島根大学学術情報リポジトリ
SWAN
Shimane University Web Archives of kNowledge

Title

Mechanisms of dietary docosahexaenoic acid-induced protection against and amelioration of impairment of memory learning ability in Alzheimer's disease model rats

Author(s)

Md. S. Hossain, Michio Hashimoto, Shinji Yamashita, Masanori Katakura, Yoko Tanabe, Hironori Fujiwara, Shuji Gamoh, Teruo Miyazawa, Noriyuki Arai, Toshio Shimada, Osamu Shido

Journal

Alzheimer’s & Dementia, Volume 4, Issue 4S_Part_8

Published

01 July 2008

URL

https://doi.org/10.1016/j.jalz.2008.05.735

この論文は出版社版でありません。
引用の際には出版社版をご確認のうえご利用ください。

Mechanisms of dietary docosahexaenoic acid-induced protection against and amelioration of impairment of memory learning ability in Alzheimer's disease model rats

Michio Hashimoto[1)], Hossain Md Shahdat[1,2)], Shinji Yamashita[3)], Masanori Katakura[1)], Yoko Tanabe[1)], Hironori Fujiwara[4)], Shuji Gamoh[1)], Teruo Miyazawa[3)], Noriyuki Arai[4)], Toshio Shimada[5], Osamu Shido[1)]

1) Department of Environmental Physiology, Shimane University Faculty of Medicine, Izumo 693-8501, Japan
2) Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh
3) Department of Food and Biodynamic Chemistry Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan.
4) Department of Geriatric Medicine, Tohoku University School of Medicine, Sendai 980-8574, Japan.
5) Department of Internal Medicine, Shimane University Faculty of Medicine, Izumo 693-8501, Shimane, Japan;

Corresponding author with complete address, including an email address:

Dr. Michio Hashimoto
Department of Environmental Physiology
Shimane University Faculty of Medicine
Izumo 693-8501,
Shimane
Japan,
Phone: +81-853-20-2110
Fax: +81-853-20-2110,
e-mail: michio1@med.shimane-u.ac.jp

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Abstract

We investigated the mechanisms of the beneficial effects of dietary docosahexaenoic acid (DHA) on the impairment of cognition ability in the amyloid beta$_{1-40}$ (Aβ_{1-40})-infused Alzheimer's disease (AD)-model rats. After the administration of DHA to AD model rats for 12 weeks, the levels of Aβ_{1-40}, and cholesterol, and the composition of fatty acids were determined in the Triton X100-insoluble membrane fractions (DIFs) of their cerebral cortex. The effects of DHA on the *in vitro* formation and kinetics of fibrillation of Aβ_{1-40} were investigated by thioflavin T fluorescence spectroscopy, and then by transmission electron microscopy and fluorescence microscopy. Dietary DHA significantly decreased the levels of Aβ_{1-40}, cholesterol and saturated fatty acids in the DIFs of AD rats. The formation of Aβ fibrils was also attenuated by their incubation with DHA, as demonstrated by the decreased intensity of thioflavin T-derived fluorescence and by electron micrographs. DHA treatment also decreased the intensity of thioflavin fluorescence in preformed-fibril Aβ peptide, demonstrating the anti-amyloidogenic and fibril-destabilizing effects of DHA. DHA not only inhibits the deposition of Aβ_{1-40} *in vivo* but also inhibits formation and destabilization of preformed Aβ fibrils *in vitro*. DHA is effective in the prevention of and a radical treatment for AD.

Key words: Docosahexaenoic acid, Alzheimer's disease, amyloid fibrillation, memory

Running title: Effects of DHA on amyloid fibrillation

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Introduction

Alzheimer's disease (AD) is pathologically characterized by insoluble neurofibrillary tangles and senile plaques of 1~40 and/or 1~42 amino acid-long small peptides called amyloid beta (Aβ) peptides that are derived from the membrane-resident amyloid precursor protein (APP). To date, several factors that may participate in the fibrillation of these membrane-released peptides and the development of AD have been proposed: high serum total cholesterol is treated as an independent risk factor for AD (Notkola et al., 1998), although it is an established risk factor in the progression of coronary artery disease (CAD); high-cholesterol diets induce AD-like Aβ deposition in the rabbit brain (Sparks et al., 1994); and elevated cholesterol, which forms lipid domains and/or so-called lipid rafts at the membrane bilayer, also facilitates the deposition of neurotoxic Aβ (Kojro et al., 1998; Casserly and Topol, 2004). Thus, the initial localization of amyloid peptides in these cholesterol-rich lipid rafts and their subsequent removal therefrom, and the underlying mechanisms are the focus of many investigations.

For Peer Review

Docosahexaenoic acid (C22:6n-3, DHA), one of the predominant n-3 polyunsaturated fatty acids in brain lipids and as a dietary supplement, is the subject of a large number of

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

investigations related to AD and the associated memory impairment. DHA is essential for the normal development of the prenatal central nervous system (CNS) (Green and Yavin E, 1998). DHA deficiency is associated with loss of learning ability (Neuringer et al., 1986); however, the loss is restored by the consumption of DHA (Moriguchi and Salem Jr., 2003). Chronic dietary administration of DHA improves learning ability in young (Gamoh et al., 1999) and old (Gamoh et al., 2001) rats and accelerates hippocampal neurogenesis in adult rats (Kawakita et al., 2006). The extent of neurogenesis in the adult hippocampus correlates with the performance of learning ability (Gould et al., 1999). Chronic administration of DHA contributes to protection against neuronal damage induced by transient forebrain ischemia in rats (Okada et al., 1996). Thus, DHA plays crucial roles in the development, function and maintenance of the CNS throughout life.

Epidemiological studies suggest that dietary intake of DHA reduces the risk for AD (Huang et al., 2005; Morris et al., 2003). It decreases the level of Aβ in detergent insoluble membrane fractions (DIFs) and reduces the amyloid burden in the hippocampus and parietal cortex of transgenic AD model mice (Lim et al., 2005). Interestingly, the DHA content in the hippocampus is reduced in AD patients (Prasad et al., 1998). Consistent with these findings,

For Peer Review

our previous reports have indicated that dietary administration of DHA prevents (Hashimoto et al., 2002) and ameliorates (Hashimoto et al., 2005a) the impairment of learning ability induced by the infusion of Aβ peptide (1-40) into the rat cerebral ventricle; however, the relevant mechanisms have remained unclear. This study is, therefore, aimed at investigating the effect of DHA on the levels of Aβ peptide and cholesterol in the DIFs of the Aβ peptide-infused rat brain *in vivo*, and whether DHA affects the formation and destabilization of Aβ fibrils *in vitro*.

Materials and Methods

Animals and diet

Rats were provided for and killed in accordance with the procedures outlined in the *Guidelines for Animal Experimentation of Shimane Medical University* (Shimane, Japan), compiled from the *Guidelines for Animal Experimentation of the Japanese Association for Laboratory of Animal Science.* Wistar rats (generation 1, G1) (*Jcl*: Wistar; Clea Japan Co., Osaka, Japan) were housed in a room under controlled temperature (23 ± 2° C), relative humidity (50 ± 10%) and light-dark cycles (light: 0800 to 2000 h: dark: 2000 to 0800 h), and provided with a fish-oil-deficient pellet diet (F-1®; Funabashi Farm, Funabashi, Japan) and

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

water *ad libitum.* The inbred third generation (G3) male rats [n= 31; 20 wk old; 384 ± 5.3 g body weight (BW)], fed the same F1 diet, were randomly divided into 4 groups: a vehicle group (n = 7), an Aβ peptide (1-40)-infused group (Aβ group) (n = 7), an Aβ+DHA group (n = 8) and a DHA group (n = 9). The DHA group was orally fed ethyl-ester 4,7,10,13,16,19-docosahexaenoate (Harima Chemicals, Inc., Tokyo, Japan) emulsified in 5% gum Arabic solution at 300 mg/kg BW/day; and the vehicle group was orally fed a similar volume of the 5% gum Arabic solution alone. The administration of emulsified DHA or gum Arabic solution was continued for 12 weeks.

Surgery for the preparation of Aβ-infused AD model rats.

The surgical techniques for preparing Aβ-infused rats were essentially the same as those described (Hashimoto et al., 2002, 2005a). Briefly, each rat was anesthetized with sodium pentobarbital (50 mg/kg BW i.p.). The skull was then exposed and two holes (right and left, relative to the bregma; 0.8 mm posterior, 1.4 mm lateral) were drilled according to the atlas of Paxinos and Watson (Paxinos and Watson 1986) using a stereotaxic frame (Narishige, Tokyo, Japan). A solvent of 35% acetonitrile plus 0.1% trifluoroacetic acid (pH 2.0) was used as the vehicle for Aβ peptide (1–40) (Peptide Inst., Osaka, Japan). As a small

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

amount of $AlCl_3$ facilitates the aggregation of Aβ peptide *in vitro*, and as the method has limited reproducibility without $AlCl_3$, we used 0.5 μg $AlCl_3$ (in 5 μL, intracerebroventricularly, 1 μL/min) before implanting the osmotic pump for continuous infusion of Aβ. This procedure greatly improved reproducibility and reliability in yielding an animal model of AD with impaired memory. A mini-osmotic pump (Alzet 2002; Durect Co., Cupertino, CA, USA), containing either Aβ peptide (1–40) solution (234 ± 13.9 μL Aβ) or the vehicle alone was quickly implanted into the backs of the rats. The outlet of the pump was inserted 3.5 mm into the left ventricle and attached to the skull with screws and dental cement. The infusion rate was 0.56 μL/h, and the total amount infused was approximately 4.9–5.5 nmol/L Aβ. Oral administration of either the DHA emulsion or the gum Arabic solution was restarted 2 days after surgery and continued until the end of the experiment.

Brain tissue preparation

After completing the dietary regime, the rats were anesthetized with sodium pentobarbital (65 mg/kg BW, i.p.), and the cerebral cortex was separated on ice, blotted gently with filter paper to remove blood and extraneous tissue fragments, then frozen with liquid N_2 and stored at −80 °C until use.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Preparation of detergent insoluble membrane fractions (DIFs).

DIFs were prepared as previously described (Nordberg et al., 2002; Hashimoto et al., 2005b) with minor modifications. Cortical tissues (80–120 mg) were homogenized in ice cold 2-(*N*-morpholino)ethanesulfonic acid (MES)-buffered saline (25 mM MES, pH 6.5, 0.15 M NaCl; MBS) containing 1% Triton X-100 and the following protease inhibitors: 1.0 μM phenylmethylsulphonile fluoride, 10 μg/mL leupeptin, 1.0 μg/mL pepstatin and 10 μg/mL aprotinin. The homogenate was centrifuged initially at 700 g for 15 min to eliminate unruptured cells and debris; the supernatant was incubated with mild agitation on a shaker at ice-cold temperature for 2 hr in a cold room, and then centrifuged at 100,000 × g for 1hr. The supernatant, the so-called detergent-soluble membrane fraction, was decanted while the pellets were disrupted with the tissue layer and washed three times with the MES-buffered saline containing Triton X-100 and various protease inhibitors. Aliquots were used for electron microscopy.

After washing, the pelleted DIFs were initially suspended by vigorous mixing in a small volume of 6.0 M guanidine hydrochloride in 50 mM Tris–HCl, pH 7.6 and centrifuged again at 150,000 × g for 1 hr. The resultant supernatant was diluted to a final concentration of

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

0.5 M guanidine–HCl and used as DIFs without further purification or further fractionation. The DIFs were subjected to ELISA and/or used for the measurement of detergent-insoluble Aβ peptide $_{1\text{–}40}$, cholesterol and fatty acid profiles.

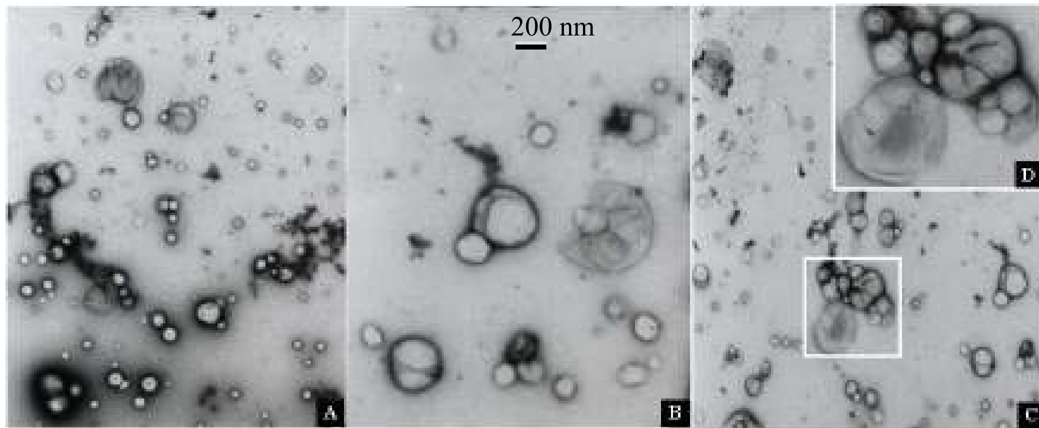

Fig. 1 Visualization of caveolae/lipid rafts containing Triton 100-X insoluble membrane lipid fractions by electron microscopy. The structures were purified from cortical whole homogenates as described in the *Materials and Methods* section. Membrane structures appear as enclosed circles. Representative micrographs of the vehicle (A) and docosahexaenoic acid (B) and Aβ (C) rats. **Inset** of C represents a clustering of the enclosed lipid rafts (D).

Electron microscopy of DIFs

The DIFs were subjected to morphological analysis by transmission electron microscopy. Briefly, a 2 µl aliquot from the sample was spread on carbon-coated 400-mesh grids, negatively stained with 1% uranyl acetate and examined under a Hitachi H-7000 electron microscope with an acceleration voltage of 75 kV. The isolated membrane fractions appeared as 25 -1500 nm encircled vesicular structures of various shapes.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Measurement of Aβ peptide$_{1–40}$ in the DIFs.

The levels of Aβ peptide$_{1–40}$ were analyzed with a colorimetric sandwich ELISA kit (Immuno-Biological Laboratories Co., LTD, Gunma, Japan).

Lipid analyses.

The cholesterol levels were measured by gas chromatography on a Model 5890II (Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and an automatic sampler (Model 7673), as previously described (Shahdat et al., 2004) with a few modifications. Briefly, 50 µL suspension of DIFs containing 100 µg protein was mixed with 50 µg of α-cholestane (1.0 µg/µL) in ethanol as an internal standard. The mixture was added to 1.0 mL saturated methanolic KOH, incubated at 80 °C for 30 min, cooled, supplemented with 1.0 mL saturated NaCl solution and 200 µL cyclohexane then vigorously shaken and centrifuged at 2000 x g. The upper organic layer was directly subjected to gas chromatography (using a 30 m × 0.25 mm inner diameter fused silica column coated with a methyl siloxane film 0.25 µm thick; HP-1, Hewlett-Packard), with helium as the carrier gas at a flow rate of 1.5 mL/min and oven temperatures programmed from 180 °C to 280 °C at 20 °C/min then at 280 °C for 10 min, and an injector splitter at 20˚C/min to a temperature of

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

290 °C.

The fatty acid profiles were determined by one-step analysis (Lepage and Roy 1988) using gas chromatography as described previously (Hashimoto et al., 2002, 2005a).

Protein concentrations were estimated by the method of Lowry et al. (Lowry et al., 1951).

Effects of DHA on Aβ fibrillation in vitro

A nonagitation-based assay was used to assemble fibrils from $A\beta_{1\text{–}40}$ solutionwith the help of nucleation-dependent polymerization *in vitro*. Trifluoroacetate salt of $A\beta_{1\text{–}40}$ (Peptide Institute, Osaka, Japan) was incubated at 37°C without shaking, then the amyloidogenic $A\beta_{1\text{–}40}$ spontaneously formed fibrils ($fA\beta_{1\text{–}40}$). Fibrillar amyloid beta ($fA\beta_{1\text{–}40}$) was initiated from fresh $A\beta_{1\text{–}40}$ solutions in a reaction volume of 600 μL containing 50 μM of $A\beta_{1\text{-}40}$, 2.3μM of $fA\beta_{1\text{-}40}$ (as nucleated seed), 50 mM phosphate buffer (pH 7.5), and 100 mM of NaCl (Naiki et al., 1998; Hasegawa et al., 1999; Ono et al., 2002a, b).

DHA (10 μM) (Sigma, St. Louis, MO) dissolved in 1% DMSO was added to 600 μl of the mixture. The reaction mixture was taken into oil-free PCR tubes (size, 0.5 mL, Takara Shuzo, Otsu, Japan); the tubes were then air-evacuated with a flush of nitrogen gas to prevent

For Peer Review

auto-oxidation, sealed with paraffin film and put into a DNA thermal cycler (PJ480; Perkin Elmer Cetus, Emeryville, CA). Starting at 4°C, the temperature was elevated at maximal speed to 37°C for 18h and the incubation was stopped by placing the tubes on ice. Aliquots (5 μL) from each tube were mixed with 250 μl of 5 μM thioflavin T (ThT) in 50 mM glycine-NaOH buffer (pH 8.5) and subjected to fluorescence spectroscopy (Hitachi F-2500 fluorescence spectrophotometer) at excitation and emission wavelengths of 445 and 490 nm, respectively. Similarly, a 4 μL aliquot was used for electron microscopy as described (Hasegawa et al., 1999). In brief, a droplet of the reaction mixture was spread on carbon-coated grids, negatively stained with 1% phosphotungstic acid (pH 7.0) and examined under a Hitachi H-7000 electron microscope with an acceleration voltage of 75 kV.

Thioflavin staining for microfluorescence study

The fibrillated A$\beta_{1\text{-}40}$ peptide was diluted ten times with 5 μM thioflavin T in 50 mM glycine-NaOH buffer (pH 8.5). An aliquot of the diluted sample was transferred onto the slide, and the droplet was allowed to air-dry for 1 min. Fluorescent signals were then visualized by the confocal laser microscope system (CLSM FV300, Olympus, Tokyo, Japan) and processed by Adobe Photoshop (Adobe Systems, Mountain View, CA, USA) or NIH

For Peer Review

image version 1.63. The fluorescence intensity was visualized in each of three random fields of the sample. As an alternative, the aggregates were allowed to air-dry on the slide, and then viewed under the microscope; however, no differences in terms of fluorescence outcome were found.

Effects of DHA on the kinetics of Aβ fibrillation

Since DHA showed significant inhibition of Aβ aggregation in the above experiments, the efficiency of DHA on the kinetics of $A\beta_{1-40}$ fibrillation was evaluated in detail by using 0 ~ 20 μM DHA. Briefly, the reaction mixture containing 50 μM $A\beta_{1-40}$, 10 μg/mL $fA\beta_{1-40}$, 50 mM phosphate buffer (pH 7.5), 100 mM NaCl or 0 ~ 20 μM DHA was dissolved in DMSO. The mixture (30 μL) was taken into oil-free PCR tubes; the tubes were air-evacuated with N_2 gas, sealed with paraffin film and incubated at 37°C for 18 hrs, as described elsewhere. The reaction was stopped by placing the tubes on ice. An aliquot (5 μL) from each tube was mixed with 250 μl of 5 μM thioflavin T in 50 mM glycine-NaOH buffer (pH 8.5) and subjected to fluorescence assay at the excitation and emission wavelengths of 445 and 490 nm, respectively.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Aβ fibril-destabilization assay

Destabilization of fAβ was assayed as described (Ono et al., 2002b). Briefly, the reaction mixture contained 25 μM preformed fresh fAβ_{1-40}, 50 mM phosphate buffer (pH 7.5), 100 mM of NaCl, and 1% polyvinyl alcohol to avoid fAβ aggregation and adsorption onto the inner wall of the tube during the reaction. DHA dissolved in DMSO was added to final concentrations of 0 μM, 5 μM, 10 μM and 20 μM. The final concentration of DMSO did not exceed 0.5 %. The mixture was incubated at 37°C in the DNA thermal cycler for 18 hrs; the reaction was then stopped on ice, and an aliquot of the mixture was mixed with 5 μM thioflavin T in 50 mM glycine-NaOH buffer (pH 8.5) and subjected to fluorescence spectroscopy as described elsewhere.

Statistical analysis.

Results are expressed as means ± S.E. For intergroup differences, the data were analyzed by one-way ANOVA. ANOVA followed by Fisher's PLSD was used for post hoc comparisons. Correlation was determined by simple regression analysis. The statistical program used was StatView® 4.01 (MindVision Software, Abacus Concepts, Inc., Berkeley, CA, USA). A level of $P < 0.05$ was considered statistically significant.

For Peer Review

Results

Effects of DHA administration on Aβ peptide$_{1–40}$ and cholesterol levels in DIFs

The level of Aβ peptide$_{1-40}$ was higher in the DIFs of the Aβ group than in those of the other three groups (P<0.05); it was significantly lower in the DIFs of the DHA group than in those of the other three groups (Table 1).

The level of cholesterol was significantly higher in the DIFs of the Aβ group than in those of the vehicle, the DHA, or the Aβ+DHA group (P<0.05); it was significantly lower in the DIFs of the Aβ+DHA and DHA groups than in those of the vehicle and the Aβ groups.

Table 1. Levels of amyloid beta peptide and cholesterol and composition of major fatty acids in cortical detergent insoluble membrane fractions (DIF)

	Vehicle	Aβ	DHA	DHA + Aβ
Amyloid β	80.5 ± 3.7^{b}	107 ± 12.2^{a}	62.0 ± 5.8^{b}	90.8 ± 11.9^{a}
Cholesterol	$141 \pm 4.2^{b, c}$	168 ± 8.5^{a}	$109 \pm 7.2^{b, c}$	126 ± 5.7^{d}
PLA	37.9 ± 0.9^{b}	41.3 ± 1.2^{a}	37.6 ± 1.0^{b}	38.0 ± 0.9^{b}
STA	31.2 ± 1.4^{a}	32.0 ± 1.0^{a}	27.7 ± 0.6^{b}	27.4 ± 1.6^{b}
AA	6.7 ± 0.2^{a}	7.04 ± 0.3^{a}	5.65 ± 0.25^{b}	6.4 ± 0.23^{a}
DHA	1.73 ± 0.09^{a}	1.41 ± 0.04^{b}	1.75 ± 0.07^{a}	1.68 ± 0.08^{a}
DHA/AA	0.259 ± 0.02^{b}	0.203 ± 0.01^{c}	0.309 ± 0.01^{a}	0.264 ± 0.01^{b}
TSFA	$70.9 \pm 1.20^{a, c}$	74.5 ± 2.15^{a}	66.6 ± 1.45^{b}	$67.8 \pm 1.20^{b, c}$
TUFA	1.92 ± 0.09^{a}	1.65 ± 0.06^{b}	1.96 ± 0.07^{a}	$1.88 \pm 0.08^{a, b}$

Values are means ± SEM. Fatty acids are expressed as mol% of the total fatty acids. n = 8 ~ 9. Means in a row with superscripts without a common letter differ, P < 0.05; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid**;** PLA, palmitic acid; STA, stearic acid; TSFA, total saturated fatty acid; TUFA, total unsaturated fatty acid.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 2. Correlation between DIF amyloid beta peptide and lipid parameters

	X						
Y	**Aβ**	**Chol**	**PLA**	**STA**	**DHA**	**AA**	**DHA/AA**
Aβ	—	0.47 (0.004)	0.41 (0.02)	0.44 (0.014)	-0.42 (0.001)	0.37 (0.04)	- 0.53 (0.001)
Chol	—	—	0.29 (0.10)	0.54 (0.014)	- 0.24 (0.20)	0.40 (0.02)	- 0.47 (0.009)

Results evaluated by a simple regression analysis. In the parenthesis are the P values. Aβ = amyloid peptide (pg/mg protein); Chol = Cholesterol (μg/mg protein). Other abbreviations are the same as Table 1.

Effects of DHA administration on the fatty acid profile of DIFs

The levels of palmitic were significantly higher in the DIFs of the Aβ group than in those of the other three groups. The levels of stearic acid were significantly decreased in the DHA-administered groups. The level of arachidonic acid was significantly lower in the DHA group than in the other three groups. The level of DHA was significantly lower in the Aβ group than in the other three groups. The ratio of DHA/AA was significantly lower in the Aβ group than in the other three groups, while it was significantly higher in the DHA group than in the other three groups. The level of total saturated fatty acid was significantly higher in the Aβ group than in the DHA and DHA + Aβ groups. The level of total unsaturated fatty acid was significantly lower in the Aβ group than in the vehicle and DHA groups (Table 1). The

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

levels of both DHA and the DHA/AA molar ratio were negatively correlated with the Aβ levels in DIFs. The levels of cholesterol, palmitic acid, stearic acid and arachidonic acids were positively correlated with the Aβ levels ($P<0.05$). The cholesterol levels were positively correlated with the levels of stearic, arachidonic and palmitic acid, but not significantly in the latter, and negatively with DHA and the DHA/AA ratio (Table 2).

Morphology of the DIF

The electron micrographs of DIFs suggested that the lipid rafts/caveolae were of aggregated structures and exhibited wide-size heterogeneity of 50 nm to 4-5 μm; however, average morphological (size) differences among different rat groups could not be detected by electron microscopy (Fig. 1).

Effects of DHA on Aβ polymerization measured by fluorescence spectroscopy, transmission electron microscopy and microfluorescence

Fluorescence spectroscopic measurements clearly indicated that the polymerization of $A\beta_{1-40}$ monomers incubated with DHA was significantly inhibited by about 40% (Fig. 2A). The florescence data were further confirmed by transmission electron microscopy. DHA (10 μM) drastically inhibited the extensive fibril formation compared with that in the control

For Peer Review

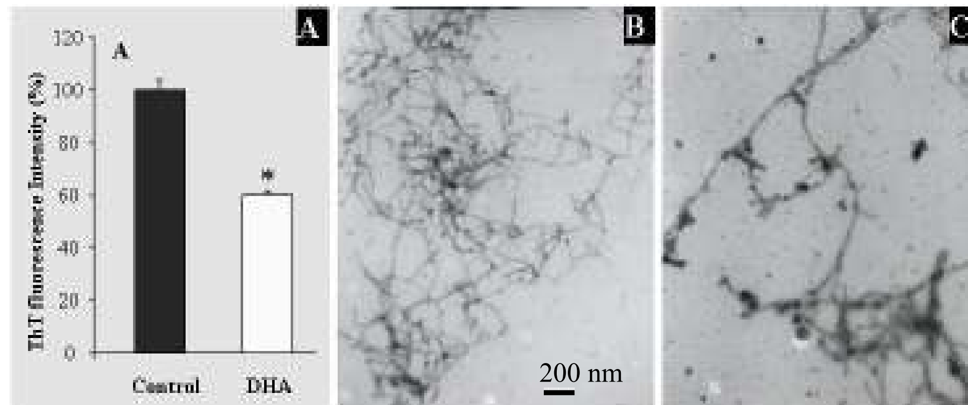

Fig. 2 The effect of docosahexaenoic acid (DHA) on Aβ fibril formation. Aβ peptide$_{1\text{-}40}$ was incubated with (white bar of A or micrograph C) or without (black bar of A or micrograph B) DHA **(**10 μM**)** for 18 hr**,** and the extent of fibril formation was estimated either spectrofluorometrically (A) and/or were visualized by transmission electron microscope, as described in *Materials and Methods***.**

For Peer Review

(Figs. 2B and 2C). Because of the massive stretch of the polymerized Aβ$_{1\text{-}40}$ threads onto the 400-mesh electron microscope grids, it was difficult to count the individual fibrils; therefore, the extent of fibril formation was further examined by microfluorescence for direct visualization (Fig. 3). The results were the same as those observed by biochemical and electron microscopic studies. Thioflavin T bound well with the fibril aggregates and emitted green fluorescence. The number of illuminated aggregates, observed randomly from different visual fields, was always smaller in the presence of DHA than in the absence of DHA (controls) (Figs. 3A and 3B).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

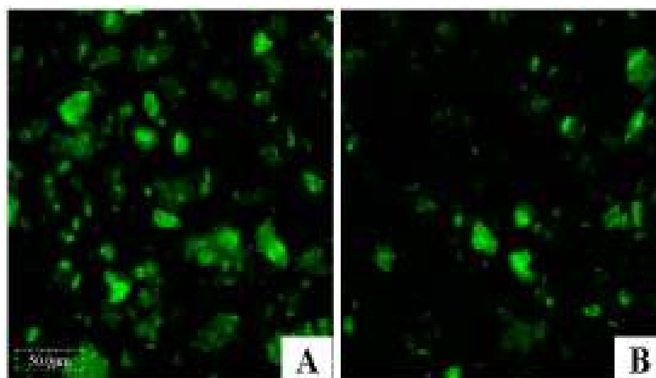

Fig. 3 Thioflavin T staining of polymerized $A\beta_{1-40}$ aggregating materials in the absence (Control, A) or presence of docosahexaenoic acid (10 μM) (B). Under fluorescence illumination with thioflavin T staining, amyloid aggregates appear green. The sample preparation was the same as for Figure 2. B shows the clear inhibitory effect of DHA on the $A\beta_{1-40}$ fibril formation, as evidenced by the smaller number of illuminated $A\beta_{1-40}$ deposits.

For Peer Review

Effects of DHA on kinetics of the formation and destabilization of Aβ fibrils

The presence of DHA significantly inhibited the formation of fibrils (Fig. 4A) and destabilized Aβ fibrils (Fig. 4B). The addition of 5, 10 and 20 μM DHA to the fibrils decreased fluorescence intensity to 63±4.0%, 42±2.0 % and 34±2.0 %, respectively.

Under the same conditions, moreover, the fibril-destabilizing efficiency of DHA was checked by electron microscopy. Preformed $A\beta_{1-42}$ fibrils diminished drastically in the presence of DHA (Fig. 5).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Discussion

According to the amyloid-cascade-hypothesis, the progressive aggregation of Aβ is a critical step in AD pathogenesis (Selkoe, 1997); therefore, agents capable of protecting against amyloidogenesis have been one of the targets of the search for pharmacological strategies for AD therapy. We have previously shown that dietary administration of DHA prevents (Hashimoto et al., 2002) and ameliorates (Hashimoto et al., 2005a) the impairment of spatial cognitive learning ability in $A\beta_{1-40}$-infused AD model rats. In the present study, we evaluated the effects of DHA on the levels of $A\beta_{1-40}$ in the DIF, where Aβ is believed to act as starter molecules for the seeding of amyloidogenesis, and correlated the efficiency of DHA with its effect on *in vitro* aggregation of $A\beta_{1-40}$. The level of $A\beta_{1-40}$ was significantly low in the DIFs of Aβ + DHA rats compared with that of Aβ rats. Thus, the dietary DHA-induced protection against and amelioration of memory impairment in AD model rats (Hashimoto et al., 2002, 2005a) is assumed to be mediated by removing $A\beta_{1-40}$ from its residence, the DIF. The direct cause-effect relationship remains to be clarified, however.

DHA increases the expression of transthyretin (Puskas et al., 2003), a transporter protein of thyroxine and retinol-binding protein. It is not unlikely that DHA by increasing the

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

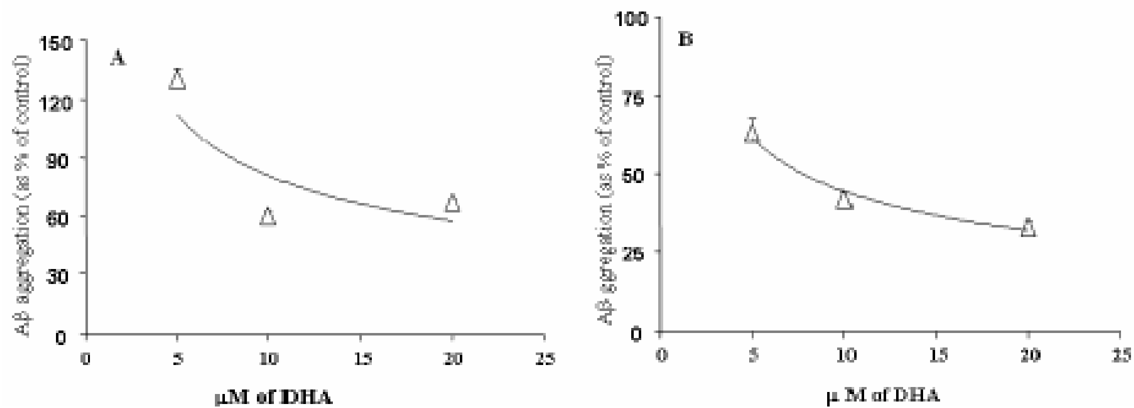

Fig. 4 Effects of docosahexaenoic acid (DHA) on the kinetics of the formation of $A\beta_{1-40}$ fibrils (**A**). Aβ aggregation was measured by thioflavin T method and expressed as a percentage of control, which was observed in the absence of DHA. Error bars represent SD (n = 5). Effects of DHA on the destabilization of $A\beta_{1-40}$ fibrils (B). Aβ aggregation was measured by thioflavin T method and expressed as a percentage of control, which was observed in the absence of DHA. Error bars represent SD (n = 5).

For Peer Review

levels of transthyretin reduces the level of $A\beta_{1-40}$ in the DIFs and indirectly prevents their aggregation (Schwarzman et al., 1994). $A\beta_{1-40}$ infusion degenerates cholinergic neurons and reduces memory-related performance (Itoh et al., 1999), while dietary DHA increases cortical acetylcholine levels with a concomitant increase in memory-related performance (Minami et al., 1997). The level of DHA in the hippocampus is low in AD patients compared with that in the brains of age-matched humans (Söderberg et al., 1991). The decreased levels of amyloid peptides in the Aβ + DHA rats are thus compatible with the observation that increased cholinergic transmission reduces amyloid deposition (Beach et al., 2001). Infusion of amyloid peptide into brain ventricles increases oxidative stress (Hashimoto et al., 2002, 2005a). Thus,

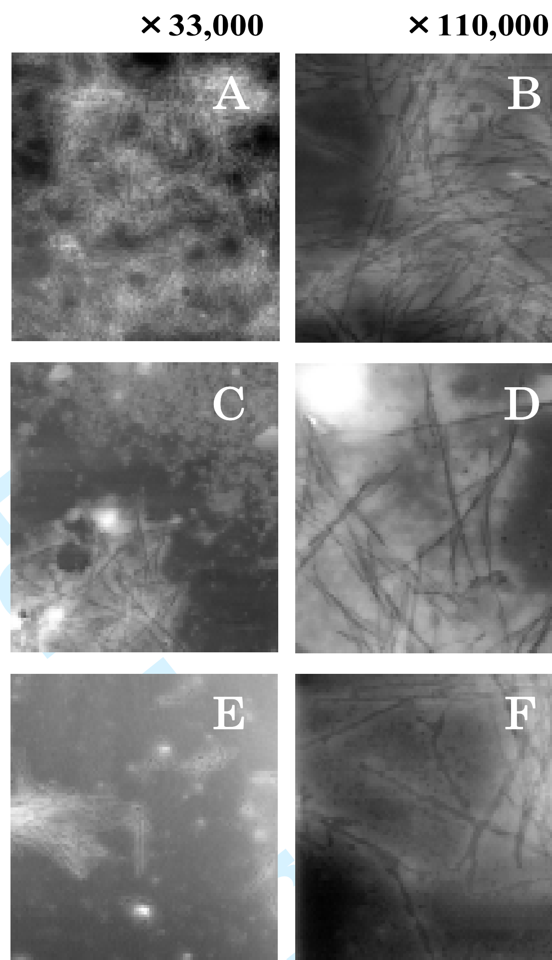

Fig. 5 Electron micrographs of destabilized amyloid fibrills. The mixtures containing 20 μM aggregated Aβ peptides with (10 μM; C and D), (50 μM; E and F) or without (0 μM; A and B) DHA were incubated at 37℃ for 30 min. Amplification = 33 K and 110 K.

DHA may, by decreasing amyloid peptide in the DIFs, provide antioxidative defense for continued smooth cholinergic neurotransmission and easy memory-related performance.

The DIF is highly enriched in the so-called lipid caveolae or lipid rafts, as visualized by electron microscopy, which act as a floating platform for the amyloidogenic seeding process. The rafts are rich in sphingomyelin, cholesterol and saturated fatty acids like

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

palmitic acid and stearic acid. These biomolecules construct a special detergent-insoluble conformational entity (thus enabling to isolate lipid rafts by the use of Triton 100-X at ice-cold temperature) and facilitate the formation and subsequent localization of amyloid. The Aβ levels were significantly higher in the Aβ rats (Table 1). The molar percentage of palmitic and stearic acids and cholesterol levels in the DIFs were higher in Aβ rats than in vehicle rats, stressing the inherent preference of Aβ peptide to localize in the lipid rafts. Palmitic and stearic acids also induce hyperphosphorylation of tau protein in neurons (Patil and Chan, 2005). Tau hyperphosphorylation is one of the critical steps leading to the formation of paired helical filament/neurofibrillary tangles, the hallmark lesion seen in the brains of AD patients. The hyperphosphorylated tau protein impairs learning ability (Sun et al., 2003). In this regard, DHA significantly decreases the raft-facilitating saturated fatty acids and cholesterol levels in DIFs. We therefore speculate that DHA constrains/inhibits the localization of Aβ somehow by decreasing the levels of saturated fatty acids and cholesterol. Our results also suggest that palmitic and stearic acids easily accumulate in the DIFs of the brains of AD model rats. The β-site amyloid-cleaving enzyme (BACE), an aspartyl protease, cleaves β-APP to generate the immediate substrate of β-secretase, then functions as the rate-limiting step in the generation

For Peer Review

For Peer Review

of Aβ. BACE tends to be directed into cholesterol-rich rafts by palmitoylation (Sidera et al., 2005). Raft proteins are not the only ones recruited to palmitoylation; instead, other fatty acids may also be involved in their acylation (Mumby, 1997). We are not certain whether Aβ $peptide_{1-40}$ remains in plamitoylated form in DIFs or whether it also remains acylated with stearic acid; as such, we speculate that DHA assists in scavenging Aβ $peptide_{1-40}$ by decreasing the amount of palmitic and stearic acids in DIFs.

Cholesterol is an essential component of plasma membranes. Recent epidemiological studies suggest the elevated cholesterol level as a significant factor in the pathogenesis of AD (Notkola et al., 1998; Sparks et al., 2005). Reduction of plasma cholesterol levels is associated with inhibited Aβ production and, subsequently, reduced symptoms of AD pathology, as demonstrated in human populations using statins, inhibitors of HMG-CoA reductase (Wolozin et al., 2000). The risk of AD is reduced by up to 70% in hypercholesterolemic patients treated with statins (Jick et al. 2000). Statins decrease the accumulation of Aβ peptides (Fassbender et al., 2001) in animals fed large quantities of dietary. Moreover, changes in membrane cholesterol levels affect the formation of Aβ from APP in cultured hippocampal cells, (Simons et al., 1998). The present study indicates that

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

dietary administration of DHA significantly decreases the level of cholesterol in the DIFs of Aβ rats. DHA decreases cholesterol levels in endothelial cells (Hashimoto et al., 1999) and neuronal cells (Shahdat et al., 2004). Whether the lowering effect of DHA on DIF-cholesterol is effective in reducing the levels of Aβ and the risk of AD has yet to be elucidated. With these data at hand, we investigated the direct effect of DHA on in vitro Aβ fibrillation.

Studies of Aβ fibrils have shown that these constructs are formed from low molecular weight Aβ (monomers or dimmers), lapse into protofibril structures, and then with time the equilibrium shifts to the formation of complex fibril structures (Walsh et al., 1999). Thioflavin T (ThT) binds rapidly and specifically to the β-sheet of the fibrils of Aβ peptide, but does not bind to monomer or oligomeric intermediates. Therefore, the amount of the cross β-pleated sheet structure of Aβ fibrils can be measured by the intensity of ThT fluorescence spectroscopy. In this study, we found that DHA inhibits the formation of Aβ fibrils (Fig. 4A) and destabilizes preformed Aβ fibrils (Fig. 4B) *in vitro*, suggesting that membrane DHA inhibits the formation of and destabilizes the preformed fibrils and thus removes the Aβ fibrils which may already have accumulated in the plasma membrane of the brain. Initially, we assumed the anti-oxidative activity of DHA to be the mechanism of these effects, at least

For Peer Review

for the destabilizing effect, because oxidized DHA did not affect the preformed Aβ fibrils (data not shown). Vitamins C and E, known as antioxidant vitamins, do not, however, destabilize preformed Aβ fibrils *in vitro* (Ono et al., 2004). Therefore, the mechanism of this effect of DHA cannot be explained merely by anti-oxidation. In the process of amyloid fibrillation, hydrophobic residues are embedded into the core of the fibrils (Pellarin and Caflisch, 2006). Thus, it is assumed that, unlike hydrophilic substances, hydrophobic or surface-active substances have a tendency to penetrate into and act at the core of the fibrils. Although further investigations are indispensable for clarifying whether DHA produces these effects, the site of DHA action may be hydrophobic residues in core components.

In conclusion, our study suggests that DHA not only inhibits the deposition of Aβ in vivo but also destabilizes preformed Aβ fibrils *in vitro*. Therefore, DHA is effective not only for the prevention of AD pathogenesis but also for the radical treatment of AD. A wide blood-brain barrier (BBB) passage and few side effects are very important in a drug for use in AD therapy. For example, while anthracycline 4'-iodo-4'-deoxydoxorubicin provides anti-aggregative activity against Aβ, it is not suitable for AD therapeutic application because of its high intrinsic toxicity and narrow BBB passage (Forloni et al., 2001). On the other hand,

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

there are very few reports on the toxicity of DHA; at least within ranges of up to 1500mg/kg, no acute toxicity has been observed (Hempenius et al., 1997). Moreover, the BBB is not a barrier to fatty acids (Dhopeshwarkar and Mead, 1973). Thus, DHA could be a very specific and safe agent against AD and may liberate people from the ravages of the disease.

For Peer Review

References

Beach T.G., Kuo T.M., Schwab C., Walker D.G., Roher A.E. (2001) Reduction of cortical amyloid beta levels in guinea pig brain after systemic administration of physostigmine. *Neurosci. Lett* **310**, 21–24.

Casserly I. and Topol E. (2004) Convergence of atherosclerosis and Alzheimer's disease: inflammation, cholesterol, and misfolded proteins. *Lancet* **363**, 1139–1146.

Dhopeshwarkar G.A. and Mead J.F. (1973) Uptake and transport of fatty acids into the brain and the role of the blood-brain barrier system. *Adv. Lipid Res.* **11**,109–142.

Fassbender K., Simons M., Bergmann C., et al. (2001) Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta42 and Abeta 40 in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* **98**,5856–5861.

Forloni G., Colombo L., Girola L., Tagliavini F., and Salmona M. (2001) Anti-amyloidogenic activity of tetracyclines: studies in vitro. *FEBS Lett.* **487**, 404–407.

Gamoh S., Hashimoto M., Sugioka K., Shahdat Hossain M., Hata N., Misawa Y., and Masumura S. (1999) Chronic administration of docosahexaenoic acid improves reference

memory-related learning ability in young rats. *Neuroscience* **93**,237–241.

Gamoh S., Hashimoto M., Hossain S., and Masumura S (2001) Chronic administration of docosahexaenoic acid improves the performance of radial arm maze task in aged rats. *Clin. Exp. Pharmacol. Physiol*. **28**,266–270.

Gould E., Beylin A., Tanapat P., Reeves A., and Shors T.J. (1999) Learning enhances adult neurogenesis in the hippocampal formation. *Nat. Neurosci.* **2**,203–205.

Green P. and Yavin E. (1998) Mechanisms of docosahexaenoic acid accretion in the fetal brain. *J. Neurosci. Res*. **52**, 129–136.

Hashimoto M., Hossain S., Shimada T., Sugioka K., Yamasaki H., Fujii Y., Ishibashi Y., Oka J., and Shido O (2002) Docosahexaenoic acid provides protection from impairment of learning ability in Alzheimer's disease model rats. *J. Neurochem*. **81**,1084–1091.

Hashimoto M., Tanabe Y., Fujii Y., Kikuta T., Shibata H., and Shido O (2005a) Chronic administration of docosahexaenoic acid ameliorates the impairment of spatial cognition learning ability in amyloid beta-infused rats. *J. Nutr.* **135**, 549–555.

Hashimoto M., Hossain S., Agdul H., and Shido O. (2005b) Docosahexaenoic acid-induced amelioration on impairment of memory learning in amyloid beta-infused rats relates to the decreases of amyloid beta and cholesterol levels in detergent-insoluble membrane fractions. *Biochim. Biophys. Acta* **1738,** 91-98.

Hashimoto M., Shinozuka K., Gamoh S., Tanabe Y., Hossain M.S., Kwon Y.M., Hata N., Misawa Y., Kunitomo M., and Masumura S. (1999) The hypotensive effect of docosahexaenoic acid is associated with the enhanced release of ATP from the caudal artery of aged rats. *J. Nutr.* **129**, 70–76.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Hashimoto M., Hossain S., Yamasaki H., Yazawa K., and Masumura S. (1999) Effects of eicosapentaenoic acid and docosahexaenoic acid on plasma membrane fluidity of aortic endothelial cells. *Lipids* **34**, 1297–1304.

Hempenius R.A., Van Delft J.M., Prinsen M., and Lina B.A. (1997) Preliminary safety assessment of an arachidonic acid-enriched oil derived from Mortierella alpina: summary of toxicological data. *Food Chem. Toxicol*. **5**, 573–581.

Huang T.L., Zandi P.P., Tucker K.L., Fitzpatrick A.L., Kuller L.H., Fried L.P., Burke G.L., and Carlson M.C. (2005) Benefits of fatty fish on dementia risk are stronger for those without APOE epsilon4. *Neurology* **65**, 1409–1414.

Itoh A., Akaike T., Sokabe M., Nitta A., Iida R., Olariu A., Yamada K., and Nabeshima T. (1999) Impairments of long-term potentiation in hippocampal slices of beta-amyloid-infused rats. *Eur. J. Pharmacol*. **382**, 167–175.

Jick H., Zornberg G.L., Jick S.S., Seshadri S., and Drachman DA (2000) Statins and the risk of dementia. Lancet 356:1627-1631 (Erratum in: Lancet 2001 **357**, 562).

Kawakita E., Hashimoto M., and Shido O (2006) Docosahexaenoic acid promotes neurogenesis in vitro and in vivo. *Neuroscience* **139**, 991–997.

Kojro E., Gimpl G., Lammich S., Marz W., and Fahrenholz F (1998) Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the alpha-secretase ADAM 10. *Proc. Natl. Acad. Sci. US.* **95**, 6460–6464.

Lepage G., and Roy C.C. (1988) Specific methylation of plasma nonesterified fatty acids in a one-step reaction. *J. Lipid Res.* **29**, 227–235.

Lim G.P., Calon F., Morihara T., Yang F., Teter B., Ubeda O., Salem N. Jr., Frautschy S.A.,

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

and Cole G.M. (2005) A diet enriched with the omega-3 fatty acid docosahexaenoic acid reduces amyloid burden in an aged Alzheimer mouse model. *J. Neurosci.* **25**, 3032–3040.

Lowry O.H., Rosebrough N.J., Farr A.L., and Randall R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193**, 265–275.

Moriguchi T., and Salem N. Jr. (2003) Recovery of brain docosahexaenoate leads to recovery of spatial task performance. *J. Neurochem.* **87**, 297–309.

Morris M.C., Evans D.A., Bienias J.L., Tangney C.C., Bennett D.A., Wilson R.S., Aggarwal N., and Schneider J. (2003) Consumption of fish and n-3 fatty acids and risk of incident Alzheimer disease. *Arch. Neurol.* **60**, 940–946.

Neuringer M., Connor W.E., Lin D.S., Barstad L., and Luck S. (1986) Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys. *Proc. Natl. Acad. Sci. USA* **83**, 4021–4025.

Nordberg A., Hellstrom-Lindahl E., Lee M., Johnson M., Mousavi M., Hall R., Perry E., Bednar I., and Court J. (2002) Chronic nicotine treatment reduces beta-amyloidosis in the brain of a mouse model of Alzheimer's disease (APPsw). *J. Neurochem.* **81**, 655–658.

Notkola I.L., Sulkava R., Pekkanen J., Erkinjuntti T., Ehnholm C., Kivinen P., Tuomilehto J., and Nissinen A. (1998) Serum total cholesterol, apolipoprotein E epsilon 4 allele, and Alzheimer's disease. *Neuroepidemiology* **17**, 14–20.

Okada M., Amamoto T., Tomonaga M., Kawachi A., Yazawa K., Mine K., and Fujiwara M (1996) The chronic administration of docosahexaenoic acid reduces the spatial cognitive deficit following transient forebrain ischemia in rats. *Neuroscience* **71**, 17–25.

Ono K., Yoshiike Y., Takashima A., Hasegawa K., Naiki H., and Yamada M (2004) Vitamin A

For Peer Review

exhibits potent antiamyloidogenic and fibril-destabilizing effects in vitro. *Exp. Neurol.* **189**, 380–392.

Patil S., and Chan C. (2005) Palmitic and stearic fatty acids induce Alzheimer-like hyperphosphorylation of tau in primary rat cortical neurons. *Neurosci. Lett.* **384**, 288–293.

Paxinos G., and Watson C (1986) The Rat Brain in Stereotaxic Coordinates, Academic Press, New York

Pellarin R, Caflisch A (2006) Interpreting the Aggregation Kinetics of Amyloid Peptides. J Mol Biol 360:882–892.

Prasad M.R., Lovell M.A., Yatin M., Dhillon H., and Markesbery WR (1998) Regional membrane phospholipid alterations in Alzheimer's disease. *Neurochem. Res*. **23**, 81–88.

Puskas L.G., Kitajka K., Nyakas C., Barcelo-Coblijn G., and Farkas T (2003) Short-term administration of omega 3 fatty acids from fish oil results in increased transthyretin transcription in old rat hippocampus. *Proc. Natl. Acad. Sci. USA* **100**, 1580–1585.

Schwarzman A.L., Gregori L., Vitek M.P., et al. (1994) Transthyretin sequesters amyloid βprotein and prevents amyloid formation. *Proc. Natl. Acad .Sci. USA*. **91**, 8368–8372.

Selkoe D.J. (1997) Alzheimer's Disease: Genotypes, Phenotype, and Treatments. *Science* **275**, 630–631.

Shahdat H., Hashimoto M., Shimada T., and Shido O (2004) Synaptic plasma membrane-bound acetylcholinesterase activity is not affected by docosahexaenoic acid-induced decrease in membrane order. *Life Sci*. **74**, 3009–3024.

Sidera C., Parsons R., and Austen B (2005) Post-translational processing of beta-secretase in Alzheimer's disease. *Proteomics* **5**, 1533–1543.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Simons M., Keller P., De Strooper B., Beyreuther K., Dotti C.G., and Simons K. (1998) Cholesterol depletion inhibits the generation of betaamyloid in hippocampal neurons. *Proc. Natl. Acad. Sci.* USA **95**, 6460–6464.

Söderberg M., Edlund C., Kristensson K., and Dallner G. (1991) Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease. *Lipids* **26**, 421–425.

Sparks D.L., Petanceska S., Sabbagh M., Connor D., Soares H., Adler C., Lopez J., Ziolkowski C., Lochhead J., and Browne P. (2005) Cholesterol, copper and Abeta in controls, MCI, AD and the AD cholesterol-lowering treatment trial (ADCLT). *Curr. Alzheimer Res*. **2**, 527–539.

Sparks D.L., Scheff S.W., Hunsaker J.C.3rd., Liu H., Landers T.,and Gross DR (1994) Induction of Alzheimer-like beta-amyloid immunoreactivity in the brains of rabbits with dietary cholesterol. *Exp. Neurol.* **126**, 88–94.

Sun L., Liu S.Y., Zhou X.W., Wang X.C., Liu R., Wang Q., and Wang J.Z (2003) Inhibition of protein phosphatase 2A- and protein phosphatase 1-induced tau hyperphosphorylation and impairment of spatial memory retention in rats. *Neuroscience 118*, 1175–1182.

Walsh D.M., Hartley D.M., Kusumoto Y., Fezoui Y., Condron M.M., Lomakin A., Benedek G.B., Selkoe D.J., and Teplow D.B. (1999) Amyloid beta-protein fibrillogenesis: structure and biological activity of protofibrillar intermediates. *J. Biol. Chem.* **274**, 25945–25952.

Wolozin B., Kellman W., Ruosseau P., Celesia G.G., and Siegel G (2000) Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. *Arch. Neurol.* **57**, 1439–1443.

For Peer Review