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Oral Supplementation with Docosahexaenoic Acid and Uridine-5'-Monophosphate Increases Dendritic Spine Density in Adult Gerbil Hippocampus

Toshimasa Sakamoto^a, Mehmet Cansev^{a,b}, and Richard J. Wurtman^{a,*}

^a*Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

^b*Department of Pharmacology and Clinical Pharmacology, Uludag University, Medical School, Bursa 16059, Turkey*

Abstract

Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid, is an essential component of membrane phosphatides and has been implicated in cognitive functions. Low levels of circulating or brain DHA are associated with various neurocognitive disorders including Alzheimer's disease (AD), while laboratory animals, including animal models of AD, can exhibit improved cognitive ability with a diet enriched in DHA. Various cellular mechanisms have been proposed for DHA's behavioral effects, including increases in cellular membrane fluidity, promotion of neurite extension, and inhibition of apoptosis. However, there is little direct evidence that DHA affects synaptic structure in living animals. Here we show that oral supplementation with DHA substantially increases the number of dendritic spines in adult gerbil hippocampus, particularly when animals are co-supplemented with a uridine source, uridine-5'-monophosphate (UMP), which increases brain levels of the rate-limiting phosphatide precursor CTP. The increase in dendritic spines (> 30%) is accompanied by parallel increases in membrane phosphatides, and in pre- and post-synaptic proteins within the hippocampus. Hence oral DHA may promote neuronal membrane synthesis to increase the number of synapses, particularly when co-administered with UMP. Our findings provide a possible explanation for the effects of DHA on behavior and also suggest a strategy to treat cognitive disorders resulting from synapse loss.

Keywords

docosahexaenoic acid; uridine; membrane synthesis; spine formation; synaptogenesis; phosphatides

1. Introduction

Nutritional supplementation with essential omega-3 fatty acids including docosahexaenoic acid (DHA) is increasingly practiced (Marszalek and Lodish, 2005). DHA, a 22-carbon compound with six double bonds, is synthesized from its precursor α -linolenic acid, which

*Correspondence should be addressed to: Richard J. Wurtman, M.D., MIT, 43 Vassar St., 46-5023B, Cambridge MA, 02139, Email: dick@mit.edu, Phone: 617-253-6731, Fax: 617-253-6882.

Scope: Neurophysiology, Neuropharmacology and other forms of Intercellular Communication

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cannot be synthesized in mammals (mammals lack the desaturase enzyme that forms the double bond at the third carbon from its methyl terminal) (Marszalek and Lodish, 2005). Since conversion from α -linolenic acid to DHA is very slow, DHA must also be obtained from dietary sources, for example fatty fish (Marszalek and Lodish, 2005). Like other fatty acids, DHA is a precursor of diacylglycerol (DAG) (Marszalek and Lodish, 2005), an essential and sometimes rate-limiting precursor for the phosphatides in cellular membranes (Araki and Wurtman, 1997). Clinical studies suggest that DHA can maintain or enhance cellular functions in various physiological systems including the nervous system (Marszalek and Lodish, 2005).

The mechanisms by which DHA affects brain functions or behaviors are poorly understood. The fatty acid readily crosses the blood-brain barrier (Spector, 2001; Hashimoto et al., 2002) and then cellular membranes, whereupon it is acylated and can be incorporated into DAG (Marszalek and Lodish, 2005). The DHA-containing DAG is preferentially utilized for membrane phosphatide synthesis (Marszalek and Lodish, 2005). Low levels of circulating or brain DHA are reportedly associated with neurodegenerative or psychiatric disorders, such as Alzheimer's disease (Soderberg et al., 1991; Schaefer et al., 2006), depression (Logan, 2004; Appleton et al., 2006) and schizophrenia (Assies et al., 2001; Reddy et al., 2004). Behavioral studies in laboratory rodents suggest that DHA is involved in learning and memory (Yoshida et al., 1997; Gamoh et al., 1999; Moriguchi et al., 2000; Calon et al., 2004; Hashimoto et al., 2002, 2006). Animals given a diet deficient in omega-3 fatty acids exhibit learning impairments (Yoshida et al., 1997; Moriguchi et al., 2000), while those receiving supplemental DHA show improved learning (Gamoh et al., 1999; Calon et al., 2004; Hashimoto et al., 2002, 2006). Various cellular mechanisms have been proposed as mediating DHA's neurobehavioral effects, for example increasing cell membrane fluidity (Hashimoto et al., 2006), promoting neurite extension (Ikemoto et al., 1997; Calderon and Kim, 2004; Marszalek et al., 2004, 2005; Darios and Davletov, 2006), inhibiting apoptosis (Hashimoto et al., 2002; Calon et al., 2004), and increasing synthesis of the phosphatides in synaptic membranes (Wurtman et al., 2006). However, there is little direct evidence that DHA affects synaptic structures in intact animals.

Uridine-5'-monophosphate (UMP) is a precursor of circulating uridine (Cansev et al., 2005). Like DHA, uridine readily crosses the blood-brain barrier and enters brain cells (Cansev, 2006). It is then phosphorylated by uridine-cytidine kinases to form uridine triphosphate (UTP), which can be further transformed by CTP synthetase to cytidine triphosphate (CTP), the usual rate-limiting precursor in phosphatide biosynthesis (Ross et al., 1997). CTP, for example, can combine with phosphocholine to form cytidine-5'-diphosphocholine (CDP-choline) (Kennedy and Weiss, 1956), which then combines with DAG to yield phosphatidylcholine (PC), the major phosphatide in neuronal membranes (Sastry, 1985). Uridine promotes neurite outgrowth in PC-12 cells treated with nerve growth factor (Pooler et al., 2005), and neurotransmitter release from the rat striatum in vivo (Wang et al., 2005, 2007). Moreover, when given with DHA, uridine increases membrane phosphatides and synaptic proteins in the adult gerbil brain (Wurtman et al., 2006). These results suggest that uridine as well as DHA can enhance phosphatide synthesis in neuronal or synaptic membranes.

Here we examined the effects of supplemental DHA and uridine on the number of dendritic spines in brains of living animals. Dendritic spines are small membranous protrusions extending from dendrites, which compartmentalize post-synaptic responses. They mediate most excitatory connections and are thought to reflect the number of excitatory synapses in the central nervous system (Matus, 2000; Hering and Sheng, 2001; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2004). We show that oral supplementation with DHA increases the number of dendritic spines in adult gerbil hippocampus, particularly when animals are also supplemented with UMP, a uridine source. Oral DHA may thus increase the number of brain synapses, particularly when co-administered with UMP. We have also examined the ability of arachidonic acid, an omega-6 fatty acid, to increase hippocampal dendritic spines. Unlike

DHA, this fatty acid has been shown (Cansev and Wurtman, In Press) not to affect brain levels of phosphatides or synaptic proteins.

2. Results

2.1. Oral supplementation with DHA increases dendritic spine density in adult gerbil hippocampus

To examine whether DHA affects the number of dendritic spines, we treated adult gerbils with the fatty acid daily for 4 weeks, in oral doses of 0, 50, 100 or 300 mg/kg. The spine density increased significantly in the primary apical dendrites of CA1 pyramidal neurons after the animals received either the 100 or 300 mg/kg/day doses (Fig. 1). The increases were 12% ($p = 0.04$) with the 100 mg/kg/day dose and 18% ($p < 0.001$) with the 300 mg/kg/day dose.

2.2. DHA-induced dendritic spine formation in adult gerbil hippocampus is enhanced by co-supplementation with UMP

We next tested the effect on dendritic spine density of providing both DHA (300 mg/kg/day, by gavage) and a uridine source, UMP (0.5%, via the standard choline-containing diet) for 4 weeks. A recent study from our laboratory had shown that oral UMP, given concurrently, enhances the DHA-induced increases in membrane phosphatide and synaptic protein levels in gerbil brain (Wurtman et al., 2006). DHA supplementation alone caused a significant, 19% ($p = 0.004$, Fig. 2B) increase in spine density. In contrast, animals that had received both DHA and UMP exhibited a 36% increase ($p < 0.001$ vs. Control), or approximately double the increase produced by DHA alone ($p = 0.008$ vs. DHA alone, Fig. 2B). Giving UMP alone did not significantly increase dendritic spine density (Fig. 2B), although it did when all dendritic protrusions (including filopodias) were included for statistical analysis (data not shown; see also Methods for the definition of dendritic spines). In another study, we examined the relation between duration of treatment with DHA and UMP and spine density; animals received the two compounds daily for 1, 2, 3 or 4 weeks. The effect of the co-supplementation on spine density was already apparent at 1 week ($p = 0.02$), and continued throughout the treatment period (Fig. 2C).

2.3. Supplementation with DHA or DHA-plus-UMP increases major membrane phospholipids in adult gerbil hippocampus

Treatment with DHA or DHA-plus-UMP daily for 4 weeks, which increased dendritic spine density (Fig. 2B), also caused major increases in membrane phosphatides within the gerbil hippocampus (Fig. 3). The increases with DHA alone were: phosphatidylcholine (PC): 8%; phosphatidylethanolamine (PE): 26%; phosphatidylserine (PS): 75%; and phosphatidylinositol (PI): 29% (all $p < 0.05$ except PC); and those with DHA-plus-UMP were: PC: 28%; PE: 59%; PS: 160%; and PI: 100% (all $p < 0.001$ vs. their controls). No significant increases were observed with UMP alone.

2.4. Supplementation with DHA or DHA-plus-UMP elevates expression of pre- and post-synaptic proteins in adult gerbil hippocampus

We further tested whether the increases in spine density following treatment with DHA and UMP were associated with increases in hippocampal levels of pre- and post-synaptic proteins. If the increased number of dendritic spines represented an increase in the number of synapses, it might be anticipated that there would be comparable increases in such proteins as PSD-95, GluR-1 (an AMPA receptor subunit) and Synapsin-1 (Greengard et al., 1993; El-Husseini et al., 2000; Kasai et al., 2003; Matsuzaki et al., 2004; Ziv and Garner, 2004; Matus, 2005). Expression levels of PSD-95 (El-Husseini et al., 2000) and GluR-1 (Kasai et al., 2003; Matsuzaki et al., 2004) are highly associated with growth of dendritic spines and levels of post-

synaptic responses. Synapsin-1, on the other hand, is expressed in pre-synaptic terminals, apparently anchoring synaptic vesicles to the actin cytoskeleton for exocytosis or synaptogenesis (Greengard et al., 1993; Ziv and Garner, 2004). Western blot analysis of aliquots of the hippocampal homogenates used for the phosphatide assays above (i.e., hippocampal homogenates obtained from the same animals as those tested for dendritic spines) demonstrated significantly elevated expression of all of these synaptic proteins after treatment with DHA alone or, to a greater extent, with DHA-plus-UMP (Fig. 4A-C). The increases in PSD-95, GluR-1 and Synapsin-1 with DHA alone were 42%, 29% and 37%, respectively (all $p \leq 0.05$); the increases after treatment with DHA-plus-UMP were 44%, 37% and 57%, respectively (all $p < 0.01$). We further examined the expression levels of two cytoskeletal proteins: Actin and β -tubulin. Actin has been demonstrated to directly regulate morphology of dendritic spines, and this effect can underlie such manifestations of synaptic plasticity as long-term potentiation (LTP) and depression (LTD) (Matus, 2000, 2005; Hering and Sheng, 2001; Nimchinsky et al., 2002; Matsuzaki et al., 2004). On the other hand, β -tubulin is a structural protein not specifically localized within synaptic structures. We found that the expression level of Actin was substantially elevated with DHA (by 60%, $p = 0.04$) and with DHA-plus-UMP (by 88%, $p = 0.004$), whereas that of β -tubulin was unaffected with the treatments (Fig. 4D and E).

2.5. Oral supplementation with an omega-6 fatty acid (arachidonic acid) does not affect spine density in adult gerbil hippocampus

To examine the specificity of DHA's effects on spine density, we determined whether arachidonic acid (ARA), an omega-6 fatty acid, also produces these effects. ARA contains 20 carbons with 4 double bonds; the first of these is situated at the sixth carbon from its methyl terminal (Marszalek and Lodish, 2005; Smith, 2005). Like DHA, ARA cannot be synthesized de novo in mammalian tissues, and must be obtained from dietary sources (Marszalek and Lodish, 2005; Smith, 2005). It also is a precursor for DAG (Marszalek and Lodish, 2005). Moreover, ARA is known to affect neurite outgrowth (Darios and Davletov, 2006) and synaptic plasticity (Williams et al., 1989; Ramakers and Storm, 2002; Feinmark et al., 2003). But unlike DHA, ARA administration daily for 4 weeks failed to affect dendritic spine density in the CA1 region of the gerbil hippocampus (Fig. 5A and B). Similarly, ARA failed to affect levels of hippocampal phosphatides or synaptic proteins (Fig. 5C and D).

2.6. Supplementation with DHA, UMP or ARA did not affect size of dendritic spines in adult gerbil hippocampus

In addition to dendritic spine number density, we investigated the effects of DHA, UMP or ARA on spine size. We measured the length and width of dendritic spines used for the spine density analysis described above. The mean length and the mean width were obtained for each dendrite and the averages of the means were compared between treatment groups. The results showed that none of DHA, UMP or ARA affected these parameters (SI Fig. 8 and 9).

3. Discussion

These studies show that oral supplementation with DHA, an omega-3 fatty acid, increases dendritic spine density in adult gerbil hippocampus (Fig. 1). This effect of DHA is approximately doubled when animals also receive UMP (Fig. 2), and is accompanied by parallel increases in membrane phosphatides (Fig. 3) and in specific pre- and post-synaptic proteins (Fig. 4). Supplementation with arachidonic acid (ARA), an omega-6 fatty acid, fails to increase spine density (Fig. 5).

Dendritic spines are small membranous protrusions extending from neuronal dendrites (Matus, 2000; Hering and Sheng, 2001; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2004). They

mediate most excitatory connections in the CNS, compartmentalizing post-synaptic responses (Matus, 2000; Hering and Sheng, 2001; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2004). In the adult mammalian cortex, all of the mature spines are thought to make synapses, with a typical spine having a single synapse at its head (Matus, 2000; Hering and Sheng, 2001; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2004). Hence, the increase in dendritic spine number that we observe may reflect an increased number of hippocampal synapses as well. DHA administration has been described as promoting cognitive functions (Gamoh et al., 1999; Hashimoto et al., 2002, 2006; Calon et al., 2004), yet its effects on synapses have been obscure. Our finding that oral DHA promotes spine formation suggests that one way through which DHA enhances cognition involves increasing the number of synapses. This hypothesis is further supported by our finding that the DHA-induced increase in spine density is accompanied by parallel increases in such pre- and post-synaptic proteins as PSD-95, GluR-1 and Synapsin-1 (Fig. 4).

DHA is a component of DAG, an immediate precursor of such phosphatides as PC, PE, PS and PI. DHA-containing DAG is formed by acetylating glycerol-3-phosphate with DHA on its sn-2 position, and with a saturated fatty acid on its sn-1 position (Marszalek and Lodish, 2005). Oral supplementation with DHA has been shown to elevate both plasma and brain DHA levels in rats (Hashimoto et al., 2002). In PC 12 cells, DHA added to the medium is rapidly internalized by the cells, and activated by acyl-CoA synthetases (Marszalek et al., 2004, 2005). It then increases cellular phosphatide levels and promotes neurite outgrowth (Marszalek et al., 2004, 2005). Given that formation of dendritic spines, like neurite outgrowth, requires a major increase in the amount of cell membrane, our present finding that DHA-induced spine formation accompanies substantial increases in the phosphatides suggests that DHA promotes spine formation by enhancing the synthesis of membrane phospholipids.

Formation of dendritic spines can be induced by synaptic inputs that induce LTP in CA1 pyramidal neurons (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). Because the induction of spine formation is blocked by inhibition of NMDA receptors, this process appears to be mediated by Ca^{2+} influx into the post-synaptic dendrite (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). In this context, membrane synthesis that realizes spine formation may be induced by Ca^{2+} or synaptic activity, as well as other related processes such as actin polymerization (Matus, 2000; Matsuzaki et al., 2004). In the present study, we tested the effects of two different classes of polyunsaturated fatty acids (i.e., DHA and ARA) on spine density. Like DHA, ARA can be acylated to the sn-2 position of DAG, and is enriched in neuronal membranes (Marszalek and Lodish, 2005). However, supplementation with ARA failed to affect brain levels of phosphatides or synaptic proteins in a previous study (Cansev and Wurtman, In Press), and failed to affect dendritic spine number or phosphatide levels (Fig. 5) in the present study. The mechanism that allows DHA but not ARA to promote spine formation or phosphatide synthesis awaits discovery, but it could involve the substrate specificities of the enzymes that regulate the utilization of the fatty acids for membrane synthesis (Marszalek and Lodish, 2005; Marszalek et al., 2005).

Our finding that oral supplementation with uridine substantially enhances DHA-induced spine formation provides confirmation that uridine can affect membrane phosphatide synthesis in neurons (Cansev, 2006; Wurtman et al., 2006; Cansev and Wurtman, In Press). Uridine, a pyrimidine nucleoside that readily crosses the blood-brain barrier, is a precursor of UTP, and subsequently of CTP (Cansev et al., 2005; Cansev, 2006), the major rate-limiting precursor in the synthesis of membrane phosphatides (i.e., PC, PE, PS and PI) (Ross et al., 1997). In brain cells, CTP reacts with phosphocholine or phosphoethanolamine to form CDP-choline or CDP-ethanolamine (Kennedy and Weiss, 1956; Sastry, 1985). These two products then react with DAG to generate PC or PE, respectively (CTP can also react directly with DAG to form CDP-DAG, which then reacts with inositol to yield PI; PS is mainly synthesized by a base exchange

mechanism in which free serine is substituted for the nitrogen base of a phosphatide) (Borkenhagen et al., 1961; Sastry, 1985). These metabolic processes can be limited by the availability of CTP or the proportion of DAG represented by a particular species (i.e., one containing a DHA or other omega-3 fatty acid). The effect on phosphatide synthesis of forming additional DHA-containing DAG would thus be maximized when uridine administration also increases CTP levels. Consistent with this hypothesis, our present study demonstrated that the promoting effects of oral DHA on spine formation and membrane synthesis are substantially potentiated by co-supplementation with UMP (a uridine source). The combined effects were more than the sums of the individual effects observed when each of the compounds was given alone (Figs. 2B and 3; see also Fig. 6).

The effects of DHA or uridine on spine formation may also be mediated by pre-synaptic mechanisms. Results from various model systems suggest that both DHA (Ikemoto et al., 1997; Calderon and Kim, 2004; Marszalek et al., 2004, 2005; Darios and Davletov, 2006) and uridine (Pooler et al., 2005; Wang et al., 2005, 2007) promote axonal growth and exocytosis. A recent study has demonstrated that DHA can activate the SNARE protein Syntaxin-3 (Darios and Davletov, 2006). Uridine, through UTP, can activate P2Y receptors (Pooler et al., 2005), which are expressed in hippocampal neurons (Abbrachio et al., 2006) and implicated in pre-synaptic induction of LTP (Price et al., 2003). Formation of dendritic spines or synaptogenesis in mammalian brains can be induced or initiated by pre-synaptic neurons (Matus, 2000; Hering and Sheng, 2001; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2004). The increases in spine density with DHA and UMP (Figs. 1 and 2) may be due to their promoting effects on pre- and/or post-synaptic mechanisms.

Various psychiatric and neurological disorders, for example, schizophrenia, mental retardation syndromes and Alzheimer's disease (AD), are accompanied by subnormal numbers of brain synapses or dendritic spines (Selkoe, 2002; Blanpied and Ehlers, 2004). Oral administration of DHA and uridine may provide a means to compensate for or prevent synapse loss in these disorders. Studies on animal models of AD have demonstrated that administration of DHA can improve their cognitive performance (Calon et al., 2004; Hashimoto et al., 2002, 2006). Promoting synapse formation may be a primary mechanism underlying the effect of DHA on behavior.

4. Experimental Procedures

4.1. Animals

Normal adult male gerbils (*M. unguiculatus*, 60~80g body weight) were given a standard rodent diet (Harlan Teklad) (Wurtman et al., 2006) and, by gavage, various doses of DHA or its diluent (5% gum arabic) daily for 4 weeks. Animals receiving UMP were given the above diet supplemented with 0.5% UMP. Animals supplemented with arachidonic acid (ARA) were treated in the same way as the DHA-supplemented animals, except that they received ARA in the place of DHA. All the diets also contained 0.1% choline. The fatty acids were purchased from Nu-Chek Prep, Inc. We used gerbils because, unlike rats, this species' pyrimidine metabolism closely resembles that of humans (Cansev, 2006).

4.2. Tissue preparation

Animals that had been treated for 4 weeks were sacrificed with CO₂ gas inhalation and then decapitated by guillotine. The hippocampi were then extracted quickly from each brain hemisphere; one was sliced at 300 μm thickness with a tissue chopper for neuronal imaging and the other was homogenized for membrane and protein assays (see below).

4.3. Neuroimaging

Immediately after slicing, tissues were fixed overnight with 4% paraformaldehyde at 4°C and then stained with a lipophilic membrane staining dye, DiI (CellTracker™ CM-DiI, Invitrogen), using a needle to embed the dye in CA1 regions of the hippocampus. Images of CA1 pyramidal neurons were obtained using a confocal microscope (Olympus, FV300). Individual slices were placed in PBS on a coverglass and imaged at 568 nm excitation to capture emission signals from DiI with a 60x water-immersion objective (Olympus, UPlan Apo). A three-dimensional image stack was obtained at 0.5 µm intervals in the vertical (Z) - axis, using a FluoView software (1024 x1024 pixels) with 3x zoom. The total depth of imaging space was mostly 5~10 µm (i.e., 10~20 optical sections). Five to 10 neurons were randomly sampled from 4~5 slices for individual animals. An individual image stack was analyzed on its two-dimensional projection using a MetaMorph software (Molecular Devices). Small protrusions extending from the primary apical dendrite of a CA1 pyramidal neuron (where CA3 axons form excitatory synapses with CA1 dendrites) were counted by drawing lines to measure the lengths of the protrusions from the edge of the dendritic shaft. Only one dendritic segment was analyzed per neuron. Protrusions whose lengths fell between 0.3 to 2 µm were defined as dendritic spines and included for analysis (Hering and Sheng, 2001; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2004). Stacked images were also inspected section by section to ensure that all the countable spines were counted in a defined length of dendrite (about 30 µm on average). The data were expressed as “spine density” (i.e., number of spines per unit length of dendrite) for statistical analysis. To confirm the reliability of the measurement, part of the spine counting was repeated by a second person, who was blind to the group identity of images. The correlation coefficient in spine density between the two observers was 0.9 (n = 30 dendrites; see SI Fig. 10). Spine width (see SI Fig. 9) was defined as the apparent diameter of a spine head.

4.4. Biochemical assays

Phosphatides were extracted and measured as described previously (Wurtman et al., 2006). Briefly, individual hippocampi were weighed and homogenized in 20 volumes of ice-cold deionized water using a 5 ml Teflon-glass homogenizer (Wheaton) with 10 strokes by hand. 1 ml of each aliquot was mixed with 3 ml of a chloroform/methanol mixture (2:1 v/v) and vortexed, followed by sequential addition of 3 ml of chloroform/methanol mixture (2:1 v/v) and 1 ml of ice-cold deionized water. The mixture was allowed to stand overnight at 4°C (18–20 h). Next day, the organic (lower) and aqueous (upper) phases of the mixture were separated by centrifugation (10 min at 4 °C; 1000 g). Aliquots (0.1–0.4 ml) of the organic phase were dried under vacuum for phosphatide analysis. Residues of 0.4-ml aliquots of the organic phase were reconstituted in 40 µl of methanol and subjected to thin-layer chromatography using silica G plates (Adsorbosil Plus-1, Alltech) and a system consisting of chloroform/ethanol/triethylamine/water (30:34:30:8) as the mobile phase. Phospholipid standards were used to identify the corresponding bands under UV light after the plates were sprayed with 0.1% diphenylhexatriene in petroleum ether. Bands for individual phosphatide classes (PC, PE, PS and PI) were scraped off the plates and extracted into 1 ml of methanol, dried under vacuum and assayed for phosphorus content. Aliquots of the hippocampal homogenates were assayed for total protein amounts by a bicinchoninic acid reagent (Perkin-Elmer).

Expression of synaptic marker proteins was assayed by Western blot. Aliquots of the hippocampal homogenates were mixed with equal volumes of KFL loading buffer and boiled for 5 min prior to gel electrophoresis. Equal amounts of protein (20 µg) were loaded for each lane and separated using SDS-PAGE (4–20%; Bio-Rad). Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). The remaining binding sites were blocked with 4% non-fat dry milk (Carnation) for 30 min in TBST buffer. Membranes were rinsed 5 times in TBST and immersed in TBST containing the antibody of interest [mouse anti-PSD-95 (Upstate); rabbit anti-GluR-1 (Chemicon); mouse anti-

Synapsin-1 (Calbiochem); and mouse anti-F-actin (Abcam). Following overnight incubation and 5 rinses in TBST, blots were incubated for 1 h with an appropriate peroxidase-linked secondary antibody. Blots were then rinsed in TBST 5 times, and protein–antibody complexes were detected and visualized by the ECL system (Amersham Biosciences) on Kodak X-AR film. Films were digitized using a Supervista S-12 scanner with a transparency adapter (UMAX Technologies). Immunoreactive bands were compared densitometrically using the Public Domain NIH Image program, Image J (available at <http://rsb.info.nih.gov/ij/>). Areas under the absorbance curves were expressed as arbitrary units, and were normalized as percentage of the average for controls in the same blot. β -tubulin was used as the loading control for the proteins tested. For this purpose, PVDF membranes were rinsed in stripping buffer (Pierce; Rockford, IL) for 30 min and then reincubated with mouse anti-tubulin beta III subunit (Chemicon).

4.5. Statistics

Data were expressed as mean \pm standard error of the mean (SEM) for each treatment group. Unless otherwise noted, one-way analysis of variance (ANOVA, SigmaPlot) was used to compare group means, with post-hoc analysis as needed. The significance (α) level was set at $p = 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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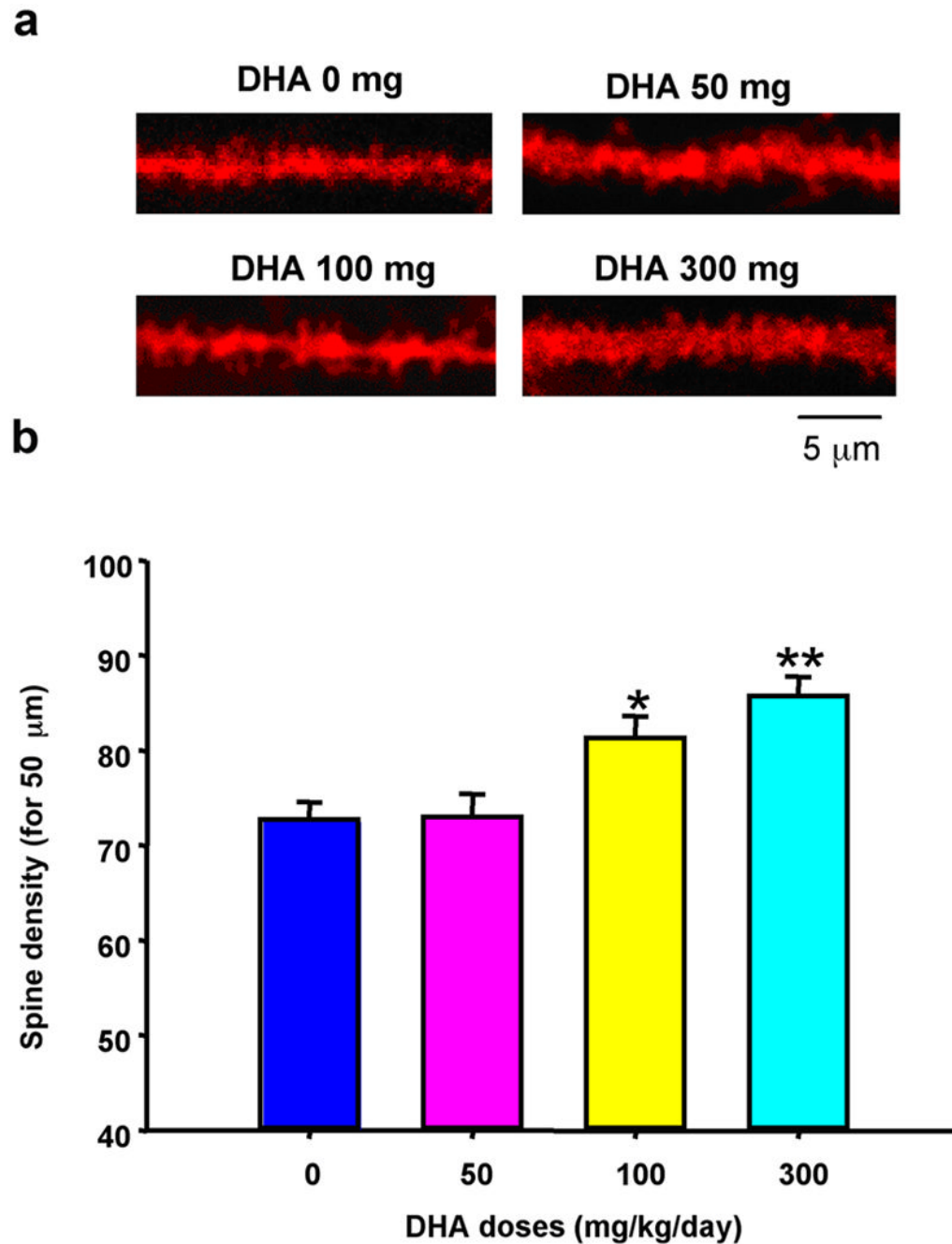


Fig. 1. Oral supplementation with DHA increases dendritic spine density in adult gerbil hippocampus

Eight animals were randomly divided into 4 groups and were supplemented with 0, 50, 100 or 300 mg/kg of DHA daily for 4 weeks. (A) Primary apical dendrites of CA1 pyramidal neurons. (B) Animals supplemented with 100 or 300 mg/kg/day showed increased spine density: a 12 % increase after the 100 mg/kg/day dose (* $p = 0.04$) and an 18 % increase after the 300 mg/kg/day dose (** $p < 0.001$ vs. 0 mg/kg/day). $n = 16 \sim 20$ neurons from 2 animals per group. One-way ANOVA followed by Tukey's test.

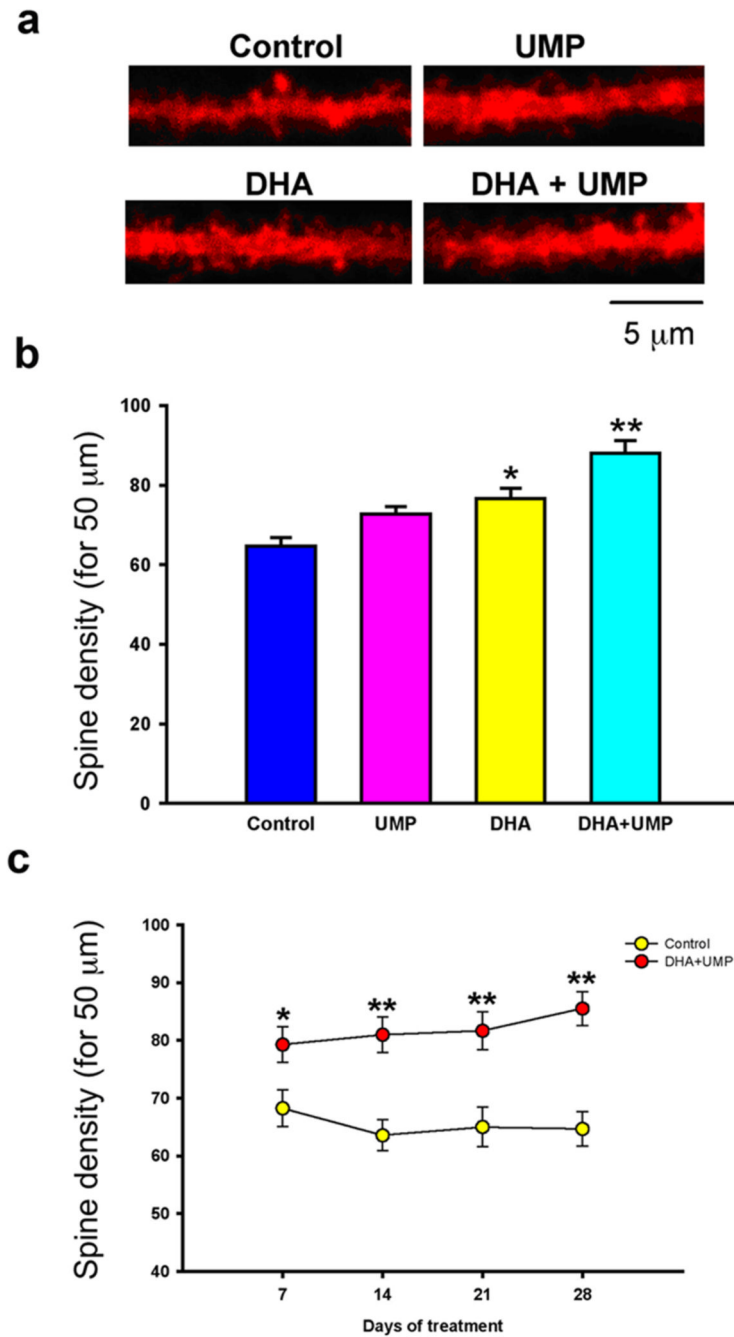


Fig. 2. DHA-induced dendritic spine formation in adult gerbil hippocampus is enhanced by co-supplementation with UMP

Animals received UMP (0.5%), DHA (300 mg/kg) or both daily for 4 weeks; control gerbils received neither. (A) Apical dendrites of CA1 pyramidal neurons. (B) Animals supplemented with DHA exhibited a significant increase in spine density (by 19%, $*p = 0.004$ vs. Control); those receiving both DHA and UMP exhibited a greater increase (by 36%, $**p < 0.001$ vs. Control or by 17%, $p = 0.008$ vs. DHA). $n = 20 \sim 25$ neurons from 4 animals per group. One-way ANOVA followed by Tukey's test. (C) The effect of DHA-plus-UMP on spine density was apparent by 1 week after the start of the treatment. The treated groups (total 8 animals) received both UMP (0.5%) and DHA (300 mg/kg) daily for 1, 2, 3 or 4 weeks; the control

groups (8 animals) were given only a regular diet. $n = 12 \sim 20$ neurons from 2 animals per group. Two-way ANOVA followed by Tukey's test. * $p = 0.02$; ** $p < 0.001$.

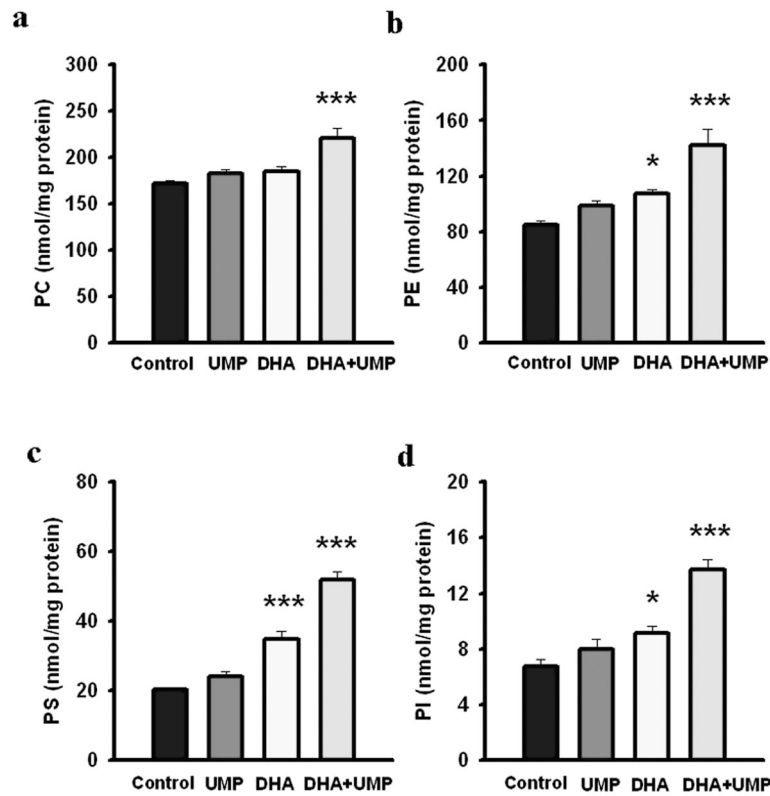


Fig. 3. Supplementation with DHA or DHA-plus-UMP increases major membrane phospholipids in adult gerbil hippocampus

(A) PC, phosphatidylcholine; (B) PE, phosphatidylethanolamine; (C) PS, phosphatidylserine; (D) PI, phosphatidylinositol. The increases ranged from 26% (PE by DHA) to 160% (PS by DHA-plus-UMP) compared with their controls. $n = 4 \sim 7$ animals per group including all the animals used for the dendritic spine analysis shown in Fig. 2B. For those animals, the hippocampus was collected from the contralateral hemisphere. The data are shown as percentage of the average of Controls. One-way ANOVA followed by Tukey's test. * $p < 0.05$; *** $p < 0.001$ vs. Controls.

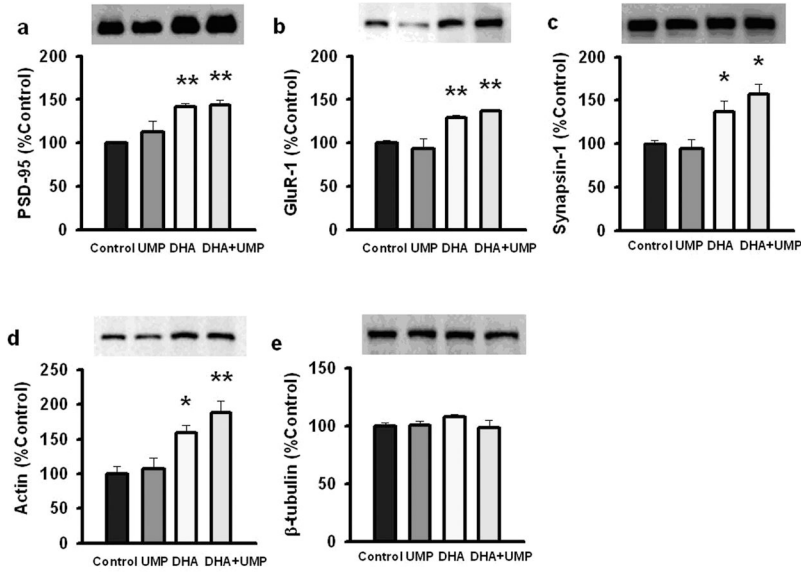


Fig. 4. Supplementation with DHA or DHA-plus-UMP elevates expression of pre-and post-synaptic proteins in adult gerbil hippocampus

(A and B) Expression levels of the post-synaptic proteins PSD-95 (A) and GluR-1 (B) were increased by 42% and 29% with DHA alone, and by 44% and 37% with DHA-plus-UMP, respectively. (C) Expression of the pre-synaptic protein Synapsin-1 was increased by 37% with DHA alone and by 57% with DHA-plus-UMP. (D and E) Expression of cytoskeletal proteins. The level of Actin (D), which can regulate dendritic spine structures, was increased by 60% with DHA alone and by 88% with DHA-plus-UMP, whereas that of β -tubulin (E), which is not specifically localized within synaptic structures, was unaffected with any of the treatments. Hippocampi used for this experiment were collected from the contralateral hemisphere in animals used for the dendritic spine analysis shown in Fig. 2B. $n = 4$ animals per group. The data are shown as percentage of the average of Controls. One-way ANOVA followed by Tukey's test. * $p \leq 0.05$; ** $p < 0.01$ vs. Controls. See also supplementary information (SI) Fig. 7.

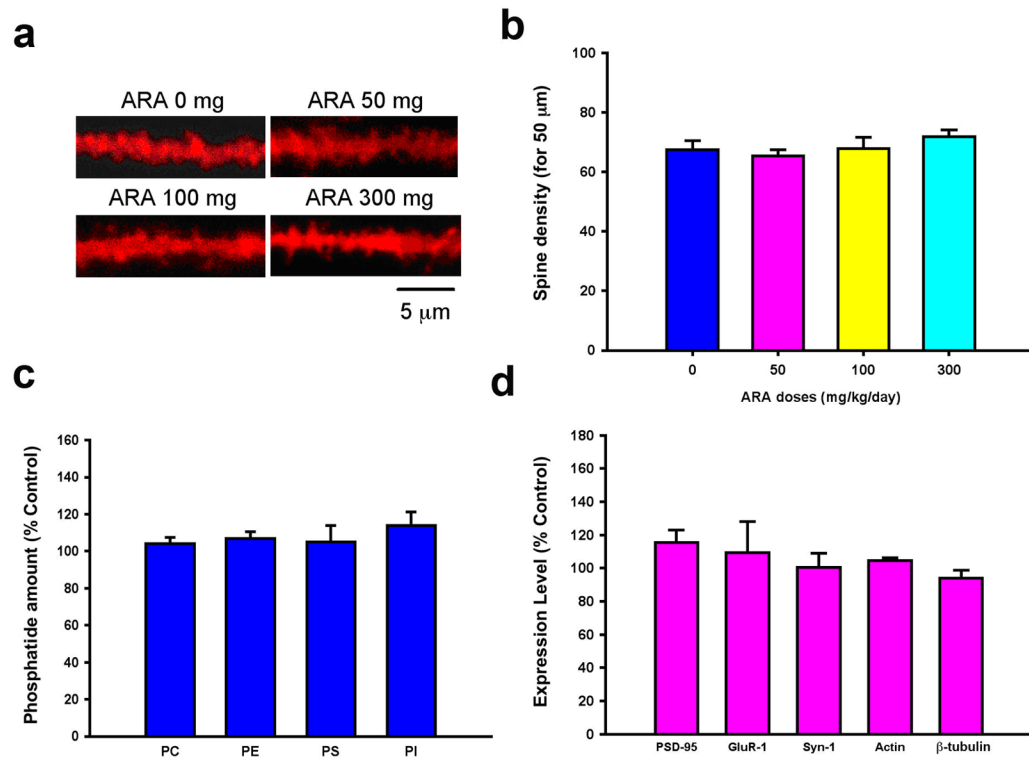


Fig. 5. Oral supplementation with arachidonic acid (ARA) does not increase dendritic spine density in adult gerbil hippocampus

(A) Primary apical dendrites of CA1 pyramidal neurons. (B) Supplementation with ARA did not change spine density in adult gerbil CA1. $n = 18 \sim 21$ neurons from 2 animals per group. (C) Relative amounts of phosphatides in the hippocampi obtained from animals treated with 300 mg/kg/day of ARA. The data are shown in percentage of the average of controls for each phosphatide. There was no statistical difference in any phosphatide between the Control and ARA groups. t -test was performed for each phosphatide (the α level was adjusted to .012 for multiple comparisons). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. (D) Western blot analysis for pre- and post-synaptic marker proteins in the same hippocampal homogenates as those used for the phosphatide assay (β -tubulin was used as a negative control). Supplementation with ARA did not significantly increase the expression of the marker proteins. Syn-1, Synapsin-1. $n = 4$ animals for each of Control and ARA groups. Tested by multiple t -tests for individual proteins ($\alpha = 0.01$).

