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Nutritional modifiers of aging brain function: Increasing the formation of brain synapses by administering uridine and other phosphatide precursors

R.J. Wurtman[1], **M Cansev**[2], **T Sakamoto**[3], and **I.H. Ulus**[4]

[1] Massachusetts Institute of Technology, Cambridge, MA

[2] Uludag University Medical School, Bursa, Turkey

[3] Kobe Gakuin University, Kobe, Japan

[4] Acibadem University Medical School, Istanbul, Turkey

Abstract

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

INTRODUCTION

Presumably, all of the information that flows through and out of the brain is mediated by neurotransmitters, released into synapses, and subsequently bound to postsynaptic receptors. Diseases of aging that, like Alzheimer's disease decrease the number of synapses thereby impair cognition[1–2] and ultimately compromise most brain functions.

No treatment strategy is available that has been shown to increase the number of synapses in brains of Alzheimer patients or, for that matter, of normal people. The agents now available for treating Alzheimer's disease act by amplifying (acetylcholinesterase inhibitors) or modulating (glutamate antagonists) the actions of particular neurotransmitters. These drugs have only small and transient therapeutic effects, and apparently do nothing either to slow synaptic loss or to accelerate the production of new synapses that might compensate for this loss. The loss is generally thought to result from the locally-toxic effects of an endogenous peptide, A-beta, or its aggregates[3–4] on synapses themselves or on their anatomic precursor, dendritic spines[3]. An extensive and often-frustrating search has been pursued for several decades to find a treatment that might block A-beta's formation, aggregation, or toxic effects, or perhaps remove the A-beta using a monoclonal antibody. No solid evidence is yet available that doing so will slow the course of Alzheimer's disease or reverse the synaptic and cognitive deficits.

Address correspondence to: Richard J. Wurtman, MD, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Bldg. 46-5023, Cambridge, MA 02139 USA, Ph: 617.253.6731; FAX: 617.253.6882, dick@mit.edu.

Since synapses are composed principally of a special type of membrane, "synaptic membrane", comprised of lipids, principally phosphatides, and a specific set of proteins, a strategy for increasing their number would require agents that increased the formation of both their lipid and protein components. It would also require amplifying the genetic instructions that cause adult neurons to differentiate to form the structures – dendritic spines and terminal boutons – which come into contact and thereby generate synapses. New studies have shown that treating animals concurrently with three particular phosphatide precursors present in the blood and formed endogeneously (uridine and choline) or derived from foods (choline and omega-3 fatty acids) can have both of these effects: It increases brain phosphatides, synaptic proteins, neurite outgrowth and the formation of dendritic spines[5]. This treatment also enhances cognition and the release of some brain neurotransmitters in the animals. Moreover, administration of the phosphatide precursors (along with additional supporting nutrients) to patients with mild Alzheimer's disease significantly improved cognition in an initial large-scale (212 patients) clinical trial, discussed below[6].

PHOSPHATIDE PRECURSORS AND SYNAPTOGENESIS

If animals are treated for several weeks with uridine, choline, and the omega-3 fatty acid docosahexaenoic acid (DHA) the quantities of membrane synthesized from these compounds increase significantly[7], both in whole brain and per brain cell. Moreover the brains also exhibit parallel changes in levels of proteins known to be associated with pre- and post-synaptic membranes[7].

The biochemical mechanisms that underlie these responses involve an unusual kinetic property of enzymes in the phosphatide-producing Kennedy cycle, i.e. – poor affinities for the substrates that they transform to intermediates in phosphatide synthesis. This property allows relatively small increases in available levels of uridine, for example, to accelerate the production of UTP and CTP; of DHA to increase brain levels of diacylglycerol molecules containing DHA; and of choline to increase brain phosphocholine. The brain is unusual among organs in the extent to which the rates of some of its most characteristic biochemical reactions are controlled by substrate levels, and thus by the extent to which a key enzyme is saturated with its physiologic substrate (and not by the enzyme's activity, per se.) Since the substrates involved are often nutrients, this dependence allows nutrient consumption to have important effects on brain composition and function. Thus, for example, the rates at which brain neurons synthesize and release the monoamine neurotransmitters serotonin[8–10], acetylcholine[11], histamine[12], and dopamine [13] can all be increased by raising brain levels of the nutrients that are their circulating precursors, i.e., tryptophan, choline, histidine, and tyrosine, respectively. And similarly, giving animals the three normally-circulating phosphatide precursors increases brain levels of their end-product, phosphatidylcholine (PC), as well as of the other major membrane phosphatides, per brain cell. This sensitivity to substrate concentrations allows phosphatide levels, the quantity of synaptic membrane, and, ultimately, the number of synapses to be affected by nutrient intake.

Synapses consist of a presynaptic terminal originating on an axon; the synaptic cleft; and the postsynaptic membrane, usually on a dendrite or cell body. Presynaptic terminals synthesize the neuron's neurotransmitter, and, generally, store it in and release it, upon depolarization, from synaptic vesicles. The locus of this release, the synaptic cleft, is a fluid-filled space between the two neurons. The neurotransmitter then either diffuses to the postsynaptic membrane or is inactivated, by enzymatic degradation (e.g. for acetylcholine, by acetycholinesterase) or by reuptake into its neuron of origin. The postsynaptic membrane contains receptors to which the neurotransmitter can bind, and additional protein molecules which transduce the functional consequences of the receptor's activation (e.g. "scaffolding" molecules like PSD-95; enzymes like adenylate cyclase). Pre- and postsynaptic membranes

contain similar lipids – principally phospholipids and cholesterol; however the membranes differ from each other and from membranes elsewhere in the brain by virtue of the high concentration of polyunsaturated omega-3 fatty acids in their phosphatides, and the specific proteins each contains, as described below.

The postsynaptic membranes on which glutamate, the most widely-used brain neurotransmitter, acts, often contain characteristic postsynaptic densities, each housing a large number of different proteins, which initiate the further transduction of biological signals generated by the transmitter-receptor complex. This transduction is accomplished by the opening or closing of protein channels in the membranes, which allows specific ions that affect the cell's voltage to pass into or out of the cell, or by activating membrane-bound enzymes coupled to G-proteins, which synthesize intracellular second messengers.

The formation of a new synapse among, for example, hippocampal neurons that use glutamate as their neurotransmitter is usually initiated by the coming together of a presynaptic element, the terminal bouton, and a postsynaptic dendritic spine, a process that is facilitated by the latter's motility[14]. A variety of environmental factors apparently can increase the number of dendritic spines, for example administration to mice of the hormone ghrelin, which also crosses the blood-brain barrier, enhances memory performance, and promotes long-term potentiation. Targeted disruption of the gene for ghrelin decreases dendritic spine numbers and memory performance[15] – thus affirming the importance of dendritic spines in hippocampal synaptic transmission. Dendritic spines are also known to be particularly vulnerable in Alzheimer's disease[3]. Among transgenic mice that overproduce A-beta, dendritic spines and synapses are diminished by local amyloid plaques[3], and cognition is thus impaired early in the course of the disease, prior to the overt loss of neurons.

It is not yet possible to quantify the effects on synaptic number of any but the most neurotoxic biochemical treatments. Thus, estimates of changes in synaptic number must, in general, be extrapolated from surrogate measurements, e.g. of numbers of dendritic spines, or concentrations of synaptic proteins, or of behaviors known to involve particular neurons. Of these surrogates the number of dendritic spines is generally believed to provide the best correlations with the actual number of synapses, since as many as 90% of dendritic spines ultimately become synapses[14–23].

Although most brain synapses are formed during pre- or early post-natal development, each survives for only days to months, and thus must be renewed periodically throughout the individual's life span[24]. This continuing necessity is probably of major importance in underlying the brain's plasticity and the individual's ability to learn, since it allows specific, perhaps newly-formed synapses to be associated with newly-learned material[19,25]. Early in development most synaptogenesis occurs independent of neuronal depolarization and neurotransmitter release[26,27]. In adulthood, however, the rate at which new synapses form, and the ways that new synaptic connections become configured, are largely governed by neuronal activity. This allows very active synapses to facilitate the formation of additional synapses[19]. Synaptogenesis can also be enhanced by the activation of particular neuronal genes, for example those for transcription factors like CREB (the cAMP response element-binding protein), which enhances synapse formation[28–30], and for MEF2, which limits the potentially-excessive formation of new synapses[19,31]. Among new neurons formed from stem cells in adult mouse hippocampus, which are making their initial synaptic contacts it can be shown[23] that new synapses start to come into being when a dendritic spine from one neuron comes into contact with a presynaptic bouton of another. Hence the rate of synaptogenesis is dependent on the numbers of dendritic spines that happen to be available, and treatments like the nutrient mixture described in this report which increase dendritic spine number can also thereby promote synaptogenesis.

EFFECTS OF URIDINE, CHOLINE, AND OMEGA-3 FATTY ACIDS ON SYNAPTIC MEMBRANE FORMATION AND SYNAPTOGENESIS

All cells utilize DHA and other fatty acids; uridine; and choline to form phosphatidycholine (PC) and the other phosphatide subunits which, when aggregated, constitute the major components of their membranes. PC, the principal such subunit in brain, is synthesized from these precursors by the CDP-choline cycle or "Kennedy Cycle"[32]; PC also provides the phosphocholine moiety needed to synthesize sphingomyelin (SM), the other major choline-containing brain phospholipid. The phosphatide phosphatidylethanolamine (PE) is also synthesized via the Kennedy Cycle, utilizing ethanolamine instead of choline, while phosphatidylserine (PS), the third major structural phosphatide, is produced by exchanging a serine molecule for the choline in PC or the ethanolamine in PE.

The CDP-choline cycle involves three sequential enzymatic reactions. In the first, catalyzed by choline kinase (CK), a monophosphate is transferred from ATP to the hydroxyl oxygen of the choline, yielding phosphocholine. The second, catalyzed by CTP: phosphocholine cytidylyltransferase (CT), transfers cytidine-5′-monophosphate (CMP) from CTP to the phosphorus of phosphocholine, yielding cytidine-5′-diphosphocholine (also known as CDP-choline or as citicoline). As discussed below, much of the CTP that the human brain uses for this reaction derives from circulating uridine[33]. The third and last reaction, catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), bonds the phosphocholine of CDP-choline to the hydroxyl group on the 3- carbon of diacylglycerol (DAG), yielding the PC. DAG molecules containing a PUFA at the 2-position are preferentially utilized for this reaction[34]. All three PC precursors must be obtained by brain entirely or in large part from the circulation, and because the PC-synthesizing enzymes that act on all three have low affinities for these substrates, blood levels of all three can affect the overall rate of PC synthesis[35,7].

Thus, choline administration increases brain phosphocholine levels in rats[36] and humans[37], because CK's Km for choline (2.6 mM)[38] is much higher than usual brain choline levels (30–60 μM)[39,40,41]. Most commonly the second, CT-catalyzed reaction most influences the overall rate of PC synthesis, either because not all of the CT enzyme is fully activated by being attached to a cellular membrane[42] or because local CTP concentrations are insufficient to saturate the CT. Thus, when brain CTP levels are increased by giving animals uridine[43], CTP's circulating precursor in human blood[33], PC synthesis is accelerated[43]. The activity of CPT and the extent to which this enzyme is saturated with DAG can also control the overall rate of PC synthesis[44]: In PC-12 cells, NGF increased DAG levels five-fold, CPT activity by 70%, and the incorporation of choline into PC by two-fold. If rodents are given a standard diet supplemented with choline and uridine (as its monophosphate, UMP) and, also, by gavage, DHA, brain PC synthesis rapidly increases[7,43], and absolute levels of PC per cell (i.e. DNA) or per mg protein rise substantially (e.g., by 30% or more after several weeks of daily treatment[7] (Table 1).

This treatment also increases the levels of each of the other principal membrane phosphatides (Table 1), as well as those of particular proteins known to be localized within presynaptic and postsynaptic membranes (for example synapsin-1, PSD-95 and syntaxin-3)[45] (Table 2), but not of β-tubulin, a ubiquitously-distributed protein[7,46].

These changes in synaptic proteins are probably mediated by an additional mechanism[47] discussed below – the activation of P2Y receptors by uridine or uridine-containing nucleotides. Administration of DHA, UMP and choline to adult gerbils also promotes the formation of hippocampal dendritic spines[48], improves hippocampus-dependent cognitive behaviors in rats[49,50] and gerbils[51], and can amplify neurotransmitter release[52,53]. Providing

supplemental UMP or DHA without the other can also increase brain phosphatide levels, however by less than when all three precursors (including choline, which is present in all of the test diets) are given.

Sources of plasma and brain uridine

Few data are available as to whether foods other than milk contain significant quantities of free uridine or uridine-containing nucleotides, or whether consumption of any naturally-occurring food, by adults, can substantially increase plasma uridine levels. What is known is that pyrimidines, as well as purines, are constituents of nucleic acids, i.e., ribonucleic acid (RNA), which contains uridine and cytidine, and deoxyribonucleic acid (DNA), which contains cytidine. Since RNA and DNA are components of all cells, any food consumed by humans that contains cells (e.g., meats, poultry, fish, vegetables, fruits, etc.) is, at least theoretically a good source of nucleic acids, and perhaps also of plasma pyrimidines. Evidence from in vitro studies suggests that, following enzymatic breakdown of dietary nucleic acids, pyrimidine compounds are taken up into the blood from the intestine, however no in vivo study has demonstrated, in adults, an actual increase in plasma uridine levels after eating an RNA- or DNA-containing food. The nucleic acids in foods or in breast milk have been shown in in vitro studies to be degraded to yield purine and pyrimidine nucleotides; nucleosides; and free bases[54,55]. In vitro, RNA is digested by ribonucleases to yield uridine nucleotides, and these can be further hydrolyzed to uridine by phosphatases in the intestinal mucosa[56].

Uridine is present as such in breast milk, but also as constituents of RNA; nucleotides (5′-UMP); and nucleotide adducts (UDP-glucose, UDP-galactose)[57,58]. The total available uridine contents of pooled milk samples from 100 European women determined by a method that simulated in vivo digestion[57] (i.e., by enzymatically degrading nucleic acids, nucleotides, and nucleotide adducts) were 32, 48, and 47 μM respectively for mothers of 2–10 day-old, 1 month-old, and 3 month-old babies. Available cytidine contents in the same samples were 86, 102, and 96 μM[57]. Synthetic infant formulas are also routinely fortified with uridine and cytidine monophosphates.

Uridine is transported across the intestinal mucosal epithelium as such[59,60], or as uracil, the free base. In the rat's small intestine cytidine derived from RNA or DNA is partly deaminated to uridine[54]. In humans this deamination in intestinal mucosa and liver is probably much greater than in rats, since exogenously administered cytidine is almost undetectable as such in human plasma[33].

The transport of pyrimidine nucleosides and bases across the small intestine is mediated by the sodium-dependent concentrative nucleoside transporters CNT1 and CNT2[61]. The kinetic properties of this uptake have not yet been determined. Following intestinal absorption, uridine and uracil are transferred via the portal vein to the liver. In rats the liver is probably the major organ modulating plasma uridine concentrations: more than 90% of the uridine that enters the liver via the portal vein is metabolized in a single pass[62]; moreover, uridine's concentration in hepatic venous plasma (1.32 ± 0.45 μM) is slightly higher than in portal (1.03 ± 0.3 μM) or arterial (1.06 ± 0.2 μM) blood, indicating that some of the uridine in the hepatic venous blood derived from de novo hepatic synthesis.

Uridine and cytidine are transported across cellular membranes in all tissues[63] including the brain, via two families of transport proteins, i.e., the Na^+-independent, low-affinity, equilibrative transporters (ENT1 and ENT2; SLC29 family) and the Na^+-dependent, high affinity, concentrative (CNT1, CNT2 and CNT3; SLC28 family). The two ENT proteins exhibit Km values for both uridine and cytidine in the high micromolar range (100–800 μM)[64]; thus they probably mediate BBB pyrimidine uptake only when plasma levels have

been elevated experimentally. In contrast, CNT2, which transports both uridine and purines like adenosine, probably mediates BBB uridine transport under physiologic conditions: its Km values for uridine (and adenosine) are in the low micromolar range (9–40 μM) whereas plasma uridine levels are subsaturating, i.e., 0.9–3.9 μM in rats, 3.1–4.9 μM in humans and around 6.5 μM in gerbils. Pyrimidines also may be taken up into brain via the choroid plexus (CP) epithelium, however, because the surface area of BBB is so much greater (i.e., in humans 21.6 m^2 versus 0.021 m^2) it is clear that the BBB is the major locus of uridine uptake.

Uridine and cytidine are phosphorylated to their respective nucleotides by various kinases. Thus, uridine-cytidine kinase (UCK) (ATP:uridine 5′-phosphotransferase, EC 2.7.1.48) converts to uridine-5′-monophosphate (UMP)[65,66]; UMP is then converted to uridine-5′-diphosphate (UDP) by UMP-CMP kinase (UMP-CMPK) (ATP:CMP phosphotransferase, EC 2.7.4.14)[67,68,69], and to UTP by nucleoside diphosphate kinases (NDPK) (Nucleoside triphosphate:Nucleoside diphosphate phosphotransferase, EC 2.7.4.6)[66]. Interconversions of uridine and cytidine and of their respective nucleotides, also occur in mammalian cells. Cytidine and CMP can be deaminated to uridine and UMP[70,71], while UTP is aminated to CTP by CTP synthase (UTP:ammonia ligase [ADP-forming], E.C. 6.3.4.2)[71].

All of the above enzymes are unsaturated with their respective nucleoside or nucleotide substrates in brain and other tissues. For example, the Km's for uridine of UCK prepared from various tissues varied between 33–270 μM[21,65,66], and from recombinant mouse brain enzyme was 40 μM[72]. Brain uridine and cytidine levels are about 22–46 pmol/mg wet weight[43,73] and 6–43 pmol/mg wet weight[43], respectively. Hence, the syntheses of UTP and CTP, and the subsequent syntheses of brain PC and PE via the Kennedy pathway, depend on the availability of their pyrimidine substrates. Indeed, an increase in the supply of uridine or cytidine to neuronal cells, in vitro[47,74,75] or in vivo[43], enhanced the phosphorylation of uridine and cytidine, elevating the levels of UTP, CTP, and CDP-choline.

Brain levels of particular uridine-containing compounds following uridine administration were examined in gerbils given a single dose of UMP (1 mmol/kg)[43] by gavage and killed between 5 min and 8 h thereafter. Thirty minutes after gavage, plasma uridine levels were increased from 6.6 ± 0.58 to 32.7 ± 1.85 μM ($P<0.001$) and brain uridine from 22.6 ± 2.9 to 89.1 ± 8.82 pmol/mg tissue ($P<0.001$). UMP also significantly increased plasma and brain cytidine levels. However, both basally and following UMP administration these levels were much lower than those of uridine, rising from 1.2 μM to 1.9 μM in plasma and from 5 pmol/mg tissue to 12 pmol/mg tissue in brain 30 to 60 minutes after gavage. (In human subjects receiving oral cytidine as CDP-choline, plasma cytidine levels did not rise detectably at all)[33]. Brain UTP, CTP and CDP-choline were all elevated in gerbils 15 min after UMP (from 254 ± 31.9 to 417 ± 50.2 ($P<0.05$); 56.8 ± 1.8 to 71.7 ± 1.8 ($P<0.001$); and 11.3 ± 0.5 to 16.4 ± 1, ($P<0.001$ pmol/mg tissue, respectively), returning to basal levels after 20 and 50 min. The smallest UMP dose that significantly increased brain CDP-choline was 0.5 mmol/kg. These results show that oral UMP, a uridine source, enhances the synthesis of CDP-choline, the immediate precursor of PC, in gerbil brain, but that the increases in nucleotides or CDP-choline are short-lived, and disappear long before increases in brain phosphatides become detectable. How, then, does repeated daily intake of supplemental uridine (as UMP in the test diet) ultimately raise brain PC? Probably, in part, via uridine's other mechanism of action, discussed below - its activation of P2Y receptors, which then elicit longer-term downstream effects.

Sources of plasma and brain choline

Choline is present in plasma as the free base[76,77]; as a constituent of phospholipids (including PC; SM; lyso-PC; choline-containing plasmalogens; and the platelet activating

factor (PAF)); and as PC's water-soluble metabolites (principally phosphocholine and glycerophosphocholine[78]. Free choline is also found in other biologic fluids[79], and concentrated within erythrocytes through the action of an uptake molecule which is unsaturated (Km=5–10 μM at normal plasma choline concentrations).

Plasma choline derives from three main sources, - dietary choline, consumed as the free base or as a constituent of phospholipids; endogenous synthesis, principally in liver; and liberation from the membrane phosphatides of all mammalian cells. Choline is present within many foods[79] (see http://www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choline.html) and also in breast milk and infant formulas[80] principally as the free molecule or as phosphatides, and its plasma levels can rapidly increase several-fold after ingestion of choline-rich foods. Thus, consumption by humans of a 5-egg omelet (containing about 1.3 g of choline) increased these levels from 9.8 μM to 36.6 μM within 4 hours. Prolonged fasting reduced human plasma choline levels from 9.5 μM to 7.8 μM after seven days. Similarly, removal of all choline-containing foods from the diet for 17–19 days gradually lowered plasma choline, from 10.6 μM to 8.4 μM in humans[81] and from 12.1 μM to 6.3 μM in rats, indicating that plasma choline can be partially but not fully sustained by release from endogenous stores.

Dietary PC is deacylated within the gut to form lyso-PC. About half of this product is further degraded to free choline within the gut or liver. The remainder is reacylated to regenerate PC[82], which is then absorbed into the lymphatic circulation[83]. Much of the dietary choline that reaches the liver via the portal circulation is destroyed by oxidation to betaine, ultimately providing methyl groups that can be used to regenerate S-adenosylmethionine (SAM) from homocysteine. The rest passes into the systemic circulation.

In 1998, the Food and Nutrition Board (FNB) of the U.S. Institute of Medicine established a dietary reference intake (DRI) for choline[81,84]. Since the FNB did not believe that existing scientific evidence allowed calculation of a Recommended Daily Allowance (RDA) for choline, it instead set an Adequate (daily) Intake level (AI), and an Upper (daily) Limit (UL) that should not be exceeded. The main criteria for determining the AI and UL were, respectively, the amount of choline needed to prevent liver damage, and the choline intake associated with choline's most sensitive adverse effect, i.e., hypotension[84]. It should be noted that subsequent studies have shown that the enzymes, described below, which synthesize and metabolize choline can be affected by common genetic polymorphisms which cause important person-to-person variations in dietary choline needs. For further details about dietary reference intakes and the choline contents of various foods, the reader is referred to the official websites of the Institute of Medicine (http://www.nap.edu/catalog/6015.html#toc) and the USDA (http://www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choline.html).

Endogenous choline is synthesized, principally in liver but also to a small extent within brain[85,86,87], by the sequential addition of three methyl groups to the amine nitrogen of PE; this forms PC, which can then be hydrolyzed to liberate the choline. The methylation reactions are catalyzed by two phosphatidylethanolamine-N-methyltransferase enzymes, (PEMT1; EC: 2.1.1.17), which converts PE to its monomethyl derivative, and phosphatidyl-N-methylethanolamine-N-methyltransferase (PEMT2; EC: 2.1.1.71), which adds the second and third methyl groups (a single enzyme may catalyze all three methylations in liver). Both enzymes utilize SAM as the methyl donor; their Km's for SAM are $2–4\times10^{-6}$ M and $20–110\times10^{-6}$ M, respectively[86], while brain SAM concentrations are 10–17 μg/g wet weight (50–85 μM assuming that about 50% of the brain mass is aqueous). Hence PEMT1 is probably fully saturated with SAM while PEMT2 is not. PEMT activity has been identified brain homogenates[87], particularly in synaptosomes[85], suggesting that nerve terminals can

synthesize choline. PE itself is formed in liver, kidney, or brain from free ethanolamine, via the CDP-ethanolamine cycle ("Kennedy Cycle"), or from the decarboxylation of PS. PS is produced, in nerve terminals[88] and elsewhere, by "base-exchange", in which a serine molecule substitutes for the ethanolamine in PE or the choline in PC.

The biosynthesis of PC, and thus of endogenous choline, by the methylation of hepatic PE is diminished among animals given inadequate amounts of vitamins required for methyl group production, i.e. B6, B12 and folate. This relationship provides a basis for administering supplemental quantities of these vitamins to subjects receiving uridine, DHA, and choline to promote membrane phosphatide formation.

Free choline is liberated from PC by the phospholipase enzymes. Phospholipase D (PLD) directly cleaves the choline/phosphate bond to generate choline and phosphatidic acid. Phospholipase A2 (PLA2) acts on the bond connecting a fatty acid to the hydroxyl-group on PC's number-2 carbon to yield that fatty acid (often arachidonic acid [AA] or DHA) and lyso-PC; the lyso-PC is then further metabolized to choline by a phosphodiesterase, or to glycerophosphocholine (GPC), then cleaved to choline by a phosphatase. Phospholipase C (PLC) acts on the bond connecting the phosphate and the hydroxyl- group on PC's number-3 carbon to yield DAG and phosphocholine; the phosphocholine can then be metabolized to free choline by a phosphatase.

It is estimated that, on average, about 15% of the free choline that enters the human blood stream derives from endogenous synthesis, the rest coming principally from dietary sources[89]. Acute or chronic liver disease or deficiencies in methionine, folic acid or vitamin B12 intake can thus lower plasma choline levels by impairing hepatic PC synthesis.

Cellular membranes contain most of the choline in the body, principally as PC and sphingomyelin. They also contain, of course, the phosphatides PS, PE, and phosphatidylinositol (PI), and specific proteins, cholesterol, and various minor lipids. The quantities of choline present in brain as PC (2–2.5 mmoles/g) or SM (0.25 mmoles/g) are orders of magnitude greater than those of free choline (30–60 μM).

"PC" is highly heterogeneous, actually representing a family of compounds with differing fatty acid compositions and, consequently, differing chemical and physical properties. The fatty acid in the C-1 position of PC tends most often to be saturated, e.g., stearic or palmitic acid), while that in position C-2 is more likely to be monounsaturated (oleic acid) or polyunsaturated (e.g., the omega-3 fatty acids DHA [22:6] and EPA [20:5]; or the omega-6 fatty acid AA [20:4]). Newly-synthesized phosphatide molecules contain relatively larger quantities of polyunsaturated fatty acids (PUFA) than the phosphatide molecules present at steady-state[90]. This reflects either faster turnover of PUFA-containing phosphatides, or their rapid deacylation followed by reacylation with more-saturated fatty acid species, or both. Membranes of retinal and brain cells are especially rich in PUFA, particularly DHA (which comprises about 20% of the total fatty acids in retinal phospholipids and about 7% of those in brain phospholipids, respectively). As described below, administration of supplemental DHA accelerates PC synthesis, and increases brain levels of PC and other phosphatides.

Dietary choline or choline secreted into the gut can be broken down by intestinal bacteria to form trimethylamine and related amine products. This process is responsible for the "fishy odor" sometimes detected in people taking large doses of choline supplements.

Because choline is, by virtue of its quaternary nitrogen atom, highly polar, it had generally been assumed that plasma choline was unavailable to the brain. And since brain cells were also thought to be incapable of synthesizing choline de novo, the ability of cholinergic neurons to maintain the intracellular choline concentrations needed for acetylcholine (ACh)

synthesis was usually attributed either to an extraordinarily effective reuptake mechanism for reutilizing choline formed from the hydrolysis of ACh, or to the uptake into brain of circulating PC or lyso-PC. And since the poor affinity of choline acetyltransferase (ChAT), the enzyme that catalyzes choline's conversion to ACh, for choline made it likely that intracellular choline concentrations would control brain ACh synthesis, it was broadly conjectured that choline's high-affinity uptake from the synaptic cleft controlled the rate of brain ACh synthesis.

It is no longer held that brain choline levels are sustained solely by circulating phosphatides or by the high-affinity uptake of free choline from synapses, or that variations in high-affinity uptake are responsible for observed variations in brain choline levels. Choline molecules (but not those of PC or lyso-PC) do readily cross the BBB[91,92], and brain cells do indeed synthesize choline de novo[85]). Physiological variations do occur in choline levels within brain neurons; however these result principally from changes in plasma choline concentrations after eating choline-rich foods[77], or from choline's metabolism.

Free choline molecules in brain derive from four known sources, - uptake from the plasma; liberation from the PC in brain membranes; high-affinity uptake from the synaptic cleft after ACh released from a cholinergic terminal has been hydrolyzed; and, probably to a minor extent, the breakdown of newly-synthesized PC formed from the methylation of PE.

The brain can obtain circulating choline via two routes: Small amounts pass from the blood to the cerebrospinal fluid through the action of a specific transport protein, organic cation transporter 2 (OCT2), present in cells lining the choroid plexus[93]. However, orders of magnitude more choline pass bidirectionally[92] between the blood and the brain's extracellular fluid (ECF) by facilitated diffusion. This process is catalyzed by a different transport protein, localized within endothelial cells that line the brain's capillaries[92–94]. Its action is independent of sodium, and can be blocked by hemicholinium-3.

This transport protein, (RBE4), exhibits a relatively low Km for choline (estimated variously as 39–42 μM or 20 μM)[91] or 220–450 μM[92,94,95]. These differences in affinities might reflect the different methods used for their measurement. But in any case, the protein would still be unsaturated at physiological plasma choline concentrations, and its net activity still affected by variations in these concentrations.

Choline can pass in either direction, based on the gradient between its blood and brain levels[96]. When plasma choline levels are elevated (e.g., to 50 μM in the rat) by eating a choline-rich meal, choline tends to enter the brain, but when plasma choline levels are low its flux is in the opposite direction. It has been estimated that the plasma choline concentration in rats required in order for the net choline flux to be from blood to brain is about 15 μM; below this concentration net choline flux is presumably from brain to blood[96]. Once circulating choline has entered the brain's extracellular fluid it can be taken up into all cells by a low-affinity transport protein (Km = 30–100 μM), or into cholinergic nerve terminals by a high-affinity uptake protein (Km = 0.1–10 μM). The high-affinity process – unlike the passage of choline across the BBB - is energy- and sodium-dependent.

The choline in membrane PC can be liberated through the actions of the phospholipase enzymes, described above. In brain the activation of each phospholipase is tightly regulated and, in general, initiated by the interaction of a neurotransmitter or other biologic signal with a receptor coupled to a G-protein. For example, the PLC enzymes (which act on PC to yield DAG and phosphocholine, or on PI) and PLD (which acts on PC to yield phosphatidic acid and choline), are all activated when ACh attaches to M1 or M3 muscarinic receptors.

The release of choline from PC can also be enhanced, and its reincorporation into PC diminished, by sustained neuronal depolarization[97]. This process has been termed "autocannibalism" when some of the choline is diverted for the synthesis of Ach[11,98]. Autocannibalism may, by decreasing the quantities of phosphatide molecules, and thus of neuronal membranes, underlie the particular vulnerability of cholinergic neurons in certain diseases. It can be blocked by providing the brain with supplemental choline.

Acetylcholine released into synapses is very rapidly hydrolyzed to free choline and acetate by the acetylcholinesterases (EC 3.1.1.7; AChE) within the cholinergic synapse. Most of the free choline liberated by the hydrolysis of ACh, is taken back up into its nerve terminal of origin by a high-affinity choline transporter, and either reacetylated to form ACh or phosphorylated for ultimate conversion to membrane PC.

Plasma and brain DHA and EPA

The omega-3 PUFAs DHA (22:6n-3) and EPA (20:5n-3), and the omega-6 PUFA AA (22:4n-6) are long-chain derivatives of α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA: 18:2n-6), respectively. ALA and LA are essential dietary constituents for vertebrates, since these animals cannot synthesize them or their polyunsaturated products de novo. Although DHA and EPA, as well as AA can be produced in humans through the elongation and desaturation of ALA and LA, respectively, the conversion of ALA to EPA or DHA is slow, since about 75% of available ALA is shunted to β-oxidation. Furthermore, the commercial oils that provide dietary ALA, like safflower, sunflower and corn oils, also contain very high proportions of LA thus yielding disproportionately large amounts of AA which then suppresses the delta-6 desaturase enzyme that would convert LA to AA. Thus, additional EPA and DHA must be obtained from the diet, particularly from high-fat fish or foods fortified with deodorized omega-3 rich oils. No authoritative body has defined a requirement for DHA[99]; intakes as great as 3 g per day, or even more, have been used to lower plasma triglyceride levels in diabetes mellitus.

The uptakes of circulating PUFAs into the brain and brain cells involve both simple diffusion (also termed "flip-flop")[100] and protein-mediated transport[101–102]. DHA, EPA and AA are then transported from the brain's ECF into cells; activated to their corresponding CoA species (e.g., docosahexaenoyl-CoA; eicosapentaenoyl-CoA; arachidonoyl-CoA); and acylated to the sn-2 position of DAG to form PUFA-rich DAG species[103] for incorporation into phosphatides. DHA is acylated by a specific acyl-CoA synthetase, Acsl6[104] which exhibits a low affinity for this substrate (Km=26 μM[105] relative to usual brain DHA levels (1.3–1.5 μM)[106]. Hence, treatments that raise blood DHA levels rapidly increase its uptake into and retention by brain cells.

EPA can be acylated to DAG by the Acyl-CoA synthetase[107] or it can be converted to DHA by brain astrocytes[108], allowing its effects on brain phosphatides and synaptic proteins, described below, to be mediated by DHA itself. Exogenously administered AA, like DHA, is preferentially incorporated into brain phosphatides[109], as well as into other lipids, e.g. the plasmalogens. AA shares some neurochemical effects with DHA, for example, the ability to activate syntaxin-3[45], and also has other important functions, e.g. as the precursor of prostaglandins. However, unlike DHA, AA administered orally to laboratory rodents without uridine and choline apparently does not promote synaptic membrane synthesis[46] nor dendritic spine[48] formation.

AA is widespread throughout the brain, and particularly abundant in PI and PC; DHA is concentrated within synaptic regions of gray matter[110], and is especially abundant in PE and PS[111]; in contrast, EPA is found only in trace amounts in brain phosphatides, mostly in PI. No significant differences have been described between the proportions of ingested omega-3

and of omega-6 PUFAs that enter the blood, nor between the rates at which radioactively-labeled circulating DHA and AA are incorporated into brain phospholipids[109,112].

P2Y receptors as mediators of uridine effects

How does exogenous uridine – a precursor for the cytidine compounds utilized in the syntheses of PC and other cellular lipids – increase levels of cellular proteins, specifically of various pre-and post-synaptic neuronal proteins? Most likely by a second mechanism in which uridine and its phosphorylated products act as ligands for P2Y receptors which then can activate protein synthesis and normal neuronal differentiation.

Extracellular nucleotides can serve as ligands for a variety of ionotropic P2X and metabotropic P2Y receptors. While P2X receptors recognize adenine nucleotides, P2Y receptors can recognize both adenine and uridine nucleotides. Members of the P2Y family, G protein-coupled receptors, are widely distributed throughout the body, including in the brain[113]. To date, eight P2Y receptors of human origin (P2Y1, 2, 4, 6, 11, 12, 13, 14) have been cloned and characterized[113].

P2Y receptors that recognize adenine but not uridine nucleotides, i.e. the P2Y1, P2Y11, P2Y12, and P2Y13 subtypes, exist principally outside the brain. P2Y2 receptors, in contrast, are abundant in brain and are activated by UTP or ATP; P2Y4 receptors are activated by UTP, and P2Y6 receptors by UDP. Their activation, through coupling to phospholipase C (PLC), increases intracellular concentrations of DAG, IP3 and calcium[114].

That uridine nucleotides affect neurite outgrowth as well as neuronal differentiation and function by stimulating P2Y receptors[115] has been demonstrated mainly using in vitro assay systems[47,116]. UTP increases neurite outgrowth by NGF-stimulated PC-12 cells[47] and the expression of neurofilament proteins and synaptic proteins (e.g. PSD-95); these effects are blocked by P2Y receptor antagonists or by apyrase, a drug that degrades extracellular nucleotides[47]. Such P2Y-receptor-mediated actions could argue for the possible utility of P2Y agonists in treating Alzheimer's disease, especially since P2Y2 receptors are known to be selectively deficient in parietal cortex of AD brains[117].

EFFECTS OF TREATMENT WITH PHOSPHATIDE PRECURSORS ON NEURITE OUTGROWTH AND DENDRITIC SPINE FORMATION

As discussed above, the formation of a new brain synapse generally follows the interaction of a highly differentiated outgrowth, a dendritic spine, from what will become the postsynaptic neuron, with a terminal bouton of a presynaptic neuron. The number of dendritic spines at steady-state in a brain region depends on genetic factors, and also on the frequency with which the neuron is depolarized or stimulated by synaptic transmission. It is also increased in hippocampus of animals treated with the uridine-DHA-choline mixture or, less so, with DHA alone. Moreover, uridine[47], DHA[45] and choline[118] alone can increase the number of neurites projecting from PC-12 cells. AA, an omega-6 PUFA, fails to increase dendritic spines in vivo[48] but does stimulate neurite outgrowth[45].

Uridine and neurite formation by PC-12 cells

PC-12 cells which had been differentiated by nerve growth factor were exposed to various concentrations of uridine, and the number of neurites that the cells produced was measured[47]. After 4 but not 2 days uridine significantly and dose-dependently increased the number of neurities per cell. This increase was accompanied by increases in neurite branching and in the levels of the neurite proteins neurofilament M and neurofilament 70. Uridine treatment also increased intracellular levels of CTP and UTP, which suggests that it

enhanced neurite output both by stimulating PC synthesis and by activating P2Y2 receptors. The increase in neurite output was mimicked by exposing the cells to UTP, and could be blocked by various drugs known to antagonize P2Y receptors (e.g., suramin; Reactive Blue 2; pyridoxal-phosphate-6-azophenyl-2′,4′ disulfonic acid [PPADS]). Treatment of the cells with uridine or UTP also enhanced their accumulation of inositol phosphates, and this effect was also blocked by PPADS. Moreover, degradation of nucleotides by apyrase blocked the stimulatory effect of uridine on neuritogenesis.

Uridine is not unique in regulating cell differentiation and metabolism via two separate mechanisms: i.e. as a receptor agonist and a bulk precursor of CTP needed for phosphatide synthesis. Diacyglycerol also acts in two ways, both as a potent “second messenger” that activates protein kinase C, and as a bulk precursor in phosphatide synthesis, the intracellular levels of which modulate the substrate-saturation of CPT[44]. The density of P2Y2 receptors, but not other P2 receptors, is, as noted above, selectively reduced in brains of patients with Alzheimer’s disease[117]. This could reflect a loss of postsynaptic structures that contain this protein (e.g., postsynaptic densities), or perhaps the action of a toxin that inhibits neurite outgrowth and ultimately suppresses synapse formation in Alzheimer brains.

As discussed above, mature dendritic spines, the small membranous protrusions extending from postsynaptic dendrites of neurons, form and then represent excitatory glutamatergic synapses. Their numbers in particular brain regions are highly correlated with numbers of synapses and it has been proposed[23] that “more than 90% of excitatory synapses occur on dendritic spines”. This suggests that processes that damage the spines (e.g, beta-amyloid; amyloid plaques[3,119,120]) or that increase spine number (treatment with uridine, DHA, and choline, discussed below[48]) will cause parallel changes in synapse number. The formation of dendritic spines in the hippocampus is induced physiologically by synaptic inputs that induce long-term potentiation in CA1 pyramidal neurons, probably mediated by enhanced calcium influx into the postsynaptic neuron[121,122].

The effects of administering the phosphatide precursors DHA (300 mg/kg) and uridine (as UMP, 0.5%) on dendritic spine number (in CA1 pyramidal hippocampal neurons) were examined in adult gerbils treated daily for 1–4 weeks; animals received one or both compounds, as well as choline[48]. DHA alone caused dose-related increases in spine density, accompanied by parallel increases in membrane phosphatides and in specific pre- and post-synaptic proteins; its effect was doubled if animals also received uridine (UMP). In contrast, administration of the omega-6 PUFA AA, with or without uridine, had no effect on spine density nor on phosphatide nor synaptic protein levels. DHA administration has been described as promoting cognition yet its effects on neurotransmission have been obscure. Perhaps its effect on cognition is mediated in part by the increases it produces in numbers of dendritic spines or synapses.

Similar studies were performed on pregnant rats and their offspring[123]. The dams consumed UMP, DHA, or both compounds for 10 days prior to parturition and 21 days while nursing. By day 21, brains of weanlings exhibited significant increases in membrane phosphatides; various pre- and postsynaptic proteins (Synapsin-1; mGluR1, and PSD 95), and in hippocampal dendritic spine density. Perhaps administering the phosphatide precursors to lactating mothers or to infants could be useful in treating developmental disorders characterized by deficient synapses.

PHYSIOLOGICAL AND BEHAVIORAL EFFECTS OF PHOSPHATIDE PRECURSORS

Consumption by rats of a diet containing uridine (as UMP) and choline can increase dopamine (DA) and ACh levels in, and – as assessed using in vivo microdialysis - their release from, corpus striatum neurons. Dietary supplementation of aged male Fischer 344 rats with 2.5% w/w UMP for 6 weeks, ad libitum, increased the release of striatal DA evoked by potassium-induced depolarization.($P<0.05$)[53]. Giving both uridine and DHA amplified uridine's effect on DA levels[124]. In general, each animal's DA release correlated with its striatal DA content, measured postmortem. The levels of neurofilament-70 and neurofilament-M proteins, two markers of neurite outgrowth, were also increased after UMP treatment[53].

In a similar microdialysis study, ACh release, basally as well as after administration of atropine (a muscarinic antagonist which blocks inhibitory presynaptic cholinergic receptors), was found to be enhanced following UMP consumption (0.5 or 2,5% for one or six weeks ($P<0.05$)[52]. Thus, giving a uridine source may enhance some cholinergic functions, perhaps by increasing the amount of synaptic membrane, or the quantities of ACh stored in synaptic vesicles. Apparently no data are available on effects of UMP plus DHA on neurotransmitter release.

Indirect evidence that treatment with UMP alone, or with UMP plus DHA, can affect brain neurotransmission also is provided by behavioral studies[49–51]. Animals received DHA (300 mg/kg) by gavage, UMP (0.5%) in the diet, or both compounds and hippocampal- and striatal- forms of memory were measured in rats exposed to environmentally-impoverished or enriched environments for 1 month starting at weaning, and consuming a choline-containing diet. Giving either DHA or UMP improved performance in the hidden version of the Morris water maze (all $P<0.05$), a hippocampal-dependent task; co-administration of both phosphatide precursors further enhanced performance among environmentally-impoverished rats ($P<0.001$); neither giving UMP or DHA alone, nor giving both compounds affected performance by rats raised in the enriched environment, nor the performance by either group on the visible version of the Morris water maze, a striatal-dependent task. Chronic dietary administration of UMP (0.1%) alone for 3 months also ameliorated this impairment among the impoverished rats[50]. In normal adult gerbils DHA plus choline improved performance on the four-arm radial maze, T-maze and Y-maze tests; co-administering UMP enhanced these increases. These findings demonstrate that a treatment that increases synaptic membrane can enhance cognitive functions in normal animals, as well as in those reared in a restricted environment.

CLINICAL APPLICATIONS

Brains of patients with Alzheimer's disease are deficient in choline[78] and in DHA[125] and exhibit selective decreases in numbers of P2Y2 receptors[117] and dendritic spines[120] and synapses[1,2]. Since the loss of dendritic spines or synapses precedes neuronal degeneration, and is associated with cognitive deficits in both patients and animal models of Alzheimer's disease, it can be hypothesized that impaired synaptic signaling is an initial process in developing the pathologic findings and behavioral characteristics of Alzheimer's disease. The loss of spines may result from toxic effects of beta-amyloid, particularly that in senile plaques[3,119,120].

Since administering a uridine-DHA-choline mixture improved cognition and increased dendritic spine number synaptic membrane levels[4], it seemed reasonable to explore whether this treatment might also improve cognition in impaired patients with Alzheimer's disease.

A randomized, controlled, double-blind, parallel group, multi-centre, multi-country clinical trial, involving 212 drug-naïve subjects with mild Alzheimer's disease and directed by Prof. Philip Scheltens[6] was thus performed to examine the effects of a mixture including DHA, UMP, choline and "Souvenaid®", and other nutrients, e.g. vitamins B6, B12 and folic acid) on a delayed verbal memory task (derived from the Wechsler Memory Scale-revised) and the item-modified ADAS-cog at 12 weeks. The trial was pre-registered with the Dutch Trial Registry (NO. ISRCTN 722254645).

In the group receiving the mixture a significant benefit was found in mild and very mild Alzheimer's disease on the verbal memory task. The unadjusted analyses showed no significant effect on the modified ADAS-cog test. However, the baseline modified ADAS-cog score was a predictor for the intervention effect, i.e. patients with a higher baseline score showed a greater effect after treatment with the mixture. Intervention with the mixture was well tolerated (compliance was 94%) and safe. This proof-of-concept study was interpreted as demonstrating that giving a drink that contains DHA, uridine, choline and other nutrients for 12 weeks can improve memory in mild and very mild Alzheimer's disease; and that further studies now in progress, are justified.

CONCLUSION

The rates at which brain neurons form new dendritic spines and then synapses depend upon brain levels of three limiting compounds – uridine; docosahexaenoic acid [DHA]; and choline – which are precursors of the phosphatides in neuronal membranes. Hence oral administration of these compounds can increase brain phosphatide levels. Moreover the uridine, acting as an agonist for P2Y2 receptors (and perhaps the DHA, via other receptors) concurrently stimulates the production of pre- and post-synaptic proteins, and activates the mechanisms that cause synaptic membrane to be shaped into neurites, dendritic spines, and, ultimately, synapses. Administration of the three precursors for several weeks can enhance cognitive functions and neurotransmitter release in experimental animals. Moreover their administration to patients with mild Alzheimer's Disease, along with the B-vitamins that promote hepatic choline synthesis, significantly improved memory in a clinical trial involving about 220 subjects. Three additional trials are underway.

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Table 1

Effects of UMP and DHA on brain phospholipid levels.

Treatments	PC	PE	SM	PS	PI
Control diet + Vehicle	152 ± 6	65 ± 4	45 ± 2	33 ± 3	21± 2
UMP diet + Vehicle	171 ± 8[a]	84 ± 8[a]	52 ± 5	35 ± 3	31± 2[b]
Control diet + DHA	185 ± 12[a]	78 ± 5[a]	56 ± 3[a]	39 ± 3	32 ± 2[b]
UMP diet + DHA	220 ± 12[c]	113 ± 6[c]	73 ± 4[c]	46 ± 6[c]	36 ± 3[c]

Gerbils consumed a control or a UMP-containing (0.5%) diet, and received orally (by gavage) DHA (300 mg/kg); or its vehicle for 28 days. On the 29th day their brains were harvested and assayed for phospholipids. Data are given as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey test.

PC = Phosphatidylcholine; PE = Phosphatidylethanolanine; SM = Sphingomyelin; PS = Phosphatidylserine; PI = Phosphatidylinositol

[a] P<0.05;

[b] P<0.01;

[c] P<0.001 when compared with the values for Control diet + Vehicle group.

Adapted from reference [7].

Table 2

Effects of UMP and DHA on synaptic protein levels.

Treatment	PSD-95	Syntaxin-3	β-tubulin
Control diet + Vehicle	100±11	100±6	100±1
UMP diet + Vehicle	116±8	116±6	100±1
Control diet + DHA	125±11[a]	120±10[b]	93±2
UMP diet + DHA	142±5[c]	131±8[c]	102±1

Gerbils consumed a control or a UMP-containing (0.5%) diet, and received orally (by gavage) DHA (300 mg/kg): or its vehicle for 28 days. On the 29th day their brains were harvested and assayed for synaptic proteins using Western Blots. In rodents receiving the Control diet + Vehicle (i.e. the control group) arbitrary values obtained from protein band intensities were normalized to 100 in order to compare data obtained from treatment groups as percents of those of the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey test.

[a] $P<0.05$;

[b] $P<0.01$;

[c] $P<0.001$ when compared with the values for Control diet + Vehicle group.

Adapted from reference [46].