

Dietary uridine enhances the improvement in learning and memory produced by administering DHA to gerbils

Sarah Holguin, Joseph Martinez, Camille Chow, and Richard Wurtman¹

Massachusetts Institute of Technology, Department of Brain and Cognitive Sciences, Cambridge, Massachusetts, USA

ABSTRACT This study examined the effects on cognitive behaviors of giving normal adult gerbils three compounds, normally in the circulation, which interact to increase brain phosphatides, synaptic proteins, dendritic spines, and neurotransmitter release. Animals received supplemental uridine (as its monophosphate, UMP; 0.5%) and choline (0.1%) *via* the diet, and docosahexaenoic acid (DHA; 300 mg/kg/day) by gavage, for 4 wk, and then throughout the subsequent period of behavioral training and testing. As shown previously, giving all three compounds caused highly significant ($P < 0.001$) increases in total brain phospholipids and in each major phosphatide; giving DHA or UMP (plus choline) produced smaller increases in some of the phosphatides. DHA plus choline improved performance on the four-arm radial maze, T-maze, and Y-maze tests; coadministering UMP further enhanced these increases. (Uridine probably acts by generating both CTP, which can be limiting in phosphatide synthesis, and UTP, which activates P2Y receptors coupled to neurite outgrowth and protein synthesis. All three compounds also act by enhancing the substrate-saturation of phosphatide-synthesizing enzymes.) These findings demonstrate that a treatment that increases synaptic membrane content can enhance cognitive functions in normal animals.—Holguin, S., Martinez, J., Chow, C., Wurtman, R. Dietary uridine enhances the improvement in learning and memory produced by administering DHA to gerbils. *FASEB J.* 22, 000–000 (2008)

Key Words: choline • omega-3 fatty acids • pyrimidines • behavior

CONSUMPTION OF CERTAIN NUTRIENTS can influence brain function, even when the nutrients are not being used to correct malnutrition syndromes (1). Supplemental DHA, an omega-3 fatty acid, reportedly can improve cognition in humans (2) and in developing (3) or aged rats (4), or rats infused intracerebrally with amyloid beta (5). This treatment also enhances the levels of membrane phosphatides and of specific proteins in synaptic membrane (6) and the density of hippocampal dendritic spines (7); it thus may enhance synaptic transmission in the hippocampus and else-

where. Oral administration of uridine monophosphate (UMP), a source of circulating and brain uridine (8), also promotes the synthesis of synaptic phosphatides and proteins (6), acting *via* its phosphorylated products uridine triphosphate (UTP) (which stimulates P2Y receptors; ref. 9) and cytidine triphosphate (CTP) (which is rate limiting in phosphatide synthesis *via* the Kennedy cycle; 10). Moreover, the effects on phosphatide synthesis of giving UMP to animals also receiving DHA and choline tend to be substantially greater than the sum of the increases observed after either treatment alone (6). Local application of UMP into the hippocampus 30 min prior to acquisition of the Y maze reportedly improved performance, as examined 48 h later (11). A UMP-enriched diet can reverse the memory impairments observed among rats reared under impoverished environmental conditions (12). Consumption of UMP along with DHA plus choline amplifies the increases in hippocampal dendritic density produced by giving choline plus DHA alone (6, 7).

The ability of DHA, uridine, and choline to increase brain phosphatide synthesis derives from the kinetic properties of the Kennedy cycle enzymes and those that produce CTP from uridine: 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 (UCK) to form UTP (13), which is transformed to CTP by the enzyme CTP synthetase (14); and DHA is taken up into neurons and acylated by fatty acyl-CoA synthetase, ultimately yielding DHA-containing diacylglycerol (15). Phosphocholine and CTP combine to form cytidine-5-diphosphocholine (CDP-choline), which then combines with diacylglycerol (DAG), preferentially that containing omega-3 fatty acids like DHA (15, 16) to form the phosphatidylcholine (PC) (10). At normal plasma and brain levels (14), the synthesis of phosphocholine, and then PC, by CK is enhanced in animals (17) or humans (18) given choline because CK is not saturated by choline (14). Similarly, the conver-

¹ Correspondence: Massachusetts Institute of Technology, 43 Vassar St., 46–5023, Cambridge, MA 02139, USA. E-mail: dick@mit.edu

doi: 10.1096/fj.08-112425

sion of uridine to UTP and CTP is enhanced in PC12 cells (9) and rodent brain (6) when the saturation of UCK (19, 20, 21, 22, 23, 24) has been increased by providing the pyrimidine (13). Normal brain CTP concentrations are insufficient to saturate CTP: phosphocholine cytidyltransferase (CT) (25), and raising available levels of DAG (26), particularly that containing DHA, also enhances its combination with CDP-choline to form PC. The phosphatide phosphatidyl ethanolamine (PE) is synthesized by the Kennedy cycle, and thus is also dependent on uridine and DHA levels. Phosphatidylserine (PS) production is indirectly dependent on these precursors, as it is formed by base-exchange from PC or PE (6).

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 the animals' brain phosphatide levels. Because uridine is the principal circulating pyrimidine in humans and gerbils but not rats, we used normal gerbils for this study. The gerbils received UMP and choline *via* the diet, and DHA by daily gavage, for 4 wk before cognitive testing on a four-arm radial maze, T maze, or Y maze, and during subsequent periods of behavioral training and testing. The radial-arm maze and T maze provide good measures in rodents of spatial learning and memory, using long fasting times and food as motivation; the radial maze allows more choices than the T maze and is more difficult to complete; the Y maze is a simple two-choice task for measuring spatial learning and memory among rodents.

MATERIALS AND METHODS

Animals

Male gerbils (*Meriones unguiculatus*) (60–80 g) purchased from Charles River Laboratories (Wilmington, MA, USA) were housed in pairs, in a climate-controlled area kept on a 12:12-h light-dark cycle (lights on at 7 AM). 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 masked to the treatments. In addition, different gerbils were used for each behavioral test to eliminate possible effects of preexposure to different mazes or of prior handling. Efforts were made to minimize animal suffering, according to National Institutes of Health guidelines. Protocols were approved by the Massachusetts Institute of Technology Committee on Animal Care (MIT's institutional animal care and use committee).

Treatments

Gerbils were allowed to eat a 16% protein chow containing 0.1% choline chloride (as described in ref. 6), or this diet supplemented with 0.5% UMP (provided by Numico Research, Wageningen, The Netherlands). The diets were prepared by Harlan-Teklad (Madison, WI, USA). Animals weighing 80 g typically consumed 4 g 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 300 mg/kg DHA (purchased from Nu-ChekPrep, Inc., Elysian, MN, USA) The DHA was not presented *via* the diet because it is readily autooxidized and is sensitive to light. Consumption of the control and UMP-containing diets, and administration of the DHA, were initiated when the animals were 3 months of age, and continued throughout all subsequent phases of training and testing. Behavioral training was initiated 4 wk after the onset of UMP and DHA administration.

Four-arm radial maze apparatus

The four-arm radial maze apparatus consisted of a plastic square platform (15×15 cm), with four enclosed plastic arms (10×37.5 cm), each with a small semicylindrical trough (7.5 cm diameter; 2 cm deep) located 2.5 cm from the distal end for placing a food reward (0.5×0.5 cm food pellet). The platform and arms were 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 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 deprived of food for 17 h overnight and then provided with food from 11 AM to 6 PM. The animals were handled daily for 4 days before testing to habituate them to routine contact, and then familiarized with the maze for an additional 4 days by placing food pellets throughout the arms and allowing 3 min for exploration. Gerbils underwent 1 trial/day, and all surfaces were sanitized between trials in this and other experiments, using 10% ethanol followed by quatricide. Arms were also rotated between each trial to ensure that gerbils did not follow each other's 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 animal 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 animals had learned the task sufficiently well (>80% accuracy for 3 consecutive days).

Working memory errors were recorded whenever a gerbil reentered 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 (15×15 cm), with three arms (10×37.5 cm) in the shape of a T, each with a small cylindrical trough (7.5 cm diameter; 2 cm deep) located 2.5 cm from the distal end for placing a food reward (0.5×0.5 cm food pellet). The platform and arms were opaque (white) and 25 cm tall. The maze was located in a testing room devoid of sound but with 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 were available *ad libitum* until the day of testing. Gerbils were handled daily for 4 days during the week

prior to testing in order to habituate them to routine contact and were familiarized with the maze for an additional 4 days by placing food pellets throughout the arms and allowing 3 min for exploration. They were then deprived of food for 17 h overnight, tested on the T maze, and provided with food from 11 A.M. to 6 P.M. They continued to receive UMP, DHA, both, or neither, throughout the remainder of training period, and were trained on the maze for 1 trial/day for 5 wk. Training consisted of placing a food pellet at the distal end of one arm in the "hat" of the T. The gerbils were then placed at the end of the base of the T and allowed 2 min to find the food pellet. Arms were also rotated between each trial to ensure that gerbils did not follow each other's scent to locate the food pellet.

Working memory errors were recorded whenever a gerbil reentered an arm that contained a food pellet, or one in which food pellet had already been consumed. 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 (25×25 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 (10×15 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 handled daily for 4 days to habituate them to routine contact; (food and water were available *ad libitum*), and the animals were not deprived of food for this test. Gerbils underwent 3 trials per day; as above, cages were rotated between each trial to ensure that gerbils did not follow each other's 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 s, 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 followed the same procedure. The door chosen during each trial was recorded.

Rotarod apparatus and testing

Male gerbils, 5 mo old, were used for this motor behavioral test. 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 a procedure described previously (27). 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 4 trials/day with a 1 h intertrial interval.

Sample collection

Gerbil brains were obtained immediately following the conclusion of each behavioral test. Animals were anesthetized

with CO₂, then decapitated, and their brains were removed and dissected. Brain tissue was weighed and homogenized in distilled water such that each sample contained the same ratio of tissue to water; *i.e.*, 20 mg tissue:1 ml water. Samples were stored at -80 C for further analysis.

Total DNA assay

Total DNA in each sample was measured as described previously (28). Briefly, known standards were diluted to 50 µg/ml with DNA buffer (50 mM KPO₄, 2 mM EDTA, 250 mM NaCl, pH 7.4); 10 µl samples of a standard or homogenized tissue extract were placed in well plates (Falcon Micro Test 96-well assay plate, optilux black; Becton Dickinson, Franklin Lakes, NJ, USA). Hoechst solution (Molecular Probes, Inc., Eugene, OR, USA) was diluted to 1 µg/µl in DNA buffer, and 200 µl was added to standard- and sample-containing wells. Following a 30-min incubation at room temperature, and in the dark, the plates were read and analyzed on Thermo Lab-system Fluoroskan Ascent using Microplate Manager software at 450 nm (Thermo Fisher Scientific, Waltham, MA, USA).

Total protein assay

The total protein in each sample was also measured using previously described techniques (28). Levels in homogenized brain tissues were compared with those in known bovine serum albumin (BSA) standards. Briefly, standards and samples were added to well plates (clear Falcon Pro-Bind 96-well assay plate; Becton Dickinson) and CuSO₄ solution diluted 1:49 in bichinonic acid was added to all standard- and sample-containing wells. Following a 30-min incubation at room temperature, the plate was read and analyzed on a Bio-Rad microplate reader, model 550 (Bio-Rad, Richmond, CA, USA), using Ascent software at 450 nm.

Total phospholipid assay

Phospholipid contents were determined by measuring phosphorus and by comparing levels in samples with those in potassium phosphate standards. The compounds were extracted using previously described methods (29): 1 ml of homogenates was mixed with 3 ml of a chloroform-methanol mixture (2:1 v/v) and vortexed for 30 s. After this mixture cooled on ice for 1 h, 1 ml of deionized water and then 3 ml of chloroform and methanol (2:1 v/v) were added. After remaining at 4 C for 18–20 h, the mixture was separated by centrifugation at 3500 rpm for 15 min at 4 C, and 100-µl aliquots of the bottom phase were dried in a Savant lyophilizer (Savant Instruments, Farmingdale, NY, USA) and then digested in 70% perchloric acid for 1.5 h at 150 C. To determine the amounts of individual phospholipids (30) in digested brain samples, the phospholipids were first separated using thin-layer chromatography (TLC). Thirty microliters of each sample was spotted onto an Alltech silica gel G-channelled plate (Alltech, Lexington, KY, USA), placed in running buffer (30 ml chloroform, 34 ml ethanol, 30 ml triethylamine, and 8 ml water) for 1.5 h, and visualized by spraying the plate with petroleum ether containing 1,6-diphenyl-1,3,5-hexatriene; relevant peaks were then viewed under UV light. Bands corresponding to each phospholipid were scraped, reconstituted in methanol, and dried overnight in a Savant lyophilizer. Following this initial separation step, samples were digested and assayed for phosphorus, as described above for total phospholipids. Individual phosphatides were measured as described previously (31): 300 µl of 15% ascorbic acid and 200 µl of 5% ammonium molybdate was added to samples and standards. These remained at room

temperature for 30 min and were then read at 450 nm using a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer (Perkin-Elmer, Waltham, MA, USA). The absorbance reading of each sample was compared with that of its standards. The calculated phospholipid contents were adjusted according to total DNA and protein levels, as determined above.

Data analysis

For all tests comparing two groups, 2-tailed Student's *t* tests were used. For comparisons involving more than one factor, or comparing more than two groups, factorial 2-way ANOVAs followed by Tukey tests were used.

RESULTS

Body weight

Body weights 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 gerbil performance on a four-arm radial maze

All groups were able to learn the four-arm radial maze task, showing decreases in the numbers of errors recorded over time (**Fig. 1**; values are means \pm SE; $n=12$). Reference memory errors were decreased by administering either UMP or DHA, but largest decreases were observed when both had been given [UMP: $F(1,12)=5.721$, $P<0.038$; DHA: $F(1,12)=12.315$, $P<0.042$; UMP \times DHA: $F(1,12)=23.659$, $P<0.001$] (**Fig. 1A**). Working memory errors were decreased by administering either UMP or DHA, but especially when both had been given [UMP: $F(1,12)=7.236$, $P<0.029$; DHA: $F(1,12)=19.145$, $P<0.035$; UMP \times DHA: $F(1,12)=17.329$, $P<0.001$] (**Fig. 1B**). The total number of errors decreased by administration of either UMP or DHA, but the largest decreases followed the administration of both [UMP: $F(1,12)=8.237$, $P<0.022$; DHA: $F(1,12)=9.658$, $P<0.034$; UMP \times DHA: $F(1,12)=18.521$, $P<0.001$] (**Fig. 1C**). These findings indicate that long-term dietary treatment with UMP or oral DHA improves gerbils' spatial memory and that spatial memory, like brain phosphatide synthesis, is further enhanced when both are given.

Effects of UMP and DHA supplementation on gerbil performance on a T maze with a delayed memory test

All groups were able to learn the T-maze task, showing decreases in the numbers of errors recorded over time (**Fig. 2A**; values are means \pm SE; $n=12$.) The number of sessions required for gerbils to reach the criterion for successful learning of the maze was decreased by administration of either UMP or DHA, with the largest decrease being observed by their coadministration

[UMP: $F(1,12)=5.764$, $P<0.038$; DHA: $F(1,12)=7.861$, $P<0.024$; UMP \times DHA: $F(1,12)=16.325$, $P<0.017$] (**Fig. 2A**). Following a 24-h delay, gerbils that had received either UMP or DHA were more likely to locate the correct arm, with those most likely to do so being those that had received both UMP and DHA [UMP: $F(1,12)=7.365$, $P<0.04$; DHA: $F(1,12)=6.295$, $P<0.036$; UMP \times DHA: $F(1,12)=18.263$, $P<0.021$] (**Fig. 2B**). These findings indicate that long-term dietary treatment with UMP or oral DHA improves gerbils' retention of spatial memory following a delay in testing and that the retention of spatial memory is further enhanced if both UMP and DHA are administered.

Effects of UMP and DHA supplementation on gerbil performance on a Y maze with a delayed memory test

All groups were able to learn the Y-maze task, showing increases in the number of times the gerbils chose the correct door to their home cage (**Fig. 3**), as indicated by a significant main effect of day (block of 4 training trials/day; $P<0.001$; values are means \pm SE; $n=12$.) No other significant main effects were observed during acquisition of the task. Following a 4-day delay, gerbils that had received either UMP or DHA were more likely to choose the correct door, and the group most likely to choose correctly was that treated with both UMP and DHA [UMP: $F(1,12)=8.326$, $P<0.012$; DHA: $F(1,12)=10.296$, $P<0.009$; UMP \times DHA: $F(1,12)=22.356$, $P<0.001$] (**Fig. 3**). These findings indicate that long-term dietary treatment with UMP or oral DHA improves gerbils' retention of spatial memory following an extended delay in testing and that retention of spatial memory is further enhanced if both UMP and DHA are administered.

Effects of UMP and DHA supplementation on gerbil performance on an accelerating rotarod test

All groups were able to learn the rotarod task, showing increases in the length of time they were able to remain on the accelerating rotarod (**Fig. 4**), as indicated by a significant main effect of day (block of 4 training trials/day; $P<0.02$; values are means \pm SE; $n=12$.) No other significant main effects were observed ($P>0.05$), 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 PC and PE levels in the gerbils' brains 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 phosphatidylinositol (PI) levels significantly, by 26, 10, 50, and 53%, respectively. Among gerbils receiving both UMP and DHA, brain PC, PE, sphingomyelin (SM), PS, and PI

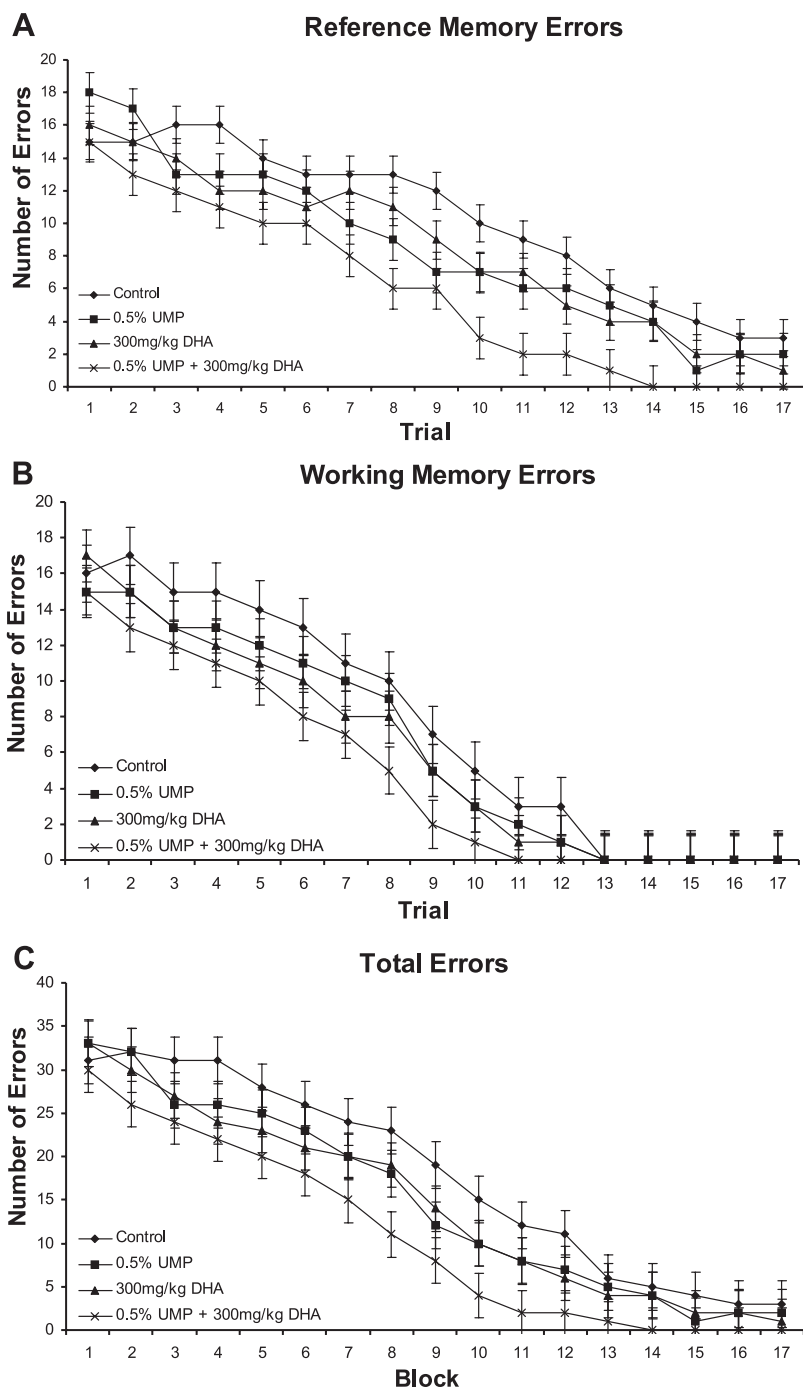


Figure 1. The effects of a UMP-supplemented diet and/or daily administration of DHA on acquisition of a four-arm radial maze with two arms baited in gerbils. Values are means \pm SE; $n = 12$. *A*) Reference memory errors (UMP, $P < 0.038$; DHA, $P < 0.042$; UMP \times DHA, $P < 0.001$). *B*) Working memory errors (UMP, $P < 0.029$; DHA, $P < 0.035$; UMP \times DHA, $P < 0.001$). *C*) Total errors (UMP, $P < 0.022$; DHA, $P < 0.034$; UMP \times DHA, $P < 0.001$).

levels rose significantly to a considerably greater extent, that is, by 66, 108, 100, 75, and 94%, respectively. Total phospholipid levels were also significantly increased among gerbils receiving both UMP and DHA, by 32% (Table 1) but not by UMP or DHA alone. Two-way ANOVA revealed a significant effect of dietary UMP or oral DHA on brain PC, PE, PS, and PI levels (all < 0.05). Two-way ANOVA also revealed a significant effect of coadministering dietary UMP and oral DHA on brain PC, PE, SM, PS, PI, and on total phospholipids levels (all $P < 0.001$). Similar results were obtained when data were expressed per microgram DNA (data not shown).

DISCUSSION

These data show that oral administration to gerbils of three compounds normally present in the circulation—UMP, DHA, and choline—can significantly improve cognitive performance. Administration of either UMP (0.5%) or DHA (300 mg/kg) to animals consuming a choline-containing diet significantly increased test scores on the four-arm radial maze, T-maze, and Y-maze tasks (Figs. 1–3), whereas coadministration of UMP and DHA further enhanced these improvements. Performance on the rotarod task, a noncognitive behavior, was not affected (Fig. 4). As observed previously, these

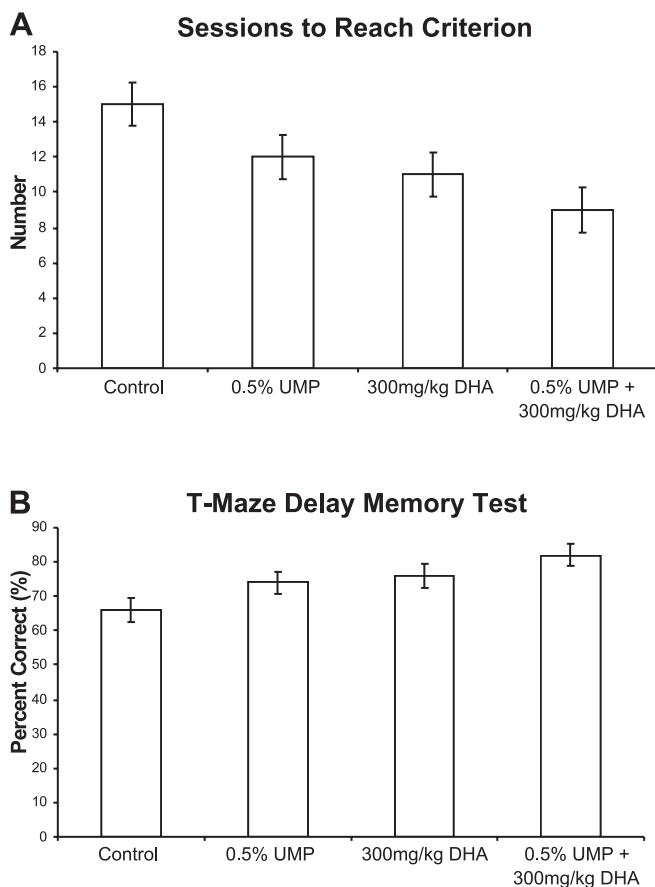


Figure 2. The effects of a UMP-supplemented diet and/or daily administration of DHA on acquisition of a T maze with one arm baited in gerbils. Values are means \pm SE; $n = 12$. *A*) Acquisition of the task was affected (sessions to reach criterion: UMP, $P < 0.038$; DHA, $P < 0.024$; UMP \times DHA, $P < 0.017$). *B*) Retention of the task was affected (percentage correct in a 24-h delay memory test: UMP, $P < 0.04$; DHA, $P < 0.036$; UMP \times DHA, $P < 0.021$).

animals also exhibited increases in membrane phosphatide levels (Table 1), and, in general, the increase in phosphatides observed in each test group correlated with its improvement in cognitive performance. A previous study in behavioral brain research demonstrated that treatment with the phosphatide precursors could restore cognitive performance that had been diminished by rearing rats in an environmentally impoverished environment (32). The present study shows that the treatment also improves performance in animals with normal cognitive function.

Previous studies have shown that consumption by rodents of a DHA-deficient diet decreases performance on tests of cognition (33), 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 (33), and impaired spatial learning and memory in the Morris water maze (34). Potential confounders were not usually evaluated, as noted by the authors of these reports, and could not be excluded as alternative explanations for many of the observations. Most behavioral tests measure a

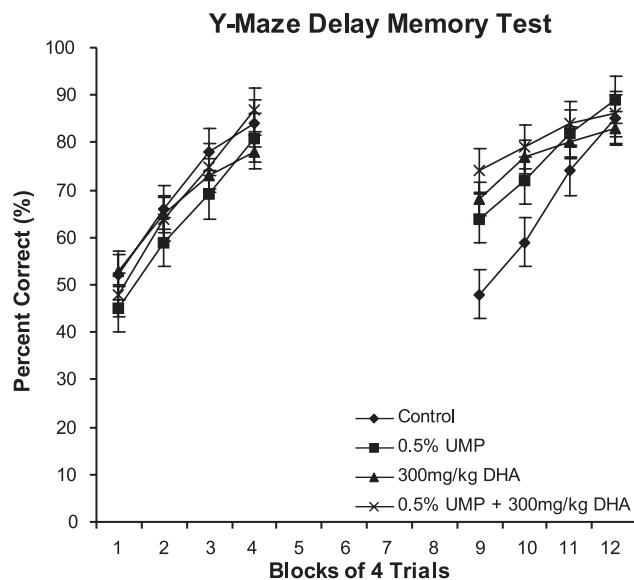


Figure 3. The effects of a UMP-supplemented diet and/or daily administration of DHA on acquisition of a Y maze with a 4-day delay memory test. Values are means \pm SE; $n = 12$. Retention of the task was affected (percentage correct: UMP, $P < 0.012$; DHA, $P < 0.009$; UMP \times DHA, $P < 0.001$).

combination of performance and behavioral characteristics (35); 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 (36) and can also be affected by diminished synthesis of saturated and monounsaturated fatty acids (37) needed as energy sources (38), and by high levels of inflammation (39). Therefore, rodents consuming diets deficient in choline

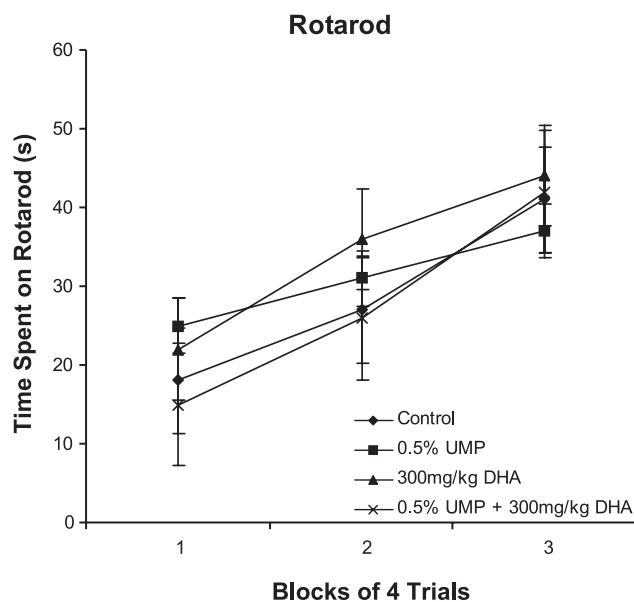


Figure 4. The effects of a UMP-supplemented diet and/or daily administration of DHA on an accelerating rotarod motor activity test. Values are means \pm SE; $n = 12$. The time spent on the rotarod was not affected by any treatment ($P > 0.05$).

Table 1. *Effects of UMP-supplemented diet and DHA 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
300 mg/kg DHA	411 ± 6	174 ± 9*	64 ± 9*	40 ± 9	36 ± 11*	26 ± 9*
0.5% UMP + 300 mg/kg DHA	452 ± 18***	229 ± 15***	121 ± 13***	74 ± 10***	42 ± 12***	33 ± 4***

Gerbils were fed a diet containing 0.5% UMP and were treated with 300 mg/kg DHA daily by gavage, both or neither for 8 wk. Brains were then obtained and their phosphatide levels were determined as described in the text. Data are presented as nmol/mg protein. PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol. Values are means ± SE, $n = 12$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control group.

or DHA may experience adverse effects not related to learning and memory, which may influence their performance on tests of cognition. In the present study, all gerbils consumed diets containing nutritionally adequate quantities of omega-3 fatty acids; hence, the likelihood that improvement in performance was based on increased locomotor ability or visual acuity was reduced.

Although some previous studies have shown that administration of DHA or UMP alone can improve memory, this to our knowledge is the first study to demonstrate that coadministration 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 and synaptic membrane synthesis (6), 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 (40), and brightness discrimination learning task (41). Chronic administration of UMP (0.1%) improved the hippocampal-dependent memory deficits associated with rearing rats in an impoverished environment (12). Results from the present study show that DHA and UMP apparently act in synergy to improve learning acquisition and retention of spatial memory, just as they do in promoting synaptic membrane and dendritic spine formation (7).

In humans, administration of DHA reportedly improves performance on tests of cognition. Feeding DHA-supplemented formulas to term infants (42) and preterm infants (43, 44) improved their performance on cognitive or behavioral tests, as well as on tests of visual acuity (42). (All of these diets and those discussed below also contained choline.) Infants of women whose breast milk contains high levels of DHA reportedly had higher scores on the National Behavior Assessment Scale Range of State cluster score, suggesting that DHA may maintain optimal arousal in infants (45). DHA supplementation of infant formula improved visual and cognitive maturation during infancy, and visual and cognitive scores at 1 yr (46) and 4 yr of age (47). Results from the present study indicate that cognition in humans may be further improved if DHA and UMP are administered in combination, and should be studied further in humans.

The changes in brain phosphatide levels that we

observed in gerbils given DHA and UMP presumably contributed to their improved cognition. Synthesis of the brain phosphatide PC, the most abundant constituent of cellular membranes (48), is increased by consumption of the uridine source UMP (6), and especially by giving UMP plus DHA. Plasma uridine crosses the blood-brain barrier (49), and sequentially increases brain uridine (8), UTP, and CTP levels, as well as those of CDP-choline, the immediate precursor of PC (25). The CDP choline then combines with a DAG, preferentially one containing a polyunsaturated fatty acid (PUFA) moiety such as DHA, to form PC (10). The UTP formed in brain from uridine acts as a ligand to activate P2Y receptors (9) that mediate neuronal differentiation and neurite outgrowth (9).

Besides increasing brain phosphatides, giving gerbils or rats DHA plus UMP also selectively increases levels of synaptic proteins (*e.g.*, synapsin-1, PSD-95, MGLUR-1, syntaxin-3) (6), and dendritic spine density in the gerbil hippocampus (7). The increases in brain phospholipids that we observed in gerbils receiving DHA plus UMP for 8–9 wk (Table 1) were significantly greater than those described previously following 4 wk of treatment (6), 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 (50), *i.e.*, and not simply slowing its degradation.

Besides enhancing phosphatide synthesis, DHA, but not analogs such as DPAn-6 (51), produces additional effects on brain tissue that could contribute to its behavioral effects. It suppresses inflammation (52), scavenges free radicals (53), and affects brain neurotransmitter levels (54), among other actions. Similarly, uridine and UTP, by activating P2Y receptors, can increase inositol phosphate turnover (9) and calcium release from intracellular stores (55); these cellular messengers also reportedly improve learning and memory in animals (56).

In summary, the present study demonstrates that increased consumption of either UMP or DHA, plus choline, improves the acquisition and retention of spatial memory by normal gerbils, concurrently increasing brain phosphatides. Largest increases in PL levels and greatest enhancement in memory occur when all three circulating phosphatide precursors, uridine, DHA, and choline, are given concurrently. FJ

We thank Lisa Teather for advice and assistance in preparing this paper, and Tim Maher for providing the rotarod testing apparatus. We also thank Rona Stephanopoulos and Paul Jaffe for their assistance with behavior and biochemistry assays. This study was supported by National Institutes of Health grant MH-28283 and the Center for Brain Sciences and Metabolism Charitable Trust.

REFERENCES

- Gilani, G. S., and Nasim, A. (2007) Impact of foods nutritionally enhanced through Bio/Technology in alleviating malnutrition in developing countries. *J. AOAC Int.* **90**, 1440–1444
- Eilander, A., Hundscheid, D. C., Osendarp, S. J., Transler, C., and Zock, P. L. (2007) Effects of n-3 long chain polyunsaturated fatty acid supplementation on visual and cognitive development throughout childhood: a review of human studies. *Prostaglandins Leukot. Essent. Fatty Acids* **76**, 189–203
- Gamoh, S., Hashimoto, M., Sugioka, K., Shahdat Hossein, M., Hata, N., Misawa, Y., and Masumura, S. (1999) Chronic administration of docosahexaenoic acid improves reference memory-related learning ability in young rats. *Neuroscience* **93**, 237–241
- Gamoh, S., Hashimoto, M., Hossain, S., and Masumura, S. (2001) Chronic administration of docosahexaenoic acid improves the performance of radial arm maze task in aged rats. *Clin. Exp. Pharmacol. Physiol.* **28**, 266–270
- Green, K. N., Martinez-Coria, H., Khashwji, H., Hall, E. B., Yurko-Mauro, K. A., Ellis, L., and LaFerla, F. M. (2007) Dietary docosahexaenoic acid and docosapentaenoic acid ameliorate amyloid-beta and tau pathology via a mechanism involving presenilin 1 levels. *J. Neurosci.* **27**, 4385–4395
- Wurtman, R. J., Ulus, I. H., Cansev, M., Watkins, C. J., Wang, L., and Marzloff, G. (2006) Synaptic proteins and phospholipids are increased in gerbil brain by administering uridine plus docosahexaenoic acid orally. *Brain Res.* **1088**, 83–92
- Sakamoto, T., Cansev, M., Wurtman, R. J. (2007) Oral supplementation with docosahexaenoic acid and uridine-5'-monophosphate increases dendritic spine density in adult gerbil hippocampus. *Brain Res.* **1182**, 50–59
- Cansev, M., Watkins, C. J., van der Beek, E. M., and Wurtman, R. J. (2005) Oral uridine-5'-monophosphate (UMP) increases brain CDP-choline levels in gerbils. *Brain Res.* **1058**, 101–108
- Pooler, A. M., Guez, D. H., Benedictus, R., and Wurtman, R. J. (2005) Uridine enhances neurite outgrowth in nerve growth factor-differentiated pheochromocytoma cells. *Neuroscience* **134**, 207–214
- Kennedy, P., and Weiss, B. (1956) The function of cytidine coenzymes in the biosynthesis of phospholipides. *J. Biol. Chem.* **222**, 193–214
- Ott, T., Grecksch, G., and Matthies, H. (1978) Retention improvement by topical application of uridine monophosphate into different brain areas. *Med. Biol.* **56**, 133–137
- Teather, L. A., and Wurtman, R. J. (2006) Chronic administration of UMP ameliorates the impairment of hippocampal-dependent memory in impoverished rats. *J. Nutr.* **136**, 2834–2837
- Suzuki, N. N., Koizumi, K., Fukushima, M., Matsuda, A., and Inagaki, F. (2004) Structural basis for the specificity, catalysis, and regulation of human uridine-cytidine kinase. *Structure* **12**, 751–764
- Spanner, S., and Ansell, G. B., (1979) Choline kinase and ethanolamine kinase activity in the cytosol of nerve endings from rat forebrain. *Biochem. J.* **178**, 753–760
- Marszalek, J. R., and Lodish, H. F. (2005) Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: Breastmilk and fish are good for you. *Annu. Rev. Cell Dev. Biol.* **21**, 633–657
- Marszalek, J. R., Kitidis, C., DiRusso, C. C., and Lodish, H. F. (2005) Longchain acyl-CoA synthetase 6 preferentially promotes DHA metabolism. *J. Biol. Chem.* **280**, 10817–10826
- Millington, W. R., and Wurtman, R. J. (1982) Choline administration elevates brain phosphorylcholine concentrations. *J. Neurochem.* **38**, 1748–1752
- Cohen, B. M., Renshaw, P. F., Stoll, A. L., Wurtman, R. J., Yurgelun-Todd, D., and Babb, S. M. (1995) Decreased brain choline uptake in older adults. An in vivo proton magnetic resonance spectroscopy study. *JAMA* **20**, 274, 902–907
- Skold, O. (1960) Uridine kinase from Erlich ascites tumor: Purification and properties. *J. Biol. Chem.* **274**, 902–907
- Orengo, A. (1969) Regulation of enzymic activity by metabolites. I. Uridine-cytidine kinase of Novikoff ascites rat tumor. *J. Biol. Chem.* **244**, 2204–2209
- Anderson, E. (1973) Nucleoside and nucleotide kinases. In: *The Enzymes* (Boyer P., ed.) pp. 49–96, Academic Press, New York
- Greenberg, N., Schumm, D. E., and Webb, T. E. (1977) Uridine kinase activities and pyrimidine nucleoside phosphorylation in fluoropyrimidine-sensitive and-resistant cell lines of the Novikoff hepatoma. *Biochem. J.* **164**, 379–387
- Ropp, P., and Traut, T. (1996) Cloning and expression of a cDNA encoding uridine kinase from mouse brain. *Arch. Biochem. Biophys.* **336**, 105–112
- Ropp, P., and Traut, T. (1998) Uridine kinase: Altered enzyme with decreased affinities for uridine and CTP. *Arch. Biochem. Biophys.* **359**, 63–68
- Ross, B. M., Moszczynska, A., Blusztajn, J. K., Sherwin, A., Lozano, A., and Kish, S. J. (1997) Phospholipid biosynthetic enzymes in human brain. *Lipids* **32**, 351–358
- Galli, C., Marangoni, F., and Galella, G. (1993) Modulation of lipid derived mediators by polyunsaturated fatty acids. *Prostaglandins Leukot. Essent. Fatty Acids* **48**, 51–55
- Nolan, M. F., Malleret, G., Lee, K. H., Gibbs, E., Dudman, J. T., Santoro, B., Yin, Dm, Thompson, R., Siegelbaum, S. A., Kandel, E. R., and Morozov, A. (2003) The hyperpolarization-activated HCN1 channel is important for motor learning and neuronal integration by cerebellar Purkinje cells. *Cell* **115**, 551–564
- Labarca, C., and Paigen, K. (1980) A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102**, 4–52
- Folch, J. Lees, M., and Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497–509
- Svanborg, A., and Svennerholm, L. (1961) Plasma total lipids, cholesterol, triglycerides, phospholipids and free fatty acids in a healthy Scandinavian population. *Acta Med. Scand.* **169**, 43–49
- Ulus, I. H., Wurtman, R. J., Mauron, C., and Blusztajn, J. K. (1989) Choline increases acetylcholine release and protects against the stimulation-induced decrease in phosphatide levels within membranes of rat corpus striatum. *Brain Res.* **484**, 217–227
- Holguin, S., Huang, Y., Liu, J., and Wurtman, R. J. (2008) Chronic administration of DHA and UMP improves the impaired memory of impoverished rats. *Behav Brain Res.* **191**, 11–16
- Fedorova, I., Hussein, N., Di Martino, C., Moriguchi, T., Hoshiba, J., Majchrzak, S., and Salem, N., Jr. (2007) An n-3 fatty acid deficient diet affects mouse spatial learning in the Barnes circular maze. *Prostaglandins Leukot. Essent. Fatty Acids* **77**, 269–277
- Xiao, Y., Wang, L., Xu, R. J., and Chen, Z. Y. (2006) DHA depletion in rat brain is associated with impairment on spatial learning and memory. *Biomed. Environ. Sci.* **19**, 474–480
- Wainwright, P. E. (2002) Dietary essential fatty acids and brain function: a developmental perspective on mechanisms. *Proc. Nutr. Soc.* **61**, 61–69
- Wainwright, P. E., Xing, H. C., Girard, T., Parker, L., and Ward, G. R. (1998) Effects of dietary n-3 deficiency on amphetamine-conditioned place preference and working memory in the Morris water-maze. *Nutr. Neurosci.* **1**, 281–293
- Brenna, J. T. (2002) Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man. *Curr. Opin. Clin. Nutr. Metab. Care* **5**, 127–132
- Sinclair, A. J., Attar-Bashi, N. M., and Li, D. (2002) What is the role of alpha-linolenic acid for mammals? *Lipids* **37**, 1113–1123
- Lopez-Garcia, E., Schulze, M. B., Manson, J. E., Meigs, J. B., Albert, C. M., Rifai, N., Willett, W. C., and Hu, F. B. (2004) Consumption of (n-3) fatty acids is related to plasma biomarkers of inflammation and endothelial activation in women. *J. Nutr.* **134**, 1806–1811
- Moriguchi, T., and Salem, N Jr. (2003) Recovery of brain docosahexaenoate leads to recovery of spatial task performance. *J. Neurochem.* **87**, 297–309
- Ikemoto, A., Ohishi, M., Sato, Y., Hata, N., Misawa, Y., Fujii, Y., Okuyama, H. (2001) Reversibility of n-3 fatty acid deficiency-

- induced alterations of learning behavior in the rat: level of n-6 fatty acids as another critical factor. *J. Lipid Res.* **42**, 1655–1663
42. Willatts, P., Forsyth, S. J., DiModugno, M. K., Varma, S., and Colvin, M. (1998) Influence of long-chain polyunsaturated fatty acids on infant cognitive function. *Lipids* **33**, 973–980
 43. Werkman, S. H., and Carlson, S. E. (1996) A randomized trial of visual attention of preterm infants fed docosahexaenoic acid until nine months. *Lipids* **31**, 91–97
 44. Carlson, S. E., and Werkman, S. H. (1996) A randomized trial of visual attention of preterm infants fed docosahexaenoic acid until two months. *Lipids* **31**, 85–90
 45. Hart, S. L., Boylan, L. M., Carroll, S. R., Musick, Y. A., Kuratko, C., Border, B. G., and Lampe, R. M. B. (2006) Brief report: newborn behavior differs with docosahexaenoic acid levels in breast milk. *J. Pediatr. Psychol.* **31**, 221–226
 46. Auestad, N., Scott, D. T., Janowsky, J. S., Jacobsen, C., Carroll, R. E., Montalto, M. B., Halter, R., Qiu, W., Jacobs, J. R., Connor, W. E., Connor, S. L., Taylor, J., Neuringer, M., Fitzgerald, K. M., and Hall, R. T. (2003) Visual, cognitive, and language assessments at 39 months: a follow-up study of children fed formulas containing long-chain polyunsaturated fatty acids to 1 year of age. *Pediatrics* **112**, e177–e183
 47. Birch, E. E., Garfield, S., Castaneda, Y., Hughbanks-Wheaton, D., Uauy, R., and Hoffman, D. (2007) Visual acuity and cognitive outcomes at 4 years of age in a double-blind, randomized trial of long-chain polyunsaturated fatty acid-supplemented infant formula. *Early Hum. Dev.* **83**, 279–284
 48. Bell, M. V., and Tocher, D. R. (1989) Molecular species composition of the major phospholipids in brain and retina from rainbow trout (*Salmo gairdneri*). Occurrence of high levels of di-(n-3) polyunsaturated fatty acid species. *Biochem. J.* **264**, 909–915
 49. Li, J. Y., Boado, R. J., and Pardridge, W. M. (2001) Cloned blood-brain barrier adenosine transporter is identical to the rat concentrative Na⁺ nucleoside cotransporter CNT2. *J. Cereb. Blood. Flow Metals* **21**, 929–936
 50. Cansev, M., and Wurtman, R. J. (2007) Chronic administration of docosahexanoic acid or eicosapentanoic acid, but not arachidonic acid, alone or in combination with uridine, increases brain phosphatide and synaptic protein levels in gerbils. *Neuroscience* **148**, 421–431
 51. Lim, S. Y., Hoshiba, J., and Salem, N Jr. (2005) An extraordinary degree of structural specificity is required in neural phospholipids for optimal brain function: n-6 docosapentaenoic acid substitution for docosahexaenoic acid leads to a loss in spatial task performance. *J. Neurochem.* **95**, 848–857
 52. Farooqui, A. A., Horrocks, L. A., and Farooqui, T. (2007) Modulation of inflammation in brain: a matter of fat. *J. Neurochem.* **101**, 577–899
 53. Green, P., Glozman, S., Weiner, L., and Yavin, E. (2001) Enhanced free radical scavenging and decreased lipid peroxidation in the rat fetal brain after treatment with ethyl docosahexaenoate. *Biochim. Biophys. Acta.* **1532**, 203–212
 54. Levant, B., Radel, J. D., and Carlson, S. E. (2004) Decreased brain docosahexaenoic acid during development alters dopamine-related behaviors in adult rats that are differentially affected by dietary remediation. *Behav. Brain Res.* **152**, 49–57
 55. Bofill-Cardona, E., Vartian, N., Nanoff, C., Freissmuth, M., and Boehm, S. (2000) Two different signaling mechanisms involved in the excitation of rat sympathetic neurons by uridine nucleotides. *Mol. Pharmacol.* **57**, 1165–1172
 56. Xia, H. J., and Yang, G. (2005) Inositol 1,4,5-trisphosphate 3-kinases: functions and regulations. *Cell Res.* **15**, 83–91

*Received for publication May 6, 2008.
Accepted for publication June 19, 2008.*