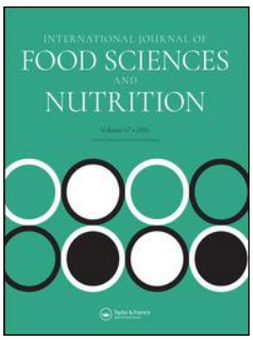

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RESEARCH ARTICLE

The effect of fish oil supplementation on brain DHA and EPA content and fatty acid profile in mice

Kelly J. Valentini, C. Austin Pickens, Jason A. Wiesinger and Jenifer I. Fenton

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI, USA

ABSTRACT

Supplementation with omega-3 (n-3) fatty acids may improve cognitive performance and protect against cognitive decline. However, changes in brain phospholipid fatty acid composition after supplementation with n-3 fatty acids are poorly described. The purpose of this study was to feed increasing n-3 fatty acids and characterise the changes in brain phospholipid fatty acid composition and correlate the changes with red blood cells (RBCs) and plasma in mice. Increasing dietary docosahexaenoic (DHA) and eicosapentaenoic acid (EPA) did not alter brain DHA. Brain EPA increased and total n-6 polyunsaturated fatty acids decreased across treatment groups, and correlated with fatty acid changes in the RBC ($r > 0.7$). Brain *cis*-monounsaturated fatty acids oleic and nervonic acid ($p < .01$) and saturated fatty acids arachidic, behenic, and lignoceric acid ($p < .05$) also increased. These brain fatty acid changes upon increasing n-3 intake should be further investigated to determine their effects on cognition and neurodegenerative disease.

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KEYWORDS
Brain; phospholipid; DHA; EPA; desaturase; fatty acid

Introduction

Phospholipids are the most fundamental component of cellular membranes with a high content of polyunsaturated fatty acids (PUFAs). The diversity in the saturated and unsaturated fatty acyl positions along the phospholipid bilayer influences the biophysical and biokinetic properties of cell membranes (Slater et al. 1994; Zerouga et al. 1995; Shaikh 2012; Turk and Chapkin 2013). Mammalian phospholipid membranes are particularly high in concentrations of the very long chain PUFAs (VLCPUFAs) docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (AA; 20:4n-6), especially in the nervous tissue, where together they comprise more than 25% of the total fatty acids in the peripheral and central nervous system (Kishimoto et al. 1969; Breckenridge et al. 1972). These VLCPUFAs are synthesised from their respective precursors, linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) in a tissue-dependent manner (Barcelo-Coblijn and Murphy 2009). LA and ALA cannot be synthesised de novo from 2-carbon fragments; therefore, an adequate intake of these fatty acids is considered essential.

The interactions and metabolism of LCPUFAs play a critical role in the development and functioning of the central nervous system. In the foetal brain, the fatty acid composition changes throughout development by progressively enriching phospholipids with DHA at the expense of other n-6 LCPUFAs (Svennerholm and Vanier 1973). An increase in DHA and the ratio of n-3/n-6 fatty acids is especially significant during the third trimester of gestation (Martinez and Mougan 1998; Makrides et al. 2010). n-3 PUFAs also play an essential role in neuronal growth in the developing human brain, with dietary DHA as the preferred fatty acid source (Anderson et al. 1990; Uauy and Dangour 2006; Lauritzen et al. 2016). During adulthood, the lipid composition of the brain is closely linked to dietary unsaturated fatty acid intake, where normal brain DHA and AA concentrations in adult rats can be maintained by the elongation, desaturation, and distribution of ALA and LA precursors in the liver (Igarashi et al. 2007). Altered metabolism of PUFAs, such as AA and DHA, in membrane phospholipids is linked to such neurological diseases as Alzheimer's Disease, Parkinson's Disease, suicide, depression, bipolar disorders, dementia, cognitive functions and mental stability (Chen et al. 2008; Whelan 2008; Zarate et al. 2017).

There is compelling evidence to support the importance of dietary lipids to improve brain

CONTACT Jenifer I. Fenton imigjeni@msu.edu Department of Food Science and Human Nutrition, Michigan State University, 208B G.M. Trout Building, East Lansing, MI 48824, USA

Supplemental data for this article can be accessed here.

development or even to prevent the conditions of neurological diseases (Innis 2007; Serini and Calviello 2016). In rodents, adequate dietary intake of n-3 PUFAs is critical in performing learning and behavioural tasks (Carrie et al. 2002; Fedorova and Salem 2006). In humans, increasing dietary unsaturated fatty acid intake, specifically n-3 and monounsaturated fatty acids (MUFAs), is associated with improved cognitive performance (Solfrizzi et al. 2010). In addition, consumption of n-3 PUFAs is linked to mild to modest improvements in cognitive function in the elderly, as well as in healthy adults (Issa et al. 2006; Yurko-Mauro et al. 2010). Adherence to a Mediterranean diet, which is high in MUFAs, is also associated with slower cognitive decline and a lower risk of developing cognitive impairment during aging (Panza et al. 2004; Feart et al. 2009). However, in other epidemiological studies, dietary fat intake was not associated with dementia, cognitive performance, or cognitive decline (Devore et al. 2009; Kroger et al. 2009).

One potential explanation for discrepancies in findings of clinical trials and epidemiological studies may be due to inability of dietary recall surveys or food frequency questionnaires to estimate circulating blood levels of LCPUFA (Hjartaker et al. 1997). Compounding these discrepancies is the lack of a universally accepted biomarker, which assesses an individual's overall body tissue LCPUFA status in relation to their lipid dietary intake. For example, how is neural tissue uptake and maintenance of fatty acid metabolism similar to the red blood cell (RBC) as the omega-3 index is used as a biomarker of dietary intake but does not always reflect target tissue levels (Fenton et al. 2016)?

The objective of this study was to (1) investigate how increasing dietary eicosapentaenoic acid (EPA)+DHA influences brain fatty acid composition, and (2) determine if the RBC, as a biomarker, reflects dietary induced fatty acid changes in the brain in mice.

Materials and methods

Experimental design and dietary regime

The primary endpoints of this study were brain EPA, DHA, and other fatty acids and to characterise the changes in brain fatty acids with increasing dietary EPA + DHA in mice. The secondary endpoints were RBC and plasma fatty acids to compare brain fatty acid levels with the plasma and RBC (the typical biomarker of fatty acid intake). Mice were utilised due to the inability to design a study like this using human subjects. All mice were bred, raised and housed under specific pathogen-free conditions in 60-square-inch plastic microisolatorcages in the Research Containment Facility at Michigan State University, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Thirty four five-week-old male and female 129-Smad3tm/par/J mice ($n = 8$–9/group) were housed and fed experimental diets for five weeks as previously described (Gurzell et al. 2014). Mice were randomised to treatment group using computer-generated simple randomisation. The rationale for the study design, diets, diet composition tables, experimental design, and sample size determination were as previously published (Gurzell et al. 2014). Briefly, AIN-93 G based premix diet without fat was purchased from Dyets Inc. (112163CCL; Bethlehem, PA, USA). Differing only in fat composition, all diets contained 7% fat by weight, providing a total of 17% Kcal of energy from fat. The AIN-93 G control diet contained 7% (wt/wt) soybean oil, while the experimental diets substituted a portion of the soybean oil with increasing amounts of EPA + DHA fish oil (1:1 ratio; wt/wt), which was formulated by combining appropriate volumes of EPA enriched and DHA enriched fish oils (EPA4E1400 + DHA4E1400 MEG-3; Ocean Nutrition, Dartmouth, Nova Scotia, Canada). Although the soybean oil contains approximately 1% of total energy from ALA, which is a precursor for DHA, it has been previously reported that there is a low conversion of dietary ALA to DHA (Pawlosky et al. 2001). Furthermore, it was shown that ALA entering the rat brain was largely β-oxidized and had little effect on brain phospholipid DHA content (Demar et al. 2005). The particular concentrations of EPA + DHA oil were chosen to represent a human diet of 2000 Kcal, which utilises 30% of the total energy from fat. The concentrations utilised in this study (250 mg, 1500 mg, 4000 mg of daily supplementation) translate to approximately 0.1%, 0.675% and 1.8% of total energy from EPA + DHA in the human diet.

After dietary treatment, mice were asphyxiated with CO_2 and exsanguinated through cardiac puncture using a heparin-coated syringe. Within five minutes post-mortem, whole brains from each animal were promptly removed and carefully rinsed in ice-cold phosphate buffered saline. Using an ice-cold ceramic platform, the left hemisphere (including cerebellum, pons, and olfactory tubercle) was cut with a horizontal slicer before freezing on dry ice. To obtain erythrocytes, whole blood was centrifuged at 1500*g* for 10 min at 4 °C and plasma carefully collected by aspiration. All samples were then transferred under dim lighting into opaque containers for storage. Besides the brief period during weighing, all tissue, plasma,

and erythrocytes remained continuously frozen in liquid nitrogen, dry ice, or −80 °C freezer until the extraction of lipids. All animal procedures were previously approved by the Michigan State University All-University Committee on Animal Care and Use (AUF 02/14-031-00).

Phospholipid extraction and preparation of fatty acid methyl esters (FAMEs)

A detailed methods section for the extraction and isolation of phospholipids was previously described by Gurzell et al. (2014). In brief, lipid extraction was performed through a modified version of Rose and Oaklander method (Rose and Oklander 1965). Isolation of phopsholipids by solid-phase extraction using Isolute-XL® SPE aminopropyl columns (500 mg; Biotage, Charlotte, NC, USA) was performed according to the modified procedures of Agren et al. (1992). FAMEs were prepared from isolated phospholipid fractions by incubation with acidified methanol, according to the modified methods of Burdge et al. (2000).

FAME analysis, identification, and quantification

Brain, plasma, and erythrocyte phospholipid fatty acid compositions were analysed using the HS-omega-3 Index® methodology (OmegaQuant, Sioux Falls, SD, USA) as described previously (Harris and Von Schacky 2004). Phospholipid FAMEs were measured by gas chromatography using a GC2010 Gas Chromatograph (Shimadzu, Duisburg, Germany) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA, USA) using hydrogen as carrier gas. Fatty acids were identified by comparison with a standard mixture of fatty acids characteristic of erythrocytes (GLC 727; Nucheck Prep, Elysian, MN, USA). Phospholipid fatty acids were calculated as a percentage of total identified fatty acids after response factor correction. Reproducibility of this method has been previously reported by Pickens et al. (2016). The omega-3 index is defined as erythrocyte EPA plus DHA expressed as weight percentage of total fatty acids (Aarsetoey et al. 2011; Von Schacky 2011). Analyses and instrumentation were quality-controlled according to DIN ISO 15189.

Statistical analysis

All statistical analyses were conducted using R v3.2.2 (Team 2015) and the GraphPad Prism 4 software (GraphPad, San Diego, CA, USA). Significant differences within and between the fatty acid composition for RBCs, plasma, and brain were determined using an ANOVA followed by a Dunn's multiple comparison test. p Values were corrected for false discovery according to Benjamini–Hochberg (1995). Spearman correlation coefficients were calculated for comparison between RBCs, plasma, and brain. $p < 0.05$ were considered statistically significant.

Results

Brain phospholipid fatty acids levels in response to increasing dietary EPA + DHA

In this study, brain phospholipid DHA levels were not significantly altered by increasing dietary EPA + DHA intake (Table 1). EPA levels in the whole brain significantly increased in a dose responsive manner from 0.02% in adult mice fed the control diet (0.0%en EPA + DHA) to 0.08% in mice fed the 1.8%en EPA + DHA diet (Table 1). A significant increase in the EPA metabolite DPA n-3 was also measured in adult mice fed the highest dose of dietary EPA + DHA, increasing from 0.12% in control mice to 0.19% in mice fed the 1.8%en EPA + DHA diet (Table 1).

Total brain n-6 VLCPUFA phospholipid percentages significantly decreased from 11.95% in control mice to 8.58% and 7.76% in mice fed the 0.675%en and 1.8%en EPA + DHA diet, respectively (Table 1). Specifically, the n-6 VLCPUFAs AA, DTA, and DPA n-6 all significantly decreased in mice fed the increasing dietary EPA + DHA. Mean brain AA percentages decreased from 8.49% in control mice to 5.68% in mice fed the 1.8%en EPA + DHA diet (Table 1). The AA metabolites DTA and DPA n-6 also decreased with dietary EPA + DHA (Table 1).

As shown in Table 1, the sum percentages of brain phospholipid *cis*-MUFAs increased significantly in a dose responsive manner from 20.74% in control mice to over 27.08% in mice fed the 1.8%en EPA + DHA diet. An evaluation of the most predominate MUFA found in cellular membranes shows that brain oleic content is significantly increased with dietary EPA + DHA, increasing from 17.35% in control mice to 21.41% in mice fed the highest dose of dietary EPA + DHA (Table 1). The oleic acid long-chain metabolite nervonic acid also significantly increased across dietary treatment groups (Table 1).

Very long chain saturated fatty acid phospholipid levels in the brain were also influenced by dietary EPA + DHA supplementation. Specifically, arachidic, behenic, and lignoceric acid all increased in a dose

Table 1. Brain phospholipid fatty acid levels (expressed as % of total fatty acids) and changes with dietary EPA + DHA[a].

Fatty acid	0.0% EPA + DHA	0.1% EPA + DHA	0.675% EPA + DHA	1.8% EPA + DHA	FDR *p* value[b]
ALA	$1e-03 \pm 2e-04^{A}$	$8e-04 \pm 3e-04^{A}$	$1e-03 \pm 6e-04^{A}$	$7e-04 \pm 3e-04^{A}$	0.277
EPA	$0.02 \pm 6e-03^{A}$	$0.02 \pm 5e-03^{AB}$	$0.04 \pm 6e-03^{BC}$	0.08 ± 0.03^{C}	**<0.0001**
DPA n-3	0.12 ± 0.02^{A}	0.11 ± 0.03^{A}	0.13 ± 0.04^{A}	0.19 ± 0.07^{A}	**<0.01**
DHA	16.24 ± 1.09^{A}	13.93 ± 3.11^{A}	12.44 ± 3.74^{A}	12.82 ± 4.76^{A}	0.152
EPA + DHA	16.27 ± 1.09^{A}	13.95 ± 3.11^{A}	12.47 ± 3.74^{A}	12.90 ± 4.78^{A}	0.154
Total n-3[c]	16.39 ± 1.11^{A}	14.06 ± 3.13^{A}	12.61 ± 3.78^{A}	13.09 ± 4.85^{A}	0.158
Total VLC n-3[d]	16.39 ± 1.11^{A}	14.06 ± 3.13^{A}	12.61 ± 3.78^{A}	13.09 ± 4.85^{A}	0.158
Linoleic	0.43 ± 0.04^{A}	0.50 ± 0.04^{B}	0.51 ± 0.04^{B}	0.49 ± 0.05^{AB}	**<0.01**
Linoelaidic	0.10 ± 0.04^{A}	0.20 ± 0.22^{A}	0.09 ± 0.03^{A}	0.22 ± 0.40^{A}	0.542
Eicosadienoic	0.16 ± 0.03^{A}	0.18 ± 0.02^{B}	0.18 ± 0.02^{B}	0.15 ± 0.02^{A}	0.064
γ-linolenic	$0.01 \pm 2e-03^{A}$	$0.01 \pm 3e-03^{A}$	$0.01 \pm 2e-03^{A}$	$0.01 \pm 2e-03^{A}$	0.129
DGLA	0.24 ± 0.02^{A}	0.25 ± 0.03^{AB}	0.27 ± 0.03^{B}	0.27 ± 0.03^{B}	**<0.05**
AA	8.49 ± 0.73^{A}	7.64 ± 0.99^{AB}	6.29 ± 1.26^{BC}	5.68 ± 1.30^{C}	**<0.0001**
DTA	2.94 ± 0.10^{A}	2.46 ± 0.59^{AB}	1.98 ± 0.59^{B}	1.77 ± 0.59^{B}	**<0.001**
DPA n-6	0.52 ± 0.12^{A}	0.38 ± 0.07^{AB}	0.31 ± 0.10^{B}	0.31 ± 0.14^{B}	**<0.01**
Total n-6[e]	12.77 ± 0.94^{A}	11.41 ± 1.62^{AB}	9.54 ± 1.89^{BC}	8.67 ± 2.01^{C}	**<0.001**
Total VLC n-6[f]	11.95 ± 0.90^{A}	10.48 ± 1.60^{AB}	8.58 ± 1.90^{BC}	7.76 ± 2.00^{C}	**<0.001**
Palmitoleic	0.27 ± 0.03^{A}	0.31 ± 0.06^{AB}	0.31 ± 0.05^{AB}	0.34 ± 0.04^{B}	**<0.05**
Palmitelaidic	0.06 ± 0.01^{A}	0.06 ± 0.01^{A}	$0.05 \pm 3e-03^{A}$	0.05 ± 0.01^{A}	0.258
Oleic	17.35 ± 0.98^{A}	20.51 ± 1.48^{B}	21.17 ± 2.21^{B}	21.41 ± 1.88^{B}	**<0.001**
Elaidic	0.12 ± 0.05^{A}	0.09 ± 0.03^{A}	0.09 ± 0.03^{A}	0.09 ± 0.03^{A}	0.284
Eicosaenoic	1.34 ± 0.35^{A}	1.68 ± 0.46^{A}	1.73 ± 0.35^{A}	1.75 ± 0.34^{A}	0.149
Nervonic	1.79 ± 0.37^{A}	2.51 ± 0.48^{AB}	3.57 ± 1.29^{B}	3.58 ± 1.41^{B}	**<0.01**
Total *cis*-MUFA[g]	20.74 ± 1.49^{A}	25.00 ± 1.99^{B}	26.78 ± 3.69^{B}	27.08 ± 3.42^{B}	**<0.001**
Palmitic	29.49 ± 1.27^{A}	30.17 ± 4.01^{A}	31.33 ± 3.37^{A}	31.70 ± 3.99^{A}	0.529
Stearic	19.17 ± 1.13^{A}	17.60 ± 0.58^{B}	17.70 ± 1.37^{AB}	17.30 ± 0.70^{B}	**<0.01**
Arachidic	0.26 ± 0.04^{A}	0.31 ± 0.08^{AB}	0.36 ± 0.08^{B}	0.36 ± 0.07^{B}	**<0.05**
Behenic	0.34 ± 0.05^{A}	0.42 ± 0.10^{AB}	0.56 ± 0.17^{B}	0.56 ± 0.17^{B}	**<0.01**
Lignoceric	0.54 ± 0.10^{A}	0.67 ± 0.17^{AB}	0.87 ± 0.26^{B}	0.88 ± 0.27^{B}	**<0.05**
Total saturated[h]	49.81 ± 2.35^{A}	49.17 ± 3.64^{A}	50.82 ± 3.02^{A}	50.80 ± 3.91^{A}	0.674
Total VLC saturated[i]	1.14 ± 0.19^{A}	1.41 ± 0.35^{AB}	1.80 ± 0.50^{B}	1.79 ± 0.50^{B}	**<0.01**

[a]Percent of total fatty acid mean ± standard deviation values displayed.
[b]ANOVA was conducted on dietary treatment group using Dunn's multiple comparison test. *p* Values were adjusted for false discovery according to Benjamini-Hochberg, and are bolded if $p < 0.05$.
[c]Total n-3 calculated as Σ ALA + EPA + DPA n-3 + DHA.
[d]Total VLC n-3 calculated as Σ EPA + DPA n-3 + DHA.
[e]Total n-6 calculated as Σ linoleic + linoelaidic + eicosadienoic + γ-linolenic + DGLA + AA + DTA + DPA n-6.
[f]Total VLC n-6 calculated as Σ AA + DTA + DPA n-6.
[g]Total *cis*-MUFA calculated as the Σ palmitoleic + oleic + eicosaenoic + nervonic.
[h]Total saturated calculated as the Σ palmitic + stearic + arachidic + behenic + lignoceric.
[i]Total VLC sat calculated as the Σ arachidic + behenic + lignoceric.
A, AB, B, BC, and C represent the multiple comparison test for each fatty acid for the 0.0%, 0.1%, 0.675%, and 1.8% EPA + DHA treatment groups.
VLC: very long chain; MUFA: monounsaturated fatty acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; DGLA: dihomo- γ-linolenic acid; AA: arachidonic acid; DTA: docosatetraenoic acid.

responsive manner with dietary EPA + DHA, as did the total VLC saturated fatty acids (Table 1). However, long chain saturated fatty acid palmitic acid and total saturated fatty acids did not significantly differ in response to dietary EPA + DHA (Table 1).

Correlations of saturated and unsaturated fatty acid levels in the RBC, plasma, and brain

The RBC omega-3 index and plasma EPA + DHA phospholipid levels were not correlated with the brain's EPA + DHA levels following dietary EPA + DHA in adult mice (Figure 1(A,B)). However, EPA levels in the brain were highly correlated with the RBC EPA levels ($r = 0.816$, $p < 0.0001$) in response to dietary EPA + DHA (Figure 1(C)). Brain DPA n-3 levels were also significantly correlated with RBC DPA n-3 levels ($r = 0.681$, $p < 0.001$) (Figure 1(D)). The results of this study demonstrate no correlation between the increase of DHA in the RBC and total brain DHA content (Figure 1(E)).

The sum of n-6 VLCPUFAs AA + DTA + DPAn-6 levels in the brain was highly correlated with the sum of these fatty acids in the RBC ($r = 0.724$, $p < 0.0001$) after dietary EPA + DHA (Figure 2(A)). Plasma AA + DTA + DPAn-6 was also correlated with brain levels of these n-6 fatty acids ($r = 0.574$, $p < 0.05$) (Figure 2(B)). In addition, when analysed individually, AA, DTA, and DPA n-6 in the brain were significantly correlated with the RBC changes in response to increasing dietary EPA + DHA (Figure 2(C–E)).

Correlations of brain cis-MUFA responses with n-3 and n-6 VLCPUFAs

As presented in Figure 3(A), changes in brain n-3 VLCPUFAs EPA + DHA phospholipids were not

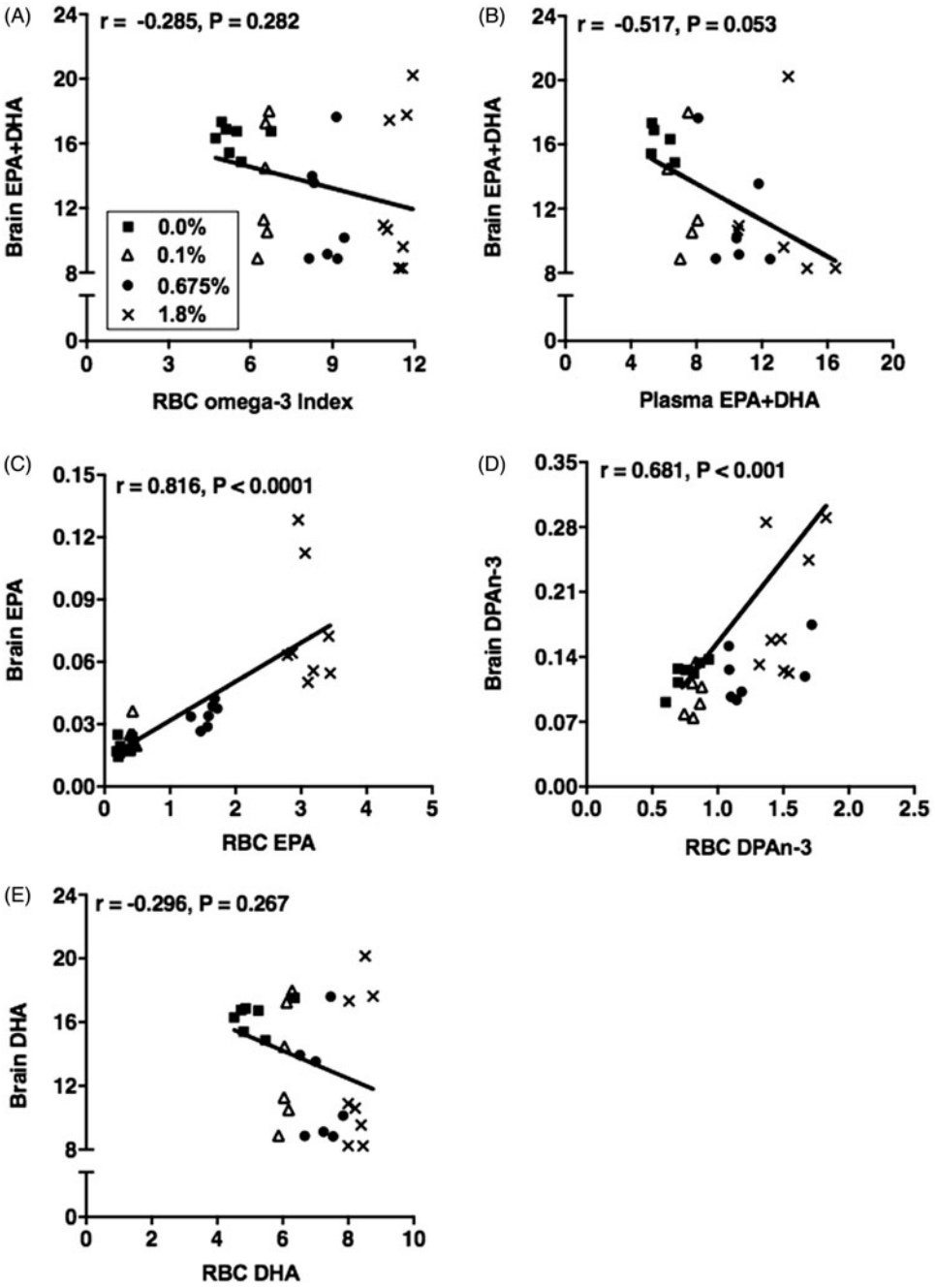

Figure 1. The relationship between the red blood cell (RBC) and tissue n-3 LCPUFAs after dietary eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake contributing to 0.0% (control), 0.10%, 0.675% and 1.8% of total daily energy intake. Values are mean EPA and DHA densities ($n = 6$–8), expressed as % of total phospholipid fatty acid composition in the RBC, plasma, and brain. Scatter-plots of Spearman correlations, which compare the rise of EPA + DHA in brain to changes in (A) RBC and (B) plasma phospholipid EPA + DHA composition. Scatter-plots of Spearman correlations comparing the RBC n-3 LCPUFAs to changes in whole brain (C) EPA, (D) DPAn-3, and (E) DHA.

correlated with increases in total *cis*-MUFAs in the brain ($r = -0.760$). Interestingly, changes in total cis-MUFAs were inversely correlated with decreased levels of brain n-6 VLCPUFAs AA + DTA + DPAn-6 ($r = -0.882$, $p < 0.0001$) (Figure 3(B)). When analysed individually, these three prominent cis-MUFAs were also inversely correlation with n-6 VLCPUFAs. Specifically, brain oleic and nervonic acid were inversely correlated with brain AA + DTA + DPAn-6 levels (Figure 3(C–E)). Importantly, the percentages of oleic and nervonic acid did not significantly change in the RBC with dietary EPA + DHA used in this study. In the plasma, nervonic acid percentages significantly increased ($p < 0.01$) in a dose responsive manner, increasing from 0.82% in control mice to 1.32% in mice fed the 1.8%en EPA + DHA diet.

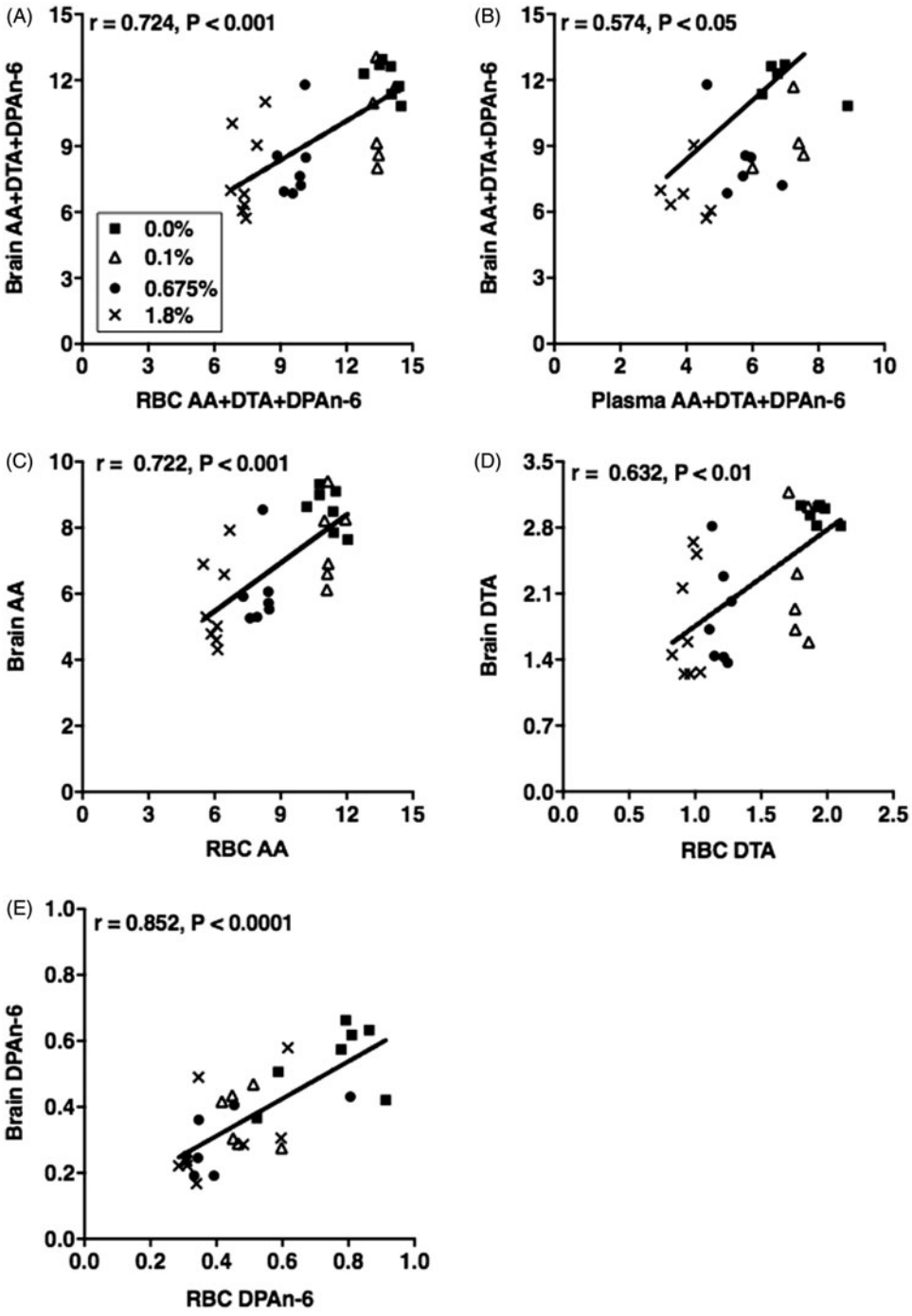

Figure 2. The relationship between red blood cell (RBC) and tissue arachidonic acid (AA), docosatetraenoic acid (DTA) & docosapentaenoic acid n-6 (DPAn-6) after dietary EPA + DHA intake contributing to 0% (CN), 0.10%, 0.675% and 1.8% of total daily energy intake. Values are mean AA + DTA + DPAn-6 densities ($n = 6$–8), expressed as % of total phospholipid fatty acid composition in the RBC, plasma, and brain. Scatter-plots of Spearman correlations comparing the reduction of sum n-6 LCPUFA in the brain to changes in the AA + DTA + DPAn-6 in the (A) RBC and (B) plasma. Scatter-plots of Spearman correlations comparing the reduction of the sum of n-6 LCPUFA in RBC to changes in whole brain (C) AA, (D) DTA, and (E) DPAn-6.

Enzyme activity estimates (EAEs) and fatty acid ratios in the brain

As shown in Table 2, SCD n-9 and D5D EAEs in the brain were significantly altered with dietary EPA + DHA supplementation. Specifically, brain SCD n-9 EAEs increased from 0.91 in the control mice compared to 1.24 in the 1.8%en EPA + DHA diet ($p < 0.001$). Brain D5D EAEs decreased significantly from 36.23 in control mice to 20.46 in mice

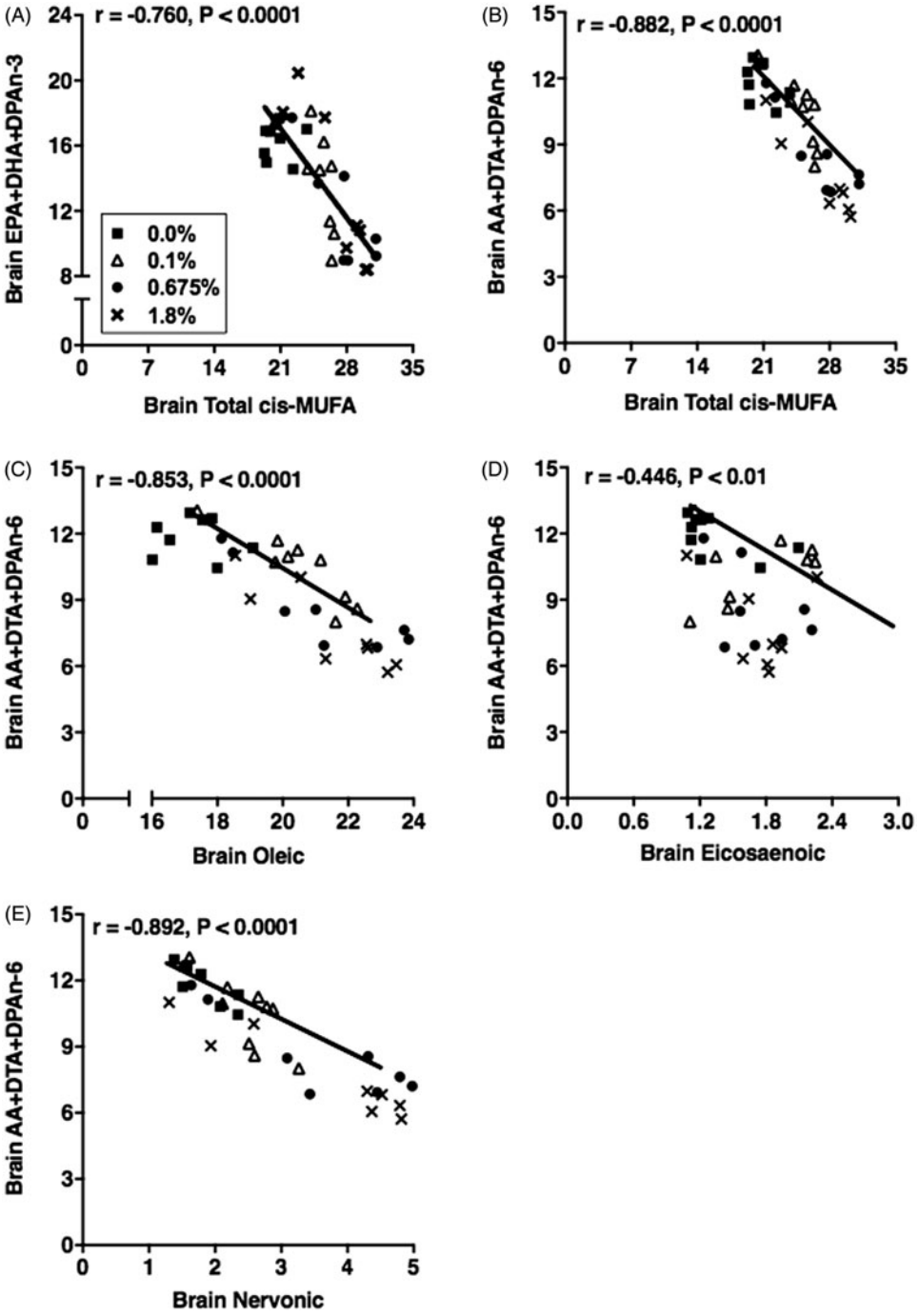

Figure 3. The relationship between whole brain cis-monounsaturated fatty acids (MUFAs), n-3 and n-6 LCPUFAs after dietary EPA + DHA intake contributing to 0% (CN), 0.10%, 0.675% and 1.8% of total daily energy intake. Mean total MUFA densities ($n = 6$–8), expressed as % of total phospholipid fatty acid composition in whole brain. Total cis MUFA = palmitoleic acid + oleic acid + eicosaenoic acid + nervonic acid. Scatter-plots of Spearman correlations comparing whole brain MUFA to changes in (A) total brain n-3 LCPUFAs and (B) total brain n-6 LCPUFAs. Scatter-plots of Spearman correlations comparing the loss of whole brain n-6 LCPUFAs to the increases in whole brain (C) oleic acid, (D) eicosaenoic acid, and (E) nervonic acid.

fed the highest dose of dietary EPA + DHA ($p < 0.0001$). On the other hand, brain SCD n-7 and D6D EAEs were not significantly altered with dietary EPA + DHA (Table 2). Several fatty acid ratios in the brain were also determined. As expected, the EPA/AA ratio significantly increased in mice fed the 1.8%en diet ($p < 0.0001$). However, the nervonic/lignoceric and eicosaenoic/arachidic ratios were not significantly altered with dietary EPA + DHA (Table 2).

Table 2. Brain phospholipid fatty acid desaturase EAEs and changes with dietary EPA + DHA[a].

Fatty acid	0.0% EPA + DHA	0.1% EPA + DHA	0.675% EPA + DHA	1.8% EPA + DHA	FDR p value[b]
SCD n-7[c]	$0.01 \pm 1e-03^{A}$	$0.01 \pm 9e-04^{AB}$	$0.01 \pm 1e-03^{AB}$	$0.01 \pm 9e-04^{B}$	0.156
SCD n-9[d]	0.91 ± 0.10^{A}	1.17 ± 0.18^{B}	1.21 ± 0.22^{B}	1.24 ± 0.15^{B}	**<0.001**
D6D[e]	0.55 ± 0.04^{A}	0.50 ± 0.07^{A}	0.54 ± 0.08^{A}	0.57 ± 0.10^{A}	0.378
D5D[f]	36.23 ± 2.95^{A}	30.50 ± 3.02^{A}	22.88 ± 2.65^{B}	20.46 ± 2.65^{B}	**<0.0001**
EPA/AA	$3e-03 \pm 7e-04^{A}$	$3e-03 \pm 9e-04^{AB}$	$6e-03 \pm 8e-04^{BC}$	$0.01 \pm 2e-03^{C}$	**<0.0001**
Eicosenoic/Arachidic	5.19 ± 0.58^{A}	5.48 ± 0.84^{A}	4.77 ± 0.51^{A}	4.96 ± 0.63^{A}	0.198
Nervonic/Lignoceric	3.30 ± 0.23^{A}	3.80 ± 0.61^{A}	4.04 ± 0.71^{A}	3.95 ± 0.67^{A}	0.087

[a]Mean ± standard deviation EAE values and fatty acid ratios displayed.
[b]ANOVA was conducted on dietary treatment group using Dunn's multiple comparison test. p Values were adjusted for false discovery according to Benjamini-Hochberg, and are bolded if $p < 0.05$.
[c]Calculated as ratio of palmitoleic acid/palmitic acid.
[d]Calculated as ratio of oleic acid/stearic acid.
[e]Calculated as ratio of dihomo-γ-linolenic acid/linoleic acid.
[f]Calculated as ratio of AA/dihomo-γ-linolenic acid.
A, AB, B, BC, and C represent the multiple comparison test for each EAE or fatty acid ratio for the 0.0%, 0.1%, 0.675%, and 1.8% EPA + DHA treatment groups.
EAE: enzyme activity estimate; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; AA: arachidonic acid; SCD n-7: stearoyl-CoA desaturase n-7; SCD n-9: stearoyl-CoA desaturase n-9; D6D: delta-6 desaturase; D5D: delta-5 desaturase.

Correlations of EAEs and fatty acid ratios in the RBC, plasma, and brain

Decreases in brain D5D EAEs were significantly correlated with decreases in the RBC ($r = 0.670$, $p < 0.001$) and plasma D5D EAEs ($r = 0.731$, $p < 0.01$) (Figure 4(A,B)). Brain D6D EAEs were not correlated with either the RBC or plasma D6D EAEs (Figure 4(C,D)). As shown in Figure 4(E), the fatty acid ratio of EPA/AA was highly correlated between the brain and RBC ($r = 0.944$, $p < 0.0001$). This ratio of EPA/AA was also strongly and significantly correlated between the brain and plasma ($r = 0.840$, $p < 0.0001$).

Summary of brain phospholipid fatty acid responses comparing control diet to 1.8% EPA + DHA

Figure 5 presents a visual summary of our results in brain phospholipid fatty acid metabolism between the control mice and the mice fed the 1.8%en EPA + DHA diet. Although only EPA and DHA dietary levels differed between these two treatment groups, there were differences in plasma phospholipid n-3, n-6, saturated, and MUFA content. Interestingly, all VLC saturated, *cis*-monounsaturated, and n-3 fatty acid percentages measured in this study increased significantly in the brain between these dietary treatments, with the exception of the DHA and eicosaenoic levels. These increases in these fatty acids are accompanied by a concomitant decrease in n-6 VLCPUFAs. Desaturase estimates were also altered in the 1.8%en EPA + DHA diet compared to control. Estimates of SCD-1 (i.e. SCD n-9 EAEs) increased in the brain, while brain D5D EAEs in the LCPUFA pathways decreased between the control and 1.8%en EPA + DHA diet.

Discussion

The primary purpose of this study was to characterise the brain's phospholipid fatty acid composition in response to dietary EPA + DHA in mice. The levels of several fatty acids were significantly altered when comparing the highest dietary intake of EPA + DHA with the control mice (Table 1). Specifically, the n-6 fatty acids AA, DTA, and DPA n-6 significantly decreased, along with the sum of these PUFAs (i.e. Total VLC n-6), resulting in over a 4% decrease in brain percentage of total n-6 fatty acids. LCMUFAs oleic and nervonic acid were significantly increased in response to increased dietary EPA and DHA. The increase in MUFA content was accompanied by a contaminant increase in VLC saturated fatty acids. The n-3 fatty acids EPA and DPA n-3 were increased 4- and 2-fold, respectively, but these PUFAs account for less than 1% of total fatty content in the brain. DHA levels were unchanged with dietary EPA + DHA intake.

Due to the difficulty of obtaining human brain tissue samples, most studies investigating brain fatty acid levels have been performed on post-mortem brains. DHA comprised approximately 15% of total fatty acid composition in healthy human brains (Fraser et al. 2010). Murine brain DHA levels in the current study were similar to humans, comprising approximately 16.2% of total fatty acids. Fraser et al. reported oleic acid comprised approximately 17.5% of the phospholipid fatty acid composition in human brains (Fraser et al. 2010), and in the current study, brain oleic acid levels in control mice were comparable with mean values at 17.4%. Similarly, DHA levels in the brain did not change after supplementation with n-3 fatty acids in rats (Gerbi et al. 1994), supporting our observation. The brain fatty percentages reported in our study are

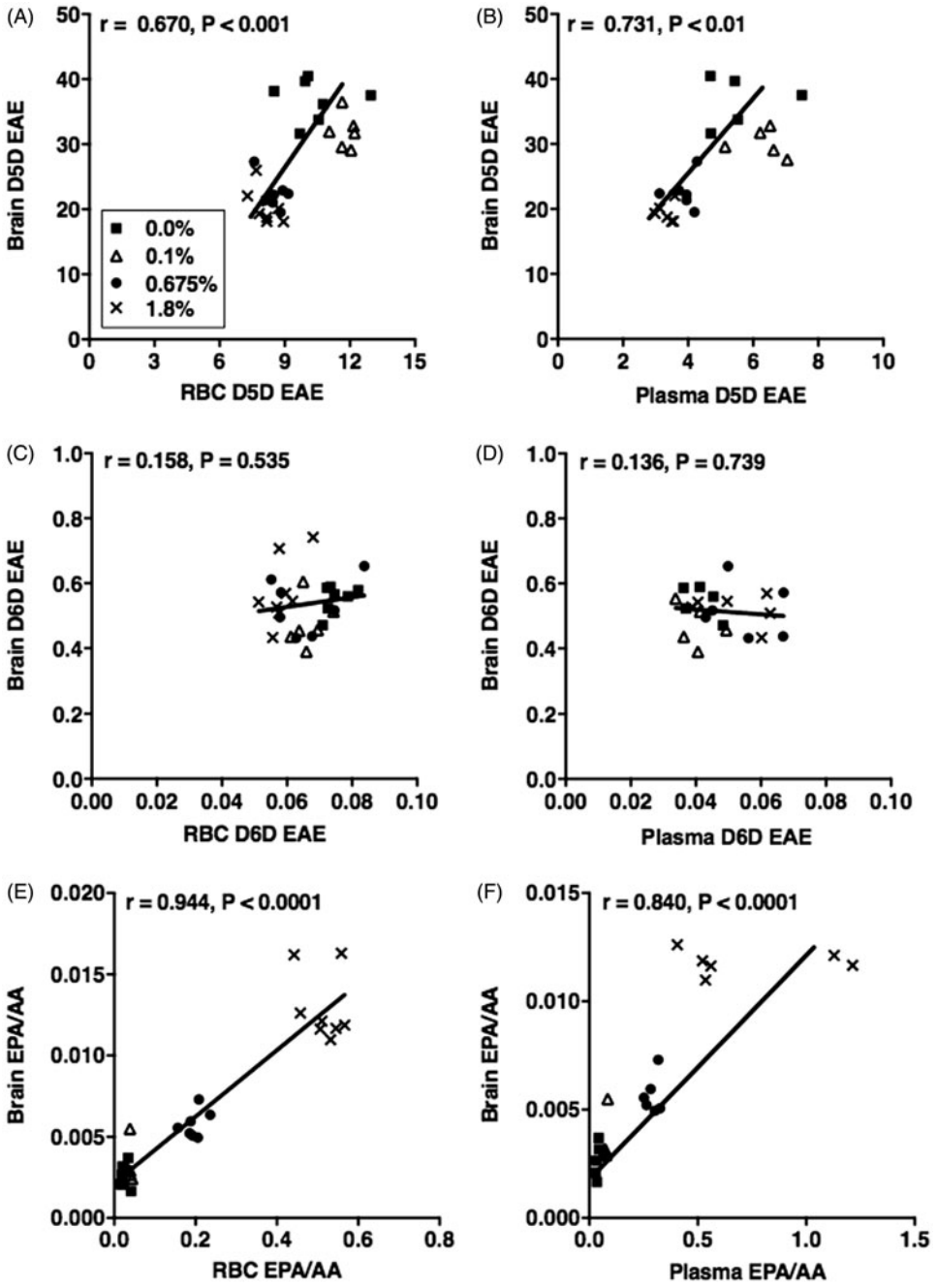

Figure 4. The relationship between whole brain enzyme activity estimates (EAEs) and fatty acid ratios after dietary EPA + DHA intake contributing to 0% (CN), 0.10%, 0.675% and 1.8% of total daily energy intake. Mean total EAEs and fatty acid ratios ($n = 6$–8), expressed. Scatter-plots of Spearman correlations comparing the delta 5 desaturase (D5D) EAEs in the whole brain and (A) red blood cell (RBC) and (B) plasma. Scatter-plots of Spearman correlations comparing the delta 6 desaturase (D6D) EAEs in the whole brain and (C) RBC and (D) plasma. Scatter-plots of Spearman correlations comparing the EPA/AA ratio in the whole brain and (E) RBC and (F) plasma. D5D calculated as ratio of AA/dihomo-γ-linoleic acid. D6D calculated as ratio of dihomo-γ-linoleic acid/linoleic acid.

also consistent with levels reported in previous studies. We report oleic acid increased by 4% in brain phospholipids when comparing the highest treatment group to controls in the current study. Oleic acid content also increased significantly in the brains of both wild type and Alzheimer's disease model mice fed diets supplemented with DHA (Bascoul-Colombo et al. 2016). Since human brain fatty acid levels are similar to those in mice, it is reasonable to assume that these observed brain fatty acid changes following dietary EPA + DHA supplementation can be extrapolated to humans.

Dietary PUFAs can cross the blood-brain barrier through mechanisms including passive diffusion and

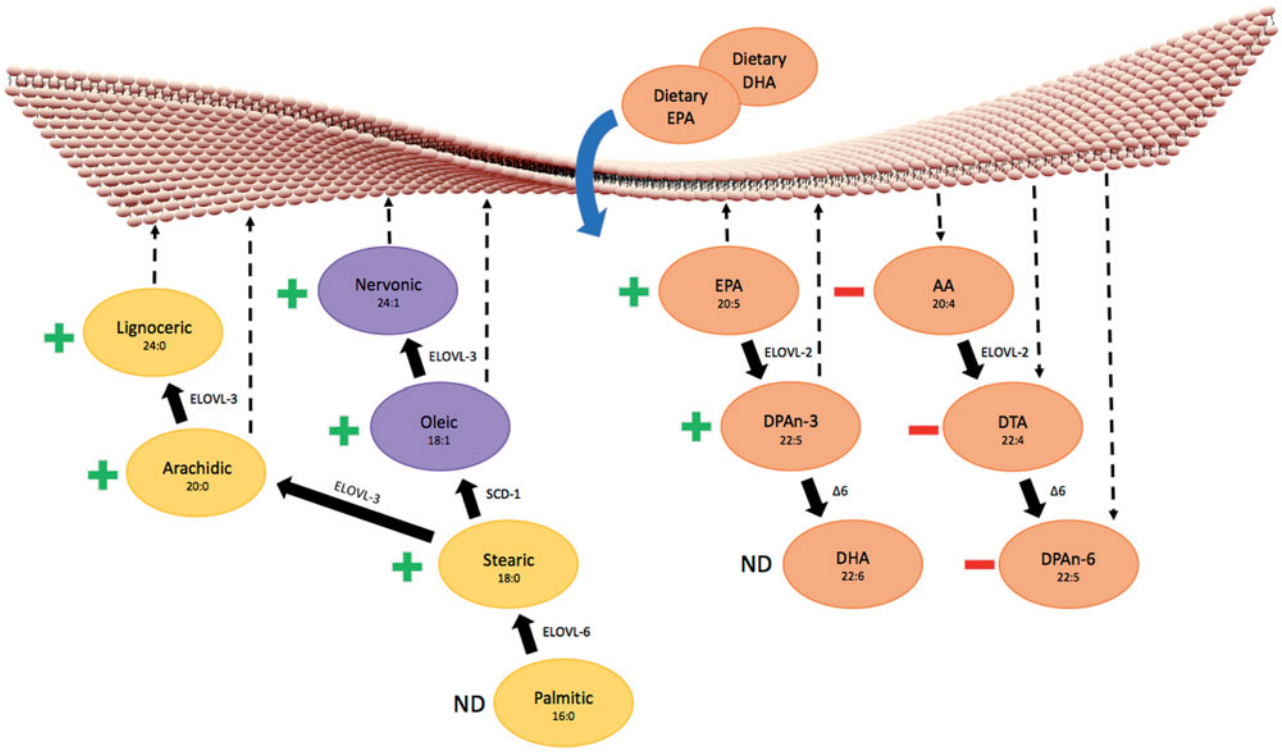

Figure 5. Summary of brain phospholipid fatty acid responses comparing control diet to 1.8% dietary EPA+DHA. Plus signs indicate significant increases, minus signs indicate significant decreases, and ND indicates no significant difference in phospholipid fatty acid levels. Saturated and monounsaturated fatty acids are displayed on the left, and polyunsaturated fatty acids are displayed on the right of the image. Solid arrows signify action of desaturating or elongating enzymes, and dashed arrows signify fatty acid incorporation into or replacement from the membrane. EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; AA: arachidonic acid; DTA: docosatetraenoic acid; ELOVL: elongation of very long chain; SCD: stearoyl-CoA desaturase; ND: no difference.

transporters depending on esterification forms. For instance, Sugasini et al. reported that the transporter Mfsd2a specially transports DHA esterified in lysophosphatidylcholine but not non-esterified DHA, and Ouellet et al. reported EPA, DHA, and AA can utilise passive diffusion from the plasma non-esterified lipid pool (Chen et al. 2008; Ouellet et al. 2009; Sugasini et al. 2017). It is widely accepted that increasing dietary EPA displaces AA from phospholipids, including in brain tissue, and that increased VLC n-3 fatty acids inhibit PUFA desaturase activity (Lo et al. 1999; Simopoulos 1999; Harel et al. 2000; Simopoulos 2008). Since n-3 fatty acids are preferred substrates for desaturases, this would explain why AA and AA-derived VLCPUFAs are lower in brain phospholipids in response to increased EPA + DHA dietary intake. Our data suggest that dietary EPA and DHA displace AA and AA-derived VLCPUFAs from phospholipids, while increasing the proportion of MUFAs and VLC saturated fatty acids (Figure 5). It is possible that n-3 fatty acids affect SCD-1 activity or expression, resulting in increased MUFA production to maintain membrane fluidity in response to the large displacement of VLC n-6 fatty acids. Although it was not within the scope of this study to directly measure desaturase enzyme expression and activity, we report that SCD n-9 enzyme activity estimates (EAEs) were significantly higher and SCD n-7 EAEs tended to be higher in mice with increased dietary EPA and DHA (Table 2).

We report MUFAs and saturated fatty acids with ≥20 carbons increased in the brain phospholipids of mice fed diets with 1.8% EPA + DHA, compared to the control mice (Table 1). ELOVL-3 is the major elongating enzyme of these VLC MUFA and saturated fatty acids (Jakobsson et al. 2006; Kitazawa et al. 2009). It is possible that increased dietary EPA and DHA alter the activity or expression of this ELOVL enzyme. However, one must consider that beta-oxidation of EPA in murine brains results in production of acetate, which may be utilised for fatty acid synthase; therefore, beta-oxidation of EPA could also contribute to the increased levels of VLC MUFA and saturated fatty acids (Chen et al. 2009). In addition, MUFA and VLC saturated fatty acid content in the liver was not significantly altered in response to increased dietary

EPA and DHA intake (Table S2), suggesting that the increase in brain MUFA and VLC saturated fatty acid content is not likely associated with altered metabolism in other tissues or fatty acid shuttling. It appears increased MUFA content in the brain is a unique phenomenon induced by a diet higher in EPA and DHA. What remains unclear is whether the displacement of VLC n-6 and contaminant increase in MUFAs and VLC saturated fatty acids has a beneficial or deleterious effect on brain function.

Increased saturated fatty acids and decreased MUFAs are associated with cognitive disease. Previous research reported brain nervonic acid levels were decreased in patients affected by demyelinating disease (Sargent et al. 1994). In addition, oleic acid levels are decreased in lipid rafts in the brains of patients with Alzheimer's disease when compared to those unaffected by the disease (Martin et al. 2010). It is possible that increased levels of brain MUFAs (i.e. nervonic and oleic acid) following increased dietary EPA + DHA intake may be beneficial in the prevention of neurological diseases. However, higher saturated fatty acid content has been associated with decreased spatial memory and learning ability in mice (Yu et al. 2010). An increased level of saturated fatty acids in the brain also decreases membrane fluidity. Therefore, there is conflicting evidence related to the effects of these fatty acid changes on membrane fluidity and cognitive function.

The secondary purpose of this study was to determine if the brain phospholipid fatty acid changes were correlated with changes in the RBC and plasma following dietary EPA + DHA. Because RBC life spans expand over 100 days, RBCs more accurately reflect an individual's long-term n-3 PUFA intake when compared to shorter-lived platelets or lipoproteins in a blood sample (Harris and Thomas 2010). We report plasma and RBC DHA levels increased with dietary EPA + DHA intake. As expected, DHA levels in the RBC and plasma were not correlated with changes in the brain. Our research adds to the growing body of evidence that DHA levels are likely tightly regulated in the brain.

Although DHA levels were not altered with dietary EPA + DHA supplementation in our study, it was recently reported that dietary DHA as lysophosphatidylcholine increases brain DHA content and improves brain function in adult mice, and these results were not observed when feeding non-esterified DHA, as it is often hydrolysed and absorbed as triglycerol (Sugasini et al. 2017). Therefore, future studies should investigate the effects of supplementing with the lysophosphatidylcholine form of DHA since this form may be more influential on the brain composition and function.

One limitation of this study is that the desaturase enzyme activities were estimated through product to precursor ratios. This is an oversimplification of the desaturase enzyme activities in the brain, and thus these estimates were used limitedly when investigating potential shifts in metabolism. Guillot et al. reported that DHA dosages exceeding one gram increased urinary isoprostane concentrations in humans, and Park et al. recently reported that DHA metabolism significantly differs between neuronal and non-neuronal cell types (Guillot et al. 2009; Park et al. 2016). Therefore, we recognise it is unknown whether oxidative stress induced by high dosages of combined EPA and DHA may have influenced our results in the brain. In addition, we acknowledge that the dose-response curves may differ between mice and humans, but the general trends in brain fatty acid composition are likely similar between these two mammalian species.

In summary, there is currently a dearth of studies that investigate altered fatty acid levels in the blood and tissues with supplementation of EPA + DHA. Unfortunately, this type of study is difficult, if not impossible, to perform in humans. However, the similar fatty acid levels between mice and human brains indicate the relevance of our model. Since there is much interest in PUFA supplementation and brain health, future studies should investigate whether PUFA induced changes in brain fatty acids, such as AA and nervonic acid, are beneficial or deleterious.

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Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

C. Austin Pickens http://orcid.org/0000-0003-1632-5777
Jenifer I. Fenton http://orcid.org/0000-0002-8875-3239

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