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Threshold changes in rat brain docosahexaenoic acid incorporation and concentration following graded reductions in dietary alpha-linolenic acid

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Abstract

Background—This study tested the dietary level of alpha-linolenic acid (α -LNA, 18:3n-3) sufficient to maintain brain ¹⁴C-Docosahexaenoic acid (DHA, 22:6n-3) metabolism and concentration following graded α -LNA reduction.

Methods—18–21 day male Fischer-344 (CDF) rats were randomized to the AIN-93G diet containing as a % of total fatty acids, 4.6% (“n-3 adequate”), 3.6%, 2.7%, 0.9% or 0.2% (“n-3 deficient”) α -LNA for 15 weeks. Rats were intravenously infused with ¹⁴C-DHA to steady state for 5 minutes, serial blood samples collected to obtain plasma and brains excised following microwave fixation. Labeled and unlabeled DHA concentrations were measured in plasma and brain to calculate the incorporation coefficient, k^* , and incorporation rate, J_{in} .

Results—Compared to 4.6% α -LNA controls, k^* was significantly increased in ethanolamine glycerophospholipids in the 0.2% α -LNA group. Circulating unesterified DHA and brain incorporation rates (J_{in}) were significantly reduced at 0.2% α -LNA. Brain total lipid and phospholipid DHA concentrations were reduced at or below 0.9% α -LNA.

Conclusion—Threshold changes for brain DHA metabolism and concentration were maintained at or below 0.9% dietary α -LNA, suggesting the presence of homeostatic mechanisms to maintain brain DHA metabolism when dietary α -LNA intake is low.

Keywords

Graded reduction; polyunsaturated fatty acids (PUFAs); docosahexaenoic acid (DHA); alpha-linolenic acid; threshold; brain; DHA metabolism

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Conflict of Interest
None to declare.

1. Introduction

Brain membrane phospholipids are highly enriched with the polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), which participate in neurotransmission [1, 2], gene transcription and the regulation of brain immunity [3–5]. The brain derives most of its DHA and AA from the plasma unesterified fatty acid pool, which is maintained by liver synthesis-secretion of DHA and AA from their dietary precursors, alpha-linolenic acid (α -LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively, or from direct dietary incorporation [6–9]. Brain endogenous synthesis of DHA and AA is low, accounting for less than 0.2% and 1% of the unesterified α -LNA and LA that enter the brain, respectively [10, 11].

Bourre et al. established that the minimum dietary α -LNA required to maintain maximal tissue DHA concentration in rats during development and adulthood, is 4% (0.4% energy) and 2.3% (0.26% energy) α -LNA of total fatty acids, respectively, when total fat content 50–60 grams per kg diet [12, 13]. Bourre et al. also reported that 0.9% dietary α -LNA of total fatty acids (0.1% energy, or 0.5 g/kg diet) is sufficient to maintain adult rat brain DHA concentrations (Table 6 of the Bourre et al paper [13]). Typically, rodent diets, such as the AIN-93G diet, are designed to provide 4.6–7.0% dietary α -LNA (of total fatty acids) throughout development and adulthood to ensure maximal DHA liver supply, and accretion by the brain and other tissues [14–18]. Total fat content of the AIN-93G diet which contains 4.8% α -LNA is 70 g per kg diet [14], whereas other similar dietary formulations contain 100 g fat per kg diet [15–18].

Studies have shown that compared to rats fed a DHA-free, “n-3 PUFA adequate” diet containing 4.6% α -LNA of total fatty acids, rats fed a DHA-free “n-3 PUFA deficient diet” (0 to 0.2% α -LNA) for 15 weeks or 2–3 generations have reduced plasma and brain DHA concentrations, increased concentrations of n-6 docosapentaenoic acid (n-6 DPA), an elongation product of AA formed in the liver, and behavioral abnormalities [15–18]. Dietary n-3 PUFA deficiency also decreased brain DHA rate of acylation-reacylation (i.e. turnover) within membrane phospholipids [19] and increased elimination half-life from 33 to 90 days [20]. This is consistent with evidence of decreased mRNA, protein or activity of DHA-metabolizing enzymes, including DHA-releasing calcium-independent phospholipase A₂ VIA (iPLA₂), cyclooxygenase (COX)-1 and 12-lipoxygenase (LOX) [21, 22]. The decrease in brain DHA metabolism is accompanied by a reciprocal increase in AA-metabolizing group IVA calcium-dependent phospholipase A₂ (cPLA₂) and COX-2 [21, 22]. While brain AA turnover or half-life did not change [23, 24], n-6 DPA concentration and turnover increased [25], suggesting that n-6 DPA replaces DHA and may act as a substrate for AA-metabolizing enzymes during chronic n-3 deficiency.

The decrease in brain DHA metabolism following dietary α -LNA deficiency suggests that homeostatic adaptations spare brain DHA metabolism. A graded dietary α -LNA study that provided 0.2%, 0.8%, 1.7%, 2.6% 3.8% or 4.6% α -LNA of total fatty acids to rats for 15 weeks showed that changes in brain DHA metabolizing enzymes and concentrations occurred at or below 1.7% dietary α -LNA, indicating adaptive changes in brain DHA metabolism [21]. Brain DHA concentration decreased and n-6 DPA concentration increased

at 0.8% dietary α -LNA relative to controls (4.6% α -LNA), whereas DHA-metabolizing iPLA₂ activity and COX-1 and 12-LOX mRNA decreased at 1.7% α -LNA [21]. Plasma unesterified DHA concentration decreased at 2.7% α -LNA, suggesting that the changes in plasma DHA concentration initiated, rather than followed changes in brain DHA and n-6 DPA concentrations and enzymes [21].

Rapoport and colleagues developed an in vivo kinetic method using radiolabeled fatty acids to quantify the incorporation coefficient (k^*) and rate (J_{in}) of plasma unesterified fatty acids into the brain [26]. Using this method, Contreras et al. reported that k^* for DHA in brain total lipid (TL), ethanolamine glycerophospholipid (EtnGpl) and phosphatidylserine (PtdSer) increased in rats following α -LNA deprivation (n-3 deficiency) for 3 consecutive generations, reflecting an increase in the brain's avidity for DHA [19]. The incorporation rate (J_{in}), reflecting net brain DHA consumption, decreased, however, due to the reduction in circulating unesterified DHA [19].

In the present study, we used the Rapoport in vivo kinetic method [26] to measure radiolabeled DHA incorporation into brain following graded dietary α -LNA reductions below 4.6% α -LNA, considered to be an n-3 PUFA adequate amount [14] that exceeds by two-fold the minimum α -LNA requirement (2.3% α -LNA of total fatty acids) established by Bourre et al. for adult rats [13]. Similar to the study by Kim et al. [21], rats received for 15 weeks the AIN-93G diet containing the recommended amount of n-6 LA, but varying levels of α -LNA, which amounted to 4.6% (Adequate), 3.8%, 2.7%, 1.8%, 0.9% and 0.2% (deficient) of total fatty acids. The rats were injected with [1 - 14 C]DHA in order to measure adaptive changes to k^* and J_{in} in response to dietary α -LNA reduction. In view of the Kim et al finding, that changes in DHA metabolizing enzymes occurred at threshold levels above 0.9% α -LNA [21], we hypothesized that adaptive increases in k^* and reductions in J_{in} will occur at or greater than 0.9% dietary α -LNA, to maintain brain DHA concentrations.

Upon completion of the study, the fatty acid composition of the diets, which were obtained from Dyets Inc., was measured by gas-chromatography. Surprisingly, the measurements revealed that the 1.8% α -LNA diet contained 5.1% α -LNA. Thus, data related to this group was removed from the analyses on the basis that the diet composition was not accurate.

Contrary to the hypothesis, we found that k^* increased and J_{in} decreased only at 0.2% α -LNA, suggesting that changes in brain DHA kinetics occur only during n-3 PUFA deficiency (0.2% α -LNA).

2. Material and methods

2.1 Animals and Diets

Experiments were conducted following the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health Publication No. 86–23) and were approved by the Animal Care and Use Committee of *Eunice Kennedy Shriver* National Institute of Child Health and Human Development. Male Fischer-344 (CDF) rat pups aged 18–21 days and their surrogate mothers were purchased from Charles River Laboratories (Portage, MI, USA). Upon arrival, the pups were weaned from their surrogate mothers and randomly

allocated to 6 diets containing 4.6% (control, n-3 PUFA adequate), 3.8%, 2.7%, 1.8%, 0.9% and 0.2% (n-3 PUFA deficient) α -LNA of total fatty acids. The calculated percent energy equivalent of the 4.6%, 3.8%, 2.7%, 1.8%, 0.9% and 0.2% α -LNA rodent diets, based on prior quantitative fatty acid gas-chromatography (GC) analysis done on the 4.6% α -LNA diet, is 0.88%, 0.74%, 0.43%, 0.18%, 0.03% and 0.002%, respectively. The macronutrient composition of the diets which were prepared by Dyets Inc. (Bethlehem, PA, USA) based on the AIN-93G formulation [14], is presented in Supplementary Table 1. The fatty acid composition of the diets (analytical details in following section) is shown in Supplementary Table 2. The diets were isocaloric and contained 10% fat. α -LNA composition was altered by substituting coconut oil with flaxseed oil which is high in α -LNA, while maintaining safflower oil (source of LA) content.

The rats were maintained on the diets for 15 weeks, with their food being replaced every 3 to 4 days. Water and food were provided ad libitum. Animals were housed in an animal facility that had regulated temperature, humidity and a 12 h light / dark cycle. They were initially housed in groups of 4 to 6 per cage and then 3 to 4 per cage a few weeks later to accommodate for cage space.

2.2 Diet fatty acid analysis

The fatty acid composition of the diets was analyzed, approximately two years after the study ended. Diets were stored at 4°C during and after the study. A few pellets from each diet were crushed using a pestle and mortar. A portion of the crushed pellets was weighed to approximately 0.2 g and transferred to glass vials (n = 3 replicates per diet). To each sample, 400 μ l toluene, 3 ml methanol and 600 μ l of 8% hydrochloric acid in methanol were added. Samples were vortexed after adding each reagent. The samples were heated at 90°C for one hour and cooled thereafter for 10–15 minutes at room temperature. Water (1 ml) and hexane (1 ml) were added to each vial. The mixture was vortexed and the phases allowed to separate for 15–30 minutes. A portion of the top hexane layer (200 μ l) from each sample was transferred to microcentrifuge tubes containing ~ 20 mg of sodium sulfate, to absorb residual water. The tube was inverted a few times and its contents allowed to settle for 10 minutes. The top hexane layer was transferred to gas-chromatography (GC) vials. Fatty acid methyl esters (FAMES) were analyzed by injecting 1 μ l of the top hexane layer into a Varian GC (see below section on FAME analysis by GC).

2.3 Surgical procedure

After 15 weeks on a given diet, the rats were anesthetized with 5% isoflurane, and polyethylene catheters (PE50, Clay Adams, Becton Dickinson, Sparks, MD, USA) filled with heparinized isotonic saline (100 IU/ml) were inserted into the right femoral artery and vein while the isoflurane was maintained at 1–3% [11]. Heparinized saline was used to prevent clogging of the arterial line [27]. The rats were allowed to recover from surgery with its hindquarters loosely wrapped and taped to a wooden block for 4 h in a temperature-controlled recovery chamber maintained at 25°C. Body temperature was checked regularly and maintained at 37°C with a rectal probe and a feedback heating element (TACT-2DF Temperature controller, Physitemp Instruments, Clifton, NJ, USA). Heart rate and blood pressure were measured prior to tracer infusion using a CyQ BPM02 system (CyQ 103/302;

CyberSense, Nicholasville, KY, USA). Surgeries were performed on 4 rats (from separate dietary groups) per day.

2.4 Tracer Infusion

[1-¹⁴C]DHA (53 mCi/mmol, >99% pure, Moravek Biochemicals, Brea, CA, USA) was dissolved in saline containing 50 mg/ml fatty acid-free bovine serum albumin by sonicating for 20 min at 37°C (Sigma-Aldrich, St. Louis, MO, USA). Four hours after surgery, the unanesthetized rat was infused intravenously for 5 min with 1.3 ml of 170 uCi/kg of [1-¹⁴C]DHA dissolved in HEPES buffer (pH 7.4) containing 50 mg/kg fatty acid-free bovine serum albumin (BSA, Sigma) at a rate of $0.223(1 + e^{-0.032t})$ ml/min ($t = \text{sec}$) with a computer-controlled variable rate infusion pump (No. 22; Harvard Apparatus, South Natick, MA, USA), to achieve a steady-state plasma specific activity within 1 min [28]. Arterial blood samples were collected at 0, 15, 30, 45, 90, 180, 240 and 300 s during infusion in order to determine plasma [1-¹⁴C]DHA and unlabeled unesterified fatty acids concentrations. Five min after starting infusion, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.v.) and subjected to head-focused microwave irradiation to stop brain metabolism (5.5 kW, 4.8 s; Cober Electronics, Norwalk, CT, USA) [29]. The brain was excised, dissected sagittally into two hemispheres and stored at -80°C until further analysis. Blood samples were centrifuged at 18,000 *g* for 30 s at room temperature to obtain plasma, which was also stored at -80°C.

2.5 Plasma lipid extraction and separation

Total lipids (TLs) were extracted from plasma (50 µl) collected at 0, 15, 30, 45, 90, 180, 240 and 300 s using 3 ml of 2:1 (v/v) chloroform / methanol and 2.5 ml 0.1M potassium chloride solution. One hundred µl of the lower organic phase was used to determine the radiolabeled unesterified plasma [1-¹⁴C]DHA concentration by liquid scintillation counting.

Concentrations of unlabeled, unesterified fatty acids were determined on 100 µl of arterial plasma collected at 5 minutes of infusion. Unesterified heptadecanoic acid (17:0) was added as an internal standard to the plasma and total lipids were extracted with 3 ml of 2:1 (v/v) chloroform / methanol and 0.75 ml 0.1M potassium chloride solution, followed by a 2 ml chloroform re-extraction wash. The lower chloroform phase containing the total lipid extract was separated by thin layer chromatography (TLC) on 60 Å silica gel plates (Whatman, Clifton, NJ) alongside phospholipid, unesterified fatty acids, triglyceride and cholesterol ester standards using the solvent system heptane: diethyl ether:glacial acetic acid (60:40:3, v/v/v). The plates were sprayed with 0.03% 6-p-toluidine-2-naphthalene-sulfonic acid in 50mM Tris-HCl buffer (pH7.4) (w/v), and the unesterified fatty acids band was identified under UV light, scraped and methylated with 1% H₂SO₄ (by vol) in anhydrous methanol for 3 h at 70°C, after adding 0.2 ml toluene. The resulting fatty acid methyl esters (FAMES) were extracted with 3 ml heptane after terminating the reaction with water 1.5 ml water. FAMES were reconstituted in 25 µl of isooctane.

2.6 Brain lipid extraction and separation

TLs were extracted from one cerebral hemisphere with the Folch method [30], and reconstituted in 5 ml of chloroform / methanol (2:1, v/v). A portion of the total lipid extract

(50 μ l) was methylated as described above with di-17:0-PC as an internal standard, or subjected to scintillation counting. Phospholipid classes (ChoGpl, choline glycerophospholipid; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; EtnGpl, ethanolamine glycerophospholipid) and neutral lipid classes (unesterified fatty acids, triacylglycerides (TGs) and cholesteryl esters (CEs)) were separated with 60 A° silica gel TLC plates in chloroform: methanol: H₂O: glacial acetic acid (60:50:4:1 by volume) and heptane: diethylether:glacial acetic acid (60:40:3, v/v/v), respectively, alongside appropriate identification standards. Bands were visualized under ultraviolet light after spraying the plates with 0.03% (w/v) 6-p-toluidine-2-naphthalene sulfonic acid in 50 mM Tris buffer (pH 7.4). Each band was scraped and used directly to quantify radioactivity by scintillation counting or to prepare FAMES following the addition appropriate quantities of di-17:0-PC as an internal standard to quantify brain esterified lipids and 17:0 (heptadecaenoic acid) to quantify brain unesterified fatty acids.

2.7 Quantification of radioactivity

Radioactivity in plasma or brain total or fractionated lipid extracts was determined using a liquid scintillation analyzer (2200CA, TRI-CARB®; Packard Instruments, Meriden, CT, USA) following reconstitution in 5 or 10 ml Cocktail mix.

2.8 FAME analysis by GC

Brain and plasma FAMES were analyzed by GC coupled to a flame ionization detector (Model 6890N; Agilent Technologies, Palo Alto, CA, USA) on a SP™-2330 fused silica capillary column (30 m $\times$ 0.25 mm inner diameter, 0.25 μ m film thickness; (Supelco, Bellefonte, PA, USA). The initial oven temperature was set at 80°C, and increased to 150°C (10°C /min) and 200°C (6°C /min), held at 200°C for 10 min, and then increased to 240°C for a total run time of 38 min. Fatty acid concentrations (nmol/g brain or nmol/ml plasma) were calculated by proportional comparison of the GC peak areas to that of the 17:0 internal standard.

Dietary FAMES were analyzed on a Varian 3800 system using a DB-23 column (Agilent Technologies, CA, USA). Helium was used as the carrier gas, at a flow rate of 1.3 ml / min. The initial temperature was set at 50°C for two minutes. It was increased to 180°C at 10°C per minute and then to 240°C at 5°C per minute. This was followed by a 5 minute hold at 240°C. The total run time was 32 minutes. Fatty acid percent composition was calculated by dividing the FAME area of each fatty acid by the total area of identified FAME peaks.

2.9 Calculations

Unidirectional incorporation coefficients, k_i^* (ml·s⁻¹·g⁻¹) of DHA, representing incorporation from plasma into brain lipid i (TL, phospholipid fractions, TG or CE), were calculated as follows:

$$k_i^* = \frac{C_{br,i}^*(T)}{\int_0^T C_{pl}^* dt} \quad (\text{Eq. 1})$$

$C_{br,i}^*(T)$ (nCi·g⁻¹) represents radioactivity of brain lipid i at time $T = 5$ min (end of infusion time), t is time after starting infusion, and C_{pl}^* (nCi·ml⁻¹) is the plasma concentration of labeled unesterified DHA during infusion. Integrals of plasma radioactivity were determined by trapezoidal integration.

The incorporation rate $J_{in,i}$ (nmol·s⁻¹·g⁻¹) of plasma unesterified DHA into brain lipid i , is calculated as follows:

$$J_{in,i} = k_i^* C_{pl} \quad (\text{Eq. 2})$$

where C_{pl} (nmol·ml⁻¹) is the concentration of unlabeled unesterified DHA in plasma.

2.10 Statistical analysis

Statistical analysis was performed on GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA, USA). The normality of distribution of the data was first determined by Shapiro-Wilk test. Since most of the data was not normally distributed, they were analyzed by Kruskal–Wallis non-parametric one-way ANOVA followed by Dunn's multiple comparison post-hoc test. Data were therefore presented as median and 25% and 75% interquartile ranges.

As reported below (results section), the 1.8% α -LNA group was excluded from the analysis because the fatty acid composition of the diet was 5.1% α -LNA, instead of the intended 1.8% amount.

Rats that died or bled during or following surgery or infusion were excluded from the analysis. Also, rats that were not infused with the full dose of the tracer, based on the attainment of plasma radioactivity concentrations three times lower than the group median following the start of infusion, were excluded from the analysis. Statistical significant was accepted at $P < 0.05$.

3. Results

3.1 Dietary fatty acid analysis

Supplementary Table 1 shows the macronutrient content of the diets. The fatty acid composition of the diets is reported in Supplementary Table 2. As indicated, α -LNA composition was 4.6%, 3.5%, 2.7%, 5.1%, 0.8% and 0% in the 4.6%, 3.8%, 2.7%, 1.8%, 0.9% and 0.2% groups, respectively. This suggested that the fatty acid composition was close to the expected amount for each diet, except for the 1.8% α -LNA diet, which contained 5.1% α -LNA. The composition of this diet was confirmed by repeating the analysis on separate pellets. Thus, rats infused with radiolabeled DHA from this group were removed from further analyses, because they did not receive the targeted amount of α -LNA.

3.2 Surgery and infusion parameters

One control rat (4.6% α -LNA diet) died after arterial catheter implantation surgery of unknown causes. One rat from the 0.2% α -LNA groups was euthanized after the catheter surgery and prior to tracer infusion because it was bleeding from the site of incision. One rat

in the 2.7% α -LNA group was excluded from further analysis because its plasma radioactivity over the 5-minute infusion period was 10–20 times lower than the median, suggesting incomplete delivery of the tracer. These three rats were therefore excluded from further measurements and analyses.

3.3 Physiologic parameters

Supplementary Table 3 shows median body weight, body temperature, heart rate and blood pressure (systolic and diastolic) at the time of surgery. Kruskal–Wallis non-parametric one-way ANOVA showed no significant difference amongst the groups.

3.4 Plasma Kinetics

Steady-state [$1\text{-}^{14}\text{C}$]DHA concentration was achieved within approximately one minute in all groups (data not shown). Median area under the curve for plasma radioactivity in TLs (nCi.s/mL), which represents the input function, did not significantly differ between the groups (4.6% α -LNA, 145833 (118862,198587); 3.8% α -LNA, 163394 (154436,193831); 2.7% α -LNA, 151649 (119008,259096); 0.9% α -LNA, 187843 (129706, 298374); 0.2 % α -LNA, 149899 (107216,174873)).

3.5 Unlabeled plasma fatty acid concentrations

Table 1 shows median plasma unesterified LA, AA, n-6 DPA, α -LNA, EPA and DHA concentrations (nmol/ml). Unesterified LA and AA did not significantly differ amongst the groups. Unesterified n-6 DPA was significantly increased by 8–12 fold in the 0.9% and 0.2% groups compared to 4.6% controls. Unesterified α -LNA and EPA were significantly reduced in the 0.9% and 0.2% α -LNA dietary groups by 85–100% compared to 4.6% α -LNA controls. Unesterified DHA was significantly reduced by 63% in the 0.2% group compared to the 4.6% α -LNA group.

3.6 Brain [$1\text{-}^{14}\text{C}$]DHA incorporation kinetics

The incorporation coefficient, k^* (Eq. 1), of [$1\text{-}^{14}\text{C}$]DHA into brain lipid compartments is shown in Figure 1. The incorporation coefficient did not significantly change in TLs, ChoGpl, PtdIns, PtdSer, TG or CE fractions (1-A, C-G). Compared with 4.6% α -LNA controls, k^* was significantly increased in the 0.2% α -LNA group by 51% in EtnGpl (1-B).

3.7 Brain [$1\text{-}^{14}\text{C}$]DHA incorporation rate

Reflecting the reduction in unesterified DHA concentration, brain DHA incorporation rate, J_{in} , into TLs and phospholipid subfractions (EtnGpl, ChoGpl, PtdIns and PtdSer), TG and CE was significantly reduced by 48–76% in the 0.2% dietary α -LNA group compared to 4.6% α -LNA controls (Figure 3).

3.8 Brain fatty acid concentrations

Table 2 shows median TL, phospholipid and unesterified brain α -LNA, LA, AA, n-6 DPA and DHA concentrations. α -LNA was measurable only in brain TLs, and its concentration did not differ significantly between the groups.

LA concentration in ChoGpls was significantly lower in the 0.9% α -LNA group than the 4.6% group. PtdSer LA was also significantly reduced in the 0.2% α -LNA group compared to the 4.6% group. LA concentration did not change significantly within TLs, EtnGpl, PtdIns and unesterified fatty acids.

ChoGpl AA concentration was lowest in the 3.8% α -LNA group (median of 2903), which differed significantly from the 0.2% α -LNA group (median of 3089). No other significant changes in AA were observed.

N-6 DPA was not detected in TLs, ChoGpls and PtdIns at 4.6%, 3.8% or 2.7% α -LNA. It was detected in these fractions when dietary α -LNA reached 0.9% and 0.2%, being 3–6 times higher in the 0.2% α -LNA group than the 0.9% α -LNA group. One way ANOVA showed that the differences between the 0.2% and 0.9% α -LNA groups were not statistically significant, although both groups differed significantly from 4.6% α -LNA controls. N-6 DPA was detectable in all groups in EtnGpl and PtdSer phospholipids. Its concentration in both fractions was significantly higher by 4–25 fold in the 0.9% and 0.2% α -LNA groups compared to 4.6% α -LNA controls. In unesterified fatty acids, n-6 DPA concentration, which was only detectable in the 0.2% and 0.9% groups, was significantly higher in the 0.2% α -LNA group compared to 4.6% α -LNA controls.

Brain TL and ChoGpl DHA concentration was significantly reduced in the 0.9% (13–16%) and 0.2% (45–52%) α -LNA groups compared to 4.6% α -LNA controls. DHA concentration was significantly reduced in EtnGpls, PtdIns and PtdSer by 40–47% in the 0.2% α -LNA group compared to 4.6% α -LNA controls. No significant differences in brain unesterified DHA were seen.

4. Discussion

This study showed statistically significant reductions in plasma unesterified and brain TL and phospholipid DHA concentrations, increased brain DHA incorporation coefficient k^* into EtnGpl, and reduced DHA incorporation rate, J_{in} , into brain TL, TG, CE and phospholipid subfractions, at or below 0.9% dietary α -LNA treatment for 15 weeks, compared to 4.6% α -LNA controls. N-6 DPA was significantly increased in plasma and brain TLs, phospholipid subfractions and unesterified fatty acids at or below 0.9% dietary α -LNA. The concentration changes in plasma and brain are largely confirmatory of the recent results of Kim et al [21], except for plasma unesterified DHA, which was reduced at 0.2% α -LNA in this study and at 2.6% α -LNA in the Kim et al study. Those authors also reported concurrent concentration reductions in n-3 PUFAs in liver, and related them to changes in brain activity of cPLA₂ IVA, COX-2 and iPLA₂ VIA.

The incorporation coefficient, k^* , was selectively increased in EtnGpl at 0.2% α -LNA, consistent with a study that found a significant increase in EtnGpl k^* following three consecutive generations of dietary α -LNA deprivation (0.2%), compared to an adequate 4.6% α -LNA diet [19]. The increase in k^* in EtnGpl, into which DHA is preferentially incorporated [31], could have occurred through increased activity of enzymes involved in DHA incorporation into membrane phospholipids [32], or esterification of DHA-CoA into

membrane phospholipids by an acyltransferase [33]. Threshold changes in acyl-CoA synthetase or acyltransferase enzyme activity following graded α -LNA reduction should be tested in future studies.

Significant reductions in plasma unesterified DHA occurred at 0.2% α -LNA, in contrast to a similar threshold study which showed significant reductions in unesterified DHA at 2.6% α -LNA [21]. The discrepancy between studies is likely due surgical stress or the use of heparinized isotonic saline to prevent the formation of blood clots within the arterial / venous catheter lines, unlike the Kim et al study in which no surgeries were conducted and blood was collected from the abdominal aorta following CO₂ asphyxiation [21]. Heparin could increase circulating unesterified DHA by increasing its hydrolysis from esterified lipid pools [34] because it activates lipase and esterase enzymes [35]. This effect, however, would have been consistent across all groups, since all animals received the same heparin treatment. It is likely that stress associated with the infusion surgery in this study may have contributed to the mobilization of unesterified fatty acids, including DHA, from adipose tissue, resulting in increased variability and loss of statistical power to detect significance at 2.6% α -LNA. The variability in plasma unesterified DHA is approximately 2 to 8 times higher in rats subjected to femoral vein / artery catheter implantation surgery, heparin injection and arterial blood withdrawals [36–40], compared to rats killed with CO₂ [21].

The variability in plasma unesterified DHA poses a limitation with the interpretation of the incorporation rate, J_{in} , values, which are derived from the product of k^* and plasma unesterified DHA. Since k^* did not significantly change (except in EtnGpl), the 48–76% reduction in brain TL, phospholipid, TG and CE J_{in} at 0.2% dietary α -LNA was driven by the significant decrease in plasma unesterified DHA. It is likely that threshold changes in J_{in} would have occurred at a higher dietary α -LNA threshold dose than 0.2% had it not been for the unesterified plasma DHA variability. Nonetheless, because the k^* values are independent of unlabeled plasma unesterified DHA concentration, the 51% reduction in EtnGpl k^* at 0.2% dietary α -LNA compared to 4.6 α -LNA controls reflects a low threshold for changes in brain DHA metabolism in response to reduced dietary α -LNA.

Compared to 4.6% dietary α -LNA controls, statistically significant reductions in brain TL and ChoGpl DHA concentrations were seen at 0.9% α -LNA, and in brain EtnGpl, PtdIns and PtdSer DHA at 0.2% α -LNA. The decrease in brain EtnGpl, PtdIns and PtdSer DHA concentrations reflects the reduction in DHA incorporation rate (J_{in}) at 0.2% dietary α -LNA, in association with reduced rate of DHA loss from membrane phospholipid [20]. The nonsignificant but 25–50% reduction in J_{in} at 0.9% dietary α -LNA, when maintained chronically for 15 weeks, could account for the significant reduction in TL and ChoGpl DHA concentrations at 0.9% α -LNA. A larger sample size may have pushed this reduction towards statistical significance.

In the present study, brain DHA metabolism was measured by infusing radiolabeled unesterified DHA to steady-state levels in plasma, and measuring DHA incorporation into the brain after correcting for its fractional uptake from the plasma [¹⁴C]DHA concentration (Equation 1) [26]. Several studies reported that DHA esterified to a lysophospholipid enters the brain via the Mfsd2a orphan transporter, and that esterified DHA is a better source of

DHA to the brain than unesterified DHA [41, 42]. In these studies, however, brain DHA uptake was assessed by quantifying the percent ^{14}C of injected radioactivity, and without correcting for the fractional uptake of ^{14}C -DHA from the plasma, since ^{14}C -DHA was not infused to steady-state levels. A recent study that quantitatively compared the incorporation of free and esterified DHA into the brain by infusing both to steady-state levels, reported that unesterified DHA is the main source of brain DHA [43].

Liver synthesis-secretion rate of DHA was reported to decrease following 15-weeks of α -LNA deprivation (0.2% or less) [6, 44], in agreement with the observed reduction in plasma unesterified DHA and brain DHA metabolism at 0.2% α -LNA. Since the liver is the main site of DHA synthesis and supply to the brain when DHA is missing from the diet, it would be worthwhile to determine threshold changes in liver DHA synthesis-secretion rates following graded α -LNA reduction [6, 44].

One surprising finding in this study is that threshold changes in brain DHA metabolism, particularly the 51% increase in $\text{EtnGpl } k^*$, occurred at a very low dietary α -LNA threshold of 0.2%. Changes in DHA-metabolizing enzymes (iPLA₂ and COX-1) occurred at a higher dietary α -LNA threshold, above 0.9% [21]. This suggests that changes in brain DHA kinetics likely follow changes in brain DHA-metabolizing enzymes, which occur in response to reduced plasma unesterified DHA concentration [21].

Many rodent studies reported deleterious effects of n-3 PUFA deficient diets (0.2% α -LNA) on brain neuropathology and behavior [15–18], particularly when challenged with trauma or a neurotoxin [3, 22, 45]. While these studies demonstrate the essentiality of dietary α -LNA for maintaining brain DHA levels and function, they are not likely to be clinically relevant because extreme n-3 PUFA deficiency may not be common in humans. We are aware of a few case studies of clinical n-3 PUFA deficiency in which patients receiving 0.02 to 0.09 % energy α -LNA (0.02% energy equals 0.66% of total fatty acids) through parenteral nutrition, developed neurological symptoms or dermatitis after a few months of being on the diet [46–49]. Estimated α -LNA intakes in North America range between 0.64 to 0.73% of total energy [50, 51], which is within the 0.6–1.2 energy % range recommended by the Institute of Medicine [52], and likely meets the 2.1–3.8 mg daily rate of brain DHA consumption in humans established with positron emitting tomography (PET) [53, 54]. Worldwide intakes of α -LNA, however, vary by region and range between 0.1 to 1.4% energy (200–3000 mg/day) [55]. Future rodent studies should explore the effects of dietary α -LNA on brain lipid metabolism and behavior with or without an experimental challenge at clinically relevant doses. It is worth noting, however, that the dietary requirements for humans and rodents are likely to differ, since omega-3 deficiency symptoms occur at 0.02 to 0.09% energy α -LNA in humans [46–49], whereas the deficiency range in rats based on this study and others is between 0.002–0.03% energy [12, 13].

A limitation of this study, aside from the low sample size, is that the 15 week duration of graded α -LNA reduction may not necessarily represent longer periods of low α -LNA intake. Supporting this suggestion is evidence of altered k^* in several brain lipid pools (increased in TLs, PtdSer and EtnGpl, and decreased in TGs) in a study which reduced α -LNA from 4.6% to 0.2% α -LNA for three consecutive generations [19], compared to increased k^* in EtnGpl

at 0.2% α -LNA in this study. It is possible that with prolonged α -LNA administration below 4.6%, k^* may change in other lipid pools, or at dietary thresholds above 0.2% α -LNA. Also, dietary α -LNA thresholds and brain DHA requirements are likely to change during development, aging, liver malfunction, or the presence of neuropathological conditions, consistent with one study which reported increased k^* for DHA in chronic alcoholics [53].

The finding that the 1.8% α -LNA diet contained 5.1% α -LNA instead of the targeted amount of 1.8% α -LNA emphasizes the importance of confirming fatty acid composition of purchased diet prior to initiating an animal trial. The lack of a 1.8% α -LNA group does not change the conclusion that threshold reductions in k^* and J_{in} occurred at 0.2% α -LNA. It also does not change the conclusion that reductions in brain DHA concentrations occurred at 0.2–0.9% α -LNA, since a similar study by Kim et al. reported no change in brain DHA concentrations at 1.7% α -LNA compared to 4.6% α -LNA controls, but significant reductions in brain DHA at 0.2 and 0.9% α -LNA [21].

In summary, threshold changes in brain DHA concentration, incorporation coefficient and incorporation rate occurred at or below 0.9% dietary α -LNA, which is at least 2.5 times below the minimum recommended amount of 2.3% α -LNA established by Bourre et al. [13]. PET imaging could be clinically considered to quantify brain DHA consumption in relation to changes in dietary α -LNA and neurological outcome, to better establish optimal human α -LNA requirements [53, 54].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

α-LNA	alpha-linolenic acid
AA	arachidonic acid
cPLA₂	Ca ²⁺ -dependent cytosolic phospholipase A ₂
iPLA₂	Ca ²⁺ -independent phospholipase A ₂
ChoGpl	choline glycerophospholipid
COX	cyclooxygenase
DHA	docosahexaenoic acid
EtnGpl	ethanolamine glycerophospholipid
FAMES	fatty acid methyl esters
GC	gas-chromatography

LA	linoleic acid
PtdIns	phosphatidylinositol
PET	positron emitting tomography
PtdSer	phosphatidylserine
PUFA	polyunsaturated fatty acids
TL	total lipids
TLC	thin layer chromatography

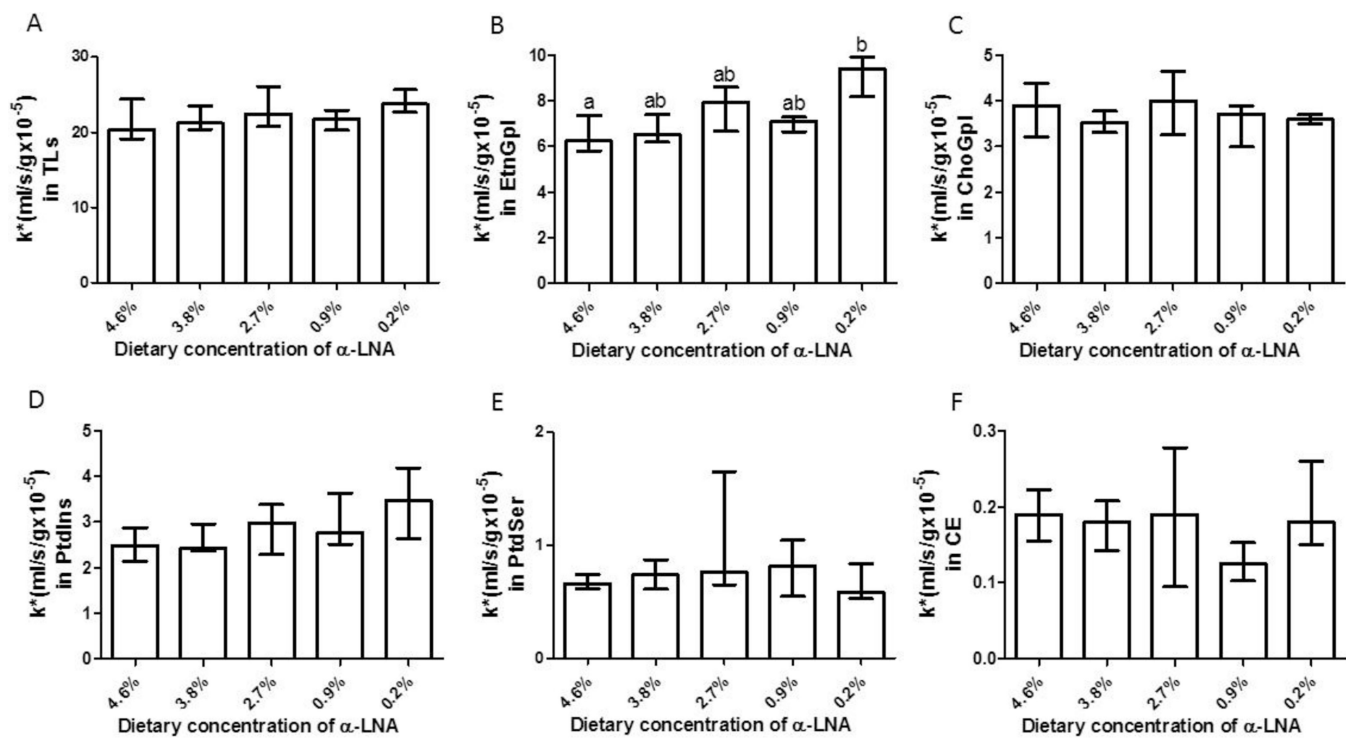
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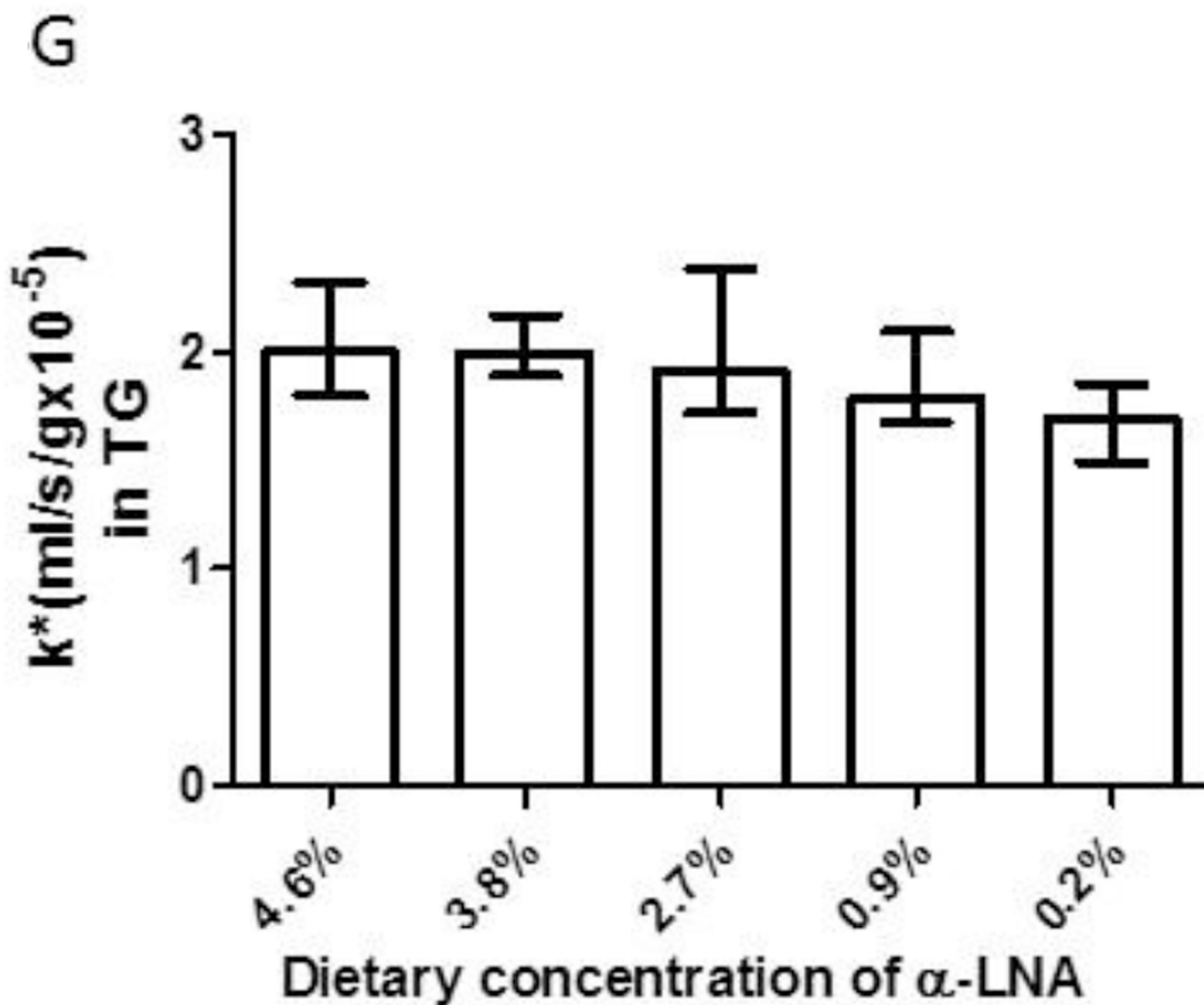
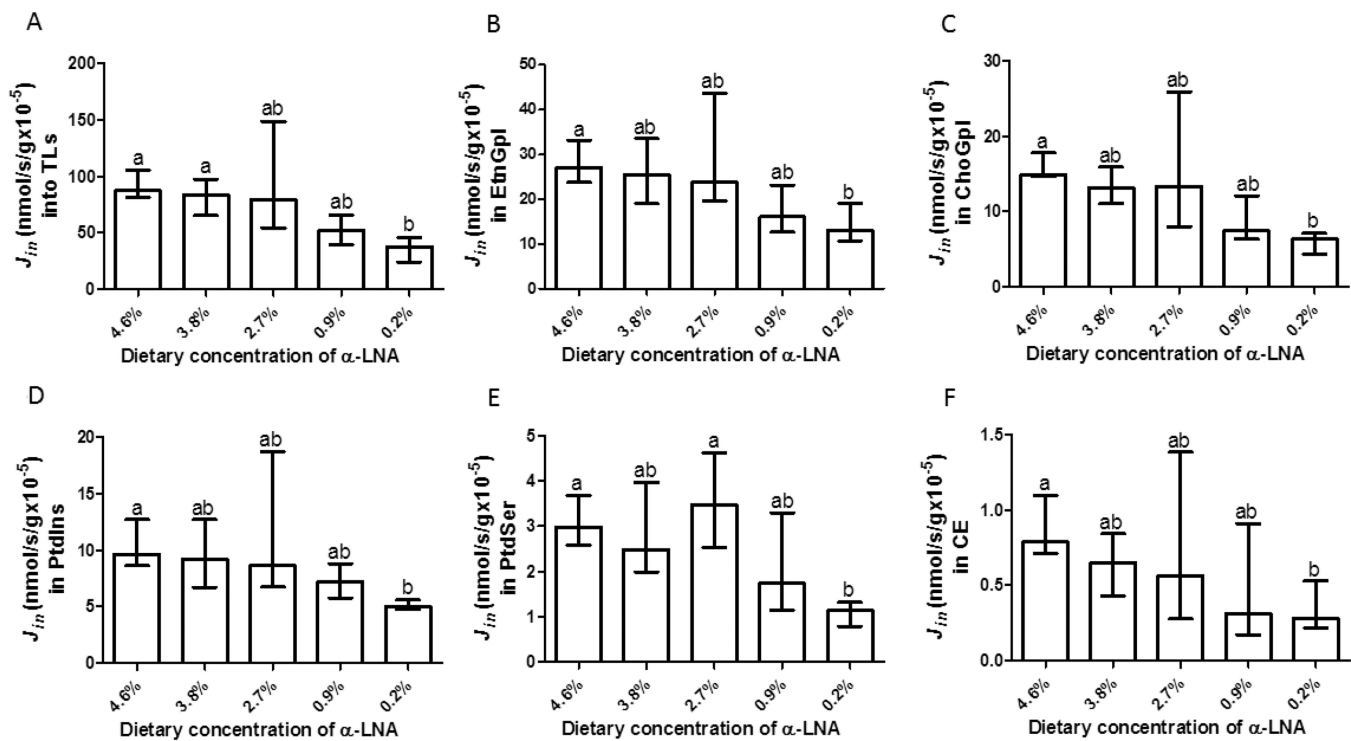


Figure 1.

Incorporation coefficient (k^*) in A) TLs, B) aqueous phase, C) EtnGpl, D) ChoGpl, E) PtdIns, F) PtdSer, G) TG and H) CE in rats fed a 4.6%, 3.8%, 2.7%, 0.9% and 0.2% α -LNA diet for 15 weeks. Data are graphed as median and interquartile ranges (error bars). The lower and upper boundaries of the error bars indicate 25th and 75th percentile values, respectively. Sample size (n) is 6–8/group. Data were analyzed by Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison post-hoc test. Different superscripts show significant differences at the $P < 0.05$ level. ChoGpl, choline glycerophospholipid; CE, cholesteryl ester; EtnGpl, ethanolamine glycerophospholipid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; TG, triglyceride; TL, total lipid.



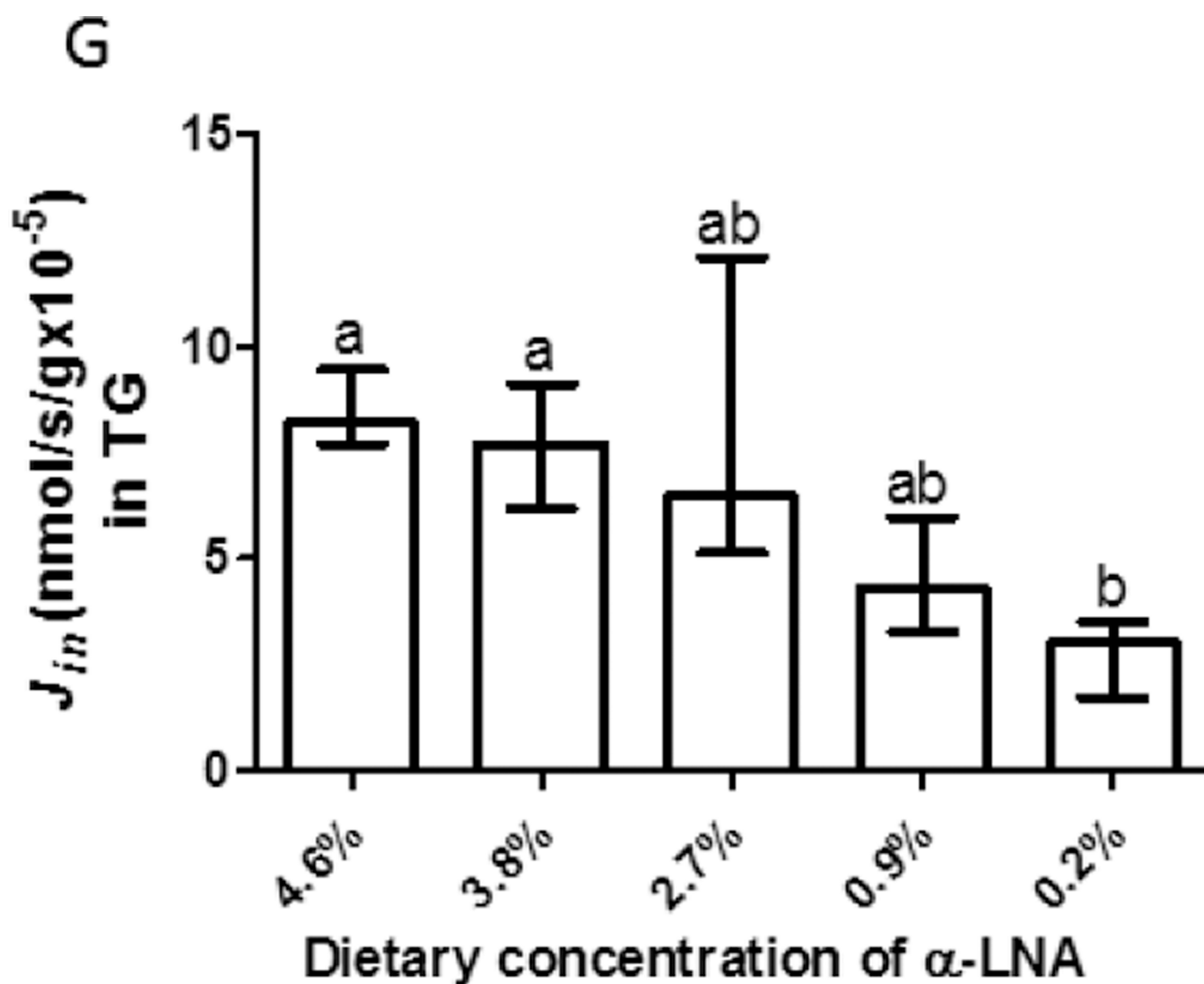


Figure 2. Incorporation rate (J_{in}) into A) TLs, B) aqueous phase, C) EtnGpl, D) ChoGpl, E) PtdIns, F) PtdSer, G) TG and H) CE in rats fed a 4.6%, 3.8%, 2.7%, 0.9% and 0.2% α -LNA diet for 15 weeks. Data are graphed as median and interquartile ranges (error bars). The lower and upper boundaries of the error bars indicate 25th and 75th percentile values, respectively. Sample size (n) is 6–8 / group. Data were analyzed by Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison post-hoc test. Different superscripts show significant differences at the $P < 0.05$ level. ChoGpl, choline glycerophospholipid; CE, cholesteryl ester; EtnGpl, ethanolamine glycerophospholipid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; TG, triglyceride; TL, total lipid.

Table 1

Plasma unesterified polyunsaturated fatty acid concentrations (nmol/ml)

α -LNA dietary content	4.6% (n=8)	3.8% (n=8)	2.7% (n=6)	0.9% (n=8)	0.2% (n=7)
n-6 fatty acids					
LA	147.6 (110.2,201.8)	132.4 (107.4,169.6)	145.7 (93.8,164.1)	123.5 (64.9,181.2)	132.2 (118.9,167.6)
AA	11.9 (9.4,14.1)	10.5 (9.8,12.3)	12.7 (11.5,17.3)	13.7 (10.1,21.6)	13.5 (11.4,17.4)
n-6 DPA	0.1 (0.0,3) ^a	0.2 (0.0,3) ^a	0.4 (0.0,4) ^{ab}	0.9 (0.6,2.1) ^b	1.4 (1.1,1.9) ^b
n-3 fatty acids					
α -LNA	22.5 (17.0,26.8) ^a	15.2 (12.8,20.1) ^{ab}	13.6 (9.1,14.4) ^{abc}	3.3 (2.5,5.4) ^{bc}	0.8 (0.7,1.2) ^c
EPA	1.8 (1.1,2.0) ^a	1.0 (0.8,1.2) ^{ab}	0.8 (0.5,1.3) ^{abc}	0.1 (0.0,6) ^{bc}	0 (0,0) ^c
DHA	4.5 (3.3,4.7) ^a	3.6 (3.1,4.1) ^a	3.8 (2.3,5.5) ^a	2.3 (1.9,3.2) ^{ab}	1.7 (0.9,1.9) ^b

Data are median (25%,75%) of n= 6–8 / group. The lower and upper boundaries within the brackets indicate 25th and 75th percentile values, respectively. Data were analyzed by Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test. Different superscripts show significant differences at the P<0.05 level. α -LNA, alpha-linolenic acid; AA, arachidonic acid; n-6 DPA, n-6 docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; DHA, docosahexaenoic acid.

Table 2

Brain fatty acid concentrations (nmol/g)

α -LNA content	4.6% (n=8)	3.8% (n=8)	2.7% (n=6)	0.9% (n=8)	0.2% (n=7)
TL					
α -LNA	751 (611,842)	787 (718,890)	737 (668,837)	664 (603,773)	796 (741,1070)
LA	1056 (1016,1079)	1017 (979,1157)	989 (955,1045)	881 (837,1012)	881 (831,1078)
AA	13184 (12861,13734)	13307 (13078,13706)	13542 (13083,14110)	13354 (13095,13818)	13809 (13608, 14332)
n-6 DPA	0 (0.0) ^a	0 (0.0) ^a	0 (0.0) ^a	1836 (1781, 1940) ^b	6341 (5957,6733) ^b
DHA	13107 (12256,13396) ^a	12929 (12661,13233) ^a	12916 (12596,13048) ^{ab}	11411 (11027,11689) ^{bc}	7190 (7066,7773) ^c
ChoGpl					
LA	457 (431,502) ^a	442 (413,480) ^{ab}	448 (420,483) ^{ab}	390 (354,409) ^b	389 (374,459) ^{ab}
AA	2968 (2879,3087) ^{ab}	2903 (2790,2911) ^a	3047 (3020, 3092) ^{ab}	2914 (2869,3138) ^{ab}	3089 (3052,3230) ^b
n-6 DPA	0 (0.0) ^a	0 (0.0) ^a	0 (0.0) ^a	250 (230,280) ^b	1000 (962,1056) ^b
DHA	2019 (1952,2097) ^a	1968 (1860,2034) ^{ab}	1986 (1907, 2054) ^{ab}	1689 (1551, 1737) ^{bc}	971 (945,1097) ^c
EnnGpl					
LA	234 (204,354)	316 (284,376)	291 (173,376)	271 (196,350)	135 (124,211)
AA	6933 (6885,7371)	7131 (6733,7882)	7310 (7012,7661)	7455 (6825,7701)	7396 (7019,7503)
n-6 DPA	181(140,219) ^a	231 (110,257) ^a	369 (348,407) ^{ab}	1257 (1173,1368) ^b	4518 (4301,4647) ^b
DHA	10708 (10268,10994) ^a	10407 (10016,10898) ^a	10766 (10503,10964) ^a	9491 (8915,9859) ^{ab}	5677 (5301,6325) ^b
PtdIns		n=7*			
LA	28 (26,41)	23 (22,32)	31 (21,45)	30 (22,34)	29 (21,33)
AA	1763 (1713,1815)	1779 (1316,1854)	1810 (1687,2004)	1712 (1676,1894)	1845 (1634,1877)
n-6 DPA	0 (0.0) ^a	0 (0.0) ^a	0 (0.0) ^a	12 (7,16) ^b	72 (61,91) ^b
DHA	178 (167,195) ^a	185 (173,194) ^a	171 (168,201) ^a	171 (141,198) ^{ab}	107 (91,113) ^b
PtdSer					
LA	62 (58,73) ^a	66 (25,73) ^a	56 (43,70) ^{ab}	25 (0,60) ^{ab}	0 (0,14) ^b
AA	513 (468,611)	523 (426,712)	517 (454,576)	502 (463,573)	538 (503,666)
n-6 DPA	177 (166,190) ^a	185 (169,219) ^a	244 (231,285) ^{ab}	725 (618,823) ^{ab}	2326 (2261,2478) ^b
DHA	4154 (3795,4546) ^a	3821 (3573,4461) ^a	3920 (3839,4656) ^a	3740 (3399,3876) ^{ab}	2235 (2104,2329) ^b

α -LNA content	4.6% (n=8)	3.8% (n=8)	2.7% (n=6)	0.9% (n=8)	0.2% (n=7)
Unesterified					
LA	3.4 (2.4,4.4)	4.1 (2.6,5.3)	3.4 (1.2,4.2)	2.9 (2.5,4.0)	4.7 (3.6,7.8)
AA	10.5 (7.5,11.8)	8.6 (7.3,12.6)	8.5 (7.4,10.4)	8.8 (8.0,10.5)	10.9 (10.7,15.4)
n-6 DPA	0 (0.0) ^a	0 (0.0) ^a	0 (0.0) ^a	0.2 (0.0,7) ^{ab}	3.7 (2.1,5.2) ^b
DHA	4.5 (1.1,7.5)	6.3 (3.6,13.3)	4.5 (1.9,8.2)	4.1 (0.8,5.1)	4.1 (2.6,5.0)

Data are median (25%,75%) of n= 6–8 / group. The lower and upper boundaries within the brackets indicate 25th and 75th percentile values, respectively.

* n=7 in PtdIns because fatty acid peak in one sample from 3.8% α -LNA group could not be detected.

Data were analyzed by Kruskal–Wallis test followed by Dunn's multiple comparison post-hoc test. Different superscripts show significant differences at the P<0.05 level. ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; TL, total lipid.