Original Article

Effect of brain DHA levels on cytoskeleton expression

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Brain docosahexaenoic acid (DHA, 22:6n–3) levels are associated with learning memory performance, but it is not known the mechanism of DHA on enhancing memory effect. The aim of this study was to examine effect of brain DHA levels on cytoskeleton expression. Rats were fed a chow or sunflower oil-based n–3 fatty acid-deficient diet supplemented with or without fish oil starting from embryo and through postnatal day 140. The various DHA levels were from 5.0% to 15.6% of total fatty acids in hippocampus, 3.9% to 13.7% in visual cortex, and 5.3% to 14.4% in olfactory bulbs. The expression of the cytoskeleton markers tyrosine tubulin, acetylated tubulin, and β-actin in the hippocampus, visual cortex and olfactory bulb was not affected by brain DHA levels.

Key Words: docosahexaenoic acid, fish oil, brain, cytoskeleton, tubulin

INTRODUCTION

Docosahexaenoic acid (DHA, 22:6n–3) plays an important role on brain function.[1] In the mammalian brain, 10% of the wet weight or 50% of the dry weight is lipid, while DHA is the major n–3 polyunsaturated fatty acids (PUFA) in neuron membranes. Brain DHA deficiency is associated with reduced learning ability in rats and memory loss in patients of Alzheimer's disease (AD).

AD is a progressing neurodegenerative disease characterized by dementia. The main pathology of AD is extracellular deposits of fibrillar amyloid β peptide, which causes phosphorylation of intracellular tau protein which is responsible for the neuronal death.[2] In AD patients, fibrillar amyloid β peptide plaques form first in the basal neocortex, then spread to the hippocampus, and later to the cerebral cortex.[3] The main function of the hippocampus is short-term memory linked to the learning process, while that of the cerebral cortex is perception, reasoning, and other higher functions. DHA levels are significantly decreased 50% in the hippocampus and frontal lobe of AD patients [4,5] and serum DHA levels in AD patients reduce with severity of clinical dementia compared to healthy age-matched controls.[6] The development of AD is delayed in patients who consume fish oil.[7]

Our previous study revealed that brain DHA deficiency was recovered back by n–3 fatty acids-rich fish oil supplementation at adulthood for 80 days, and the recovery of brain DHA levels did improve spatial reference and working memory (unpublished data). However, it is still not known the mechanism of DHA action on enhancing memory function.

Neuronal degeneration in AD is caused by phosphorylated tau protein, a neuronal microtubule-associated protein, one of the components of the cytoskeleton. The cytoskeleton is connected to the cell membranes, and DHA is one of the major components of the neuron membrane. It is therefore of interest to study the effect of DHA on the cytoskeleton. In addition, DHA levels in the normal brain are region-specific. It is important to further verify the effect of DHA on the cytoskeleton in different brain regions.

The aim of this study was to determine whether regional brain DHA levels may affect the cytoskeleton. We showed that DHA levels in different brain regions ranged from 3.9% to 15.6% of total fatty acids in animals fed sunflower oil-based n–3 fatty acid-deficient diet or chow diet supplemented with or without fish oil starting from embryo and through postnatal day 140. However, fish oil supplementation of normal or n–3 fatty acid-deficient animals did not alter the expression of tyrosine tubulin as a dynamic marker, acetylated tubulin as a stable marker, or β-actin as a filament component in the hippocampus, olfactory bulb and visual cortex. It is concluded that the selected markers of cytoskeleton were not affected by brain DAH levels.

MATERIALS & METHODS

Animals and study design

Pregnant Sprague-Dawley rats (Crl:SD) at 2 days of gestation were obtained from BioLasco Taiwan Co. Ltd (Taipei, Taiwan), a technology licensee of Charles River Laboratories in Taiwan, and were immediately randomly assigned to three experimental diets. The pregnant dams were given a normal chow diet (5001, LabDiet Inc, USA) (control group), chow diet supplemented by oral gavage with 0.15 mL of fish oil (Leiner Health Products, LLC, CA, USA) (FO group), or a sunflower oil-based n–3 fatty acid-deficient diet prepared in our laboratory (n–3D group).

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After weaning, pups of the control and FO groups were maintained on the same diet as their dams, while the FO pups were kept on the chow diet, then, at age of 60 days old, were supplemented with 0.1 ml of fish oil by oral gavage once a day until sacrificed at postnatal day 140. In addition, at postnatal day 60, half of the males in the n–3D group were assigned to the n–3DF group and were supplemented with fish oil until sacrificed. The animals were sacrificed at postnatal day 140.

All rats were housed in a humidity-controlled room at 24±1°C on a 12 hour light-dark cycle with free access to tap water and diet. The protocols and animal treatments used in this study were approved by the Animal Care and Use Committee of the National Taiwan University College of Medicine.

Diet composition

The ingredients of the n–3 deficient diet and the fatty acid composition of chow diet, sunflower oil, and fish oil are presented in Table 1 and 2. Sunflower oil containing 63% linoleic acid (18:2n–6) and no n–3 fatty acids was used as the n–3 fatty acids deficient diet. The n–3 fatty acids deficient diet ingredients were obtained from MP Biomedicals, LCC (Ohio, USA), except methionine and choline, which were from Sigma-Aldrich Inc (MO, USA), and the sunflower oil, corn starch, and sucrose, which were purchased from a local supermarket.

Lipid analysis

The animals were anesthetized with CO_2 and decapitated.

Table 1. Ingredients of the n–3 fatty acid-deficient diet

Ingredient	Amount (g / kg diet)
Sunflower oil	200
Casein	238
Methionine	3.5
Corn starch	150
Sucrose	294
Alphacel	58.8
AIN 76 vitamin mix	11.8
AIN 76 mineral mix	41.2
Choline chloride	2.4

Table 2. Fatty acid composition of the diets and oils (%wt of total fatty acids)

% wt	Chow diet	n–3 def diet	Fish oil
Total sat.	36.6±1.5	12.1±0.3	41.1±1.6
Total mono.	28.1±0.9	24.8±0.2	23.7±3.6
18:2n–6	33.1±0.5	63.0±0.2	1.5±0.6
Total n–6	33.1±0.4	63.0±0.3	5.8±1.3
22:6n–3	0.5±0.3	n.d.	7.8±0.2
Total n–3	2.2±0.6	n.d.	29.5±3.3

The data are presented as the mean±SEM (n=3). n.d.: not detectable

The brain was rapidly removed and brain regions, including the hippocampus, olfactory bulb, and visual cortex were dissected on ice, frozen in liquid nitrogen, and stored in a -80°C freezer until analysis. Total lipids were extracted from aliquots of tissue homogenate according to the method of Blight and Dyer [8], then converted to their methyl esters [9] and run in a Hewlett-Packard 5890 gas chromatography using flame ionization detection on a DB-1 fused silica capillary column (60 m x 0.25 mm x 0.1 um, Agilent, Inc, Palo Alto, CA, USA) with nitrogen as carrier gas. The oven temperature program was set at 60°C for 2 minutes, then increased at 10°C per minute to 170°C, then at 3°C per minute to 270°C, and held at 270ºC for 15 minutes. The fatty acid peaks were identified by comparison of the retention times with standard mixture of 68A (Nu-Chek Prep, Elysian, MN USA), 37 FAME, PUFA2, and PUFA3 (all from SUPELCO, Bellefonte, PA, USA). The fatty acid composition was expressed as the weight % of total carbon 14 to carbon 22 fatty acids.

Expression of cytoskeletal proteins by Western blot analysis

The hippocampus, visual cortex and olfactory bulb were homogenized in a sonicator in lysis buffer, 10 mM Tris-HCl, pH 7.3, containing 150 mM NaCl, 1mM EDTA, 1% Triton X-100, and a protease inhibitor cocktail mix (Sigma, Missouri, USA). The homogenate was centrifuged at 14,000g for 15 min at 4°C, and the supernatant was collected as soluble protein. The pellet was resuspended with SDS-Urea buffer, centrifuged and the supernatant was collected as insoluble protein. The soluble and insoluble protein was used for Western blotting. Protein concentrations were measured using a Bio-Rad Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were denatured by heating at 95°C for 5 min in SDS sample buffer and aliquots containing 10-20 ug of protein were separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (Bio-Rad) by electrotranfer at 200 mA for 4 h, which was then blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline, 0.1% Tween 20 (blocking buffer). The membranes were incubated overnight at 4°C with mouse monoclonal anti-tyrosine tubulin (clone TUB-1A2, 1:2000, Sigma), anti-acetylated tubulin (clone 6-11B-1, 1:5000, Sigma), anti-β-actin (clone AC-74, 1:5000, Sigma), or anti-GAPDH (1:1000, Novus Biologicals, Inc CO, USA) antibodies in blocking buffer. GAPDH was used as loading control. Bound antibodies were detected using horseradish peroxidase-conjugated anti-mouse IgG antibody in blocking buffer and an enhanced chemiluminescence Western blot detection reagent (ECL, Amersham Biosciences, Buckinghamshire, England). Quantification of the data was performed using a UVP BioDoc-IT Imaging system.

Statistical analysis

Data are presented as the mean ± SEM. Statistical differences between the four groups were determined by one-way ANOVA followed by Fisher's least significant difference test between groups.

Table 3. Fatty acid composition of different brain regions in rats fed a chow diet or n–3-deficient diet with or without fish oil supplemenation[1,2] (% weight of total fatty acids)

wt%	Control	FO	n–3D	n–3DF
Total saturated				
Hippocampus	55.3±2.0^{a}	44.4±1.75^{c}	47.7±3.3^{bc}	47.7±1.0^{b}
Olfactory bulb	49.7±2.4^{a}	46.0±2.4^{b}	47.2±2.0^{b}	46.8±2.2^{b}
Visual cortex	50.8±0.59^{b}	53.5±1.2^{ab}	51.6±2.4^{b}	54.0±1.3^{a}
Total mono				
Hippocampus	18.9±1.1	18.3±1.4	19.2±0.6	17.9±0.5
Olfactory bulb	26.0±1.0^{a}	23.0±0.8^{b}	20.9±0.9^{b}	22.2±0.4^{b}
Visual cortex	21.9±0.6^{a}	20.9±0.5^{a}	19.3±0.8^{ab}	18.2±0.2^{b}
20:4n-6				
Hippocampus	11.7±0.7^{b}	16.0±0.8^{a}	14.6±1.1^{a}	15.5±0.3^{a}
Olfactory bulb	10.4±0.7	11.0±1.1	12.7±1.2	12.3±0.5
Visual cortex	10.2±0.2	9.8±0.5	12.9±0.4	11.6±0.5
22:4n–6				
Hippocampus	2.2±0.2^{b}	3.8±0.5^{a}	3.6±0.7^{a}	3.5±0.2^{a}
Olfactory bulb	2.0±0.6^{b}	2.6±0.6^{a}	3.3±0.3^{a}	3.2±0.4^{a}
Visual cortex	2.4±0.1^{c}	2.3±0.2^{c}	4.2±0.4^{a}	3.2±0.3^{b}
22:5n–6				
Hippocampus	0.1±0.1^{c}	0.4±0.1^{c}	8.2±1.4^{a}	3.1±0.3^{b}
Olfactory bulb	0.4±0..1^{c}	0.2±0.1^{c}	7.8±0.4^{a}	2.3±0.2^{b}
Visual cortex	0.1±0.1^{c}	0.1±0.0^{c}	6.3±0.4^{a}	2.3±0.3^{b}
22:6n–3 (DHA)				
Hippocampus	10.3±1.3^{b}	15.6±1.6^{a}	5.0±0.9^{c}	11.4±0.4^{b}
Olfactory bulb	10.3±0.5^{b}	14.4±1.3^{a}	5.3±0.9^{c}	10.3±1.0^{b}
Visual cortex	13.7±0.5^{a}	12.2±0.9^{a}	3.9±0.5^{c}	9.4±0.7^{b}

[1.] The data are presented as the mean±SEM (n=4/group). [2.] Statistical differences between the four groups determined by one-way ANOVA followed by Fisher's least significant difference test between groups.

RESULTS

DHA levels in brain regions

The animals were fed chow diet or a sunflower oil-based n–3 fatty acid-deficient diet with or without fish oil supplementation.

The fatty acid composition of the three brain regions is shown in Table 3. In rats fed the chow diet, DHA levels were 10.3, 13.7 and 10.3% of total fatty acids in the hippocampus, olfactory bulb and visual cortex, respectively.

In the n–3D, n–3DF, control, and FO groups, hippocampal DHA levels were, respectively, 5.0, 11.4, 10.3, and 15.56% of total fatty acids. The decreased hippocampal DHA levels were replaced by docosapentaenoic acid (22:5n-6) levels of 8.2, 3.1, 0.4, and 0.1% of total fatty acids.

Expression of cytoskeletal proteins in the visual cortex and olfactory bulb

Expression of the cytoskeleton markers acetylated tubulin, tyrosine tubulin, and β-actin in the visual cortex olfactory bulb and hippocampus was not significantly different between the four groups (Fig. 1).

DISCUSSION

DHA is essential for neuron function, but the mechanism is unclear. Tubulin and actin are components of the cytoskeleton which is important in supporting nutrient transport by axons from the neuron cell body to the synapse, in maintaining the shape of the neuron, and in the growth of neurons. Tubulins and β-actin account, respectively, for about 20% and 10% of the total protein in brain and have been suggested to play an important role in neuron function.[10] Tubulins must be in the dynamic state to support the function of axons.[11] The marker of dynamic tubulin is tyrosine tubulin, while detyrosinated and acetylated tubulin are markers of stabilized tubulin.[12] It is interesting that fish oil supplementation of the chow or n–3-deficient diet altered DHA levels in the hippocampus, visual cortex and olfactory bulb, but had no effect on the expression of tyrosine tubulin, acetylated tubulin, or β-actin.

The various brain regional DHA levels ranged from 3.9 ~ 15.6% of total fatty acids were created by feeding

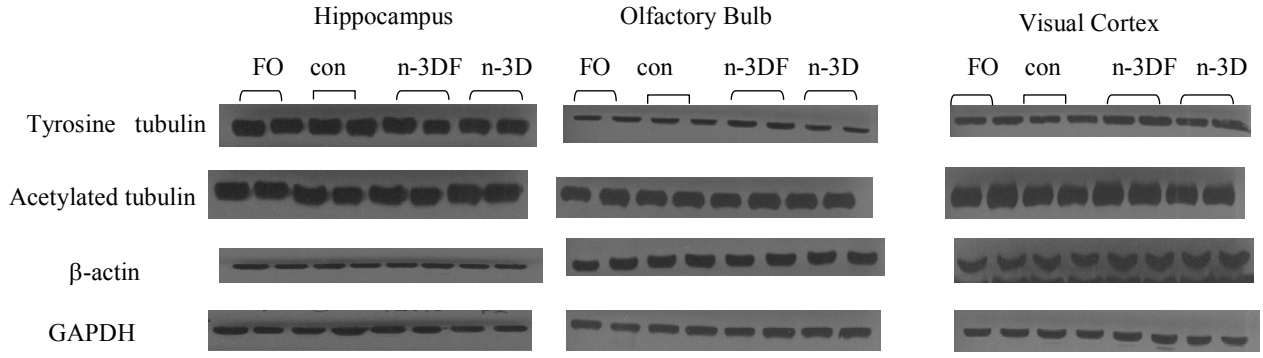

Figure 1. Effect of fish oil supplementation of the chow diet or n–3 deficient diet on cytoskeletal protein expression in the hippocampus, olfactory bulb and visual cortex. Western blot analysis was performed with the indicated antibodies. GAPDH was used as the loading control. No significant difference in expression of tyrosine tubulin, acetylated tubulin, or as β-actin was seen between the four groups (n=4 rats/group) by one-way ANOVA.

sunflower oil based n-3 fatty acids deficient diet or chow diet supplemented with or without fish oil. The deficiency of hippocampal and olfactory bulbs DHA levels were fully recovered back by giving fish oil supplementation at adulthood for 80 days, while only 69% of DHA was recovered in visual cortex. By giving fish oil supplementation to chow diet feeding animals, more DHA were significant accumulated in hippocampus and olfactory bulbs but not in visual cortex.

In summary, the various brain regional DHA levels were created by the designed four experimental diets. However, the expression of the selected markers for cytoskeleton in hippocampus, olfactory bulbs, and visual cortex were not affected by its DHA levels.

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AUTHOR DISCLOSURES

Ann-Che Hasio, Jen-Jui Chen, Wan-Ling Chung and Hui-Mi, no conflicts of interest.

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