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Abstract **Because brain membranes contain large amounts of docosahexaenoic acid (DHA, 22:6n-3), and as (n-3) PUFA dietary deficiency can lead to impaired attention, learning, and memory performance in rodents, we have examined the influence of an (n-3) PUFA-deprived diet on the central cholinergic neurotransmission system. We have focused on several cholinergic neurochemical parameters in the frontal cortex and hippocampus of rats fed an (n-3) PUFA-deficient diet, compared with rats fed a control diet. The (n-3) PUFA deficiency resulted in changes in the membrane phospholipid compositions of both brain regions, with a dramatic loss (62–77%) of DHA. However, the cholinergic pathway was only modified in the hippocampus and not in the frontal cortex. The basal acetylcholine (ACh) release in the hippocampus of deficient rats was significantly (72%) higher than in controls, whereas the KCl-induced release was lower (34%). The (n-3) PUFA deprivation also caused a 10% reduction in muscarinic receptor binding. In contrast, acetylcholinesterase activity and the vesicular ACh transporter in both brain regions were unchanged. Thus, we evidenced that an (n-3) PUFA-deficient diet can affect cholinergic neurotransmission, probably via changes in the phospholipid PUFA composition.**—Aïd, S., S. Vancassel, C. Poumès-Ballihaut, S. Chalon, P. Guesnet, and M. Lavialle. **Effect of a diet-induced n-3 PUFA depletion on cholinergic parameters in the rat hippocampus. *J. Lipid Res.* 2003.** 44: **1545–1551.**

Supplementary key words α-linolenic acid deficiency • docosahexaenoic acid • acetylcholine • frontal cortex • microdialysis

Brain membranes are rich in the polyunsaturated fatty acids (PUFAs), arachidonic acid (AA, 20:4n-6), and docosahexaenoic acid (DHA, 22:6n-3). Mammals must obtain the linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) from which they are derived from their diet. These long-chain PUFAs may be important for the structure and function of many membrane proteins, including receptors, enzymes, and active transport molecules (1, 2). Dietary α-linolenic acid deficiency that reduces the brain DHA contents has been directly linked to impaired central nervous system (CNS) function (3, 4). Rodents subjected to a chronic (n-3) PUFA dietary deficiency suffer from impaired attention and learning ability (5, 6), so that their performance in shock avoidance tasks (3), olfactory learning (6), and exploratory (7) and flexibility behavior (8) are poor. We have investigated the dopaminergic pathway in order to explain these cognitive deficits (9–14), and found marked changes in the synthesis and release of dopamine in the mesocorticolimbic system. Although cholinergic pathways are also important for cognitive function, the effect of PUFA on acetylcholine (ACh) neurotransmission has not been studied. Several studies have demonstrated that ACh is critical for processes underlying arousal, attention, learning, and memory (15, 16). Others have shown that attention and learning are correlated with the release of ACh in the cortex and hippocampus (17–19).

This lack of data on the effects of a chronic α-linolenic acid-deficient diet on cholinergic neurotransmission prompted us to examine several parameters of cholinergic function to determine the nature and extent of changes in cholinergic synapse function in rats deprived of (n-3) PUFA. We analyzed those brain areas that have prominent cholinergic inputs from the basal forebrain (frontal cortex and hippocampus) (20, 21). Microdialysis was used to monitor basal and KCl-stimulated ACh concentrations in (n-3)-PUFA-deficient rats and in rats fed a diet with an adequate (n-3) PUFA supply. Muscarinic receptors were investigated by autoradiography and vesicular acetylcholine transporter (VAChT) binding sites by the in vivo binding of [^{125}I]benzovesamicol (IBVM). Cholinergic catabolism was assessed by measuring acetylcho-

Abbreviations: AA, arachidonic acid; ACh, acetylcholine; AChE, acetylcholinesterase; CNS, central nervous system; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; IBVM, iodobenzovesamicol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; ROD, relative optical density; VAChT, vesicular acetylcholine transporter.

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linesterase (AChE) activity. The data on the cholinergic neurochemical parameters were correlated with the phospholipid fatty acid compositions of the hippocampal and cortical membranes.

MATERIALS AND METHODS

Animals and diets

Two generations of female Wistar rats were fed a diet containing 6% fat in the form of African peanut oil (deficient in α-linolenic acid), giving about 1,200 mg linoleic acid and <11 mg α-linolenic acid per 100 g diet (deficient diet) (**Table 1**). Two weeks before mating, a second generation of deficient females was divided into two groups. The first group was fed the deficient diet, and the second group was fed a control diet (peanut oil and rapeseed oil) containing about 1,200 mg linoleic acid and 300 mg α-linolenic acid per 100 g diet. At weaning, the males from each litter were housed two per cage with free access to the same diet as their mothers, and under controlled temperature (22 ± 1°C), humidity (50 ± 10%), and light cycles (7 AM to 7 PM). Experiments were performed on 2- to 3-month-old rats. The experimental protocol complied with the European Community guidelines (directive 86/609/EEC).

Fatty acid analysis of phospholipid classes

Rats were killed by decapitation. Their brains were quickly removed, and the hippocampus and the frontal cortex were dissected out on ice, weighed, and frozen in liquid nitrogen. Total lipids were extracted by a modification of the Folch method (22). Phospholipid classes (phosphatidylcholine, PC; phosphatidylethanolamine, PE; and phosphatidylserine, PS) were separated from total lipids on an aminopropyl-bonded silica gel cartridge (BAKERBOND spe™ Amino) by the method of Alessandri and Goustard-Langelier (23). The fatty acids were then transmethylated with 10% boron trifluoride (Fluka, Socolab, France) at 90°C for 20 min (24), and the composition of each phospholipid class was determined by gas chromatography (Carlo Erba) (25). The fatty acid methyl esters were 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. Differences between control and deficient rats were analyzed by one-way ANOVA followed by a post hoc Bonferroni *t*-test. The significance of differences between cerebral regions within a single dietary group was analyzed by paired Student's *t*-test. Significance was set at $P < 0.01$.

TABLE 1. Composition of the experimental diets

	Control	(n-3) PUFA Deficient
	(g/100 g diet)	
Casein (vitamin free)	22	22
DL-methionine	0.2	0.2
Cellulose	2	2
Mineral mix[a]	4	4
Vitamin mix[a]	1	1
Corn starch	42.6	43.2
Sucrose	21.3	21.6
Fats[b]	*(g/100 g diet)*	
African peanut oil	3.8	6.0
Rapeseed oil	2.2	—
Fatty acid composition	*(mg/100 g diet)*	
18:2n-6	1,194	1,200
18:3n-3	296	11

The diet provided about 16.5 MJ/kg diet. Lipids provided 13.5% of total calories. Oils were kindly supplied by Lesieur-Alimentaire (Coudekerque, France).

[a] According to (10).

[b] Total dietary lipids: 6 g/100 g diet.

Microdialysis

Rats were anesthetized at 10 AM with urethane (1.5 mg/Kg ip), and commercially supplied probes (4 mm-long membrane, polycarbonate, 35 kDa cut-off; MAB 6, Sweden) were stereotaxically implanted in the left lateral hippocampus [−5.6 mm anterior to the bregma, 4.4 mm lateral, −7.5 mm from the dura (26)]. Body temperature was maintained at 37°C using a thermostatically controlled heating blanket (CMA 150, CMA, Microdialysis, Sweden). Ringer solution (147 mM NaCl, 4 mM KCl, 3.4 mM $CaCl_2$) containing 0.5 μM neostigmine (Sigma, France) was perfused through the probe at 2 μl/min. All studies included a 90 min washout period prior to collecting 20 min dialysates. Three dialysate samples were then collected and defined as basal samples. Potassium chloride (KCl, 100 mM) was then added to the perfusion buffer for 40 min. Return to basal ACh level was recovered for the next 1 h 40 min (five samples). Samples of dialysate were stored at –80°C.

The rats were decapitated immediately after the microdialysis. Their brains were removed and quickly frozen. The probe location was checked on coronal cryosections. Any results obtained with an incorrectly located probe were discarded. With careful handling and storage, a probe could be reused for as many as five acute experiments. The recovery of ACh through the probe was tested in vitro prior to each experiment using Ringer's solution containing 500 nM ACh.

Determination of ACh

Perfusate samples (20 μl) were assayed for ACh by HPLC with electrochemical detection. ACh was separated on a reverse-phase analytical column (C18 Superspher, 100 mm × 2 mm, 4 μm, Macherey-Nagel, France) using a mobile phase (flow rate 0.3 ml/min) of 50 mM KH_2PO_4, 0.5 mM tetramethylammonium chloride, 2.5 mM heptane-sulfonic acid, and 0.01% (v/v) bactericide (pH 7). ACh was then enzymatically converted to hydrogen peroxide in a postcolumn solid phase reactor containing covalently bound AChE and choline oxidase, and the resulting H_2O_2 was detected electrochemically using a platinum electrode operating at a potential of +500 mV. The detection limit was 50 fmol/20 μl dialysate. The signal was recorded and the ACh was quantified by comparison with ACh standard solutions and corrected for the in vitro recovery of the probe. The mean basal ACh release for each animal was estimated by averaging the three samples collected during the 1 h preceding KCl perfusion, and expressed as pmol per 20 μl dialysate. ACh release is expressed as a percentage of the baseline value for each animal. Statistical differences between dietary groups for each collection time were tested using Student's *t*-test and were considered significant when $P < 0.05$.

Autoradiography of muscarinic receptors

Animals were killed by decapitation, and their brains were rapidly removed and frozen in isopentane at −35°C. Coronal cryosections (20 μm) were cut, thaw-mounted on gelatin-coated microscope slides, and stored at −80°C. The [^{3}H]scopolamine binding assay was performed according to Albin et al. (27). Sections were incubated for 30 min at room temperature with buffer (pH 7.4) containing 137 mM NaCl, 3 mM KCl, 1 mM EDTA, 8 mM $NaHPO_4$, 1.5 mM KH_2PO_4, and 5 nM [^{3}H]scopolamine (70 Ci/mmol, Amersham, France) with (nonspecific binding) or

without (total binding) 20 μM atropine (Sigma). The sections were then rinsed for 10 min in 4°C buffer, and for 30 s in 4°C distilled water. They were dried and exposed to Biomax MS film (Kodak, France) in an autoradiography cassette for 2 weeks at −80°C. The films were developed, fixed, and the autoradiographs analyzed with an imaging system (Biocom, Visioscan, France). Relative optical densities (RODs) are expressed as the ratio of optical density measured in a region of interest over the optical density of a reference region, the corpus callosum. Measurements were performed on six serial sections for each brain region of eight control and nine deficient rats. RODs were compared between diets for each cerebral region, and between cerebral regions for each dietary group, by Student's *t*-test. Significance was set at $P < 0.05$.

VAChT distribution by brain uptake of IBVM

IBVM was prepared at INSERM U316 (Tours, France) (28) and purified by HPLC. The resulting labeled compound had a specific activity of 2,200 Ci/mmol. Groups of six deficient and six control rats (380–400 g) were injected with a (50.1–61.8 μCi) bolus of IBVM via the tail vein and killed 2 h later by decapitation. Their brains were quickly removed, and the hippocampus and frontal cortex were dissected out and weighed. The radioactivity in the tissues was measured in a γ counter (LKB1282 Compugamma), calculated per gram of tissue, and referred to the injected dose (ID). Statistical differences were tested using two-way ANOVA (brain region × diet) followed by a Bonferroni *t*-test, and were considered significant when $P < 0.05$.

Determination of AChE activity

Rats were decapitated, their brains were quickly removed, and the frontal cortex and hippocampus were dissected out on ice, weighed, frozen in liquid nitrogen, and stored at −80°C. AChE activity was determined by the method of Ellman et al. (29). Tissues were homogenized in 0.1 M phosphate buffer (pH 8.0). The reaction mixture consisted of a 0.4 ml aliquot of homogenate, 2.6 ml phosphate buffer, and 0.1 ml 0.01 M dithiobis-nitrobenzoate (Sigma). Substrate [0.02 ml 0.075 M acetylthiocholine iodide (Sigma)] was added to the reaction mixture and the absorbance at 412 nm was recorded for at least 6 min in a UVIKON spectrophotometer (Kontron Instrument, UVK LAB Service, France). Protein content was determined by the Bradford procedure (30). The differences in enzymatic activity between dietary groups and between brain regions were analyzed by two-way ANOVA (brain region × diet), followed by the Bonferroni *t*-test. Differences were considered significant when $P < 0.05$.

RESULTS

Fatty acid compositions of phospholipid classes in the frontal cortex and hippocampus

The overall concentrations of saturated and monounsaturated fatty acids in all three phospholipid classes in the rats fed the (n-3) PUFA-deficient diet and the control rats were essentially the same (**Table 2**). The (n-3) PUFAs in the controls were almost entirely 22:6n-3, and their concentration was greatest in the PE (24.5 ± 1.5% of total fatty acids in the frontal cortex; 21.6 ± 1.2% in the hippocampus) and the PS (23.7 ± 2.7% in the frontal cortex; 11.7 ± 1.0% in the hippocampus). But the 22:6n-3 accounted for less than 6% of the total in the PC. The DHA concentrations in the three phospholipid classes were significantly higher in the frontal cortex than in the hippocampus ($P < 0.01$). The major (n-6) PUFA was 20:4n-6. Its concentration was greatest in PE (12.7 ± 1.1% in the frontal cortex; 13.7 ± 1.1% in the hippocampus), followed by PC (6.5 ± 0.1% in the frontal cortex; 8.6 ± 0.3% in the hippocampus) and PS (less than 3%).

The DHA levels in (n-3) PUFA-deficient rats were much lower than in controls, with a decrease of 77% and 68% for PE, 76% and 62% for PC, and 64% and 71% for PS, as compared with respective measures in control rats in the frontal cortex and hippocampus, respectively ($P < 0.01$). Concomitantly, the total (n-6) PUFA levels were increased in PE by 96% and 77%, in PC by 48% and 51%, and in PS by 378% and 119%, in the frontal cortex and hippocampus, respectively ($P < 0.01$). This large increase was mainly due to

TABLE 2. Main fatty acid compositions of phospholipid classes

	Frontal Cortex		Hippocampus	
	Control Rats	(n-3) PUFA-Deficient Rats	Control Rats	(n-3) PUFA-Deficient Rats
	mg/100 mg fatty acids			
PC				
16:0	44.2 ± 0.5^{b}	44.8 ± 0.5^{b}	42.6 ± 0.7^{a}	42.6 ± 1.2^{a}
18:0	12.1 ± 0.2	11.6 ± 0.1^{a,*}	12.3 ± 0.1	12.3 ± 0.2^{b}
18:1n-9	21.1 ± 0.3^{b}	19.9 ± 0.2^{b,*}	19.8 ± 0.3^{a}	18.6 ± 0.3^{a,*}
20:4n-6	6.5 ± 0.1^{a}	7.0 ± 0.2^{a,*}	8.6 ± 0.3^{b}	10.2 ± 0.8^{b,*}
22:4n-6	0.8 ± 0.1	1.1 ± 0.0^{a,*}	0.8 ± 0.1	1.2 ± 0.0^{b,*}
22:5n-6	0.1 ± 0.0	3.1 ± 0.3*	0.1 ± 0.0	3.0 ± 0.3*
Σ (n-6) PUFA	8.3 ± 0.2^{a}	12.3 ± 0.4^{a,*}	10.3 ± 0.3^{b}	15.6 ± 0.8^{b,*}
22:6n-3	5.4 ± 0.3^{b}	1.3 ± 0.1^{a,*}	4.0 ± 0.1^{b}	1.5 ± 0.1^{b,*}
Σ (n-3) PUFA	5.5 ± 0.3^{b}	1.3 ± 0.1^{a,*}	4.4 ± 0.2^{a}	1.5 ± 0.1^{b,*}
PE				
16:0	4.8 ± 0.1	5.2 ± 0.2^{b,*}	4.5 ± 0.5	4.5 ± 0.5^{a}
18:0	16.4 ± 0.2^{b}	17.2 ± 0.9^{b}	14.9 ± 1.1^{a}	15.4 ± 1.0^{a}
18:1n-9	9.3 ± 0.6	8.5 ± 0.3	9.8 ± 0.8	9.0 ± 1.0
20:4n-6	12.7 ± 1.1	15.0 ± 0.6*	13.7 ± 1.1	15.8 ± 1.4*
22:4n-6	4.9 ± 0.2^{a}	6.4 ± 0.4*	5.4 ± 0.2^{b}	6.6 ± 0.4*
22:5n-6	1.1 ± 0.8	16.0 ± 0.7*	1.0 ± 0.6	13.2 ± 2.6*
Σ (n-6) PUFA	19.4 ± 1.8	38.1 ± 1.2*	20.9 ± 1.7	37.0 ± 4.6*
22:6n-3	24.5 ± 1.5^{b}	5.7 ± 1.0*	21.6 ± 1.2^{a}	7.0 ± 2.6*
Σ (n-3) PUFA	25.0 ± 1.6^{b}	6.4 ± 0.9*	22.4 ± 1.0^{a}	7.3 ± 2.6*
PS				
16:0	6.3 ± 0.1^{a}	4.8 ± 1.1^{a}	13.9 ± 2.4^{b}	13.4 ± 1.4^{b}
18:0	38.2 ± 0.7	38.6 ± 0.3^{b}	36.9 ± 2.3	36.8 ± 1.2^{a}
18:1n-9	19.4 ± 3.4	16.0 ± 1.8^{a}	19.5 ± 2.0	20.0 ± 1.7^{b}
20:4n-6	2.2 ± 0.2^{a}	2.9 ± 0.1*	3.0 ± 0.2^{b}	2.5 ± 0.9
22:4n-6	1.5 ± 0.3	2.7 ± 0.4^{b,*}	1.3 ± 0.3	2.2 ± 0.0^{a,*}
22:5n-6	0.6 ± 0.1	19.2 ± 2.2^{b,*}	0.7 ± 0.4	8.0 ± 0.1^{a,*}
Σ (n-6) PUFA	5.4 ± 0.6	25.8 ± 2.3^{b,*}	6.2 ± 1.1	13.6 ± 0.6^{a,*}
22:6n-3	23.7 ± 2.7^{b}	8.5 ± 0.5^{b,*}	11.7 ± 1.0^{a}	3.4 ± 0.1^{a,*}
Σ (n-3) PUFA	23.9 ± 2.7^{b}	8.9 ± 0.5^{b,*}	13.0 ± 0.4^{a}	4.3 ± 0.4^{a,*}

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid. Σ (n-6) PUFA is the sum of 18:2n-6, 18:3n-6, 20:3n-6, 22:4n-6, and 22:5n-6; Σ (n-3) PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. Values are means ± SD (mg/100 mg fatty acids). Minor saturated and monounsaturated fatty acids accounting for less than 5 mg/100 mg fatty acids have not been reported.

^{a,b} For each fatty acid, a significant difference exists between cortical and hippocampal regions in the same dietary group (paired Student's *t*-test; $P < 0.01$).

* For each fatty acid, significantly different from control group in the same cerebral region (one-way ANOVA followed by posthoc Bonferroni *t*-test; $P < 0.01$).

22:5n-6 (DPA), whose levels in PC was increased 30-fold and 29-fold, in PE 13-fold and 12-fold, and in PS 31-fold and 10-fold in the frontal cortex and hippocampus, respectively ($P < 0.01$). The amount of 20:4n-6 was also increased in the frontal cortex and hippocampus, respectively, in PE by 18% and 15%, in PC by 8% and 19% ($P < 0.01$). The amount of 20:4n-6 in PS was increased by 32% in the frontal cortex ($P < 0.01$), and it was decreased by 17% in the hippocampus, but this decrease was not statistically significant.

Effect of (n-3) PUFA deficiency on ACh release

The basal concentrations of extracellular ACh in the frontal cortex (**Fig. 1A**, insert) and hippocampus (Fig. 1B, insert) were estimated by averaging the three values obtained before KCl perfusion. The basal ACh concentration in controls were 1.72 ± 0.3 pmol/20 μl dialysate for the frontal cortex and 1.94 ± 0.2 pmol/20 μl dialysate for the hippocampus. The basal ACh concentration in the hippocampus of the deficient rats was 72% higher than in control rats ($P < 0.05$), but the concentration in the frontal cortex of test and control rats was not different.

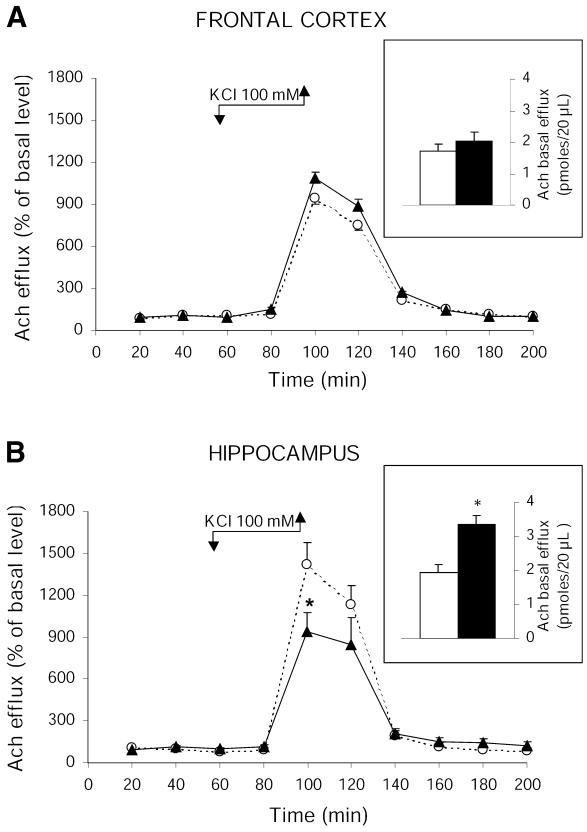

Fig. 1. Acetylcholine (ACh) efflux in the frontal cortex (A) and the hippocampus (B) of control (open circles, n = 6 to 8) and (n-3) PUFA-deficient rats (black triangles, n = 6 to 9). ACh is expressed as a percentage (mean ± SEM) of the baseline value. Data were corrected for individual probe recovery. Double arrows indicate the KCl perfusion period. Insert: Basal ACh release in control (open bars) and (n-3) PUFA-deficient rats (black bars). Values are expressed as the average (pmol/20 μl of dialysate) of the three 20 min dialysate collections immediately preceding 100 mM KCl perfusion. * Significantly different between dietary groups (Student's *t*-test; $P < 0.05$).

We infused 100 mM KCl through the dialysis probe to mimic neurone activation. KCl perfusion caused the extracellular ACh concentration to increase 9-fold (1.72 ± 0.3 vs. 15.67 ± 0.6 pmol/20 μl) in the frontal cortex (Fig. 1A) and 11-fold (1.94 ± 0.2 vs. 22.7 ± 3.4 pmol/20 μl) in the hippocampus (Fig. 1B) of controls. In the (n-3) PUFA-deprived rats, the KCl-stimulated release of ACh in the hippocampus was 34% lower than in the (n-3) PUFA-adequate animals ($P = 0.048$), while there was no difference between the two dietary groups in the release in the frontal cortex.

Effect of (n-3) PUFA deficiency on muscarinic receptors

The muscarinic receptor binding sites in the hippocampus and frontal cortex of control and (n-3) PUFA-deprived rats were assayed by [^{3}H]scopolamine binding. Less [^{3}H]scopolamine was bound to the muscarinic sites in the frontal cortex (1.35 ± 0.51 ROD) than to those in the hippocampus (2.08 ± 0.19 ROD, $P < 0.01$) of control rats. The scopolamine binding to muscarinic sites in the hippocampus of (n-3) PUFA-deficient rats (1.87 ± 0.12 ROD) was significantly lower than the binding to the same brain region of control rats (2.08 ± 0.19 ROD, $P = 0.012$), whereas the binding to the frontal cortex sites of deprived (1.64 ± 0.39 ROD) and control rats (1.35 ± 0.51 ROD, $P = 0.052$) was not statistically different.

Effect of (n-3) PUFA deficiency on VAChT

The distributions of IBVM in the frontal cortex and hippocampus of rats were evaluated 2 h after injection. In the control group, significantly more injected IBVM was bound to the frontal cortex [0.203 ± 0.051% of ID per gram of tissue (ID/g)] than to the hippocampus (0.156 ± 0.029%, $P < 0.05$). The amounts of IBVM specifically bound to control and (n-3) PUFA-deficient rat brain areas were the same; they were 0.203 ± 0.051% and 0.189 ± 0.014% of ID/g for the frontal cortex of control and test rats, and 0.156 ± 0.029% and 0.158 ± 0.019% of ID/g for the hippocampus.

Effect of (n-3) PUFA deficiency on AChE activity

No difference was found in the protein content of the homogenate from cerebral areas studied between rats from the two dietary groups (data not shown). The AChE activities in homogenates of the frontal cortex (64.6 ± 13.7 μmol/g prot/min) and hippocampus (73.7 ± 12.5 μmol/g prot/min) of controls were similar (**Fig. 2**). But the AChE activity in the frontal cortex of (n-3) PUFA-deficient rats was significantly lower (63.71 ± 13.6 μmol/g prot/min) than in the hippocampus (82.17 ± 12.7 μmol/g prot/min; $P < 0.05$). The deficiency had no effect on the enzyme activity in either the frontal cortex or the hippocampus.

DISCUSSION

Very few data are available on the relationship between (n-3) PUFAs and in vivo cholinergic neurotransmission. Minami et al. (31) reported higher ACh concentrations in

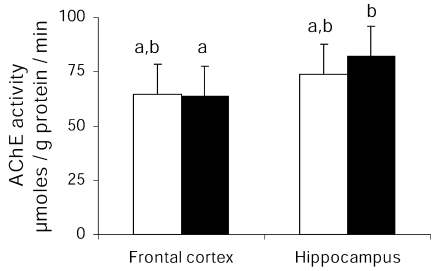

Fig. 2. Acetylcholinesterase (AChE) activity in the frontal cortex and the hippocampus of control (open bars; n = 9) and (n-3) PUFA-deficient rats (black bars; n = 9). AChE is expressed as means ± SD of µmol acetylthiocholine hydrolyzed per min × g protein. a, b: Significantly different between dietary groups and cerebral regions (two-way ANOVA followed by Bonferroni *t*-test; $P < 0.05$).

homogenates of the hippocampus from hypertensive rats fed a DHA supplement. Recently, Favrelière et al. (32) found that a DHA-enriched phospholipid diet enhanced the spontaneous and evoked release of ACh by the hippocampus of aging rats. The present report demonstrates that some parameters of the cholinergic neurotransmission in the hippocampus are specifically affected by a diet-induced lack of neuronal (n-3) PUFA. The basal release of ACh in the hippocampus of (n-3) PUFA-deficient rats was significantly higher (72%) than in controls, whereas the KCl-induced release was lower (34%) than in controls. Since an intact septo-hippocampal cholinergic system is crucial for learning and memory (21), changes in hippocampal ACh flow could be involved in the reduced performance of rats fed a low-(n-3) PUFA diet (4, 5).

The increased basal ACh efflux in the hippocampus of deficient rats could be due to increased release or to decreased catabolism of the neurotransmitter in the synaptic cleft. We found no alteration in the AChE activity in the hippocampus of the deficient rats, although a previous study reported that a low-(n-3) PUFA diet altered the thermotropic behavior of AChE activity in the synaptosomal membranes of the rat brain (33). However, the two studies differed as to the length and time of dietary treatment, the protocols used to measure AChE activity, and especially in that Foot, Cruz, and Clandinin used total brain-synaptosomal fractions, while we used specific material from the cortex and hippocampus.

The increased release of ACh could be due to changes in synthesis, storage, or exocytosis. The diet-induced changes in neuronal lipid composition could result in an increased spontaneous release of ACh (without neurone depolarization), which would in turn deplete the vesicular store. Thus, the pool of newly synthesized ACh, which is mainly mobilized during neuronal activation, would be reduced in the hippocampus of the (n-3) PUFA-deficient rats, leading to lower stimulated outflow. We showed that KCl produced an 11-fold increase in extracellular ACh in control rats, while this effect was reduced by 34% in the hippocampus of (n-3) PUFA-deficient rats. Yoshida et al. (34) reported that rats suffering from a long-term α-linolenate deficiency had a lower density of synaptic vesicles in the hippocampus following a brightness discrimination task. We assessed the vesicular storage of ACh by measuring the in vivo binding of the specific vesicular transporter inhibitor IBVM to VAChT sites. The binding of this specific presynaptic marker of cholinergic neurons (35) showed no difference between (n-3) PUFA-deprived rats and controls, suggesting that vesicular storage was not altered by the diet.

We also observed a slight reduction in the specific binding to muscarinic sites in the hippocampus of (n-3) PUFA-deficient rats. These changes could be due to a direct effect of diet on receptors or a change in ACh release. [^{3}H]scopolamine labels a large population of muscarinic receptor subtypes that are present in different densities in the brain regions (36, 37), where they regulate the cholinergic system in different ways. The modification in the [^{3}H]scopolamine binding to muscarinic sites we observed in the hippocampus of the deficient rats could involve one or all receptor subtypes. This is reinforced by a recent study that suggested that an (n-3) PUFA dietary supplement specifically enhances the binding to M1 muscarinic receptors in the hippocampal formation (38). Muscarinic receptors are also present in noncholinergic cells and could participate in the regulation of other neurotransmitter systems (39). Lastly, there may be functional interactions between cholinergic and monoaminergic systems that are altered by an (n-3) PUFA deficiency (10, 12). Both systems contribute to normal hippocampal function and the regulation of behavior (40, 41). Dopamine may inhibit septo-hippocampal cholinergic activity (42). The diet-induced cholinergic changes we observed could then result from the effects of a lack of DHA on dopaminergic and cholinergic systems.

The chronic dietary (n-3) PUFA deficiency led to dramatic changes in the PUFA compositions of neuronal membrane phospholipids in the frontal cortex and hippocampus. The (n-3) PUFA levels, exclusively represented by 22:6n-3 (DHA), were all drastically reduced in the major phospholipid classes (PE, PC, and PS), and these reductions were offset by increases in (n-6) PUFAs, especially 22:5n-6. These findings confirm the particular abundance of DHA and the greater sensitivity to (n-3) PUFA dietary deficiency of the frontal cortex compared with the hippocampus (7, 10, 11, 43). However, this specificity does not seem to be related to changes in cortex cholinergic function. This could be partly due to the specific fatty acid composition or to the particular cholinergic innervation of the frontal cortex. The diet-induced increase in (n-6) PUFA levels must also be taken into account, since recent work has shown that the various PUFAs do not all have the same physical properties [as reviewed in ref. (44)]. For example, AA may act on the cholinergic system, since its level in the membrane phospholipids of (n-3) PUFA-deficient rats is increased slightly. AA is presumed to act as a facilitatory retrograde messenger in cholinergic muscarinic transmission (45), and could facilitate ACh release from cholinergic nerve terminals in the (n-3) PUFA-deficient rats.

In conclusion, we have shown that a chronic α-linolenic

acid-deficient diet causes changes in several cholinergic parameters, with an elevated spontaneous release of ACh in the hippocampus, and a lower evoked release associated with reduced binding to muscarinic receptors. These effects can result from different responses to the loss of neuronal DHA and the enrichment in (n-6) PUFAs, specifically by the cholinergic system but also by overall neurotransmitter release processes. Given that the septo-hippocampal cholinergic system plays a major role in the regulation of cognitive functions, these modifications might contribute to the cognitive impairment that occurs in (n-3) PUFA-deficient rats.

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