Consuming a Diet Enriched with Choline, UMP, and DHA Improves Memory in Rodents when these Compounds Increase Phospholipids

By

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Abstract

A new treatment tested in laboratory animals increases synaptic membrane and cholinergic neurotransmission. This treatment involves giving three compounds; DHA, a phosphatide precursor; choline, another phosphatide precursor present in the rodents' diets but not varied in this study; and UMP, a precursor of CTP, the rate-limiting compound in Kennedy Cycle production of phosphatides. Their administration increases the quantities of phosphatides, the major constituent of neuronal membrane, per brain cell, as well as specific synaptic proteins, the number of dendritic spines; and the expression of genes related to hippocampal glutamatergic neurotransmission. Since such membrane is the predominant component of synapses, the treatment might also ameliorate the loss of synapses occurring in the brains of Alzheimer's patients. My study involves assessing the behavioral effects of the treatment in individual rats or gerbils subsequently shown to manifest its neurochemical effects (i.e., increased phosphatides). My present findings show that the treatment improves performance on various tests of memory function. The largest improvements in memory function and increases in phosphatide levels are observed when DHA, choline, and UMP are consumed in combination.

Thesis Supervisor: Richard J Wurtman
Title: Cecel H Green Distinguished Professor of Neuropharmacology

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Finally, I dedicate this thesis to my brother, Ray Holguin, whose battle with mental illness continues to drive my passion for research. Your unyielding love is priceless to me.

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Introduction

Our laboratory has been interested in the ability of several circulating compounds, uridine monophosphate [UMP], docosahexaenoic acid [DHA], and choline - to increase the production of membrane phospholipids in brain and, concurrently, to increase brain levels of pre- and post- synaptic proteins (1) and the density of dendritic spines in the hippocampus (2). Hence I undertook a series of experiments to determine whether this treatment could also enhance various cognitive functions in experimental animals. My initial studies utilized gerbils, because pyrimidine metabolism in this species (but not in rat) is similar to that in humans (1).

Briefly, gerbils consumed UMP through their diet, and received DHA by daily gavage, and effects on learning and memory were assessed via the four arm radial maze, T-maze, and Y-maze tasks. I found that gerbils consuming UMP outperformed their control counterparts in every task. Likewise, gerbils administered DHA outperformed both the control, and the UMP-fed groups. Finally, gerbils receiving both UMP and DHA outperformed all other groups tested (Chapter 1). In subsequent studies on rats receiving dietary UMP and gavaged DHA, I compared the effects of these phosphatide precursors on animals that had been exposed to enriched or impoverished environments; learning and memory were assessed using the hidden and visible versions of the Morris water maze. I found that giving UMP plus DHA protected environmentally-impoverished rats from the cognitive decline usually associated with impoverishment; in contrast, the UMP plus DHA did not enhance memory among environmentally-enriched rats (Chapter 2).

Measurements of whole brain phospholipid levels in gerbils and rats revealed that the increases in these levels caused by UMP and DHA correlated with their

improvements in memory functions (Chapter 1, 2). Interestingly, rats exposed to an enriched environment had significantly higher whole brain phospholipid levels than those exposed to an impoverished environment; likewise otherwise-untreated environmentally-enriched rats outperformed impoverished rats on the hidden version of the Morris water maze (Chapter 2).

I elected to test learning and memory in gerbils using the radial maze, T-maze, and Y-maze because these methods allow for simultaneous recordings of both working and reference memory. Preliminary experiments also indicated that UMP and DHA can not be shown to affect cognitive function using high thoroughput assays, so I used protocols that allowed for one trial a day thus increasing the intertrial delay. The Morris water maze, a more standard test of memory, was not used to study gerbils because this species had difficulty learning how to swim, and thus the test itself would have been a stressor.

I chose to test the cognitive affects of UMP and DHA in impoverished and enriched rats using both the hidden and the visible versions of the Morris water maze because these tasks test two different types of memory. The hidden version measures hippocampal-dependent memory, and relies on spatial cues; the visible version measure striatal-dependent memory, and relies on habitual learning. Altering the rats' environmental conditions was incorporated in my studies because doing so provided a way to induce cognitive impairment (impoverished conditions) or cognitive enhancement (enhanced conditions), and UMP had previously been found in our laboratory to enhance cognition in impoverished rats (3). We hypothesized that UMP plus DHA would improve the memory functions of rats, including both impoverished and enriched rats,

since giving UMP plus DHA increases phospholipid levels and enhances cognition in normal adult gerbils (Chapter 1).

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Chapter 1:

Dietary Uridine Enhances the Improvement in Learning and Memory Produced by Administering DHA plus Choline to Gerbils

Sarah Holguin, Joseph Martinez, Camille Chow, Richard Wurtman

Abstract

Docosahexaenoic acid (DHA) and choline, two circulating precursors for brain phosphatides, can each improve cognitive abilities when administered to humans and experimental animals: DHA consumption enhances learning and memory in premature infants, and also in aged rats or rats infused intracerebrally with amyloid beta. Choline administration early in life improves learning and memory in adult gerbils, and choline also does so in malnourished humans. In the present study, we show that oral DHA, co-administered to gerbils with choline chloride, improves performance on various tests of learning and memory, including the four arm radial maze ($p < 0.001$); T-maze ($p< 0.021$); and Y-maze ($p < 0.001$); as described previously this treatment concurrently increases brain phosphatide levels ($p < 0.001$). Co-administering a uridine source [uridine monophosphate (UMP)] with DHA plus choline further enhances the increases in cognitive scores and phosphatide levels. The uridine probably acts by generating CTP, which can be limiting in phosphatide synthesis, and UTP, which activates P2Y receptors.

Introduction

Consumption of certain nutrients can influence brain function, even when the nutrients are not being used to correct malnutrition syndromes (1). Consumption of omega 3 fatty acids can improve cognition in animals or humans (2) or when given to developing (3), or aged rats (4), or to rats infused intracerebrally with amyloid beta (5). Aged rats consuming DHA for three weeks exhibited improved performance when tested on an 8-arm radial maze (4); dentate gyri basally contained reduced DHA levels, which were returned to normal after they consumed DHA-enriched diet for eight weeks (6). The DHA may have acted by increasing brain phosphatide levels, thus enhancing synaptic transmission (7; 8) and thereby improving cognition (9). Pups of dams fed a DHA-enriched diet exhibited accelerated neurologic development (10), similarly treated animals performed better on the Morris water maze as adults (11). DHA administered chronically to rats reversed the behavioral impairment caused by infusing beta-amyloid into the right ventricle (12), and rats fed a DHA-deficient diet had longer escape latencies than control animals when tested on the Morris water maze (11).

Raising circulating and brain DHA levels can enhance the levels ofphosphatides and of specific proteins in synaptic membrane (7), and the density of hippocampal dendritic spines (13); this increase in dendritic spines could enhance synaptic transmission in the hippocampus. Oral administration of UMP, a source of circulating and brain uridine (14), also promotes the synthesis of synaptic phosphatides and proteins (7), acting via its phosphorylated products UTP (which stimulates P2Y receptors [15]) and CTP (which is rate-limiting in phosphatide synthesis via the Kennedy cycle [16]). Moreover, the effects on phosphatide synthesis of giving UMP to animals receiving DHA and

choline tends to be substantially greater than the sum of the increases after either treatment alone (7).

Numerous investigations have described improvements in learning and memory among animals given a uridine source: local application of UMP into the hippocampus thirty minutes prior to acquisition of the Y-maze reportedly improved performance as examined forty-eight-hours later (17). Also, consuming a diet enriched with UMP reversed the memory impairment usually observed among rats reared under impoverished environmental conditions (18). Consumption of UMP along with DHA plus choline amplified the increases in hippocampal dendritic density produced by givingUMP or DHA plus choline alone (7, 13).

The ability of DHA, uridine, and choline to increase brain phosphatide synthesis derives from the kinetic properties of the enzymes involved: all are of low affinity, and thus unsaturated with their substrates. Choline is phosphorylated by choline kinase (CK) to form phosphocholine, uridine is phosphorylated by uridine–cytidine kinase (CDK) to form uridine triphosphate (UTP) (19), which is transformed to cytidine triphosphate (CTP) by the enzyme CTP synthetase (20); DHA is taken up into and acylated by fatty acyl-CoA synthetase, in neurons (21). Phosphocholine and CTP combine to form cytidine- 5-diphosphocholine (CDP-choline), which combines with diacylglycerol (DAG), preferentially that containing polyunsaturated fatty acids (PUFA) like DHA (21, 22) to form phosphatidylcholine (PC) (16). At normal plasma and brain levels (20), the synthesis of PC by choline kinase (CK) is enhanced in animals (23) or humans (24) given choline because CK is not saturated by choline (20). The conversion of uridine to UTP and CTP is enhanced in PC12 cells (15) and rodent brain (7) when the saturation of

uridine-cytidine kinase (UCK) (25, 26, 27, 28, 29, 30), has been increased by providing the pyrimidine (19). Plasma and brain CTP concentrations do not saturate CTP: phosphocholine cytidylyltransferase (CT) (31), or the uptake into brain (32). Giving animals DHA and choline plus uridine increases enzyme saturation by all three of these precursors, and thus maximally enhances the synthesis of PC and other phosphatides (7).

We examined the effects on cognitive functions of giving gerbils UMP, DHA or both, plus a choline-supplemented diet, and correlated those effects with the increases in their produced brain membrane phosphatides. In humans, uridine is the principal circulating pyrimidine; however, in rats, the principal circulating pyrimidine is cytidine, not uridine (33). Pyrimidine metabolism in gerbils more closely resembles that in humans, hence, we used Mongolian gerbils and not rats in this study. The gerbils received UMP and choline via the diet and DHA by gavage, for four weeks prior to cognitive testing on a four arm radial maze, T-maze or Y-maze.

Materials and Methods

Animals

Male gerbils (*Meriones unguiculatus*) (60-80 g) purchased from Charles River Laboratories, Wilmington, MA, were housed in pairs, in a climate-controlled area kept on a 12:12 light cycle (lights on at 7:00 h). An enriched environment was provided by placing toys in the cage, providing a fresh paper towel for shredding twice a week, and housing the animals in pairs. Each experiment was repeated at least 3 times and by at least 2 different experimenters who were blind to the treatments. Efforts were made to minimize animal suffering, according to NIH guidelines. Protocols were approved by the Massachusetts Institute of Technology Committee on Animal Care (MIT's Institutional Animal Care and Use Committee [IACUC]).

Treatments

Gerbils were allowed to eat 16% protein chow, or the same diet supplemented with 0.5% UMP (Harlan-Teklad, Madison, WI). All diets contained 0.1% choline chloride. Gerbils weighing 80g. typically consumed 4g. of food per day. In addition, gerbils were gavaged daily with either a vehicle solution containing 5% arabic gum in saline or the same vehicle supplemented with 300mg/kg DHA. At 3 months of age, gerbils consumed UMP by dietary supplementation and DHA by gavage for four weeks prior to behavioral training, and throughout all phases of behavioral testing.

4-Arm radial maze apparatus

The 4-arm radial maze apparatus consisted of a plastic square platform (15 cm x

15 cm), with 4 enclosed metal arms (10 cm x 37.5 cm), each with a small semi-
cylindrical trough (7.5 cm diameter x 2 cm deep) located 2.5 cm from the distal end 105.
for placing a food reward (.5 cm x .5 cm food pellet). The platform and arms were 106.
opaque (white) and 25 cm tall, without a top. The maze was located in a testing room
devoid of sound but with ample spatial cues including counters, chairs, etc. A 108.
constant level of ceiling illumination was provided throughout the study.

Radial maze training and behavioral measures

Food and water were available ad libitum until the day of experimental testing, at which point gerbils were first fasted for 17 hours overnight and then provided with food from 11AM to 6PM. Gerbils were handled daily for 4 days before testing to habituate them to routine contact. They were familiarized with the maze for an additional 4 days by placing food pellets throughout the arms and allowing 3 min for exploration. Gerbils received 1 trial/day, and all surfaces were sanitized with 10% ethanol followed by quatricide between trials. Arms were also rotated between each trial to ensure that gerbils did not follow each others' scent to locate the food pellet. Training consisted of placing a food pellet at the distal end of the same 2 arms for all trials. The gerbil was placed in the center of the maze and allowed 2 min to find the food pellets. Testing continued at the same time each day until gerbils had learned the task sufficiently well (>80% accuracy for 3 consecutive days).

Working memory errors were recorded whenever a gerbil re-entered an arm which contained a food pellet and which had previously been visited during a trial. Reference memory errors were recorded whenever a gerbil entered an arm that had

not contained a food pellet during previous trials. The time it took to find both food pellets and the path length chosen to obtain the pellets were also recorded.

T-maze apparatus

The T- maze apparatus consisted of a square platform (15 cm x 15 cm), with three arms (10 cm x 37.5 cm) in the shape of a "T", each with a small cylindrical trough 131. (7.5 130. cm diameter x 2 cm deep) located 2.5 cm from the distal end for placing a 132. food reward (.5 cm x .5 cm food pellet). The platform and arms are opaque (white) 133. and 25 cm tall. The maze was located in a testing room devoid of sound but with 134. ample spatial cues including counters, chairs, etc. A constant level of ceiling illumination was provided throughout the study.

T-maze training and behavioral measures

Food and water was available ad libitum until the day of testing. Gerbils consumed 0.5% UMP-supplemented diet or control diet were given 300mg/kg of 139. DHA (or its vehicle) daily by gavage at approximately three months of age, four 140. weeks prior to behavioral training. Gerbils were handled daily for four days the week prior to testing to habituate them to routine contact and were familiarized with 142. the maze for an additional four days by placing food pellets throughout the arms and 143. allowing three minutes for exploration. They were fasted for seventeen hours overnight, tested on the T-maze, and then provided with food from 11 a.m. to 6 p.m. 145. They continued to receive UMP and/or DHA throughout the remainder of training, 146. approximately nine weeks total. They received one trial/day for five weeks. Training

consisted of placing a food pellet at the distal end of one arm in the "hat" 148. of the T. The gerbils were placed at the end of the base of the T and allowed two 149. minutes to find the food pellet. All surfaces were sanitized with quatricide and 10% 150. ethanol between trials. Arms were also rotated between each trial to ensure that gerbils did not follow each others' scent to locate the food pellet.

Working memory errors were recorded whenever a gerbil re-entered an arm that contained a food pellet and after consuming the food pellet. Reference memory errors were recorded whenever a gerbil entered an arm that did not contain a food pellet during previous trials. The time required to find the food pellets and the path length used to obtain the pellets were also recorded.

Y-maze apparatus

The Y-maze apparatus consisted of a wooden square platform (25 cm x 25 cm), with one removable wall opening into the corner of a 120 ° triangular region (side length 60 cm). All walls were 25 cm tall without a top. Two hinged doors (10 cm x 15 cm), were located symmetrically (10 cm from edge) on the wall opposite the square platform. Behind one door was the gerbil's home cage, and behind the other door was an identical clean cage. The maze was located in a testing room devoid of sound but with ample spatial cues including counters, lamps, etc. A consistent level of ceiling illumination was provided throughout the study.

Y-maze training and behavioral measures

Animals were first handled daily for 4 days to habituate them to routine contact.

Food and water were available ad libitum. Gerbils began to eat UMP-supplemented 169. chow at 3 months of age, 2 weeks prior to behavioral training, and continued to do 170. so throughout the remainder of training. Animals were not fasted for this test. Gerbils underwent 3 trials per day, and all surfaces were sanitized with 10% ethanol 172. between trials. Cages were also rotated between each trial to ensure that gerbils did 173. not follow each others' scent to locate the food pellet. Training took place for 4 consecutive days, followed by 4 days of no training, and 4 days of training . Training consisted of placing a gerbil in the square platform for 5 sec, removing the removable wall, and allowing the gerbil 3 min to choose which door to enter. Animals were not able to re-enter the testing chamber after entering a door. Trials 178. followed the same procedure. The door chosen during each trial was recorded.

Rotarod apparatus and testing

The rotarod apparatus consisted of a 3.2-cm-diameter rod (RRAC-3002; O'Hara & Company, Tokyo, Japan). The rotarod test was performed according to the procedure described previously (34). Male gerbils, 5 months old, were used for motor behavioral tests. During the training period, gerbils were placed on the rotating rod starting at 4 rpm and gradually accelerated to 40 rpm at a rate of 0.15 rpm/s. The latency to fall (retention time) was measured with a cutoff time of 4 min. Gerbils were trained for 3 consecutive days, receiving four trials per day with a 1 h intertrial interval.

Sample Collection

Gerbil brains were obtained immediately following the conclusion of each behavioral test by CO_2 anesthesia followed by decapitation and immediate dissection of the whole brain. All brain tissue was weighed and homogenized in DI water such that each sample contained the same ratio of tissue to water; i.e., 20mg tissue:1mL water. Samples were stored at -80 C for further analysis.

Total DNA Assay

To determine the total DNA in each sample, previously described techniques (35) were used. Briefly, known standards were diluted 50 μg/mL in DNA buffer (50mM KPO4, 2mM EDTA, 250mM NaCl, pH=7.4); 10 μL of standards and homogenized tissue were placed in well plates (Falcon Micro Test 96-well Assay Plate, optilux Black). Hoechst solution was diluted to 1μg/μL in DNA buffer, and 200μL was added to standard- and sample-containing wells. Following a thirty minute incubation at RT and in the dark, the plate was read and analyzed on Thermo Labsystem Fluoroskan Ascent using Micro plate Manager Software at 450 nm.

Total Protein Assay

To determine the total protein in each sample, previously described techniques (35) were used. Homogenized brain tissues were compared to known BSA standards. Briefly, BSA standards and samples were added to well plates (clear Falcon Pro-Bind 96 well assay plate); CuSO4 solution was diluted 1:49 in bichinconinic acid and added to all standard- and sample-containing wells. Following a thirty-minute incubation at RT, the plate was read and analyzed on a 210. Bio Rad micro plate reader, model 550, using

Ascent Software at 450 nm.

Total Phospholipids Assay

Total phospholipid content was determined by comparing samples to potassium phosphate standards. Phosphatides were extracted using previously described methods (36): 1 mL homogenates were mixed with 3 mL of chloroform and methanol mixture (2:1 v/v) and vortexed for 30 seconds. After cooling on ice for 1 hour, the mixture was added to 1 mL deionized water, and then added to 3 mL of chloroform and methanol (2:1 v/v). After remaining at -4C for 18-20 hours, the mixture was separated by centrifugation at 3500 rpm for fifteen minutes at 4C; 100 μL aliquots of the bottom phase are dried in a Savant lyopholizer and then digested in 70% perchloric acid for 1.5 hours at 150 C. Phosphatides were measured as described previously (37): 300 μL of 15% ascorbic acid and 200 μL of 5% ammonium molybdate were added to samples and standards. These remained at RT for thirty minutes, and were then read at 450 nm on a Perkin-Elmer Lambda 3B 224. UV/VIS.spectrophotometer. The absorbency reading of each sample was compared 225. to the absorbency reading of the standards to determine the phospholipid content in samples. This value was adjusted according to the total DNA and protein determined previously.

Separation of Phospholipids

To determine the amount of individual phospholipids, previously described methods (38) were used. Digested samples were separated using thin layer chromatography (TLC); 30 μL of each sample was spotted onto Alltech silica gel G

channeled plates, placed in running buffer (30 mL chloroform, 34 mL ethanol, 30 mL triethylamine, and 8 mL water) for 1.5 hours, and visualized by spraying plates with petroleum ether containing 1,6-diphenyl-1,3,5-hexatriene and viewing under UV light. The bands corresponding to individual phospholipids were scraped, reconstituted in methanol, and dried overnight in a Savant lyopholizer. Following this initial separation step, samples were digested and assayed for phosporus as described for total phospholipids above.

Data analysis

For all tests comparing 2 groups, two-tailed t-tests were used. For comparisons involving more than one factor, or comparing more than 2 groups, factorial ANOVA 242. was used.

Results

Body wt.

Body weight did not differ between UMP-supplemented, DHA gavaged, and control groups (data not shown), indicating that gerbils probably were eating equivalent amounts of diet with or without UMP or DHA supplementation.

Effects of UMP and DHA Supplementation on Gerbils Performance on a Four Arm Radial Maze.

All groups were able to learn the four arm radial maze to some degree, showing a decrease in the number of errors recorded over time (figure 1A, B, C). Values are mean ± S.E.M, n=12. Reference memory errors were decreased by administration of either UMP or DHA, the largest decrease was observed by co-administering UMP and DHA; UMP [$F(1,12) = 5.721$, $p<.038$], DHA [$F(1,12) = 12.315$. $p< .042$, and UMP x DHA [$F(1,12) = 23.659$, $p< .001$] (figure 1A). Working memory errors were decreased by administration of either UMP or DHA, the largest decrease was observed by co-administering UMP and DHA; UMP [$F(1,12) = 7.236$, $p< .029$], DHA [$F(1,12) = 19.145$, $p < .035$, UMP x DHA [$F(1,12) = 17.329$, $p < .001$] (figure 1B). The total errors were decreased by administration of either UMP or DHA, the largest decrease was observed by co-administering UMP and DHA; UMP [$F(1,12) = 8.237$, $p<.022$], DHA [$F(1,12) = 9.658$, $p< .034$], UMP x DHA [$F(1,12) = 18.521$, $p<.001$) (figure 1C). These results indicate that long-term dietary treatment with UMP or oral DHA improves gerbils' spatial memory; their spatial memory is further enhanced when UMP and DHA are co-administered.

Effects of UMP and DHA Supplementation on Gerbils Performance on a T-Maze with a Delayed Memory Test.

All groups were able to learn the T- maze to some degree, showing a decrease in the number of errors recorded over time (figure 2A). Values are mean ± S.E.M, n=12. The number of sessions required for gerbils to reach the criterion to have successfully learned the maze were decreased by administration of either UMP or DHA, the largest decrease was observed by co-administering UMP and DHA; UMP [$F(1,12) = 5.764$, $p<.038$], DHA [$F(1,12) = 7.861$, $p < .024$, and UMP x DHA [$F(1,12) = 16.325$, $p< .017$] (figure 2A). Following a 24 hour delay, gerbils administered with either UMP or DHA were more likely to locate the correct arm; the gerbils most likely to locate the correct one were those administered both UMP and DHA; UMP [$F(1,12) = 7.365$, $p < .04$], DHA [$F(1,12) = 6.295$, $p < .036$, UMP x DHA [$F(1,12) = 18.263$, $p< .021$] (figure 2B). These results indicate that long-term dietary treatment with UMP or oral DHA improves gerbils' retention of spatial memory following a delay in testing; their retention of spatial memory was further enhanced when UMP and DHA were co-administered.

Effects of UMP and DHA Supplementation on Gerbils Performance on a Y-Maze with a Delayed Memory Test.

All groups were able to learn the Y- maze to some degree, showing an increase in the number of times gerbils chose the correct door to their home cage (figure 3), and indicated by a significant main effect of day (block of four training trials/day) ($P<.001$). Values are mean ± S.E.M, n=12. No other significant main effects were determined during acquisition of the task. Following a four day delay, gerbils administered either UMP or DHA were more likely to choose the correct door, the

group most likely to choose the correct door were the gerbils co-administered UMP and DHA; UMP [$F(1,12) = 8.326$, $p<..012$], DHA [$F(1,12) = 10.296$, $p < .009$, and UMP x DHA [$F(1,12) = 22.356$, $p< .001$] (figure 3). These results indicate that long-term dietary treatment with UMP or oral DHA improves gerbils' retention of spatial memory following an extended delay in testing; their retention of spatial memory was further enhanced when UMP and DHA were co-administered.

Effects of UMP and DHA Supplementation on Gerbils Performance on an Accelerating Rotarod Test

All groups were able to learn the rotarod task, showing an increase in the length of time they are able to remain on the accelerating rotarod (figure 4), and indicated by a significant main effect of day (block of four training trials/day) ($p<.02$). Values are mean ± S.E.M, n=12. No other significant main effects were determined ($p's > .05$), suggesting that a UMP-supplemented diet and/or oral DHA has little to no effect on motor activity.

Effects of a UMP- supplemented Diet alone or in Combination with DHA Administration on Brain Phosphatide Levels

Chronic consumption of UMP (0.5%) increased gerbils' brain PC and PE levels significantly, by 18%, and 33%, respectively (table 1). Administration of DHA (300 mg/kg) to gerbils consuming control diet increased gerbils' brain PC, PE, PS, and PI levels significantly, by 26%, 10%, 50%, and 53%, respectively. Among gerbils receiving both UMP and DHA, brain PC, PE, SM, PS, and PI levels rose significantly by 66%, 108%, 100%, 75%, and 94%, respectively. Total phospholipid levels were also

significantly increased in gerbils administered both UMP and DHA, by 32% (table 1). Two-way ANOVA revealed a significant effect of dietary UMP or oral DHA on brain PC, PE, PS, and PI levels (all $< .05$). Two way ANOVA also revealed a significant effect of co-administering dietary UMP and oral DHA on brain PC, PE, SM, PS, PI, and total phospholipids levels (all $P < .001$). Similar results were obtained when data were expressed per μg DNA (data not shown).

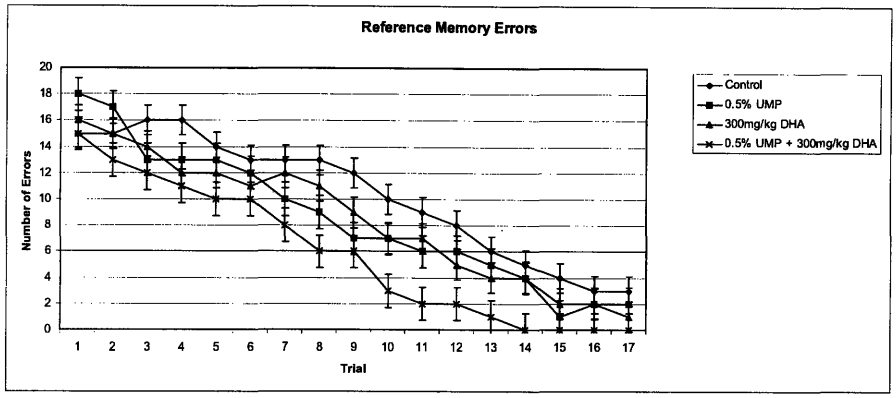
Table 1. Effects of giving UMP-supplemented diet (0.5%) and DHA (300 mg/kg) on phosphatide levels in whole brain samples.

Treatment	Total PL	PC	PE	SM	PS	PI
Control	343 ± 12	138 ± 11	58 ± 7	37 ± 8	24 ± 8	17 ± 7
0.5% UMP	364 ± 15	164 ± 12*	77 ± 10*	42 ± 5	32 ± 8	24 ± 7
300mg/kg DHA	411 ± 6	174 ± 9*	64 ± 9*	40 ± 9	36 ± 11*	26 ± 9*
0.5% UMP + 300mg/kg DHA	452 ± 18***	229 ± 15***	121 ± 13***	74 ± 10***	42 ± 12***	33 ± 4***

319. Gerbils were administered a UMP-containing (0.5%) diet, and received DHA (300
320. mg/kg) daily by gavage for 8 weeks. Values are mean ± S.E.M, n=12. Brains were
321. then obtained and their phosphatide levels determined as described in the text. Data
322. are presented as nmol/mg protein.
323. * $p<.05$ compared to control group
324. ** $p<.01$ compared to control group
325. *** $p<.001$ compared to control group

Figure 1

A.



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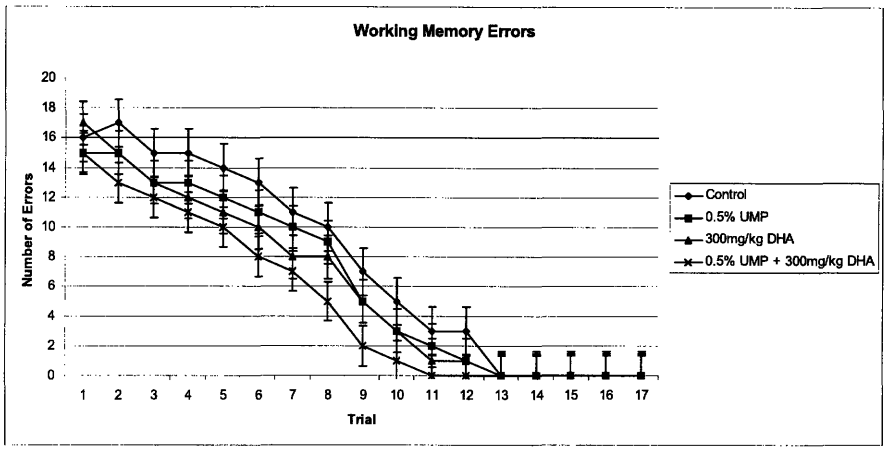

C.

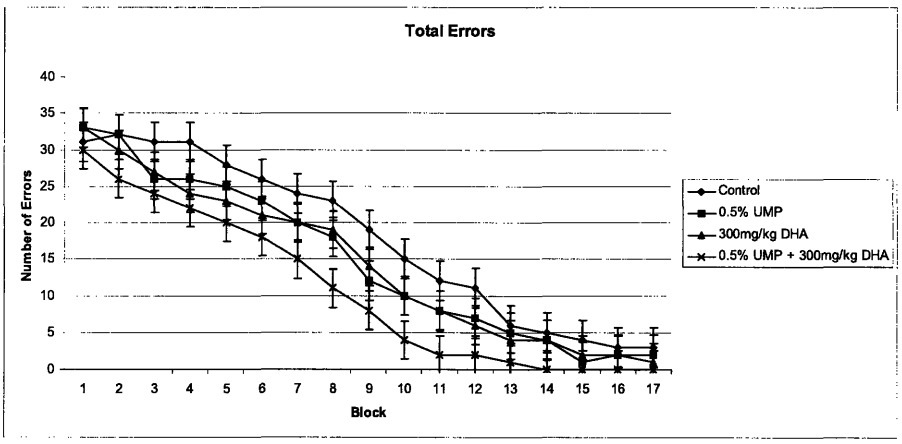

Figure 1. The effects of a UMP-supplemented diet and/or daily administration of DHA on acquisition of a four arm radial maze with two arms baited in gerbils. Values are mean ± S.E.M, n=12. A. Reference memory errors (UMP $p < .038$, DHA $p < .042$, UMP x DHA $p < .001$). B. Working memory errors (UMP $p < .029$, DHA $p < .035$, UMP x DHA $p < .001$). C. Total errors (UMP $p < .022$, DHA $p < .034$, UMP x DHA $p < .001$).

Figure 2

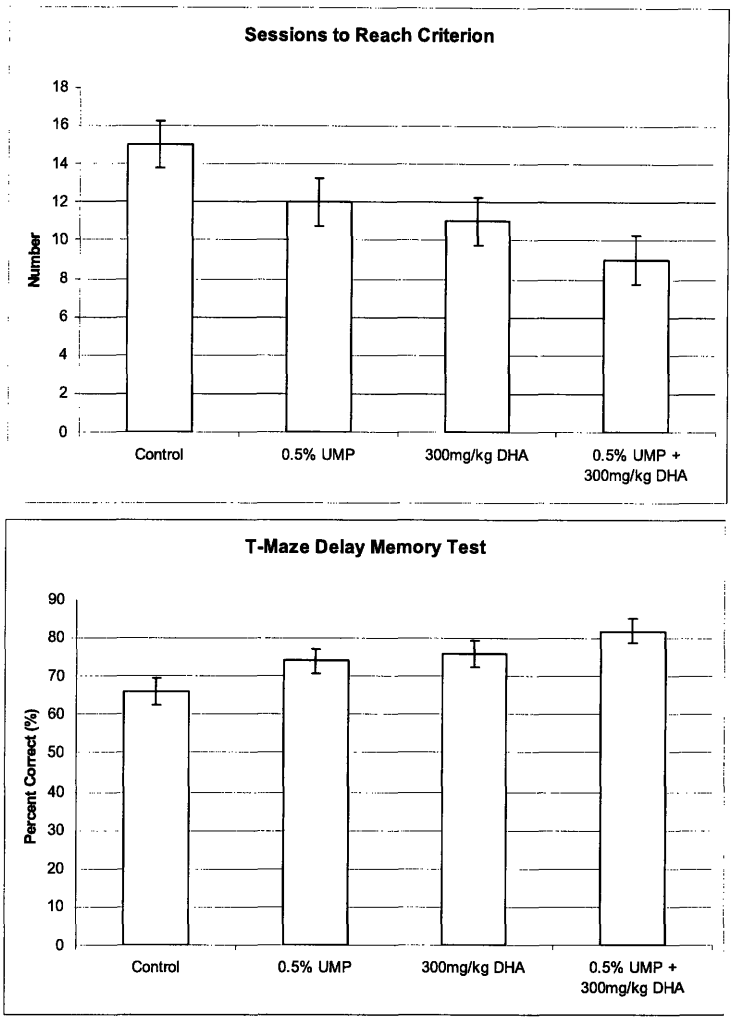

Figure 2. The effects of a UMP-supplemented diet and/or daily administration of DHA on acquisition of a T-maze with one arm baited in gerbils. Values are mean ± S.E.M, n=12. A. Acquisition of the task was affected sessions to reach criterion (UMP $p < .038$, DHA $p < .024$, UMP x DHA $p < .017$). B. Retention of the task was affected percent correct in a 24 hour delay memory test (UMP $p < .04$, DHA $p < .036$, UMP x DHA $p < .021$).

Figure 3

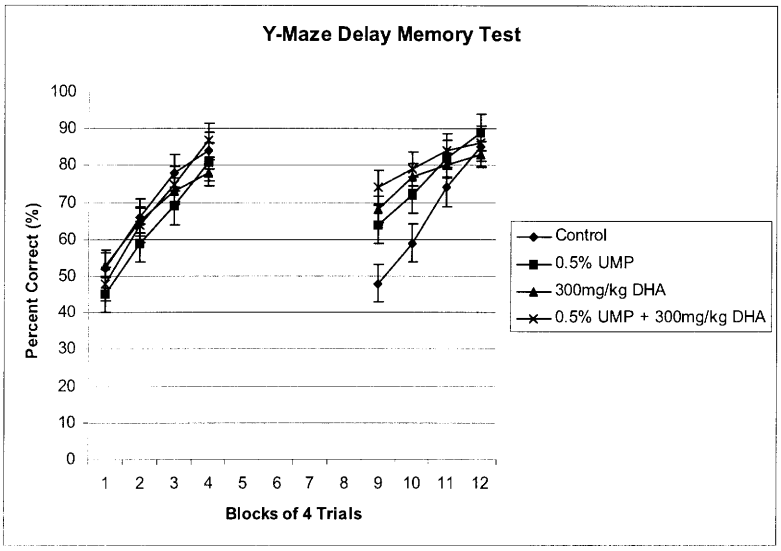

Figure 3. The effects of a UMP-supplemented diet and/or daily administration of DHA on acquisition of a Y-maze with a four day delay memory test. Values are mean ± S.E.M, n=12. Retention of the task was affected percent correct (UMP $p < .012$, DHA $p < .009$, UMP x DHA $p < .001$).

Figure 4

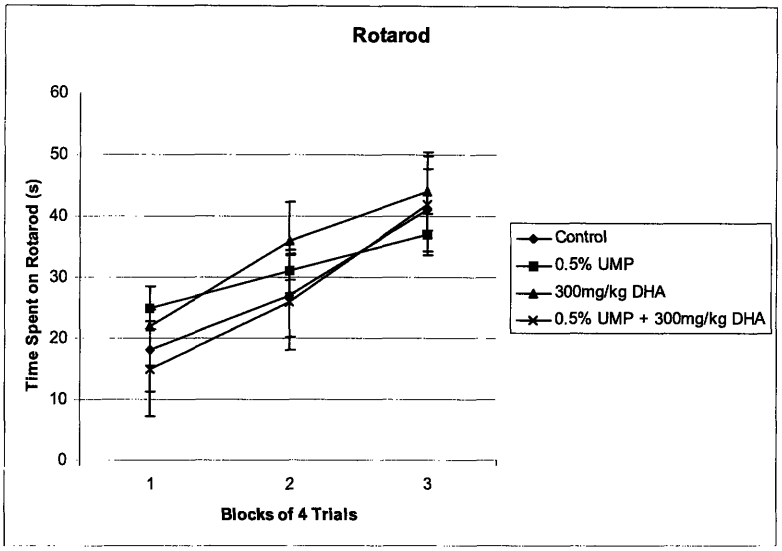

Figure 4. The effects of a UMP-supplemented diet and/or daily administration of DHA on an accelerating rotarod motor activity test. Values are mean ± S.E.M, n=12. The time spent on the rotarod was not affected (p's.> .05).

Discussion

These data show that oral administration to gerbils of either dietary UMP (0.5%) or gavaged DHA (300 mg/kg) causes gerbils to exhibit improved performance on various tests of cognition, including a four arm radial maze, T-maze, and Y- maze (figures 1, 2, 3). Co-administration of the UMP and DHA further enhance the improvements in performance on these tasks. Our data also confirm that administration of either UMP or DHA increases levels of brain phosphatides (PC and PE), concurrent with improving the cognitive behaviors. Moreover, co-administration of UMP and DHA increases levels of all of the other brain phospholipids measured (PC, PE, SM, PS, PI), and total phospholipid levels (table 1). The increases in the brain phosphatide levels correlated with improved performance on tests of cognition.

Previous studies have shown that consumption by rodents of a DHA-deficient diet decreases performance on tests of cognition (39), although not all of the effects described could be attributed to deficits in learning and memory. Rats fed a diet deficient in DHA exhibited impaired spatial learning in the Barnes circular maze (39), and impaired spatial learning and memory in the Morris water maze (11). Potential confounders were not usually evaluated, as noted by the authors of these papers, and could not be excluded as alternative explanations for many of the observations. Most behavioral tests measure a combination of performance and behavioral characteristics (40); the results from commonly used water and radial maze tests are highly dependent on locomotor ability and visual recognition of cues. Rodents consuming a DHA restricted diet can develop poor visual function (41), and also suffer from diminished synthesis of the saturated and monounsaturated fatty acids (42) needed as energy sources (43), and from high levels of

inflammation (44). Therefore, rodents consuming diets deficient in choline or DHA may experience adverse affects not related to learning and memory, which may influence their performance on tests of cognition. In the present study, all gerbils consumed diets that did not restrict their consumption of DHA, hence the likelihood that improvement in performance was based on locomotor ability or visual acuity was reduced.

Although some previous studies have shown that administration of DHA or UMP given alone can improve memory, this to our knowledge is the first study to demonstrate that co-administration of UMP further enhances the effects of DHA. This probably reflects the fact that, prior to the 2006 demonstration that the two compounds potentiate each others' effects on phosphatide synthesis (7), there was no compelling reason to examine their possible interaction in promoting cognition. DHA administration to rodents was previously shown to improve performance on tests of cognition that measure spatial learning such as the radial maze (3,4), Morris water maze (45), and brightness discrimination learning task (46). Chronic administration of UMP (0.1%) improved the hippocampal-dependent memory deficits associated with rearing rats in an impoverished environment (18). Results from the present study show that DHA and UMP apparently act in synergy to produce further improvements in learning acquisition and in retention of spatial memory.

In humans, administration of DHA can also improve performance on tests of cognition. DHA supplemented formulas fed to term infants (47) and pre-term infants (48, 49) resulted in improved performance in cognitive or behavioral tests, and on visual acuity (47). Infants of women who have higher levels of DHA in their breast milk have higher scores on the NBAS Range of State cluster score, suggesting that the DHA may maintain optimal arousal in infants (50). DHA supplementation of infant formula improved visual and cognitive

maturation during infancy, and visual and cognitive outcomes at 1 year (51) and 4 years of age (52). Results from the present study indicate that cognition in humans may be further improved if DHA and UMP are administered in combination.

Changes in brain phosphatide levels following administration of DHA and UMP may contribute to improved cognition in gerbils. Synthesis of the brainphosphatide phosphatidylcholine, the most abundant constituent of cellular membranes (53), is increased by consumption of the uridine source UMP (7). Plasma uridine crosses the blood-brain barrier (BBB) (54), and increases brain uridine levels (14). This uridine is converted, via UTP, to cytidine triphosphate (CTP), which combines with phosphocholine (formed by the phosphorylation of choline) to yield cytidine-5' diphosphate choline (CDP-choline), the immediate precursor of PC (55). The CDP-choline then combines with a diacylglycerol (DAG), preferentially one containing a polyunsaturated fatty acid (PUFA) moity such as DHA, to form PC (16). The UTP also acts as a ligand for P2Y receptors (15).

DHA and UMP supplementation increased phospholipid content and synaptic protein levels (7), and increased dendritic spine density in the gerbil hippocampus (13). In the present study, we confirmed that total phospholipid levels in the brain are increased with chronic consumption of DHA, and that co-administration with UMP further amplifies those effects (table 1). We also observed that the level of phospholipids in gerbil brain following 8 weeks of DHA-UMP-choline administration is significantly higher than that described previously following 4 weeks of treatment (7); implying that an asymptote has not yet been reached. The likely mechanism by which DHA increases brain PC content is by increasing the synthesis of the phosphatide, inasmuch as levels of PC's immediate precursor, CDP-

choline, are simultaneously depleted when DHA is administered (56).

Besides enhancing phosphatide synthesis, DHA but not analogs such as DPAn-6 (57) produces additional effects on brain that could contribute to its behavioral effect. It lowers levels of inflammation (58), has free radical scavenging capabilities (59), and affects brain neurotransmitter levels (60), among other actions. The actions of UMP may also, as mentioned above, be mediated in part through activation of P2Y receptors. Uridine and UTP activate P2Y receptors in PC12 cells, and can thereby increase turnover of inositolphosphate (IP) (15), and calcium release from intracellular stores (61); these cellular messengers also reportedly improve learning and memory in animals (62).

In summary, the present study demonstrates that increased consumption of either UMP or DHA plus choline increases brain phospholipid content and improves the acquisition and retention of spatial memory by gerbils. The largest increases in phospholipids and enhancements in memory occur when choline, DHA, and UMP are administered in combination.

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Chapter 2:

Chronic Administration of DHA and UMP Improves the Impaired Memory of Impoverished Rats

Sarah Holguin, Yi Huang, Jenny Liu, Richard Wurtman

Abstract

Living in an enriched environment (EC) during development enhances memory function in adulthood; living in an impoverished environment (IC) impairs memory function. Compounds previously demonstrated to improve memory among IC rats include CDP-choline and uridine mono phosphate (UMP). Brain phosphatidylcholine (PC) synthesis utilizes both the pyrimidine formed from CDP-choline or UMP, and the choline formed from CDP-choline. It also uses the polyunsaturated fatty acid (PUFA) DHA, a precursor for the diacylglycerol used in PC synthesis. DHA administration also improves cognition in young and aged rodents and humans. We examined the effects of co-administering DHA and UMP on hippocampal- and striatal- dependent forms of memory among rats exposed to EC or IC for 1 month starting at weaning. In the present study, we show that IC rats receiving either dietary UMP (0.5%) or gavaged DHA (300 mg/kg) exhibit improved performance on the hidden version of the Morris water maze (all $p<0.05$), a hippocampal-dependent task. Co-administration of UMP and DHA further enhances the IC rats' performance of this task ($p<0.001$). UMP plus DHA administration does not affect EC rats performance on the hidden version of the Morris water maze ($p>0.05$), nor the performance of IC or EC rats on the visible version of the Morris water maze (all $p>0.05$), a striatal-dependent task. We have also confirmed that co-administration of UMP and DHA to rats increases brain levels of the phosphatides PC, PE, SM, PS, PI, and total phospholipid levels (all $p<0.05$), and that animals reared in the enriched environment have brain PC, PS, and PI levels that are 57%, 25%, and

41% higher, respectively (all $p<0.01$) than those of animals reared in an IC environment. Total phospholipid levels were also significantly higher in EC control rats than in IC control rats by 25% ($p<0.01$). These results suggest that giving DHA plus UMP can ameliorate memory defecits associated with rearing under impoverished conditions, and that this effect may be mediated through enhanced synthesis of brain membranes.

Introduction

Changes in an animal's environment, and the ways in which that animal reacts to those changes, can have significant long-term effects on the brain and behavior [1]. Laboratory rodents are normally reared under standard conditions (SC), during which rats are housed individually, with few novel objects to interact with. Recently, rodents have been reared under enriched (EC) or impoverished conditions (IC) to determine differences in cognition acquired through increased or decreased environmental stimuli. Rats reared under EC are typically housed in larger cages, with more cage mates, and novel objects to interact with. Rodents reared under IC are housed in smaller cages, isolated from other rodents, and in a cage devoid of all unnecessary objects [2]. Rats exposed to EC conditions have improved memory compared to rats reared under SC [3]; rats exposed to IC conditions have impaired memory [4].

Enrichment enhances memory performance in various learning tasks. Enriched mice performed better on a water maze test of spatial memory [5, 6], and enriched rats performed better on a T-maze [7] EC enhanced spatial learning in adult and aged rats [8], and improved learning in rats with traumatic brain injury (TBI) [9]. The hippocampus, which is essential in learning and memory [1], is the brain region most profoundly effected by exposure to an enriched or impoverished environment [10].

Impoverishment impairs memory function in various learning tasks; some compounds can protect rodents from memory impairments. IC rats demonstrated deficits when tested on a T-maze [3], and Morris water maze [11]. Rats exposed

to IC have hippocampal-dependent memory deficits [4]. Compounds previously demonstrated to improve the memory of IC rats include CDP-choline and uridine mono-phosphate (UMP). Long-term dietary supplementation with either CDP-choline, a source of choline and cytidine (in rats) or uridine (in humans), prevented memory impairment in IC rats tested on a hippocampal- dependent spatial task [12, 13].

PC synthesis requires the use of three circulating compounds; choline, a pyrimidine such as uridine, and a polyunsaturated fatty acid (PUFA) such as docosahexaenoic acid (DHA) [14]. Furthermore, consumption of DHA, one of the precursors to PC, improves learning and memory when given during development [15], or to aged rats [16]. Chronic administration of either UMP or DHA increased brain phosphatide levels, the largest increase occurred when DHA and UMP were administered in combination [14].

DHA, uridine, and choline administration increase phosphatide synthesis because the enzymes involved are of low affinity and unsaturated by their substrates. Choline is phosphorylated by choline kinase (CK) to form phosphocholine, uridine is phosphorylated by uridine–cytidine kinase (CDK) to form uridine triphosphate (UTP) [17], which is transformed to cytidine triphosphate (CTP) by the enzyme CTP synthetase [18]. DHA is acylated by fatty acyl-CoA synthetase [19]. Phosphocholine and CTP combine to form cytidine-5′-diphosphocholine (CDP-choline), which combines with diacylglycerol (DAG) to form phosphatidylcholine (PC) [20]. DAG containing polyunsaturated fatty acids (PUFA) such as DHA is preferentially incorporated into PC [19, 21]. The

synthesis of phosphocholine by choline kinase (CK) is enhanced in animals [22] or humans [23] given choline because CK is not saturated by its substrate, choline at normal plasma and brain levels [18]. Uridine conversion to UTP and CTP is enhanced in PC12 cells [24] and rodent brain [14] when uridine-cytidine kinase (UCK) [25, 26, 27, 28, 29, 30] is saturated following pyrimidine administration [17]. Also, the concentrations of plasma and brain CTP do not saturate CTP: phosphocholine cytidylyltransferase (CT) [31], or its uptake into brain [32]. Giving animals DHA and choline plus uridine increases enzyme saturation by all three of these precursors, and thus maximally potentiates synthesis of PC and other phosphatides [14].

Increased brain phosphatide levels may enhance learning and memory [33]. We thus examined the effect of DHA and UMP on hippocampal- and striatal- dependent forms of memory among rats exposed to EC or IC for 1 month starting at weaning, using the hidden and visible versions of the Morris water maze. Subsequently, brain samples were analyzed for their phospholipid content and a correlation determined between increased brain phosphatides and improved performance on the water maze task.

Materials and Methods

Rats and Diet.

Male Sprague Dawley rats, 4 weeks of age, were purchased from Charles River Laboratories, Wilmington, MA, and were housed in a climate-controlled area kept on a 12:12 light cycle (lights on at 7:00 h). At this time, rats were matched according to body wt and assigned to either the IC or EC group. Subgroups of IC rats were given either control diet, 0.5% UMP supplemented chow, 300 mg/kg DHA daily by gavage, or 0.5% UMP and 300 mg/kg DHA daily by gavage. Subgroups of EC rats were also given either control diet, 0.5% UMP supplemented chow, 300 mg/kg DHA daily by gavage, or 0.5% UMP and 300 mg/kg DHA daily by gavage. Rats were weighed weekly to ensure that treated and untreated rats were eating equivalent amounts of food. Efforts were made to minimize animal suffering, according to NIH guidelines. Protocols were approved by the Committee on Animal Care [Massachusetts Institute of Technology's Institutional Animal Care and Use Committee (IACUC).]

Environmental Conditions

Rats were housed in the same rack in plastic cages with wire lids. Bedding and water were regularly changed, and rats had ad libitum access to food and water. EC rats were housed in groups of 2, in cages containing plastic toys (blocks, balls, PVC tubing, etc.). Toys were rotated between groups twice a week; new toys were introduced weekly. EC rats were exposed to a ''playroom'' measuring 5 ft x 5 ft every other day for 45 min. The "playroom" contained

several toys including plastic tubing, small balls, plastic boxes, wire brushes, and paper towels to shred. The IC rats were housed individually, without toys, and handled 3 times/week. IC rats were allowed to exercise 3 times/week for 15 min in an empty room measuring 5 feet x 5 feet, with only the experimenter present.

Water Maze Apparatus

The water maze was a galvanized circular tank, 6 feet in diameter and 2 feet in height. The tank was filled with water maintained at RT to a depth of 1 foot, and located in a dimly lit room containing several extramaze cues. Four starting positions were spaced around the perimeter of the tank to divide the pool into 4 equal quadrants. For the visible platform version of the water maze, a white flag was attached to the top of the submerged platform and protruded above the water surface. We mounted a video camera directly above the maze; this camera was linked to a computer with video tracking software to automatically record the escape latency (time to reach the platform), distance traveled (length of swim path taken to find the platform), and swim speed of all rats (HVS Image).

Behavioral Test

All behavioral training was carried out as described previously [12, 13] between 1400 and 1800 h, each experiment was repeated at least 3 times and by at least 2 different experimenters who were blind to the treatments. Briefly, rats were given 4 trials/day for 4 days to locate the hidden platform (1 cm below the water surface), which remained in the same position for all trials for individual

rats (within 1 of 4 quadrants). If a rat did not find the platform within 90 sec, it was guided to the escape platform by the experimenter. After mounting the platform, rats were allowed to remain on the platform for 20 sec. Following each trial, rats were removed from the maze and placed in a holding cage for a 30 sec intertrial interval. On the fifth day, rats were given a probe test; the platform was removed and the swim path and time spent searching in the quadrant of the pool that previously contained the platform were measured over 60 sec.

In the visible version of the Morris water maze, rats were given 4 trials/day for 4 days to locate the visible platform; the visible escape platform was placed in a different quadrant on each of the 4 trials. If a rat did not escape within 90 sec, it was manually guided to the escape platform by the experimenter. After mounting the platform, rats remained on the platform for 20 sec. Following each trial, rats were removed from the maze and placed in a holding cage for a 30 sec intertrial interval.

Rotarod apparatus and testing

The rotarod apparatus consisted of a 3.2-cm-diameter rod (RRAC-3002; O'Hara & Company, Tokyo, Japan). The rotarod test was performed according to the procedure described previously [34]. Rats were tested on the rotarod following the completion of all water maze testing. During the training period, rats were placed on the rod, and rotation started at 4 rpm and gradually accelerated to 40 rpm at a rate 0.15 rpm/s. The latency to fall (retention time) was measured with a cutoff time of 4 min. Rats were trained for 3 consecutive days,

receiving four trials per day with a 1 h intertrial interval.

Sample Collection

Rat brains were obtained immediately following the conclusion of each behavioral test by CO2 anesthesia followed by decapitation and immediate dissection of the whole brain. All brain tissue was weighed and homogenized in DI water such that each sample contained the same ratio of tissue to water; i.e., 20mg tissue:1mL water. Samples were stored at -80 C for further analysis.

Total DNA Assay

To determine the total DNA in each sample, previously described techniques [35] were used. The DNA in homogenized brain tissue was compared to standards. Briefly, known standards were diluted 50 μg/mL in DNA buffer (50mM KH2PO4, 2mM EDTA, 250mM NaCl, pH=7.4); 10 μL of standards and homogenized tissue were placed in well plates (Falcon MicroTest 96-well Assay Plate, optilux Black). Hoechst solution was diluted to 1μg/μL in DNA buffer, and 200μL was added to standard- and sample-containing wells. Following a thirty minute incubation at RT and in the dark, the plate was read and analyzed on Thermo Labsystem Fluoroskan Ascent using Ascent software, at excitation 355 nm./emission 460 nm.

Total Protein Assay

To determine the total protein in each sample, previously described

techniques [35] were used. Homogenized brain tissues were compared to known BSA standards. Briefly, BSA standards and samples were added to well plates (clear Falcon Pro-Bind 96 well assay plate); CuSO4 solution was diluted 1:49 in bichinconinic acid and added to all standard- and sample-containing wells. Following a thirty-minute incubation at RT, the plate was read and analyzed on a Bio Rad microplate reader, model 550, using Microplate Manager software, at 550 nm.

Total Phospholipid Assay

Total phospholipid content was determined by comparing samples to potassium phosphate standards. Phosphatides were extracted using previously described methods [36]: 1 mL homogenates were mixed with 3 mL of chloroform and methanol mixture (2:1 v/v) and vortexed for 30 seconds. After cooling on ice for 1 hour, the mixture was added to 1 mL deionized water, and then added to 3 mL of chloroform and methanol (2:1 v/v). After remaining at -4C for 18-20 hours, the mixture was separated by centrifugation at 3500 rpm for fifteen minutes at 4C; 100 μL aliquots of the bottom phase was dried in a savant lyopholizer and then digested in 70% perchloric acid for 1.5 hours at 150 C. Phosphatides were measured as described previously [37]: 300 μL of 15% ascorbic acid and 200 μL of 5% ammonium molybdate was added to samples and standards. These remained at RT for thirty minutes, and were then read on a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer, at 810 nm. The absorbency reading of each sample was compared to the absorbency reading of

the standards to determine the phospholipid content in samples. This value was adjusted according to the total DNA and protein determined previously.

Phospholipid Separation

To determine the amount of individual phospholipids, previously described methods [38] were used. Digested samples were separated using thin layer chromatography (TLC); 30 μL of each sample was spotted onto Alltech silica gel G channeled plates, placed in running buffer (30 mL chloroform, 34 mL ethanol, 30 mL triethylamine, and 8 mL water) for 1.5 hours, and visualized by spraying plates with petroleum ether containing 1,6-diphenyl-1,3,5-hexatriene and viewing under UV light. The bands corresponding to individual phospholipids were scraped, reconstituted in methanol, and dried overnight in a savant lyopholizer. Following this initial separation step, procedures were the same as those described for the total phospholipid assay above.

Data analysis

For all tests comparing 2 groups, two-tailed t-tests were used. For comparisons involving more than one factor, or comparing more than 2 groups, factorial ANOVA was used.

Results

Body wt.

Body weight did not differ between UMP, DHA, and environmental conditions (data not shown), indicating that rats were eating equivalent amounts of diet regardless of dietary supplementation or environmental conditions.

Effects of UMP, DHA, and Environmental Conditions on Rats Performance on a Hippocampal-dependent Water Maze Test

All groups were able to learn the hidden version of the Morris water maze to some degree, showing a decrease in the number of errors recorded over time (figure 1A, B) and indicated by a significant main effect of day (block of 4 training trials per day) ($p<0.001$). Values are mean ± S.E.M, n=12 for each group. A main effect of environment ($p<0.001$) and an environment x diet interaction were also observed ($p<0.05$). IC rats treated with either UMP, DHA, or UMP and DHA had decreased escape latencies compared to the IC control rats, the largest decrease was observed in IC rats co-administered UMP and DHA; UMP [$F(1,12) = 7.563$, $p<.042$], DHA [$F(1,12) = 13.253$. $p< .035$], and UMP x DHA [$F(1,12) = 27.635$, $p< .001$] (figure 1A). EC rats treated with either UMP, DHA, or UMP and DHA did not acquire the task at a faster rate than did IC control rats ($p>0.05$) (figure 1B). These results indicate that long-term dietary treatment with UMP or oral DHA improves the spatial memory deficits normally associated with impoverished conditions; spatial memory is further improved when UMP and DHA are co-administered.

The results of the 60 sec probe test indicated that rats spent more time in

the quadrant that originally contained the platform, suggesting that all experimental groups used spatial cues to locate the hidden platform (figure 1C).The percentage of swim time in the 4 quadrants during the probe test was affected by environment ($p<0.042$), quadrant ($p<0.001$), and diet x environment interaction ($p<0.05$). IC rats treated with UMP, DHA, or UMP and DHA spent more time in the correct quadrant than did the IC control rats, UMP [$F(1,12) = 7.845$, $p<0.025$], DHA [$F(1,12) = 12.374$, $p< 0.021$], UMP x DHA [$F(1,12) = 22.428$, $p<0.001$) (figure 1C). EC rats treated with UMP, DHA, or UMP and DHA did not spend more time in the correct quadrant than EC control rats ($p>0.05$).

Effects of UMP, DHA, and Environmental Conditions on Rats Performance on a Striatal-dependent Water Maze Test

All groups were able to learn the visible version of the Morris water maze to some degree, showing a decrease in the number of errors recorded over time (figure 2A, B) and a main effect of day (block of 4 training trials per day) ($p<0.001$). Values are mean ± S.E.M, n=12. No other significant effects were determined, suggesting that environment, UMP, and DHA have no effect on striatal-dependent learning and memory.

Effects of UMP and DHA Supplementation on Rats Performance on an Accelerating Rotarod Test

All groups were able to learn the rotarod task, showing an increase in the length of time they are able to remain on the accelerating rotarod (figure 3), and indicated by a significant main effect of day (block of four training trials/day)

($p<.015$). Values are mean ± S.E.M, n=12. No other significant effects were determined ($p's > .05$), suggesting that environment, UMP, and DHA have no effect on motor activity.

Effects of a UMP- supplemented Diet alone or in Combination with DHA Administration on Brain Phosphatide Levels in EC and IC Rats

Chronic consumption of UMP (0.5%) increased IC rats' brain PC, PS, and PI levels significantly, by 23%, 28%, 46%, and 27%, respectively (Table 1). Administration of DHA (300 mg/kg) to IC rats consuming control diet increased rats' brain PC, SM, PS, and PI levels significantly, by 26%, 49%, 71%, and 59%, respectively. Among IC rats receiving both UMP and DHA, brain PC, PE, SM, PS, and PI levels rose significantly by 60%, 97%, 86%, 138%, and 100%, respectively. Total phospholipids levels were also significantly increased in IC rats administered DHA by 19%, and in IC rats administered both UMP and DHA, by 29% (table 1). Two-way ANOVA revealed a significant effect of dietary UMP or oral DHA on IC rats' brain PC, PE, PS, and PI levels (all $p< .05$). Two-way ANOVA also revealed a significant effect administering DHA ($p<.05$) or co-administering dietary UMP and oral DHA on IC rats brain total phospholipids levels ($p< .001$). Similar results were obtained when data were expressed per μg DNA (data not shown).

Administration of DHA (300 mg/kg) to EC rats consuming control diet increased rats' brain SM, PS, and PI levels significantly, by 66%, 30%, and 19%, respectively (table 1). Among EC rats receiving both UMP and DHA, brain PC, PE, SM, PS, and PI levels rose significantly by 18%, 46%, 100%, 107%, and

55%, respectively. Total phospholipids levels were significantly in EC rats co-administered UMP and DHA ($p<0.05$), but were not significantly increased in EC rats administered either UMP or DHA, or the combination of UMP and DHA (all $p>0.05$) (table 1). Two-way ANOVA revealed a significant effect of oral DHA on EC rats' brain SM, PS, and PI levels (all $p< .05$). Two-way ANOVA also revealed a significant effect of dietary UMP and oral DHA on EC rats' brain PC, PE, PS, and PI levels (all $p< .05$). Similar results were obtained when data were expressed per μg DNA (data not shown).

An enriched environment increased rats' brain PC, PS, and PI levels by 57%, 25%, and 41%, respectively. Total phospholipid levels were also significantly increased in EC control rats compared to IC control rats by 25%. Two-way ANOVA revealed a significant effect of enriched environment on EC rats' brain PC, PS, and PI levels (all $p< 0.01$). Two-way ANOVA also revealed a significant effect of enriched environment on EC control rats compared to IC control rats ($p<.01$).

Table 1. Effects of giving UMP-supplemented diet (0.5%) and DHA (300 mg/kg) on phosphatide levels in whole brain samples.

Treatment	Total PL (nmol/mg protein)	PC	PE	SM	PS	PI
IC-Control	420 ± 9	168 ± 6	74 ± 7	49 ± 8	24 ± 4	22 ± 3
IC-0.5% UMP	442 ± 12	206 ± 8	95 ± 9*	52 ± 6	35 ± 5***	28 ± 5*
IC-300mg/kg DHA	501 ± 7*	211 ± 11*	78 ± 6	73 ± 5***	41 ± 5***	35 ± 5***
IC-0.5% UMP + 300mg/kg DHA	543 ± 15*	269 ± 9***	146 ± 10***	91 ± 6***	57 ± 6***	44 ± 4***
EC-Control	524 ± 11a	223 ± 8aaa	88 ± 9	47 ± 5	30 ± 5a	31 ± 6aa
EC-0.5% UMP	529 ± 9	212 ± 12	98 ± 11	49 ± 7	31 ± 4	28 ± 4
EC-300mg/kg DHA	539 ± 14	227 ± 7	77 ± 5	78 ± 8***	39 ± 6**	37 ± 7*
EC-0.5% UMP + 300mg/kg DHA	542 ± 12*	263 ± 11***	129 ± 8	94 ± 5	62 ± 4*	48 ± 9***

IC and EC rats were administered a UMP-containing (0.5%) diet, and received DHA (300 mg/kg) daily by gavage for 6 weeks. Values are mean ± S.E.M, n=12. Brains were then obtained and their phosphatides levels determined as described in the text. Data are presented as nmol/mg protein.

* $p<.05$ compared to control group

** $p<.01$ compared to control group

*** $p<.001$ compared to control group

a $p<0.05$ EC control compared to IC control

aa $p<0.01$ EC control compared to IC control

aaa $p<0.001$ EC control compared to IC control

Discussion

These data show that IC rats administered either dietary UMP (0.5%) or oral DHA (300 mg/kg) have improved performance on the Morris water maze, a hippocampal-dependent task. Co-administration of UMP and DHA further enhances the IC rats' improved performance on this task. We have also confirmed that co-administration of UMP and DHA increases the level of brain PC, PE, SM, PS, PI, and total phospholipid levels in rats. The increase in the rats' brain phosphatide levels occurs in correlation with their improved performance on tests of cognition demonstrating enhanced learning acquisition and retention of spatial memory.

There are many models of cognitive impairment; we chose to use environmental impoverishment because rats reared under IC produces cognitive deficits [3, 4] that can be prevented by long-term dietary supplementation with either CDP-choline or UMP [12, 13]. The cellular mechanisms associated with impaired cognition in IC rats are similar to the mechanisms following damage or degeneration in the central nervous system [39]. Thus, compounds that are able to improve the memory of IC rats may also benefit patients with neuronal damage [40].

We propose that UMP and DHA protect against cognitive impairment in IC rats by increasing the formation of membrane, and possibly slowing the degradation of membrane in the case of EC rats; thereby promoting neurotransmission. Rats reared under IC display reduced phosphatide levels in the brain (table 1), and reduced glutamatergic hippocampal transmission [3].

UMP and DHA administration increase the synthesis of phosphatides in the brain [14], and increase the density of dendrites in the hippocampus [41], which utilizes more synaptic membrane. DHA administration reversed the decline in GluR2 and NR2B glutamate receptor subunits, thereby improving glutamatergic transmission in the hippocampus [42]. In support of this interpretation, we confirmed Wurtman R. et al., 2006 findings that administration of UMP and DHA increases brain phosphatide levels (Table 1) and demonstrated that IC reared rats treated with UMP plus DHA protected rats from memory impairment (Figure 1).

UMP and DHA may protect the brains of IC reared animals by restoring neuronal function to levels normally observed in EC brains. Rats exposed to IC conditions have decreased brain weight and size following 300 days in IC [43]; DHA deficiency decreases brain weight and size, while DHA administration increases brain weight and size [44]. IC reared rats have decreased neurogenesis [45] and synaptogenesis [46]; DHA promotes neurite outgrowth in hippocampal neurons [47] and uridine promotes neurite outgrowth in PC12 cells [24]. DHA supplementation increased brain-derived neurotrophic factor (BDNF) levels in rats [48]; consuming a diet deficient in DHA decreased brain BDNF levels [49]. BDNF expression induces neurogenesis in the dentate gyrus of the hippocampus [50].

There are many similarities between the neural effects of EC and of DHA or UMP plus DHA administration. EC rodents have improved spatial memory [8]; while administration of DHA improves cognitive impairment in mouse models of mild cognitive dysfunction, brain lesions, and AD [51]. EC rats have

increased cell survival in C57BL/6 learning impaired mice [52], while DHA increases cell survival in retinal photoreceptors [53]. EC rodents have increased (nerve growth factor) NGF expression [54]; likewise DHA administation increases expression of NGF [55]. EC rodents have increased BDNF mRNA expression [8]; BDNF modulates synapsin 1 levels during learning [56]. Administration of UMP plus DHA increases synapsin 1 levels in gerbil brain [14]. EC rodents have increased release of acetylcholine [57]; likewise DHA supplementation increases the release of potassium-evoked acetylcholine [58]; UMP supplementation increases the release of acetylcholine [59].

In summary, the present study demonstrates that increased consumption of either UMP or DHA plus choline increases brain phospholipid content and prevents memory impairment in IC reared rats. The largest increase in phospholipids and protection from memory impairments occurs when choline, DHA, and UMP are administered in combination. EC is implicated as a possible course of treatment to prevent memory impairment in several diseases, including traumatic brain injury (TBI) [40, 60], prenatal hypoxia and alcohol [61], epilepsy [62], stroke [63], Huntington's Disease [64, 65], and depression [66]. Co-administration of DHA and UMP may aid in the treatment of these patients as well.

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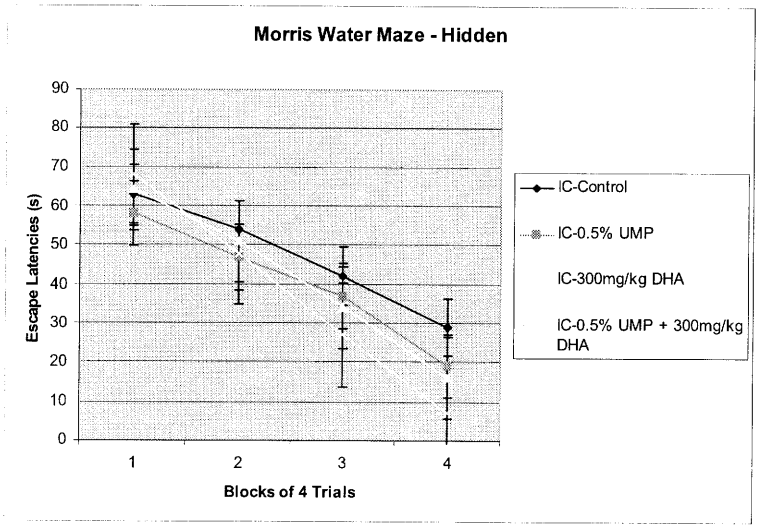
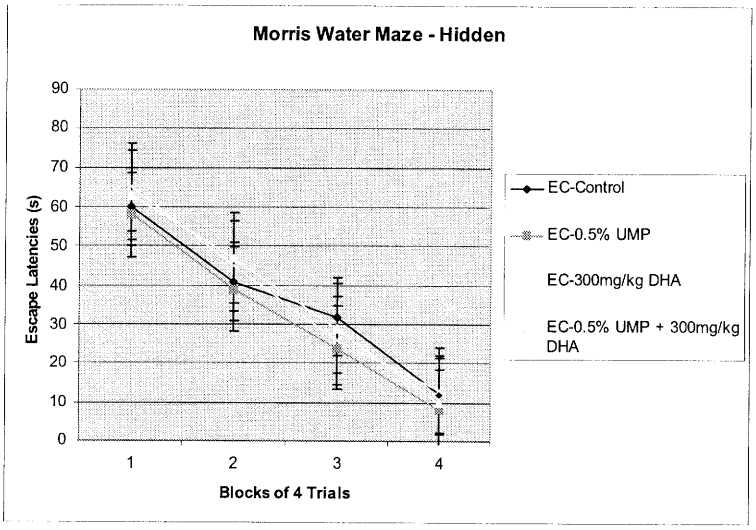
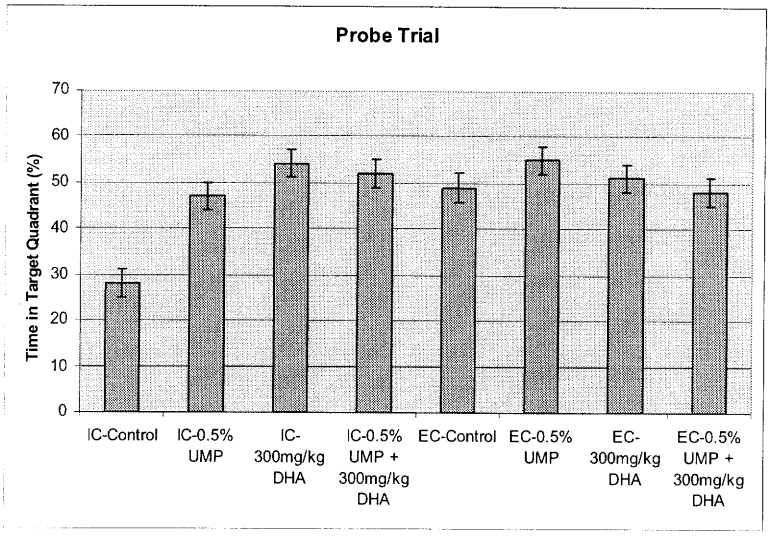
Figure 1


Morris Water Maze - Hidden
Escape Latencies (s)
90
80
70
60
50
40
30
20
10
0
1
2
3
4
Blocks of 4 Trials
IC-Control
IC-0.5% UMP
IC-300mg/kg DHA
IC-0.5% UMP + 300mg/kg DHA

A.


Morris Water Maze - Hidden
Escape Latencies (s)
90
80
70
60
50
40
30
20
10
0
1
2
3
4
Blocks of 4 Trials
EC-Control
EC-0.5% UMP
EC-300mg/kg DHA
EC-0.5% UMP + 300mg/kg DHA

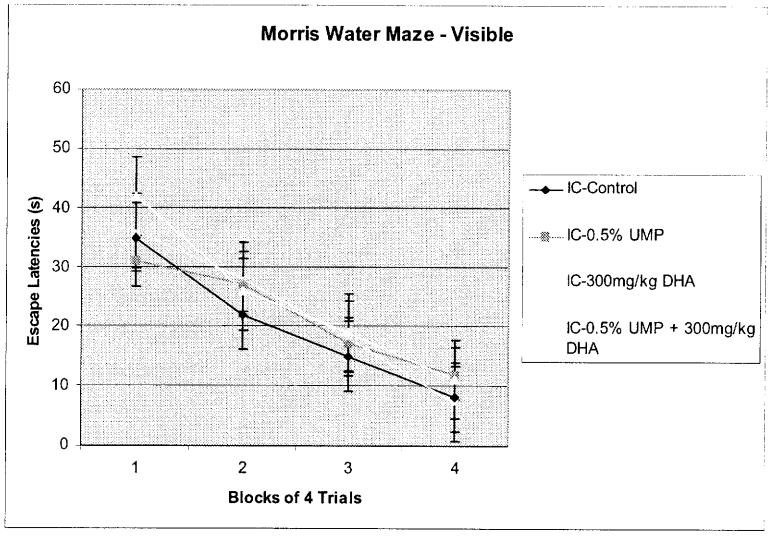
B.



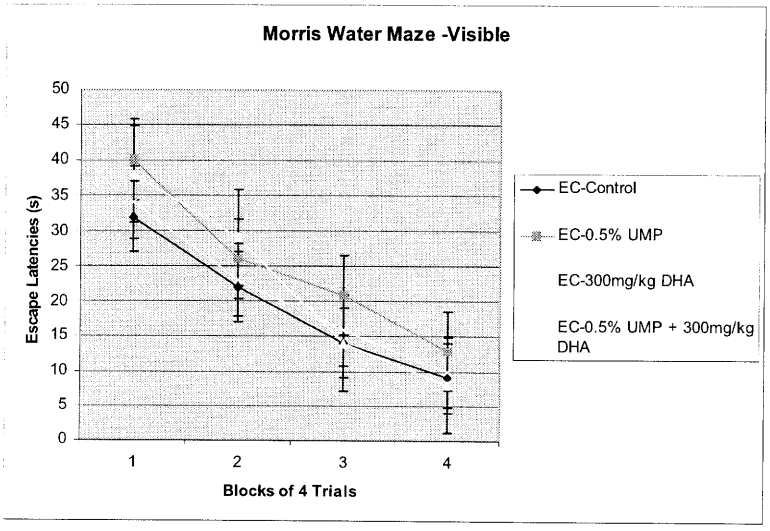
C.

Figure 1. The effects of environment, UMP, and DHA administration on memory for a hippocampal-dependent hidden platform water maze in rats reared under EC or IC conditions for 1 month immediately postweaning. Values are means ± SEM, n=12. A. IC rats administered UMP, DHA, or UMP and DHA had decreased escape latencies compared to the IC control rats (all $p < 0.05$). B. EC rats administered UMP, DHA, or UMP and DHA did not have decreased escape latencies compared to the EC control rats (all $p > 0.05$) C. The 60 sec probe test was affected by environment ($p < 0.042$), quadrant ($p < 0.001$), and diet x environment interaction ($p < 0.05$).

Figure 2


Morris Water Maze - Visible
Escape Latencies (s)
60
50
40
30
20
10
0
1
2
3
4
Blocks of 4 Trials
IC-Control
IC-0.5% UMP
IC-300mg/kg DHA
IC-0.5% UMP + 300mg/kg DHA

A.


Morris Water Maze -Visible
Escape Latencies (s)
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
Blocks of 4 Trials
EC-Control
EC-0.5% UMP
EC-300mg/kg DHA
EC-0.5% UMP + 300mg/kg DHA

B.

Figure 2. The effects of environment, UMP, and DHA administration on memory for a striatal-dependent visible platform water maze in rats reared under EC or IC conditions for 1 month immediately postweaning. Values are means ± SEM, n=12. A. IC rats administered UMP, DHA, or UMP and DHA did not have decreased escape latencies compared to the IC control rats (all p> 0.05). B. EC rats administered UMP, DHA, or UMP and DHA did not have decreased escape latencies compared to the EC control rats (all p>0.05).

Figure 3

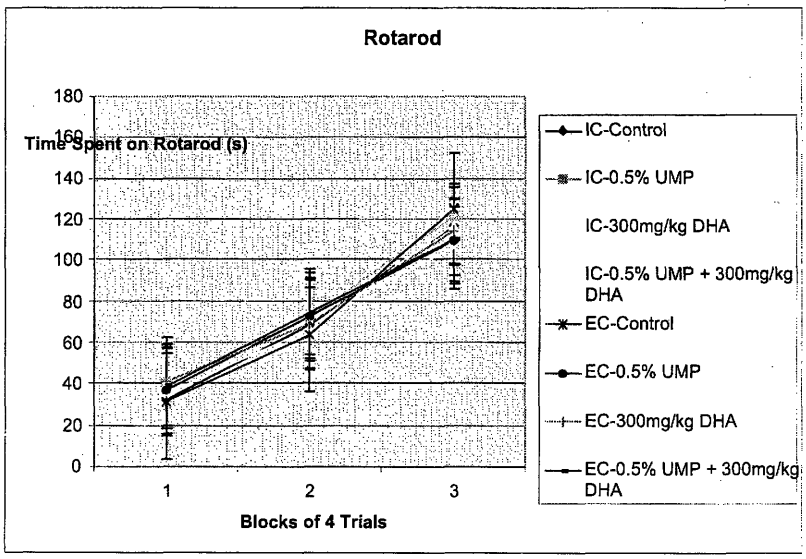


Figure 3. The effects of a UMP-supplemented diet and/or daily administration of DHA on IC and EC rats tested on an accelerating rotarod motor activity test. Values are mean ± S.E.M, n=12. The time spent on the rotarod was not affected (p's.> .05).

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Conclusion

As described in the two manuscripts, I have shown that increasing brain phospholipid levels by administering the phosphatide precursors UMP plus DHA also enhances certain cognitive behaviors:. Gerbils consuming UMP and/or DHA perform better on the radial arm maze, T-maze, and Y-maze than control, untreated gerbils (Manuscript 1). Moreover, giving UMP plus DHA also protects impoverished rats from the cognitive decline associated with living in an impoverished environment (Manuscript 2). The increases in brain phospholipid levels, in both gerbils and rats, caused by administering these compounds correlate with the improvements in behavior. (Manuscript 1, 2).

Although I did not measure the numbers of hippocampal dendritic spines or the levels of synaptic proteins in my test animals, abundant published evidence indicates that treatment with UMP plus DHA increases both (1, 2). . Therefore, I suggest that by increasing phospholipid levels in brain, UMP plus DHA may increase synaptic membrane, and the size and number of functional synapses, thus enhancing synaptic transmission and contributing to the enhanced cognition observed in these studies.

Alzheimer's brains are deficient in DHA (3), and in synapses. Hence supplementation with DHA could conceivably delay the progression of this disease, particularly if given along with a uridine source like UMP. This treatment may also prove useful in treating other neurological disorders associated with the loss of synapses; e.g. stroke, cerebral palsy, Parkinson's disease, brain injury.

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