal choline, Meck & Williams, 1997b; Blusztajn, 1998). These lasting cognitive changes are accompanied by facilitation of hippocampal long-term potentiation (LTP) (Jones *et al.*, 1999; Pyapali *et al.*, 1998) and enhanced acetylcholine release (Cermak *et al.*, 1998).

One mechanism that may contribute to the enduring effect of prenatal choline nutrition on memory may be changes in hippocampal development. Compared to fetuses of rodent mothers receiving a choline sufficient diet, fetuses of mothers supplemented with choline on embryonic days (ED) 12–17 show, on ED 17–20, more cell division and neurogenesis in the fetal hippocampus and less apoptotic cell death (Albright *et al.*, 1999; Craciunescu *et al.*, 2003). Similarly, fetuses of deficient mothers, compared to fetuses of mothers fed a choline sufficient diet, show less cell division and more cell death (Craciunescu *et al.*, 2003; reviewed in Zeisel & Niculescu, 2006). In the present study, we examined whether the changes in hippocampal neurogenesis that arise as a function of prenatal choline availability and that are evident during development persist into adulthood.

The neurogenic properties of the adult hippocampus are well documented (Altman & Das, 1965; Kaplan & Hinds, 1977; Kaplan & Bell, 1984; Cameron *et al.*, 1993; Cameron & McKay, 2001; van Praag *et al.*, 2002; Kempermann *et al.*, 2003) and regulated by a variety of factors, many of which (environmental enrichment, hormones, stress, ageing) have similar effects on neurogenesis and learning (reviewed in Gould *et al.*, 2000; Christie, & Cameron, 2006). Such correlational evidence is part of a growing literature that implicates adult dentate gyrus neurogenesis in hippocampally dependent learning and memory (Shors *et al.*, 2001, 2002; Schinder & Gage, 2004; Snyder *et al.*, 2005; Aimone *et al.*, 2006). Thus, changes in this feature of hippocampal plasticity may underlie the

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effects of prenatal choline supply on spatial memory across the lifespan.

Because experience modulates neurogenesis, we examined the neurogenic response of the hippocampus to environmental enrichment as a function of prenatal choline availability and found that in prenatally choline sufficient, but not deficient rats enrichment increased neurogenesis. Remarkably, prenatally choline supplemented rats that did not receive enrichment had increased levels of neurogenesis that were similar to supplemented and sufficient rats that did receive enrichment. To follow up on the increase in neurogenesis in prenatally choline supplemented rats that remained in standard pair house laboratory conditions during the study and because neurotrophic factors regulate hippocampal neurogenesis (reviewed in Cameron *et al.*, 1998), we measured levels of brain-derived neurotrophic factor (BDNF) in the hippocampus in a cohort of animals treated in the same way and raised to a similar age. Our findings are the first to report that a short period of dietary change during fetal development can lead to long-lasting alterations in ongoing hippocampal neurogenesis and in the neurogenic response to the environment.

## Materials and methods

### Animals and diet

All rats were housed in clear polycarbonate cages (27.9 × 27.9 × 17.8 cm) that were individually ventilated, and the colony was maintained at 21 °C with a 12-h light : 12-h dark cycle, with lights on at 07:00 h. Forty timed-pregnant Sprague–Dawley rat dams (CD strain, Charles River Breeders, Kingston, NY) arrived in the colony on day nine of gestation (ED 9). Until ED 11, all dams had *ad lib* access to a choline sufficient diet (AIN76A with 1.1 g/kg choline chloride substituted for choline bitartrate; Dyets Inc, Bethlehem, PA). From the evening of ED 11 to the morning of ED 18, some dams ( $n = 8$ ) received a version of the diet that contained 5 g/kg of choline chloride (approximately 4.5 times the amount in the sufficient diet) or a version of the diet completely deficient of choline ( $n = 8$ );

the remaining dams ( $n = 24$ ) continued to receive the sufficient diet. Before ED 11 and after ED 18, the choline sufficient version of the semisynthetic diet was given to all dams and offspring. Thus, the only period in which our rats received different diets was ED 12–17. Supplementation during this time frame has been shown to cause a lasting improvement in memory function (reviewed in Meck & Williams, 2003; McCann *et al.*, 2006; see Berger-Sweeney, 2003). These variations in the availability of choline in the pregnant rat's diet alter the concentration of choline metabolites in the brain of the fetus (see Garner *et al.*, 1995).

On the day after birth, all pups were removed from dams, permanently marked to indicate prenatal choline diet, and cross-fostered to dams that had consumed the choline sufficient diet. Litters were composed of five male and five female offspring with each diet condition represented. Pups were weaned at 24 days of age and housed in same-sex, mixed-diet treatment pairs. For the present study, 52 female offspring of mothers fed the choline-supplemented (SUP;  $n = 18$ ), choline-sufficient (SFF;  $n = 17$ ), or choline-deficient (DEF;  $n = 17$ ) diet were selected from the 24 foster mothers. At approximately 200 days of age, all rats were placed on a feeding schedule in which they were fed a daily 25-g allotment of food each evening. This schedule maintained rats at approximately 85–90% of free feeding weight values, adjusted for growth and was instituted to encourage rats to search for food during maze exploration. Rats were kept on this feeding schedule for the remainder of the study. Also at this time, each diet group was divided into two; those that received daily opportunities to explore a maze (EXPL, SUP,  $n = 8$ ; SFF,  $n = 7$ ; DEF,  $n = 6$ ) and rats that did not receive daily experience on a maze (NoEXPL, SUP,  $n = 6$ ; SFF,  $n = 6$ ; DEF,  $n = 7$ ). Twelve additional rats ( $n = 4$  per diet condition) that were pair housed for the duration of the study were used for analysis of BDNF protein content in hippocampus.

### Experimental procedures

Figure 1 outlines the experimental design and the timeline of the procedures used in this study and described in the sections below. All

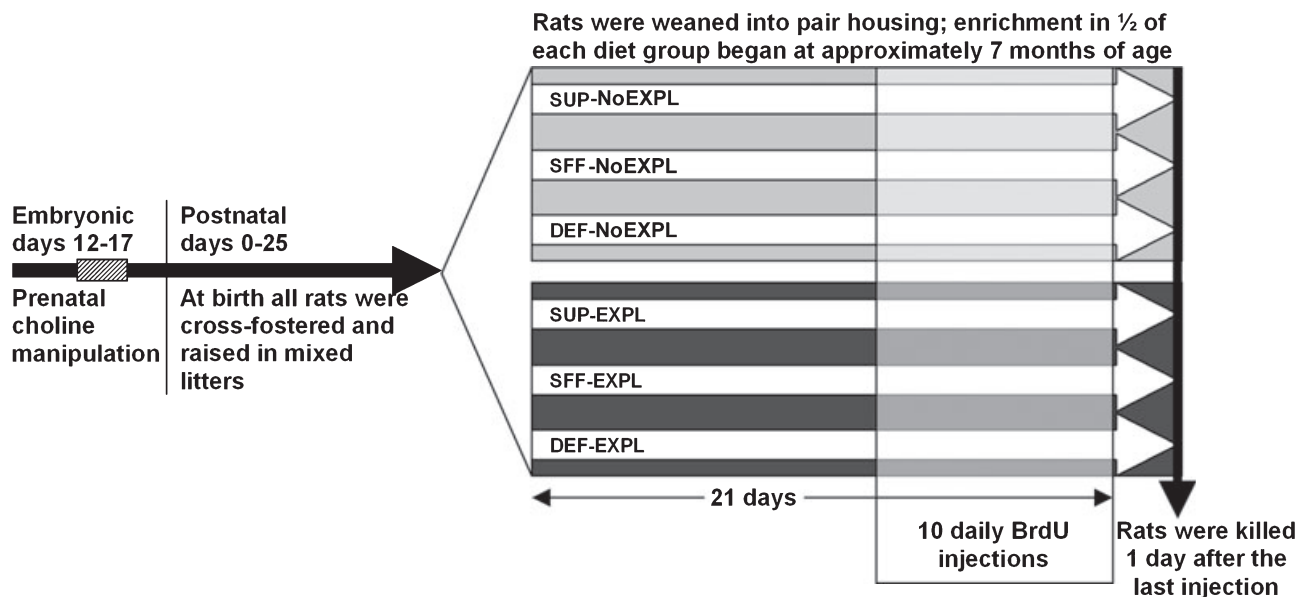


FIG. 1. Rats that were prenatally choline supplemented (SUP), sufficient (SFF), and deficient (DEF) were divided into two groups at 7 months of age; half of each diet condition underwent daily maze exploration for 21 days (EXPL) and the other half did not (NoEXPL). All rats received ten daily injections of BrdU and were killed 24 h after the last injection.

procedures were performed as approved by and according to the guidelines of the Institutional Animal Care and Use Committee of Duke University.

#### Maze exploration

The maze was made of sealed wood, consisting of a central platform 60 cm in diameter, elevated 120 cm from the floor, with 12 arms (8 × 90 cm) extending radially. It was located in a well-lit room with a variety of different extramaze cues present. Rats in the EXPL group were placed on the maze once daily for 21 consecutive days. Before each maze session, 20–25 small, sweetened cereal flakes were scattered over the centre area and 12 arms. At the start of each session, the animal was placed on the central platform and allowed to explore the maze and eat the cereal. Each session lasted 10 min. The first six of the 21 EXPL days served to acclimate rats to the procedures and teach them that food was available over the entire maze. On the first of those 6 days rats were placed on the maze in groups of four or six. On the next 2 days, rats were placed on the maze in pairs. On the next 3 days rats were placed on the maze individually and, if necessary, were encouraged and shaped to explore the maze arms. All rats were freely exploring the maze, individually, after the six training days.

These maze procedures were designed to equate all diet-treated animals in overall time and exploration of the maze. Because we have found that choline availability alters memory as assessed by radial-arm maze performance (Meck *et al.*, 1988, 1989; Meck & Williams, 1997b, 1999), we did not want to use a standard radial-arm maze memory task in this study. If rats were trained to find food in the arm ends, SUP rats would make fewer errors than SFF or DEF rats even on the first days of training (e.g. Meck *et al.*, 1988, 1989) and thus spend less time on the maze than the other groups. To avoid this confound, we chose to encourage all rats to explore/forage for small cereal pieces for a 10-min duration. Electrophysiological recordings from hippocampal place cells (O'Keefe & Dostrovsky, 1971) in animals freely moving about on a radial arm maze (Shapiro *et al.*, 1997) suggest that our procedure should actively engage hippocampal function. Numerous other reports have demonstrated that female rats treated with prenatal choline supplementation in the same way as in the present study show enhanced spatial memory at many different ages, both younger (Williams *et al.*, 1998), similar (Loy *et al.*, 1991), and older (Meck & Williams, 2003) than the female rats in this study.

Analysis of rats' activity on the maze during the final 15 days confirmed that the procedures we used to ensure that all diet groups were exploring the maze equally were successful. There were no significant differences between groups in daily maze exploration (main effect of DIET  $F < 1$ ), and only a modest decline over days (main effect of BLOCK  $F_{2,34} = 4.187$ ,  $P = 0.014$ ), with no significant interaction between DIET and BLOCK ( $F_{4,34} = 1.218$ ,  $P = 0.321$ ). On average, all three groups of rats entered 14 or 15 arms during the first 5 days of maze exploration, which declined to 12–13 arms by the last 5 days of exploration for all groups.

#### 5-bromo-2-deoxyuridine administration

On the final 10 days of maze exploration, all rats, EXPL and NoEXPL, received one daily injection of the cell division marker, 5-bromo-2-deoxyuridine (BrdU; 100 mg/kg, i.p.; Sigma). This injection regime was based on past research designed to capture the impact of a variety of manipulations on cell proliferation and survival in the hippocampus (Kempermann *et al.*, 1997; Lee *et al.*, 2002a; Rao *et al.*, 2005). Injections were administered approximately 30 min after a maze session, and groups of NoEXPL rats were yoked to EXPL rats for the timing of their injections. Twenty-four hours after the final

injection, rats were anaesthetized using ketamine (i.p. 80mg/kg) and xylazine (i.p. 10mg/kg) and perfused transcardially with ice-cold phosphate-buffered saline (PBS; 1 M), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were immersed in the fixative for a minimum of 24 h then transferred to 0.1% solution of sodium azide in 1 M PB and stored at 4 °C. Brains were sectioned on a vibratome and two series of every fifth, 55- $\mu$ m section were obtained for immunohistochemistry.

#### BrdU immunohistochemistry

Immunohistochemical procedures for BrdU-labelling were based on the methods of Kuhn *et al.* (1996). One series of free-floating sections was rinsed with Tris-buffered saline (TBS, pH 7.3) followed by 30 min in 0.6% hydrogen peroxide in TBS at room temperature to reduce nonspecific staining. After rinsing again in TBS, tissue was treated for 2 h in 50% formamide and 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65 °C, rinsed in 2 × SSC for 10 min, incubated in 2 M HCl for 30 min at 37 °C, and rinsed in 0.1 M boric acid (pH 8.5) for 15 min. Sections were rinsed in TBS, incubated in 0.1% Triton X-100 (TTX; Sigma) and 3% normal horse serum (Vector Laboratories, Burlingame, CA) in TBS for 30 min at room temperature, and then incubated with the primary antibody (monoclonal mouse anti-BrdU, 1 : 400; Boehringer Mannheim, Indianapolis, IN) for 48 h at 4 °C. Following this, the tissue was rinsed with TBS and incubated with the secondary antibody (biotinylated horse anti-mouse, 1 : 200; Vector Laboratories) for 2 h at room temperature. The tissue was then rinsed in TBS, incubated in an avidin-biotin complex (ABC, Vector Laboratories) for 1 h at room temperature, rinsed again in TBS, and treated for peroxidase detection with diaminobenzidine (Vector Laboratories, nickel intensified) for 10 min. Stained sections were mounted on gelatin-coated slides, dehydrated, counterstained with cresyl violet, and coverslipped.

#### Doublecortin immunohistochemistry

An adjacent series of tissue sets was processed for doublecortin (DCX) immunohistochemistry. These procedures were based on the methods of Rao & Shetty (2004). Free-floating sections were rinsed in TBS, treated for 30 min in 0.6% hydrogen peroxide, incubated in 0.1% TTX and 3% normal horse serum in TBS for 30 min at room temperature, and then incubated with the primary antibody (affinity purified polyclonal goat antibody raised against a peptide mapping at the carboxy terminus of human DCX, 1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at room temperature. Following this, the tissue was rinsed with TBS and incubated with the secondary antibody (biotinylated horse anti-goat, 1 : 200; Vector Laboratories) for 2 h at room temperature. The tissue was then rinsed in TBS, incubated in an avidin-biotin complex (ABC, Vector Laboratories) for 1 h at room temperature, rinsed again in TBS, and developed with vector grey (Vector Laboratories).

#### Quantification of BrdU- and DCX-labelled cells using unbiased stereology

BrdU-labelled and DCX-labelled cells in each dentate gyrus were counted using the optical fractionator method (West, 1993, 1999; Mouton, 2002). We sampled every fifth section through the rostral-caudal extent of the dentate gyrus and restricted the sampling region to the dorsal and ventral blades, including the granule cell layer and subgranular zone. StereoInvestigator (Microbrightfield Inc, Williston, VT) was used to systematically sample through the designated region and count numbers of labelled cells. We used a 100 × 100  $\mu$ m counting frame and 15–20 sites per section were analysed in

10–12 sections, yielding 150–240 frames per rat. These parameters ensured adequate sampling through the dentate gyrus to compensate for the sporadic distribution of labelled cells. For analysis we set an optical dissector height of 20  $\mu\text{m}$  with a 2- $\mu\text{m}$  guard zone and counted stained cells in each frame using a 40 $\times$  objective lens.

We also obtained estimates of the volume of dentate gyrus using the same sections that were examined for BrdU and DCX labelling. Volume estimates were made using Cavalieri's principle (e.g. Mouton, 2002). For each section examined, the area of the dentate gyrus was calculated by the StereoInvestigator software and was based on the boundaries of the contour tracings. Volume estimates were obtained by multiplying the section area estimates with the spacing between sampled areas. Spacing was derived by multiplying the measured, prehistology thickness of each sample by the number of sections examined.

#### BDNF ELISA

At 8 months of age, a second cohort of female offspring of mothers fed the choline-supplemented, choline-sufficient, or choline-deficient diets were anaesthetized, as above, and the hippocampi were removed rapidly on ice. Whole tissue extracts were prepared by adding lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet NP-40, 10% glycerol, 2 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 1  $\mu\text{g}/\text{mL}$  leupeptin, 2  $\mu\text{g}/\text{mL}$  aprotinin, 2  $\mu\text{g}/\text{mL}$  pepstatin) to tissue, followed by gentle sonication, incubation on ice for 15 min, and a brief centrifugation to clear. The ChemiKine™ BDNF sandwich ELISA kit (Chemicon Int, Inc.) was used to assay the BDNF levels in hippocampal lysates. Lysates were diluted 1 : 5 with sample or standard diluent (Chemicon). BDNF levels were measured according to manufacturer's instructions and are shown as ng/g wet weight of hippocampus.

#### Statistical analyses

For rats in the EXPL group, the number of arm entries made on the last 15 of the 21 days was averaged over three blocks of 5 days for each diet condition. These data were analysed using a 3  $\times$  3 mixed factorial analysis of variance (ANOVA) with prenatal condition (DIET; SUP, SFF, or DEF) as the between-subjects variable and block of days (BLOCK; Days 7–11, Days 12–16, Days 17–21) as the repeated measures variable and the findings are reported above in the 'Maze exploration' section. The numbers of BrdU-labelled and DCX-labelled cells estimated with the optical fractionator and the volumes of dentate gyrus that were estimated using Cavalieri's principle were analysed using 2  $\times$  3 (EXP (EXPL or NoEXPL)  $\times$  DIET), factorial between-subjects ANOVA. Levels of BDNF concentration in hippocampus were analysed using a single-factor between-subjects ANOVA with DIET as the treatment variable. Statistically significant main effects were followed by pairwise comparisons (Tukey HSD) to look for statistically significant differences between overall treatment means. Otherwise, where applicable, statistically significant differences between group means were evaluated using planned, pairwise, single-d.f. comparisons ( $F_{\psi}$ : compares actual difference between two group means while using an estimate of error variability from the overall ANOVA; Keppel & Wickens, 2004). Results of all statistical tests were considered significant with  $P < 0.05$ . Note that rats contained within each of the treatment groups were obtained from different biological mothers and were randomly cross-fostered to foster mothers for rearing. Thus, we have taken the necessary precautions to be sure that our findings are not contaminated by a lack of within litter variability.

## Results

To detect the numbers of cells and neurons born and surviving over a 10-day period in our choline-treated animals and to gauge their neurogenic response to enriching exploration, we visualized cells positive for the cell division marker, BrdU, and the neuronal marker, DCX, in the dentate gyrus of each rat. Figures 2 and 3 show photomicrographs of BrdU and DCX labelling, respectively, in the hippocampus of representative SUP, SFF, and DEF rats that received daily opportunities to explore a maze (EXPL) or did not have that opportunity (NoEXPL). BrdU-labelled cells were evident throughout the rostral-caudal extent of the dentate gyrus and in both the dorsal and ventral blades. Cells were evident in the subgranular zone, indicating that many of the cells were newly generated. BrdU+ and DCX+ cells were also visible in the granule cell layer; indicating that some cells had survived for several days and were migrating from the subgranular zone. DCX+ cells were visible with processes in various stages of development. Both markers clearly showed that, overall, SUP rats had enhanced cell proliferation and neurogenesis, and EXPL increased cell proliferation and neurogenesis in SFF rats.

Using unbiased stereology, we estimated the number of BrdU-labelled cells for each rat and these data are shown in Fig. 4A. The main finding was that, overall (main effect of DIET;  $F_{2,34} = 5.357$ ,  $P = 0.009$ ), SUP rats had significantly more BrdU-labelled cells than SFF ( $P = 0.026$ ) or DEF ( $P = 0.003$ ) rats, which were not significantly different ( $P = 0.426$ ). Although the interaction between EXP and DIET failed to achieve statistical significance ( $F_{2,34} = 2.388$ ,  $P = 0.107$ ), it was clear that the diet groups had different responses to the exploration experience. Daily maze exploration increased cell proliferation in the SFF rats compared to SFF rats without maze exploration ( $F_{\psi,1,34} = 5.464$ ,  $P = 0.025$ ). In contrast, maze exploration did not increase the number of BrdU+ cells in the DEF group, nor did this experience further increase cell proliferation in the SUP rats ( $F_s < 1$ ). Further analyses revealed that SUP EXPL rats were not statistically different from SUP NoEXPL rats because this latter group were already showing high numbers of labelled cells, significantly more than SFF NoEXPL rats ( $F_{\psi,1,34} = 7.053$ ,  $P = 0.011$ ), and more, though not significantly more, than DEF NoEXPL rats ( $F_{\psi,1,34} = 3.439$ ,  $P = 0.072$ ).

To determine whether changes in numbers of new neurons were contributing to the effects of prenatal choline and maze exploration on cell proliferation, we used adjacent tissue sections to examine the expression of the microtubule-associated phosphoprotein, DCX, that is transiently expressed in newly born neurons that are still in the process of migrating and differentiating (Brown *et al.*, 2003; Rao & Shetty, 2004). Figure 4B shows the number of DCX-labelled cells in SUP, SFF, and DEF, EXPL and NoEXPL rats. Unbiased stereological estimates of DCX-labelled cells in the dentate gyrus of each rat revealed that the overall pattern of DCX labelling was strikingly similar to that of BrdU labelling (see Fig. 4). There was a significant main effect of DIET ( $F_{1,34} = 14.394$ ,  $P = 0.001$ ); SUP rats had significantly more DCX-labelled cells than SFF ( $P = 0.001$ ) or DEF ( $P = 0.001$ ) rats, which were not significantly different ( $P = 0.205$ ) from each other. The interaction between EXP and DIET was not significantly different ( $F_{2,34} = 1.937$ ,  $P = 0.160$ ), but planned, pairwise comparisons revealed that SFF EXPL rats had significantly more DCX-labelled neurons than SFF NoEXPL rats ( $F_{\psi,1,34} = 4.922$ ,  $P = 0.033$ ), whereas there were no statistically significant differences between EXPL and NoEXPL rats in either the SUP or DEF groups ( $F_s < 1$ ). As with BrdU-labelling, the SUP EXPL rats were not different from SUP NoEXPL rats because this latter group was already showing high numbers of DCX-labelled neurons, significantly more

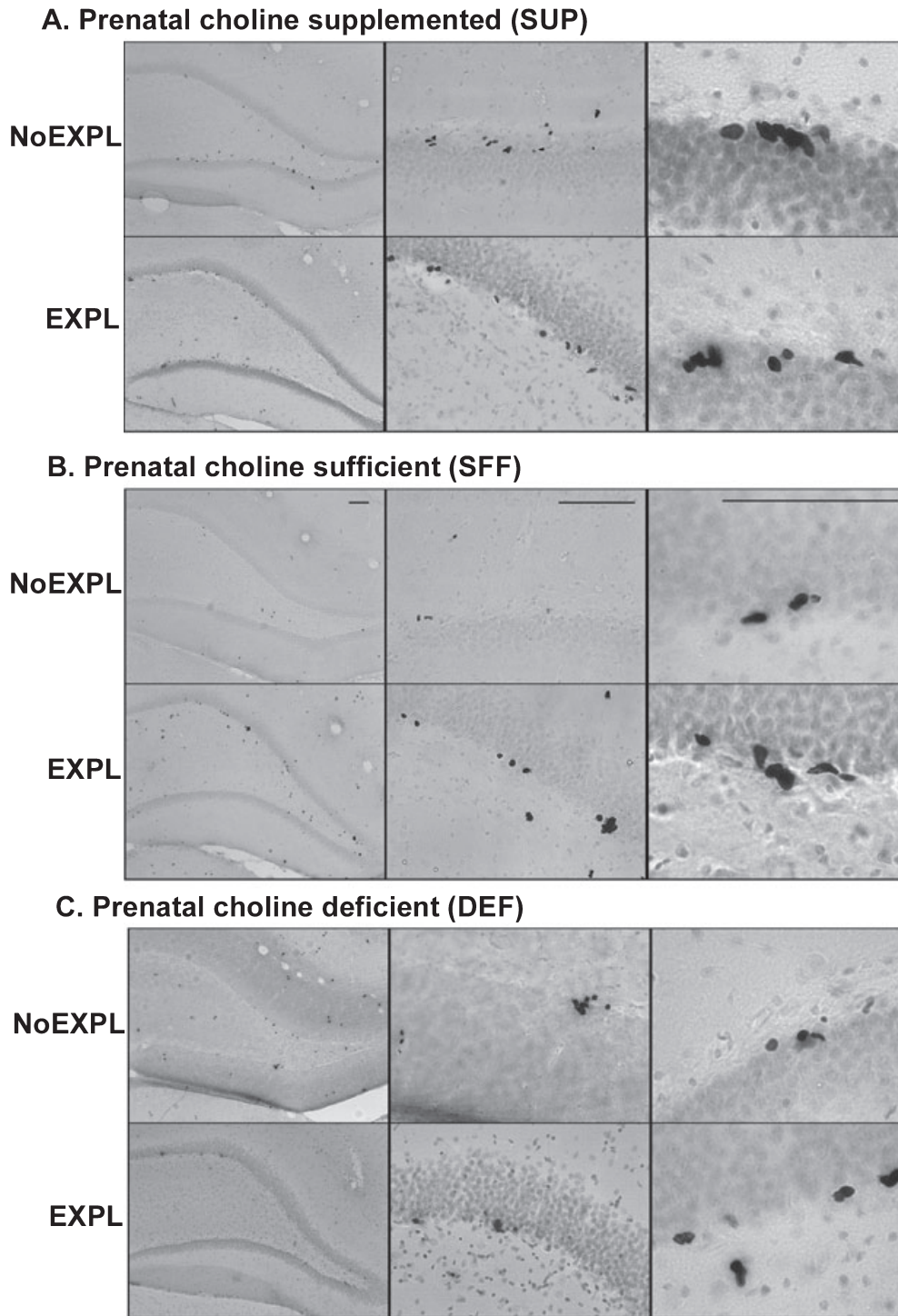


FIG. 2. Photomicrographs showing the pattern of BrdU-labelling in the dentate gyrus in (A) SUP, (B) SFF and (C) DEF rats that underwent daily maze exploration (EXPL) or not (NoEXPL). Photomicrographs in the left panels of each set were taken with a 10 $\times$  objective, middle panels with a 40 $\times$  objective, and right panels with 100 $\times$  oil objective. Bars in each image of (B) NoEXPL represent 25  $\mu$ m and apply to all images in the corresponding columns (left column at 10 $\times$ ; middle column at 40 $\times$ ; right column at 100 $\times$ ) of A, B and C.

than SFF ( $F_{\psi_{1,34}} = 14.029$ ,  $P = 0.001$ ) and DEF ( $F_{\psi_{1,34}} = 13.121$ ,  $P = 0.001$ ) NoEXPL rats.

To determine whether a larger overall size of structure could account for the higher numbers of new cells detected in the SUP EXPL and NoEXPL rats or the SFF NoEXPL rats, we estimated the volume of the dentate gyrus in each rat and did not detect any

statistically significant differences between any treatment groups ( $F_s < 1$ ).

The expression of growth factors is thought to be an important mediator of hippocampal neurogenesis (Cameron *et al.*, 1998; also see Lee *et al.*, 2002a, 2002b), and BDNF, in particular, is up-regulated in response to enriching experiences (Young *et al.*, 1999). Thus, to

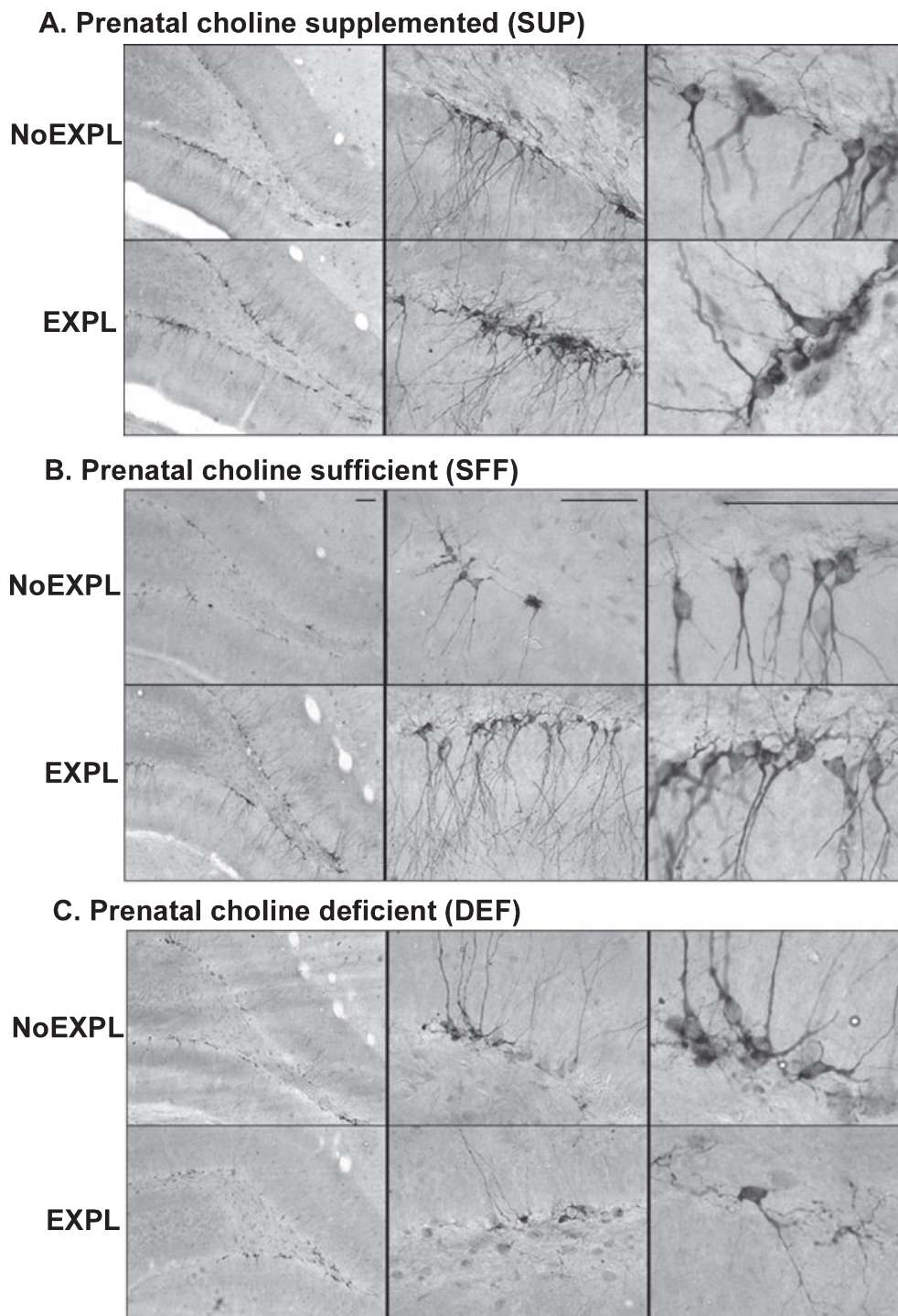


FIG. 3. Photomicrographs showing the pattern of DCX-labelling in the dentate gyrus in (A) SUP, (B) SFF and (C). DEF rats that underwent daily maze exploration (EXPL) or not (NoEXPL). Photomicrographs in the left panels of each set were taken with a 10 $\times$  objective, middle panels with a 40 $\times$  objective, and right panels with 100 $\times$  oil objective. Bars in each image of (B) NoEXPL represent 25  $\mu$ m and apply to all images in the corresponding columns (left column at 10 $\times$ ; middle column at 40 $\times$ ; right column at 100 $\times$ ) of A, B and C.

determine whether the effects on neurogenesis in our pair-housed NoEXPL rats that we report here might be occurring via a neurotrophic mechanism, we measured levels of BDNF protein in the hippocampus of additional groups of SUP, SFF, and DEF home-caged rats. Figure 5 shows these data. There were statistically significant differences between the diet conditions ( $F_{2,9} = 13.37$ ,  $P = 0.002$ ); SUP rats had significantly more BDNF content than

either SFF ( $P = 0.005$ ) or DEF ( $P = 0.003$ ) rats, which were not significantly different ( $P = 0.956$ ).

## Discussion

We report that choline availability during pregnancy has an enduring impact on hippocampal neurogenesis of the offspring. Prenatal

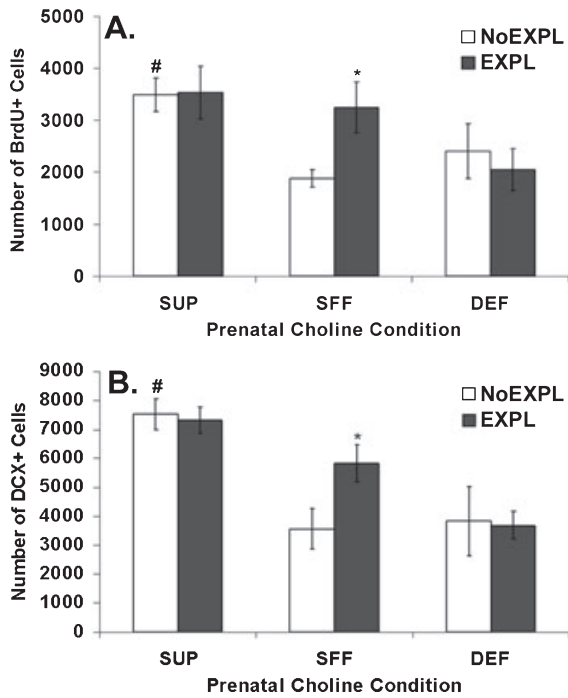


FIG. 4. Mean ( $\pm$  SEM) numbers of (A) BrdU-labelled cells or (B) DCX-labelled cells detected in the dentate gyrus of SUP, SFF, and DEF rats that explored a maze daily (EXPL; dark grey bars) or not (NoEXPL; white bars). There were significantly more BrdU-labelled cells in SUP rats, overall, compared to SFF and DEF rats ( $P_s < 0.05$ ). SUP and SFF EXPL rats had more BrdU-labelled cells than DEF NoEXPL and EXPL rats. SUP NoEXPL rats showed as many new cells as SFF and SUP EXPL rats ( $P_s > 0.05$ ). \*statistically different from SFF NoEXPL and DEF EXPL; # in (A) is statistically different from SFF NoEXPL; # in B is statistically different from SFF and DEF NoEXPL.

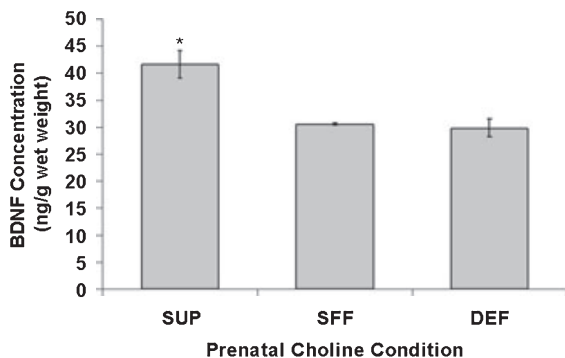


FIG. 5. Mean ( $\pm$  SEM) concentration of BDNF in the hippocampus of SUP, SFF, and DEF rats. SUP rats show significantly more BDNF content than SFF or DEF rats ( $*P < 0.05$ ).

supplementation with choline led to an enhancement in the base rate of cell proliferation in adult rats that were pair-housed and living under standard laboratory conditions. This change in the base rate of cell proliferation was detected after 8 months of identical housing conditions. In addition, we found that daily maze exploration did not stimulate hippocampal cell proliferation in adult rats that were offspring of mothers that ate a choline deficient diet during ED 12–17, while the same enriching experience increased cell proliferation in offspring of mothers eating a standard, choline-sufficient diet. This latter finding is consistent with previous reports demonstrating that

environmental factors increase adult neurogenesis (reviewed in Olson *et al.*, 2006). Paralleling the higher numbers of new neurons in SUP rats overall, we found that prenatal choline supplementation, compared to prenatal choline sufficiency or deficiency, increased BDNF levels in the hippocampus of adult rats.

The finding that SUP rats housed in standard laboratory conditions showed almost a two-fold increase in new neurons in the dentate gyrus compared to SFF and DEF rats was particularly striking. The only manipulation given to these rats compared to our SFF rats was a 4.5-fold increase in choline in their mothers' diet during ED 12–17. These prenatal choline supplemented rats did not receive any enriching environmental experience, and we have confirmed that SUP rats are not simply more active than SFF or DEF rats in a small activity box or in a large open field (Glenn & Williams, 2006), on the central platform of the radial arm maze when food is absent at the ends of arms (Meck & Williams, 2007) or on the entire radial arm maze when food is randomly available to encourage exploration (present study). Therefore, it is unlikely that a general increase in activity levels in the home cages of pair-housed SUP rats led to their increased neurogenesis compared to pair-housed SFF and DEF rats (e.g. van Praag *et al.*, 1999).

Our finding of increased adult neurogenesis with prenatal choline supplementation is consistent with past data showing that SUP rats make fewer errors (i.e. repeat visits to previously entered arms) on the radial-arm maze from very early in training (e.g. Meck *et al.*, 1988); prenatal choline supplemented female rats reared in the same fashion as in the present study, also show improved episodic memory on the radial arm maze when they are 8 months of age (Loy *et al.*, 1991). Because there is accumulating evidence that learning-induced increases in new hippocampal neurons benefit later learning (Shors *et al.*, 2001; Snyder *et al.*, 2005; but see Meshi *et al.*, 2006; reviewed in Leuner *et al.*, 2006), it is possible that SUP rats are essentially primed via up-regulated neurogenesis *prior* to an initial learning event. SUP rats also show an enhanced ability to time multiple events compared to SFF rats (Meck & Williams, 1997b), which is consistent with a new hypothesis (Aimone *et al.*, 2006) that increases in adult neurogenesis could improve encoding of time in new memories.

In terms of understanding the mechanism by which prenatal choline availability could modulate plasticity in the adult hippocampus, we offer two hypotheses; (i) choline could act on neural processes that directly impact rates of cell division and/or enhance the likelihood that stem cells in the subgranular zone commit to a neuronal phenotype, and/or (ii) choline could act indirectly on these processes by altering how the animal behaves and interacts with its world, effects that themselves enhance plasticity. In support of the first hypothesis we report here that BDNF levels in hippocampus were increased in SUP rats. This finding is consistent with past work showing that prenatal choline supplementation increases the production of several neurotrophic growth factors in young adult rats, including IGF2, FGF2, BDNF (Mellott *et al.*, 2005), and NGF (Sandstrom *et al.*, 2002), and extends these results by revealing that increased BDNF levels in the hippocampus are still evident at 8 months of age. Because these factors play a critical role in hippocampal neurogenesis (reviewed in Cameron *et al.*, 1998; Olson *et al.*, 2006), prenatal choline supplementation may enhance this feature of hippocampal plasticity in adulthood through a persistent increase in their expression.

One developmental mechanism by which dietary choline availability during prenatal development may impact the regulation of cell cycle events in the adult is through an epigenetic process. Choline is the precursor to the methyl donor betaine, and nutritional methyl status may be important in influencing the availability of methyl groups necessary for the methylation of CpG sites in DNA, and thus regulation of gene expression. A choline-deficient diet in rats

reportedly causes hypomethylation of the CpG sites in the liver (Tsujiuchi *et al.*, 1999); and feeding pregnant pseudoagouti *Avy/a* mouse dams a choline methyl-supplemented diet altered epigenetic regulation of agouti expression in the offspring, resulting in increased agouti/black coat colouration (Wolff *et al.*, 1998).

Other recent findings also provide support for the first hypothesis. Prenatal choline supplementation enhances the phosphorylation of CREB (Mellot *et al.*, 2004), and CREB may be making critical contributions to the differentiation and survival of new cells (Bender *et al.*, 2001; Giachino *et al.*, 2005). Indeed, CREB-related factors regulate BDNF transcription (Tao *et al.*, 1998). Choline is a precursor of phosphatidylcholine and sphingomyelin, which are vital structural components to biological membranes (for overview see Blusztajn, 1998). This feature of choline function makes it key to early neural development (reviewed in Zeisel, 2004, 2006) and supplemental levels of choline during this period may establish a higher baseline of progenitor production (e.g. Craciunescu *et al.*, 2003) that is maintained into adulthood.

Evidence for the second hypothesis, that prenatal choline supplementation may be acting indirectly to enhance hippocampal neurogenesis by altering neural processes that affect other, noncognitive behaviours, is less abundant, but compelling. It is possible that SUP rats are more interactive with their environments and less anxious under novel or stressful challenges, and we have data that support this position (Glenn & Williams, 2006; Meck *et al.*, 2007), although we do not yet know if prenatal choline deficiency increases anxiety or decrease exploration. Neurogenesis is increased in animals that live in enriched environments (Kempermann *et al.*, 1997), engage in hippocampally dependent learning tasks (Shors *et al.*, 2002), or are raised by mothers that exhibit high levels of maternal care behaviours (Bredy *et al.*, 2003). Thus, if prenatal choline supplementation alters how animals interact with and respond to their environment it could thereby produce corresponding, lifelong changes in the way their experiences modify neural function.

Our findings do not support the notion that the neurogenic effects of prenatal choline supplementation and maze exploration are additive. SUP rats that did not receive daily maze experience showed as much neurogenesis as SUP rats that did. One parsimonious explanation is that we simply did not provide sufficient enrichment to drive neurogenesis beyond that achieved by prenatal choline supplementation alone. In fact, it is notable that a mere 10 min of exploration per day significantly increased neurogenesis in the SFF rats. On the other hand, it is possible that there is a balance of plasticity in the hippocampus that prevents an aberrant up-regulation of new cells to a point where it is disruptive to normal function. For example, seizures are known to produce abnormally high and disruptive levels of hippocampal neurogenesis (Parent *et al.*, 1997). Even before the maze exposure, the SUP rats may already be approaching or at an optimal level for neurogenesis. Thus, it may be construed as positive that this optimal level was not exceeded by exposure to both the diet and exploration manipulations.

Interestingly, we did not find a typical 'dose-response' effect of choline availability on hippocampal plasticity. Instead, DEF rats displayed a very different profile of neurogenic response than SUP rats. First, while DEF-NoEXPL rats had fewer new neurons than SUP-NoEXPL rats (and likely fewer newly proliferating cells overall, though this did not reach statistical significance), their baseline rate of neurogenesis was not significantly different from SFF-NoEXPL rats. There are at least two potential reasons that we did not see lower baseline neurogenesis in the DEF-NoEXPL rats. All rats in our study ate the sufficient choline diet (except during ED 12–17 when the altered diets were given to the rat dams), which may contain an amount of choline that was able to maintain proliferation levels in

prenatally deficient rats that were similar to that of prenatally choline sufficient rats. That is, if DEF rats developed hippocampi that were well adapted for a choline deficient environment, the SFF diet may have served as a supplement for this group. Further work will be needed to test this hypothesis. Alternately, DEF rats may have developed hippocampi that produce a normal rate of new cell birth under standard conditions, but are unable to maintain this production into old age or to change proliferation in response to environmental demands. In fact, our data, showing that DEF rats failed to up-regulate neurogenesis with daily exploration provide some support for this latter view.

We also did not see a dose-response increase in the SUP-EXPL rats compared to SFF-EXPL rats; both had the same high level of cell proliferation. Both this finding and the fact that DEF-EXPL rats do not up-regulate neurogenesis with enriched experiences fit well with the effects of prenatal choline deficiency and supplementation on spatial memory function. Under standard testing conditions on a radial-arm maze, DEF rats perform as well as (see Meck & Williams, 2003) or better than (Meck & Williams, 1999) SFF rats. However, when massed trials are given to increase the cognitive demands of the task, DEF rats fare much worse than SFF rats (Meck & Williams, 1999). This is in stark contrast to SUP rats. They show fewer errors than DEF and SFF rats when trials are once per day and they show no decrement in performance when trials are massed. If one considers that the changes in neurogenesis that we report here may contribute to hippocampal and spatial memory function, it is not surprising that prenatal choline deficiency failed to inhibit hippocampal neurogenesis as under ordinary conditions DEF rats actually show normal cognitive function. Similarly, the failure of this group to up-regulate neural production in response to enriching experiences is consistent with their inability to maintain good performance under conditions of high cognitive demand. Furthermore, prenatal choline deficiency increases the induction threshold for LTP in the hippocampus (Pyapali *et al.*, 1998), suggesting that this brain structure may be less plastic in these animals and perhaps less responsive to environmental challenges. If our SUP rats maintain up-regulated hippocampal neurogenesis through 24 months of age, this preservation of brain plasticity into advanced age may be one way that prenatal choline supplementation sustains cognitive function over the lifespan.

Our experimental approach of administering the cell division marker for ten consecutive days does not permit us to ascertain whether prenatal choline supplementation is specifically enhancing cell proliferation or cell survival, or both, and follow-up studies that resolve these questions are underway. However, it is clear that the survival of newborn neurons in the dentate gyrus and their successful integration into the existing circuitry will be essential if they are to make a lasting contribution to hippocampal function that is an attribute of prenatally choline supplemented animals.

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## Abbreviations

BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2-deoxyuridine; DCX, doublecortin; DEF, choline-deficient; ED, embryonic day; SFF, choline sufficient; SUP, choline-supplemented; TBS, Tris-buffered saline.

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