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REVIEW ARTICLE

Docosahexaenoic acid: one molecule diverse functions

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ABSTRACT

Docosahexaenoic acid (DHA, C22:6, ω-3) is a highly polyunsaturated omega-3 fatty acid. It is concentrated in neuronal brain membranes, for which reason it is also referred to as a "brain food". DHA is essential for brain development and function. It plays an important role in improving antioxidant and cognitive activities of the brain. DHA deficiency occurs during aging and dementia, impairs memory and learning, and promotes age-related neurodegenerative diseases, including Alzheimer's disease (AD). For about two decades, we have reported that oral administration of DHA increases spatial memory acquisition, stimulates neurogenesis, and protects against and reverses memory impairment in amyloid β peptide-infused AD rat models by decreasing amyloidogenesis and protects against age-related cognitive decline in the elderly. These results demonstrate a robust link between DHA and cognitive health. Rodents that were fed a diet low in ω-3 polyunsaturated fatty acids, particularly those that were DHA-deficient, frequently suffered from anxiety, depression and memory impairment. Although the exact mechanisms of action of DHA in brain functions are still elusive, a host of mechanisms have been proposed. For example, DHA, which inherently has a characteristic three-dimensional structure, increases membrane fluidity, strengthens antioxidant activity and enhances the expression of several proteins that act as substrates for improving memory functions. It reduces the brain amyloid burden and inhibits *in vitro* fibrillation and amyloid-induced neurotoxicity in cell-culture model. In this review, we discuss how DHA acts as a molecule with diverse functions.

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DHA; learning and memory; antioxidative defense; hypertension; Alzheimer's disease; neurogenesis

Introduction

Docosahexaenoic acid (DHA, C22:6, ω-3) is an ancient nutrient for the modern human brain [1]. Isotope dating studies, relating diet to the evolution of the large human brain, led to a hypothesis that the evolution of the modern human brain with expanded cortex coincided with the inclusion of seafood in the diet [2,3] of our ancestor dwellers. This view supports the proposition that the civilization of modern human race began at the land–sea interface or more precisely at the mountain–sea interface (a dramatic landscape shaped by interaction with mountains and sea). Seafood brought DHA into the ancient dietary culture. However, not all human cultures are known to consume high levels of seafood. For example, people living in landlocked geographical regions without access to seafood, as well as vegetarians or vegans who strictly avoid all animal products, may not receive adequate levels of DHA from their diets. Such individuals may have developed other mechanisms to increase their DHA levels. Indeed, alpha-linolenic acid (α-LNA) from plants can be used as a precursor for ω-3 DHA production, and it might be an appropriate dietary source to increase DHA levels [4]. The conversion of α-LNA to DHA is catalyzed by D6D (delta 6 desaturase); however, whether D6D expression/activity is upregulated in individuals without access to dietary DHA remains unclear. We previously reported that plasma DHA levels in individuals from Mongolia, a landlocked country, were half of those in Japanese individuals [5]; moreover, in Nepal, another landlocked country, women only had trace amounts of DHA in their mature breast milk [6]. Similarly, the breast milk of women living far from coastal areas in Brazil reportedly contains low DHA levels [7]. Thus, the conversion of α-LNA to DHA seems inefficient in such populations. In other words, if D6D was upregulated in individuals consuming α-LNA, we would expect that DHA levels would have been relatively high and/or comparable to those found in people living in coastal areas. It is known that the expression and/or activity of D6D is downregulated

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by numerous factors, including high dietary ω6/ω3 ratios, fatty acid unsaturation [8–16], low intake of essential micronutrients (such as Mg, Zn, vitamin C, B2, and B6) [17], age [18] and gender [19]. Therefore, such factors may subsequently affect an individuals' ability to convert α-LNA to DHA. Importantly, conversion rates of isotope-labeled α-LNA to DHA in human adults have been found to be very low (only 0–0.04%) [20]. In addition, supplementation of pregnant women with α-LNA was shown to have no effect on DHA levels [21]. Furthermore, vegetarians who were given α-LNA supplements showed no change in their DHA levels [22,23]. Thus, incorporating direct sources of DHA may be an important consideration, particularly for those with increased needs (e.g. pregnant and lactating women) and those who are at a greater risk of poor conversion (e.g. elderly people, people with neurological disorders and premature infants). Finally, regardless of age and gender, to ensure physical, mental and neurological health, vegetarians/vegans and those who do not consume adequate amounts of seafood are advised to include direct sources of DHA in their diet [12]. Phytoplankton, single-celled creatures living in the upper levels of the ocean and using solar energy to biosynthesize DHA molecules, are the primary producers of DHA. Zooplankton, feeding on phytoplankton, also accumulates [24–26]. Fish and marine animals have limited ability to convert shorter fatty acid chains to DHA [27]. Consequently, fish and marine animals rely on DHA uptake by the plankton (Figure 1). Therefore, DHA is found concentrated in fish and marine animals feeding on plankton. DHA is nutritionally active and is a critical molecule for maintaining health and nutrition and preventing diseases. The objective of this review is to describe the factors by which DHA influences numerous biological and physiological activities in the body, including the brain. Finally, the areas considered on in this review are as follows:

- Physiochemical properties of DHA: attributes to membrane fluidity and membrane-related functions;
- Antioxidant activities of DHA, while it is itself a highly polyunsaturated fatty acid;
- Effects of DHA on systems/tissues other than the brain: May have beneficial effects on brain functions;
- DHA improves memory, affects important molecular substrates, and contributes to memory formation;
- Effects of DHA on neurogenesis, which participates in learning and memory;
- Alzheimer's disease (AD) pathology and effects of DHA on it;
- Effect of DHA on lipid rafts, which act as organizing centers for the assembly and trafficking of signaling molecules;
- *In vitro* amyloid fibrillation and the effect of DHA on it: Illustrates how DHA may inhibit *in vivo* amyloid fibrillation;
- DHA can act as a signaling molecule: How DHA-derived docosanoids work physiologically;
- Epidemiological studies: DHA and eventual cognitive decline; and
- Conclusion.

Physiochemical properties of DHA

DHA, a highly polyunsaturated fatty acid of the ω-3 series with 22 carbon atoms and six *cis* double bonds

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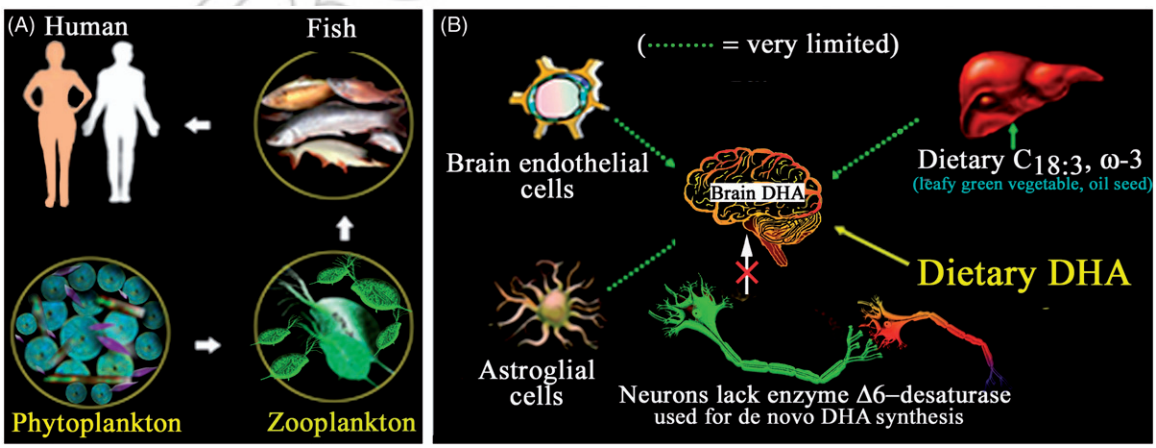

Figure 1. (A) Flow of docosahexaenoic acid from phytoplankton to humans. Humans obtain DHA from marine/riverine fish that live on phytoplankton and zooplankton. (B) Brain endothelial cells and astroglial cells have only limited capacity to biosynthesize DHA. Dietary ω-3 alpha-linolenic acid (α-NLA, C18:3, ω-3) that comes from green, leafy vegetables and plant seed oil can be used as a precursor; however, the pathway, is very slow and limited. Neurons lack delta desaturase that is required for the *de novo* synthesis of DHA. Thus, preformed DHA is the ultimate source of brain DHA.

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(Figure 2(A)), has some unique physicochemical characteristics, including multiple configurations. The presence of six *cis* double bonds results in a folding on the fatty acyl axis and allows DHA to form a curved (kinked or bent) structure [28] (Figure 2(B–D)). Kinked DHA molecules cannot fit well with straight-chain saturated fatty acids or planar and rigid cholesterol molecules when they are aligned in a membrane bilayer leaflet. The omega end bends up to the aqueous interface [29] and confers on DHA a spring-like vibrational motion. These conformational properties of DHA result in a greater degree of disorder during lipid packing or fluidity of the membrane. The molecular volume of DHA is 355.112 $Å^3$ and the molecule has 14 rotatable bonds as determined by the Molecular Dynamic Software (Molinspiration). DHA-containing phospholipids have a higher volume per unit area than other (un)saturated fatty acid-containing phospholipids (Figure 2(E)). The presence of numerous double bonds results in a lowering of the melting point of DHA such that it is highly fluid and in liquid form at low temperature. Inclusion of DHA in the membrane decreases the phase transition temperature of the bilayer, a property conducive to increased membrane fluidity and flexibility. We have previously reported the effects of *in vitro* treatment of DHA on rat thoracic endothelial cells [30] and age-associated decrease in membrane fluidity of endothelial cells [31]. We observed that dietary administration of DHA in rats increased fluidity of platelet membranes [32], neuron-synaptosomal membranes [33] and liver canalicular plasma membranes [34]. (Hashimoto et al., 2001). Consistent with our results, inclusion of DHA in artificial bilayer membranes augmented the fluidity of the membrane. Many membrane associated functions, including drug permeability [29,34], carrier-mediated transport [35,36], activities of membrane-bound enzymes [37,38] and neurotransmission [39] are modulated by increased plasma membrane fluidity. All of these membrane activities require microaggregation and conformational changes of receptors/enzymes in the membrane surface, which are eased by the increased fluidity of the lipid bilayer. Because lipid bilayers serve as the common "solvent" for membrane proteins, altered fluidity of the membrane plausibly alters protein functions. The modifications of the bilayer physical properties were more pronounced with DHA than with other polyunsaturated fatty acids [30,40]. Absorption of fatty acids in the intestine is enhanced by increased concentrations of polyunsaturated fatty acid [41,42]. These results suggest that DHA is an important structural fatty acid in bilayer membranes, such as synaptic plasma membranes, retinal outer segment membranes, and bile canalicular plasma membranes [43] and sperm tails of humans [44], monkeys [45] and mice [46]. The importance of these cells or cellular structures for the survival of animal species, including humans, is well known. DHA-induced alteration of membrane fluidity may affect the neuronal function, leading to changes in the brain function. Yehuda et al. [47] reported that polyunsaturated fatty acids may affect brain functions by modifying: (i) membrane fluidity, (ii) activity of membrane-bound enzymes, (iii) number and affinity of receptors, (iv) function of ion channels, (v) production and activity of neurotransmitters, and (vi) signal transduction, which control the activity of neurotransmitters and

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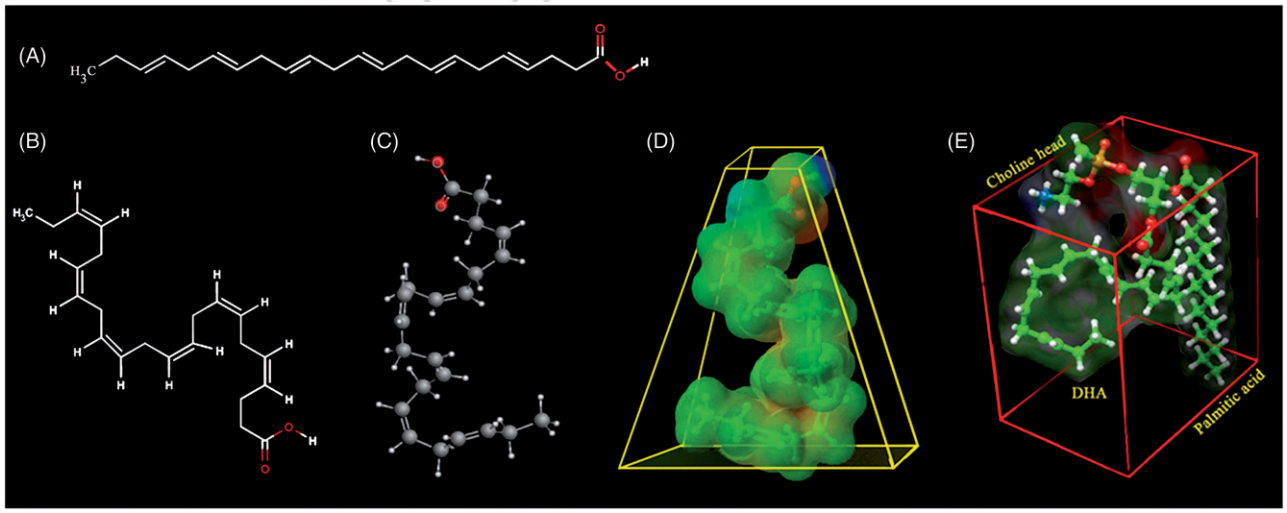

Figure 2. DHA has 22 carbon atoms and six double bonds. (A) (stick structure): straight-chain DHA that contains an unsaturated bond, originating at the third carbon from the methyl end. (B) 2D bent stick structure of DHA. (C) 3D conformer of DHA. (D) Van der Waals surface area with a cone-shaped DHA that gives the bilayer membrane a negative curvature. (E) Inter-molecular distance in the membrane leaflet is increased because of the extended conformation of DHA, which ultimately affects membrane fluidity-dependent receptor/enzyme activities.

neuronal growth factors. Studies have shown that DHA supplementation modifies both the structure and function of membranes. In particular, DHA-containing phospholipids help maintain proper membrane fluidity in neuronal cells, which is important for signal transduction and membrane permeability [48]. Moreover, an increased incorporation of DHA into synaptic membranes reportedly improves signal transduction involving phospholipase A2 and/or C [49], enhances glutamatergic [50] and dopaminergic [51] synaptic activities, and enhances [^{3}H]-noradrenaline release in SH-SY5Y cultured cells [52]. These studies suggest that DHA-induced changes in membrane fluidity affect various membrane functions, such as binding of hormone and growth factor receptors, activity of membrane-bound enzymes, transport of ions, and release and uptake of neurotransmitters of nerve cells; together, these changes ultimately influence the underlying brain function. Neuronal membrane fluidity is also crucial for receptors on the synaptic membranes to be able to recognize neurotransmitter-containing vesicles and transmit the messages that they contain. If the nerve cell membrane becomes too rigid, receptors on the membrane become less competent of recognizing neurotransmitters and transmitting signals to the nerve cell. Thus, membrane composition and fluidity status influence the ability of nerve cells to communicate with each other, which is essential for proper brain function. In concordance, we previously reported that neurobehavioral effects, particularly avoidance-related memory function, are associated with neuronal plasma membrane fluidity [32,33].

Antioxidant activities of DHA

Although it is a highly polyunsaturated fatty acid, astonishingly, DHA can act as an antioxidant in the brain (Figure 3). An increase in the number of double bonds renders cells more susceptible to damage by oxidation [53]. This notion may hold for auto-oxidation or *in vitro* oxidation. The brain accounts for less than 2% of the total body weight, whereas it accounts for approximately 20% of the total oxygen demand of the body. The antioxidative defense of the brain is poorer than that of the other organs of the body, including heart, liver and kidneys. The brain uses an uninterrupted supply of oxygen for continuous neurotransmission activity. The cells of the brain begin to die if it does not receive oxygen for only 3 min. Approximately 30–50% of total human cerebral dry weight is lipid,containing about 70% phospholipids, and 30–40% of the phospholipids are related to DHA. Under these vulnerable conditions, why is the brain enriched with a relatively large amount of DHA? Nature never selects detrimental elements

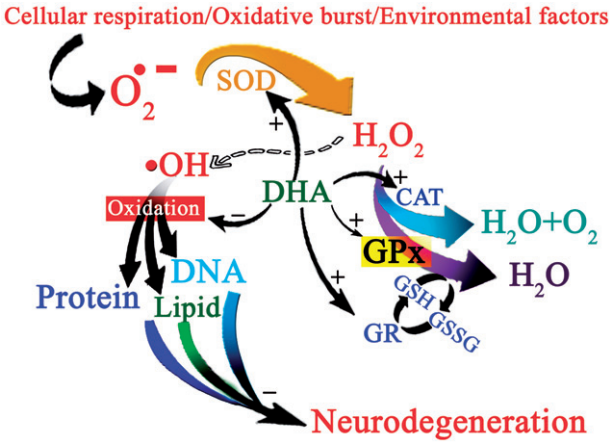

Figure 3. The figure depicts the antioxidant activity of DHA in brain tissues. Cellular oxidation and/or oxidative bursts/environmental factors lead to oxidation of O_2 and generation of superoxide anion ($O_2^{\bullet-}$). Superoxide dismutase (SOD) neutralizes $O_2^{\bullet-}$ to another reactive oxygen species, H_2O_2, which after extraction of another electron produces a highly reactive hydroxyl radical ($^{\bullet}$OH) species. Hydroxyl radical oxidizes cellular components, including proteins, lipids, and DNA, leading to neurodegeneration. DHA inhibits the neurodegenerative process by increasing antioxidant activity, including catalase, glutathione peroxidase and glutathione reductase.

without evolutionary consequences. So, why the discrepancy between the expected high oxidizability of the DHA molecule, owing to its high degree of unsaturation, and experimental results showing no change or even decreased lipid peroxidation when brain tissue is abundant in DHA? These support the view that the *in vivo* results might be quite different from the *in vitro* results. Interestingly, we have previously reported that the mere presence of DHA in brain, liver or endothelial cells does not predispose the membranes to oxidative stress but rather ameliorates oxidative stress. We inferred that the presence of ω-6 acids, such as arachidonic acid (AA, C20:4, ω-6), is attributable to the increased tendency of these cells to undergo oxidative insults. AA, which is also active in signal transduction pathways in a wide variety of cells, plays a major role in the increased production of lipid peroxide (LPO), an indicator of oxidative stress [54]. The concentration of AA was positively correlated with levels of LPO; however, the concentration of DHA was negatively correlated with levels of LPO. We found that the molar ratio of DHA/AA acted as an indicator of antioxidative defense. The cause–effect relationship between DHA and oxidability is thus far from clear. Oral administration of DHA was accompanied with an increase in the antioxidant activities, such as catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) enzyme activities [55,56]. We also found that dietary DHA increases mRNA expression of catalase and GPx in skeletal muscles of rats (unpublished data). There are

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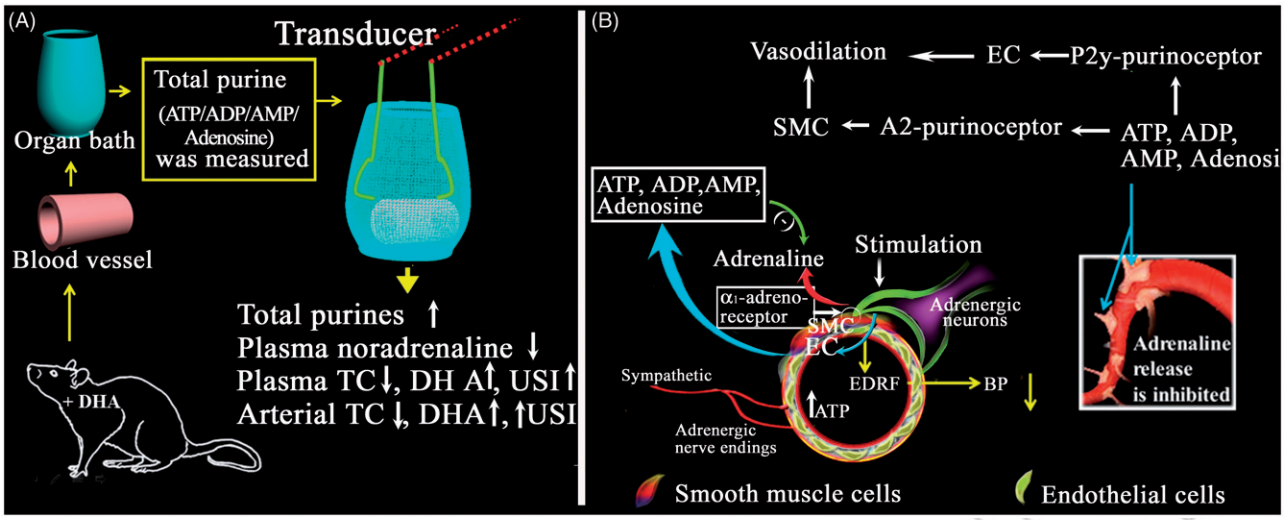

Figure 4. Effects of oral administration of DHA on hypertension and associated mechanism of actions of DHA in rats. (A) After chronic administration of DHA for 12 weeks, blood pressure was monitored. At the end of the dietary regimen, the rats were killed and the thoracic blood vessels were cleaned and subjected to organ bath/transducer. Then, stimulated and basal levels of total purines (ATP, ADP, AMP and adenosine) were measured. Plasma and arterial levels of DHA and total cholesterol were measured. Plasma noradrenaline levels were also determined by HPLC. (B) Proposed mechanism of action of DHA. Increased release of total purines inhibited the release of adrenaline from the sympathetic adrenergic nerve endings with a concurrent increase in the level of endothelial derived relaxing factor (EDRF) and all these finally reduced the blood pressure. The membrane DHA increased the membrane fluidity, which is believed to ameliorate membrane associated functions involved in the regulation of blood pressure.

also a few reports of the effect of DHA on the genetic expression of antioxidative enzymes. DHA increases expression of GPx in the brain hippocampus [57]. Dietary polyunsaturated fatty acids also increase the mRNA levels of catalase and glutathione peroxidase in hepatic tissues [58]. Finally, DHA being a member of the highly unsaturated fatty acid family can act as an antioxidant even in the oxidatively vulnerable organs including the brain.

Effect of DHA on systems/tissues other than the brain: may have beneficial effects on brain function

Hypertension is emerging as an important risk factor for dementia and Alzheimer's disease (AD) [59]. Both experimental animals and epidemiological studies suggest a role of vascular disease in the pathology of AD [60]. Moreover, risk factors for CVD and AD are generally shared [61], and risk factors for CVD are known to accelerate AD [62]. For example, ischemic white matter increases with an increase in blood pressure and appears to co-occur with AD. Therefore, addressing CVD risk factors is an important and reasonable approach for reducing the risk of AD and dementia. One of the most important risk factors for CVD is low intake of marine (ω-3) fatty acids, which is typical of Western diets [63–65]. In addition, dietary DHA may be beneficial, as it increases cerebral levels of the vasodilator acetylcholine, and thus, may reduce hypertension. Indeed, DHA has been shown to improve passive avoidance ability in stroke-prone spontaneously hypertensive rats [66]. We have previously reported that oral administration of DHA decreases blood pressure in the rats. The beneficial effects were attributed to decreased release of noradrenaline from the peripheral blood vessels [67] (Figure 4). The decreased release of noradrenaline from blood vessels was accompanied by an increased release of purine compounds, including ATP, ADP, AMP and adenosine. We hypothesized that the increased release of purines was associated with a DHA-induced increase in the membrane fluidity of endothelial and smooth muscle cells [67]. To test this hypothesis, we incubated endothelial cells, derived from rat thoracic aorta, with DHA in culture medium to enrich the plasma membrane with DHA [30]. DHA significantly increased plasma membrane fluidity with concurrent increases in the levels of DHA and total unsaturation index and decreases in the levels of cholesterol in the plasma membranes of endothelial cells. DHA also increased the plasma membrane fluidity of smooth muscle cells (yet unpublished), platelets, liver cells, concurrently with inhibition of platelet aggregation [32,33], canalicular-plasma membrane bound Mg^{+2}-ATPase and 5-nucleotidase enzyme activities; these results agree well with the proposition that DHA-induced increases in membrane fluidity,

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at least partially, affect membrane-related functions that influence hypertension, platelet aggregation and other related cardiovascular functions. With the results of these investigations, we also showed that polyunsaturated fatty acid DHA might provide beneficial effects other than those provided by its anti-hypercholesterolemic/anti-hypertriglyceridemic effects. Furthermore, in a meta-analysis of 48 studies of more than 100,000 subjects, fish oil consumption statistically improved cardiovascular health and overall health [68]. These benefits have been attributed mainly to its positive effects on triglyceride, lipoprotein metabolism, healthy blood flow, platelet function, vasodilation and vascular tone [69]. Finally, if hypertension is definitely a risk factor for AD or shares the same pathophysiology, it is reasonable to expect that measures, such as increasing the intake of dietary DHA, directed at hypertension control will enhance cognitive function. This might be an important public health goal of DHA.

DHA improves memory

Memory, which denotes the recall of past events or information in the absence of the original, can be measured by testing the changes in an animal's behavior during/after learning processes. The hippocampus and the cerebral cortex are referred to as the key structures of memory formation [70]. To our knowledge, direct beneficial effects of DHA on memory were first reported by Gamoh et al. [71] at our laboratory. DHA (300 mg/kg/day, for 10 weeks) fed to male Wistar rats (tested by radial maze tasks and/or active shuttle avoidance apparatus) (Figure 5(A), (A1) and/or (B,B1)) significantly ameliorated learning-related memory in DHA-deficient rat groups. Although the mechanism is unclear, corticohippocampal enrichment of DHA was positively correlated with improvement of memory [71,72]. Lim and Suzuki H [73] also reported that dietary administration of DHA to young mice for 4–7 months improved their spatial cognition learning ability. Although at that time we lacked

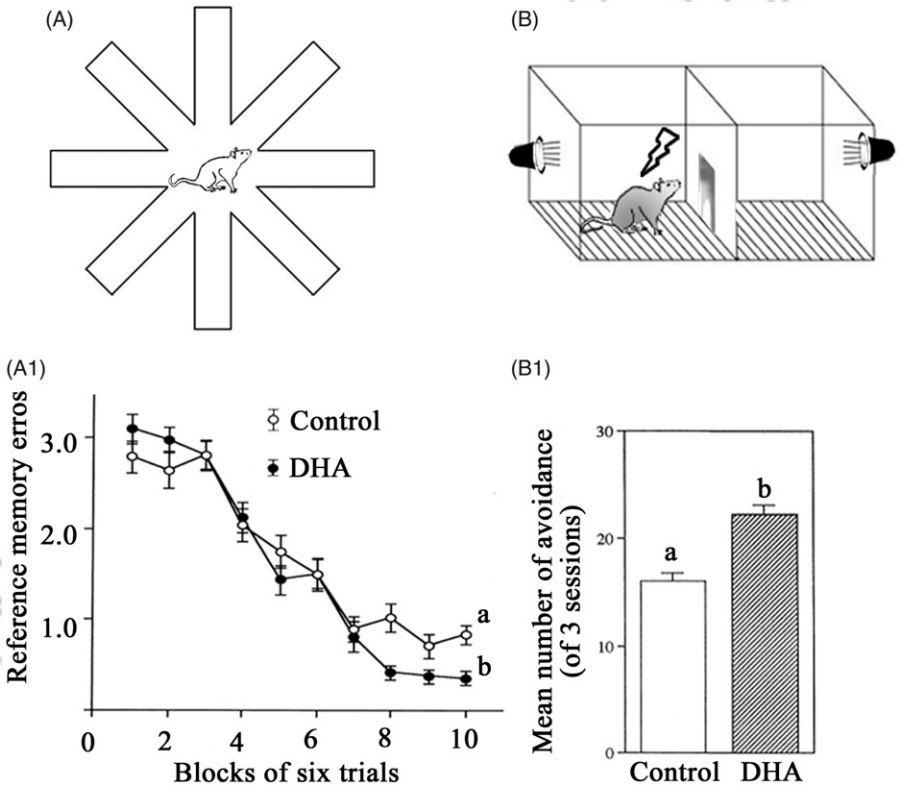

Figure 5. (A) Evaluation of learning-ass…m radial maze experimental paradigm. Memory (long-term) was measured by d… …ors (RMEs) (repeated entry into baited/unbaited arms) of young rats in the radial maze task [71]. (A1) The number of RMEs over blocks of trials. Each value denotes the number of RMEs made until the rat acquired all the rewards; results are mean ± SE in each block of six trials. (B) Evaluation of memory of DHA-fed rats by active shuttle avoidance apparatus [72]. The performance of eac… …tomatically recorded at each trial, and learning ability was determined as the number (#) of avoidance responses/sessio… …sponse latency in avoiding and escaping/UCS shock. The upper the number of avoidance responses, the higher the learning ability. One session consisted of 10 trials. Each rat had a total of three sessions, at days 7, 14 and 21 after surgery. (B1) Mean total number of "avoidance responses" at 7, 14 and 21 days after the commencing of surgery. Values are mean ± SE for each group of 30 trials. DHA was administered at 300 mg/kg/day.

Alzheimer's disease (AD) model

for AD

data describing the mechanism(s) of action of DHA, our investigation supported the notion that oral administration of DHA ameliorates learning-related memory of rats [71]. The hippocampus plays a vital role in learning and memory [74], and synaptic plasticity of the hippocampus promotes to the acquisition and retention of memories [75,76]. To reveal the mechanism of action of DHA, we assessed the levels of some important proteins responsible for memory formation along with some of their mRNA expression levels.

DHA affects important molecular substrates and contributes to memory formation

The mechanism(s) of action that underlies learning and memory is changes in synaptic plasticity (synaptic connectivity between neurons) with experience. Synaptic plasticity is umpired by long-term potentiation (LTP) [77], which involves an interaction between an extracellular ligand and membrane-bound receptors and a series of downstream signaling events in postsynaptic neurons, very often followed by retrograde signals to the presynaptic cells. The purpose of all these events is to make (synthesize) new proteins and sculpt new synapses, and finally to increase the connectivity among neurons. Accordingly, new synaptic infrastructures are formed for a given activity (memory) by changing the numbers and shape of the synapses or functions over periods of time that might last for a few seconds, minutes or hours or even for a lifetime. It is then said that a memory has been formed. Depending on time, it is referred to as a short-term, long-term or other kind of memory. Plasticity thus describes how experiences restructure neural pathways in the brain. Long-lasting functional changes in brain neurons occur when we learn new things or memorize new information for a longer period of time, and vice versa. For the above reasons, LTP is said to be the foundation of memory formation. LTP can be induced by the activation of the NMDA receptor (NMDAR). Inhibitors of NMDARs such as AP5 [78] stop the induction of LTP in the hippocampus. Transgenic mice with increased NMDAR expression, showed increased memory [79]. The NMDAR subunits NR2A and NR2B are associated with activity of the receptors. Disruption of hippocampal NR2A and NR2B subunits is associated with impairment of LTP and memory [80–82], signifying that expression of both NR2A and NR2B subunits is important for memory formation. How does an increase in the level of neuronal DHA affect synaptic function? Dietary supplementation with DHA restores neurotransmitter release and impairment in expression of LTP. DHA is required for induction of LTP [83,84]. We accordingly investigated the effect of chronic oral administration of DHA on the NMDAR-subunit proteins, including NR2A and NR2B and other synaptosome-associated proteins. This included presynaptic synaptophysin and presynaptic density protein-95 (PSD-95), and brain derived neurotrophic factor (BDNF) and BDNF's receptor tyrosine protein kinase B (TrKB) (Figure 6). The mRNA levels of both NR2A and NR2B significantly increased in the hippocampus of DHA-fed rats, compared with those in control rats. The oral administration of DHA to rats increased the expression of NR2A, whereas the expression of NR2B and TrKB was decreased in the cortex. At present, we are not certain about the (differential) effect of DHA on the expression patterns of NR2A/NR2B and/or TrKB in the brain. Literature reviews, however, suggest that these four subunits of NMDARs are distinct in terms of their distribution, properties and regulation. Thus, the reason why DHA exhibited a differential effect on the expression of these proteins remains unresolved. If the roles of NMDAR appear to be valid, our data suggest that dietary supplementation with DHA can modulate LTP, hence can help to form memory. The impairment of memories of control rats also coincided with a significant decrease

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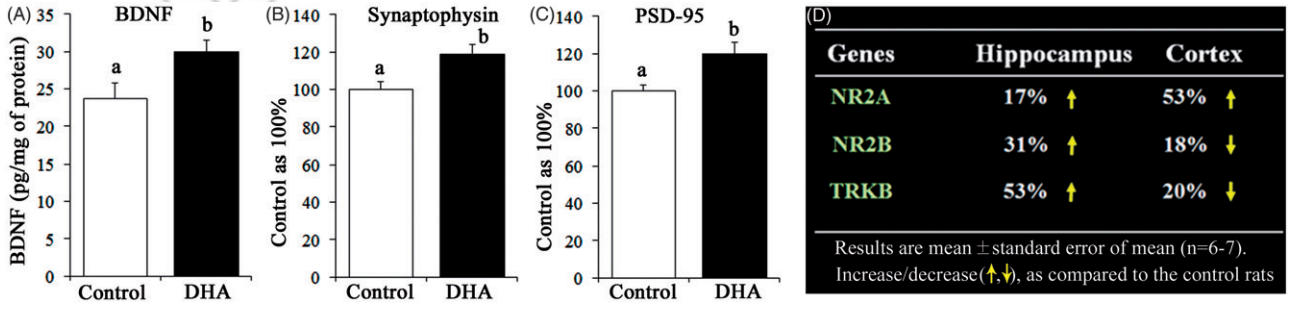

(D)

Genes	Hippocampus	Cortex
NR2A	17% ↑	53% ↑
NR2B	31% ↑	18% ↓
TRKB	53% ↑	20% ↓

Results are mean ± standard error of mean (n=6-7).
Increase/decrease(↑,↓), as compared to the control rats

Figure 6. Effect of oral administration of DHA on the relative protein levels of brain-derived neurotrophic factor (BDNF) (A), synaptophysin (B) and post-synaptic density protein-95 (PSD-95) in the hippocampus. (D) Effect of oral administration of DHA on the mRNA levels of NMDA-receptor subunits NR2A and NR2B and tyrosine receptor kinase B (TrKB), the receptor of the BDNF in both the cortex and hippocampus. The DHA significantly increased both the translation (protein levels) and transcription (mRNA levels) of important cognition-related proteins.

in the mRNA levels of the TrKB, and protein levels of the PSD-95, synaptophysin and BDNF in the hippocampus and cortex. NMDARs interact with the BDNF/TrkB pathway to support synaptic plasticity [85]. NMDARs remain anchored to PSD-95, aiding in signal trafficking of NMDARs and LTP regulation [86]. BDNF/TrKB plays important roles in consolidation of memories [87]. The presynaptic membrane-associated protein synaptophysin increases spatial memory [88] and is also involved in the regulation of the kinetics of synaptic vesicle endocytosis [89]. Taken together, the results of our DHA-study indicate that decreased levels of these memory-related protein-substrates in control rats may have accounted for the decreased or poor expression of memory. Consistent with the results of other studies, DHA increased the levels of BDNF [90], NR2B [91], and TrKB [90] in the hippocampus. Therefore, the DHA-instigated increased expressions of TrKB, NR2A/NR2B subunits of NMDAR and BDNF, synaptophysin, and PSD-95 levels may have been responsible for the increased memory of DHA-fed rats. We have previously reported that dietary DHA increases the expression of hippocampal Fos protein [92], encoded by the immediate early gene c-fos, a transcription factor and a functional marker of neuronal activity. In awake rats, a rapid increase in the level of Fos-related protein is associated with LTP generation in the dentate gyrus [93]. All the findings suggest that DHA directly or indirectly regulates the expression of various genes and may exert increasing effects on learning and memory.

Effects of DHA on neurogenesis and improvement of memory

The dentate gyrus is a part of the hippocampus and is critical for forming/storing spatial memories. It is one of the regions in the brain where neural progenitor cells constantly produce new neurons (i.e. undergo neurogenesis), which then integrate into the new neural network and form new synapses with other numerous neurons. Although the exact mechanisms remain unknown, neurogenesis is believed to participate in learning and memory [94]. Therefore, we studied whether DHA affects the differentiation of neural stem cells (NSCs) both *in vitro* and *in vivo* conditions [95]. NSCs isolated from 15.5-day-old rat embryos were propagated as neurospheres and cultured with or without DHA for the periods of 4 and 7 days. DHA significantly elevated the number of Tuj1-positive neurons when compared with that of the control on both 4 and 7 culture days, and the newborn neurons in the DHA group were morphologically more mature than those in the control (Figure 7, left panel). Thus, DHA stimulates the differentiation of neural stem cells into neurons by helping the exit from cell cycle and suppressing

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Right panel

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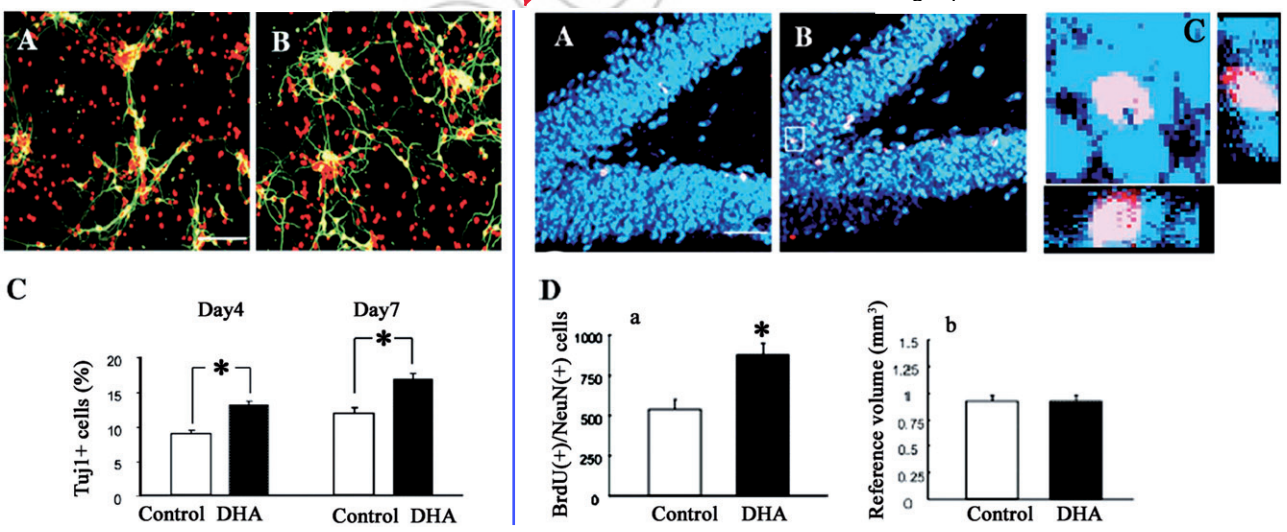

Figure 7. Left panel: (A) confocal images of Tuj1 immunostaining in control (A) and DHA groups (B) on day 7, Tuj1 (green), PI (red). Scale bar, 100 µm. (C) Quantification of Tuj1 immunoreactive cells in control and DHA groups on days 4 and 7. Data are shown as means ± SEM obtained from five to six independent cultures. Seven random fields were counted in each culture. $p < 0.0005$. Right panel: (A) neuronal identification of newly-divided cells in the adult rat DG. (A, B): confocal images of DG in vehicle (A) and DHA-treated (B) rats. BrdU (red), NeuN (blue). Scale bar +50 µm. (C) BrdU(+)/NeuN(+) newborn neuron in the white box in B. (D) Quantitative analysis of the number of newborn neuron (a) and reference volume (b) in the entire granule cell layer of the dentate gyrus (DG) in the control and the DHA rats. Data are shown as means ± SEM obtained from six hemispheres in three animals. $p < 0.005$ (with permission of Elsevier).

5-BrdU(+)/NeuN(+) newborn neurons in the granule-cell layer of the dentate gyrus in the adult rats (Figure 7, right panel). These results indicate that DHA efficiently stimulates neurogenesis process both *in vitro* and *in vivo* conditions, suggesting that it modulates hippocampal function regulated by neurogenesis [95]. Therefore, DHA-induced enhancements of (spatial) memory might be mediated by DHA-induced escalations in neurogenesis in the hippocampus. The molecular mechanism of DHA-induced neurogenesis is complicated and remains to be clarified. For differentiation, neural cells must be arrested at the G1 phase, and has to arrive at the G0 phase without passing the cell-cycle restriction-point. The repressor-type bHLH transcription factors, including Hes1 and Hes5 support NSCs in the undifferentiated state and/or delay neuronal differentiation [96,97]. On the other hand, the activator-type bHLH transcription factors, including neurogenin, Mash1 and NeuroD enhance neuronal differentiation. Katakura et al. [98] reported that DHA increases the differentiation of neural stem cells by stimulating activator-type transcription factors (e.g. neurogenin, Mash1, NeuroD) by arresting the cell cycle at the G0 phase, with concomitant inhibition of the repressor-type transcription factors, including Mes1, which otherwise inhibits the transcription/translation of the activator-type transcription factors (Neurogenin, Mash1, NeuroD) (Figure 8). These results thus show that DHA influences progenitor cells, directing their differentiation and transformation into new neurons leading to maturity, which in turn, *in vivo*, form synapses to increase synaptic connectivity (circuitry), and thereby contribute to new learning and memory.

Alzheimer's disease pathology and effects of DHA on it

Since AD is a progressive neurodegenerative disorder, regeneration of neurons from neural stem cells would thus have possible therapeutic values. If DHA could act as a stimulus of neurogenesis in the brain, it would be an ultimate brain food. Usually, AD is characterized by a deterioration of memory and cognition [99]. Neuropathologically, AD is identified by three major signs: amyloid-β plaques (Aβ), neurofibrillary tangles (NFT), and synaptic loss [100]. The amyloid beta peptides that are the main components of amyloid aggregates are $A\beta_{1-42}$, $A\beta_{1-40}$ and $A\beta_{25-35}$. The purified amyloids are commercially available, enabling us to prepare model rats by directly infusing amyloid beta peptides into the rat brain ventricle, from which Aβs diffuse into the surrounding hippocampus and cortical tissues, mimicking the deposition of Aβ seen in AD patients (Figure 9, left panel). We used third-generation DHA-deficient rats to generate AD model rats, with each generation fed on fish oil-deficient diets [71]. These

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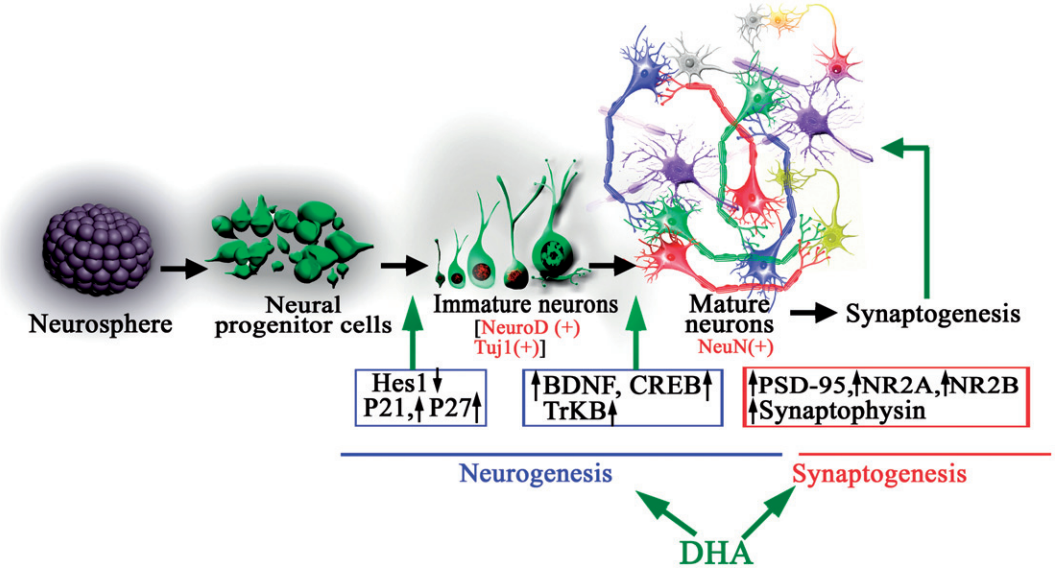

Figure 8. Schema of the effect of DHA on the cell cycle of neural stem cell progenitors. DHA inhibits the repressor-type transcription factor Hes1 and stimulates activator-type transcription factors including NeuroD. DHA also increases the brain-derived neurotrophic factor (BDNF) and its receptor TrKB. DHA-instigated downstream signal from BDNF-TrKB activation may have activated the cAMP-bound response-element binding protein (CREB), initiating the transcription and translation of other effector/relay proteins. These proteins may be the pre-/post-synaptic proteins (e.g. synaptophysin/PSD-95) required for new synaptogenesis or receptors such as NMDA-receptor subunits NR2A and NR2B. Addition of DHA to the stem cell culture and/or oral administration of dietary DHA to rats significantly ameliorated these neurogenesis/synaptogenesis-associated proteins, with a concurrent amelioration of learning and memory of elderly/Alzheimer's disease model rats.

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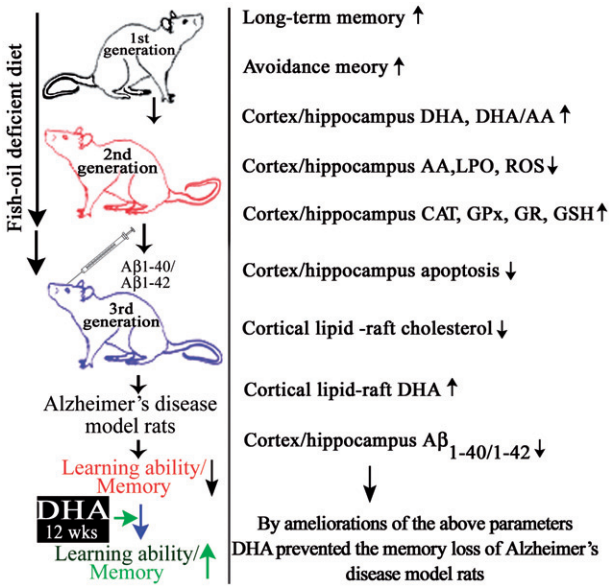

Figure 9. Left panel: preparation of amyloid beta peptide-infused AD model rats. A mini-osmotic pump (alzet 2002; Durect Co., Cupertino, CA), containing either Aβ(1–40/42) solution or the vehicle-alone was quickly inserted in the upper-backs of the rats. The opening of the pump was inserted 3.5 mm into the left ventricle (right and left, relative to Bregma; 0.8 mm posterior, 1.4 mm lateral) and attached to the skull with small screws and dental glue. Oral administration of either DHA emulsion and/or gum Arabic solution (vehicle of DHA) was restarted 2 days after surgery and continued until the end of the experiment. Right panel: at the end of the behavioral experiments (8-arm radial maze/shuttle avoidance apparatus), the rats were killed and several parameters (as shown in the figure) were measured. The oral administration of DHA significantly ameliorated these parameters. A downward arrow indicates a decrease and an upward arrow an increase.

produced DHA deficiency in both brain and serum of the rats. Under these conditions, the effect of oral administration of DHA was prominent in normal aged rats. However, such a direct manipulation of human brain DHA concentration is not possible, for ethical reasons. These results stimulated us to investigate the effect of oral administration of DHA on cognitive impairment of Aβ-infused AD model rats [72,101]. After 12-week oral administration of DHA, increases in brain DHA levels were significantly associated with amelioration of learning-related memory of the rats. These results provided us with an ample opportunity to study the effect of DHA in AD model rats maintained in DHA-deficient conditions for three generations. The oral administration of DHA for 12 weeks to Abeta-infused AD model rats significantly improved memory loss. The mechanism of the ameliorative effect was associated with: (i) increases in the levels of DHA and decreases in levels of arachidonic acid in both brain cortex and hippocampus, with resulting increases in the molar ratios of DHA/AA; (ii) decreases in the levels of LPOs in the cortex–hippocampus of DHA-fed AD model rats; (iii) decreases in reactive oxygen species (ROS) levels in synaptosomal plasma membranes; (iv) decreases in the levels of histone-associated DNA fragments, an apoptosis marker; (v) decreases in cortical lipid-raft cholesterol; (vi) increases in lipid-raft DHA levels and (vii) decreases in the amyloid burden in the cortex of AD model rats. Several studies have reported the beneficial effects of DHA in AD model animals (Figure 9, right panel). Dietary supplementation of DHA in an APP/PS1 transgenic rat model reduced behavioral deficits and Aβ pathology, with concurrent reductions in prefibrillar toxic oligomers [102]. Moreover, DHA supplementation decreased Aβ accumulation in the APP/PS1 transgenic mouse models [103,104], particularly at the earlier stages of disease progression [105–107]. The anti-Aβ effects of DHA supplementation have been primarily ascribed to its capability to reduce Aβ production via various mechanisms, including modulating APP localization and reducing α- and β-secretase enzyme activity [103], reducing PS1 levels [106], or reducing β- and γ-secretase enzyme activity and increasing α-secretase enzyme activity [108]. All these data were compatible with the expected positive effects of DHA on the AD model rats. In agreement with other studies, there is a link between DHA and brain cognition in AD.

Effect of DHA on lipid rafts

Lipid rafts or caveolae are specialized membrane structures consisting of saturated fatty acid- and cholesterol-rich membrane-invaginated floating microdomains. They harbor many key proteins and serve as signaling platforms to facilitate the transfer of substrates and protein–protein and protein–lipid interactions to facilitate specific signal transduction in living cells (Figure 10(C)). Functionally, lipid rafts are also involved in intracellular trafficking of proteins, lipids, secretory-endocytotic pathways, signal transduction, inflammatory and proteolytic signals [109]. The enrichment of DHA in these lipid-raft domains, concurrently with expulsions of cholesterol and saturated fatty acid, has been attributed to the beneficial effects of DHA on signal transduction in retinal endothelial cells and immunoresponse by T cells [110,111]. The augmented presence of Aβ in blood plasma is a potential noninvasive diagnostic marker for AD [112,113]. Aβ has previously been shown to be capable of binding to RBCs in *in vitro* as well as *in vivo* animal studies [114]. Similarly, in humans, Aβ in blood plasma may readily contact RBCs in the circulating blood and impair their oxygen binding capacity [115,116]. Indeed, we (Hashimoto et al., 2015) and others [114,117] have found that Aβ can bind to RBCs to

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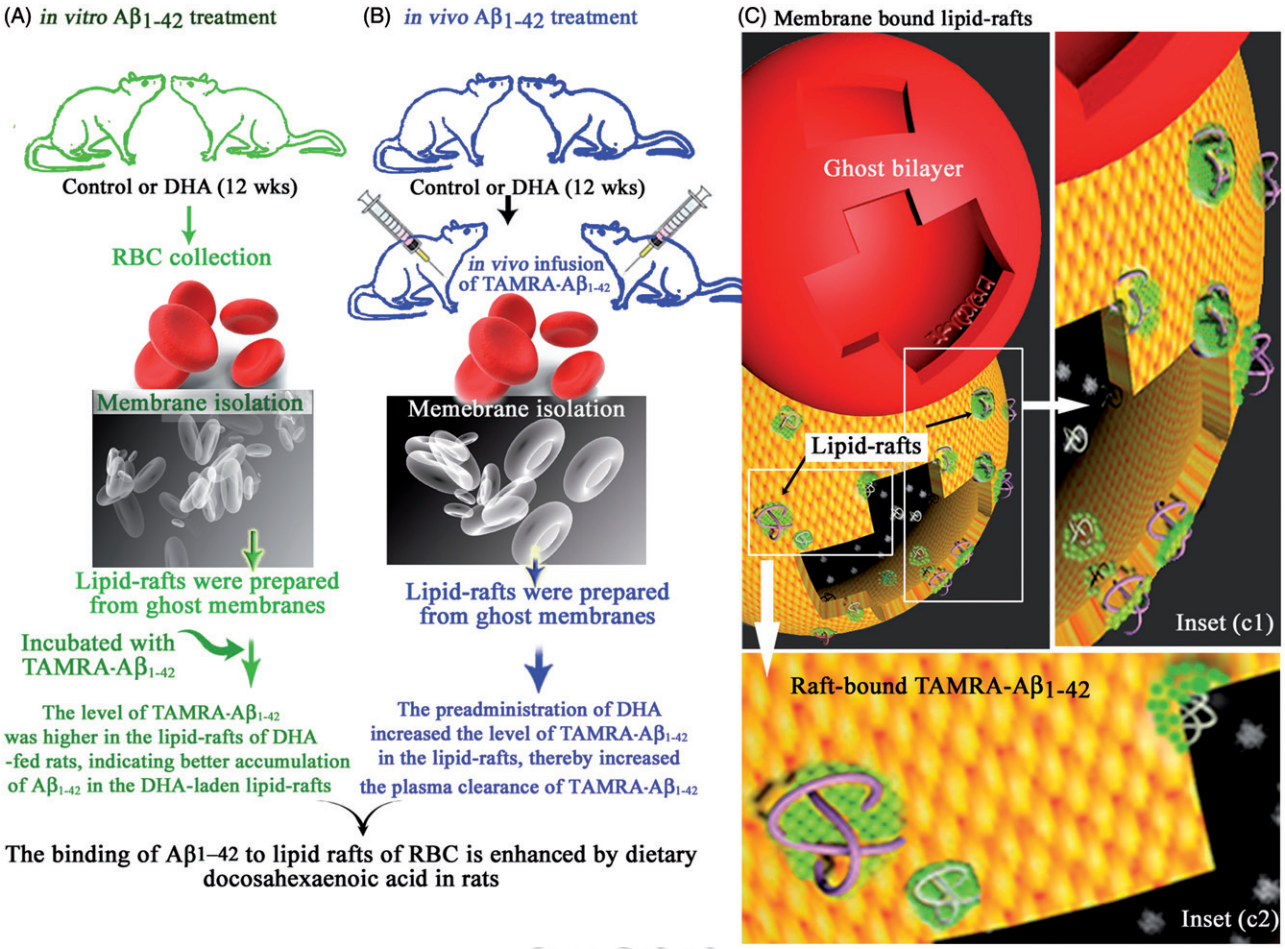

Figure 10. Effect of oral administration of DHA on the raft-driven clearance of $A\beta_{1-42}$. DHA pre-administration significantly increased the accumulation of fluorescently labeled Aβ1–42 (TAMRA-Aβ1–42) in the lipid-rafts of RBCs ghost membranes both *in vitro* (A) and *in vivo* conditions (B). (C) Schema of ghost bilayer, lipid-rafts (c1) and lipid-raft-bound Aβ1–42 (c2).

induce oxidative injury. Moreover, Aβ induces the binding of erythrocytes to endothelial cell layers and decreases endothelial viability [118]. Together, these studies suggest that Aβ plays a key role in the blood and oxidatively impairs the function of RBCs, which is important for adequate O_2 supply to the brain. Kiko et al. [119] reported that human RBC-$A\beta_{1-40}$ and -$A\beta_{1-42}$ levels increased with aging and imply a pathogenic role for RBC-Aβ. RBC membranes in AD patients are injured by unavoidable exposure to Aβ [119]. The reasoning is that once amyloid β peptides (Aβs) of Alzheimer's disease build in the blood circulation, they are capable of binding RBCs and inducing hemolysis. The mechanisms of the interaction between RBC and Aβ are largely unknown. Very recently, we investigated whether $A\beta_{1-42}$ interacts with caveolin-1-containing detergent-resistant membranes (DRMs) of RBCs and whether the interaction could be modulated by dietary pre-administration of DHA. DHA pre-administration to rats inhibited hemolysis by $A\beta_{1-42}$ (Hashimoto et al., 2015). This activity was accompanied by increased DHA levels and membrane fluidity and by decreased cholesterol levels, lipid peroxidation, and reactive oxygen species in the RBCs of the DHA-pretreated rats, suggesting that the antioxidant activity of DHA rescues RBCs from oxidative damage by $A\beta_{1-42}$. Furthermore, to supply adequate oxygen to the brain, RBCs must deform as they pass through the narrow pores of capillaries in the brain, and this deformability decreases when Aβ is bound to them [116]. Therefore, the interaction of Aβ with RBCs may decrease blood flow, impair oxygen delivery to the brain, and contribute to brain hypoxia, thereby potentially facilitating AD. RBC deformability is also impaired by reduced membrane fluidity, which is reduced by decreased membrane fatty acid unsaturation [120] and increased by lipid peroxidation [121] and/or membrane cholesterol. Moreover, increased RBC-membrane cholesterol is accompanied with reduced oxygen unloading to the tissues [122]. Thus, DHA-induced improvements of these parameters in RBCs may improve the detrimental effects of Aβ on RBCs and subsequently enhance the brain function in patients with AD. The level of caveolin-1 was

increased in the DRMs of DHA-preadministered rats. Binding between $A\beta_{1-42}$ and DRMs of RBC significantly increased in DHA-pretreated rats (Figure 10(A)). When fluorescently labeled $A\beta_{1-42}$ (TAMRA-$A\beta_{1-42}$) was directly infused into the bloodstream, it again occupied the caveolin-1-containing detergent resistance membrane (DRMs) of the RBCs from the DHA-preadministered rats to a larger extent, indicating that circulating Aβs interact with the Caveolin1-rich rafts of DRMs and that the interaction is stronger in DHA-enriched RBCs (~~Hashimoto et al., 2015~~) (Figure 10(B)). We described the mechanisms as follows: DRM vesicles displayed Aβs bound onto their surface. Aβ might also bind with caveolin1-containing lipid-rafts of RBCs. Then, the bound-Aβ is subjected to protease-degrading enzymes present on their surfaces via their raftal pockets, which deliver the Aβs to the liver for detoxification by liver proteolytic enzymes such as cathepsin D (~~Hashimoto et al., 2015~~). Whatever the mechanism, DHA may help in the clearance of circulating Aβs by increased raft-dependent degradation pathways and implicate to therapies in Alzheimer's disease. The results of this study are in agreement with the hypothesis that the enhancement of DHA in RBCs decreases the plasma burden of amyloids. Finally, alterations in morphology initiated from modifications caused by toxic interactions of oligomeric Aβ with RBCs, and these interactions involved caveolin-1-rich lipid-rafts. However, these RBC-disrupting actions were improved by the preadministration of DHA, leading to antioxidation, amyloid clearance and changes in the membrane properties of RBC.

Amyloid fibrillation *in vitro* and the effects of DHA

The chronic oral administration of DHA, besides playing a beneficial role in cardiovascular system, improves the memory-related learning ability in rats, and the level of DHA is depleted in the brains of AD [~~123,124~~], which frequently exhibits a decline in learning-related memory. Dietary administration of DHA protects against memory loss [72] and improves the impairment of memory-related learning ability of Aβ-infused AD model rats [101]. AD is characterized by aggregation of misfiled Aβs, including $A\beta_{1-42}$, $A\beta_{1-40}$ and $A\beta_{25-35}$ in affected brains. The mechanism of Aβ-fibrillation and the relationship of Aβ fibers and AD pathology are not clearly known, but involve a series of stages, including α-helix to β-sheet transformation, nucleation, oligomerization, beading of oligomers to matured fibers, and finally, coalescence of fibers into larger aggregates [~~125~~]. As we have previously reported, dietary DHA decreases the brain amyloid burden [72] or even helps in plasma clearance of amyloid levels by the RBC lipid-raft-driven mechanism (~~Hashimoto et al., 2015~~). Thus, we also wished to determine whether DHA directly inhibits the degree of fibrillation conducted in *in vitro* conditions. DHA (5.0–20 μM) significantly inhibited the *in vitro* fibrillation of $A\beta_{1-42}$, $A\beta_{1-40}$, and $A\beta_{25-35}$, as determined by ThT-fluorescence fluorospectrometry, laser-confocal microfluorescence and transmission electron microscopy (TEM) (Figure 11) [~~126–128~~]. By Western blotting, it was found that DHA inhibits the $A\beta_{1-40/42}$ at the di-to-tetramer species [~~126,128~~], while $A\beta_{25-35}$ was inhibited at the monomer level during their route to matured fibers [~~127~~]. Recent findings suggest that soluble Aβ oligomers, rather than matured-fibrils, correlate intensely with neuronal dysfunction, damage and AD symptoms [~~129~~]. If $A\beta_{1-42}$-oligomers could be inhibited *in vivo*, as they are *in vitro*, again, DHA would be a worthy therapeutic agent against Aβ-induced AD. DHA is an essential brain nutrient and can easily cross the blood–brain–barrier, with risk of its cytotoxic side effects being minimal. Finally, using antioligomer antibody, it was shown that DHA can inhibit the oligomers of the $A\beta_{1-42}$ amyloid species, the most toxic species that affects the brains of AD patients. If so, it is reasonable to conclude that Aβ-induced toxicity imparted to neuronal SH-S5Y5 cells would also be inhibited in the presence of DHA. As expected, DHA led to significant anti-amyloidogenic toxicity, as indicated by higher MTT reduction efficiency, than that in untreated cells [~~128~~]. Cells treated with $A\beta_{1-42}$ for 48 h displayed altered neuritic budding with dystrophic axonic/dendritic systems, whereas $A\beta_{1-42}$+DHA-treated cells showed well-defined axonic/dendritic sprouting processes and high viability, including full and spherical somas [~~128~~]. Therefore, the *in vitro* inhibitory effect of DHA on fibrillation (or intermediate species – during fibrillation) and the associated anti-neurotoxicity was also manifested in the *in vitro* cell culture model. Although there have been few *in vivo* reports on the effects of DHA on Aβ-aggregation, some results have indicated that oral dietary DHA supplementation may decrease the brain concentration of toxic Aβ oligomers, as measured using a conformation-specific anti-oligomer antibody (A11) in transgenic rat (APP/PS1) [102] and mouse (3xTg-AD) [106] models of AD.

DHA can act as a signaling molecule

DHA is now recognized as an important signaling molecule, particularly in brain function. Eicosanoids such as prostaglandins, thromboxanes and leukotrienes, are

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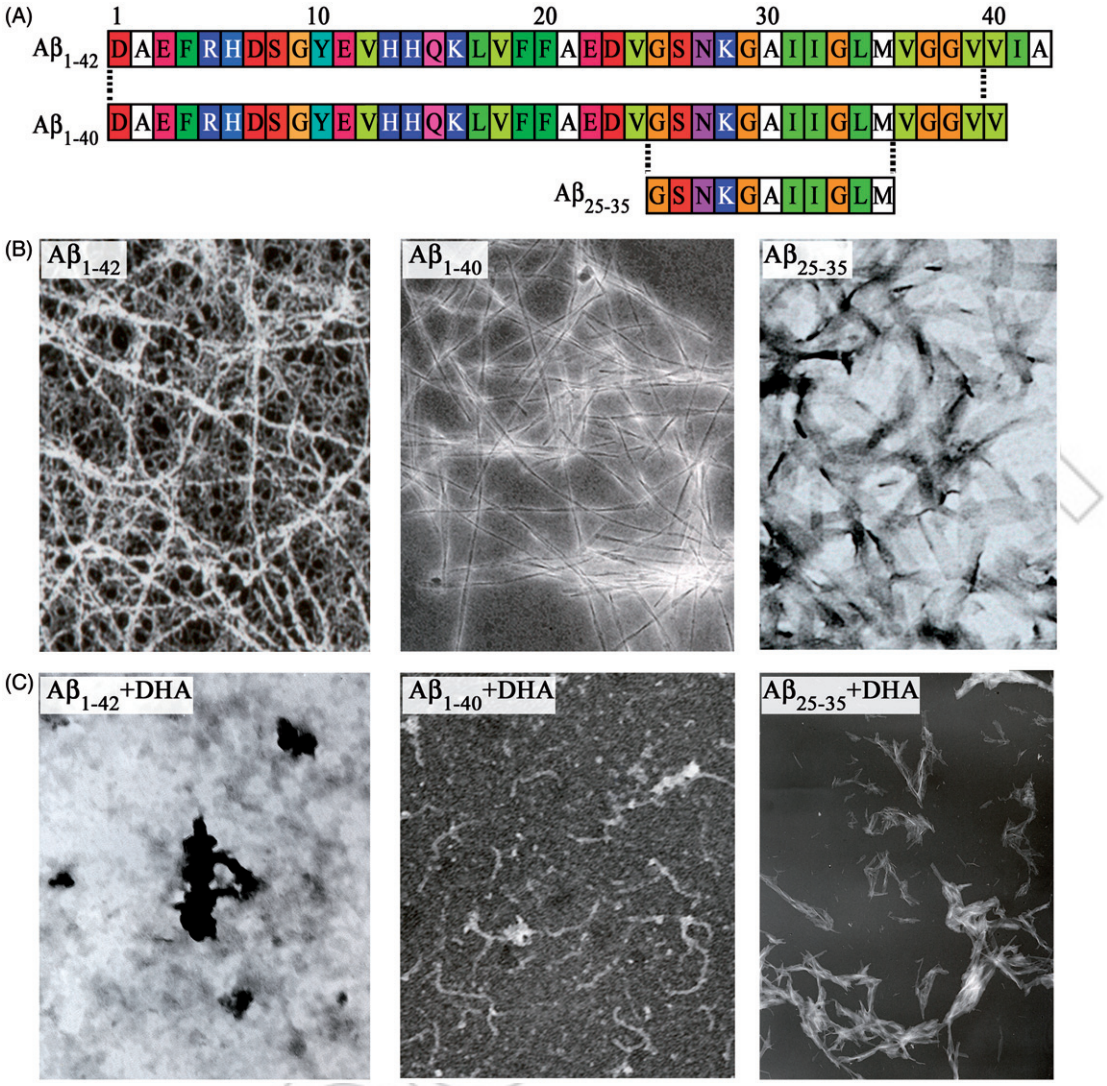

Figure 11. (A) Primary sequence of $A\beta_{1-42}$, $A\beta_{1-40}$ and $A\beta_{25-35}$. (B) Transmission electron micrographs (TEM) of $A\beta_{1-42}$, $A\beta_{1-40}$ and $A\beta_{25-35}$ fibers. (C) Effect of DHA (20 μM) on the *in vitro* fibrillation of $A\beta_{1-42}$, $A\beta_{1-40}$ and $A\beta_{25-35}$. DHA significantly decreased the amount of fibers, including oligomers (data not shown).

signaling molecules synthesized from the essential fatty acid arachidonic acid (AA), regulating blood clotting and important immune functions [130]. EPA, the precursor of DHA, can also act as a substrate for AA-cascade enzymes, but it induces the production of alternative eicosanoids such as 3-series prostanoids and 5-series leukotrienes, which are considered to be anti-inflammatory and/or less proinflammatory than AA-derived metabolites [131]. However, endogenous signaling by DHA-derived mediators (docosanoids) and their roles in brain circuitry have recently being reported, following the surprising discovery that a rapid increase in free DHA pool size occurs at the onset of seizures or brain injury. This phenomenon was later related to a bioactive docosanoid, namely neuroprotectin D1 (NPD1), formed from free DHA through 15-lipoxygenase-1 (15-LOX-1) [132]. Recently, we reported that a concentrated n-3 fatty acid formulation containing EPA and DHA could improve the learning ability of aged rats and whether this specific outcome had any relationship with the brain levels of EPA-derived eicosanoids and DHA-derived docosanoids. The rats were tested for reference memory errors (RMEs) and the working memory errors (WMEs) in an eight-arm radial maze. The fatty acid profile was analyzed by GC, whereas brain eicosanoids/docosanoids were measured by LC–ESI-MS–MS analysis. DHA-derived mediators showed a significant negative correlation with the number of RMEs, whereas EPA-derived mediators showed no relationship (Hashimoto et al., 2015). The question may arise as to how DHA-mediators affect memory-related brain activity and whether DHA-induced ameliorative effects on memory (in aged or AD model rats) underlie the phenomena. This question awaits further investigation.

The ligand-activated transcription factor peroxisome proliferator-activated receptor-γ (PPARγ), which regulates lipoprotein metabolism, adipogenesis and insulin sensitivity, has been implicated in AD [133,134]. Fatty acids bind to and activate peroxisome proliferator activated receptors, which control the expression of multiple genes affecting whole body fatty acid oxidation, storage, and inflammation. PPARγ activation triggers some of DHA's anti-inflammatory actions [134]. Moreover, PPARγ is a potential NPD1 target, given that it has a fatty acid binding pocket for polyunsaturated fatty acids [134] and their derivatives, including DHA [135]. DHA-derived docosanoids such as 10,17*S*-docosatriene also targets and affects the expression of nuclear factor κB (NFκB), which controls the production of proteins involved in inflammation and immunity, is seen during brain strokes, and plays inflammatory roles [136]. The DHA-derived mediator neuroprotectin D1 (NPD1; 10R, 17S-dihydroxy-docosa-4Z,7Z,11E,15E,19Z-hexaenoic acid) has been ascribed to decreased Aβ42 release [137], NPD1 downregulates inflammatory signaling, amyloidogenic APP cleavage and apoptosis in neurodegeneration [138]. We conclude that DHA can also regulate gene expression, governing the types of protein cells made and can thus regulate changes in gene expression that affect metabolism, inflammation, cell growth and development, and memory formation.

Epidemiological studies

On the basis of the results of the basic science, neural cell and animal studies, numerous epidemiological studies have been conducted. Soderberg et al. [123] and Prasad et al. [124] independently reported that DHA in the hippocampus is significantly lower in AD than in healthy controls. Numerous studies have reported a relationship between DHA and cognitive decline [139,140]. Gillette Guyonnet et al. [141] suggested that fish oil might protect the elderly from developing neurodegenerative diseases including AD. Literature reviews suggest that the beneficial role of ω-3 fatty acids in the prevention and progression of AD is still contradictory, as both "positive" and "no-effect" results on cognitive performance have been noted. The effects of ω-3 PUFAs have been reported both in mild cognitive impairment (MCI), a precursor condition or state of AD, and in AD. After a randomized double-blinded placebo-controlled trial, Chiu et al. [142] reported a significant improvement in cognition score in patients with MCI after DHA (0.72 g)+EPA (1.08 g) supplementation. Kotani et al. [143] demonstrated that DHA (240 mg/day) supplementation significantly ameliorated scores of immediate memory and attention in adults with MCI, but not in the AD patients who were provided with the same dose of supplementation for the same period. One study reported no significant prevention of cognitive decline in older people with MCI given DHA over six months [144]. Lopez et al. [145], in their dietary intervention study, reported that fish intake was associated with lower odds of developing AD, but this did not reach statistical significance. Indeed, numerous ω-3 supplementation studies [146–149] in AD patients have reported no significant improvement in AD measures. These investigators reported that supplementation with DHA (1.72 g)+EPA (600 mg) per day for six months did not show any improvement in cognitive deterioration in AD patients. However, in a very small subgroup of patients identified with the slightest form of AD, a significant reduction in the cognitive decline rate was observed in comparison with the placebo group. In summary, results from controlled studies suggest that intervention with ω-3 fatty acids is beneficial only in the early stages of cognitive impairment and that patients with well-established AD show no cognitive improvement with either low or high doses of ω-3 fatty acids. Both encouraging and unpromising data are available with respect to the link between DHA and cognitive deficit in AD patients. Thus, it is conceivable that the link between DHA intake and brain DHA is more complex than anticipated. Given that DHA synthesis from α-LNA and β-oxidation are both extremely low in humans [11], preformed DHA intake plays a significant role in human whole body DHA homeostasis. In principle, the effects of DHA should be experimentally evaluated under DHA deficient conditions. The brain's extraordinary and tenacious ability to keep the concentration of DHA constant has posed a serious problem. Following a 2-year randomized, double-blind, placebo-controlled trial, we have also reported that long-term daily dietary DHA (also EPA) supplementation exerts beneficial effects against age-related cognitive deterioration in otherwise healthy elderly Japanese with very mild dementia [150].

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In addition, we have more recently reported that DHA-enriched meal protects against age-related cognitive decline, and also improves apathy and caregiver burden for the oldest-elderly Japanese with cognitive impairment, such as dementia [153].

oproteins, cognitive and emotional health in elderly people and AD patients, using diverse routes to control multiple facets of cell metabolism, division and differentiation.

Disclosure statement

The authors declare no conflict of interest.

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