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Research Report

Synaptic proteins and phospholipids are increased in gerbil brain by administering uridine plus docosahexaenoic acid orally

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ABSTRACT

The synthesis of brain phosphatidylcholine may utilize three circulating precursors: choline; a pyrimidine (e.g., uridine, converted via UTP to brain CTP); and a PUFA (e.g., docosahexaenoic acid); phosphatidylethanolamine may utilize two of these, a pyrimidine and a PUFA. We observe that consuming these precursors can substantially increase membrane phosphatide and synaptic protein levels in gerbil brains. (Pyrimidine metabolism in gerbils, but not rats, resembles that in humans.) Animals received, daily for 4 weeks, a diet containing choline chloride and UMP (a uridine source) and/or DHA by gavage. Brain phosphatidylcholine rose by 13–22% with uridine and choline alone, or DHA alone, or by 45% with the combination, phosphatidylethanolamine and the other phosphatides increasing by 39–74%. Smaller elevations occurred after 1–3 weeks. The combination also increased the vesicular protein Synapsin-1 by 41%, the postsynaptic protein PSD-95 by 38% and the neurite neurofibrillar proteins NF-70 and NF-M by up to 102% and 48%, respectively. However, it had no effect on the cytoskeletal protein beta-tubulin. Hence, the quantity of synaptic membrane probably increased. The precursors act by enhancing the substrate saturation of enzymes that initiate their incorporation into phosphatidylcholine and phosphatidylethanolamine and by UTP-mediated activation of P2Y receptors. Alzheimer's disease brains contain fewer and smaller synapses and reduced levels of synaptic proteins, membrane phosphatides, choline and DHA. The three phosphatide precursors might thus be useful in treating this disease.

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Abbreviations:

DHA, docosahexaenoic acid
 UMP, uridine-5'-monophosphate
 UTP, uridine triphosphate
 CTP, cytidine triphosphate
 CDP-choline, cytidine-5'-
 diphosphocholine
 CDP-ethanolamine, cytidine-5'-
 diphosphoethanolamine
 PtdCho, phosphatidylcholine
 PtdEtn, phosphatidylethanolamine
 PtdSer, phosphatidylserine
 PtdIns, phosphatidylinositol
 SM, sphingomyelin
 PUFA, polyunsaturated fatty acid
 NF-M, neurofilament-M
 NF-70, neurofilament-70

1. Introduction

The synthesis in brain of phosphatidylcholine (PtdCho) and other membrane phosphatides can utilize, besides glucose, three compounds obtained from the circulation (Kennedy and Weiss, 1956): choline; a pyrimidine like uridine; and a polyunsaturated fatty acid (PUFA) like docosahexaenoic acid (DHA) (Rapoport, 2001; Marszalek and Lodish, 2005); all three readily cross the blood–brain barrier (Cornford et al., 1978; Li et al., 2001; Spector, 2001; Hashimoto et al., 2002). The choline is phosphorylated to form phosphocholine through the action of choline kinase (CK), a low-affinity enzyme that is unsaturated with choline at normal brain choline levels (Spanner and Ansell, 1979; Millington and Wurtman, 1982). The uridine is phosphorylated by uridine–cytidine kinase (Suzuki et al., 2004) to uridine triphosphate (UTP), which is further transformed by the enzyme CTP synthetase (Genchev and Mandel, 1974) to cytidine triphosphate (CTP), the rate-limiting precursor in PtdCho synthesis (Ross et al., 1997). Both of these latter enzymes are also low affinity, hence giving a single oral dose of uridine-5'-monophosphate (UMP), a uridine source sequentially increases brain uridine, UTP and CTP (Cansev et al., 2005). The phosphocholine and CTP combine to form cytidine-5'-diphosphocholine (CDP-choline), which then combines with diacylglycerol (DAG), including species containing DHA or another PUFA, to yield the PtdCho. The Kennedy cycle similarly synthesizes phosphatidylethanolamine (PtdEtn) from uridine and PUFA like DHA, but starting with ethanolamine, instead of choline.

Although DHA is found in PtdCho, PtdEtn and other brain membrane phosphatides (Marszalek and Lodish, 2005; Knapp and Wurtman, 1999), apparently no information is available concerning the effects of DHA's oral administration on brain phosphatide levels in vivo. Moreover, while a single dose of UMP has been shown to increase brain CDP-choline levels (Cansev et al., 2005), suggesting that it also accelerates PtdCho synthesis (Lopez-Coviella et al., 1995), no direct evidence is available that any treatment regimen involving uridine also affects brain PtdCho levels. We now show that oral administration of DHA or UMP, given alone for several weeks to animals consuming a choline-containing diet, can

increase brain PtdCho and other major membrane phosphatides. Moreover, the effect of giving both DHA and UMP tends to be greater ($P < 0.01$ for SM) than the sum of the effects observed when each is given separately. This increase may include synaptic membranes inasmuch as the treatment also increases levels of presynaptic and postsynaptic proteins.

2. Results

2.1. Effects of UMP and/or DHA on brain phosphatides

Animals received just the control diet or one of the three experimental treatments for 4 weeks; brain samples were assayed as described in Experimental Procedures, and phosphatide levels were given as nmol/mg protein. Addition of UMP to the standard diet without concurrent DHA treatment significantly increased brain levels of PtdCho, PtdEtn and PtdIns by 13%, 29% and 48%, respectively (Table 1A). Administration of DHA, without UMP, also significantly increased brain levels of these phosphatides (by 22%, 20% and 52%, respectively), as well as of sphingomyelin (by 24%). Combining both treatments (dietary UMP plus gavigated DHA) increased all of the phospholipids by more than the sum of the increases produced by UMP or DHA alone (Table 1A) ($P < 0.01$ for SM).

In a related experiment, we examined the time course of these increases among animals receiving UMP alone or both UMP and DHA (Table 1B). After 1 week of treatment, UMP supplementation produced no significant effects, while giving both UMP and DHA caused small but significant increases in brain PtdCho (21%) and PtdSer (38%). Treatment with both UMP and DHA for 3 weeks caused significant increases (by 21–48%) in all five of the phospholipids; UMP alone caused smaller but still significant increases (Table 1B).

In both experiments, essentially similar findings were made whether data were expressed per mg protein or per μg DNA (data not shown). For example, treatment for 4 weeks with both UMP and DHA increased brain PtdCho by 45% when data were expressed per mg protein and by 41% when expressed per μg DNA.

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

Treatments (groups)	Total PL (nmol/mg prt)	PtdCho (nmol/mg prt)	PtdEtn (nmol/mg prt)	SM (nmol/ mg prt)	PtdSer (nmol/mg prt)	PtdIns (nmol/mg prt)
A						
Control diet + Vehicle	351 ± 8	152 ± 6	65 ± 4	45 ± 2	33 ± 3	21 ± 2
UMP diet + Vehicle	367 ± 22	171 ± 8*	84 ± 8*	52 ± 5	35 ± 3	31 ± 2**
Control diet + DHA	392 ± 20	185 ± 12*	78 ± 5*	56 ± 3*	39 ± 3	32 ± 2**
UMP diet + DHA	442 ± 24***	220 ± 12***	113 ± 6***	73 ± 4***	46 ± 6***	36 ± 3***
B						
Control + Vehicle	403 ± 23	155 ± 8	69 ± 3	47 ± 3	34 ± 1	20 ± 2
One week						
UMP + Vehicle	390 ± 21	154 ± 6	63 ± 4	49 ± 3	39 ± 1	20 ± 4
UMP + DHA	436 ± 15	188 ± 8*	79 ± 6	57 ± 6	47 ± 1***	23 ± 1
Three weeks						
UMP + Vehicle	479 ± 6**	199 ± 5***	87 ± 4*	70 ± 4*	42 ± 2*	25 ± 1
UMP + DHA	502 ± 12***	217 ± 5***	102 ± 4***	73 ± 5**	41 ± 1*	27 ± 1*

Groups of 8 gerbils were given either a control or a UMP-containing (0.5%) diet and received orally (by gavage) DHA (300 mg/kg; in a vehicle of 5% gum Arabic solution) or just its vehicle for 28 days (A) or for 7 or 21 days (B). On the 29th (A) or 8th and 22nd days (B), their brains were harvested and assayed for phospholipids as described in the text. Data are given as means ± SEM. Statistical analysis was performed using two-way ANOVA followed by Tukey test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with the values for control diet plus vehicle group. For the 4-week study, two-way ANOVA revealed a significant effect of adding UMP to the control diet [$F(1,28) = 7.64$; $P < 0.05$]; [$F(1,28) = 14.46$; $P < 0.001$] and [$F(1,28) = 10.75$; $P < 0.01$] on brain levels of PtdCho; PtdEtn; and PtdIns, respectively. Two-way ANOVA also revealed a significant effect of gavaged DHA, in animals consuming the control diet [$F(1,28) = 16.37$; $P < 0.001$], [$F(1,28) = 8.96$; $P < 0.01$] and [$F(1,28) = 14.81$; $P < 0.001$] on brain levels of these phosphatides. For the 1- and 3-week study, two-way ANOVA revealed a significant effect of adding UMP to the control diet [$F(1,21) = 23.6$; $P < 0.001$]; [$F(1,21) = 15.3$; $P < 0.001$]; [$F(1,21) = 12.3$; $P < 0.01$]; and [$F(1,21) = 8.4$; $P < 0.01$] on brain levels of PtdCho; PtdSer; SM; and PtdEtn, respectively. Two-way ANOVA also revealed a significant effect of gavaged DHA, in animals consuming the control diet [$F(1,21) = 6.1$; $P < 0.05$] on brain PtdEtn levels.

2.2. Effects of DHA on brain CDP-choline and CDP-ethanolamine levels

To determine whether the increases in brain phosphatides caused by giving DHA, with or without UMP, might reflect increases in phosphatide synthesis, we also measured brain levels of CDP-choline and CDP-ethanolamine, the immediate precursors of PtdCho and PtdEtn. We postulated that, if DHA acted by generating more of the DHA-containing DAG and if this compound then combined with endogenous CDP-choline or CDP-ethanolamine to form more PtdCho or PtdEtn, then DHA administration might reduce brain CDP-choline and CDP-ethanolamine levels while increasing those of PtdCho and PtdEtn. This was indeed observed among animals treated with DHA for 4 weeks, both among those receiving just the DHA and those receiving both DHA and UMP (Fig. 1): CDP-choline and CDP-ethanolamine levels in DHA-treated gerbils fell by 26% and 21% (compared with those receiving just the control diet and DHA's vehicle), respectively, and CDP-choline and CDP-ethanolamine levels in animals receiving both UMP-containing diet and DHA fell by 21% and 11% (compared with those receiving UMP-containing diet and DHA's vehicle), respectively (Fig. 1). In a related study comparing animals receiving UMP plus DHA with those receiving UMP alone for 3 weeks, adding the DHA similarly reduced brain CDP-choline and CDP-ethanolamine levels (data not shown).

2.3. Effects of UMP, with or without DHA, on synaptic protein levels

Brain levels of the two specific synaptic proteins measured, Synapsin-1 and PSD-95, expressed as the percents of those in

control animals, were significantly increased among animals receiving both UMP and DHA, rising by 38% and 41%, respectively, after 3 weeks and by 35% or 25% after 1 week (Figs. 2A and B). The neurofilament proteins NF-70 and NF-M, concentrated in neurites, showed similar responses, rising by 43% or 102%, and by 19% or 48%, respectively, after 3 or 4 weeks of treatment (Fig. 3). Levels of the cytoskeletal protein beta-tubulin levels did not change significantly (Fig. 2C).

3. Discussion

These data show that administering DHA by gavage or UMP via the diet, daily for 4 weeks, significantly increases brain membrane PtdCho levels (Table 1A) among gerbils consuming a standard, choline-containing diet. These effects are observed whether the PtdCho is expressed per mg protein or per cell (DNA), indicating that each brain cell, on average, contains more of the membrane phosphatide. Moreover, when all three of the potentially limiting circulating precursors (i.e., choline, uridine and DHA) are provided, the resulting increases in brain phosphatides tend to be greater (significantly for SM; $P < 0.01$) than the sum of the increases observed with DHA + choline or UMP + choline alone (Table 1A). Significant but smaller effects are observed after 3 weeks of treatment (Table 1B) and, for PtdCho, even after 1 week (Table 1B).

Interestingly, these increases in phosphatide levels are accompanied by parallel increases in the amounts of brain proteins known to be localized within synaptic structures, the postsynaptic density protein PSD-95 (Fujita and Kurachi, 2000) (Fig. 2A); the vesicular protein Synapsin-1 (Ferreira and Rapoport, 2002) (Fig. 2B); and two neurofilament proteins,

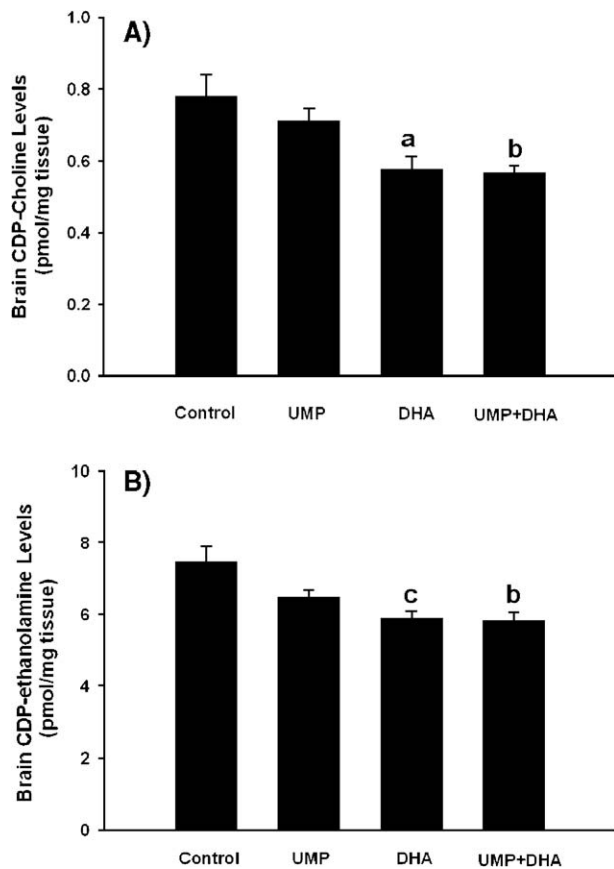


Fig. 1 – Effects of DHA on brain CDP-choline or CDP-ethanolamine levels. Groups of 8 gerbils received either a control or a UMP-containing (0.5%) diet and, by gavage, DHA (300 mg/kg; in a vehicle of 5% gum Arabic solution) or just its vehicle, for 28 days. On the 29th day, their brains were harvested and assayed for (A) CDP-choline or (B) CDP-ethanolamine as described in the text. Data are given as means \pm SEM. Statistical analysis was performed using two-way ANOVA followed by Tukey test. ^a $P < 0.05$ and ^c $P < 0.01$ when compared with the values for control diet plus vehicle group; ^b $P < 0.05$ when compared with values for UMP diet plus vehicle group. Two-way ANOVA revealed significant effects of DHA [$F(1,28) = 31.7$; $P < 0.001$] and [$F(1,28) = 26.1$; $P < 0.001$] on CDP-choline and CDP-ethanolamine levels.

NF-70 and NF-M (Fig. 3), which are enriched in neurites (Lee et al., 1982). This finding raises the possibility that providing the phosphatide precursors can affect the quantity of synaptic membranes.

The mechanism by which supplemental choline affects phosphatide synthesis probably involves increasing the substrate saturation of CK, the enzyme that initiates its incorporation into PtdCho. This enzyme has a K_m for choline of 2.6 mM (Spanner and Ansell, 1979), which is substantially higher than usual brain choline concentrations (35–100 μ M; Cohen and Wurtman, 1976). Moreover, choline administration is known to increase brain phosphocholine levels in rats (Millington and Wurtman, 1982) and humans (Babb et al., 2004).

The utilization of circulating DHA to form the DHA-containing DAG molecules preferentially used for phosphatide synthesis (Marszalek and Lodish, 2005; Marszalek et al., 2005) involves several steps: the fatty acid leaves the extracellular space by partitioning into the external leaflet of a cell's plasma membrane; it then "flip-flops" to the inner leaflet, allowing it to interact with intracellular fatty-acid-binding proteins and with an Acyl-CoA synthetase (Acsl6; Marszalek et al., 2005) which attaches it to the *sn*-2 position of glycerol-3-phosphate. This product then attaches a saturated fatty acid at the *sn*-1 position to become phosphatidic acid, which subsequently is dephosphorylated to form DAG. In vivo, orally administered DHA is known to elevate both plasma and brain DHA levels (Hashimoto et al., 2002), and in PC12 cells engineered to overexpress the Acsl6 protein, [¹⁴C]DHA added to the medium is rapidly acylated to [¹⁴C]DHA-CoA and incorporated into phospholipids and triglycerides (Marszalek et al., 2005). That exogenous DHA increases brain PtdCho and PtdEtn levels by increasing the phosphatide's synthesis is further supported by the finding that its administration concurrently raised PtdCho and PtdEtn levels (Table 1A) while lowering those of CDP-choline and CDP-ethanolamine (Fig. 1), the immediate precursors for PtdCho and PtdEtn with which the DHA-containing DAG presumably combined.

The mechanisms by which supplemental uridine increases phosphatide synthesis probably include two processes—increased substrate saturation of the enzymes that convert uridine, via UTP and CTP, to endogenous CDP-choline and CDP-ethanolamine and activation by UTP of P2Y receptors that affect neurite outgrowth. A single dose of uridine, given by gavage, has been shown to cause sequential increases in blood and brain uridine then in brain UTP, CTP and CDP-choline (Cansev et al., 2005). This effect is short-lived and insufficient to produce reliable increases in brain PtdCho or PtdEtn. However, as observed in the present study, the chronic consumption of a uridine source (via the diet) for 3 or 4 weeks is able to raise brain PtdCho and PtdEtn, even though the increases it produces in plasma uridine are small and transient [unpublished observations]. In vitro, uridine in concentrations of 50 μ M or greater increases the size of neurites (Pooler et al., 2005) generated by NGF-stimulated PC12 cells (Araki and Wurtman, 1997); it also accelerates phosphatide synthesis (Richardson et al., 2003) and increases neurofilament protein levels in these cells (Pooler et al., 2005). That the stimulatory effect of uridine on neuritogenesis involves activation by its product UTP of P2Y receptors was shown by the demonstration that a lower concentration of UTP (10 μ M) than of free uridine can enhance neurite outgrowth (Pooler et al., 2005), and by the blockade of uridine's or UTP's effects by apyrase, a drug that degrades nucleotides, or by P2Y antagonists (Pooler et al., 2005).

The tendency of combined treatment with dietary choline, UMP, and DHA to produce larger increases in brain phosphatides than the sum of the increases caused by the individual precursors probably reflects the operation of all of the above mechanisms: providing each of the three circulating precursors decreases the likelihood that its intracellular levels will become limiting when levels of the other precursors have been increased, and providing the uridine source enhances the

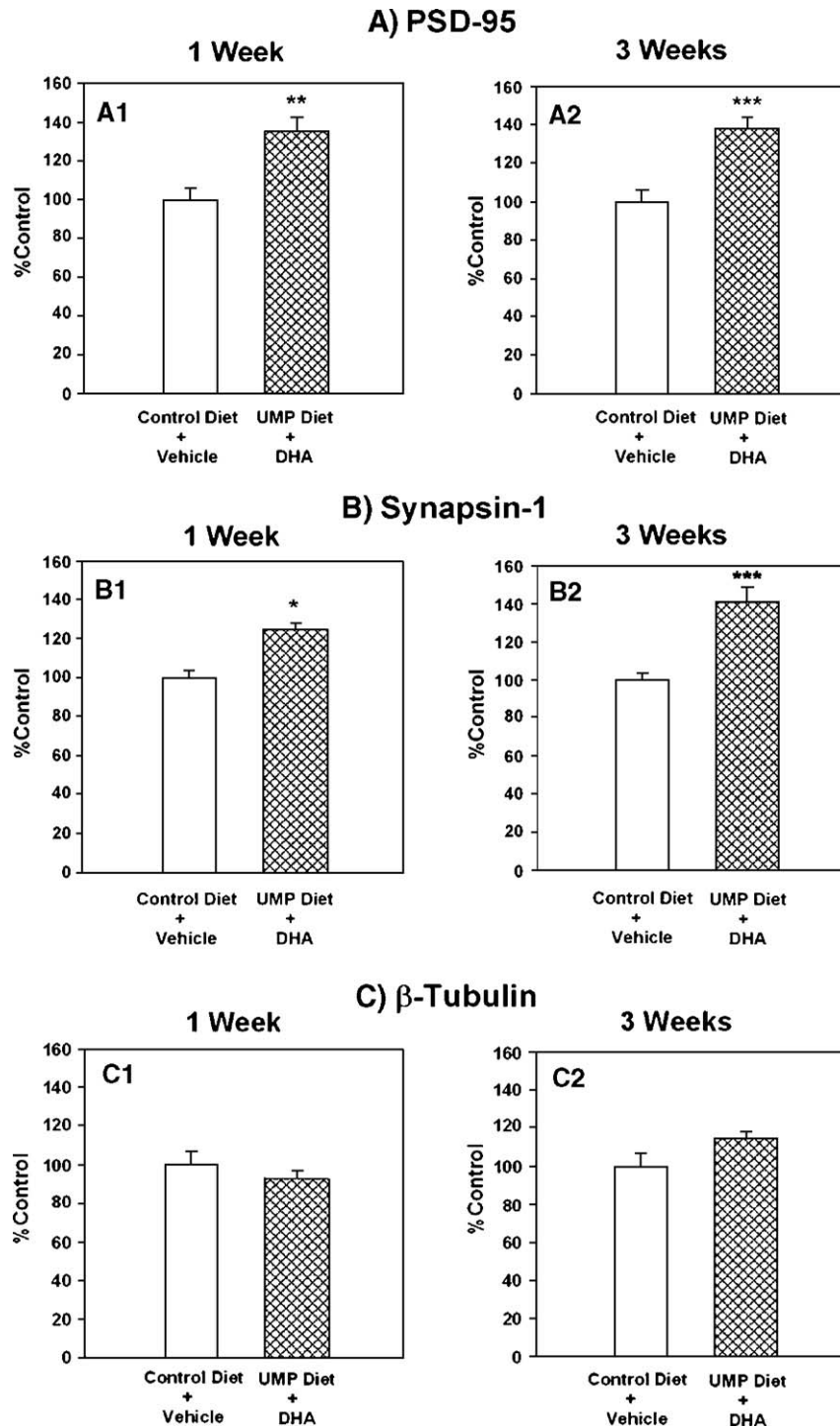


Fig. 2 – Effects of UMP diet and DHA on brain PSD-95, Synapsin-1 and beta-tubulin levels. (A) Groups of 8 gerbils received either a control diet plus, by gavage, DHA's vehicle, or a UMP-containing (0.5%) diet plus, by gavage, DHA (300 mg/kg) dissolved in the vehicle (5% gum Arabic solution) for 7 (A1) or 21 (A2) days. On the 8th and 22nd days, their brains were harvested and assayed for PSD-95 as described in the text. Values represent means \pm SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey test. ** $P < 0.01$; *** $P < 0.001$ when compared with values for control diet plus vehicle group. (B) Brains from animals prepared as described in panel A were harvested on the 8th and 22nd days and assayed for Synapsin-1 as described in the text. Values represent means \pm SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey test. * $P < 0.05$; *** $P < 0.001$ when compared with values for control diet plus vehicle group. (C) Brains from animals prepared as described in panel A were harvested on the 8th and 22nd days and assayed for beta-tubulin as described in the text. Values represent means \pm SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey test. No significant differences in beta-tubulin levels in treatment groups compared to those in control groups were observed.

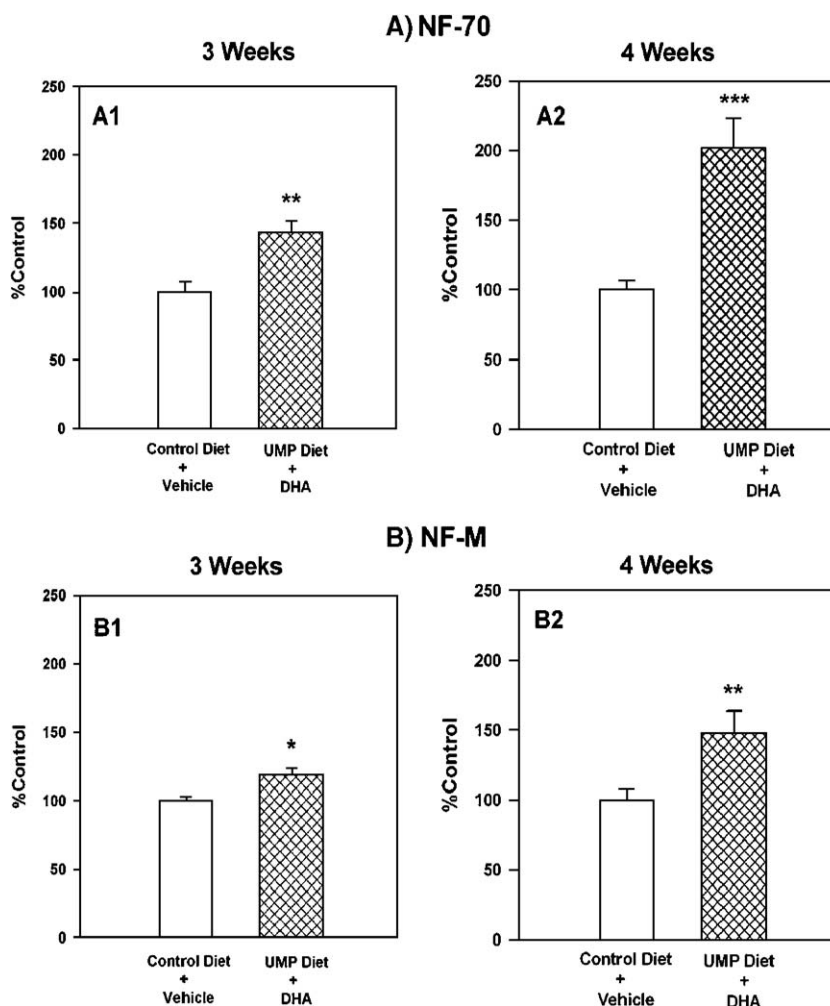


Fig. 3 – Effects of UMP diet and DHA on brain NF-70 and NF-M levels. (A) Groups of 8 gerbils received either a control diet plus, by gavage, DHA's vehicle, or a UMP-containing (0.5%) diet plus, by gavage, DHA (300 mg/kg) dissolved in the vehicle (5% gum Arabic solution) for 21 (A1) or 28 (A2) days. On the 22nd and 29th days, their brains were harvested and assayed for NF-70 as described in the text. Values represent means \pm SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey test. ** $P < 0.01$; *** $P < 0.001$ when compared with values for control diet plus vehicle group. (B) Groups of 8 gerbils received either a control diet plus, by gavage, DHA's vehicle, or a UMP-containing (0.5%) diet plus, by gavage, DHA (300 mg/kg) dissolved in the vehicle (5% gum Arabic solution) for 21 (B1) or 28 (B2) days. On the 22nd and 29th days, their brains were harvested and assayed for NF-M as described in the text. Values represent means \pm SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey test. * $P < 0.05$; ** $P < 0.01$ when compared with values for control diet plus vehicle group.

activation of relevant P2Y receptors by UTP. These receptors could include any of the pyrimidine-sensitive receptors (P2Y2, P2Y4 or P2Y6) in the P2Y family. At present, no drugs are available that allow selective blockade of one particular receptor type.

Giving the three precursors concurrently increased PtdCho by 21% (nmol/mg protein) after 1 week and by 40% or 45% after 3 or 4 weeks; corresponding increases expressed as nmol/ μ g DNA were by 20%, 34% and 41%, respectively. (Similar or greater changes were noted in PtdEtn.) It is conceivable that even greater increases might be produced by administering larger doses of the precursors, or by extending this treatment for longer periods. The amounts of choline that we provided were standard for gerbil chow; UMP is not normally included in such chows, however, it is present in mother's milk and included in infant formulas in amounts intended to provide up

to 0.3 mg/100 J (Yu, 2002); DHA is not a constituent of gerbil chow but is variably present in human diet depending in large part on the quantities of seafoods – particularly higher-fat fish like herring and salmon (Harris, 2005) – that a person elects to eat. Consumption of up to 3 g/day of DHA and other omega-3 fatty acids (e.g., eicosapentaenoic acid) is Generally Regarded as Safe (GRAS) by the U.S. Food and Drug Administration, and doses of 4 g/day, or 50–60 mg/kg, are sometimes used in the treatment of hypertriglyceridemia (Stalenhoef et al., 2000). The precise ratios of supplemental choline to uridine to DHA for optimal enhancement of brain phosphatide levels await determination, as does the question of whether other PUFA besides DHA that are present in phosphatides should also be included. Studies are in progress examining possible effects of other PUFA and of PUFA mixtures on levels of brain phosphatides and synaptic proteins.

As noted above, the concurrent administration of the three PtdCho precursors produced reliable increases in the levels of four brain proteins associated with the synaptic structures, PSD-95 (Fig. 2A), a postsynaptic protein abundant in the postsynaptic synaptosomal fraction (Fujita and Kurachi, 2000); Synapsin-1, a vesicular protein (Ferreira and Rapoport, 2002) (Fig. 2B); and NF-70 and NF-M (Fig. 3), neurofilament components concentrated in neurites (Lee et al., 1982). In contrast, levels of the cytoskeletal protein beta-tubulin did not change significantly (Fig. 2C). These increases—which were of about the same magnitude as the increases in phosphatide levels—suggest that the combined treatment affected, among other cellular membranes, those associated with synapses. This conclusion must be viewed as tentative in the absence of anatomic data indicating that, for example, the size and/or number of dendritic spines also increased. However, it may be relevant that chronic (6 weeks) consumption, by rats, of choline plus a precursor of brain CTP – without DHA or another PUFA – did enhance the evoked release of dopamine (Wang et al., 2005), as well as the spontaneous and evoked release of acetylcholine (Wang et al., 2004) within the corpus striatum. In these studies, the precursors were provided by adding either UMP or CDP-choline (which is metabolized to choline plus uridine in gerbils or humans [Wurtman et al., 2000; Cansev and Wurtman, 2005] or to cytidine in rats [Lopez-Coviella et al., 1995]) to a choline-containing diet. Similarly, aged rats (Teather and Wurtman, 2003) or rats reared in a socially deprived environment (Teather and Wurtman, 2005) exhibited significant improvement in learning and memory after consuming a CDP-choline-containing diet for 8 weeks. Administration of DHA or mixtures of DHA plus other omega-3 PUFA can improve cognitive functions in experimental animals (Hashimoto et al., 2002) and in humans (McCann and Ames, 2005). At present, no data are available on possible behavioral effects in humans of receiving the DHA along with a uridine source and choline. Brain levels of choline, ethanolamine (Nitsch et al., 1992) and DHA (Soderberg et al., 1991) are known to be subnormal in patients with Alzheimer's disease, and such patients also exhibit characteristic decreases in synaptic size and number (Terry et al., 1991; Coleman et al., 2004; Selkoe, 2002), as well as increases in the phosphatide breakdown products glycerophosphocholine and glycerophosphoethanolamine (Nitsch et al., 1992). If, as discussed above, the ability of the circulating precursors for PtdCho and PtdEtn to enhance both brain phosphatide and synaptic protein levels also enables them to increase synaptic membranes, and if brains of patients with Alzheimer's disease remain capable of responding, it is conceivable that the precursors may confer some therapeutic benefit in this disease.

4. Experimental procedures

4.1. Drugs and chemicals

DHA was purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). UMP was kindly provided by Numico Research (Wageningen, Netherlands). Control and UMP-containing diets were

prepared by Harlan-Teklad (Madison, WI, USA). Standards for phospholipids were purchased from Sigma Chemicals (St. Louis, MO, USA). Mouse anti-NF-70, mouse anti-tubulin beta III subunit, and rabbit anti-NF-M were purchased from Chemicon (Temecula, CA, USA); mouse anti-PSD-95 from Upstate (Lake Placid, NY, USA); and mouse anti-Synapsin-1 from Calbiochem (San Diego, CA, USA). Bicinchoninic acid reagent was purchased from Perkin-Elmer, Norwalk, CT, USA.

4.2. Animals

We used gerbils (*Meriones unguiculatus*) for these studies because the metabolism of pyrimidines in this species more closely resembles that in humans than does pyrimidine metabolism in rats: plasma levels of uridine are higher than those of cytidine, both basally and after giving a uridine (Cansev et al., 2005) or cytidine (Wurtman et al., 2000; Cansev and Wurtman, 2005) source, in humans and gerbils but not in rats (Lopez-Coviella et al., 1995).

Adult male gerbils weighing 60–80 g (4–6 months old) were exposed to light between 7 AM and 7 PM. Control animals were given access to a standard choline-containing (0.1%) gerbil diet (Harlan-Teklad, Madison, WI, USA) (Table 2). Experimental groups were given (1) this diet supplemented with UMP (0.5%) or (2) the unsupplemented diet plus DHA (300 mg/kg, by gavage) or (3) both the UMP-supplemented diet and the gavaged DHA. (Animals not receiving DHA were gavaged daily with its vehicle, 5% gum Arabic solution.) DHA was divided into daily doses, pipetted into light-protecting glass vials under a nitrogen gas flow that replaced the oxygen to avoid DHA's decomposition and kept at –80 °C until given by gavage. Animals each consumed an average of 5 g of food per day, so that their average intakes of uridine, choline, and DHA were 240, 80, and 300 mg/kg/day, respectively, among those having access to these compounds. None of the groups exhibited significant changes in body weight during the course of the experiment (data not shown). All experiments were carried out in accordance with 1996 *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health) and Massachusetts Institute of Technology policies.

Table 2 – Composition of control diet

Proximate analysis (%)	
Protein	16.7
Carbohydrate	60.9
Oil, fiber, ash	13.7
Choline	0.1
Fatty acids (g/kg)	
Saturated	7.34
Unsaturated	
C18:1n-9 oleic acid	8.96
C18:2n-6 linoleic acid	23.12
C18:3n-3 linolenic acid	1.53

The control diet lacked UMP or DHA, but contained 0.1% choline, 2.3% linoleic acid (C18:2n-6) and 0.15% linolenic acid (C18:3n-3), all of which are essential nutrients. The UMP-containing diet was prepared by addition of 0.5% UMP to the control diet.

The diets were consumed for 7, 21 or 28 days, and on the morning of the 8th, 22nd, or 29th day, animals were sacrificed under Telazol (80 mg/kg; i.m.) anesthesia by dipping their heads into liquid nitrogen and then decapitating them by guillotine. Brains were quickly removed and kept on dry ice; subsequently, they were homogenized in deionized water, and aliquots of whole homogenates were used for the assays described below. Bloods were placed in a chilled water bath and centrifuged to separate their plasmas which were saved for future assays. Gastric contents were examined and found in all cases to contain undigested food. Experimental groups consisted of 8 gerbils except as noted.

4.3. Assays

Brain phosphatides were extracted according to the method of Folch et al. (1957) and measured as described previously (Ulus et al., 1989; Lopez-Coviella et al., 1995). Briefly, frozen brain hemispheres were weighed and homogenized in 100 volumes of ice-cold deionized water using a tissue degrader (Polytron PT 10-35, Kinematica AG, Switzerland); 1 ml aliquots was then mixed with 3 ml of chloroform + methanol mixture (2:1 v/v) and vortexed vigorously for 30 s. After cooling for about 1 h on ice, the mixture was mixed sequentially with 3 ml of chloroform + methanol mixture (2:1 v/v) and 1 ml of ice-cold deionized water. The mixture was vortexed vigorously and allowed to stand overnight in the cold room (18–20 h). The organic (lower) and aqueous (upper) phases of the mixtures were separated by centrifugation (10 min at 4 °C; 1000×g). An aliquot (2 ml) of the upper (aqueous) phase was used for determination of CDP-choline and CDP-ethanolamine (see below), and aliquots (0.1–0.4 ml) of the lower (organic) phase were dried under vacuum for phospholipid analysis. Residues of 0.1-ml aliquots of the lower phase were assayed for total phospholipid content by measuring phosphorus (Svanborg and Svennerholm, 1961). Residues of 0.4-ml aliquots of the lower phase were reconstituted in 40 µl 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 phospholipid classes (PC, PE, SM, PS and PI) were scraped off the plates and extracted into 1 ml of methanol, dried under vacuum and assayed for phosphorus content (Svanborg and Svennerholm, 1961).

Aliquots (2 ml) of the upper (aqueous) phase were dried under a vacuum, reconstituted and injected into an HPLC for CDP-choline assay as described by Richardson et al. (2003). CDP-ethanolamine was assayed similarly, except that its elution time was shorter. Aliquots of whole brain homogenates were assayed for protein using a bicinchoninic acid reagent (Perkin-Elmer, Norwalk, CT, USA), and for DNA by a fluorometric method (Labarca and Paigen, 1980). Synaptic proteins were assayed by slot-blot and by Western blot. For Western blotting, the aliquots of brain homogenates were mixed with KFL loading buffer and boiled for 5 min prior to gel electrophoresis. Equal amounts of protein were loaded and separated using SDS-PAGE (4–20%; Bio-Rad, Hercules, CA,

USA), hence data on levels of specific proteins were normalized to those in control animals. Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA). The remaining binding sites were blocked with 4% non-fat dry milk (Varnation, Glendale, CA, USA) for 30 min in TBST. For slot blotting, two sets of aliquots (18–21 µl; containing 20 µg of protein) from brain homogenates in deionized water were blotted directly onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA) by vacuum filtration, using a slot-blot microfiltration apparatus (Minifold® II Slot Blot System (SCR 072/0); Schleicher and Schuell, Inc., Keene, NH, USA). The remaining binding sites were blocked with 4% non-fat dry milk (Carnation, Glendale, CA, USA) for 30 min in TBST. Membranes (from slot blots and Western blots) were then rinsed five times in TBST buffer and immersed in TBST solution containing the antibody of interest (mouse anti-NF-70, rabbit anti-NF-M, mouse anti-PSD-95, mouse anti-Synapsin-1 and mouse anti-beta-tubulin). Following overnight incubation and five rinses in TBST buffer, blots were incubated for 1 h with the appropriate peroxidase-linked secondary antibody. Blots were then rinsed in TBST buffer five times, and protein-antibody complexes were detected and visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA) and Kodak X-AR film. Films were digitized using a Supervista S-12 scanner with a transparency adapter (UMAX Technologies, Fremont, CA, USA). Immunoreactive bands were compared densitometrically by using the Public Domain NIH Image program available on the Internet at <http://rsb.info.nih.gov/nih-image/>. Areas under the absorbance curve were expressed as arbitrary units and were normalized as percentages of those generated in control groups in the same blot.

4.4. Statistics

Data were expressed as means ± SEM. The statistical significance of effects on brain phospholipid levels of UMP, DHA and their interaction was assessed using two-way ANOVA followed by Bonferonni test. Statistical comparisons of the means between groups for synaptic proteins were made using one-way ANOVA followed by Tukey test. *P* values less than 0.05 were considered significant.

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