

# Dietary docosahexaenoic acid supplementation alters select physiological endocannabinoid-system metabolites in brain and plasma

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**Abstract** The endocannabinoid metabolome consists of a growing, (patho)physiologically important family of fatty-acid derived signaling lipids. Diet is a major source of fatty acid substrate for mammalian endocannabinoid biosynthesis. The principal long-chain PUFA found in mammalian brain, docosahexaenoic acid (DHA), supports neurological function, retinal development, and overall health. The extent to which dietary DHA supplementation influences endocannabinoid-related metabolites in brain, within the context of the circulating endocannabinoid profile, is currently unknown. We report the first lipidomic analysis of acute 2-week DHA dietary supplementation effects on the physiological state of 15 fatty-acid, *N*-acylethanolamine, and glycerol-ester endocannabinoid metabolome constituents in murine plasma and brain. The DHA-rich diet markedly elevated DHA, eicosapentaenoic acid, 2-eicosapentaenoylglycerol (EPG), and docosahexanoylethanolamine in both compartments. Dietary DHA enhancement generally affected the synthesis of the *N*-acyl-ethanolamine and glycerol-ester metabolites to favor the docosahexaenoic and eicosapentaenoic vs. arachidonoyl and oleoyl homologs in both brain and plasma. The greater overall responsiveness of the endocannabinoid metabolome in plasma versus brain may reflect a more circumscribed homeostatic response range of brain lipids to dietary DHA supplementation. ■ The ability of short-term DHA enhancement to modulate select constituents of the physiological brain and plasma endocannabinoid metabolomes carries metabolic and therapeutic implications.—Wood, J. T., J. S. Williams, L. Pandarinathan, D. R. Janero, C. J. Lammi-Keefe, and A. Makriyannis. **Dietary docosahexaenoic acid supplementation alters select physiological endocannabinoid-system metabolites in brain and plasma.** *J. Lipid Res.* 2010. 51: 1416–1423.

**Supplementary key words** anandamide • 2-arachidonoylglycerol • lipidomics • metabolomics • mouse • nutritional fatty-acid supplementation • omega-3 fatty acids

The endocannabinoid system includes two G-protein coupled cannabinoid receptors (CB1 and CB2), their endogenous lipid ligands (endocannabinoids), and the enzymes and transporters that help regulate cannabinergic tone (1). Endocannabinoid signaling is ubiquitous in mammals, being particularly critical in brain, where it modulates neurotransmitter release and exhibits neuroprotective effects (2, 3). Although the first two endocannabinoids identified, *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (AG), have been extensively characterized, the (patho)physiological impact of endocannabinoid signaling has prompted the search for additional endocannabinoids and their related metabolites/biosynthetic precursors, creating an evolving network of chemical species collectively termed the endocannabinoid metabolome, few of which have been functionally annotated (1, 3–9). Although the translational and diagnostic aspects of endocannabinoid signaling are well appreciated (10, 11), factors that influence and regulate the endocannabinoid metabolome profile among various tissues and compartments remains incompletely described and understood (1, 10), as are the metabolome's relationship to lipid pathways outside of the endocannabinoid signaling system (3, 9).

This work was supported by the National Institutes of Health NIH/NIDA grant P01 DA9158 (A.M.); training grant T32 DA7312 (A.M.); Nestec, Ltd. (C.J.L.K.); Loders-Croklaan (C.J.L.K.) and the National Fisheries Institute (C.J.L.K.). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health or other granting agencies.

Manuscript received 19 September 2009 and in revised form 13 January 2010.

Published, JLR Papers in Press, January 13, 2010

DOI 10.1194/jlr.M002436

Abbreviations: AA, 20:4[ $\omega$ -6], arachidonic acid; AEA (anandamide), *N*-arachidonoylethanolamine; AG, 2-arachidonoylglycerol; CB, cannabinoid receptor; DHA, 22:6[ $\omega$ -3], docosahexaenoic acid; DHEA, docosahexanoylethanolamine; DHG, 2-docosahexanoylglycerol; EPA, 20:5[ $\omega$ -3], eicosapentaenoic acid; EPG, 2-eicosapentaenoylglycerol; OEA, oleoylethanolamine; OG, 2-oleoylglycerol.

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Defining the extent to which specific dietary components can modulate the physiological endocannabinoid metabolome has been highlighted as an important contemporary research goal (10, 12–15). Of the two principal long-chain PUFAs in mammalian brain, docosahexaenoic acid ([22:6( $\omega$ -3)]; DHA) is preferentially incorporated into brain tissue relative to arachidonic acid ([20:4( $\omega$ -6)]; AA) (14, 16–18), from which AEA and AG are derived (1, 3). AA and DHA are supplied to the brain from the circulation, either from dietary sources or as synthesized primarily in the liver from shorter-chain essential fatty acids (19, 20). Dietary fatty acids thus exert particularly stringent substrate control over brain phospholipid production and are principal determinants of neuronal phospholipid composition (18–24). Adequate intake of DHA and/or eicosapentaenoic acid ([20:5( $\omega$ -3)]; EPA) is critical to normal brain development, structure, and function, and supports health (25–27). Nutritional DHA supplementation has also been shown to improve neurological status in psycho-behavioral disorders such as female postpartum depression and schizophrenia (28, 29).

The devastating inflammatory effects of certain AA-derived metabolites (30) and the health benefits of nutritive  $\omega$ -3 fatty acid supplementation in the central nervous system and periphery (21, 22, 25–29) have prompted *in vivo* fatty acid feeding studies that generally corroborate a positive association between DHA, EPA, or AA intake and the level of that respective fatty acid or their endocannabinoid-related ethanolamide and monoglyceride derivatives in various tissues and/or plasma (12, 13, 16, 23, 24, 31–33). The impact of DHA consumption on the various endocannabinoid-related metabolites in compartments where endocannabinoid signaling carries particular (patho)physiological and/or diagnostic import, such as brain and the blood circulation, has not been comprehensively examined *in vivo* (3, 29, 34, 35). To address this issue, we have applied our targeted LC-MS/MS lipidomics approach (35, 36) in a mouse model to quantify the coordinate effect of short-term (2-week) dietary DHA supplementation on 15 fatty acid, *N*-acyl-ethanolamine, and glycerol-ester constituents of the physiological brain and plasma endocannabinoid metabolomes. The results define *in vivo* metabolite- and compartment-selective responses of diverse endocannabinoid-related lipid species to dietary DHA enhancement that may be of functional and therapeutic significance.

## MATERIALS AND METHODS

### Materials

Fatty-acid free BSA was purchased from EMD Chemicals (San Diego, CA). HPLC-grade methanol and HPLC-grade chloroform were purchased from Fisher Scientific (Pittsburg, PA). High-purity gases and liquid nitrogen were purchased from Med Tech Gases (Medford, MA). Ethanol (200 proof), HPLC-grade acetone, and PBS powder were from Sigma-Aldrich (St. Louis, MO). Other reagents, including deuterated standards for LC-MS/MS analysis, were purchased from commercial sources or synthesized in-house, as previously described (36).

### Diets

For the DHA-supplemented experimental diet, DHA-rich fish oil (8:1 DHA:EPA by weight; Loders-Crooklaan, Channahon, IL) was added to 5P00 Prolab RMH 3000 mouse chow (Purina Mills, St. Louis, MO) to replace the pork fat in the standard control diet. The  $\omega$ -3 fatty acid content of the control diet was 0.25% compared with 1.45% in the experimental diet. The total fat content of the two diets was identical: 13.7% of total calories provided as fat. The grains used for both chow formulations were from the same lot to ensure that all other nutrient components would be identical. Both diets were stored at room temperature.

### Feeding procedure

Healthy male CD1 mice (Charles River Laboratories, Wilmington, MA), initially weighing 16–18 g, were acclimated to vivarium conditions for 1 week prior to experimentation; water and food were provided *ad libitum*. On experimental day 1, each animal was placed into a metabolism cage and supplied with water and either the control or the DHA-rich chow *ad libitum*. To prevent consumption outside the food trough and ensure accurate measurement of food intake, all chow was ground into a powder just prior to feeding. Prewedged amounts were introduced into each feeding trough on alternate days after recovering any residual food remaining from the prior feeding period. Food intake was monitored throughout the study, and animal body weight was determined at the beginning and end of the 2-week study period. This protocol was approved by the Northeastern University Institutional Animal Care and Use Committee in accordance with public health and safety policies.

### Sample collection

At the end of 2 weeks, mice were euthanized by cervical dislocation followed by decapitation and rapid collection of trunk blood and brain tissue. Plasma, immediately isolated by centrifugation, and brain samples were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. This method of euthanization, plasma/tissue collection, sample processing, and storage prevents artifactual changes in the lipid analytes, as reported for AEA and AG and validated by our laboratory for the other metabolites quantified (35, 36).

### Plasma extraction

As previously detailed (35, 36), frozen plasma was rapidly thawed, and a known amount of deuterated internal standard mixture was added. Plasma proteins were precipitated with one volume ice-cold acetone and centrifugation (20,000 g, 5 min,  $4^{\circ}\text{C}$ ). The supernatant was recovered, and the acetone was evaporated under nitrogen. Liquid-liquid phase extraction was performed on the remaining supernatant with PBS (pH 7.4):methanol:chloroform (1:1:2 by volume). The resulting two phases were separated by centrifugation (20,000 g, 5 min,  $4^{\circ}\text{C}$ ), and the lower organic layer was recovered quantitatively and evaporated to dryness under a nitrogen stream. Dried lipid extracts were reconstituted in ethanol, vortexed, sonicated briefly, and centrifuged (20,000 g, 5 min,  $4^{\circ}\text{C}$ ) prior to analysis.

### Brain extraction

Each frozen mouse brain was weighed prior to addition of deuterated internal standard mixture and homogenization in ice-cold acetone:PBS, pH 7.4 (3:1, by volume). After centrifugation (10,000 g, 10 min,  $4^{\circ}\text{C}$ ), the supernatant was recovered and processed as described above for plasma.

### LC-MS/MS metabolomic analysis

Multiple reaction monitoring of the endocannabinoid metabolome and the corresponding deuterated internal standards was

performed as previously described (8, 36) using a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA) with an Agilent 1100 HPLC on the front end (Agilent Technologies, Wilmington, DE). In brief, analytes were separated on an Agilent Zorbax SB-CN column (2.1 × 50 mm, 5 μm) with gradient elution using 10 mM ammonium acetate (pH 7.3 using ammonium hydroxide; solvent A) and 100% methanol (solvent B). Eluted peaks were ionized via atmospheric pressure chemical ionization and detected by their respective selected reaction monitoring transitions.

## Data analysis

The significance ( $P < 0.05$ ) of the differences between the control and DHA-supplemented animals in either the brain or plasma concentration for each endogenous metabolite analyzed was assessed with the *t*-test using Prism, version 4 (GraphPad Software, San Diego, CA).

## RESULTS

### Endocannabinoid metabolome profiling

A total of 15 fatty acid, *N*-acyl-ethanolamine, and acylglycerol species currently recognized as constituents of the endocannabinoid metabolome were analyzed simultaneously by our quantitative LC-MS/MS-based metabolomics approach (35, 36). Standard curves for all 15 analytes were linear with regression values of  $>0.98$ , except those for DHA and AA, which had linear regression values of 0.85 (data not shown). Extraction efficiencies were  $>90\%$  as determined by comparing the area ratios (analyte:internal standard) of each extracted BSA analyte to the unextracted standards in ethanol (36). Of the 15 endocannabinoid-related metabolites studied, all but eicosapentaenoylethanolamine were readily detected in both brain and plasma (Fig. 1).

### Dietary parameters, animal weight, and DHA/EPA intake

Mice fed a diet supplemented with DHA-rich fish oil for 2 weeks consumed the same amount of food as mice fed the nonsupplemented control chow ( $P > 0.05$ ; Table 1). Weight gain over the 2-week experimental period was also comparable in the two dietary groups ( $P > 0.05$ ). Based on the average amount of food ingested, mice fed the DHA-supplemented chow received 59.0 mg DHA and 8.9 mg EPA/day.

### Brain and plasma endocannabinoid responses to dietary DHA

The absolute mean concentrations and percent changes in the analyzed endocannabinoid metabolome constituents of brain and plasma between the control and DHA-supplemented animals are summarized in Table 2 along with statistical analysis of the significance of the mean difference for each lipid within either compartment. These results, examined in detail for each metabolic class below, demonstrate that 2-week dietary DHA enhancement altered select endocannabinoid metabolites in brain and/or plasma.

### Fatty acids

In response to the DHA-enriched diet, all fatty acids analyzed displayed a significant change in either or both compartments examined (Fig. 2). Dietary DHA supplementation increased the DHA content of both brain and plasma. Although the DHA-supplemented diet contained relatively low levels of EPA, the EPA content of brain increased significantly, as did that of plasma. The DHA-enriched diet reduced plasma AA but did not affect brain AA content.

### *N*-acyl-ethanolamines

Of the 5 *N*-acyl-ethanolamine species analyzed, only docosahexaenoylethanolamine (DHEA) significantly in-

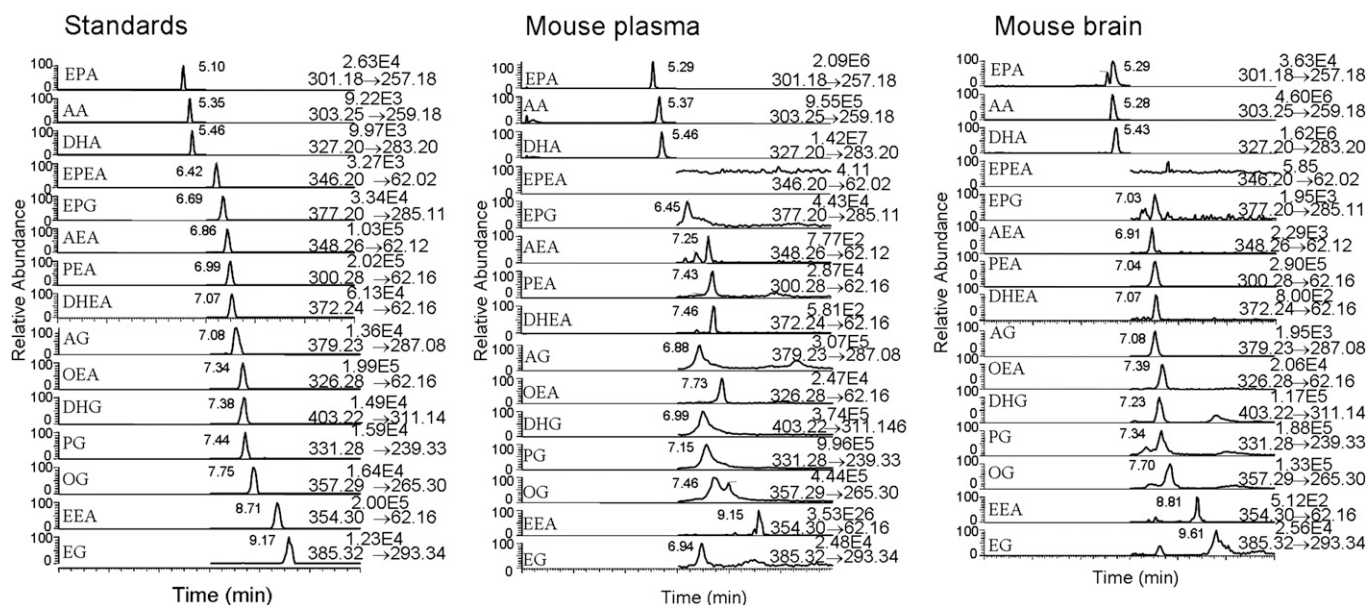


Fig. 1. Multiple reaction monitoring LC-MS chromatograms of the metabolite standards and physiological endocannabinoid metabolome constituents analyzed in mouse plasma and brain.



TABLE 1. Food consumption and weight gain

Parameter	Control Diet	DHA-Supplemented Diet
Food eaten (g/day)	5.52 ± 0.26	5.27 ± 0.51
Weight gained (g)	5.10 ± 1.70	5.79 ± 1.86

creased in both brain and (particularly) plasma upon dietary DHA supplementation (**Fig. 3**). The DHA-enriched diet decreased AEA in brain but not in plasma. The tissue specificity was opposite for oleylethanolamine (OEA): plasma levels decreased significantly, while brain content remained unchanged. Neither the brain nor plasma concentrations of palmitoylethanolamine (PEA) or eicosanoylethanolamine (EEA) were significantly altered by the DHA-supplemented diet.

### Glycerol-esters

Of the 6 glycerol-ester endocannabinoid metabolome constituents quantified, dietary DHA-supplementation did not significantly influence brain or plasma concentrations of 2-docosaheptaenoylglycerol (DHG), 2-palmitoylglycerol (PG), or 2-eicosanoylglycerol (EG) (**Fig. 4**). Concentrations of AG and 2-oleoylglycerol (OG) were likewise unaffected in brain but declined significantly in plasma. Despite the low levels of EPA in the DHA-supplemented diet, 2-eicosapentaenoylglycerol (EPG) concentrations increased markedly in both brain and plasma.

### ω-3, ω-6, and ω-9 metabolites

Because the two principal endocannabinoids in mammalian systems, AEA and AG, are derived from the ω-6 fatty acid, AA (1, 3), we directly compared the amounts of AEA and AG in brain and plasma relative to their corresponding DHA-derived homologs, DHEA and DHG. In both compartments, dietary DHA supplementation significantly increased the DHEA/AEA and DHG/AG ratios. Mean ratio increases were more pronounced in plasma (7.9 ± 1.0 vs. 2.9 ± 0.4;  $P < 0.01$  for DHEA/AEA and

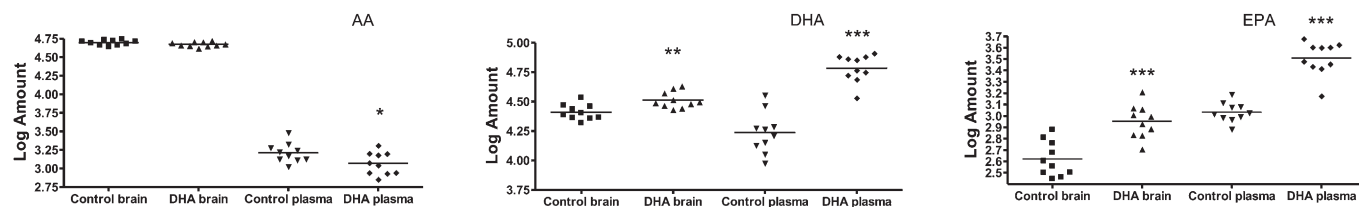
3.1 ± 0.2 vs. 1.4 ± 0.1;  $P < 0.001$  for DHG/AG) than in brain (3.3 ± 0.3 vs. 2.1 ± 0.1;  $P < 0.01$  for DHEA/AEA and 0.52 ± 0.04 vs. 0.42 ± 0.03;  $P < 0.05$  for DHG/AG). Similarly, with respect to ω-9 fatty acids, dietary DHA-supplementation significantly increased the ratio of DHEA/OEA in both compartments (0.73 ± 0.13 vs. 0.17 ± 0.04;  $P < 0.0001$  for plasma and 0.15 ± 0.03 vs. 0.11 ± 0.04;  $P < 0.05$  for brain), but the DHG/OG ratio increased only in plasma (1.5 ± 0.4 vs. 0.58 ± 0.18;  $P < 0.0001$ ).

## DISCUSSION

Simultaneous analysis of multiple metabolites by a high-sensitivity LC-MS/MS-based lipidomics approach is recognized as an experimental platform that can provide valuable chemical and quantitative information about the steady-state profile of specific lipid molecular species in complex biological matrices and the coordinate responses of these species to pathological, pharmacological, or nutritional provocation (33, 37, 38). Our targeted lipidomic analysis in a murine model constitutes the initial profiling of short-term dietary DHA-supplementation on 15 brain and plasma endocannabinoid-related lipids (36). The results demonstrate that 2 weeks of dietary DHA enrichment increases the DHA, EPA, DHEA, and EPG concentrations in both brain and plasma and exerts compartment- and/or metabolite-selective influence on other endocannabinoid-related species. MS-based experimental approaches such as ours require that analytes first be baseline resolved by in-line LC and then ionized for MS detection (37). In the present study, these technical requirements could not be met by select lipids of potential interest, particularly some precursors implicated in endocannabinoid formation (i.e., oleic and palmitic acid), which did not form sufficient negatively charged fragments to allow for their reliable quantification (8). Nonetheless, our analysis extends far beyond the two prototypical endocannabinoids, AEA and AG, to encompass an unprecedented chemical variety

TABLE 2. Concentrations (mean ± SEM; n = 10) and mean percent changes in endocannabinoid metabolome constituents in mice fed a DHA-supplemented diet relative to mice fed the control diet. *P*-values for significant ( $P < 0.05$ ) dietary DHA-induced changes are italicized.

Analyte	Brain				Plasma			
	Control Diet (ng/g)	DHA Diet (ng/g)	Mean % Change	<i>P</i>	Control Diet (ng/ml)	DHA Diet (ng/ml)	Mean % Change	<i>P</i>
<b>Fatty acids</b>								
DHA	25,900 ± 3,990	33,000 ± 5,500	27	<i>0.0026</i>	18,600 ± 8,150	62,800 ± 15,100	238	<i>&lt;0.0001</i>
EPA	443 ± 167	943 ± 321	113	<i>0.0001</i>	1,100 ± 220	3,370 ± 990	207	<i>&lt;0.0001</i>
AA	50,000 ± 3,900	47,500 ± 3,540	-5	0.1688	1,700 ± 570	1,240 ± 430	-27	<i>0.0366</i>
<b>N-acyl-ethanolamines</b>								
DHEA	3.77 ± 0.66	4.62 ± 0.74	22	<i>0.0129</i>	1.25 ± 0.28	2.34 ± 0.58	87	<i>&lt;0.0001</i>
AEA	1.88 ± 0.46	1.47 ± 0.42	-22	<i>0.0324</i>	0.55 ± 0.38	0.38 ± 0.26	-32	0.1757
OEA	41.1 ± 30.9	31.9 ± 7.1	-22	0.4334	7.48 ± 2.16	3.25 ± 0.74	-57	<i>&lt;0.0001</i>
PEA	55.8 ± 21.2	65.5 ± 23.2	18	0.3092	7.06 ± 2.30	6.05 ± 1.32	-14	0.3245
EEA	4.59 ± 1.04	4.37 ± 1.00	-5	0.6275	0.30 ± 0.26	0.21 ± 0.21	-30	0.2692
<b>Glycerol-esters</b>								
DHG	2,500 ± 1,470	2,970 ± 1,640	19	0.4613	2,150 ± 990	2,550 ± 1,030	19	0.3694
PG	1,970 ± 990	2,060 ± 1,120	5	0.954	8,560 ± 2,120	7,160 ± 1,890	-16	0.1674
EG	1,080 ± 210	1,230 ± 220	14	0.1757	208 ± 45	237 ± 67	14	0.2678
AG	5,700 ± 2,100	5,480 ± 1,950	-4	0.8164	1,570 ± 750	876 ± 442	-44	<i>0.0227</i>
OG	1,980 ± 560	2,010 ± 670	1	0.9211	4,270 ± 2,580	1,830 ± 840	-57	<i>0.0144</i>
EPG	54.1 ± 20.3	205 ± 77	278	<i>&lt;0.0001</i>	204 ± 113	485 ± 309	137	<i>0.0034</i>

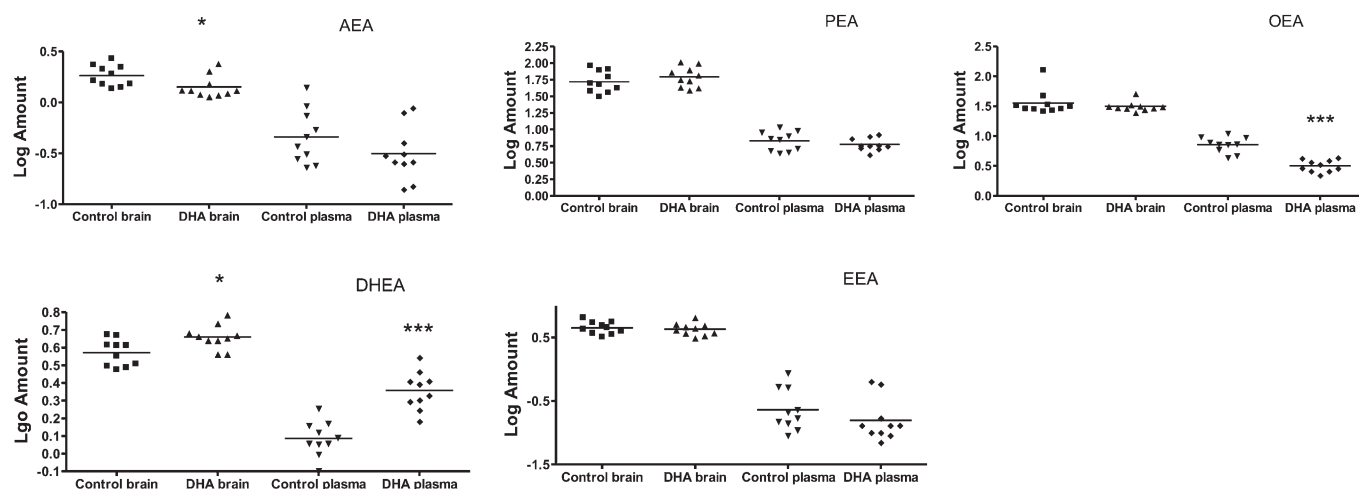


**Fig. 2.** Fatty acid levels in brain and plasma. Plotted values are log transformations of ng/g brain and ng/ml plasma for each individual animal. The number of stars represents the significance of the mean differences for each metabolite between animals fed the control versus the DHA-supplemented diet for each respective compartment examined; one to three stars represent *t*-test *P*-values < 0.05, 0.01, and 0.001, respectively.

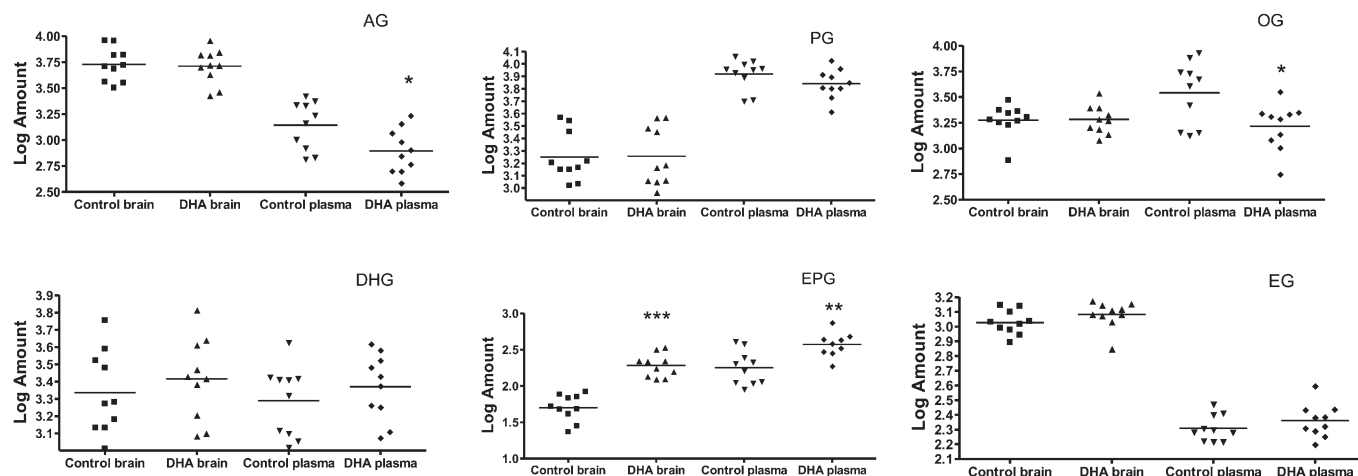
of 15 endocannabinoid-related lipids. The multiplicity and partial redundancy of the putative routes for AEA and AG synthesis, the ill-defined nature of the biosynthetic pathways for many other endocannabinoid-related species, and the ongoing identification of new intrinsic cannabinergic mediators make the endocannabinoid metabolome an evolving entity that will likely defy complete biochemical and functional characterization for some time (1, 3, 9, 39). Consequently, additional endocannabinoid-related lipids other than those examined herein await discovery and analysis. As exemplified by a recently published LC-MS method for *N*-acyl-phosphatidylethanolamines (40), future technological advances should prove enabling to this effort.

The acute, 2-week duration of our dietary DHA regimen is based upon previous literature findings. Lim and Suzuki (32) established that dietary DHA-ethyl ester supplementation increased brain DHA concentration in Crj:CD-1 mice within 2 weeks, an increase that was sustained over the subsequent 3 months with continued supplementation. DHA and EPA plasma concentrations, on the other hand, continued to increase throughout the 3 months. Another study reported that 1 week of increased DHA consumption did not alter brain levels of AEA, AG, eicosapentaenoylethanolamine, and DHEA in the rat (12).

The species difference notwithstanding, these combined studies (12, 32) suggest that, although the plasma lipid profile may be more responsive acutely to dietary DHA enhancement, the brain fatty acid complement and derived metabolites can establish a new steady-state within 2 weeks. While these results provide a rationale for our 2-week DHA supplementation protocol, it is conceivable that other levels and/or diet duration could elicit changes in the endocannabinoid metabolome different from those we have described (41). In the current study, DHA-induced increases in the plasma concentrations of DHA and its *N*-acyl-ethanolamine (DHEA) were associated with increased brain concentrations of these species, but brain DHA and DHEA levels did not increase to the same extent as they did in plasma. The greater acute sensitivity of the plasma versus brain lipid complement (including endocannabinoid species) to dietary DHA supplementation that we and others (12, 17, 32) have noted suggests that a plateau effect may operate physiologically to maintain brain lipids within a comparatively more stringent homeostatic profile in the face of dietary fatty acid enhancement. Further support for this suggestion comes from the observation that inhibition of intestinal fatty acid absorption by genetic CB26 transporter ablation in mice decreased plasma but not brain OEA (42).



**Fig. 3.** *N*-acyl-ethanolamine levels in brain and plasma. Plotted values are log transformations of ng/g brain and ng/ml plasma for each individual animal. The number of stars represents the significance of the mean differences for each metabolite between animals fed the control versus the DHA-supplemented diet for each respective compartment examined; one to three stars represent *t*-test *P*-values < 0.05, 0.01, and 0.001, respectively.



**Fig. 4.** Glycerol-ester levels in brain and plasma. Plotted values are log transformations of ng/g brain and ng/ml plasma for each individual animal. The number of stars represents the significance of the mean differences for each metabolite between animals fed the control versus DHA-supplemented diet for each respective compartment examined; one to three stars represent *t*-test *P*-values < 0.05, 0.01, and 0.001, respectively.

The fish oil used in our DHA-supplemented chow contained 8-fold more DHA than EPA. Yet the levels of EPA in the brain and plasma of the DHA-supplemented group were notably elevated relative to the control group fed standard chow. This result implies that at least some of the EPA increase upon dietary DHA supplementation might reflect DHA retroconversion to EPA (43, 44). Although EPA is efficiently  $\beta$ -oxidized in mouse brain (45), some DHA-derived EPA could have escaped that catabolic route to serve as substrate for the downstream increase of brain EPA glycerol-ester (i.e., EPG) with enhanced DHA intake.

Supplemental  $\omega$ -3 fatty acid intake (including DHA) can reduce cell and tissue levels of AA and AA-derived downstream metabolites, including AEA and/or AG potentially due to substrate competition among biosynthetic enzymes (12, 16, 23, 24, 32, 46, 47). Alternatively, diets low in  $\omega$ -3 and rich in  $\omega$ -6 fatty acids (including AA) have been shown to increase AA-derived AEA and AG in mouse and piglet brain (24, 31). Our acute dietary DHA regimen affected brain and plasma *N*-acyl-ethanolamine and glycerol-ester endocannabinoid metabolites to favor the formation of  $\omega$ -3 docosahexaenoic and eicosapentaenoic derivatives over their  $\omega$ -6 arachidonoyl and  $\omega$ -9 oleoyl analogs. Specifically, DHEA and EPG concentrations increased in both plasma and brain, whereas AEA decreased in brain and OEA, AG, OG, and AA levels decreased in plasma. Collectively, these data suggest that competition among lipid biosynthetic enzymes utilizing fatty acids as substrates could represent an important influence on the formation of downstream lipid metabolites, including their derived endocannabinoid metabolome constituents.

The causative factors underlying the endocannabinoid metabolome changes we have documented in vivo are likely multifactorial and not limited to substrate competition among lipid biosynthetic enzymes. Although we did not analyze the changes in the fatty acid composition of phospholipid precursors for fatty acid ethanolamines and

glycerol esters, increased availability of DHA for acylation of endocannabinoid precursors such as membrane phospholipids, *N*-acyl-phosphatidylethanolamines, and 1,2-diacylglycerol is perhaps the most direct candidate explanation for the shift we observe toward DHA-derived brain and plasma *N*-acyl-ethanolamine and glycerol-ester endocannabinoid metabolites over their respective arachidonoyl and oleoyl species with short-term dietary DHA enrichment. Dietary fatty acids can exert multiple, indirect influences on lipid metabolism across  $\omega$ -3,  $\omega$ -6,  $\omega$ -9 pathways through various processes, including tissue fatty acid uptake, distribution, and oxidation; fatty acid circulation time (in lipoproteins), inter- and trans-compartmental fluxes, and clearance/elimination rates; and acyltransferase activity/selectivity and desaturase activity (20, 23, 48–51). With respect to  $\omega$ -3 PUFAs, dietary DHA or EPA supplementation rapidly shunts palmitate and oleate away from esterification and storage in tissue/circulating substrate lipid pools that could be mobilized for synthesis of downstream products (such as OEA and its glycerolipid precursors) by potentiating their mitochondrial oxidation (48). Reciprocal cross-talk among nonmetabolically related fatty acids in the  $\omega$ -3 and  $\omega$ -6 pathways in brain can also be elicited in vivo by various dietary or genetic manipulations (23). Emken et al. (51) have shown that dietary linoleic acid ( $\omega$ -6) competes with linolenic acid ( $\omega$ -3) for conversion to respective downstream  $\omega$ -6 and  $\omega$ -3 metabolites and their incorporation into or partitioning between membrane and plasma lipid stores. The present study thus offers guidance for directly investigating the underlying metabolic basis in vivo for specific DHA-induced changes in the endocannabinoid metabolomes of brain and plasma by employing suitably tagged molecular probes (51).

Endocannabinoids and their metabolites have been implicated in such basic physiological processes as neuroprotection, energy balance, food consumption, and cardio-metabolic health (3, 10, 11, 38). Our results invite specula-

tion that at least some of the changes we have identified in the brain and circulating endocannabinoid metabolomes could have a physiological impact as a result of dietary DHA supplementation. For example, our DHA-enriched diet significantly decreased circulating levels of both the anorectic lipid OEA as well as AG, an endocannabinoid that promotes eating (15, 52, 53). Our observation that acute dietary DHA supplementation did not influence either food consumption or weight gain may reflect a counterbalancing effect between the coordinate, DHA-induced decreases in circulating OEA and AG that allowed maintenance of homeostatic feeding behavior. Alternatively, because a local OEA increase in the small intestine is sufficient to suppress appetite to an extent similar to that elicited by systemic OEA administration (15, 52–54), changes in plasma/brain OEA per se (vs. changes in small intestine OEA) may not be reliable correlates to or indices of food consumption, which is a complex process involving multiple, interactive nutritional, bioenergetic, and metabolic signaling pathways (55, 56).

Shifts in lipid (including endocannabinoid-related species) production consequent to nutritive changes in fatty acid supply may have beneficial consequences with therapeutic potential. Diets rich in  $\omega$ -3 PUFAs dampened the inflammatory response and reduced ectopic triacylglycerol accumulation in the obese Zucker rat, an effect attributed, at least in part, to the accompanying reduction in membrane phospholipid AA ester substrate for proinflammatory molecules and AEA/AG (47). Although appropriate caution should be exercised in extrapolating these findings in a genetic rat model of obesity and related metabolic dysfunction (i.e., type-2 diabetes) to ours in healthy mice, the aggregate data of Batetta et al. (47) and the present study suggest that dietary  $\omega$ -3 supplementation may shift AA availability in vivo to modulate endocannabinoid tone for therapeutic benefit. This suggestion is reinforced by the positive correlations observed between plasma AG and triacylglycerol levels in abdominally obese men (57) and in mice whose plasma AG had been pharmacologically elevated (58). Whether brain or circulating triacylglycerol levels are modified by our dietary DHA-enrichment regime remains to be explored. A longer period of DHA supplementation may be required, as suggested by the demonstration that  $\omega$ -3-rich diets can lower plasma triacylglycerol (usually by 10–20%) in various strains of healthy mice but only after 4–16 weeks of supplemental  $\omega$ -3 fatty acid intake (59–61).

In summary, we report the first focused lipidomic analysis of the effects of acute (2-week) dietary DHA supplementation on the physiological state of 15 fatty-acid N-acyl-ethanolamine and glycerol-ester constituents of the endocannabinoid metabolome in murine plasma and brain. The DHA-rich diet markedly elevated DHA, EPA, DHEA, and EPG in both compartments. Dietary DHA enhancement shifted the physiological balance of N-acyl-ethanolamine and glycerol-ester endocannabinoid-related metabolites to favor  $\omega$ -3 over  $\omega$ -6 and  $\omega$ -9 species, i.e., docosahexaenoyl and eicosapentaenoyl versus arachidonoyl and oleoyl derivatives. The plasma endocannabinoid

metabolome was more responsive to the dietary manipulation than that of brain. The ability of dietary DHA enhancement to moderately select constituents of the physiological endocannabinoid metabolomes of brain and plasma has metabolic and therapeutic implications. These data provide a foundation for future studies to define potentially beneficial relationships between the short-term effects of enhanced DHA intake on endocannabinoid-related lipid species and indices of psychobehavioral and physical health. **BB**

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