Imaging plasma docosahexaenoic acid (dha) incorporation into the brain *in vivo*, as a biomarker of brain DHA: Metabolism and neurotransmission

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Abstract: *Docosahexaenoic acid (DHA) is critical for normal brain structure and function, and its brain concentration depends on dietary DHA content and hepatic conversion from its dietary derived n-3 precursor, α-linolenic acid (α-LNA). We developed an* in vivo *method in rats using quantitative autoradiography to image incorporation into brain of unesterified plasma DHA, and showed that the incorporation rate equals the rate of brain metabolic DHA consumption. Thus, quantitative imaging of DHA incorporation from plasma into brain can be used as a* biomarker *of brain DHA metabolism and neurotransmission. The method has been extended to humans with the use of positron emission tomography (PET). Furthermore, imaging in unanesthetized rats using DHA incorporation as a biomarker in response to N-methyl-D-aspartate (NMDA) administration confirms that regional DHA signaling is independent of extracellular calcium, and likely mediated by a calcium-independent phospholipase A_2 ($iPLA_2$). Studies in mice in which $iPLA_2$-VIA (β) was knocked out confirmed that this enzyme is critical for baseline and muscarinic cholinergic signaling involving DHA.*

Key words: *docosahexaenoic acid, $iPLA_2$, $cPLA_2$, brain, biomarker, imaging, positron, arachidonic, calcium, rat, human, PET*

Docosahexaenoic acid (DHA, 22:6n-3), an n-3 polyunsaturated fatty acid (PUFA), is essential for maintaining normal brain structure, function and metabolism, and its brain concentration depends on dietary DHA content and liver synthesis of DHA from its shorter chain dietary precursors, particularly α-linolenic acid (α-LNA (18:3n-3)) (Gao *et al.*, 2009a). DHA participates in signal transduction, gene transcription and other important functions, and is a precursor for neuroprotectins, resolvins and other antiinflammatory products (Bazan, 2009; Salem *et al.*, 2001).

Multiple rodent studies have demonstrated that prolonged dietary n-3 PUFA deprivation, from 15 weeks to as long 3 generations, results in reduced brain DHA content within individual phospholipids, associated with increased brain concentrations of docosapentaenoic acid (DPA)n-6 (22:5n-6) largely derived from liver biosynthesis (Salem *et al.*, 2001). These studies generally involved euthanization and direct chemical analysis of brain lipid content, limiting our ability to interpret *in vivo* brain DHA metabolism and kinetics. In view of conflicting evidence that low dietary intake of DHA-containing fish products is correlated with multiple human brain diseases, including Alzheimer disease, and that dietary DHA supplementation may be helpful in some of these conditions (Conquer *et al.*, 2000; Quinn *et al.*, 2010), it would be useful to have an *in vivo* biomarker of regional brain DHA consumption that could be used in awake animals and in humans to test the efficacy of DHA supplementation and other experimental or clinical conditions. Further, it would be useful to understand DHA participation in signal transduction, thus if DHA incorporation into brain might be used as a biomarker of regional brain metabolism and neurotransmission in health and disease.

Neither arachidonic acid (AA, 20:4n-6) nor DHA can be synthesized *de novo* from 2 carbon chains in vertebrates (Holman, 1986). Both long-chain PUFAs enter the brain from the circulation, but quantitation of their rates of entry as well as of their disposition within brain have remained controversial. In a study involv-

Abbreviations

DHA	docosahexaenoic acid
AA	arachidonic acid
DPA	docosapentaenoic acid
α-LNA	α-linolenic acid
EPA	eicosapentaenoic acid
PLA_2	phospholipase A_2
$cPLA_2$	cytosolic PLA_2
$sPLA_2$	secretory PLA_2
$iPLA_2$	calcium-independent PLA_2
NMDA	N-methyl-D-aspartate
PUFA	polyunsaturated fatty acid
PET	positron emission tomography

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ing feeding a radiolabeled fatty acid to rats (Purdon *et al.*, 1997), we showed that only after its hydrolysis from circulating lipoprotein was its brain uptake measurable, and at a rate equivalent to the rate following injecting the unesterified fatty acid intravenously, thus arguing for uptake of the unesterified fatty acid. This finding was supported by studies in mice genetically lacking lipoprotein receptors (Chen *et al.*, 2008). Calculations of on-off kinetics of unesterified fatty acids bound to serum albumin indicate that about 5% of the fatty acid is removed from albumin as blood passes through brain (Robinson *et al.*, 1992).

Once having entered brain, unesterified DHA is largely (>80%) and selectively delivered *via* an acyl-CoA synthetase and acyltransferase to the stereospecifically numbered *sn*-2 position of phospholipids, while its precursors α-LNA or eicosapentaenoic acid (EPA, 20:5n-3) are largely β-oxidized within mitochondria after transfer by carnitine acyltransferase (Chen *et al.*, 2011; DeGeorge *et al.*, 1991; DeGeorge *et al.*, 1989; DeMar *et al.*, 2005; Gavino and Gavino, 1991). Additionally, elongases and desaturases that can convert the n-3 precursors to DHA, while present, have very low brain activities and are unaffected by dietary n-3 PUFA deprivation (Igarashi *et al.*, 2007).

It is possible to calculate and image the rate of incorporation of unesterified unlabeled DHA in a single study in an unanesthetized rodent, by infusing radiolabeled [1-^{14}C]DHA intravenously and measuring regional incorporation coefficients k* into brain using quantitative autoradiography, then multiplying the unlabeled concentration of unesterified plasma DHA by the incorporation coefficient to calculate the incorporation rate, J_{in}, of unlabeled DHA, where the asterisk identifies labeled plasma or brain concentrations of DHA (Robinson *et al.*, 1992).

$$k^* = \frac{C^*_{brain(DHA)}}{\int_0^T C^*_{plasma(DHA)}}$$

$$J_{in} = k^* \, C_{plasma(DHA)}$$

Doing this gives a whole brain incorporation rate of DHA equal to about 0.19 μmol/gram brain per day in the adult rat (Contreras *et al.*, 2000). Confirmation that this calculated incorporation rate from a single study equals the rate of whole brain DHA consumption was derived from separate studies in multiple rats in which [4,5-^{3}H]DHA was injected into the cerebral ventricles and brain DHA radioactivity and concentrations were followed in animals killed animals from 0 to 60 days thereafter. A whole brain half-life of 33 days was calculated for DHA, giving a daily rate of DHA consumption of 0.25 μmol/g/day (DeMar *et al.*, 2004), equivalent to the single injection value considering the variance of the data. The efficiency and simplicity of the single injection measurement, using quantitative autoradiography, makes it ideal for measuring whole brain DHA consumption.

Additional measurements with intracerebroventricular [4,5-^{3}H]DHA showed that 15 weeks of dietary n-3 PUFA deprivation prolonged the DHA half-life in brain to 90 days and reduced brain DHA consumption to 0.06 μmol/g/day, while downregulating expression of calcium-independent phospholipase A_2 ($iPLA_2$)-VIA (β) (Rao *et al.*, 2007). *In vitro* studies indicate that this enzyme is selective for the hydrolysis of DHA from membrane phospholipid (DeMar *et al.*, 2004; Garcia and Kim, 1997; Strokin *et al.*, 2004). The observed downregulation of enzyme expression with would be expected to help to preserve brain DHA. As DHA metabolites, many of which are antiinflammatory and include neuroprotectins and resolvins, are the major pathways of DHA metabolic loss in brain (Bazan, 2009), these results provided a basis for the reduced resistance to neuroinflammation and cognitive dysfunction in animals subjected to dietary n-3 PUFA deprivation (DeMar *et al.*, 2006; Farooqui *et al.*, 2007).

The equivalence between J_{in} for DHA calculated from an intravenous infusion injection at a single time point, and the DHA consumption rate calculated by intracerebroventricular injection followed by sampling brain from multiple animals over a 60-day period, indicates that the single time point measurement *represents* a biomarker of brain DHA consumption. Accordingly, we synthesized positron-labeled [1-^{11}C]DHA and conducted studies using positron emission tomography (PET) to quantitatively image incorporation of unesterified plasma DHA into the brain of adult healthy human volunteers (Channing and Simpson, 1993; Umhau *et al.*, 2009). Values of incorporation coefficients k* for DHA were higher in gray than white matter brain 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 mg/day *(figure 1)*. This net rate, approximating the net rate of DHA consumption by brain, is less than the suggested human diet DHA supplementation 200 mg per day of DHA (Kris-Etherton *et al.*, 2000).

In an as yet unpublished study (Kim *et al.*, submitted), we subjected rats post-weaning for 15 weeks to DHA-free diets having graded reductions in α-LNA content below a dietary ''sufficient'' level containing 4.6% α-LNA (DeMar *et al.*, 2004). While plasma DHA fell in rough proportion to the reduction in dietary α-LNA, the brain DHA concentration surprisingly was maintained down to 1.7% dietary α-LNA, suggesting that brain DHA does not track plasma DHA until quite large reductions in plasma concentration arise. In this regard, plasma DHA is reduced by 50% in vegetarians compared with omnivores (Rosell *et al.*, 2005), despite there being no difference in overall mortality or mortality from any general cause between the two groups (Key *et al.*, 2009). Together, the rodent and human data suggests that using the blood DHA concentration as a biomarker of brain DHA integrity may be incorrect. Thus, it would be of importance to determine whether brain consumption of DHA, measured with our intravenous infusion method in rodents or humans, compared with the brain concentration of DHA, measured by direct determination in rodents, is the major determinant of brain functional integrity by measuring consumption in relation to plasma DHA content in rats or in humans with the intravenous infusion method.

One approach that as has not been exploited sufficiently is the use of radiolabeled DHA to image its role in regional brain signal transduction and neuroplasticity using quantitative autoradiography *in vivo*. For example, 3 months after removing one eye in a rat, DHA incorporation from plasma into the contralateral brain regions, e.g. superficial gray matter of the superior colliculus and dorsal lateral geniculate nucleus, that normally were innervated by the eye

that was removed, was significantly reduced, emphasizing a role for DHA in signaling and neuroplasticity that deserves to be exploited (Wakabayashi *et al.*, 1995). DHA incorporation also was altered in an L1210 leukemia cells implanted in rat brain, suggesting a role for DHA in tumor metabolism (Nariai *et al.*, 1994).

Several groups of PLA_2 enzymes have been identified in the mammalian brain, and their specificity has been characterized based *in vitro* studies (Six and Dennis, 2000). Questions remain about their locations and functions in the intact organism, however. These include (1) AA-selective calcium-dependent cytosolic $cPLA_2$ type IVA, which can be activated *via* multiple G-protein-coupled neuroreceptors, including serotonergic $5\text{-}HT_{2A/2C}$ receptors (Berg *et al.*, 1998; Qu *et al.*, 2005), and muscarinic $M_{1,3,5}$ receptors (Bayon *et al.*, 1997), and the ionotropic N-methyl-D-aspartate (NMDA) receptor which when activated allows extracellular calcium into the cell (Basselin *et al.*, 2006); (2) secretory presynaptic $sPLA_2$ which requires a high calcium concentration (20 mM) for activation, and (3) calcium-independent $iPLA_2$, which is considered DHA-selective, and can be activated through both muscarinic and serotonergic receptors (DeGeorge *et al.*, 1991; Garcia and Kim, 1997). Both $cPLA_2$ and $iPLA_2$ have postsynaptic locations in mammalian brain (Ong *et al.*, 1999; Ong *et al.*, 2005).

Recognizing the *in vitro* enzyme selectivity of $cPLA_2$ and $iPLA_2$ for AA and DHA, respectively, we confirmed their *in vivo* dependencies on extracellular-derived calcium in unanesthetized rats by showing that NMDA administration increased incorporation of intravenously injected radiolabeled AA but not of radiolabeled DHA into the brain (Ramadan *et al.*, 2010) *(figure 2)*. The results suggest that greater AA than DHA release during glutamate-induced excitotoxicity could cause brain cell damage since high concentrations of AA and its metabolites are considered to be neurotoxic and proinflammatory (Bazan *et al.*, 1981).

We also confirmed a role for $iPLA_2$-VIA ($iPLA_2\beta$) in brain DHA signaling *in vivo* (Basselin *et al.*, 2010; DeGeorge *et al.*, 1991), consistent with evidence that this enzyme can be activated by calcium derived from intracellular calcium stores of the endoplasmic reticulum, thereby

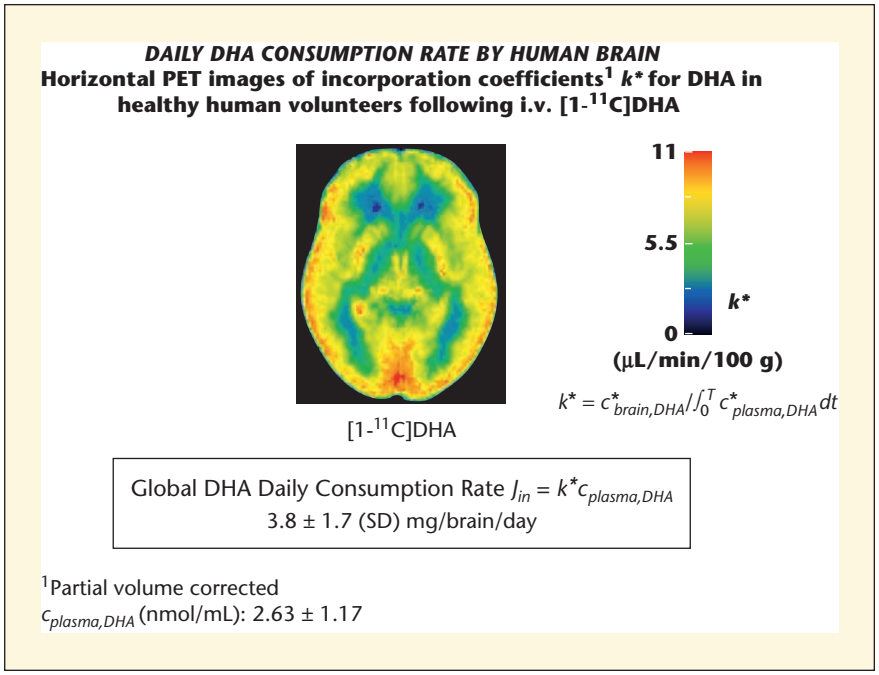

Figure 1. *Daily DHA consumption rate by human brain. Measurements were performed by injecting [1-^{11}C]DHA intravenously in volunteers and using positron emission tomography (Umhau* et al.*, 2009).*

displacing it from calmodulin (Rosa and Rapoport, 2009). Mutations in the PLA2G6 gene encoding the enzyme occur in patients with idiopathic neurodegeneration plus brain iron accumulation and dystonia-parkinsonism without iron accumulation (Kurian *et al.*, 2008), whereas mice lacking PLA2G6 show

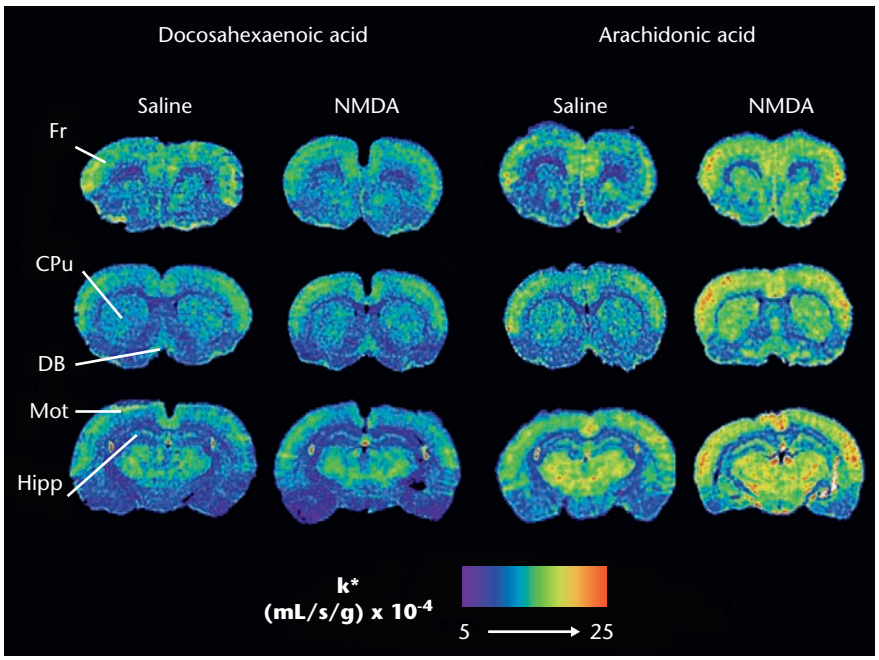

Figure 2. *NMDA (25 mg/kg i.p.) initiates arachidonic but not docosahexaenoic acid signal in rat brain. Coronal autoradiographs of brains from rat injected with NMDA compared to animals injected with saline. FR, frontal cortex; CPU, caudate-putamen, DB, diagonal band; Mot, motor cortex; Hipp, hippocampus. Incorporation coefficients k* are color-coded. From (Ramadan* et al.*, 2010).*

neurological dysfunction and significant neuropathology after 13 but not 4 months of age. We hypothesized that brain DHA metabolism and signaling in response to the cholinergic muscarinic $M_{1,3,5}$ agonist, arecoline (DeGeorge *et al.*, 1991) would be reduced in 4-month-old iPLA$_2$β-deficient mice without overt neuropathology. Saline or arecoline (30 mg/kg) was administered to unanesthetized (homozygous, heterozygous or wildtype mice iPLA$_2$β (-/-), (+/-), or (+/+) mice), and [1-^{14}C]DHA was infused intravenously. DHA incorporation coefficients and rates representing DHA metabolism were determined using quantitative autoradiography in 81 brain regions. iPLA$_2$β(-/-) and (+/-) mice compared with iPLAβ (+/+) mice showed widespread and significant baseline reductions in k* and J_{in} for DHA. Arecoline increased both parameters in brain regions of iPLA$_2$β(+/+) mice but quantitatively less so in iPLA$_2$β(-/-) and iPLA$_2$β (+/-) mice (Basselin *et al.*, 2010). Consistent with iPLA$_2$β's reported ability to selectively hydrolyze DHA from phospholipid *in vitro*, a genetic iPLA$_2$β deficiency reduced brain DHA metabolism and signaling *in vivo* at baseline and following $M_{1,3,5}$ receptor activation by arecoline. Positron emission tomography might be used to image disturbed brain DHA metabolism in patients with PLA2G6 mutations.

Conclusion

Brain imaging of DHA incorporation (consumption) as a biomarker of DHA metabolism following a single intravenous injection of radiolabeled DHA may prove useful in studying the role of DHA in health and disease, and in investigating the influence of diet, in animal and humans. As noted above, in a study in which plasma DHA was reduced in relation to reduced dietary α-LNA in rats subjected to 15 weeks of a DHA-free diet containing different α-LNA content starting with 4.6% DHA (adequate diet), brain DHA did not follow plasma DHA but fell only after dietary DHA had declined by more than half, to 1.7% α-LNA (Kim *et al.*, submitted). Thus brain DHA did not track plasma DHA when the latter was reduced markedly by diet. A similar lack of tracking may occur in humans a well.

Another important issue is that brain DHA content and metabolism depend not only on diet, but also on the ability of the liver to synthesize DHA from circulating α-LNA, making it critical to be able to assess liver synthesis under different dietary conditions. To address this issue, we have developed a method involving a constant intravenous infusion of heavy isotopically labeled precursor [U-^{13}C]α-LNA for 2 h in rats on a DHA containing diet, while measuring labeled and unlabeled n-3 PUFA in arterial plasma using negative chemical ionization GC-MS (Gao *et al.*, 2009b). Newly synthesized esterified [^{13}C]DHA, [^{13}C]EPA, and [^{13}C]DPA (22:5n-3) appeared in arterial plasma after 60 min of infusion, then their concentrations rose in an S-shaped manner. Esterified concentration × plasma volume data were fit with a sigmoidal equation, whose peak first derivatives provided synthesis rates of unlabeled EPA, DPA, and DHA. The DHA synthesis rate exceeded the published daily rat brain DHA consumption rate by 30-fold, suggesting that liver synthesis from α-LNA could maintain brain DHA homeostasis were DHA absent from the diet. Conversion rate from infused isotopically labeled EPA also were measured (Gao *et al.*, 2009b). More recently, we showed that the synthesis rates from α-LNA were markedly elevated when DHA was absent from the diet, as was expression of appropriate liver elongases and desaturases (Gao *et al.*, unpublished). This stable isotope infusion method could be used to quantify whole-body DHA synthesis rates in humans in relation to consumption by brain and other organs, and fill out the equation that whole body DHA is the sum of dietary and hepatic inputs.

The ability to image labeled DHA incorporation into brain with quantitative autoradiography in rodents or with PET in humans provides an opportunity as a biomarker that remains to be exploited under different experimental and clinical conditions.

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