Effects of a n-3 PUFA deficient diet on the expression of retinoid nuclear receptors, neurogranin and neuromodulin in rat brain

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Abstract: *A lot of studies performed in rodents revealed that n-3 polyunsaturated fatty acid (PUFA) deficient diets could induce deficits of learning capacities but the mechanisms involved are not well known. Retinoic acid (RA) and its nuclear receptors (RAR and RXR) play a central role in the maintenance of cognitive processes and synaptic plasticity via its action on target genes that are neurogranin (RC3) and neuromodulin (GAP43). Given some interferences were described between the retinoid and fatty acid signaling pathways, we investigated the effects of a α-linolenic acid (18:3 n-3) deficient diet on retinoic acid nuclear receptors (RAR, and RXR), on GAP43 and RC3, and on blood and brain fatty acid composition in rats at three times of diet: 3, 9 and 18 weeks. In blood and brain of these animals, we observed a severe n-3 PUFA deficit (18:3 n-3, 20:5 n-3 and particularly 22:6 n-3) associated with an increase in the n-6 PUFA content (mainly 22:5 n-6). Real-time PCR and western blot analysis allowed us to note that retinoid signaling, GAP43 and RC3 expression were affected in the striatum of the n-3 PUFA deprived rats.*

Key words: *n-3 PUFA deficient diet, retinoic acid nuclear receptors, brain, neurogranin, neuromodulin*

Introduction

Brain is a tissue rich in lipids (about 50-60 percent of its dry weight). Cerebral membranes are especially rich in n-6 and n-3 PUFAs; arachidonic (20:4 n-6, AA) and docosahexaenoic acids (22:6 n-3, DHA) are their main representatives. These PUFAs are specifically present in membrane phospholipids such as phosphatidylethanolamine (PE).

According to literature [1], AA and DHA play fundamental roles, notably the second which is essential for structure and functional development of brain during prenatal and early life.

Since several years, the involvement of PUFAs in brain functioning is well studied. For instance, some studies performed in rodents fed with variable and controlled contents of n-6 and n-3 PUFAs evidenced an influence of PUFAs on cognitive processes [2, 3]. It emerges that decreased cerebral amounts of DHA resulting from dietary n-3 deficiency are associated with impaired cognitive function such as decreased learning performances and disorders of memory and attentiveness. In addition, data obtained in human [review in 4] reported that some neurological disorders (autism, schizophrenia, depression) could be related to a membrane PUFA deficiency.

Multiple roles have been described for PUFAs within the central nervous system (CNS) [5]. In addition to their impact on the membrane biophysical properties (with consequences on membrane fluidity, ionic transport and interaction with membrane proteins), and their involvement in regulation of neurotransmitter release and synthesis of biologically active oxygenated derivatives, PUFAs could have also a transcriptional action. To modulate nuclear receptor-mediated transcription of genes [6], the nuclear receptors responsive to fatty acids (PPAR, peroxisome proliferator-activated receptors) have to form a functional transcriptional unit upon heterodimerization with RXR (retinoid X receptor), one of the nuclear receptors of retinoids.

Vitamin A and retinoic acid (RA), its most potent natural metabolite, play a significant role within the CNS, not only during brain development but also in the function of the mature brain [7-9]. In brain, RA controls, via its nuclear receptors RAR (retinoic acid receptor) and RXR, the expression of genes involved in synaptic plasticity, memory [10] and cognitive processes [11]. Among RA target genes, there are those coding for two identified neuron-specific protein kinase substrates implicated in molecular mechanisms underlying synaptic plasticity and memory formation: the neurogranin or RC3 [12] and the neuromodulin or GAP43 [13]. These two proteins are expressed on both sides of the synaptic cleft and are considered as good markers of dendrite spine density.

In this context, we hypothesized that a modification of the bioavailability of the nuclear modulators that are PUFA (by establishing a n-3 PUFA deficiency), could induce modifications of nuclear receptor (RAR and RXR) expression patterns. These modified profiles were described in our laboratory as able to cause modifications of the expression of genes involved in synaptic plasticity (RC3 and GAP43) with consequences on the synaptic plasticity state. Alterations of this state have been described as responsible, at functional level, for disorders of memory performances.

Thus, the retinoid nuclear receptor (RAR and RXR) expression and that of neuromodulin and neurogranin were measured in striatum, a brain area involved in memory processes. Levels of mRNA and proteins were respectively measured by real time RT-PCR and western blot analysis. At last, plasma, red blood cell membrane and brain fatty acid patterns were investigated.

Materials and methods

Experimental protocol

Our study was performed with male rat pups at weaning (Wistar), randomly divided into two experimental groups designated as n-3 adequate (n = 22) and n-3 deprived (n = 20) diets. The first group received a n-3 PUFA adequate diet which consisted of a mixture of peanut and rapeseed oils in the same proportions (50/50, v/v). The 18:2n-6/18:3n-3 (LA/ALA) ratio amounted to 5 and was conformed to the current French recommendations. The second group fed a α-linolenic acid (18:3 n-3) deficient diet, made of peanut oil; the LA/ALA ratio was equivalent to 232. In the two diets, lipids represented 5% of the ration and globally exhibited the same proportions of SFA, MUFA and PUFA (*table 1*). All animals were fed and given water ad libitum. Each rat was weighed three times weekly; food intake was recorded daily. Rats fed the diets during three different periods: 3, 9 and 18 weeks. At the end of each time, rats were sacrificed by decapitation. Blood and brain were rapidly

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removed, and individual brain sections (striatum) were dissected out and then stored at – 80 °C for subsequent analysis.

RNA extraction and reverse transcription

Total RNA was extracted from striatum by using TRIzol reagent (Invitrogen, France) according to the manufacturer's protocol. Purified RNA was quantified and assessed for purity by UV spectrophotometry. RNA samples were reverse-transcribed as previously described [14] with minor modifications: reverse-transcription was conducted from 1 μg of total RNA and each target gene was co-reverse-transcribed with PPIB (cyclophilin B) as reference gene.

Analysis of gene expression by using real-time PCR

The polymerase chain reaction (PCR) was carried out involving a LightCycler system (Roche Diagnostics, Mannheim, Germany), and by using LightCycler DNA Master SYBR Green according to the manufacturer's instructions, as previously described [15].

Forward and reverse primers sequences used were as follows: PPIB sense 5′-GTT CTGGAAGGCATGGATGT-3′, antisense 5′-TCC CCGAGGCTCTCTCTACT-3′; RARβ sense 5′-CAGCTGGGTAAATACACCACGAA-3′, antisense 5′-GGGGTATACCTGGTACAAATTCTGA-3′; RXRβγ sense 5′-AGGCAGGTTTGCCAAG CTTCTG-3′, antisense 5′-GGAGTGTCTCCAA TGAGCTTGA-3′; RC3 sense 5′-GCTCCAAG CCAGACGACGATATTC-3′, antisense 5′-CAC TCTCCGCTCTTTATCTTCTTC-3′; GAP43 sense 5′-AGAAAGCAGCCAAGCTGAGGAGG-3′, antisense 5′-CAGGAGAGACAGGGTTCAGGTGG-3′.

Quantification data were analyzed using the LightCycler Relative Quantification Software, 3.5. (Roche Diagnostics, Mannheim, Germany). The interest of this software is illustrated in Féart *et al.* [15]. In our case, the calibrator was chosen among the rats fed the n-3 adequate diet.

Western blot analysis

Western blot analysis was performed on striatum of rats fed the two experimental diets for 18 weeks, according to the procedure described by Husson *et al.* [16] for the experiment concerning RC3 and β-actin, and as decribed by Husson *et al.* [17] for the expression of GAP43. The staining intensity of protein bands was determined using an image analyser (Quantity One, Biorad Laboratories, USA). The relative levels of RC3, GAP43 and β-actin proteins were determined as percent of RC3, GAP43 and β-actin respectively of n-3 adequate rats.

Lipid analyses

Extraction of brain lipids

Total lipids of brain were extracted by using the method of Folch *et al.* [18], with 20 volumes of chloroform/methanol (2/1, by vol.) per g of tissue. Extraction was made under agitation at room temperature; after 1 h, 0.2 volumes of KCl (0.8% in water) were added per volume of extraction mixture. Hydroalcoolic and chloroformic phases were separated by centrifugation. The hydroalcoolic phase was removed and the chloroformic phase was washed with "upper phase". After centrifugation, the chloroformic phase was filtered and the pellet washed with chloroform/methanol (2/1, by vol.); then solvents were evaporated under vacuum, at room temperature with a rotary evaporator. The lipid extract was taken again with chloroform and filtered to obtain a chloroformic solution. The solvent was evaporated under nitrogen and dry extract was taken again with chloroform/methanol (2/1, by vol.). The final solution called "Folch extract" was stored at – 20 °C.

Table 1. *Lipid composition of n-3 PUFA adequate and deficient diets.*

	Adequate	Deficient
Lipid content (g/100 g diet)	5	5
Oil composition (g/100 g diet)		
Rapeseed oil	2,5	0
Peanut oil	2,5	5
Lipid contributions	1,25	1,25
SFA[1] (g/day)	0,17	0,24
MUFA (g/day)	0,76	0,72
PUFA (g/day)	0,32	0,29
18:2n-6 (mg/day)	273	290
18:3n-3 (mg/day)	50	1,25
18:2n-6/18:3n-3	*5*	*232*

[1]SFA, saturated; MUFA, monounsaturated; PUFA.

Isolation of brain phosphatidylethanolamine (PE)

Solvent of "Folch extract" was evaporated to dryness under a stream of nitrogen. Lipids were taken up in an appropriate volume of chloroform/methanol (2/1, v/v). Total phospholipids of brain were separated by thin layer chromatography (TLC) with using plates pre-coated with 0,35 mm silica gel 60H (Merck, France). A volume of "Folch extract" was deposed on silica gel; the solvent system used for separation was a mixture of chloroform/methanol/acetic acid/water (75/45/12/6, by vol). After migration and revelation by DCF (0.2% in ethanol), the silica gel area corresponding to PE was visualized under U.V. (254 nm), removed from the TLC plate and transferred in a glass tube for preparation of fatty acid methyl esters.

Preparation of fatty acid methyl esters (FAME)

Total fatty acids of brain PE were methylated according to the method of Morrison and Smith [19]. 1mL of boron trifluoride methanol solution (14%; w/v) (SigmaChemical Co.) was added to the silica gel area corresponding to PE in a glass tube, maintained for 20 min at 90°C after closing. After addition of 1 mL of NaOH (5N), FAME obtained were extracted three times with 2 mL of hexane. Hexanic phases were concentrated, washed with 1 mL of water and stored at – 20 °C.

Plasma lipids

Total fatty acids of plasma were methylated according to the method of Lepage and Roy [20]. 2 mL of methanol/benzene (4/1, v/v) were added to 400 μL of plasma. Then, under agitation and at 0 °C, 200 μL of acetyl chlorure were added, and this mix was maintained for 1 hour at 100 °C in a closed tube. To stop the reaction, 5 mL of Na_2CO_3 6% (w/v) were added to the mixture. After centrifugation, the upper phase containing FAME was removed and stored at – 20 °C.

Analyses of FAME

Analyses of total FAME were carried out on gas chromatograph equipped with a flame-ionization detector and a split injector. A fused-silica capillary column (BPX 70, 60 m × 0.25 mm i.d., 0.25 μm film; SGE, France) was used with H_2 as a carrier gas (inlet pressure : 1 bar). The split ratio was 1:70. The column temperature was programmed from 150 C to 200 °C at 1.5 °C/min for 25 min, then from 200 °C to 225 °C at 20 °C/min and held at 225 °C until completion of the analysis (20 min). The injection port and the detector were maintained at 250 and 280 °C respectively. The gas chromatography (GC) peaks were integrated using a SP 4400 integrator

(Spectra Physics, San Jose, CA). Identification of each fatty acid methyl ester was made by comparison of retention time of authentic standards (Sigma Chemical Co.).

Statistical analysis

Values are given as means and standard errors of the mean (SEM). The statistical significance of differences between means was calculated by ANOVA followed by Student's t-test ($P < 0.05$) using Statgraphics Plus 5.1. software.

Results and discussion

Retinoic acid nuclear receptor expression

The retinoic acid nuclear receptor expression was studied in the striatum of rats fed the deficient diet for 3, 9 and 18 weeks.

In comparison with animals of the adequate group, animals fed the deficient diet exhibited i) after 3 weeks, no significant variation of expression of any nuclear receptors ; ii) after 9 weeks, a strongly decreased expression of RARβ (– 28.2%, $p < 0.001$); iii) after 18 weeks, no significant variation of RARβ expression.

Data have shown in many animal tissues that retinoid signaling pathway was susceptible to fatty acid supply [21] and consequently to the level of activity of their signaling pathway. So the deficient diet could lead to a decrease of the expression of retinoid receptors, as observed after 9 weeks, which would indicate a hypoactivity of the retinoid signaling pathway.

Synaptic plasticity marker expression (table 2)

The expression of neurogranin (RC3) and neuromodulin (GAP43) was investigated at the mRNA and protein levels. The n-3 deprived rats displayed i) after 3 and 9 weeks, no different RC3 and GAP43 mRNA contents between the two experimental groups; ii) after 18 weeks, a decreased expression of the two synaptic plasticity markers: – 14% (at mRNA level) and –18% (at protein level) for RC3; – 24% (at protein level) for GAP43.

Considering the involvement of RARβ in the regulation of RC3 and GAP43 expression, the lack of decreased expression of these two synaptic plasticity markers after 9 weeks is difficult to explain. This result suggests a possible regulation of RC3 and GAP43 expression by another signaling pathway as it has been reported by Guadano-Ferraz *et al.* [22] about the transcriptional induction of RC3 by thyroid hormone. Concerning the difference of expression between GAP43 mRNA and protein, Namgung and Routtenberg [23] have previously suggested a post-transcriptional regulation of this synaptic plasticity marker.

Regarding the consequences of such results, if we consider some bibliographic data showing that RC3 knockout mice have impaired synaptic plasticity and spatial learning [24], and as well as that decreased GAP43 expression was associated with reduced neuronal plasticity and learning [25], we can suppose that the n-3 deficiency could lead to similar cognitive alterations.

Table 2. *Influence of the deficient diet on the mRNA and protein expression (% expression/n-3 adequate diet) of neurogranin and neuromodulin in striatum.*

		3 weeks	9 weeks	18 weeks
RC3[1]	mRNA	98,18 ± 4,55	94,85 ± 6,19	86,21 ± 3.5*
	protein	ND[2].	ND[2].	82.03 ± 12.91***
GAP43[1]	mRNA	90,82 ± 6,12	92,93 ± 4,04	108,33 ± 2,08
	protein	ND[2]	ND[2]	76,42 ± 10,45*

Data represent the mean ± SEM of measures performed on 6 rats.
[1] RC3, neurogranin; GAP43, neuromodulin.
[2] ND, not determined.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, significantly different from adequate diet.

Effects of the deficient diet on plasma and brain PE fatty acid composition

Plasma polyunsaturated fatty acid composition (table 3)

The plasma n-6 and n-3 PUFA composition of rats fed the deficient diet changed all along the study; these modifications consisted in i) an important decrease (– 80%) of the total n-3 PUFA proportions, for α-linolenic acid (18:3 n-3) as much as for long-chain derivatives (eicosapentaenoic 20:5 n-3, docosapentaenoic 22:5 n-3, and docosahexaenoic 22:6 n-3 acids); ii) a slight increased percentage (+10%) of total n-6 PUFAs, specially arachidonic (20:4 n-6) and docosapentaenoic (22:5 n-6) acids. Nevertheless, the linoleic acid (18:2 n-6) proportion was diminished, despite the same supply of both experimental diets. This suggests that this fatty acid is used for synthesis of the n-6 long-chain derivatives (20:4 and 22:5), because of the natural competition phenom-

Table 3. *Influence of the deficient diet on plasma polyunsaturated fatty acid composition (% of total fatty acids).*

	3 weeks		9 weeks		18 weeks	
Polyunsaturated fatty acids[1]	**Adequate**	**Deficient**	**Adequate**	**Deficient**	**Adequate**	**Deficient**
18:2 n-6	12,33 ± 0,29	11,24 ± 0,36*	12,31 ± 0,22	10,78 ± 0,25***	13,33 ± 0,33	12,02 ± 0,35*
20:4 n-6	22,98 ± 0,74	27,39 ± 1,03*	19,76 ± 0,85	22,31 ± 0,31*	18,72 ± 0,57	21,76 ± 0,72**
22:5 n-6	0,09 ± 0,01	1,82 ± 0,12***	0,07 ± 0,01	1,32 ± 0,12***	0,06 ± 0,01	1,41 ± 0,13***
Total n-6 PUFA	36,31 ± 0,72	41,67 ± 0.74***	32,96 ± 0,75	35,55 ± 0,44*	33,27 ± 0,67	36,52 ± 0,61**
18:3 n-3	0,7 ± 0,05	0,04 ± 0.00***	0,87 ± 0,04	0,06 ± 0,01***	0,68 ± 0,03	0,03 ± 0,00***
20:5 n-3	0,66 ± 0,05	0,03 ± 0.00***	0,75 ± 0,07	nd[2***]	0,59 ± 0,04	nd.[2***]
22:5 n-3	0,54 ± 0,04	0,09 ± 0,01***	0,49 ± 0,04	0,06 ± 0,01***	0,43 ± 0,02	0,09 ± 0,01***
22:6 n-3	3,43 ± 0,11	0,84 ± 0,04***	2,98 ± 0,11	0,55 ± 0,03***	2,56 ± 0,07	0,8 ± 0,02***
Total n-3 PUFA	5,33 ± 0,10	1,00 ± 0.005***	5,09 ± 0,09	0,66 ± 0.02***	4,27 ± 0,09	0,72 ± 0,03***

Data represent the mean ± SEM of measures performed on 6 rats.
[1] Polyunsaturated fatty acid abbreviations: 18:2n-6, linoleic; 20:4n-6, arachidonic; 22:5n-6, docosapentaenoic; 18:3n-3, α-linolenic; 20:5n-3, eicosapentaenoic; 22:5n-3, docosapentaenoic; and 22:6n-3, docosahexaenoic acids. PUFA, polyunsaturated fatty acids.
[2] nd., not detected.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, significantly different from adequate diet.

Table 4. *Influence of the deficient diet on brain phosphatidylethanolamine (PE) polyunsaturated fatty acid composition (% of total fatty acids).*

	3 weeks		9 weeks		18 weeks	
Polyunsaturated fatty acids[1]	**Adequate**	**Deficient**	**Adequate**	**Deficient**	**Adequate**	**Deficient**
18:2 n-6	0,28 ± 0,02	0,21 ± 0,01*	0,2 ± 0,00	0,17 ± 0.01***	0,39 ± 0,02	0,36 ± 0,02
20:4 n-6	10,35 ± 0,17	10,9 ± 0,21	9,2 ± 0,07	10,35 ± 0,09***	9,43 ± 0,09	10.27 ± 0.13***
22:5 n-6	0,91 ± 0,03	3,88 ± 0,12***	0,46 ± 0,02	4,16 ± 0,07***	0,41 ± 0,01	4,52 ± 0,10***
Total n-6 PUFA	17,56 ± 0,19	21,5 ± 0,35***	15,2 ± 0,09	20,98 ± 0,15***	15,56 ± 0,11	21,45 ± 0,21***
22:5 n-3	0,26 ± 0,01	0,12 ± 0,00***	0,23 ± 0,01	0,06 ± 0,00***	0,18 ± 0,00	0,04 ± 0,00***
22:6 n-3	17,42 ± 0,49	14,49 ± 0,35***	16,62 ± 0,31	12,88 ± 0.20***	14,91 ± 0,15	10,33 ± 0,20***
Total n-3 PUFA	17,88 ± 0,49	14,61 ± 0,35***	16,88 ± 0,31	12,93 ± 0,20***	15,09±0,15	10,37 ± 0,20***

Data represent the mean ± SEM of measures performed on 6 rats.
[1] Polyunsaturated fatty acid abbreviations: 18:2n-6, linoleic; 20:4n-6, arachidonic; 22:5n-6, docosapentaenoic; 22:5n-3, docosapentaenoic; and 22:6n-3, docosahexaenoic acids. PUFA, polyunsaturated fatty acids.
* P< 0.05; *** P< 0.001, significantly different from adequate diet.

enon between n-6 and n-3 fatty acids towards enzymes of the fatty acid metabolism; in the deficient group, preference is given to the n-6 fatty acids.

At the same time, we studied the total fatty acid composition of red blood cell membranes, as described previously [26]. This membrane model gives indications about the incorporation ability of dietary fatty acids into cell membranes. The same patterns as those observed in plasma were obtained for n-6 and n-3 PUFAs.

The study of the plasma total fatty acid composition is of very particular interest because it is an indicator of fatty acids usable by brain [27]. Some authors [28] reported that low plasma DHA content was a significant risk factor for the development of Alzheimer disease and appeared to be common in cognitive impairment with aging. Others [29], in epidemiological studies, evidenced in old subjects, that a higher proportion of n-6 PUFAs and a lower n-3 PUFA content in erythrocyte membrane were associated with a greater risk of cognitive decline, diverse neuropsychiatric and neurodegenerative diseases.

Brain phosphatidylethanolamine polyunsaturated fatty acid composition (table 4)

Phosphatidylethanolamine (PE), with phosphatidylcholine, is the most abundant phospholipid in rat cerebral membranes but especially the richest in DHA.

Like in plasma, total n-6 and n-3 PUFA proportions were modified by the deficient diet but in a different way, i) the n-6 DPA (22:5 n-6) percentage was extensively increased as the survey went (+ 326% after 3 weeks and + 1002% after 18 weeks); ii) concomitantly, the DHA proportion was diminished (– 17% and – 31%, after 3 and 18 weeks respectively). Data of the literature mentioned that a declined brain DHA percentage was balanced by an increase of the n-6 DPA rate in order to maintain the membrane insaturation rate.

Thus, in the experimental conditions tested, cerebral membranes were impoverished in DHA. Considering its involvement in brain functioning and its ability to bind RXR [30], the loss of DHA could have neurological consequences. This assumption is in accordance with some results [31] that described poorer performance in spatial tasks concomitantly to a loss of brain DHA. Others [32] demonstrated, by studying the effects of a n-3 PUFA deprived diet in rat, that the resulting disturbed brain PUFA metabolism (elevated n-6 DPA and reduced DHA proportions) may be involved in human depression, aggression, and bipolar disorder.

These data are just preliminary results of a wide work about the effects of PUFA dietary contributions on the brain vitamin A action. Other measures of nuclear receptor expression have to be done in striatum and in another brain area implicated in memory processes (hippocampus).

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