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TRANSLATIONAL STUDIES ON REGULATION OF BRAIN DOCOSAHEXAENOIC ACID (DHA) METABOLISM *IN VIVO*

Stanley I. Rapoport*

Brain Physiology and Metabolism Section, National Institute on Aging, National Institutes of Health, Bethesda, MD, USA

Abstract

One goal in the field of brain polyunsaturated fatty acid (PUFA) metabolism is to translate the many studies that have been conducted *in vitro* and in animal models to the clinical setting. Doing so should elucidate the role of PUFAs in the human brain, and effects of diet, drugs, disease and genetics. This review briefly discusses new *in vivo* radiotracer kinetic and neuroimaging techniques that allow us to do this, with a focus on docosahexaenoic acid (DHA). We illustrate how brain PUFA metabolism is influenced by graded reductions in n-3 PUFA content in unanesthetized rats. We also show how kinetic tracer techniques in rodents have helped to identify mechanisms of action of mood stabilizers used in bipolar disorder, how DHA participates in neurotransmission, regulated by calcium-independent iPLA₂β. In humans, regional rates of brain DHA metabolism can be measured with positron emission tomography and following intravenous injection of [1-¹¹C]DHA.

Keywords

docosahexaenoic acid; iPLA₂; cPLA₂; brain; biomarker; imaging; positron emission tomography; arachidonic; human; PET; diet; PUFA; phospholipase A2

INTRODUCTION

A challenge in the field of brain polyunsaturated fatty acid (PUFA) metabolism is to translate results from studies that have been conducted in the test tube, in cells *in vitro* and in animal models, to the clinical setting. Doing so may clarify human brain function involving lipid metabolism, and help to identify effects of diet, drugs, disease and genetics on this function. Sophisticated lipidomic analytical methods have been developed in pre-clinical models and applied to human body tissues (plasma, cerebrospinal fluid, or postmortem brain) in the identification of novel biological mediators and their receptor targets of therapeutic relevance [1, 2]. Genetic studies also have identified defects in PUFA metabolizing enzymes that underlie some human brain diseases [3-5].

Additionally, *in vivo* brain imaging and kinetic techniques have been elaborated in rodent and primate studies, and can be translated to address clinically relevant questions. In this paper, I discuss briefly these *in vivo* approaches and their potential applications, particularly

*Corresponding address: Brain Physiology and Metabolism Section National Institute on Aging Building 9, room 1S128 National Institutes of Health Bethesda, MD 20892, USA sir@helix.nih.gov.

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with regard to docosahexaenoic acid (DHA, 22:6n-3). DHA is found in high concentrations in the stereospecifically numbered (*sn*)-2 position of brain membrane phospholipids and is critical for maintaining normal brain structure, function and metabolism. It influences brain signal transduction, gene transcription, and membrane stability, and is a precursor for neuroprotectins, resolvins and other antiinflammatory products [6-8].

DIETARY EFFECTS ON BRAIN α -LNA, DHA AND DPAn-6

Clinical evidence

The brain concentration of DHA depends on the dietary n-3 PUFA content and on hepatic synthesis and secretion of DHA from its circulating shorter-chain nutritionally essential precursor, α -linolenic acid (α -LNA, 18:3n-3), as well as from circulating eicosapentaenoic acid (EPA, 20:5n-3) [9, 10], [11]. Epidemiological studies have suggested that a low dietary DHA + EPA intake, due to low intake of fish or fish products, is associated with a number of human neuropsychiatric diseases, including Alzheimer disease, bipolar disorder and depression, and that dietary EPA and/or DHA supplementation may be helpful in some of these conditions. Studies also have reported reduced DHA levels in blood or postmortem brain tissue in Alzheimer disease, bipolar disorder and other brain disorders [12-15]. Supporting these clinical results are rodent studies, involving dietary n-3 PUFA depletion for as long as 3 generations but as short as 15 weeks in a single generation, that indicate that n-3 PUFA dietary deficiency can disturb brain function and behavior [16, 17]. However, some of the clinical observations have not been confirmed [18-21], and the deprivation studies in rodents may have been too extreme to be clinically relevant.

Graded dietary n-3 PUFA reductions in rats—To more closely consider effects of dietary n-3 PUFA deficiency on brain function and metabolism, we determined plasma, liver and brain PUFA concentrations and brain and liver expression of PUFA-metabolizing enzymes in rats, in relation to graded dietary n-3 PUFA reductions for 15 weeks after weaning at 21 days. We chose a reference diet containing 4.6% (of total fatty acid) α -LNA, free of DHA or arachidonic acid (AA, 20:4n-6) [22]. This diet is nutritionally “adequate” for maintaining normal body organ function and DHA metabolism in rats [23, 24].

Compared with their concentrations in rats fed the n-3 PUFA “adequate” diet, plasma unesterified and esterified α -LNA and DHA concentrations fell progressively with the reduction in dietary α -LNA (Figure 1). The threshold (indicated as arrows in Figure 1) that produced a statistically significant reduction in unesterified plasma α -LNA or DHA concentration compared to control concentration was 2.8% dietary α -LNA, whereas the threshold for significant reductions in total (mainly esterified) plasma α -LNA and DHA concentrations was 1.7% dietary α -LNA. Plasma levels of unesterified and total AA were not changed significantly by any dietary α -LNA reduction, whereas the plasma esterified concentration of docosapentaenoic acid (DPA 20:5n-6) was elevated at 1.7% dietary α -LNA, while unesterified plasma DPAn-6 was first elevated at 0.8% α -LNA. These DPA changes reflected liver synthesis and secretion (Figure 2) [25].

Brain DHA concentration remained unchanged down to 1.7% dietary α -LNA (Figure 2), at which point activity of DHA-selective calcium independent phospholipase A₂ (iPLA₂ VIA(β)) was downregulated [22] and activity of calcium dependent cPLA₂ IVA was upregulated. Since brain and liver AA concentrations were unaffected at all dietary α -LNA levels, it cPLA₂ IVA may hydrolyze DPAn-6 as well as AA from brain phospholipid. In any case, homeostatic mechanisms maintained a normal brain DHA concentration down to 1.7% dietary α -LNA, despite reduced plasma unesterified and esterified DHA concentrations. They also suggest that brain DHA fell significantly only when only following its displacement by circulating liver-derived DPAn-6.

Translational relevance—The observations using graded deprivation in rats indicate that the brain DHA level is related nonlinearly to the plasma esterified and unesterified DHA concentrations. Establishing these relations could lead to using these peripheral concentrations as “biomarkers” of the brain DHA level and metabolism [26]. Reports that overall mortality or mortality from any cause does not differ significantly between vegetarians and omnivores, despite a 50% low blood DHA concentration in vegetarians [27, 28], support further clarifying the relation of plasma DHA to brain DHA functional integrity and metabolism. This might be accomplished with the help of positron emission tomography (PET) (see below).

MEASURING *IN VIVO* BRAIN DHA KINETICS

New kinetic paradigms

Despite the unchanged brain DHA concentration at 1.7% dietary α -LNA in the face of reduced plasma DHA concentrations in the graded study, rates of brain DHA metabolism and of formation of downstream DHA metabolites [29] may have been reduced. They are reduced following after feeding the 0.2% α -LNA diet (see below) [30]. This could be tested by applying new *in vivo* kinetic methods and models in unanesthetized animal models and to some extent in humans. These *in vivo* kinetic approaches include: (1) quantitation of PUFA turnover due to deacylation-reacylation in brain phospholipids (Figure 3) [31, 32]; (2) quantitation of PUFA flows (fluxes) from plasma into different brain and chemically identifiable lipid compartments, including the acyl-CoA pool and individual phospholipids, triacylglycerol and cholesteryl esters; (3) quantitative imaging of net PUFA incorporation into different brain regions [33-36]. Some of these approaches, particularly those involving neuroimaging, have been translated with the help of PET for human studies (see below) [37-39].

Unesterified PUFA is taken up preferentially from plasma into brain

In vertebrates, the long-chain PUFAs, AA and DHA, cannot be synthesized *de novo* from 2-carbon chains [40]. They must be obtained from the diet or *via* liver synthesis from their circulating nutritionally essential shorter-chain precursors, α -LNA or linoleic acid (LA, 18:2n-6), respectively. Fatty acids enter the brain from plasma mainly in their unesterified form, as was shown in a study in which a radiolabeled fatty acid was fed to rats [41]. The labeled fatty acid appeared soon thereafter when esterified in triglycerides of circulating lipoproteins, but it was taken up by brain incorporation started only after it had been hydrolyzed to the unesterified form, at a rate reported following direct intravenous injection (see below). This conclusion is supported by studies showing that mice genetically lacking lipoprotein receptors show no difference in whole brain PUFA concentrations [42].

Fatty acid incorporation into brain phospholipid

Once having entered brain from plasma, unesterified DHA is largely (> 80%) and selectively delivered *via* an acyl-CoA synthetase and acyltransferase to the *sn*-2 position of membrane phospholipids, particularly ethanolamine glycerophospholipid and phosphatidylcholine (Figure 3). In contrast, its shorter-chain circulating unesterified precursors, α -LNA and EPA, that enter brain are largely (> 99%) oxidized [43-47]. The activities of conversion enzymes, elongases 2 and 5 and Δ 5 and Δ 6 desaturases, are much lower in brain than in liver, and unlike the liver enzymes are not upregulated by dietary n-3 PUFA deprivation [48, 49].

Measuring incorporation rates into brain phospholipid in vivo

It is possible to image rates of incorporation of unesterified unlabeled plasma DHA (or AA) into the brain of unanesthetized rodents. These rates equal the rates of consumption and are independent of cerebral blood flow (see below). For example, radiolabeled DHA bound to serum albumin (e.g., [1-¹⁴C]DHA) is infused intravenously for T = 5 min, after which regional brain radioactivity is measured on sections of frozen brain using quantitative autoradiography. Regional incorporation coefficients k^* are calculated by normalizing radioactivity to the integrated plasma radioactivity during infusion (input function) (Eq. 1). Multiplying k^* by the unlabeled plasma unesterified DHA concentration gives incorporation rates, J_{in} , of unlabeled unesterified DHA (where the asterisk identifies labeled DHA) [33, 50],

$$k^* = \frac{c_{brain(DHA)}^*}{\int_0^T c_{plasma(DHA)}^* dt} \quad \text{Eq. 1}$$

$$J_{in} = k^* c_{plasma(DHA)} \quad \text{Eq. 2}$$

Measurements also can be made on whole rodent brain that has been subjected to rapid high energy microwaving to prevent post-mortem release fatty acid hydrolysis [51]. The ratio of specific activity of the brain DHA-CoA pool to that of plasma DHA (dilution coefficient λ) then can be used to calculate DHA turnover and half life in individual brain phospholipids,

$$\text{Turnover} = J_{in} / \lambda c_{brain(DHA)} \quad \text{Eq. 3}$$

$$\text{Half-life} = 0.693 / \text{turnover} \quad \text{Eq. 4}$$

PUFA half-lives within mammalian brain phospholipids can be a few hours or less [34, 52, 53], and may be the only measurable lipid endpoint demonstrating significant drug or diet effects. For example, the hypothesis that mood stabilizers approved for treating patients in bipolar disorder downregulate brain AA metabolism was proposed from showing that lithium, carbamazepine and valproic acid, when given chronically to rats, reduced AA but not DHA or palmitate turnover in brain phospholipids [54-56]. “Metabolic leakages” associated with turnover in brain phospholipids are the formation of bioactive eicosanoids such as prostaglandin E₂ (PGE₂) and thromboxane B₂ (TXB₂) for AA and docosanoids for DHA, and β -oxidative or other catabolic pathways [57].

PUFA turnover and half-life due to deacylation-reacylation (Eqs. 3 and 4) differ from turnover and half-life due to overall metabolic loss and reincorporation from plasma [30, 58, 59]. The latter are related to the corresponding deacylation-reacylation parameters by the dilution factor λ of the acyl-CoA pool (cf. Eq. 4) [60]. Thus,

$$\text{DHA turnover due to metabolic loss and synthesis} = J_{in} / c_{brain(DHA)} \quad \text{Eq. 5}$$

PLA₂ INFLUENCE IN VIVO BRAIN DHA METABOLISM

Relevant enzymes that regulate deacylation-reacylation include (1) AA-selective calcium-dependent cytosolic cPLA₂ type IVA, which can be activated *via* G-protein-coupled neuroreceptors such as serotonergic 5-HT_{2A/2C} receptors [61, 62], dopaminergic D₂-like neuroreceptors [63] and cholinergic muscarinic M_{1,3,5} receptors [64], as well as of

ionotropic N-methyl-D-aspartate (NMDA) receptors that promote extracellular calcium entry into the cell [65]; (2) secretory presynaptic sPLA₂, which requires a high calcium concentration (20 mM) for activation; and (3) DHA selective calcium-independent iPLA₂, which can be activated through muscarinic or serotonergic neuroreceptors [44, 66, 67]. cPLA₂ and iPLA₂ have post-synaptic as well as other cellular locations in neurons, and are found in astrocytes [67-70], and are coupled to and be colocalized with downstream enzymes, cyclooxygenases, lipoxygenases and cytochrome p450 epoxygenases, within the AA and DHA cascades [57, 71].

DHA-selective calcium-independent iPLA₂β

There are two iPLA₂ isoforms in the mammalian brain, iPLA₂β and iPLA₂γ [67]. Mutations in the *PLA2G6* gene for iPLA₂ VIA (iPLA₂β) contribute to a number of human neurodevelopmental and neurodegenerative diseases, including idiopathic neurodegeneration plus brain iron accumulation and dystonia-parkinsonism without iron accumulation [3, 5]. Although not activated by calcium *in vitro* or by entry of extracellular calcium into the cell *in vivo* [72-74], iPLA₂ may be activated by calcium released from the endoplasmic reticulum, thereby displacing it from calmodulin [67].

Mice lacking the *PLA2G6* show neurological dysfunction and significant neuropathology at 13 but not 4 months of age [75]. Nevertheless, showed with *in vivo* imaging that DHA metabolism and signaling are markedly disturbed in 4-month old iPLA₂β-deficient mice, and that brain expression of iPLA₂γ was not upregulated [76, 77]. Saline or the cholinergic muscarinic M1,3,5 agonist, arecoline, was administered to unanesthetized homozygous, heterozygous or wildtype mice (iPLA₂β (−/−), (+/−), or (+/+) respectively). [1-¹⁴C]DHA was infused intravenously followed by neuroimaging using quantitative autoradiography. DHA incorporation coefficients and rates in iPLA₂β (−/−) and (+/−) mice compared with iPLA₂β (+/+) mice were markedly reduced at baseline. Arecoline increased both parameters in the iPLA₂β (+/+) mice, but significantly less so in iPLA₂β (−/−) and iPLA₂β (+/−) mice. PET thus might be used, with preclinical justification, to image brain DHA metabolism in patients with *PLA2G6* mutations (see below).

IMAGING BRAIN DHA CONSUMPTION

The rate of incorporation of unesterified plasma DHA into brain phospholipids of unanesthetized adult rats, determined following an intravenous injection of radiolabeled DHA (Eq. 2), equaled 0.19 μmol/gram brain per day [78]. We confirmed that this rate equals the rate of DHA metabolic loss in a direct experiment, by injecting [4,5-³H]DHA into the brain and measuring brain radioactivity and DHA concentrations in rats killed during the following 60 days [59]. This study gave a whole brain DHA half-life of 33 days and consumption rate of 0.25 μmol/g/day [30], equal to the DHA incorporation obtained with the single time point by intravenous injection. The simplicity of the single time point injection procedure, when applied with quantitative autoradiography (e.g. Figure 4), makes it ideal for rapidly measuring brain DHA consumption in unanesthetized rodents using quantitative autoradiography, or in humans with PET. Quantitative imaging can be performed in different brain activation or neuropathological states, since AA or DHA incorporation from plasma is independent of changes in cerebral blood flow [79, 80].

Additional measurements with intracerebroventricular [4,5-³H]DHA showed that feeding the deficient 0.2% α-LNA diet (see above) for 15 weeks prolonged the DHA metabolic half-life to 90 days and reduced consumption to 0.06 μmol/g/day. Also, iPLA₂β was downregulated, which would be expected to help to preserve brain DHA. We now are determining whether the 1.7% α-LNA diet, which did not reduce brain DHA but nevertheless reduced plasma DHA (Figs. 1 and 2), also prolongs DHA loss half life and

reduces DHA consumption. This could increase risk for neuroinflammation and cognitive dysfunction [17].

The equivalence between J_{in} for DHA calculated using a single intravenous injection, and the DHA consumption rate calculated by intracerebroventricular injection followed by sampling brain from many animals over a 60-day period [30, 59], suggests that injection-derived images can be used as biomarkers of brain DHA consumption [26]. Accordingly, we synthesized positron-labeled [$1-^{11}\text{C}$]DHA and used PET to image DHA brain incorporation in adult human volunteers [37, 81]. Incorporation coefficients k^* for DHA were higher in gray than white matter regions. For the entire human brain, the net DHA incorporation rate J_{in} , the product of k^* and the unesterified plasma DHA concentration, equaled 3.8 ± 1.7 (S.D.) mg/day (Figure 5).

SUMMARY AND CONCLUSIONS

A challenge in the field of brain PUFA metabolism is to translate the studies that have been conducted *in vitro* and in animal models to the clinical setting. This should help to estimate effects of aging, diet, drugs, disease and genetics on consumption. In this review, we discuss new *in vivo* radiotracer kinetic and neuroimaging techniques that allow us to do this, while focusing on DHA.

Brain DHA concentration depends on dietary n-3 PUFA content and liver metabolism. Our study in adult rats, using 15-week graded reductions in dietary α -LNA content below a reference level (4.6% α -LNA) considered nutritionally adequate, showed that the plasma DHA concentration fell below control before the brain DHA concentration declined significantly, at which point DHA was displaced by circulating DPAn-6.

We have outlined in this study how kinetic approaches involving tracer can be used to measure AA or DHA incorporation, turnover and half-lives in brain. This incorporation rate, the product of the incorporation coefficient and unesterified plasma concentration, is independent of brain blood flow and equivalent to the rate of metabolic consumption. It can be measured in humans with the help of PET. Imaging of DHA incorporation under different experimental or clinical conditions should further elucidate its role in health and disease, and identify specific effects of drugs, genetics or dietary manipulation.

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Abbreviations

DHA	docosahexaenoic acid
AA	arachidonic acid
α-LA	linoleic acid
α-LNA	α -linolenic acid
DPA	docosapentaenoic acid
EDP	epoxy-docosapentaenoic acid
EPA	or eicosapentaenoic acid
PLA₂	phospholipase A ₂

cPLA₂	cytosolic PLA ₂
sPLA₂	secretory PLA ₂
iPLA₂	calcium-independent PLA ₂
NMDA	N-methyl-D-aspartate
PUFA	polyunsaturated fatty acid
PET	positron emission tomography

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Figure 1. Plasma unesterified and total fatty acid concentrations in rats fed different α -LNA containing diets for 15 weeks

Values are mean $\pm$ SD (n = 6 per group). Superscripts show significant differences at $p < 0.05$ from mean at 4.6% dietary α -LNA. Arrows indicate threshold difference from control. From [22].

Figure 2. Total fatty acid concentrations in liver and brain of rats fed different α -LNA containing diets for 15 weeks

Values are mean $\pm$ SD (n = 6 per group). Superscripts show significant differences at $p < 0.05$ from mean at 4.6% dietary α -LNA. Arrows indicate threshold difference from control. From [22].

BRAIN DOCOSAHEXAENOIC ACID CASCADE INITIATED BY SYNAPTIC $iPLA_2$ ACTIVATION

Figure 3. Model of brain DHA cascade

DHA at the *sn*-2 position of a phospholipid is hydrolyzed by receptor-mediated activation (star) of calcium-independent phospholipase A_2 ($iPLA_2$) at the synapse. A small fraction of liberated DHA is converted to bioactive docosanoids. The remainder diffuses to the endoplasmic reticulum while bound to a fatty acid binding protein (FABP), from where it is converted to DHA-CoA by acyl-CoA synthetase with the consumption of 2 ATPs, then re-esterified by an acyltransferase. Unesterified DHA in the endoplasmic reticulum exchanges freely and rapidly with unesterified DHA in plasma, into which labeled DHA (DHA*) has been injected. Equations for calculating kinetic parameters are shown in right lower corner.

Figure 4. Reduced baseline and arecoline-initiated DHA signals in 4- month old iPLA₂β (VIA) knockout mice

Autoradiographs of coronal brain sections showing effects of genotype and arachidonic on regional DHA incorporation coefficients k^* in wildtype (+/+), heterozygous (+/-) and homozygous (-/-) iPLA₂β knockout mice. Incorporation coefficient k^* given on color scale. Abbreviations: CPu, caudate-putamen; Hb, habenular nuclei; Hipp, hippocampus; Mot, motor cortex; SN, substantia nigra; Vis, visual cortex. From [76].

DAILY DHA CONSUMPTION RATE BY HUMAN BRAIN
 Horizontal PET images of incorporation coefficients¹ k^* for DHA in
 healthy human volunteers following i.v. [$1\text{-}^{11}\text{C}$]DHA

Global DHA Daily Consumption Rate $J_{in} = k^* c_{\text{plasma,DHA}}$

3.8 ± 1.7 (SD) mg/brain/day

¹Partial volume corrected

$c_{\text{plasma,DHA}}$ (nmol/ml): 2.63 ± 1.17

Figure 5. Imaging daily DHA consumption rate by human brain

Measurements were performed by injecting [$1\text{-}^{11}\text{C}$]DHA intravenously in volunteers and using positron emission tomography. From [37].