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Long Chain-Polyunsaturated Fatty Acids Modulate Membrane Phospholipid Composition and Protein Localization in Lipid Rafts of Neural Stem Cell Cultures

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ABSTRACT

Rat neural stem cells/neural progenitors (NSC/NP) are generally grown in serum-free medium. In this study, NSC/NP were supplemented with the main long-chain polyunsaturated fatty acids (PUFAs) present in the brain, arachidonic acid (AA), or docosahexaenoic acid (DHA), and were monitored for their growth. Lipid and fatty acid contents of the cells were also determined. Under standard conditions, the cells were characterized by phospholipids displaying a highly saturated profile, and very low levels of PUFAs. When cultured in the presence of PUFAs, the cells easily incorporated them into the phospholipid fraction. We also compared the presence of three membrane proteins in the lipid raft fractions: GFR and connexin 43 contents in the rafts were increased by DHA supplementation, whereas Gβ subunit content was not significantly modified. The restoration of DHA levels in the phospholipids could profoundly affect protein localization and, consequently, their functionalities. J. Cell. Biochem. 110: 1356–1364, 2010.

KEY WORDS: NEURAL STEM CELLS; POLYUNSATURATED FATTY ACIDS; DOCOSAHEXAENOIC ACID; LIPID RAFTS

Lipids are more than inert structural components delimitating the extra- and intracellular compartments. By the numerous properties that they display, they can influence and modify many cellular functions: the fatty acid composition of phospholipids determines the biophysical properties of cell membranes, and protein functionalities or localizations. Fatty acids are also precursors of signaling derivatives, and ligands for membrane or nuclear receptors [Piomelli et al., 2007].

The brain is particularly rich in lipids, and is characterized by high levels of omega-3 and -6 polyunsaturated fatty acids (PUFAs). Many experimental data demonstrate that lipid composition, and particularly n-3 PUFA, can affect cerebral functions [Alessandri et al., 2004; Dyall and Michael-Titus, 2008].

In this study, we have checked the PUFA composition of neural stem cells/neural progenitor cells (NSC/NP). Indeed, like in other organs, niches of dividing undifferentiated cells can be identified in the brain. These cells, once isolated, can be amplified in vitro when grown in the presence of mitogenic factors such as bFGF and epidermal growth factor (EGF) [Reynolds and Weiss, 1992], and contrary to most cultured cells are not proliferating in the presence of serum, because serum would induce premature differentiation [Ray and Gage, 2006]. Therefore, they are deprived of any supplying of essential unsaturated fatty acids. NSC cultures have been widely developed and used in determining their properties, characteristics, or the mechanisms difficult to study in vivo [Imura et al., 2003; Kim et al., 2006; Garza et al., 2008]. They are also used with the eventual goal of transplantation in regenerative biology [Walton et al., 2006].

We determined the PUFA composition of standard cultures, and supplemented them with arachidonic acid (AA) or docosahexaenoic acid (DHA), the PUFAs most abundant in the brain. We monitored the changes in cell growth, membrane phospholipid composition, and the localization in the lipid rafts of a set of three membrane proteins, involved in different physiological functions. We studied the lipid rafts contents of the receptor of EGF (EGFR), since EGF is a constituent of the culture medium, Gβ subunit as an example of a membrane protein involved in the protein G signaling pathway, and connexin 43 (Cx 43), which is involved in intercellular communication, cell growth, and migration.

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MATERIALS AND METHODS

REAGENTS

The sodium salts of AA, and DHA were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). The primary antibodies were purchased from the following companies: anti-caveolin 1 (Cav 1), anti-Gβ, and anti-GAPDH, Santa Cruz; anti-EGFR and anti-phospho EGFR (Ab 869), GenScript; anti-Cx 43, Abcam; anti-beta III tubulin, anti-CNPase, anti-Neu N, and anti-FABP7, Millipore; anti-O4 and anti-GFAP, Sigma–Aldrich. The secondary antibody anti-rabbit-HRP used in the Western blot procedure was purchased from Cell Signaling, and the anti-mouse-HRP was purchased from Jackson ImmunoResearch. Secondary antibodies used in the immunocytochemistry were purchased from Sigma–Aldrich.

ANIMALS

One-day-old Wistar rats were used in this study, either for the cell culture, or for the lipid analysis reported below. "Deficient" and "supplemented" pups referred to in Tables I and II were obtained according to the following protocol: the brood mothers of the experimental animals were fed diets providing either a deficient amount in n-3, or an enriched one, for 2 weeks before breeding. Diets contained 6.6 g lipids/100 g. The "deficient" diet provided 0.01% of total energy in ALA (5 mg/100 g diet), and the "enriched" diet provided high levels of DHA (1.0% total energy, 500 mg/100 g diet). Diets were prepared using mixtures of rapeseed and tuna fish oils [Pifferi et al., 2005].

PRIMARY CULTURES

The method used was a modification of the protocol described by Reynolds and Weiss [1992]. Brains from 1-day-old Wistar rats (n = 5) were chopped and enzymatically dissociated in 10 ml trypsin–EDTA 0.1% (Invitrogen) at 37°C for 14 min, mixed with 5 ml ice cold 4% BSA in Earle's-Balanced Salt Solution (EBSS), pH 7.5. The homogenate was then passed through a 70 μm cell strainer and centrifuged at 400*g* for 5 min. The cell pellet was suspended in 10 ml ice cold 0.9 M sucrose in 0.5 × Hank's Balanced Salt Solution, pH 7.5, and centrifuged at 750*g* for 10 min. The cells were then suspended in 2 ml 4% BSA in EBSS solution, pH 7.5, placed on top of 10 ml of the same solution, and centrifuged at 400*g* for 7 min. The supernatant was removed and cell pellet was cultured in 75-cm^2 low-adherence flask (Nunc GmbH, Langenselbold, Germany) in "complete" Neurocult NS-A proliferation medium (StemCell Technologies, Grenoble, France), containing 20 ng/ml of human recombinant EGF, 10 ng/ml human recombinant basic fibroblast growth factor (Sigma–Aldrich) and 2 μg/ml heparin (Stemcell Technologies). The cultures were placed in a 5% CO_2 incubator at 37°C. Typically, the cells were dissociated every 3–4 days. They were spun at 30 g for 5 min. The supernatant was removed and 250 μl accutase (PAA Laboratories, Pasching, Austria) were added. The cell pellet was gently triturated at least 10 times and incubated 10 min at 37°C. When the spheres were entirely dissociated, complete medium was added. The cultures were used up until the fourth passage, after which complete cell-to-cell dissociation was more difficult.

IMMUNOCYTOCHEMISTRY

Immunolabeling of the neurospheres was realized according to the method described by Singec et al. [2006] and visualized with an Apotome (Zeiss).

For differentiation, neurospheres were spun and suspended in low amounts of complete medium, in which the growth factors had been omitted. The cells were seeded on poly ornithine/laminin (P/L)-coated glass coverslips [Gage et al., 1995]. After the spheres had started to adhere, more medium was added, along with PUFAs at the desired concentrations. The cells were maintained for 8 days in

TABLE I. EPG Fatty Acid Composition (%TFA) of Neurospheres Incubated for 72 h With 0, 2, 5 μM DHA or AA, and Newborn Pups Brains (mean ± SE; n = 3 Flasks per Treatment; n = 6 for Brain Samples)

	Neurospheres EPG							
		DHA supplementation		AA supplementation		AA + DHA	Brain EPG	
	Ctrl	2 μM	5 μM	2 μM	5 μM	2 μM each	n-3 deficient	n-3 supplemented
16:00	20.9 ± 1.2^{a}	23.6 ± 0.6^{a}	18.2 ± 1.2^{a}	19.5 ± 2.3^{a}	14.0 ± 0.9^{b}	17.8 ± 1.0^{a}	10.2 ± 0.2^{c}	9.8 ± 0.1^{c}
18:00	7.5 ± 0.3^{c}	5.8 ± 0.9^{c}	13.5 ± 0.5^{b}	8.5 ± 0.3^{c}	9.5 ± 0.7^{c}	12.5 ± 1.0^{b}	17.8 ± 0.9^{a}	16.9 ± 0.4^{a}
Total SFA	31.4 ± 0.9^{a}	33.6 ± 1.2^{a}	34.1 ± 2.0^{a}	33.2 ± 2.7^{a}	29.5 ± 0.8^{b}	35.2 ± 3.3^{a}	28.8 ± 1.2^{b}	27.4 ± 0.3^{b}
16:1n-9	2.9 ± 0.2^{a}	3.3 ± 0.5^{a}	$1.8 \pm 0.3^{a,b}$	$2.5 \pm 0.3^{a,b}$	$1.9 \pm 0.3^{a,b}$	1.0 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0^{b}
16:1n-7	$2.3 \pm 0.2^{a,b}$	4.2 ± 1.0^{a}	$1.4 \pm 0.1^{b,c}$	$3.9 \pm 0.8^{a,b}$	$1.4 \pm 0.2^{b,c}$	$2.8 \pm 0.4^{a,b}$	0.6 ± 0.1^{c}	0.6 ± 0^{c}
18:1n-9	23.7 ± 0.6^{a}	$20.2 \pm 1.2^{a,b}$	24.1 ± 0.8^{a}	$20.5 \pm 1.5^{a,b}$	16.7 ± 1.2^{b}	$21.4 \pm 1.3^{a,b}$	6.3 ± 0.1^{c}	6.2 ± 0.1^{c}
18:1n-7	8.9 ± 0.5^{c}	6.2 ± 0.4^{a}	6.0 ± 0.6^{a}	3.7 ± 0.3^{b}	2.4 ± 0.5^{b}	$5.6 \pm 0.7^{a,b}$	1.7 ± 0.1^{d}	1.5 ± 0^{d}
Total MUFA	39.2 ± 1.3^{a}	34.8 ± 1.3^{b}	34.3 ± 1.4^{b}	31.4 ± 1.7^{b}	23.2 ± 2.0^{c}	30.7 ± 1.9^{b}	9.3 ± 0^{d}	9.1 ± 0^{d}
20:3n-9	6.7 ± 0.3^{a}	3.0 ± 0.1^{b}	2.0 ± 0.1^{b}	4.4 ± 0.3^{b}	3.0 ± 0.5^{b}	0.2 ± 0.1^{c}	0.48 ± 0.12^{c}	0.5 ± 0^{c}
20:4n-6	4.6 ± 0.0^{c}	3.6 ± 0.1^{c}	1.2 ± 0.1^{d}	12.8 ± 1.6^{b}	20.3 ± 1.2^{a}	8.9 ± 0.5^{b}	18.6 ± 0.9^{a}	$15.8 \pm 0.2^{a,b}$
22:4n-6	0.6 ± 0.2^{c}	0.3 ± 0.0^{c}	0.1 ± 0.0^{c}	0.3 ± 0.1^{c}	1.6 ± 0.1^{b}	1.1 ± 0.2^{b}	5 ± 0.2^{a}	3.6 ± 0.1^{a}
22:5n-6	1.3 ± 0.0^{c}	$0.7 \pm 0.0^{c,d}$	0.1 ± 0.0^{d}	1.6 ± 0.2^{c}	4.6 ± 0.3^{b}	1.2 ± 0.3^{c}	13 ± 0.4^{a}	1.9 ± 0.3^{c}
Total n-6 PUFA	$8.9 \pm 1.1^{a,c}$	$5.8 \pm 0.2^{a,d}$	2.8 ± 0.1^{d}	16.6 ± 1.9^{c}	28.2 ± 1.2^{b}	18.3 ± 0.9^{c}	37.2 ± 1.2^{a}	$22 \pm 0.3^{b,c}$
20:5n-3	0.5 ± 0.1^{a}	0.2 ± 0.0^{a}	1.9 ± 0.1^{b}	1.2 ± 0.1^{b}	1.2 ± 0.3^{b}	0.4 ± 0.2^{a}	0 ± 0^{a}	0.3 ± 0^{a}
22:5n-3	0.2 ± 0.0^{a}	0.5 ± 0.1^{a}	1.7 ± 0.1^{b}	0.3 ± 0.0^{a}	0.5 ± 0.1^{a}	0.6 ± 0.0^{a}	0.2 ± 0^{a}	0.7 ± 0^{a}
22:6n-3	2.5 ± 0.5^{c}	13.1 ± 0.4^{b}	20.6 ± 0.9^{a}	1.8 ± 0.2^{c}	0.7 ± 0.0^{c}	0.8 ± 0.5^{c}	11 ± 0.5^{b}	26.4 ± 0.6^{a}
Total n-3 PUFA	3.9 ± 1.2^{c}	15.2 ± 0.6^{b}	25.5 ± 0.7^{a}	5.1 ± 0.5^{c}	3.8 ± 0.6^{c}	9.1 ± 0.5^{c}	11.3 ± 0.6^{b}	27.4 ± 0.6^{a}

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Means in a row with superscripts without a common letter differ, $P < 0.05$.

TABLE II. PC Fatty Acid Composition (%TFA) of Neurospheres Incubated for 72 h With 0, 2, 5 μM DHA or AA, and Newborn Pups Brains (mean ± SE; n = 3 Flasks per Treatment; n = 6 for Brain Samples)

	Neurospheres PC							
		DHA supplementation		AA supplementation		AA + DHA	Brain PC	
	Ctrl	2 μM	5 μM	2 μM	5 μM	2 μM each	n-3 deficient	n-3 supplemented
16:00	43.1 ± 0.4^{b}	37.7 ± 2.3^{b}	39.2 ± 3.2^{b}	41.3 ± 1.1^{b}	42.4 ± 0.3^{b}	41.8 ± 3.9^{b}	49.7 ± 0.8^{a}	48.9 ± 0.3^{a}
18:00	2.2 ± 0.2^{c}	2.7 ± 0.4^{c}	2.6 ± 0.5^{c}	3.2 ± 0.7^{bc}	2.2 ± 0.1^{c}	11.9 ± 1.5^{a}	5.0 ± 0.2^{b}	5.2 ± 0.4^{b}
Total SFA	47.5 ± 0.5^{b}	51.1 ± 3.6^{b}	45.8 ± 1.5^{b}	49.6 ± 0.5^{b}	49.4 ± 0.2^{b}	59.8 ± 4.1^{a}	59.0 ± 0.4^{a}	58.9 ± 0.6^{a}
16:1n-9	8.1 ± 0.4^{a}	8.4 ± 1.5^{a}	10.9 ± 1.5^{a}	8.0 ± 0.6^{a}	8.8 ± 0.4^{a}	2.9 ± 0.9^{b}	$5.2 \pm 0.3^{a,b}$	$5.6 \pm 0.3^{a,b}$
16:1n-7	3.9 ± 0.2	3.6 ± 0.3	4.3 ± 0.3	3.8 ± 0.3	3.4 ± 0.0	1.8 ± 0.7	3.2 ± 0.3	3.4 ± 0.2
18:1n-9	25.0 ± 0.2^{a}	$21.0 \pm 1.3^{a,b}$	19.4 ± 2.0^{b}	$22.2 \pm 0.6^{a,b}$	20.3 ± 0.1^{b}	19.2 ± 2.2^{b}	16.2 ± 0.5^{b}	16.1 ± 0.4^{b}
18:1n-7	10.2 ± 0.0^{a}	7.7 ± 0.5^{b}	5.2 ± 0.7^{c}	6.2 ± 0.5^{c}	5.8 ± 0.1^{c}	6.2 ± 0.5^{c}	4.4 ± 0.2^{c}	4.5 ± 0.2^{c}
Total MUFA	47.6 ± 0.2^{a}	41.1 ± 2.3^{b}	39.9 ± 1.2^{b}	39.8 ± 0.7^{b}	39.1 ± 0.3^{b}	30.8 ± 1.1^{c}	29.2 ± 0.3^{c}	29.9 ± 0.2^{c}
20:3n-9	1.2 ± 0.1^{a}	0.9 ± 0.1^{a}	0.5 ± 0.1^{b}	1.6 ± 0.3^{a}	1.2 ± 0.1^{a}	0.1 ± 0.1^{c}	0.0 ± 0.0^{c}	0.1 ± 0.1^{c}
20:4n-6	0.7 ± 0.0^{c}	0.9 ± 0.2^{c}	$1,0 \pm 0.2^{c}$	2.2 ± 0.2^{b}	4.9 ± 0.0^{a}	1.7 ± 0.4^{b}	6.8 ± 0.3^{a}	5.6 ± 0.3^{a}
22:4n-6	0.2 ± 0.1^{b}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.1 ± 0.0^{b}	0.4 ± 0.0^{a}	0.2 ± 0.2^{b}	0.6 ± 0.1^{a}	0.5 ± 0.0^{a}
22:5n-6	0.3 ± 0.1^{c}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.4 ± 0.0^{b}	0.9 ± 0.0^{a}	0.2 ± 0.1^{c}	1.4 ± 0.1^{a}	0.2 ± 0.0^{c}
Total n-6 PUFA	2.0 ± 0.4^{c}	2.5 ± 0.7^{c}	2.6 ± 0.6^{c}	4.0 ± 0.1^{b}	7.3 ± 0.1^{a}	5.9 ± 1.9^{b}	10.1 ± 0.4^{a}	7.5 ± 0.4^{a}
20:5n-3	0.0 ± 0.1^{b}	0.0 ± 0.0^{b}	1.0 ± 0.2^{a}	0.7 ± 0.1^{a}	$0.3 \pm 0.0^{a,b}$	$0.4 \pm 0.3^{a,b}$	0.0 ± 0.0^{b}	0.1 ± 0.0^{b}
22:5n-3	0.0 ± 0.1^{c}	0.0 ± 0.0^{c}	0.8 ± 0.1^{a}	0.1 ± 0.0^{c}	0.3 ± 0.1^{b}	0.1 ± 0.1^{c}	0.0 ± 0.0^{c}	0.1 ± 0.0^{c}
22:6n-3	0.7 ± 0.1^{c}	2.6 ± 0.3^{b}	7.21 ± 0.9^{a}	0.1 ± 0.0^{c}	0.2 ± 0.0^{c}	0.7 ± 0.4^{c}	$1.3 \pm 0.1^{b,c}$	2.9 ± 0.2^{b}
Total n-3 PUFA	1.1 ± 0.2^{c}	3.4 ± 0.5^{b}	10.4 ± 1.7^{a}	3.5 ± 0.6^{b}	$2.3 \pm 0.2^{b,c}$	3.0 ± 1.1^{b}	1.4 ± 0.1^{c}	3.5 ± 0.2^{b}

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Means in a row with superscripts without a common letter differ, $P < 0.05$.

growth factors-deprived medium, and the medium was changed every other day.

Cells were fixed in 4% paraformaldehyde for 10 min, and permeabilized in 0.25% Triton for 10 min. They were incubated in 3% BSA in phosphate-buffered saline (PBS), and with the following primary antibodies: nestin and FABP7 (specific markers of undifferentiated cells), beta III tubulin (marker of neurons), GFAP (astrocytes), O4 and CNPase (oligodendrocytes). After several rinses in PBS, specific antibody binding was revealed by incubation with the corresponding secondary antibodies, conjugated to different fluorochromes.

Nuclei were counterstained by incubation with 0.5 μg/ml DAPI (Sigma–Aldrich).

Coverslips were mounted in DAKO fluorescent mounting medium (Dako, Trappes, France), and the observations were recorded using a fluorescence microscope, equipped with a camera (Olympus, France). The pictures were analyzed using the Image J software.

PUFA SUPPLEMENTATION

Proliferating cells in suspension were dissociated and seeded at a density of 2×10^6 cells/25-cm^2 flasks in 10 ml of complete medium, supplemented or not with 2 or 5 μM AA or DHA. When the cells were grown in the presence of both PUFAs, the concentration was 2 μM each. After 3 days of incubation, the cells were pelleted, and analyzed either for lipid composition, or protein contents in the raft fraction, or whole cell lysates.

MONITORING OF CELL GROWTH

We measured cell proliferation under the different conditions of supplementation by using the Click-it EdU imaging kit (Invitrogen). Cells proliferating in suspension were dissociated, and grew for 3 days in medium supplemented with EGF and bFGF, as described above, with or without 5 μM AA or DHA. EdU (5-ethynyl-2′-deoxyuridine) (10 μg/ml, final concentration) was incorporated in the medium in the last 16 h of incubation. The neurospheres were allowed to adhere on P/L glass coverslips in the last 12 h. The cells were fixed with 4% paraformaldehyde for 10 min, and the treatments of the samples followed the manufacturer's instructions. The number of dividing cells, labeled with EdU, was compared to the total number of cells, counterstained with DAPI. On each coverslip, at least 500 cells were counted.

FATTY ACID ANALYSIS

Membrane fatty acid compositions of proliferating cells were determined from triplicate flask analysis. The composition of each flask was determined by capillary gas chromatography of the ethanolamine-phosphoglycerolipids (EPG) and the phosphatidylcholine (PC) classes.

Dissociated cells were seeded at a density of 2×10^6 cells/25-cm^2 flasks, grown for 3 days in "complete" neurocult NS-A proliferation medium containing 0, 2, 5 μM of the sodium salt of DHA or AA. The cells were harvested by centrifugation, then washed with PBS. Total lipids were extracted from 1 vol of cell homogenate with 4 vol of chloroform/methanol (2:1, v/v) in the presence of 0.005% (by wt) butylhydroxytoluene [Folch et al., 1957]. The lipid bottom phase was washed, dried, solubilized in chloroform, and stored at −80°C until separation. The EPG and PC fractions were purified by solid-phase extraction on a 500-mg prepacked aminopropyl cartridges (J.T. Baker, Deventer, The Netherlands) according to a method previously detailed [Langelier et al., 2005]. Briefly, the cartridges were equilibrated beforehand with eluent I (isopropanol/chloroform, 1:2, v/v). Each sample of total lipids was dried under nitrogen, solubilized in 250 μl of eluent I and deposited onto a single cartridge, which was immediately eluted successively with 3 ml of eluent I, 3 ml of diethyl ether/acetic acid (98:2), 1 ml of acetonitrile, 8 ml of acetonitrile/n-propanol (3:1) to recover the PC fraction, 2 ml

of acetonitrile/n-propanol (1:1), and 3 ml of methanol to recover the EPG fraction. The fractions were then transmethylated for 4 h with 10% HCl and 4% dimethoxypropane in methanol at 70°C [Berry et al., 1965]. The methyl esters were then injected on gas chromatograph (Carlo Erba) equipped with a CP-WAX 52 CB bonded fused-silica capillary column of a 0.32 mm i.d. and 60-m length (Varian, Les Ulis, France), identified by comparison with commercial standards of equivalent chain lengths and quantified by integration using the Nelson Analytical Program System (SRA, France). Results are expressed as the percentage of total fatty acids (%TFA).

LIPID RAFT EXTRACTION

The isolation of lipid rafts was performed as previously described [Heberden et al., 2006]. Briefly, an adaptation of the detergent-free method described by Smart et al. [1995] was used. In this detergent-free method membrane extracts are obtained after isolation on a Percoll gradient, and are then sonicated, and submitted to two steps of Optiprep gradients. An opaque band above the interface of the 5% Optiprep phase of the second gradient is recovered and consists of the lipid rafts. The proteins were precipitated by the TCA-acetone method, solubilized 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 kit Bio-Rad DC protein assay (Bio-Rad).

WESTERN BLOTTING

Whole cell lysates or raft extracts were dissolved in Chaps buffer described above. The proteins were separated by sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE, resolving gel: gradient 4–15%, Bio-Rad). The migration buffer was 25 mM Tris base, pH 8.3, 192 mM glycine, 0.1% SDS. They were then electrophoreticaly transferred to a polyvinyldenfluoride membrane (PVDF, Immobilon P, Millipore) in the same buffer, with 10% ethanol, at 35 V for 3 h.

The free sites on the membrane were blocked by immersing it 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 immunoreactive proteins were revealed by enhanced chemiluminescence (ECL kit, Amersham). EGFR was detected as a band of ~110 kDa, Cx 43, 43 kDa, Caveolin 1, 20 kDa, GAPDH, 35 kDa, and Gβ 30 kDa. Densitometry of the blots was analyzed using a camera connected to a computer and image analysis software (AIDA, Raytest, Courbevoie, France).

STATISTICAL ANALYSES

The lipid data were analyzed using SigmaStat statistical software (version 3.1, SPSS) by one-way ANOVA, followed by the multiple comparison Student–Newman–Keuls test. If the equal variance or normality test failed, the data were analyzed using the nonparametric Kruskal–Wallis test. The data from Western blots were pooled from at least three experiments in duplicates, and analyzed by Students' *t*-test. Data are expressed as means ± SE.

RESULTS

CHARACTERIZATION OF THE CELLS

The cells isolated by our procedure were able to divide and proliferate in suspension and formed neurospheres after 4–5 days in culture. The proliferating cells were positive for nestin and FABP7 (Fig. 1).

Once allowed to adhere on P/L surfaces, and deprived of mitogenic stimulus for 8 days, they differentiated into neurons, astrocytes, and oligodendrocytes. Indeed, the ICC observations showed the presence of βIII tubulin and Neu N positive cells (neurons), GFAP and S100 positive cells (astrocytes) and O4 and CNPase positive cells (oligodendrocytes) (Fig. 1).

Although upon cell differentiation, the glial lineage was predominant, the percentage of neurons was nevertheless relatively high, representing 15–30%, depending on the culture (Fig. 2). This percentage tended to decrease after the fourth passage (~20 days in vitro) (result not shown).

Therefore, the cultures displayed the characteristics classically described for NSC/NP: detection of markers specific for undifferentiated cells, and the ability to generate glial and neuronal differentiated daughter cells [Ray and Gage, 2006].

PHOSPHOLIPID COMPOSITION OF PROLIFERATING NSC AND OF RAT PUP BRAINS

We analyzed the composition of the fatty acid present in EPG and PC: PC is usually the most abundant class of phospholipid in the cells, and EPG is the fraction where the percentage of PUFAs over TFA is highest. The results are reported in Table I for EPG and Table II for PC.

Our data showed that control neurospheres were rich in saturated fatty acids, since they represent 29–34% in EPG and 46–51% in PC. Second in abundance were the monounsaturated fatty acids, mainly 18:1n-9, with 23–39% in EPG and 39–47% in PC. The cells cultured under the standard conditions were highly deficient in PUFAs from both families, with percentages varying between 2% and 9% for the n-6 family, and 1% and 4% for the n-3 family. In both classes of phospholipids, AA and DHA were the most abundant of their respective family. Consistent with these results, the percentage of 20:3n-9 (trienoic fatty acid) was high (6.7% in EPG and 1.2% in PC compared to 0.1–0.5% in the rat pup brains): the presence of this fatty acid is a well-known marker of a PUFA deficiency.

When cells were exposed to increasing doses of AA or DHA, the levels were restored in a dose-dependent manner. The incorporation in the PL was significant at doses as low as 2 μM, and concomitantly, a decrease in the level of 20:3n-9 was registered. When the cells were exposed to 5 μM, the level of AA or DHA were close to 20% of the TFA of the EPG. The profiles were then very similar those obtained from brains derived of 1-day-old rat pups. The supplementation with 5 μM DHA or AA permitted to rectify the deficiency of control cultures to a level similar to the in vivo situation.

The data also showed that the cells were capable of metabolizing both PUFAs. When the cells were exposed to the highest dose of DHA, the percentage of EPA (20:5n-3) in EPG rose from 0.5% in the controls to 1.9% since this fatty acid arose from a retroconversion of

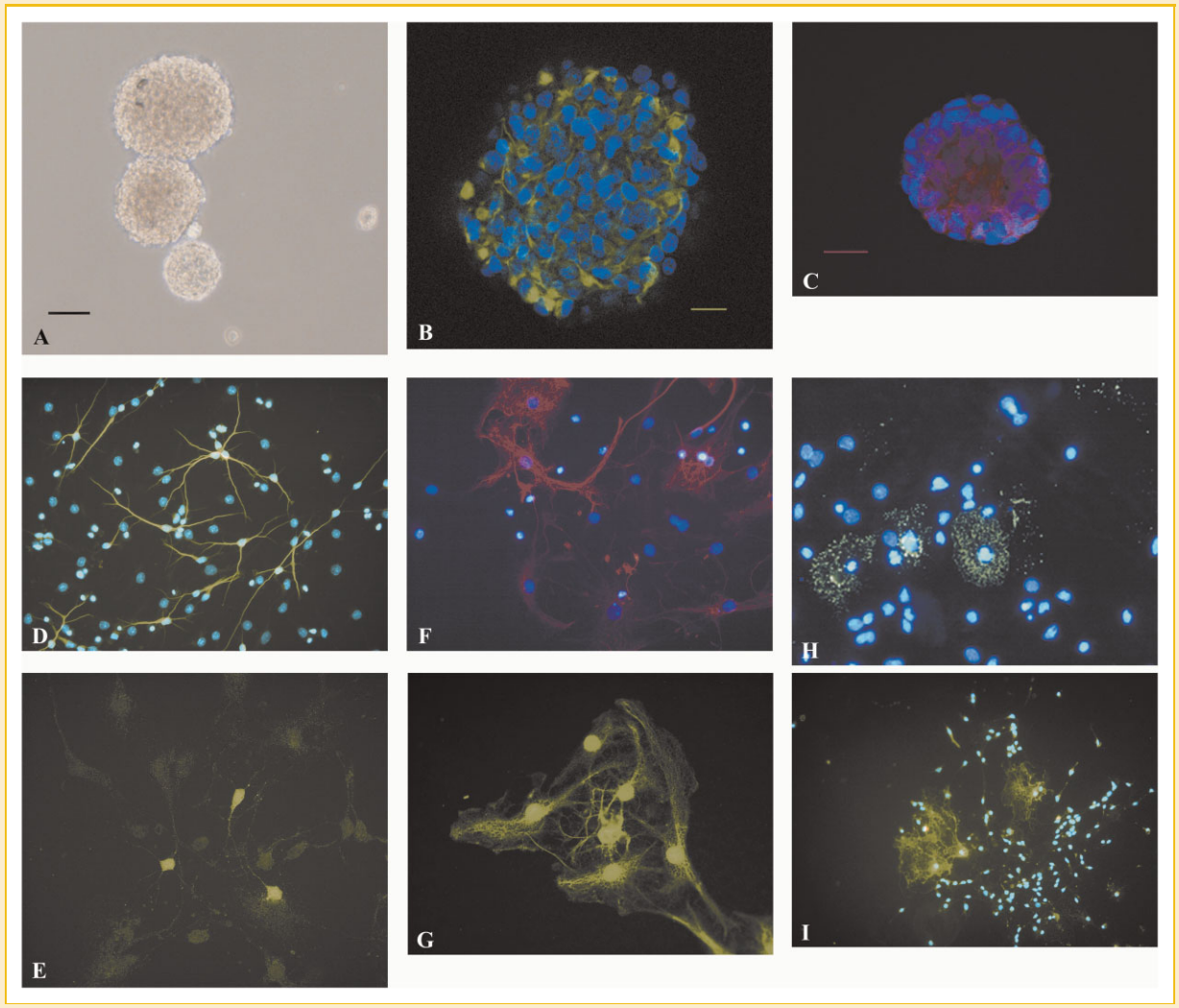

Fig. 1. Characterization of the cultures of NSC/NP. The proliferating cells formed neurospheres after 4–5 days of culture (A; scale bar 100 μm), and were positive for nestin (B; scale bar 10 μm) and FABP7 (C; scale bar 20 μm). When the cells were allowed to differentiate on P/L matrices in medium devoid of growth factors, the three types of cells were present: neurons, positive for βIII tubulin (D) and NeuN (E), astrocytes, positive for GFAP (F) and S100 (G), and oligodendrocytes, positive for O4 (H) and CNPase (I).

the DHA. In the same idea, DPA (22:5n-3) that was present at 0.2% in the control EPG, was found at 1.9% in the cells exposed to the highest dose of DHA; this derivative was synthesized by elongation of 20:5n-3. Similarly, when the cells were exposed to the highest dose of AA, 22:4n-6 and 22:5n-6 were synthesized, a sign that the peroxysomial β-oxidation was active in the cells. When the cells were supplemented with both PUFAs, at 2 μM each, the PC composition was very close to the composition of the n-3 supplemented brains. Therefore, the addition of PUFAs in the medium could restore a composition of phospholipids closer to the values observed in vivo in the brain.

CELL GROWTH AND PUFA SUPPLEMENTATION

The growth of the cells differently supplemented was monitored using EdU incorporation. The results are reported in Figure 3. The growth of the cells indifferently supplemented with 5 μM AA or DHA, or both (2 μM each), were significantly superior to the growth of control cells. For technical reasons, EdU was supplemented in the last 16 h of a 3-day incubation and yet the growth of the supplemented cells was still 25% higher than the growth of the control cells. Thus, PUFA supplementation enhanced cell proliferation significantly.

PROTEIN LOCALIZATION IN LIPID RAFTS OF CELLS GROWN IN THE ABSENCE OF EGF

Since the proliferation of supplemented cells was higher in the presence of mitogenic growth factors, this prompted us to question whether the increased growth was associated with a modification of the localization of growth factor receptors in the membrane: we thus turned to the analysis of the localization of EGFR and two other proteins in the membrane microdomains, since several studies showed that fatty acid composition could modify the membrane organization.

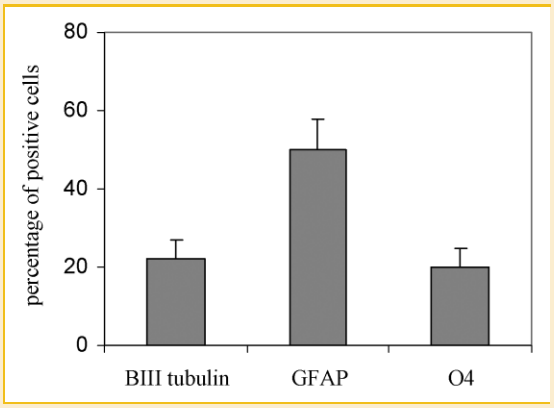

Fig. 2. NSC/NP differentiation. The cells grown in neurospheres were allowed to adhere on P/L glass coverslips and to differentiate for 8 days in medium devoid of growth factors. The cultures were fixed with paraformaldehyde, and labeled for specific markers: βIII tubulin for neurons, GFAP for astrocytes, O4 for oligodendrocytes. The cells were counterstained with DAPI, and the percentages of positive cells were established. At least 500 cells per experiment were counted.

We checked the presence of EGFR in the microdomain fraction. Since EGFR is rapidly internalized after EGF exposure, and therefore undetectable in the membrane, EGF was omitted in the culture medium for this set of experiments. The Western blot results are summed up in Figure 4.

EGFR content in the raft fraction was normalized to the caveolin 1 (Cav 1) content. EGFR was slightly but significantly more abundant in the microdomains in DHA-treated cells. The other treatments did not lead to consistent modifications. We checked whether addition of PUFAs modified the content of EGFR in whole

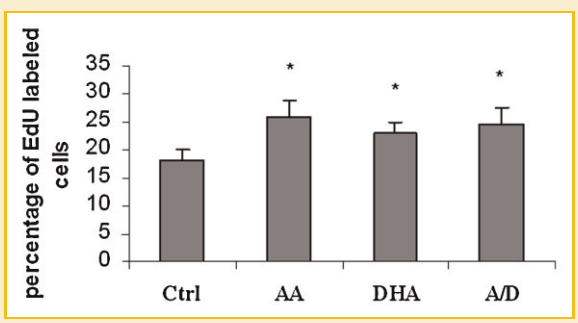

Fig. 3. Analysis of cell proliferation in the presence or absence of PUFAs. The neurospheres were dissociated, and 2×10^6 cells were allowed to grow for 3 days in complete medium, in the absence (Ctrl) or presence of 5 μM AA (AA), or 5 μM DHA (DHA), or both PUFAs at 2 μM each (A/D). EdU (10 μg/ml, final concentration) was incorporated in the medium in the last 16 h of incubation. The neurospheres were allowed to adhere on P/L glass coverslips in the last 12 h. The cells were fixed with 4% paraformaldehyde for 10 min, and the treatments of the samples followed the manufacturer's instructions. The number of dividing cells, labeled with EdU, was compared to the total number of cells, counterstained with DAPI. On each coverslip, at least 500 cells were counted.

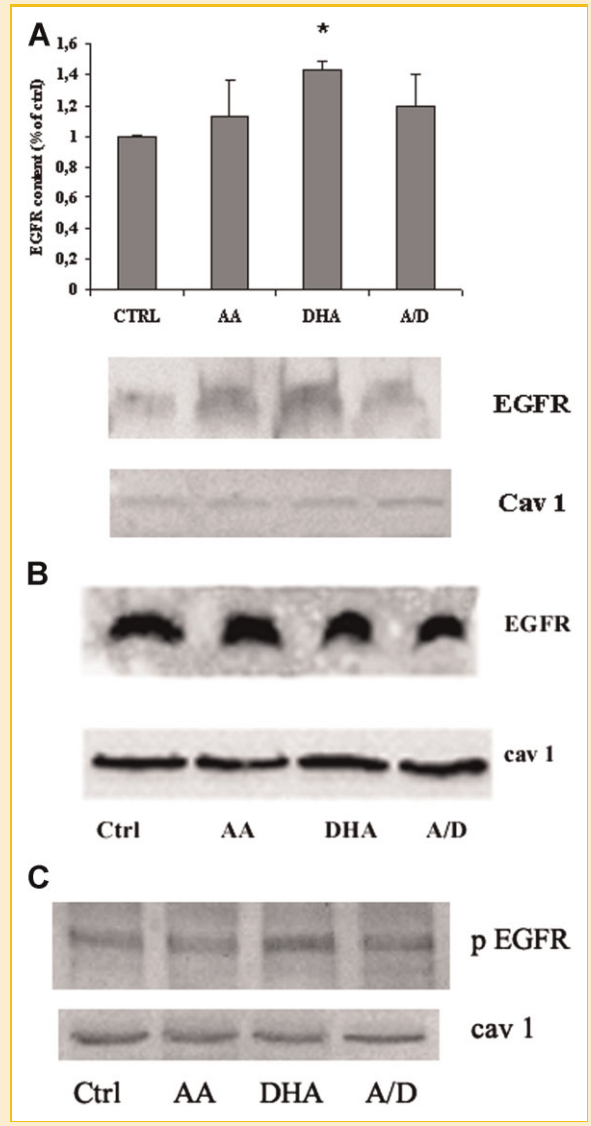

Fig. 4. Analysis by Western blotting of the localization of EGFR in the microdomains. After dissociation, 2×10^6 cells were grown for 3 days in medium deprived of EGF, in the absence (Ctrl), or presence of 5 μM AA (AA), or 5 μM DHA (DHA), or both PUFAs at 2 μM each (A/D). Panel A: Lipid rafts were extracted according to the method described above, and analyzed by Western blotting. EGFR content was normalized to Cav 1 content. The results of three separate experiments are shown in the histogram. $^*P < 0.05$ Students' *t*-test. Panel B: EGFR and Cav 1 contents in whole cell lysates. Panel C: phosphorylated EGFR and Cav 1 contents in whole cell lysates, recorded after a 5-min exposure to 50 ng/ml EGF.

cell lysates, and we could not detect any change under these conditions (Fig. 4B). We checked the phosphorylation status of EGFR consequently to EGF exposure, and determined that it was slightly increased in DHA-treated cells (Fig. 4C).

We also analyzed the presence of other proteins known to be included in the raft fractions: Cx 43 was slightly increased in PUFA-supplemented cells, although the increase was only significant for the DHA supplementation. Gβ subunit was not modified (Fig. 5).

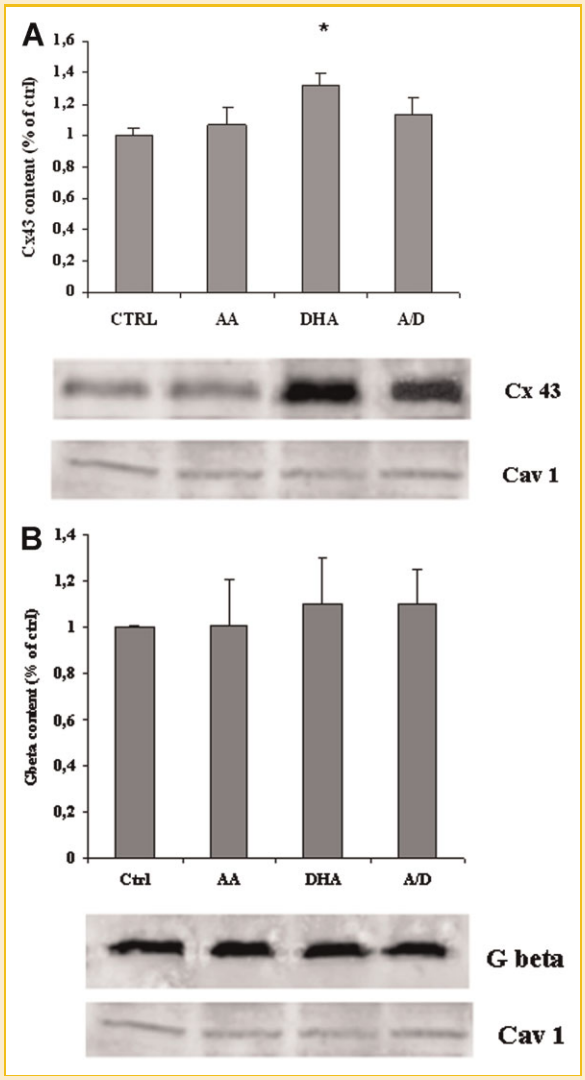

Fig. 5. Localization of Cx 43 and Gβ in the membrane microdomains in the absence of EGF. Panel A: The cells were treated similarly as described in Figure 3, that is, grown in the absence of EGF, and lipid rafts were analyzed for the presence of Cx 43. Treatments with PUFAs and legends are similar to the ones described in Figures 2 and 3. The results of three separate experiments are summed up in the histogram, Cx 43 content normalized to Cav 1 content. $^{*}P < 0.05$ Students' *t*-test. Panel B: The cells were grown in the absence of EGF, and assayed for the presence of Gβ subunit in the membrane microdomains. Treatments with PUFAs and legends are similar to the one described in Figures 2 and 3. The results of three separate experiments are summed up in the histogram, Gβ content normalized to Cav 1 content.

PROTEIN LOCALIZATION IN LIPID RAFTS OF CELLS GROWN IN THE PRESENCE OF EGF

The contents of Gβ and Cx 43 in rafts were also studied in cells grown in complete medium, that is, in the presence of EGF. The results are presented in Figure 6. The data were normalized to the Cav 1 content: we noticed that, in the presence of EGF, the Cav 1 content of the rafts was higher than in the absence of EGF (Figs. 4 and 6). Gβ remained not significantly modified by either treatment (not shown), but the increase in Cx 43 protein was observed again in

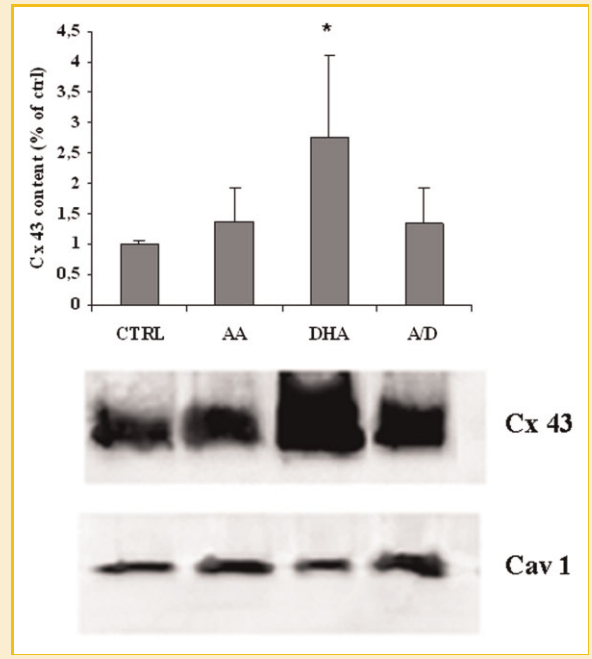

Fig. 6. Localization of Cx 43 in the membrane microdomains in the presence of EGF. After dissociation, 2×10^6 cells were grown for 3 days in complete medium, that is, in the presence of EGF, and in the absence (Ctrl), or presence of 5 μM AA (AA), or 5 μM DHA (DHA), or both PUFAs at 2 μM each (A/D). The lipid rafts were extracted and analyzed for the Cx 43 content. The histogram shows the results of three separate experiments, Cx 43 content normalized to Cav 1 content. $^{*}P < 0.05$ Students' *t*-test.

DHA-treated cells, and was even more markedly present than previously noticed in the absence of EGF (Figs. 5 and 6).

We checked the variations of the selected proteins in whole cell lysates, to verify if the supplementation would change their total contents. The results are shown in Figure 7: the total amounts of Gβ,

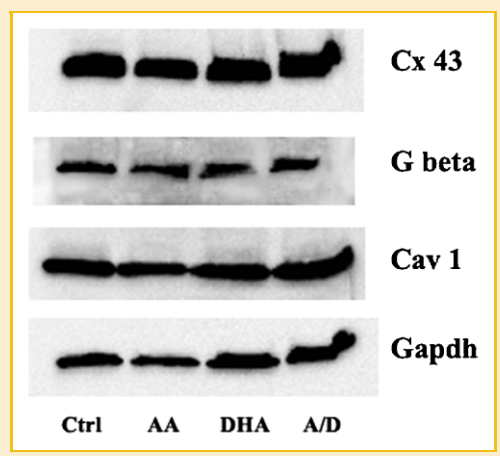

Fig. 7. Variations of total contents of Cx 43, Gβ, Cav 1, and Gapdh in cells supplemented or not with PUFAs; A fraction of the cells treated as described in Figure 5 was lysed and examined for the total contents of Cx 43, Gβ, Cav 1, and Gapdh. The legends are similar to the ones described in Figure 5. Gapdh content was used as a control for equal gel loading.

Cx 43, and Cav 1 were not modified by either treatment. Thus, the changes seen in rafts of DHA-supplemented cells may originate from a different position and placement of the proteins in the membrane.

DISCUSSION

The lipid analyses show that under standard conditions, the cells display profiles characterized by a high deficiency in PUFAs, a situation already reported for other cell models such as the neuroblastoma SH SY5Y, or astrocytes [Langelier et al., 2005; Champeil-Potokar et al., 2006]. Most of the cell cultures are performed in the presence of serum, which is then the vector for fatty acids. NSC/NPs necessitate growing in the presence of mitogens, since serum would induce premature differentiation [Ray and Gage, 2006]. Therefore, the NSC/NPs are found highly deficient in PUFAs, to a much higher extent than what was already reported, with profiles very different from those recorded in vivo, as shown by the data obtained from rat pup brains.

Yet they incorporate and metabolize PUFAs readily, at a very low concentration and the plateau of incorporation is reached at 5 μM for both PUFAs. This very low concentration is close to the concentrations of free fatty acids present in the brain—since it has been estimated that nonesterified DHA is present at 1.5 μM in this organ [Demar et al., 2005]. Also, it has been reported that AA could be released intracellularly at micromolar doses by the action of phospholipase A2 [Brash, 2001]. Membrane phospholipid fatty acid composition can therefore be easily modified to meet the values observed in the brain, with low doses of PUFAs, close to the in vivo situation.

We compared the proliferation of NSC/NP cells under the different conditions of supplementation, and noticed that the cell growth was higher when cells were grown in PUFA-containing medium. Because of the enrichment in PUFA, and the resulting probable modifications in membrane properties, we checked the presence in the lipid rafts of a set of proteins, known to be included in these domains. EGFR was analyzed first, because of the higher growth noted in complete medium, that is, in the presence of EGF and bFGF. We also chose to focus on EGFR because this receptor is especially important in the developing brain. Neural progenitors overexpressing EGFR acquire an undifferentiated and migrating phenotype [Boockvar et al., 2003; Aguirre et al., 2005]. Overexpression of EGFR in the subventricular zone also expands oligodendrocytes progenitors [Aguirre et al., 2007], and following brain injury, NSC are expanded via an increase in EGF responsiveness [Alagappan et al., 2009]. EGFR has been shown to be included in microdomains, but is not detectable after EGF exposure because it is internalized [de Laurentiis et al., 2007]. Therefore, we omitted EGF in the medium in this set of experiments. The only significant modification was observed in samples from DHA-supplemented cells, and EGFR was slightly increased. Thus, the increase in cell growth could not be attributed to a change in receptor localization, although it is not to be excluded that the enrichment in EGFR in DHA-treated cells could favor its accessibility to the ligand and further down signaling. Yet, this result invited us to look for other proteins, known to be present in microdomains.

Cx 43 plays a particularly interesting role in the developing brain and NSC [Elias et al., 2007; Kunze et al., 2009]. This protein has long been known to provide intercellular communication. Cell-to-cell exchanges of small molecules are the first and only type of cell communication in the developing brain, well before the synaptogenesis. Furthermore, Cx 43 is now recognized as an adherent protein, favoring cell migration, a crucial property during the migration of the neuroblasts along the radial glia [Elias et al., 2007; Kunze et al., 2009]. Cx 43 is present in the microdomains, and its lipid raft localization determines its role in cell migration [Schubert et al., 2002; Langlois et al., 2008; Leaphart et al., 2008]. We show here that its presence in the rafts is enhanced in DHA-supplemented cells. We noticed that the increase was even greater when EGF was included in the medium (Fig. 4). This can be connected to the observation of the study of Orlichenko et al. [2006], showing that EGF could promote the phosphorylation of caveolin1, and consequently, the formation of caveolae. Cx 43 is known to interact with caveolin1 [Schubert et al., 2002; Langlois et al., 2008], and therefore, with EGF present in the medium, it is likely that its incorporation in microdomains could be improved. In contrast, the Gβ levels in the rafts were not modified in either conditions, that is, with or without EGF, or under either PUFA treatment.

The only significant modifications in the levels of EGFR and Cx 43 in the rafts were noticed in DHA-treated cells. AA did not have the same implications for the microdomain compositions, although its supplementation led to a modification of the phospholipid compositions. This observation is in agreement with studies showing that DHA, because of its length and unsaturation, distinguishes from other fatty acids, and plays a particular role in membrane organization and fluidity. Recently, it has been reported in the rat model of Smith–Lemli–Optiz syndrome, that reduced retinal rod outer segment membrane fluidity was associated with a severe reduction in DHA content [Boesze-Battaglia et al., 2008]. DHA with its six double bonds possesses an extremely flexible structure, which makes DHA-containing phospholipids incompatible with close contact to cholesterol, and therefore create lateral segregation of fluid DHA-rich zones, and of rigid cholesterol-containing domains [Wassall and Stillwell, 2008].

In summary, we have shown that NSC cultures are severely deprived of PUFA under standard culture conditions, and that the cell membranes display highly deficient PUFA compositions, highly different from in vivo profiles. The restoration of the levels of PUFA can be easily achieved through a low-dose supplementation of the culture medium. We noticed variable consequences on the protein localization in lipid rafts. Our data suggest that, DHA particularly, could profoundly affect the protein properties and modulate their cell functions, through a different organization of the cell membrane, and this is consistent with its well-acknowledged effect on membrane fluidity.

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