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Journal of Nutritional Biochemistry 24 (2013) 380–387

Journal of Nutritional Biochemistry

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

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Received 29 March 2012; received in revised form 20 July 2012; accepted 1 August 2012

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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http://dx.doi.org/10.1016/j.jnutbio.2012.08.001

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.

2.6. Western blot analysis

Differentiated cells from deficient or supplemented animals were lysed in Chaps buffer (0.02 M Tris–HCl, pH 7.6, 1% Chaps) containing a protease inhibitor cocktail (Complete-EDTA free, Roche Diagnostics, Meylan, France), and the protein content was measured using the Bio-Rad DC protein assay kit (Bio-Rad, Meylan, France). The migration buffer contained 25 mM Tris base, pH 8.3, 192 mM glycine, and 0.1% sodium dodecyl sulfate. The proteins were then electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon P, Millipore, Molsheim, France) in the same buffer supplemented with 10% ethanol at 35 V for 3 h. The non-specific binding sites were blocked by immersing the membrane in 10 mM Tris buffer, pH 7.5, containing 150 mM NaCl and 5% skim milk. The membranes were incubated overnight at 4°C with the different antibodies and the immunoreactive proteins were revealed by enhanced chemiluminescence (ECL kit, Amersham, GE Healthcare, Vélizy-Villacoublay, France). The PPARα and RXRα proteins were detected as bands corresponding to a mass of 55 kDa, and GAPDH, as a band corresponding to a mass of 35 kDa.

2.7. Statistical analysis

The results are reported as the mean±S.D. The data analysis was performed using the Sigma Stat statistical software (version 3.1, SPSS). The differences between the NSCs were tested for significance by a non-parametric one-way analysis of variance (ANOVA) followed by a Kruskal-Wallis post hoc test, except for genomic data from differentiated cells. The data for differentiated cells were compared using a two-way ANOVA and Bonferroni *t* test with a significance level of .05 ($P<.05$).

3. Results

3.1. Cell growth and differentiation

The proliferation of the cells derived either from the deficient or supplemented rat pups was analyzed by labeling of the cells in S phase with EdU, as previously described in the methods section.

The supplemented cells displayed a higher proportion of EdU-positive cells than the deficient cells (Fig. 1A). This higher rate of proliferation was maintained for the duration of the culture, and the differences in growth rate were noted up until 40 days in culture.

The differentiation of the two cultures was also examined. There were no differences observed in the proportions of the glial or neuronal lineages (Supplemental Figure sF1). However, when we examined neuritogenesis by measuring the cellular neurite length, we noticed a significant difference between the deficient and the supplemented cells; the neurite lengths of supplemented cells were slightly longer than those of deficient cells. In addition, the neurites had significantly increased in length in the deficient cells when either AA or DHA was added to the medium (Fig. 1B). The supplemented cells did not display this response; the neurite lengths remained similar regardless of the treatment conditions. This behavior was

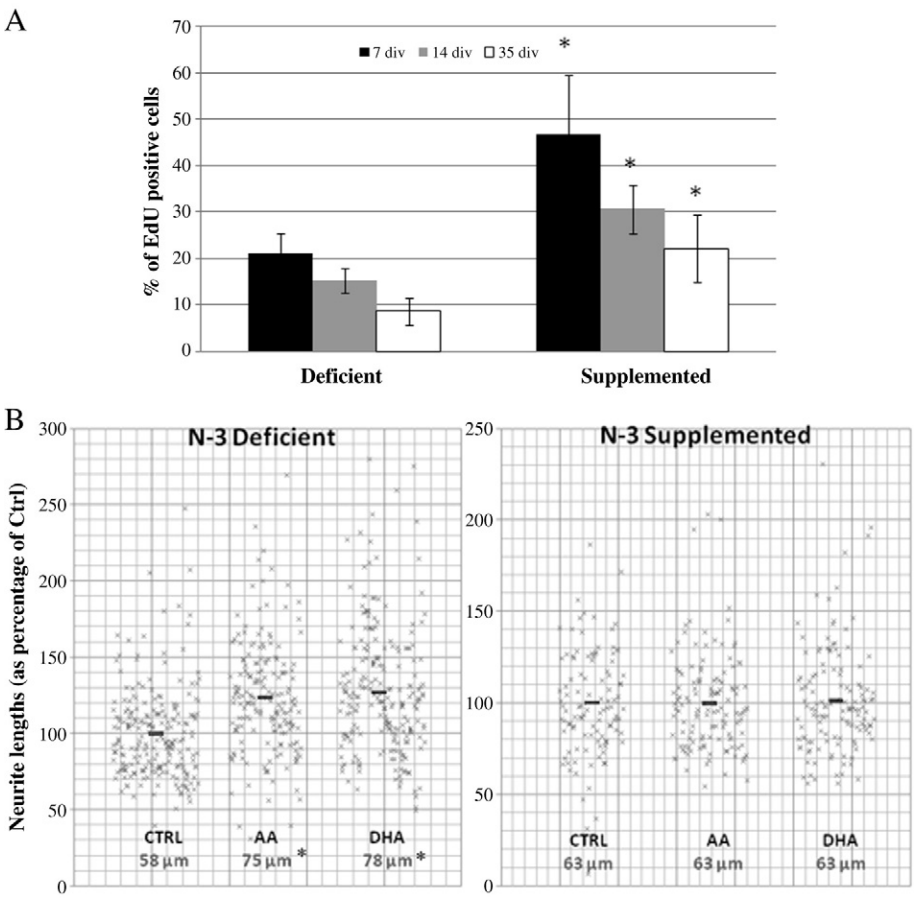

Fig. 1. The growth and differentiation characteristics of the deficient and supplemented cells. NSCs from deficient and supplemented pups were tested for proliferation and differentiation throughout the duration of the culture. (A) At 24 hours after dissociation with accutase, the cells were exposed to 10 µM EdU for 6 h; 24 h later, they were allowed to adhere onto P/L glass coverslips, and they were fixed with 4% paraformaldehyde. Staining for EdU was performed according to the manufacturer's guidelines. The cells were counterstained with 0.5 µg/ml DAPI. The cells were counted using Image J; at least 500 cells were counted for each coverslip. DIV, days in vitro. The asterisk denotes a significant difference at $P<.05$ (Student's *t* test). (B) The cells from deficient or supplemented pups were allowed to differentiate on P/L glass coverslips for 8 days in the absence of growth factors. The cells were fixed with 4% paraformaldehyde, immunolabeled with β-III tubulin antibody and counterstained with DAPI. The neurite lengths were measured using Image J. The figure compiles the results from cells that were examined from 20 to 35 DIV. The numbers at the bottom of the figure express the average length in µm recorded for cells in each treatment condition. The asterisk denotes a significant difference at $P<.05$.

Table 1
PC and EPG fatty acid composition (%TFA) of proliferating neural stem cells

	EPG			PC		
	Total n-6 PUFA	DHA	Total n-3 PUFA	Total n-6 PUFA	DHA	Total n-3 PUFA
11 DIV						
Deficient	14.22±3.96[a]	1.87±0.57[a]	3.16±0.52[a]	2.75±0.04[a]	0.11±0.10[a]	1.10±0.03[a]
Supplemented	14.64±2.96[a]	4.65±1.16[b]	6.01±1.14[b]	3.02±0.18[a]	0.57±0.01[b]	1.77±0.21[a]
18 DIV						
Deficient	12.47±0.11[a]	0.77±0.03[a]	1.95±0.14[a]	2.20±0.04[a]	0.05±0.04[a]	1.30±0.33[a]
Supplemented	11.57±2.67[a]	1.21±0.25[a]	2.24±0.34[a]	2.63±0.37[a]	0.19±0.08[a]	1.12±0.20[a]
25 DIV						
Deficient	10.47±2.43[a]	0.83±0.07[a]	2.89±0.45[a]	2.29±1.39[a]	0.09±0.13[a]	2.25±0.30[b]
Supplemented	11.40±5.20[a]	0.91±0.57[a]	2.67±0.04[a]	2.46±0.25[a]	0.29±0.27[a]	2.42±0.13[b]

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids.
Means with a "b" superscript are significantly different from means with a "a" superscript, $P<.05$.

consistently observed throughout the duration of the culture, which extended to 35 days.

Given that the cells were derived from animals with different n-3 PUFAs statuses, we questioned the changes in the composition of the membrane phospholipids during the culture, since the culture medium is deprived of PUFAs [26]. The results of this analysis are presented in Table 1. After 8 days of culture in vitro, DHA in the EPG of supplemented cells was higher than in deficient cells, but the DHA content declined over time to reach a level similar to that obtained for the deficient cells already after 18 days in vitro. The change in DHA levels in the PC fraction was also similar.

3.2. Differential mRNA and miRNA expressions in proliferating cells

Given the differences observed in the rate of proliferation and the neuronal maturation, we selected 96 genes that are involved in the cell cycle, proliferation or growth factor signaling and are receptors or neuronal or glial markers and examined their expressions by using TLDA. A complete list of these genes is presented in supplemental Table sT1. The results in Fig. 2 were calibrated with deficient cells that were proliferating (PD cells) as the baseline. The threshold of biological significance was established at 2.00 for an enhancement and 0.5 for a reduction.

Under these conditions, in the proliferating supplemented (PS) cells, a group of genes was differentially expressed, and genes related to the cell cycle, transcription factors and neuronal/glial lineages were minimally represented in this group. Genes that encode growth factors and receptors showed strong differential expression. The expression levels of *egf* and its receptor, *egfr*, were increased in the supplemented cells. By contrast, the expression level of *fgf2* was significantly reduced. Importantly, the highest expression levels of *fabp7* and *pparα* were found in the supplemented cells, and the proteins both bind to DHA. *drd2* was also highly expressed in the supplemented cells compared to the deficient cells.

We examined the global miRNA expression profiles of the deficient and supplemented cells by miRNA array. The results generated by this analysis are reported in supplemental Figures sF2A and sF2B. In light of the data obtained from the miRNA array, we selected 3 miRNAs to be individually investigated: miR-9 and miR-124, which are known to be expressed in the brain [28–30], and miR-210, which appears to be differentially expressed in the array (Supplemental Figure sF2A). The results of these investigations are reported in Fig. 3. We found that the expression of miR-9, miR-124 was similar in the PD and PS cells and that miR-210 was more highly expressed in the PS cells than in the PD cells.

3.3. Differential mRNA expressions and protein contents in differentiating cells

A similar panel of genes was examined in the differentiated cells that were deficient (DD) or supplemented (DS). The results are summarized in Fig. 4A and are normalized to the expression observed

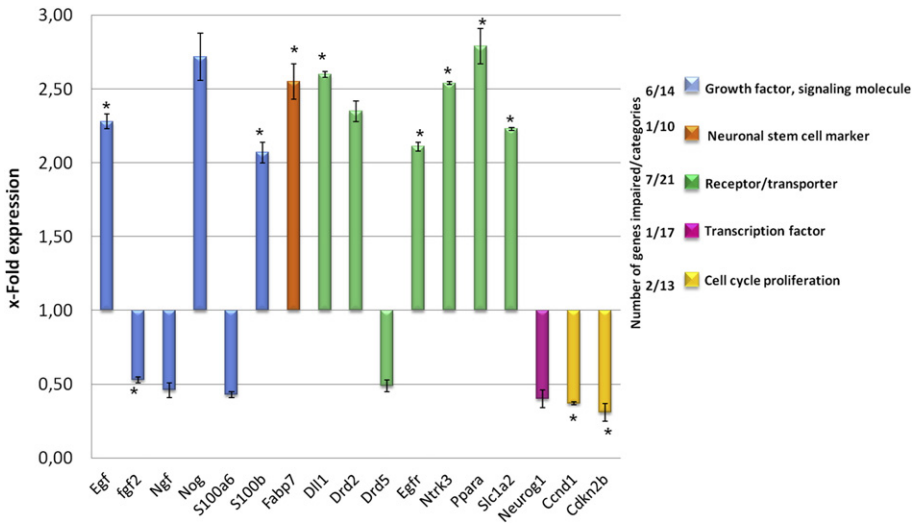

Fig. 2. Differential expression of genes in proliferating cells. Gene expression was quantified by TLDA. The complete list of genes is also reported in the supplementary information. The cells were examined at 25 DIV. The expression of each gene is shown relative to *gapdh* expression. PD cells were used as the calibrator. A twofold change was considered to be biologically significant for enhancement, and a 0.5 change was considered to be biologically significant for reduction. The asterisk denotes a difference that was statistically significant at $P<.05$.

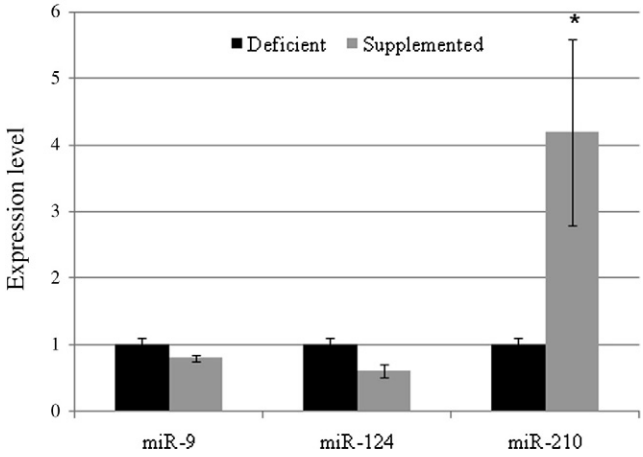

Fig. 3. miRNA qPCR. The expression levels of 3 miRNAs in the cells at 25 DIV were determined by qPCR and were normalized to the expression level of U6 snRNA. The PD cells were used as the calibrator.

in the PD cells. More genes were found to vary in expression in this analysis, including families of genes involved in synaptogenesis. This finding is consistent with the observed changes associated with cell differentiation. Some genes (e.g., *pparα* and *drd2*) also behaved similarly to those in the proliferating cells, whereas some (e.g., *fgf2*) exhibited the opposite expression profiles, i.e., the expression was increased compared with proliferating cells. Although statistical significance was not always achieved, the increases tended to be consistently higher in the DS cells and the reductions were less prominent relative to those observed in the DD cells.

Some markers of differentiation (*map2* and *s100b*) were more highly expressed in the DS cells, and the genes involved in synaptogenesis were also slightly increased in expression.

Overall, these modifications seemed to indicate an enhanced potential for differentiation of the DS cells.

Given that the addition of PUFAs to the medium modified neuronal maturation (Fig. 1B), we compared the gene expression levels in the presence or absence of AA or DHA addition in the culture medium. We noticed a common pattern of expression upon DHA addition in vitro or DHA supply in utero in a set of 17 genes. These results are shown in Fig. 4B and Table 2.

Results presented in Fig. 4B are calibrated against DD cells as the baseline. The level of expression of these genes was higher in the DS cells than in the DD cells. When the DD cells differentiated in the presence of DHA, they displayed gene expression levels similar to those observed in the DS cells. This increase was not noted in the presence of AA, which indicates a specific role of DHA either in vivo (in DS cells) or in vitro (when added to DD cells). In contrast, the DS cells that differentiated in the presence of DHA and AA displayed decreased levels of expression. The roles of the genes analyzed in this group are very important for neurogenesis, as these genes encode transcription factors (e.g., *tlx*), VEGFa, histone deacetylases and DHA binding proteins (Table 2).

We examined the protein quantities of PPARα and RXRα in the DS and DD cells. Our results are reported in Fig. 5. PPARα and RXRα were more abundant in the differentiated cells that originated from the supplemented animals. Therefore, the expression patterns observed in the protein levels were consistent with the patterns found for the corresponding mRNAs.

4. Discussion

Dietary modifications over the past 40 years have led to the predominant consumption of n-6 PUFAs. Epidemiological studies and animal experimental models have indicated that this change is detrimental to neural functions. The n-3 PUFAs have been a focus of investigation in numerous studies of brain physiology. Adult neurogenesis is enhanced by high quantities of n-3 PUFAs in the brain. Thus far, these studies have mainly focused on the direct consequences of differential n-3 PUFA intake on the regulation of adult neurogenesis [14,15,21] or on neurodevelopment [12,13].

Our study focused on the consequences of n-3 PUFA maternal dietary intake on the NSC properties of the offspring; we showed that the proliferation and differentiation capacity of NSCs are intrinsically modified by the nature of the maternal diet. Our observations led us to three conclusions:

1. Elevated n-3 PUFA levels in the maternal diet can modify NSC properties in the offspring in a way that is beneficial to cell renewal because n-3 PUFAs favor proliferation and differentiation.
2. n-3 PUFAs can specifically modify the expression of a particular set of genes both in vitro and in vivo.
3. The modifications observed in the intrinsic cell properties induced in utero by the maternal n-3 PUFAs were persistent throughout the duration of the culture.

4.1. NSC from deficient and supplemented animals behave differently in vitro

Our study showed that NSCs derived from n-3 PUFAs supplemented animals had a greater propensity to proliferate and differentiate. Proliferation was increased amongst cells that were derived from supplemented pups. The differentiation of these cells was also found to be favored. Specifically, the cells derived from the supplemented animals expressed their full differentiation abilities, without the need for the addition of PUFAs in vitro. Indeed, it has been shown that PUFA in vitro could enhance neuronal maturation, by enhancing neuritogenesis [31], but the addition of AA or DHA remained without effect on neurite lengths in supplemented cells, whereas they induce the expected effect on deficient cells. The analysis of differential RNAs expression showed that several genes were differently expressed. *egf* and *egfr* were up-regulated in the supplemented cells and so was 210 miRNA, a result consistent with the higher growth rate observed in the supplemented cells. Indeed, EGF is a well known mitogen, and miRNA-210 up-regulation has been recently associated with cell proliferation [32].

PUFA phospholipid compositions of the cell membranes in the rat pups are directly linked to the dietary PUFA intakes of their mothers [26]. This composition also varied during the culture, since the classical culture medium used for the proliferation of NSC is deprived of fetal calf serum, and therefore is devoid of fatty acids. To determine whether phospholipid compositions could contribute to these differences observed in the proliferation and differentiation, we analyzed the proportions of PUFAs in EPG and PC, the two main PLs in the membranes. Indeed, the composition of the PL changes during the culture, and free PUFAs can be released from within the cell, bind to receptors and ultimately modify gene transcription. Yet, the differences in the cell properties persisted up to 35–40 days of in vitro culture, and we have observed that the PL compositions were similar between the cultures after 18–25 days of culture (Table 1). Therefore, these changes were not related to the differences in PL composition and must have originated from the different exposures to n-3 PUFAs in utero.

4.2. Some genes are modulated by n-3 PUFAs and not by n-6 PUFAs.

Because of the differences observed in cell proliferation and differentiation, we selected a group of 96 genes that are involved in

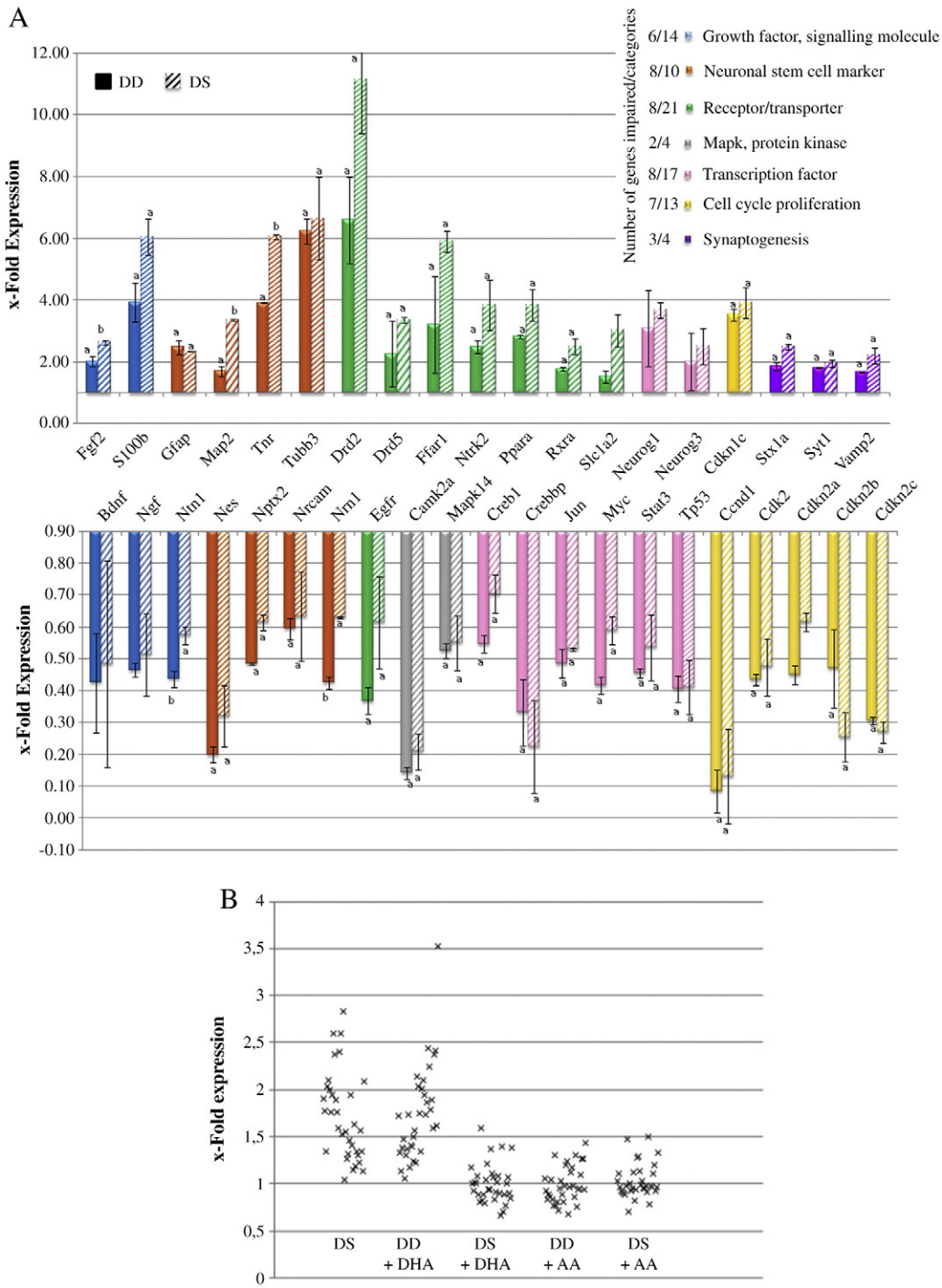

Fig. 4. Differential expression of genes in the differentiated cells. Panel A: The expression level of each gene is reported relative to the *gapdh* expression level. PD cells were used as the calibrator. The cells were examined at 25 DIV. A twofold change was considered to be biologically significant for enhancement, and a 0.5-fold change was considered to be biologically significant for reduction. The bars without a common letter were statistically significantly different, $P<.05$. (B) The calibrator was DD cells. Each of the 17 genes exhibited a higher level of expression in the DS cells than the DD cells. The expression profile was modified in a similar manner when either AA or DHA was added to the differentiation medium (see text for details). The genes and their expression levels are reported in Table 2.

cell growth and neurogenesis. We compared their expression in the deficient and supplemented cells. We showed that in the proliferating and differentiated states, some genes were differentially expressed; in particular, the genes encoding the receptors and growth factors were the most consistently represented in this set of genes.

Interestingly, a group of genes showed a common pattern of expression in the differentiated cells, and were modified specifically by DHA (Fig. 4B and Table 2). Among these genes, some are recognized to play a fundamental role in neurogenesis, and to our knowledge, have not been described as specific targets of DHA so far. *vegfa*, for instance, was the most overexpressed gene in the supplemented cells. VEGFa has been shown to play a functional role in neurogenesis through its action on angiogenesis. However, recent reports have suggested that this growth factor may directly promote neurogenesis [33–36]. Another interesting gene present in this group is the gene coding for the transcription factor TLX. This factor is highly expressed in

Table 2
Relative quantitation of genes specifically modified by DHA addition in vitro or DS, differentiated supplemented cells; DD, differentiated deficient cells

Genes	DS	DD+DHA	DS+DHA	DD+AA	DS+AA
Fabp3	1.47±0.44	1.87±0.15	1.14±0.04	1.13±0.07	1.16±0.13
Fabp7	1.66±0.12^{b}	1.54±0.12^{b}	1.04±0.03^{a}	1.08±0.15^{a}	1.12±0.02^{a}
Fgf2	1.30±0.04^{b}	1.23±0.11^{ab}	0.87±0.05^{a}	0.76±0.08^{a}	0.98±0.01^{a}
Hdac4	1.68±0.36	1.70±0.31	0.97±0.06	0.93±0.04	0.98±0.05
Hdac7	1.78±0.32	1.79±0.31	1.01±0.06	0.99±0.04	0.93±0.05
Map2	1.97±0.02	1.50±0.45	1.08±0.01	1.11±0.04	0.93±0.03
TLX	1.58±0.17	1.52±0.21^{b}	0.89±0.01^{a}	0.9±0.07^{a}	0.93±0.05^{a}
Ntrk2	1.54±0.39	1.62±0.25	0.74±0.07	0.81±0.05	0.94±0.05
Ntrk3	2.12±0.48	2.09±0.35	1.49±0.01	1.31±0.01	1.33±0.02
Pax6	1.78±0.59	1.71±0.54	0.76±0.06	0.76±0.01	0.74±0.01
PRARα	1.62±0.28	1.60±0.19	0.89±0.01	0.88±0.06	0.99±0.02
Robo1	1.53±0.23^{b}	1.64±0.24^{b}	0.79±0.02^{a}	0.83±0.11^{a}	0.94±0.02^{a}
RXRα	1.41±0.18^{b}	1.41±0.17^{b}	1.03±0.02^{a}	1.02±0.07^{a}	1.03±0.02^{a}
GLT1 (a2)	1.98±0.42	1.94±0.44	0.93±0.07	1.15±0.12	1.16±0.06
Slit2	1.97±0.63	1.99±0.43	0.92±0.02	1.07±0.19	0.87±0.05
Vamp2	1.32±0.19	1.43±0.19	0.90±0.05	0.87±0.07	0.96±0.02
Vegfa	2.46±0.20^{b}	2.84±0.37^{b}	1.37±0.01^{a}	1.21±0.23^{a}	1.30±0.03^{a}

Means in a row with superscripts without a common letter differ, $P<.05$.

adults, and it contributes to the maintenance of proliferation [37–39]. Interestingly, a common feature of VEGFa and TLX is that their syntheses are both reduced during aging, and these reductions could play a role in the decline of adult neurogenesis in aged organisms. It would therefore be interesting to determine whether n-3 PUFA intake could counteract the detrimental effect of aging by up-regulating them [33,39]. The HDACs were also modified specifically by DHA; they have been shown to play a role in the control of neurogenesis and are important in epigenetic processes. We also found a few genes that were directly related to neuronal differentiation and synaptogenesis (*vamp2*, *glt1*, *map2*, and *pax6*) or neuronal migration (*robo1* and *slit2*). *ntrk3*, encoding a neurotrophin receptor tyrosine kinase, is highly expressed in the supplemented cells. A decrease in the expression of this receptor has recently been linked to schizophrenia and psychotic disorders [40]. Furthermore, the nuclear receptors or cytoplasmic proteins that have PUFAs as potential ligands (RARα, RXRα and FABP7) were also up-regulated in the supplemented cells. This up-regulation may have rendered these cells more sensitive to DHA exposure.

4.3. The modifications are persistent throughout the duration of culture.

Our study shows that NSCs from deficient or supplemented pups behave differently ex vivo and that the modifications in proliferation and differentiation persist throughout the duration of the culture. Steady and persistent changes in cells or organisms are frequently

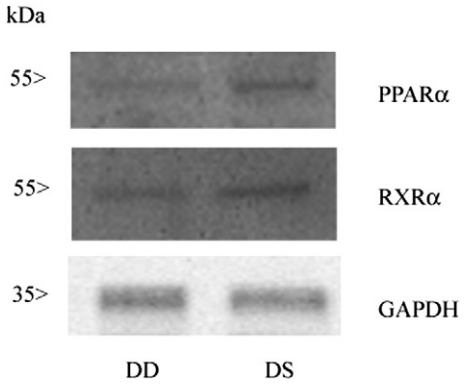

Fig. 5. The PPARα and RXRα protein levels in differentiated cells. Cells at 25 DIV were lysed and analyzed for PPARα and RXRα contents by western blotting. These experiments were repeated in triplicate and yielded similar results.

associated with epigenetic modifications, and the current literature provides numerous pieces of evidence to support the hypothesis that NSCs are prone to such modifications. The NSCs are influenced by both intrinsic and external cues, depending on their location in the cerebral structures [41]. Moreover, the developmental shift from neurogenesis to gliogenesis is regulated by epigenetic mechanisms [42]. Stress, through the action of glucocorticoids, can modulate NSC proliferation and inhibit proliferation in vitro [43]. In addition, diet can be at the origin of these epigenetic modifications. For example, dietary choline deficiency [44,45] and folic acid treatment [46] can induce epigenetic modifications. Alcohol intake can also inhibit NSC differentiation by acting on DNA methylation [47]. Furthermore, PUFAs are now thought to induce transgenerational consequences. A recent study of metabolic syndrome has shown that diets that are differentially enriched in the two families of PUFAs could have transgenerational effects [48]. Epigenetic regulation induced by PUFAs has been shown [24] or suggested [49]. Therefore, that the persistent modifications of NSC properties originate from possible epigenetic influences of the maternal n-3 PUFAs is an appealing hypothesis, which would deserve further attention.

In conclusion, our study suggests that supplementation with n-3 PUFAs could have effects that might extend far beyond neurodevelopment, since the intrinsic NSC properties are modified durably. The mechanisms underlying these changes require further investigation.

Supplementary materials related to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.08.001.

Acknowledgments

This work was supported by a grant obtained from the Groupe Lipides Nutrition (Paris, France), and by a grant from the Agence Nationale de la Recherche (France) (ANR-09-Alia-006-01 “Neuromega3”). The TLDA were performed at the ICE facility (Jouy en Josas, France).

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