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Oral administration of circulating precursors for membrane phosphatides can promote the synthesis of new brain synapses

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Abstract Although cognitive performance in humans and experimental animals can be improved by administering omega-3 fatty acid docosahexaenoic acid (DHA), the neurochemical mechanisms underlying this effect remain uncertain. In general, nutrients or drugs that modify brain function or behavior do so by affecting synaptic transmission, usually by changing the quantities of particular neurotransmitters present within synaptic clefts or by acting directly on neurotransmitter receptors or signal-transduction molecules. We find that DHA also affects synaptic transmission in mammalian brain. Brain cells of gerbils or rats receiving this fatty acid manifest increased levels of phosphatides and of specific presynaptic or postsynaptic proteins. They also exhibit increased numbers of dendritic spines on postsynaptic neurons. These actions are markedly enhanced in animals that have also received the other two circulating precursors for phosphatidylcholine, uridine (which gives rise to brain uridine diphosphate and cytidine triphosphate) and choline (which gives rise to phosphocholine). The actions of DHA aere reproduced by eicosapentaenoic acid, another omega-3 compound, but not by omega-6 fatty acid arachidonic acid. Administration of circulating phosphatide precursors can also increase neurotransmitter release (acetylcholine, dopamine) and affect animal behavior. Conceivably, this treatment might have use in patients with the synaptic loss that characterizes Alzheimer's disease or other neurodegenerative diseases or occurs after stroke or brain injury.

Keywords: Phosphatide; Uridine; Docosahexaenoic acid; Precursor; synaptic membrane; Dendritic spine; Alzheimer's disease

1. Introduction

Although theories abound as to the precise pathologic mechanisms that diminish the numbers of brain synapses in patients with Alzheimer's disease, there seems to be little doubt that these reductions do invariably occur, and that they are a major factor causing patients to develop cognitive disturbances [1]. If it were possible to cause the surviving neurons in damaged brain regions to make more or larger synapses, would this restore neurotransmission, and would it ameliorate the behavioral symptoms of the disease? It has never been possible to test this hypothesis, because no method has been known that reliably increases synaptic number or size. However, now a treatment has been identified that increases the quantities of synaptic membrane in [2] and the numbers of dendritic spines on [3] hippocampal cells of normal animals. Although it remains to be determined whether this treatment also affects synapses in brains of normal humans, much less patients with Alzheimer's disease, the compounds it uses all occur normally in the blood and apparently are benign. Hence, it might be useful to determine whether their administration is beneficial to patients with Alzheimer's disease.

The circulating compounds involved are three essential precursors needed to synthesize phosphatidylcholine (PC), the major phosphatide in neuronal membranes [4], as well as the other principal phosphatides, ie, the polyunsaturated omega-3 fatty acid docosahexaenoic acid (DHA), a uridine

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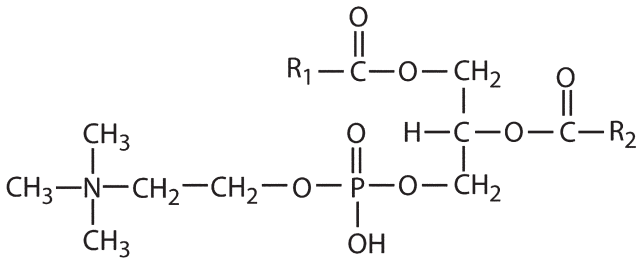

Fig. 1. Structure of phosphatidylcholine.

source and a choline source. As described below, each of these compounds can be limiting in controlling the overall rate of PC synthesis (because their levels in brain are insufficient to saturate the brain enzymes that catalyze the reactions involved in PC synthesis), and the effects of giving all three together tend to be greater than the summed effects of giving each alone. Uridine might also promote membrane synthesis via the activation of P2Y receptors by its fully phosphorylated product uridine triphosphate (UTP) [5], and DHA's effects might also involve alternative sites of action [6], including, for example, activation of brain proteins serving as receptors [7]. Perhaps surprisingly, when the three precursors are administered chronically, not only do brain levels of phosphatides, a lipid moiety, increase but also those of certain presynaptic and postsynaptic proteins [2,8], and major structural changes occur, an increase in the number of dendritic spines [3].

This article summarizes available information on the biochemical mechanisms that mediate the effects on synaptic membrane of exogenous DHA, uridine, and choline and on the known consequences of these effects. It also provides a rationale for testing these compounds to treat Alzheimer's disease or other diseases characterized by synaptic loss.

2. Biosynthesis of membrane phosphatides

All cells use DHA and other fatty acids, uridine, and choline to form the phosphatide subunits (eg, PC) (Figure 1) that, when aggregated, constitute the major components of their membranes. PC, the principal such subunit in brain, which is synthesized from these precursors by the cytidine diphosphate (CDP)–choline cycle or Kennedy cycle [9] (Figure 2), also provides the phosphocholine moiety needed to synthesize sphingomyelin, the other major choline-containing brain phospholipid. The phosphatide phosphatidylethanolamine (PE) likewise is synthesized via the Kennedy cycle by using ethanolamine instead of choline, whereas phosphatidylserine (PS), the third major structural phosphatide, is produced by exchanging a serine molecule for the choline in PC or the ethanolamine in PE [4].

The CDP-choline cycle involves three sequential enzymatic reactions (Figure 2). In the first, catalyzed by choline kinase (CK), a monophosphate is transferred from adenosine triphosphate (ATP) to the hydroxyl oxygen of the choline, yielding phosphocholine. The second, catalyzed by CTP:phosphocholine cytidylyl transferase (CT), transfers cytidylyl monophosphate (CMP) from cytidine-5′-triphosphate (CTP) to the phosphorus of phosphocholine, yielding cytidylyldiphosphocholine (also known as CDP-choline or as citicoline). As discussed below, much of the CTP that the human brain uses for this reaction derives from circulating uridine [10]. The third and last reaction, catalyzed by CDP-choline:1,2-diacylglycerol choline phosphotransferase (CPT), bonds the phosphocholine of CDP-choline to the hydroxyl group on the 3-carbon of diacylglycerol (DAG), yielding the PC. All three PC precursors must be obtained by brain entirely or in large part from the circulation, and because the PC-synthesizing enzymes that act on all three have low affinities for them, blood levels of all three can affect the overall rate of PC synthesis [2,11].

Thus, choline administration increases brain phosphocholine levels in rats [12] and humans [13], because CK's Michaelis-Menten constant (Km) for choline (2.6 mmol/L [14]) is much higher than usual brain choline levels (30 to 60 μmol/L) [15–17]. Most commonly the second, CT-catalyzed reaction is most rate-limiting in PC synthesis, either because not all of the CT enzyme is fully activated by being attached to a cellular membrane [18] or because local CTP

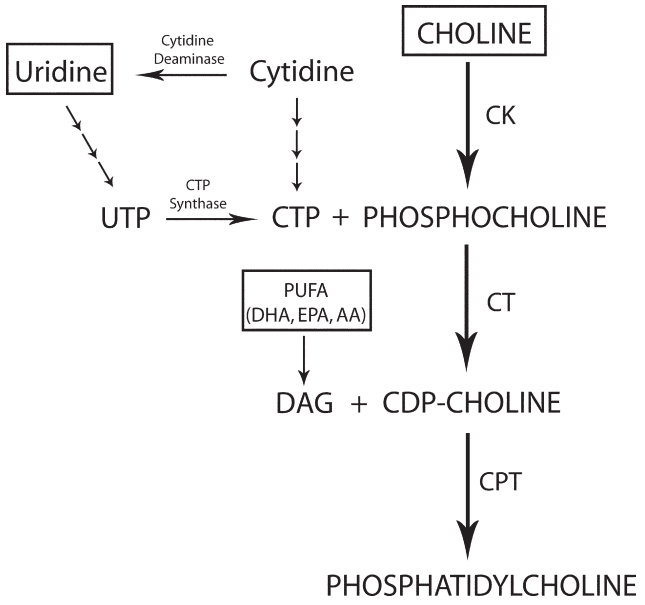

Fig. 2. PC biosynthesis via the Kennedy cycle [17]. In rats, cytidine is the major circulating pyrimidine [95]; in humans [19] and in gerbils [20] the primary circulating pyrimidine is uridine. Only small amounts of circulating cytidine are converted to brain CTP, because the BBB high-affinity transporter for pyrimidines (CNT2) has a very low affinity for cytidine [96–98]; uridine, in contrast, readily enters the brain via CNT2, yielding UTP that can be converted to CTP by CTP synthase [89]. CTP then reacts with phosphocholine to form CDP-choline, which combines with DAG, preferentially species containing PUFAs like DHA, EPA, or AA to form PC. Boxes indicate the compounds that are obtained from the circulation. Synthesis of PE via the Kennedy cycle uses ethanolamine instead of choline. Reprinted with permission from Cansev and Wurtman [8].

Table 1
Effects of UMP-containing diet and/or DHA on brain phospholipid levels

Treatments	Total PL	PC	PE	SM	PS	PI
Control diet + vehicle	351	152	65	45	33	21
UMP diet + vehicle	367	171*	84*	52	35	31**
Control diet + DHA	392	185*	78*	56*	39	32**
UMP diet + DHA	442***	220***	113***	73***	46***	36***

NOTE. Data are presented as nmol/mg protein. Reprinted with permission from Wurtman et al [2].
* $P < .05$, ** $P < .01$, and
*** $P < .001$ compared with values from Control diet + Vehicle group.

concentrations are insufficient to saturate the CT [17]. Thus, when brain CTP levels are increased by giving uridine to animals [11], CTP's circulating precursor in human blood [10], PC synthesis is accelerated [11]. The activity of CPT and the extent to which this enzyme is saturated with DAG can also control the overall rate of PC synthesis, as has been demonstrated in, for example, permeabilized HeLa cells exposed to glycerol-3-phosphate and acyl-CoA [19] or in PC12 cells extending neurites after exposure to nerve growth factor (NGF) [20]. In PC-12 cells, NGF increased DAG levels five-fold, CPT activity by 70%, and the incorporation of choline into PC by two-fold. DAG species containing DHA or other polyunsaturated fatty acid (PUFA) on the middle carbon apparently are preferentially used for phosphatide synthesis as opposed to triglyceride synthesis [21]. (This does not explain why giving DHA, thereby presumably increasing, at least transiently, the proportions of PC molecules that contain this PUFA, would also increase the absolute levels of PC in a cell [Table 1]).

If rodents are given a standard diet supplemented with choline and uridine (as its monophosphate, UMP) and also DHA by gavage, brain PC synthesis rapidly increases [2,11], and absolute levels of PC per cell (DNA) or per mg protein increase substantially (eg, by 40% to 50% after several weeks of daily treatment [2]) (Table 1). This treatment also increases the levels of each of the other principal membrane phosphatides (Table 1), as well as the levels of particular proteins known to be localized within presynaptic and postsynaptic membranes (for example, synapsin-1 [22], PSD-95 [23], and syntaxin-3 [7]), but not a ubiquitously distributed brain protein, β-tubulin (Figure 3) [2,8]. Treatment with DHA, UMP, and choline also promotes the formation of dendritic spines in adult gerbil hippocampus [3] (see section 6) and improves hippocampus-dependent cognitive behaviors in rats reared in a socially deprived environment [24] (see section 7). Thus, the production and levels of brain phosphatides and synaptic proteins are found to depend to a surprising extent on blood levels of PC's three circulating precursors. Providing supplemental UMP or DHA without the other can also increase brain phosphatide levels, but by less than when all three precursors are presented. (Choline is included in all of the test diets).

In studies designed to affirm that the increases in brain phosphatide levels caused by giving DHA, with or without UMP, reflect actual increases in phosphatide synthesis (and not, for example, inhibition of phosphatide degradation), brain levels of CDP-choline and CDP-ethanolamine, the immediate precursors of PC and PE, also were measured. It was postulated that if DHA acted by generating more DHA-containing DAG, and if this compound then combined with endogenous CDP-choline or CDP-ethanolamine to form additional PC or PE, then DHA administration would concurrently reduce brain CDP-choline or CDP-ethanolamine while increasing brain levels of their PC products and PE. This expectation was confirmed [2]. Among animals receiving either DHA alone or DHA plus UMP, CDP-choline and CDP-ethanolamine levels decreased significantly, whereas those of PC and PE increased (Figure 4).

3. Properties of the enzymes that mediate brain phosphatide synthesis

The ability of each of the three circulating phosphatide precursors to affect the rate of phosphatide synthesis results principally from the low affinities of these enzymes for their substrates. This unusual property is described below.

3.1. Choline kinase

The synthesis of PC (Figure 2) is initiated by the phosphorylation of choline, in which CK (EC 2.7.1.32) catalyzes the transfer of a monophosphate group from ATP to the hydroxyl oxygen of the choline. In some neurons choline is also used to synthesize the neurotransmitter acetylcholine (ACh), the enzyme choline acetyltransferase (ChAT) transferring an acetyl group from acetyl-CoA to the hydroxyl oxygen of the choline. Like CK, ChAT has a very low affinity for its choline substrate [25,26]. The Km's of these enzymes in brain (which describe the choline concentrations at which the enzymes operate at only half-maximal velocity) are reportedly 2.6 mmol/L [14] and 540 μmol/L [27], respectively, whereas brain choline levels are only about 30 to 60 μmol/L [15–17]. Hence, the syntheses of both phosphocholine and ACh are highly responsive to treatments that raise or lower brain choline levels.

The ability of choline administration to increase brain phosphocholine levels was first noted in 1982 [12] and its similar effect on ACh in 1975 [28,29]. It had previously been shown that the production of another brain neurotrans-

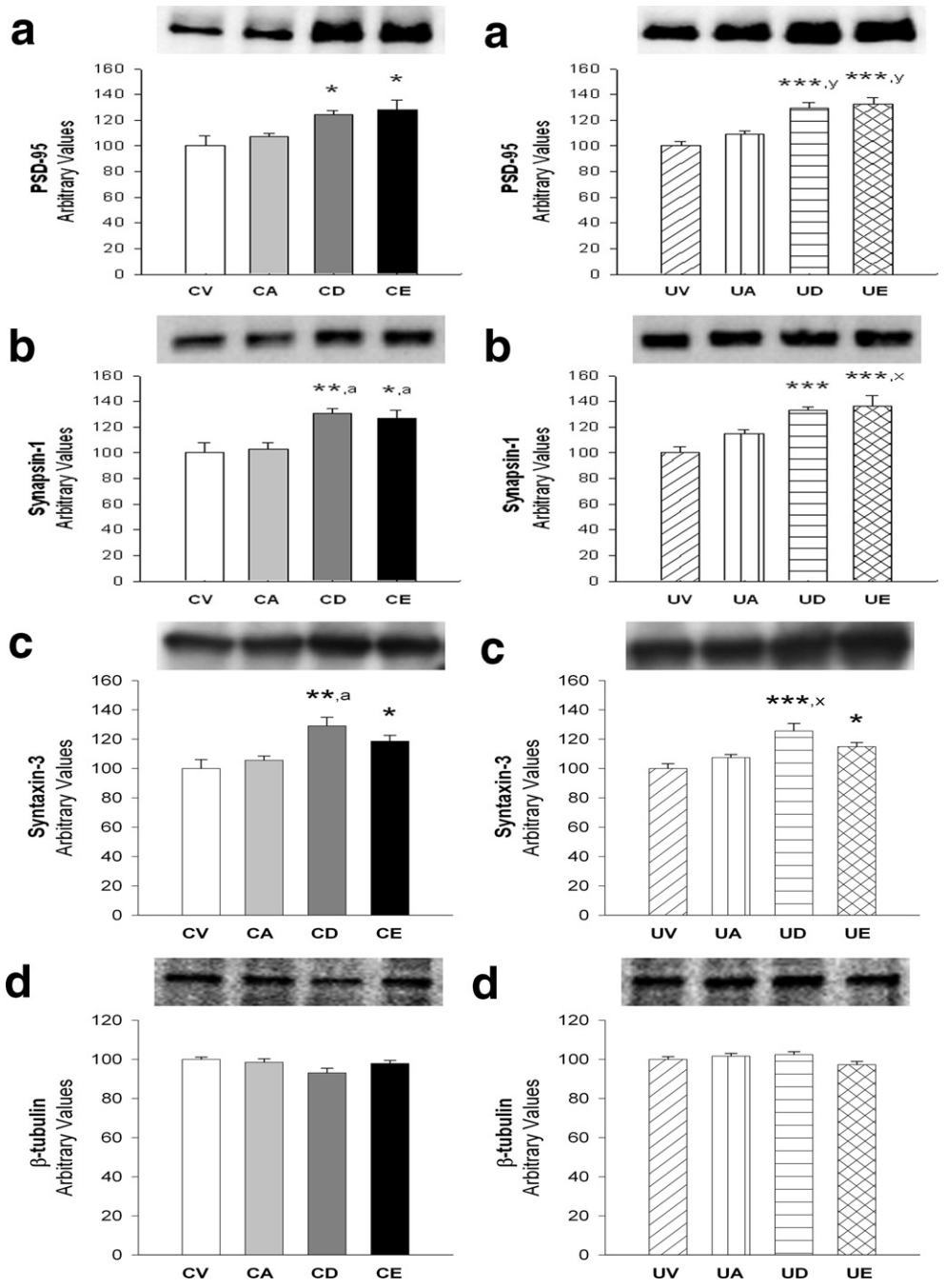

Fig. 3. Effects of AA, DHA, or EPA, alone or in combination with a UMP-supplemented diet, on levels of the presynaptic or postsynaptic proteins PSD-95 (a1, a2); synapsin-1 (b1, b2), and syntaxin-3 (c1, c2). CV, control diet + vehicle; CA, control diet + AA; CD, control diet + DHA; CE, control diet + EPA; UV, UMP-supplemented diet + vehicle; UA, UMP-supplemented diet + AA; UD, UMP-supplemented diet + DHA; UE, UMP-supplemented diet + EPA. $^{*}P < .05$; $^{**}P < .01$; and $^{***}P < .001$ compared with CV, and $^{a}P < .05$ compared with CA on the left-sided columns (a1, b1, and c1) with one-way analysis of variance (ANOVA). $^{*}P < .05$; $^{**}P < .01$; and $^{***}P < .001$ compared with UV, and $^{x}P < .05$; and $^{y}P < .01$ compared with UA on the right-sided columns (a2, b2, and c2) with one-way ANOVA. Reprinted with permission from Cansev and Wurtman [8].

mitter, serotonin, was increased among animals receiving physiologic doses of its circulating precursor, tryptophan [30,31]. This was shown to be because tryptophan hydroxylase, the enzyme that determines the overall rate at which tryptophan is converted to serotonin, has a very low affinity for this substrate. Inasmuch as the affinities for choline of CK and ChAT, measured in vitro, were also known to be low relative to choline availability, it seemed reasonable to inquire as to whether giving choline could also increase phosphocholine or ACh synthesis.

Even though brain choline concentrations shared with those of tryptophan the ability to control the rates at which

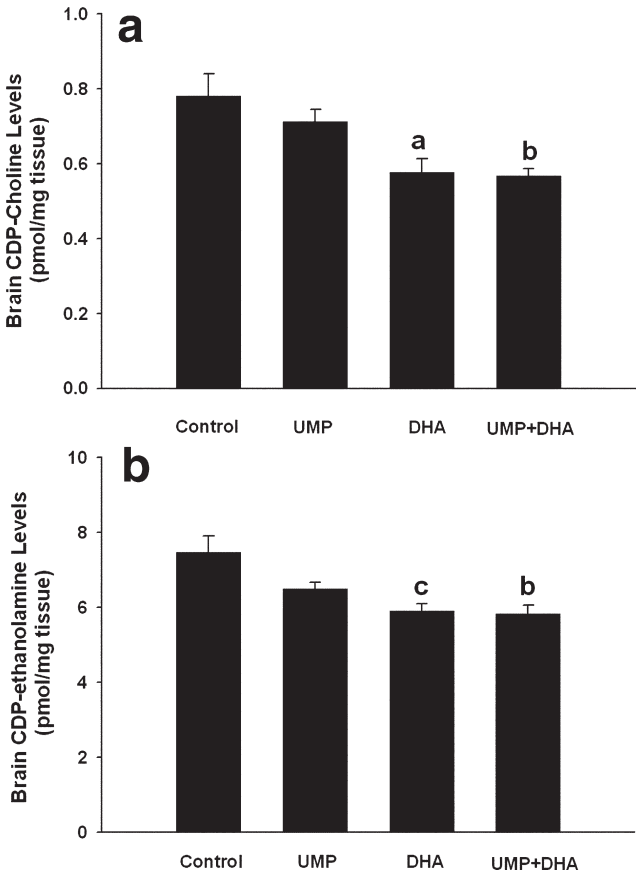

Fig. 4. Effects of DHA on brain CDP-choline or CDP-ethanolamine levels. Groups of 8 gerbils received either a control or a UMP-containing (0.5%) diet and, by gavage, DHA (300 mg/kg; in a vehicle of 5% gum Arabic solution) or just its vehicle for 28 days. On the 29th day their brains were harvested and assayed for (A) CDP-choline or (B) CDP-ethanolamine. $^{a}P < .05$ and $^{c}P < .01$ when compared with the values for control diet plus vehicle group; $^{b}P < .05$ when compared with values for UMP diet plus vehicle group. Reprinted with permission from Wurtman et al [2].

the precursor is used to synthesize a neurotransmitter, choline and tryptophan differed in an important respect: Both are used by certain neurons for two purposes, tryptophan for conversion to serotonin and incorporation into proteins and choline for conversion to ACh and incorporation into phospholipids. However, in the case of tryptophan these two processes are segregated into different parts of the neuron, the nerve terminal and perikaryon, whereas for choline both can take place within the nerve terminal (because that structure contains both ChAT and CK). Hence, the acetylation and the phosphorylation of choline sometimes compete for available substrate [32,33]. When cholinergic neurons are forced to fire frequently and maintain the rapid release of ACh, choline's incorporation into PC decreases [32], and the breakdown of membrane PC increases ("autocannibalism"), both processes liberating additional choline for ACh synthesis [34–36]. However, when the utilization of choline to form PC is increased (by providing supplemental uridine and an omega-3 fatty acid), ACh synthesis is not diminished, probably because so little choline is used for phosphatide formation relative to the amount used to produce ACh [33].

3.2. CTP:phosphocholine cytidylyltransferase

CTP:phosphocholine cytidylyltransferase (CT) (EC 2.7.7.15) catalyzes the condensation of CTP and phosphocholine to form CDP-choline (Figure 2). CT is present in both the soluble and particulate fractions of the cell [37]; the cytosolic form is reportedly inactive, and the membrane-bound form is active [18,38]. Increases in the association of CT with membranes reportedly correlate with increases in CT activity and in the net synthesis of PC in vitro [39–41]. Some other lipids (eg, PS) [42] and DAG [39,43] also stimulate the translocation of CT from the cytosol to membranes in vitro, thereby activating the enzyme [44]. The phosphorylation state of CT affects its net activity [45], as does its substrate saturation with CTP and perhaps with phosphocholine. The Km's of CT for CTP and phosphocholine in brains of laboratory rodents and humans are reportedly 1 to 1.3 mmol/L and 0.30 to 0.31 mmol/L [17,46], respectively, whereas brain levels of these compounds are only 70 to 110 μmol/L [11,47,48] and 0.32 to 0.69 mmol/L [12,16,49], respectively. Hence, brain CT normally is highly unsaturated with CTP and only about half-saturated with phosphocholine in vivo, suggesting that its degrees of substrate saturation, particularly with CTP, exert important limiting roles in PC synthesis. In fact, treatments that increase cellular CTP (eg, administration of a uridine or cytidine source) have been shown to enhance CDP-choline and PC synthesis in poliovirus-infected HeLa cells [50], undifferentiated PC12 cells [51,52], slices of rat corpus striatum [53], and gerbil brain in vivo [11].

3.3. CDP–choline:1,2-diacylglycerol choline phosphotransferase

CDP–choline:1,2-diacylglycerol cholinephosphotransferase (CPT) (EC 2.7.8.2) catalyzes the final reaction in the Kennedy cycle; it transfers the phosphocholine moiety from CDP-choline to DAG, thus yielding PC (Figure 2). CPT, an integral membrane protein, is present primarily in the endoplasmic reticulum [54]. The enzyme protein has been solubilized and partially purified from microsomes of rat liver [55,56], rat brain [57], and hamster liver [58]. A human cDNA has been isolated that codes for an enzyme with both choline phosphotransferase and ethanolamine-phosphotransferase (EPT) activities (hCEPT1 [59]), and a different human cDNA has also been isolated, the product of which exhibits only choline phosphotransferase–specific activity (hCPT1) [60]. CPT might be a reversible enzyme, synthesizing CDP-choline from PC and CMP in microsomal preparations from liver [61,62] or brain [63–65].

The choline phosphotransferase reaction also is unsaturated with the enzyme's substrates. Its Km values for CDP-

choline and DAG in rat liver are 200 μmol/L and 150 μmol/L, respectively [66], whereas the concentrations of these compounds in liver are approximately 40 μmol/L [67] and 300 μmol/L [68]. (A DAG concentration of at least 1,000 μmol/L thus would probably be needed to saturate the enzyme). Brain CDP-choline and DAG levels are even lower, ie, about 10 to 30 μmol/L [11,69] and 75 μmol/L [48], respectively. Levels of cellular DAG have been shown to limit PC synthesis in permeabilized HeLa cells [19], cultured rat hepatocytes [70], and PC12 cells [11]. None of these studies distinguished between the enzyme that acts on both choline and ethanolamine (PECT1) and the enzyme that acts only on choline (PCT1). A more recent report, with cloning and expression methods, described the Km of human PECT1 for CDP-choline as being 36 μmol/L [71], which would probably still be too high for it to be saturated with this substrate in brain. The Km of PCT for its substrates might also be affected by the fatty acid composition of the DAG molecule [72]; however, no data are available demonstrating that the fatty acids (eg, DHA) that are most able to promote PC synthesis [73] do so because they selectively enhance cellular levels of DAG species that are preferentially bound to or acted on by CPT.

3.4. Uptake of uridine into brain and its conversion to UTP and CTP

Because circulating uridine elevates brain CTP levels, thus modulating DHA's effects on the formation of synaptic membrane, the enzymes and uptake proteins that mediate blood uridine's effect on brain CTP are discussed here.

Uridine and cytidine are transported across cell membranes, including the blood-brain barrier (BB), via two families of transport proteins, ie, the Na^+-independent, low-affinity, equilibrative transporters (ENT1 and ENT2) [74] and the Na^+-dependent, high-affinity, concentrative (CNT1, CNT2, and CNT3) [75] nucleoside transporters [76]. The two ENT proteins, which transport uridine and cytidine with similar affinities, have been cloned from rat [77] and mouse [78]. Inasmuch as their Km values for the pyrimidines are in the high micromolar range (100 to 800 μmol/L [79]), they probably mediate BBB pyrimidine uptake only when plasma levels of uridine and cytidine have been elevated experimentally. In contrast, CNT2, which transports both the pyrimidine uridine and such purines as adenosine, probably does mediate uridine transport across the BBB under physiologic conditions. Km values for the binding of uridine and adenosine to this protein (which has been cloned from rat BBB [80]) are in the low micromolar range (9 to 40 μmol/L in kidney, intestine, spleen, liver, macrophage, and monocytes [81]), whereas plasma uridine levels are subsaturating, ie, 0.9 to 3.9 μmol/L in rats [82], 3.1 to 4.9 μmol/L in humans [82], and around 6.5 μmol/L in gerbils [11]. Cytidine has not been thought to be a substrate for CNT2 [75]; however, recent studies suggested that CNT2 can also transport this compound, however, with a much lower affinity than that for uridine [83–85].

It should be noted that although both of the pyrimidines, uridine and cytidine, are present in the blood of laboratory rats, human blood contains unmeasurably low quantities of cytidine [82]; even among individuals consuming a cytidine source like oral CDP-choline [10], the cytidine is quantitatively deaminated to uridine in the human liver. Hence, in humans, circulating uridine, and not cytidine, is the precursor of the brain CTP used for phosphatide synthesis. Gerbil blood contains both of the pyrimidines but proportionately less cytidine than blood of rats; hence, gerbils are often used as a model for studying the effects of exogenous uridine sources on the human brain [86].

Like other circulating compounds, pyrimidines might also be taken up into brain via the epithelium of the choroid plexus (CP) and the ENT1, ENT2, and CNT3 transporters [74,75]; all of these proteins have been found in CP epithelial cells of rats [77,87,88] and rabbits [89,90]. However, the surface area of BBB is probably 1,000 times that of the CP epithelium (ie, 21.6 vs 0.021 m^2 in humans [91]); hence, the BBB is the major locus at which circulating uridine enters the brain.

Uridine and cytidine are converted to their respective nucleotides by successive phosphorylations catalyzed by various kinases. Uridine-cytidine kinase (UCK) (ATP:uridine 5′-phosphotransferase; EC 2.7.1.48) phosphorylates uridine and cytidine to form UMP and CMP, respectively [92–94]. UCK activity is regulated by cellular UTP and CTP levels. At relatively low UTP and CTP levels, uridine taken up into brain cells is phosphorylated, initially by UCK to form uridine nucleotides. At higher UTP and CTP concentrations, UCK's activity is inhibited, thus suppressing uridine's phosphorylation [95]. Several different forms of UCK exist, possibly as isoenzymes [96,97]. Humans have two such isoenzymes, UCK1 and UCK2, both of which have been cloned [98,99]. UMP-CMP kinase (UMP-CMPK) (ATP:CMP phosphotransferase, EC 2.7.4.14) [100–102] then converts UMP or CMP to uridine diphosphate (UDP) or CDP. These nucleotides in turn are further phosphorylated to UTP and CTP by nucleoside diphosphate kinases (NDPKs) (nucleoside triphosphate:nucleoside diphosphate phosphotransferase, EC 2.7.4.6) [103,104]. mRNAs for UCK1 [99] and UMP-CMPK [105] have been described in brain [106,107], as has NDPK activity.

Various interconversions between uridine and cytidine and between their respective nucleotides are known to occur in mammalian cells. Cytidine and CMP can be deaminated to uridine and UMP [108], whereas UTP is aminated to CTP by CTP synthase (UTP:ammonia ligase [adenosine diphosphate–forming], E.C. 6.3.4.2) [109,110]. This enzyme acts by transferring an amide nitrogen from glutamine to the C-4 position of UTP, thus forming CTP [111]. CTP synthase activity has been demonstrated in rat brain [112].

Table 2
Effects of various PUFAs, given with a control diet, on gerbil brain phosphatide levels

	Total PL	PC	PE	PS	PI
Control diet + vehicle	322	113	63	251	15
Control diet + AA	326	114	65	281	16
Control diet + DHA	344	133*	77*	32***	18*
Control diet + EPA	347	125	76*	32**	19***,†
UMP diet + vehicle‡	332	131*	701	29*	16

NOTE. Data are given as means ± standard error of the mean. Data are presented as nmol/mg protein. Reprinted with permission from Cansev and Wurtman [8].

* $P < .05$, $P < .01$, and

† $P < .05$ compared with control diet + AA group by one-way ANOVA.

‡ Data from gerbils receiving UMP diet but no PUFA are included to illustrate that uridine alone also affects phosphatide levels.

*** $P < .001$ compared with control diet + vehicle group.

All of the enzymes described above apparently are unsaturated with their respective nucleoside or nucleotide substrates in brain and other tissues. For example, the Km's for uridine and cytidine of UCK prepared from various tissues varied between 33 to 270 μmol/L [93,94,113,114], and the Km for uridine of recombinant enzyme cloned from mouse brain was 40 μmol/L [115,116]. Brain uridine and cytidine levels are about 22 to 46 pmol/mg wet weight [11,117] and 6 to 43 pmol/mg wet weight [11,118], respectively. Hence, the syntheses of UTP and CTP and the subsequent syntheses of brain PC and PE via the Kennedy pathway depend on available levels of their pyrimidine substrates. Indeed, increasing the supply of uridine or cytidine to neuronal cells, in vitro [5,52,53] or in vivo [11,86], enhanced the phosphorylation of uridine and cytidine and elevated levels of UTP, CTP, and CDP-choline.

4. Availability of DHA and other PUFAs to brain cells

The omega-3 PUFAs DHA and eicosapentaenoic acid (EPA) and the omega-6 fatty acid arachidonic acid (AA) are essential for humans and other animals and thus must be obtained from the diet either as such or as their also essential precursors, alpha-linolenic acid (ALA) and linoleic acid (LA). Although the processes by which circulating PUFAs are taken up into the brain and, subsequently, into brain cells await full characterization, they are thought to include both simple diffusion (also termed "flip-flop" [119]) and protein-mediated transport [120]. One such transport protein (B-FATP) [121] has been cloned [122]. DHA, EPA, and AA are then transported from the brain's extracellular fluid into cells and can be activated to their corresponding CoA species (eg, docosahexaenoyl-CoA; eicosapentaenoyl-CoA; arachidonoyl-CoA) and acylated to the sn-2 position of DAG [123] to form PUFA-rich DAG species [124,125]. DHA is acylated by a specific acyl-CoA synthetase, Acsl6 [126], which exhibits a low affinity for this substrate Km = 26 μmol/L [127] relative to usual brain DHA levels (1.3 to 1.5 μmol/L) [128]. Hence, treatments that raise blood DHA levels rapidly increase its uptake into and retention by brain cells.

EPA can be acylated to DAG by the acyl-CoA synthetase [129], or it can be converted to DHA by brain astrocytes [130], allowing its effects on brain phosphatides and synaptic proteins to be mediated by DHA itself. Exogenously administered AA, like DHA, is preferentially incorporated into brain phosphatides [131,132], as well as into other lipids, eg, the plasmalogens [133,134]. AA shares with DHA the ability to activate syntaxin-3 [7]; however, its oral administration to laboratory rodents apparently does not promote synaptic membrane synthesis or dendritic spine formation.

DHA and AA are major components of brain membrane phospholipids [135]. Whereas AA is widespread throughout the brain and is abundant in phosphatidylinositol (PI) and PC, DHA is concentrated in synaptic regions of gray matter [136] and is especially abundant in PE and PS [137]. In contrast, EPA is found only in trace amounts in brain phosphatides, mostly in PI [138]. No significant differences have been described between the relative proportions of ingested omega-3 and omega-6 PUFAs that actually enter the systemic circulation [139,140]. Moreover, the rates at which radioactively labeled DHA and AA are taken up into brain and incorporated into phospholipids after systemic injections also are similar [131,141]. (To our knowledge, no study has compared the brain uptake of EPA with that of another PUFA in rodents or humans; however, exogenously administered EPA is known to increase brain EPA levels in vivo [142]). On the other hand, the half-lives of the omega-3 PUFAs in the blood (20 ± 5.2 hours for DHA and 67 ± 14 hours for EPA [143]) are substantially higher than that for AA (3.8 seconds [144]). Similarly, the half-life of DHA in brain PC (22.4 ± 2.9 hours), but not in PI or PE, is much longer than that of AA (3.79 ± 0.12 hours) [145]. Thus, a considerable proportion of AA might be cleared from plasma or oxidized before it is used for PC synthesis, or, once incorporated into phosphatides, it might be liberated by hydrolysis (mediated by phospholipase A_2 [146]) and then oxidized.

Table 3
Effects of various PUFAs, given with a UMP-supplemented diet, on gerbil brain phosphatide levels

	Total PL	PC	PE	PS	PI
UMP diet + vehicle	332	131	70	29	16
UMP diet + AA	379	132	81	31	20
UMP diet + DHA	384*	147***,†	88**	39**	22**
UMP diet + EPA	407***	148**,†	91***	41**,‡	25***

NOTE. Data are given as means ± standard error of the mean. Data are presented as nmol/mg protein. Reprinted with permission from Cansev and Wurtman [8].

* $P < .05$, † $P < .01$ and

‡ $P < .05$ compared with UMP diet + AA group by one-way ANOVA.

** $P < .01$, and

*** $P < .001$ compared with control diet + vehicle group.

It should be noted that the ability of orally administrated DAG, given daily for several weeks, to increase brain phosphatide levels does not necessarily imply that, concurrent with such increases, the quantities of DHA in the phosphatides, relative to the quantities of other fatty acids, also are increased. Indeed, this has not been demonstrated. Conceivably, DHA-rich DAG is preferentially used for PC synthesis, but once the DAG-containing PC is formed, it is rapidly hydrolyzed to form lyso-PC lacking DHA and then reacylated to PC by addition of a different fatty acid [146].

5. Effects of DHA and other PUFAs on synaptic protein and phosphatide levels in gerbils

In experiments designed to compare the effects of administering each of the three PUFAs, DHA, EPA, or AA, on brain phosphatide levels, animals received 300 mg/kg daily by gavage of one of the fatty acids for 4 weeks and consumed a choline-containing diet that did or did not also contain UMP. Giving DHA without uridine increased PC, PI, PE, and PS levels significantly by 18%, 20%, 22%, and 28%, respectively (Tables 2 and 3) throughout the brain (eg, in cortex, striatum, hippocampus, brain stem, and cerebellum) (Table 4). Giving EPA also increased brain PE, PS, and PI levels significantly by 21%, 24% and 27%, respectively (Tables 2 and 3). In contrast, AA administration failed to affect brain levels of any of the phosphatides (Tables 2 and 3) [8].

Consuming the UMP-supplemented diet alone increased brain PS and PC levels significantly (by 15% and 16%, respectively) (Tables 2 and 3) compared with those in control gerbils. Among gerbils receiving both UMP and DHA, brain PC, PE, PS, and PI levels rose significantly by 12%, 26%, 34%, and 38%, respectively (Tables 2 and 3). Similarly, among gerbils receiving both UMP and EPA, brain PC, PE, PS, and PI levels rose significantly by 13%, 30%, 41%, and 56%, respectively (Tables 2 and 3). In contrast, giving UMP with AA failed to increase levels of any brain phosphatide above those found in gerbils receiving UMP alone (Tables 2 and 3). Total brain phospholipid levels were

Table 4
Effects of giving UMP-supplemented diet (0.5%) and DHA (300 mg/kg) on phosphatide levels in different gerbil brain regions

	Cortex	Striatum	Hippocampus	Brain Stem	Cerebellum
Total PL					
Control diet + vehicle	267	265	264	450	270
UMP diet + DHA	316**	339***	314**	521**	317**
PC					
Control diet + vehicle	94	100	102	114	98
UMP diet + DHA	122***	126*	117***	139***	111***
PE					
Control diet + vehicle	58	60	61	117	64
UMP diet + DHA	80**	85***	81***	156***	85***
PS					
Control diet + vehicle	24	24	24	30	24
UMP diet + DHA	30***	29*	28***	35***	29**
PI					
Control diet + vehicle	10.6	7.6	8.8	9.3	10.4
UMP diet + DHA	13.2**	11.9***	11***	11.8*	11.5*

NOTE. Data are presented as nmol/mg protein. Reprinted with permission from Cansev and Wurtman [8].

* $P < .05$, ** $P < .01$, and

*** $P < .001$ compared with control diet + vehicle group by using Student t test.

also elevated significantly by 16% and 23% after treatment with UMP plus DHA or with UMP plus EPA, respectively (Tables 2 and 3), but not by treatment with UMP plus AA [8]. Essentially similar results were obtained whether data were expressed per μg DNA or per mg protein (data not shown).

Giving the gerbils DHA or EPA alone significantly increased brain levels of the postsynaptic density protein PSD-95 by 24% or 28% (Figure 3a1). When this treatment was combined with dietary UMP, the observed increases in PSD-95 were 29% or 33% greater than those found after UMP-supplementation alone (Figure 3a). AA failed to affect brain PSD-95 levels, either when given alone or in combination with the UMP-supplemented diet (Figure 3a). Similar to PSD-95, levels of synapsin-1, a presynaptic vesicular protein, were significantly increased by 31% or 27%, respectively, by DHA or EPA treatment alone (Figure 3b1) or by 33% or 36% when the PUFA was combined with UMP (Figure 3b2). Again, AA failed to affect brain synapsin-1 levels when given alone or concurrent with a UMP-supplemented diet (Figure 3b).

Also similarly to PSD-95 and synapsin-1, brain levels of syntaxin-3, a plasma membrane soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) protein that reportedly mediates the stimulation by PUFAs of neurite outgrowth [7] and exocytosis [147] in cultured cells, were significantly increased in animals receiving DHA or EPA by 29% or 19%, respectively (Figure 3c1), whether or not they also received UMP, but AA was without effect if given alone or in combination with UMP (Figure 3c).

None of the PUFAs, given alone or with UMP, changed brain levels of the structural protein β-tubulin, perhaps reflecting its ubiquity in brain; hence, β-tubulin was used as the loading control for Western blot assays of synaptic proteins (Figure 3d).

The mechanism that allows the omega-3 fatty acids DHA and EPA, but not the omega-6 fatty acid AA, to increase synaptic membrane is unclear. Exogenously administered AA, like DHA, is preferentially incorporated into brain phosphatides [131,132], as well as into other brain lipids (eg, the plasmalogens [133,134]), and AA shares with DHA the ability to activate syntaxin-3 in vitro [7].

Mechanisms that could underlie the differential effects of omega-3 and omega-6 PUFAs on membrane synthesis might include, among others, different efficacies for their uptakes into brain or their acylation, different half-lives in the circulation, different affinities for enzymes that control their incorporation into DAG and phosphatides (apparently not the case [8]), differences in the rates at which the PUFAs are removed from phosphatides by deacylation, the differential activation of genes encoding proteins needed for membrane synthesis [148], or the tendency of AA to be incorporated into phospholipids by the acylation of 1-acyl-2-lyso-sn-glycerophospholipids, not via the Kennedy cycle [149].

6. Effects of DHA and other PUFA on dendritic spine formation and synaptogenesis

Dendritic spines are small membranous protrusions extending from postsynaptic dendrites in neurons, most of which form synapses with presynaptic axon terminals. The dendritic spines compartmentalize postsynaptic responses, and their numbers are thought to reflect the numbers of excitatory synapses within regions of the central nervous system [150–152]. Oral supplementation with DHA to adult gerbils increases the number of dendritic spines in the hippocampus, particularly if the animals are also supplemented with UMP [3] (Figure 5). This treatment also increases the levels of membrane phosphatides and of various presynaptic and postsynaptic proteins [2]. Oral DHA might thus increase the number of brain synapses, particularly when co-administered with UMP.

Gerbils that received daily doses of DHA for 4 weeks (100 or 300 mg/kg by gavage) exhibited increased dendritic spine density (ie, the number of spines per length of dendrite) in CA1 pyramidal neurons (Figure 6); the increases were 12% ($P = .04$) with the 100 mg/kg/day dose and 18% ($P < .001$) with the 300 mg/kg/day dose. These effects were amplified when gerbils received both DHA (300 mg/kg/day by gavage) and UMP (0.5% via the standard choline-containing diet) for 4 weeks; DHA supplementation alone increased spine density by 19% ($P < .004$; Figure 5), and administration of both precursors did so by 36% or approximately double the increase produced by DHA alone ($P = .008$) (Figure 5). (Giving UMP alone did not affect dendritic spine density significantly [Figure 5]; however, it did increase spine density when all dendritic protrusions were included for statistical analysis, including the filopodia, which are precursor forms of dendritic spines). The effect on dendritic spine density of giving both DHA and UMP was already apparent after 1 week of treatment ($P = .02$) and continued for as long as animals were treated (4 weeks) (Figure 5). DHA plus UMP did not affect the length or width of individual dendritic spines, only their number.

In the above experiments the increases in hippocampal phospholipids after DHA alone were PC, 8%; PE, 26%; PS, 75%; and PI, 29% (all $P < .05$ except for PC) and after DHA plus UMP were PC, 28%; PE, 59%; PS, 160%; and PI, 100% (all $P < .001$ vs their controls). Comparable increases were noted in the presynaptic and postsynaptic proteins examined in the contralateral hippocampus of the same animals. Expression levels of PSD-95 [153] and GluR-1 [154,155] are known to be highly associated with the growth of dendritic spines and also with the intensity of the physiologic responses of the postsynaptic neurons. Synapsin-1, on the other hand, is expressed in presynaptic terminals and apparently anchors synaptic vesicles to the actin cytoskeleton for exocytosis or synaptogenesis [156,157]. The increases in PSD-95, synapsin-1, and GluR-1 (a subunit of the glutamatergic AMPA receptor) after treatment with

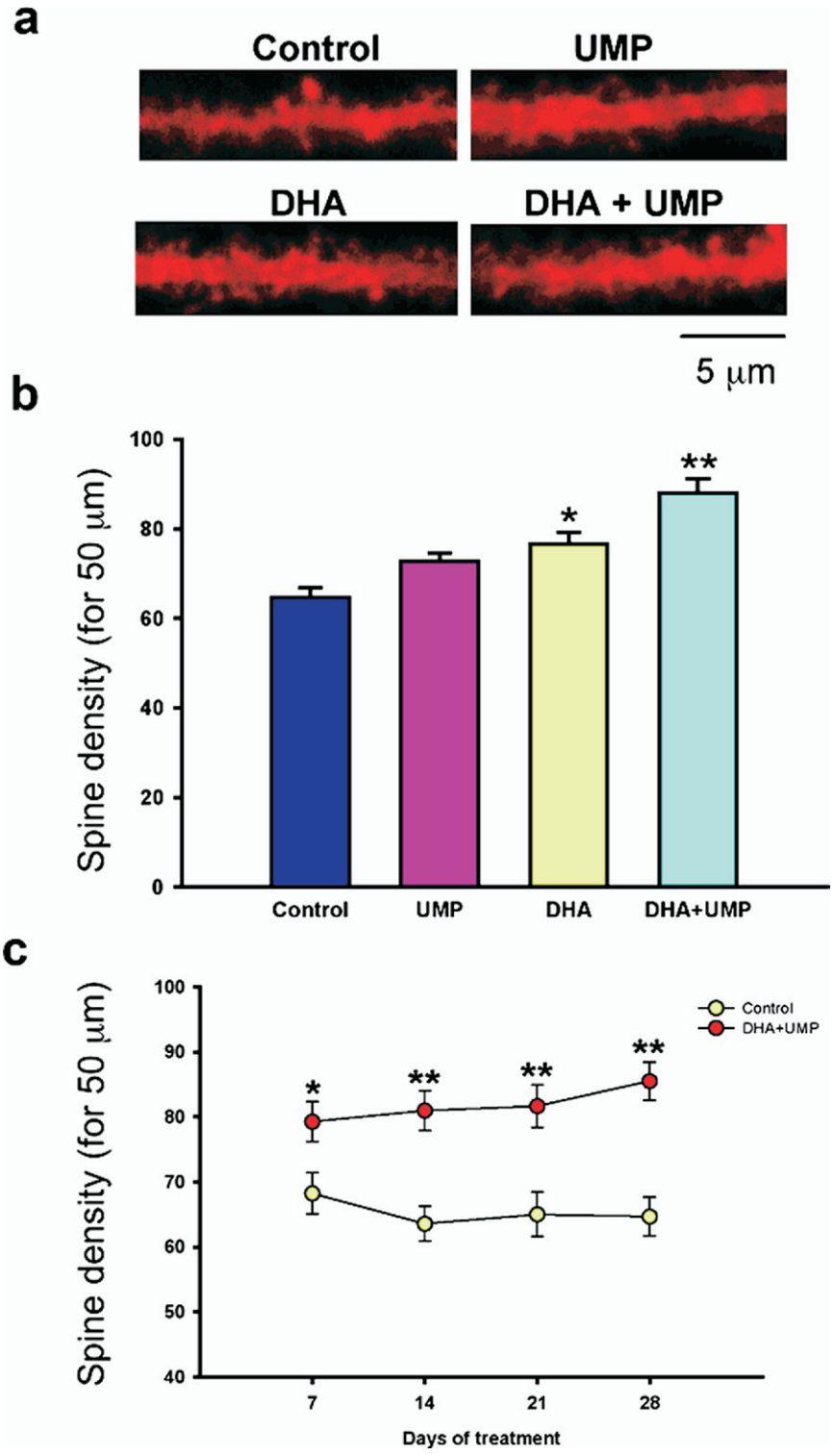

Fig. 5. Effects of DHA, alone or in combination with a UMP-supplemented diet, on dendritic spine formation in adult gerbil hippocampus. Animals received UMP (0.5%), DHA (300 mg/kg), or both daily for 4 weeks; control gerbils received neither. (a) Apical dendrites of CA1 pyramidal neurons. (b) Animals supplemented with DHA exhibited a significant increase in spine density (by 19%, $*P = .004$ vs Control); those receiving both DHA and UMP exhibited a greater increase (by 36%, $**P < .001$ vs Control or by 17%, $P = .008$ vs DHA). n = 20 ~ 25 neurons from 4 animals per group. One-way ANOVA followed by Tukey test. (c) Effect of DHA plus UMP on spine density was apparent by 1 week after the start of the treatment. The treated groups received both UMP (0.5%) and DHA (300 mg/kg) daily for 1, 2, 3, or 4 weeks; the control groups were given only a regular diet. n = 12 ~ 20 neurons from 2 animals per group. Two-way ANOVA followed by Tukey test. $*P = .02$; $**P < .001$. Reprinted with permission from Sakamoto et al [159].

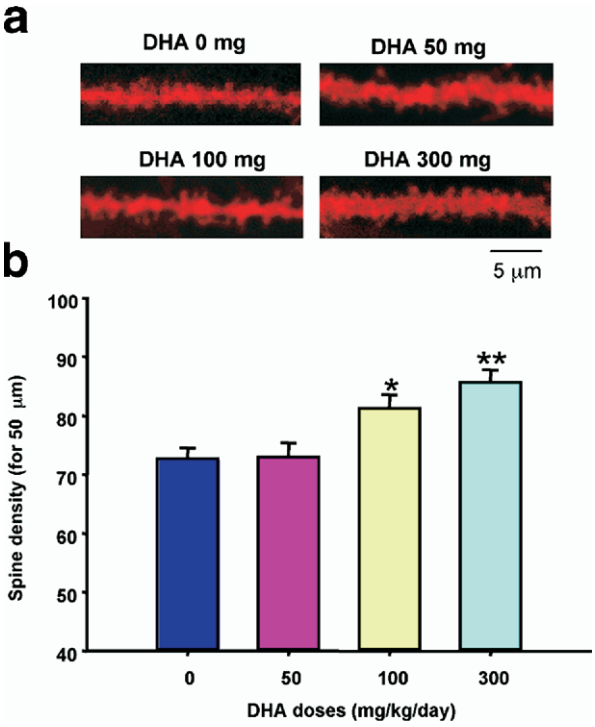

Fig. 6. Effects of oral supplementation with various doses of DHA on dendritic spine density in adult gerbil hippocampus. Animals were supplemented with 0, 50, 100, or 300 mg/kg of DHA daily for 4 weeks. (a) Apical dendrites of CA1 pyramidal neurons. (b) Animals supplemented with 100 or 300 mg/kg/day showed increased spine density, a 12 % increase after the 100 mg/kg/day dose (*P = .04) and an 18% increase after the 300 mg/kg/day dose (**P < .001 vs 0 mg/kg/day). n = 16 ~ 20 neurons from 2 animals per group. Reprinted with permission from Sakamoto et al [159].

DHA alone were 42%, 37%, and 29% (all $P \leq .05$), whereas the increases after treatment with DHA plus UMP were by 44%, 57%, and 37%, respectively (all $P < .01$). Treatment with DHA or with DHA plus UMP also elevated brain levels of actin, a cytoskeletal protein that can directly regulate the morphology of dendritic spines and that is implicated in such manifestations of synaptic plasticity as long-term potentiation and depression [150–152,155,158]. Actin levels increased by 60% after DHA and by 88% in animals receiving DHA plus UMP [159].

In contrast, levels of β-tubulin, a cytoskeletal protein that is not specifically localized within synaptic structures, were unaffected by the treatments [2].

Oral supplementation with AA failed to affect dendritic spine density in the CA1 region of the adult gerbil hippocampus, even though, like DHA, AA does affect synaptic plasticity in cultured neurons [160–162]. AA also failed to affect hippocampal levels of phosphatides or of synaptic proteins [159].

The mechanisms through which DHA, with or without uridine, increases dendritic spine formation might also involve presynaptic processes. Results from various model systems indicate that both DHA [7,163,164] and uridine [5,165,166] can promote axonal growth and exocytosis in cultured cells. DHA can activate the SNARE protein syntaxin-3 [7], whereas uridine through UTP can activate P2Y receptors [5], which are expressed in hippocampal neurons [167] and are implicated in presynaptic induction of long-term potentiation [168]. Formation of dendritic spines and synaptogenesis in mammalian brains can be induced or initiated by presynaptic neurons, and this process might involve calcium [150–152,169]. The increases in spine density with DHA and UMP treatment (Figure 5) might thus result from potentiation of presynaptic or postsynaptic mechanisms.

7. Effects of uridine on neurotransmitter release and of UMP plus DHA on behavior

Consumption by rats of a diet containing uridine (as UMP) and choline can increase dopamine (DA) and ACh levels in and—as assessed with in vivo microdialysis—their release from corpus striatum neurons. Apparently no data are available on the effects on neurotransmitter production or release of giving DHA alone or with the other two phosphatide precursors. Dietary supplementation of aged male Fischer 344 rats with 2.5% UMP for 6 weeks, ad libitum, increased the release of striatal DA that was evoked by potassium-induced depolarization from 283% ± 9% in control rats to 341% ± 21% in those receiving the UMP ($P < .05$) [165]. In general, each animal's DA release correlated with its striatal DA content, measured postmortem. The levels of neurofilament-70 and neurofilament-M proteins, two markers of neurite outgrowth, were also increased after UMP treatment to 182% ± 25% of control levels for neurofilament-70 ($P < .05$) and to 221% ± 34% ($P < .01$) for neurofilament-M [165].

In a similar microdialysis study, ACh release, basally as well as after administration of atropine (a muscarinic antagonist that blocks inhibitory presynaptic cholinergic receptors), was found to be enhanced after UMP consumption. Among aged animals consuming a UMP-containing diet (2.5%, w/w) for 1 or 6 weeks, baseline ACh levels in striatal microdialysates rose from 73 to 148 fmol/min after 1 week of treatment ($P < .05$) and to 197 fmol/min after 6 weeks ($P < .05$) [166]. Dietary UMP (0.5%, 1 week) also amplified the increase in ACh release caused by giving atropine (10 μmol/L via the artificial cerebrospinal fluid); atropine alone increased ACh concentrations from 81 to 386 fmol/min in control rats and from 127 to 680 fmol/min in those consuming UMP ($P < .05$). Young rats eating the UMP-containing diet exhibited similar responses. These data suggest that giving a uridine source might enhance some cholinergic functions, perhaps by increasing the amount of synaptic membrane or the quantities of ACh stored in synaptic vesicles.

Additional evidence that treatment with UMP alone or with UMP plus DHA can affect brain neurotransmission

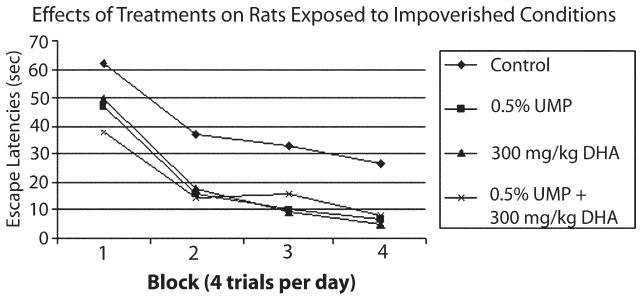

Fig. 7. Rats were allowed to eat 16% protein food or the same diet supplemented with 0.5% UMP and/or by gavage 300 mg/kg DHA. Rats began to eat the UMP-supplemented food and to receive the daily gavage of DHA at approximately 4 weeks of age, 4 weeks before testing in a water maze, which continued throughout testing. Also beginning at 4 weeks of age and continuing for duration of 4 weeks, rats were placed in impoverished conditions (IC). IC required that rats be housed individually without toys and exercised 3 times a week for 15 minutes in an empty room to avoid weight gain. After exposure to 4 weeks of impoverished environmental conditions, rats' learning and memory were tested with the hidden version of the Morris water maze (8 animals in each group).

comes from a few behavioral studies [24,170]. Among socially impoverished rats, DHA (300 mg/kg by gavage) or DHA plus dietary UMP (0.5%) treatment for 4 weeks reversed the deficits in hippocampal-dependent learning and memory performance [24] (Figure 7). Chronic dietary administration of UMP (0.1%) alone for 3 months also ameliorated this impairment among the impoverished rats [170].

8. Conclusions

Brain phosphatide synthesis requires three circulating compounds: DHA, uridine, and choline. Oral administration of these phosphatide precursors to experimental animals increases the levels of phosphatides and synaptic proteins in the brain and per brain cell, as well as the numbers of dendritic spines on hippocampal neurons. AA fails to reproduce these effects of DHA. If similar increases occur in human brain, giving these compounds to patients with diseases like Alzheimer's disease that cause the loss of brain synapses could be beneficial.

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