Choline Availability During Embryonic Development Alters Progenitor Cell Mitosis in Developing Mouse Hippocampus

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ABSTRACT

Previously, we reported that dietary choline influences development of the hippocampus in fetal rat brain. It is important to know whether similar effects of choline occur in developing fetal mouse brain because interesting new experimental approaches are now available using several transgenic mouse models. Timed-pregnant mice were fed choline-supplemented (CS), control (CT) or choline-deficient (CD) AIN-76 diet from embryonic day 12 to 17 (E12–17). Fetuses from CD dams had diminished concentrations of phosphocholine and phosphatidylcholine in their brains compared with CT or CS fetuses (P < 0.05). When we analyzed fetal hippocampus on day E17 for cells with mitotic phase–specific expression of phosphorylated histone H3, we detected fewer labeled cells at the ventricular surface of the ventricular zone in the CD group (14.8 ± 1.9) compared with the CT (30.7 ± 1.9) or CS (36.6 ± 2.6) group (P < 0.05). At the same time, we detected more apoptotic cells in E17 hippocampus using morphology in the CD group (11.8 ± 1.4) than in CT (5.6 ± 0.6) or CS (4.2 ± 0.7) group (P < 0.05). This was confirmed using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin anti-digoxigenin fluorescein conjugate antibody nick end-labeling (TUNEL) and activated caspase-3 immunoreactivity. We conclude that the dietary availability of choline to the mouse dam influences progenitor cell proliferation and apoptosis in the fetal brain.

KEY WORDS: • choline • phosphatidylcholine • brain development • mice • hippocampus

Maternal dietary choline supplementation in rats results in lifelong cognitive and memory enhancement in the offspring. The underlying physiologic and biochemical bases for these lifelong alterations are just beginning to be understood. Certain anatomical regions of the hippocampus (e.g., dentate gyrus) play critical roles in learning and acquisition of memory. In rats, prenatal choline supplementation increased the sensitivity of CA1 hippocampal neurons to stimulation of long-term potentiation (LTP) (1), and increased working memory spatial memory (2–6), whereas prenatal choline deficiency increased the threshold for LTP and retarded temporal processing (7). We previously reported that, in rats, these physiologic and behavioral changes may be related to neuroanatomical changes in regions of fetal brain hippocampus and basal forebrain that are known to regulate memory (8–10). The recent development of genetically modified mice that have defects in phosphatidylcholine (PtdCho) synthesis (11) and in folate metabolism (12) could enhance the elucidation of the underlying mechanisms for the choline effect on brain development. However, mouse metabolism and brain development differ substantially from those of rats, and it has not yet been proven that maternal dietary choline influences fetal brain development in mice.

The neuroanatomical development of the hippocampus is dictated by the tight regulation of proliferation in progenitor-type stem cells [mainly within two relatively thin layers of tissue lining the primitive ventricular cavities; the ventricular zone (vz) and the subventricular zone (svz) (13)], and their subsequent migration and differentiation to form specialized regions of the hippocampus (14). In the developing fetal rat hippocampus, changing the level of choline in the maternal diet altered neurogenesis (8,9). These effects were mediated in part by altered expression of p15Ink4B and p27Kip1 cyclin-dependent kinase inhibitors (CDKI), proteins that inhibit cell cycling (10). Choline deficiency in fetal rat brain on embryonic day 18 (E18) reduces cell proliferation and increases apoptosis (8,9). Here we report that
choline deficiency reduces cell proliferation and increases apoptosis in developing mouse hippocampus on day E17.

MATERIALS AND METHODS

Animals. Timed-pregnant C57 BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and used in all experiments. The day after mating was considered day E1. The mice were housed individually in cages in a temperature-controlled room at 24°C and expelled a 12-h light-dark cycle. In all experiments, unless otherwise indicated, the mice consumed AIN-76A purified diet (15) with 1.1 g/kg choline chloride replacing 2 g/kg choline bitartrate (ingredient, g/kg: casein, 200.00; L-threonine, 3.00; cornstarch, 150.00; sucrose, 500.90; cellulose, 50.00; corn oil, 50.00; salt mix #20000, 35.00; vitamin mix #30050, 10.00) (Dyets, Bethlehem, PA). The AIN-76A diet was chosen because it was used in all of the earlier published studies on the effects of choline on rat brain development (8–10). The mice consumed their experimental diet and water ad libitum until d 12 of gestation. On E12, the pregnant mice were randomly assigned to one of the three feeding groups (n = 6/group). The choline-deficient (CD) group received the AIN-76A diet containing 0.0 g/kg choline chloride, the control (CT) group received the AIN-76A diet containing 1.1 g/kg choline chloride and the choline-supplemented (CS) group received the AIN-76A diet containing 4.95 g/kg choline chloride. These pregnant mice received their special diets from the morning of day E12 until the morning of day E17.

Tissue collection. On day E17, pregnant mice were anesthetized with a single subcutaneous injection of 0.03 mL ketamine (100 mg/L) and 0.02 mL xylazine (20 mg/L) (Henry Schein, Melville, NY) and kept on a heating pad to maintain body temperature. The uterine horns were exposed by a midline abdominal incision and the fetuses kept on a heating pad to maintain body temperature. The uterine horns were removed individually for perfusion. The chest cavity of the fetus was opened and the heart was cannulated with a 23G needle. The perfusion fluid was a solution of 4% formaldehyde and 0.2% glutaraldehyde (Polysciences, Louis, MO). Antigen retrieval was performed for 20 min in 20 mm Tris-buffered glycerol, pH 7.0 (21) and a #1 thickness coverglass. Images were acquired using a Zeiss Confocal Laser Scanning Microscope LSM 210 (Carl Zeiss, Thornwood, NY) as described below.

Assessment of apoptosis. We used a combination of classical apoptotic morphology, active caspase-3 immunoreactivity and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin anti-digoxigenin fluorescent antibody nick end-labeling (TUNEL) to detect apoptotic cells in fetal brain. Apoptotic cells exhibit chromatin condensation and hypersegmentation of nuclear chromatin within shrunken cells, changes that are characteristic of end-stage apoptosis (22). These apoptotic cells were detected hemi-laterally in each of the selected sections after hematoxylin and eosin staining using a light microscope as described previously (9). Apoptotic indices for the fetal mouse brain hippocampus of the different mouse groupings are presented as the number of apoptotic cells/section of the hippocampal hemispheres. Four hippocampal hemispheres from two consecutive serial sections were averaged to obtain a single hemispherical value/hippocampal section/mouse. For double labeling of TUNEL and active caspase-3, the DNA terminal labeling, conducted according to the manufacturer's protocol (S 7111 Apoptag Plus Fluorescein In Situ Apoptosis Detection Kit, Serologicals, Norcross, GA), was followed by overnight incubation with the primary antibody to cleaved (active) caspase-3 (Asp175) (Cell Signaling Technology, Beverly, MA). Then, fluorescein-antidigoxigenin conjugate and goat rhodamine-anti-rabbit IgG (Calbiochem, San Diego, CA) were applied for 2 h at room temperature to display the TUNEL-positive nuclei and active caspase-3. DAPI (0.1 mg/L for 20 min) was used to counterstain nuclear DNA. The TUNEL and active caspase-3 positive cells were identified and scored by a trained observer (C.N.C.), who was unaware of the maternal diet grouping, based upon the presence of green fluorescent nuclear staining for TUNEL and red fluorescent nuclear staining for active caspase-3. Stained nuclei were usually but not always condensed, and intense blue fluorescent chromatin was often visible inside, consistent with fragmented DNA.

Image analysis. For apoptosis studies using morphological criteria, image acquisition was performed on a Nikon FXA microscope (Nikon, Garden City, NY) equipped with an Optronics TEC-470 CCD Video Camera System (Optronics Engineering, Goleta, CA) and the public domain NIH Image program version 1.61 as described previously (10).

For the mitosis assessment, TUNEL and active caspase-3 evaluation, the image analysis of fetal brain slices was performed on a Zeiss Confocal Laser Scanning Microscope LSM 210 (Carl Zeiss) equipped with an Optronics DEI 750 low light level integrating CCD camera (Optronics Engineering) connected to an Apple Macintosh G3 computer utilizing a Scion CGG image capture card, for digital image capture of standard and epifluorescence images, and the public domain NIH Image program version 1.61. All images were captured by using 5, 20 or 40X objectives and fluorescent filters optimized for observing DAPI (blue), fluorescein isothiocyanate (green) and rhodamine-Cy3 conjugates (red) signals, respectively. Images obtained from the same field in the different fluorescent probes were subsequently overlapped or merged.

Analysis of brain and hepatic choline metabolite concentrations. Choline and its metabolites, glycerophosphocholine (GPCCho), phos-
phosphocline (PCho) and PtdCho, were analyzed using GC/MS as previously described (23).

Statistical differences among group means for mitotic cells [phospho-histone H3(+) cells/region of interest in one section], apoptotic cell number (apoptotic cells/hippocampal section per mouse), and choline and its metabolites in liver and whole brain were assessed as follows. We tested that variances were equal using O’Brien, Brown-Forsythe, Levene, and Bartlett tests (JMP Version 3; SAS Institute, Cary, NC). For observed significance probabilities > 0.05 (considered evidence of equal variances across the levels), we used comparisons for each pair using Student’s t test, for all pairs using Tukey-Kramer Honestly Significant Difference test, and comparison with control using Dunnett’s Method as indicated in the figures. If the tests of equal variances revealed that the group variances were significantly different (the probability of obtaining by chance alone an F-value larger than the one calculated if, in reality, the variances are equal across all levels), the Welch ANOVA for the means was used. We used the Welch test, for all pairs using Dunnett’s test. We used the Welch test, for all pairs using Dunnett’s test, for all pairs using Dunnett’s test. The number of apoptotic cells counted using morphological criteria (Fig. 2C), TUNEL or activated caspase-3 immunoreactivity (Fig. 2D) in the whole hippocampal area was more than 100% higher in the CD group than in the CT and CS groups (P < 0.01) (Fig. 2A).

RESULTS

Choline and its metabolites in dam liver and fetal liver and brain. After 5 d of CD or CS diet intake by pregnant dams, we detected some significant differences from the CT group in concentrations of choline and its metabolites in dam livers and fetal livers and brains (Table 1). In livers of CD dams, all choline metabolite concentrations were lower than in CT dams, whereas in livers of CS dams, concentrations of choline, GPCho and PCho were greater than in controls. In livers of CD fetuses, choline, GPCho and PCho concentrations were lower than in CT fetuses. PCho and PtdCho concentrations were lower in brain of CD fetuses than in controls.

Mitosis. We measured the accumulation of phospho-H3-labeled cells at the ventricular surface of the hippocampal vz, adjacent to the fimbria (Fi) (Fig. 1B, C), primordial dentate gyrus (DG) and Ammon’s horn (AH) (Fig. 1B, D). On day E17, a significantly lower proportion of the cells at the ventricular surface of the hippocampal vz were phospho-H3-labeled in the CD group compared with the CT or CS groups. When analyzed by single regions, the CD group had fewer phospho-H3-labeled cells in all areas (Fi, DG, AH) compared with the CT group; however, the CS group had more phospho-H3-labeled cells in one region (Fi) than the CT group (Fig. 1A).

Apoptosis. In the hippocampus on day E17, apoptotic cells were located mainly in the regions of developing fimbria and dentate gyrus (Fig. 2B). The number of apoptotic cells counted using morphological criteria (Fig. 2C), TUNEL or activated caspase-3 immunoreactivity (Fig. 2D) in the whole hippocampal area was more than 100% higher in the CD group than in the CT and CS groups (P < 0.01) (Fig. 2A).

DISCUSSION

Dietary choline intake by pregnant mice during d 12–17 of gestation influenced fetal brain development. On day E17, fewer hippocampal cells had phosphorylated histone-3 in CD compared with CT and CS brains (Fig. 1), consistent with decreased progenitor cell proliferation in the choline-deprived fetal hippocampus. CD mice had more incidence de-
Maternal dietary choline deficiency in timed-pregnant mice fed choline-supplemented (CS), control (CT) or choline-deficient (CD) AIN-76 diet from embryonic day 12 to 17 (E12–17) decreases mitosis in embryonic mice on day E17 at the ventricular surface of the ventricular zone of the hippocampus. All mice were killed on E17 and coronal sections were prepared from the brains of fetuses from each group for the analysis of mitosis using the mitosis-specific marker anti-phospho-histone H3 as described in Materials and Methods. The DAPI nuclear DNA counterstaining is blue, whereas the Cy3 conjugated secondary antibody bound to the anti-phospho-histone H3 (Ser10) primary antibody stains red. Panel A: In CD fetal hippocampus compared with CT, there were fewer phospho-histone H3 positive cells at the ventricular surface of the ventricular zone adjacent to fimbria (Fl), dentate gyrus (DG) and Ammon’s Horn (AH), and this was reflected in the calculated values for the whole hippocampal section length of ventricular zone (H). Compared with the CT group, the CS group had a higher incidence of phospho-histone H3-labeled cells at the ventricular surface of the ventricular zone only in the fimbria (Hi). The graph insert shows the equivalence of the sections in terms of total hippocampal ventricular zone length. Values are means ± SEM of at least 6 pups per group from 6 dams. Means without a common letter differ, *P < 0.05 (for each pair using Student’s t test, for all pairs using Tukey-Kramer HSD test, and comparison with control using Dunnett’s Method). Panel B shows a representative fetal hippocampus at a magnification of 50X with the regions of interest marked. Panels C and D are 400X magnifications of the boxed regions in panel B, and show representative labeled cells in the hippocampal regions.

We described previously how choline availability modulates apoptosis signaling pathways in the hepatocytes, PC12 cell and cultured fetal hippocampal neuron (8,9,29,30). Apoptosis plays a critical role in determining the size of neuronal subpopulations, and thus, morphogenesis, in developing brain regions (31). Newly generated cells in the fetal hippocampus can reenter the cell cycle (i.e., remain as stem cells), commit to differentiation or undergo apoptosis. Previous studies in developing rat brain demonstrated that CD increased, whereas CS decreased, apoptosis in day E18–20 hippocampus (9). Here we show that apoptosis in the hippocampus of developing mice, like rats, is increased by maternal dietary choline deprivation.

It is interesting that maternal dietary choline deficiency did not alter choline concentrations but rather decreased PCho and PtdCho concentrations in the fetal mouse brain (Table 1). We reported previously that PCho is the storage form for choline in tissues and is the first indicator of diminished choline status in liver tissue (32). PtdCho is an important component of membranes, and changes in brain concentrations could be mediating changes in apoptosis and cell proliferation (29,33). Choline metabolism is closely interrelated to methyl group and folate metabolism (34), and changes in methylation of genes or histones also could be the mechanisms of action (35).

The demonstration of a choline effect on the development of the mouse brain is important because it makes possible new studies using new mouse models that have been created with genetic manipulations in enzymes of methyl group metabolism including methylenetetrahydrofolate reductase (36), methionine adenosyltransferase (37), cystathionine β-synthase (38) and phosphatidylethanolamine methyltransferase (39). We reported recently that methylenetetrahydrofolate reductase knockout mice and phosphatidylethanolamine methyltransferase knockout mice become choline deficient (40,41). These mouse models could be very helpful in unraveling how choline modulates neurogenesis in fetal brain. The studies we report here show that the mouse replicates many of the choline-mediated changes previously described in rats and suggest that...
use of transgenic mouse models would be appropriate in future investigations.

LITERATURE CITED