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Maternal dietary choline availability alters the balance of netrin-1 and DCC neuronal migration proteins in fetal mouse brain hippocampus

Craig D. Albright, Mei-Heng Mar, Corneliu N. Craciunescu, Jiannan Song, and Steven H. Zeisel*

Department of Nutrition, CB#7461, Room 2212 McGavran-Greenberg Hall, School of Public Health and School of Medicine, University of North Carolina-Chapel Hill, Chapel Hill, NC 27599, USA

Abstract

Alterations in maternal dietary choline availability during days 12–17 of pregnancy led to an increase in the level of immunoreactive netrin-1 and a decrease in the level of DCC protein in the developing fetal mouse brain hippocampus compared with controls. Changes in the expression of cell migration cues during development could account for some of the lifelong consequences of maternal dietary choline availability for cognitive and memory processes.

Keywords

Brain development; Chemoattraction; Choline; Hippocampus; Pregnancy

Maternal dietary choline availability during pregnancy plays a critical role in the establishment and lifelong functioning of cognitive and memory processes in rodent offspring. The underlying choline-sensitive physiological and biochemical processes (e.g., threshold for long-term potentiation; cholinergic activity) affecting temporal and spatial memory in offspring are beginning to be understood [13,20,22]. However, the neuroanatomical basis for these behavioral changes is less well understood.

The neuroanatomical development of the fetal hippocampus is influenced by the proliferation, survival, and migration of precursor cells generated in the ventricular zone. We previously showed that, depending on the availability of maternal dietary choline, precursor cells in ventricular zone undergo proliferation and commit to differentiate into neuronal cell types (e.g., calretinin-containing cells) [8] known to regulate memory, or undergo apoptosis. We previously described how choline availability affects pro-apoptotic signal transduction in neuronal-type cells [18,28] and hepatocytes [1,4,6,7,9]. We are beginning to understand how maternal dietary choline availability during pregnancy affects not only the generation, but also the targeted migration of precursor neuronal-type cells in the fetal hippocampus [2,3,14]. Targeted migration of precursor cells in the developing nervous system is regulated in part by interactions between the binding of netrin-1, a secreted glycoprotein, and to DCC, a transmembrane receptor [16,17]. These interactions can exert repulsive and attractive effects on precursor-type cells in the developing nervous system [19,21]. In addition to steering the migration of precursor cells in developing brain, interactions between netrin and DCC have also been implicated in apoptosis signaling [15]. In order to better understand the neuroanatomical basis for the effects of maternal dietary choline on the establishment of a functioning of cognitive and memory processes in rodent offspring, we investigated whether

* Corresponding author. E-mail address: steven_zeisel@unc.edu (S.H. Zeisel)..

choline availability in the diet of pregnant mice alters the localization of netrin-1 and DCC proteins that are known to direct the migration of precursor cells in developing brain.

Timed-pregnant C57BL/6 mice (Jackson Lab., Bar Harbor, ME) obtained on days 4–9 of gestation were maintained in a climate-controlled environment and exposed to a 12-h light/dark cycle daily. The animals were provided with AIN-76A diet containing 1.1 g/kg choline chloride (Dyets, Bethlehem, PA) and water ad libitum. Pregnant mice on day 12 of gestation were divided into three groups: supplemented, control, and deficient. Control (CT) animals received an AIN-76A standard diet containing 1.1 g/kg choline chloride. The choline supplemented (CS) mice received an AIN-76A diet with 4.5 g/kg choline chloride. The choline-deficient (CD) group received an AIN-76A diet without choline. These treatments continued until gestational day 17 (E17). All experimental animal procedures were performed according to NIH guidelines and were approved by The University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Timed-pregnant mice on day E17 from all treatment groups were anesthetized by injection of 0.03 ml ketamine and 0.02 ml xylazine (Henry Shein Inc., Melville, NY), kept on a heating pad to maintain body temperature. The uterine horns were exposed by a midline abdominal incision and the fetuses were removed individually for perfusion. The chest cavity of the fetus was opened and, for in situ fixation, ~2 ml of perfusion fixative containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.2 M phosphate buffer (Polysciences, Warrington, PA) was injected into the fetal heart. The fetuses were decapitated and the fetal skull was opened for postfixation overnight. The fetal brains were subsequently embedded in paraffin and coronal sections (5 μ m) of fetal brain containing the hippocampus (Fig. 1, panel A) were deparaffinized, antigen retrieval was performed at room temperature for 20 min in 20 μ mol/L proteinase K (Sigma, St. Louis, MO) and for 40 min in 10 mg/ml sodium borohydride (Sigma, St. Louis, MO), and nonspecific sites were blocked with 2% non-immune donkey serum (Chemicon, Temecula, CA) in 1 $\times$ PBST. Brain sections were incubated in goat anti-netrin-1 antibody (Clone N-18, Santa Cruz Biotechnology, Santa Cruz, CA) or in rabbit anti-DCC antibody (Clone H-205, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 in PBST overnight at 4 $^{\circ}$ C; these antibodies detect single bands by Western blotting [15,20]. Sites of netrin-1 and DCC were detected using donkey anti-rabbit AlexaFluor 594 (Molecular Probes Inc., Eugene, OR) and donkey anti-goat AlexaFluor 488 (Molecular Probes), respectively, at 1:500 dilution for 2 h at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). A negative control was prepared by replacing the primary antibody with non-immune serum; this resulted in no staining. In addition, netrin-1 and DCC immunoreactivity were not detected in adult mouse liver. Analysis of netrin-1 and DCC immunoreactivity was also performed using cortical neuronal precursor cells obtained at day E14 from C57BL/6 mice (QBM Cell Science, Germantown, MD) and maintained in cell culture as described previously [26].

Image analysis of fetal brain slices and neural precursor cells was performed on a Nikon FXA microscope equipped with an Optronics DEI 750 low light level integrating CCD camera (Optronics Engineering, Goleta, CA) and the public domain NIH Image program version 1.61 as described previously [4]. Digital fluorescence images of netrin-1 and DCC-labeled brain sections or neuronal precursor cells were acquired using narrow band-pass emission filters, gray scale images were prepared and Scion image software were then used to measure the integrated optical density of netrin-1 and DCC immunoreactivity. The integrated optical density of netrin-1 and DCC labeling was determined from $n = 5$ measurements of each brain region in each of 5 fetal brains per treatment group. This experiment was replicated twice. For neuronal precursor cells, the integrated optical density of netrin-1 and DCC was determined from $n = 5$ to 7 neurospheres per treatment group. This experiment was independently replicated.

Results are presented as mean $\pm$ standard error of the mean (SEM). Statistical differences from group means were calculated using two-way analysis of variance (ANOVA) and Scheffé's *F* test (JMP Version 2, SAS). For the analysis of neuronal precursor cells, data were analyzed by *t* test.

DCC protein was found in the cytoplasm of precursor cells in the ventricular zone and the developing fimbria, primordial dentate gyrus, and CA1–CA3 regions of Ammon's horn (Fig. 1, panels B–F). The intensity of DCC fluorescence immunoreactivity in the hippocampal ventricular zone was 64–77% decreased in the CD group, and 74–89% decreased in the CS group compared to controls ($P < 0.01$, Fig. 2). The intensity of fluorescent antibody labeling of DCC was similarly decreased in CA1–CA3 regions of Ammon's horn in the CD and CS groups; however, other regions of the hippocampus were unaffected.

In day E17 fetal mouse brain hippocampus, the intensity of netrin-1 fluorescent antibody labeling in the hippocampal ventricular zone was 200–300% increased in the CD group, whereas in the CS group, the intensity of netrin-1 labeling was increased 100–750% compared to controls ($P < 0.01$, Fig. 3). Importantly, in the primordial dentate gyrus, but not fimbria or Ammon's horn, the intensity of netrin-1 labeling was increased more than 200% in the CD and CS group compared to controls ($P < 0.01$, Fig. 3). Double-label immunofluorescence studies which were performed on the same coronal sections of day E17 mouse hippocampus showed that, compared to controls (CT), the CD and CS groups exhibited altered colocalization of these guidance proteins, due primarily to reciprocal changes in the expression of DCC (Fig. 2) and netrin-1 protein (Fig. 3). In order to assess the mechanism of regulation of netrin-1, non-radioactive in situ hybridization was performed. Consistent with previous reports [11], strong expression of netrin-1 mRNA was detected in fetal mouse brain septum; however, very weak expression of netrin-1 mRNA (not shown) was detected in the fimbria region of hippocampus and mRNA levels were unaffected by changes in the level of maternal dietary choline. We previously showed that pups from dams with deficient choline intake during days E12–17 generated fewer precursor neuronal type cells in the ventricular zone, resulting in decreased numbers of newly generated cells in the primordial dentate gyrus [3]. Thus, these differences in the expression of netrin-1 and DCC on day E17 could be related to differences in neurogenesis. However, peak expression of some forms of netrin mRNA can occur as early as day E13.5 and decrease by day E18 [27]; thus, we cannot rule out that expression of netrin-1 (or DCC) is regulated by choline at the mRNA level early in fetal development.

Similar changes in netrin-1 and DCC were observed in VZ neuronal precursor cells and cells committed to undergo differentiate and migrate to adjacent functional regions of the fetal hippocampus. Recent studies implicate diffusible factors released by differentiated cells in neuronal precursor cell development in the ventricular zone [25]. Therefore, we examined the response to choline in undifferentiated embryonic neuronal precursor cells in culture. As with VZ precursor neuronal-type cells in day E17 fetal hippocampus, DCC and netrin-1 protein were colocalized in the cell bodies of embryonic neuronal precursor cells in culture (Fig. 4, panels A–F). Consistent with our in vivo studies, the intensity of DCC fluorescent antibody labeling was decreased and netrin-1 labeling increased in cells that had been switched for 48 h to control or CD medium ($P < 0.01$, Fig. 4, panel G). These findings suggest that VZ neuronal precursor cells are sensitive to diet choline-induced alterations in guidance cues that could play an important role in establishing patterns of differentiation in developing brain. For example, studies show that DCC colocalizes with calbindin, which colocalizes with another calcium-binding protein, calretinin, in fetal mouse forebrain [10,24]. Previously, we showed that maternal dietary choline increases, whereas supplementation decreases the expression of calretinin in developing mouse hippocampus [8]. The differences we report in netrin-1 and DCC guidance proteins might be related to changes in the appearance and localization of differentiated neurons.

Surprisingly, netrin-1 and DCC showed similar changes in the CD and CS groups. This is in contrast to mitotic inhibition, apoptosis, and cell proliferation in fetal developing hippocampus which do not track in the same direction in the CD and CS groups [2–4,7,14], suggesting not only that each of these components of neurogenesis has a different choline dose-response, but also that the consequences of netrin-DCC signaling for precursor cell migration are dependent on additional molecular interactions. For example, recent reports suggest a critical role for cyclic nucleotides in regulating the response to guidance cues. Thus, when the level of cAMP was reduced, netrin-1 repelled neuronal-type cells which were normally attracted by this guidance cue [23]. Importantly, in CD-hepatocytes, an increase in the level of cAMP prevented apoptosis and restored cell coupling via connexin43 (Cx43) [5], a protein thought to synchronize the development of populations of precursor cells in the ventricular zone of developing brain [12]. These studies highlight the notion that maternal dietary choline availability affects molecular interactions that could influence the functional role for netrin/DCC during neuronal development.

In addition, a decrease in the level of intact DCC, or a doubling of the level of netrin-1, is known to activate caspase-3-mediated apoptosis, resulting in decreased survival of neuronal precursor cells [15,19]. We found that choline deficiency-induced apoptosis in neuronal-type cells or developing brain involves activation of caspase-3 [14,28]. Taken together, these results suggest maternal dietary choline availability could act on netrin-1 and DCC resulting in modulation of neurogenesis, cell survival, and differentiation in developing fetal hippocampus.

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Fig. 1.

Effects of choline availability on the localization of DCC and netrin-1 in day E17 mouse hippocampus. Panel A: regions of day E17 mouse hippocampus chosen for measurement of DCC and netrin-1 immunoreactivity ($\times 50$) illustrated in panels B–F. (B) Fimbria; (C) ventricular zone adjacent to dentate gyrus; (D) dentate gyrus; (E) ventricular zone adjacent to CA1–CA3; (F) CA1–CA3. Arrows: colocalization of DCC and netrin-1. Green: DCC immunoreactivity (AlexaFluor 488); red: netrin-1 immunoreactivity (AlexaFluor 594); blue: nuclear DNA (DAPI) ($\times 200$); DG: dentate gyrus; Fi: fimbria; VZ: ventricular zone.

Fig. 2.

Effects of choline availability on the level of DCC protein in choline deficient (CD, white bars), control (CT, gray bars), or choline-supplemented (CS, black bars) day E17 mouse hippocampus Ammon's horn (AH) and adjacent ventricular zone (VZ) (Panel A), (B) dentate gyrus (DG) and adjacent VZ (Panel B), and fimbria (Fi) and adjacent VZ (Panel C). Means with different letters differ, $P < 0.01$ by ANOVA and Scheffé's test.

Fig. 3.

Effects of choline availability on the level of netrin-1 protein in a choline-deficient (CD, white bars), control (CT, gray bars), or choline-supplemented (CS, black bars) day E17 mouse hippocampus Ammon's horn (AH) and adjacent VZ (Panel A), dentate gyrus (DG) and adjacent VZ (Panel B), and fimbria (Fi) and adjacent VZ (Panel C). Means with different letters differ, $P < 0.01$ by ANOVA and Scheffé test.

Fig. 4.

Effects of choline availability on the levels of DCC and netrin-1 embryonic neuronal stem cells. Panels A and B: localization of DCC protein in CD (A) versus CT (B) neurospheres. Panels C and D: localization of netrin-1 in CD (C) versus CT (D) neurospheres. Panels E and F: colocalization of DCC and netrin-1 in CD (E) versus CT (F) neurospheres. Green: DCC; red: netrin; blue: DAPI. A–F: $\times 400$. Panel G: The graph shows the levels of DCC (gray bars) and netrin-1 protein (black bars) measured in CD (5 μM choline) and control (CT, 70 μM choline) neurospheres (* $P < 0.01$ by t test).