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Rat neural stem cell proliferation and differentiation are durably altered by the in utero polyunsaturated fatty acid supply

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Abstract

We isolated neural stem cells/neural progenitors (NSC) from 1-day-old rat pups born to mothers fed diets that were deficient or supplemented with n-3 polyunsaturated fatty acids (PUFAs) and compared their proliferation and differentiation in vitro.

The cells isolated from the n-3PUFA-deficient pups consistently proliferated more slowly than cells that were isolated from n-3PUFA-supplemented pups, despite the fact that both were cultured under the same conditions. The differences in the proliferation rates were evaluated up until 40 days of culture and were highly significant.

When the cells were allowed to differentiate, the deficient cells exhibited a higher degree of neuronal maturation in response to the addition of PUFAs in the medium, as demonstrated by an increase in neurite length, whereas the neurons derived from the supplemented pups showed no change. This result was consistent, regardless of the age of the culture.

The properties of the NSC were durably modified throughout the length of the culture, although the membrane phospholipid compositions were similar. We examined the differential expression of selected mRNAs and micro RNAs. We found significant differences in the gene expression of proliferating and differentiating cells, and a group of genes involved in neurogenesis was specifically modified by n-3 PUFA treatment.

We conclude that n-3 PUFA levels in the maternal diet can induce persistent modifications of the proliferation and differentiation of NSCs and of their transcriptome. Therefore, the n-3 supply received in utero may condition on a long-term basis cell renewal in the brain.

Keywords: Docosahexaenoic acid; Adult stem cells; Neurogenesis; Nutrition

1. Introduction

Adequate maternal nutrition and supply of nutrients during intrauterine life are necessary for proper fetal brain development. Polyunsaturated fatty acids (PUFAs), especially linoleic acid (18:2 n-6, LA), α-linolenic acid (18:3 n-3, LNA), arachidonic acid (20:4 n-6, AA), and docosahexaenoic acid (22:6 n-3, DHA), are major elements of fetal nutritional requirements. Indeed, LA and LNA are the precursors of the n-6 and n-3 families of PUFA, respectively, and must be obtained from dietary sources because they cannot be synthesized by mammals. They are converted by the same enzymes through a succession of desaturations and elongations in the corresponding long-chain PUFA molecules (LC-PUFAs), AA and DHA for the n-6 and n-3 families, respectively. The brain is rich in LC-PUFA. These molecules represent approximately 30% of the brain's dry weight. The retina and brain have the highest proportion of DHA in the body. The accumulation of DHA in the fetal brain occurs very early in development. DHA and AA are thought to be supplied by the maternal blood because the rate of conversion of the precursors is very low in the embryo [1,2]. LC-PUFAs are key constituents of the membrane phospholipids, but their role also extends to other functions. PUFAs can be converted enzymatically into active derivatives (eicosanoids and docosanoids), and they can bind to nuclear or membrane receptors and modulate gene expression [3].

The Western diet is characterized by a high fat intake and an imbalance between the two families of PUFAs. Current dietary intake favors the n-6 family; indeed, the n-6 PUFAs content in western diets has increased considerably over the past four decades, whereas the n-3 PUFAs content has remained stable. Currently, the dietary intake of the n-3 LC-PUFAs almost exclusively relies on seafood and fish consumption. The LA/LNA ratio in the diets of the French people, for instance, has increased fourfold since the 1970s and is now approximately 10–15-fold above the recommended ratio (n-6/n-3≤5) [1,4–6]. The changes in the n-6/n-3 PUFA status of the western population are reflected in blood, breast milk and adipose tissue samples and have been described in developed countries [4,7,8]. This dietary shift translates into an overrepresentation of the n-6 series in the phospholipids, through its main component, AA. Consequently, DHA is less represented in the cerebral structures. The importance of an adequate representation of the n-3 PUFA in the brain membranes has been emphasized by impairments linked to their deficiency. In infants, a maternal diet that is low in DHA was associated with low visual acuity [9]. In animal models that mimic the n-3 PUFA

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deficiency, which is prevalent in the western diet, there are alterations in learning and spatio-temporal memory. A deficiency in n-3 PUFAs has also been linked to depression and detrimental effects on neural function and emotional responses [10,11].

A balanced maternal dietary intake of n-3 PUFA insures proper fetal neurodevelopment [12,13] and improves upon neurogenesis in the adult as well [14,15]. Adult neurogenesis refers to the process by which new functional neurons are generated from neural stem cells (NSC) in the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus. The neuroblasts generated in the SVZ migrate to the olfactory bulb and integrate into the neuronal network in the CA3 region of the DG. This process is regulated by many positive and negative external and internal factors. Stimuli that improve the performance on cognitive tasks increase neurogenesis, while negative stimuli decrease neurogenesis [16–18]. A link between neurogenesis and depression has frequently been made, and it has been recently shown that active neurogenesis can prevent the vulnerability to depression [19,20].

There are numerous pieces of experimental evidence indicating the fundamental role of DHA in brain physiology, and the contribution of DHA to maintenance of active neurogenesis [14,15,21], although the mechanisms involved are not completely understood. It is known that NSC are highly sensitive to the modifications of their environment and are prone to epigenetic regulations [22,23]. Furthermore, a recent study has demonstrated that in utero DHA could alter the placental global DNA methylation patterns in rats [24].

Therefore, in this study, we questioned whether NSC from animals with different PUFAs status behaved similarly in vitro, either under basal conditions or under PUFAs supplementations. We isolated and grew NSC from the brain of 1-day-old pups. The mothers had been fed one of two different diets: “deficient” (rich in n-6, mimicking the western diet) and “enriched” (supplemented with preformed DHA). Our objective was to determine the effect of the n-3 PUFA maternal supply on the modifications of offspring NSC intrinsic properties by studying the effects on NSC proliferation, differentiation and transcriptome expression.

For clarity, we will now refer to the cells derived from rat pups bred from mothers fed on a deficient or supplemented diet as “deficient” and “supplemented” cells, respectively.

2. Methods

2.1. Reagents

The AA and DHA sodium salts, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF or FGF2), 4′,6-diamidino-2-phenylindole (DAPI) polyornithine and laminin were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The anti-β III tubulin antibody was purchased from Millipore (Molsheim, France), and the secondary antibodies used for immunocytochemistry were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Anti-peroxisome proliferator-activated receptor alpha (PPARα), anti-retinoid X receptor alpha (RXRα) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies, and the corresponding secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Neurocult medium was purchased from StemCell technologies (Grenoble, France), and EdU (5-ethynyl-2′-deoxyuridine) was obtained from Invitrogen (Life Technologies, Villebon-sur-Yvette, France). Accutase was purchased from PAA (Cölbe, Germany).

2.2. Animal diets

Wistar rats obtained by local breeding were used in this study, in accordance with the official French regulations (No 87848 and 03056) and National Institutes of Health guidelines (No 85–23). The 8-week-old mothers of the 1-day old rat pups had been fed diets containing a deficient or enriched amount of n-3PUFAs from two weeks before breeding up until delivery. The diets contained 6.6 g lipids/100 g. The “deficient” diet provided 0.01% of the total energy as LNA (5 mg/100 g diet), and the “supplemented” diet provided high levels of DHA (1.0% of the total energy, 500 mg/100 g diet). The diets were prepared using mixtures of rapeseed, sunflower and tuna fish oils [25], kindly provided by Lesieur (Asnières-sur-Seine, France).

2.3. Cell proliferation and differentiation

The NSCs were isolated from five to ten 1-day-old female rat brains, from two to three litters. Briefly, the cells were grown as neurospheres and displayed specific markers for neural stem cells/progenitors. After adhering onto poly-ornithine/laminin (P/L) matrices in the absence of growth factors, the NSCs differentiated into neurons (20–30%), astrocytes (50%) and oligodendrocytes (20-30%), as previously described [26].

The cell proliferation was assessed by EdU incorporation. Twenty-four hours after accutase dissociation, the proliferating cells were incubated for 6 h in medium containing 10 mg/ml EdU. At the end of the incubation, the cells were pelleted and rinsed twice in phosphate-buffered saline to remove any unincorporated EdU. After 1 day, the cells were allowed to adhere onto P/L coverslips. Once the cells became adherent, they were fixed in 4% paraformaldehyde, permeabilized with 0.25% TritonX-100 and stained for EdU, according to the manufacturer's instructions (Invitrogen, Life technologies, Villebon-sur-Yvette, France). Subsequently, the cells were counter-stained with DAPI, and the EdU-labeled nuclei and total cell number were counted using a fluorescence microscope.

For differentiation assays, the cells were allowed to adhere onto the P/L coverslips in the absence of growth factors in the culture medium. After 8 days, the cultures were fixed and the neurons were labeled with the β III tubulin antibody [26]. Lastly, the neurite lengths were measured using Image J.

2.4. Phospholipid analysis

The fatty acid compositions of the two main classes of membrane phospholipids, the ethanolamine-phosphoglycerolipids (EPG) and phosphatidylcholine (PC), were analyzed by gas chromatography, as previously described [26].

2.5. RNA extraction and Taqman Low Density Arrays

The total RNA from the proliferating and differentiating cells were extracted using the mirVana miRNA isolation kit according to the manufacturer's instructions (Ambion, France). The RNA quantity was routinely assessed on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Illkirch, France). The RNA quality was determined using the Bioanalyzer 2100 (Agilent Technology, Paris, France).

The total RNAs were reverse-transcribed with the high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Courtaboeuf, France) in a final volume of 100 µl containing 1×RT buffer, 1×RT random primers, 4 mM dNTP mix and 250 U multiscribe reverse transcriptase.

The quantitative polymerase chain reactions (PCRs) were performed using the ABI Prism 7900 HT sequence detection system (Applied Biosystems, Courtaboeuf, France). The Taqman Low Density Array (TLDA) was designed to measure the expression of 96 genes, which are either involved in the cell cycle, cell proliferation or cell differentiation of NSCs. The complete list is included as part of the supplemental information in Table sT1.

Briefly, 600 ng of cDNA per sample-loading port was loaded, with each port allowing for 48 q-PCR reactions as per the manufacturer's instructions (10 min at 94.5°C followed by 40 cycles of 30 s at 97°C and 1 min at 59.7°C). The gene expression levels were normalized relative to the GAPDH expression level [26], and the analysis was performed with biological replicates. Data were analyzed using SDS RQ manager v1.2 software on the ABI system followed by DataAssist 3.0 Software, which utilizes the ΔΔ Ct method [26].

The miRNA expression analysis was performed using TLDA (Applied Biosystems, Courtaboeuf, France). The global profiling for miRNA expression was performed using the TaqMan Rodent MicroRNA Array Card A in a 384-well format. Card A contains 380 TaqMan MicroRNA Assays, which enables the simultaneous quantification of 360 rodent miRNAs in addition to endogenous controls. In brief, total RNA was first reverse-transcribed with the Multiplex RT pool set through a RT reaction using the Megaplex TM RT Rodent Primers Pool and the TaqMan MicroRNA Reverse Transcription Kit, according to the manufacturer's instructions. A total of 6 µl RT products were added to 444 µl nuclease-free water and mixed with 450 µl TaqMan Universal MasterMix. The samples were then dispensed into the 384 wells by centrifugation. In addition, real-time PCR was performed using the ABI PRISM 7900 System. The raw miRNA array data were analyzed using the SDS RQ manager v1.2 software on the ABI system, followed by DataAssist 3.0 Software, which utilizes the ΔΔ Ct method [27]. The endogenous small nucleolar control RNA, U6, was used for normalization.

The miRNA expression analysis involved individual TaqMan assays (Applied Biosystems). Individual TaqMan assays were used to analyze the expression of the following mature rat miRNAs: miR-9, miR-124 and miR-210. A total of 10 ng total RNA was used in each RT reaction. The RT mix included 50 nM stem-loop RT primers, 1×RT buffer, 0.25 mM each dNTP, 10 U/µl MultiScribe reverse transcriptase and 0.25 U/µl RNase inhibitor. The 7.5-µl reaction mixture was then incubated for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. The cDNA was then used for subsequent PCR amplification using the TaqMan 2× Universal PCR Master Mix. Mammalian U6 expression was assayed for normalization. All of the reactions were performed in quadruplicate and the relative miRNA expression was normalized against the endogenous controls using the comparative delta-delta Ct method.