Anandamide and diet: Inclusion of dietary arachidonate and docosahexaenoate leads to increased brain levels of the corresponding *N*-acylethanolamines in piglets

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Endogenous ligands of cannabinoid receptors have been discovered recently and include some *N*-acylethanolamines (NAEs; e.g., *N*-arachidonoylethanolamine) and some 2-acylglycerols (e.g., *sn*-2-arachidonoylglycerol). Previously, we found these compounds to be active biologically when administered *per os* in large quantities to mice. In the present work, piglets were fed diets with and without 20:4*n*−6 and 22:6*n*−3 fatty acid precursors of NAEs, in levels similar to those found in porcine milk, during the first 18 days of life, and corresponding brain NAEs were assessed. In piglets fed diets containing 20:4*n*−6 and 22:6*n*−3, there were increases in several biologically active NAEs in brain homogenates—20:4*n*−6 NAE (4-fold), 20:5*n*−3 NAE (5-fold), and 22:5*n*−3 and 22:6*n*−3 NAE (9- to 10-fold). These results support a mechanism we propose for dietary long-chain polyunsaturated fatty acids influences on brain biochemistry with presumed functional sequelae. This paradigm will enable targeted investigations to determine whether and why specific populations such as infants, elderly, or persons suffering from certain clinical conditions may benefit from dietary long-chain polyunsaturated fatty acids.

arachidonic acid | docosahexaenoic acid | essential fatty acids

Cannabinoids (CBs) have been used for at least 4,000 years to treat migraine, muscle spasm, seizures, glaucoma, pain, and nausea. Identification of the endogenous lipids *N*-acylethanolamines [NAEs; e.g. *N*-arachidonoylethanolamine (anandamide)] and 2-acylglycerols [e.g., *sn*-2-arachidonoylglycerol (2-AG)], as ligands of CB receptors is one of the most important developments in CB research in recent years (1–9). Polyunsaturated NAEs such as anandamide exert their biological effects principally by means of binding to G protein-coupled CB1 receptors, but also bind to CB2 receptors. The CB1 receptor is found predominantly in brain, with highest densities in hippocampus, cerebellum, and striatum. The CB2 receptor is found predominantly in spleen and hematopoietic cells, with 44% overall nucleotide sequence identity with CB1 receptor.

Recently, there has been interest in effects of orally derived NAEs. In 1996, *N*-arachidonoyl NAE and other NAEs were identified in chocolate [a non-arachidonic acid-containing food (10)] and were purported to be responsible for "rewarding" properties of chocolate in lay and scientific media (11). We, however, failed to validate the finding of 20:4*n*−6 NAE in chocolate (12). Non-*N*-arachidonoyl NAEs were found in plant foods, and 2-AG was present in milks, but not at levels having typical cannabimimetic activity. Available data suggest most CB receptor-binding lipids in foods would be degraded in the intestine and liver (13), although such lipids could have biological activity on binding to gut CB receptors. The possibility remains, however, that dietary long-chain polyunsaturated fatty acids (LCPUFA) consumption could lead to higher cellular levels of corresponding NAEs, monoacylglycerols (MAGs), and primary amides by influencing NAE precursor lipid pools of 20:4*n*−6 and 22:6*n*−3 in whole brain and brain regions. In this study, this hypothesis was tested in piglets that were fed diets with and without 20:4*n*−6 and 22:6*n*−3 at levels similar to those found in porcine milk, and brain levels of corresponding NAEs were assessed. Remarkable differences were observed in 20:4*n*−6, 22:6*n*−3, and some related NAEs. These results have important implications for resolving the many controversies still surrounding the nutritional influences of *n*−3 and *n*−6 LCPUFA. They provide a rational basis for explaining previously observed dietary influences on neural and gastrointestinal function and will help to orient future research on the value of LCPUFA in the nutrition of infants and the elderly, and in specific clinical populations.

Experimental Procedures

Animals and Diets. Newborn male piglets weighing >1 kg at birth and <12 h old (Kintail Meats, Langley, BC, Canada) were fed liquid formulas based on the macro- and micronutrient composition of pig's milk that differed only in fat composition ($n = 6$ piglets per group; Table 1). In a separate feeding study, piglets were fed identical diets that had been stored at −80°C under N_2, and individual brain sections were analyzed for NAEs ($n = 6$ per group). Formulas contained 8.30% of total calories (en %) 18:2n−6 and 0.80 en % 18:3*n*−3, and were supplemented optionally with 0.20 en % 20:4*n*−6 and 0.16 en % 22:6*n*−3 (0.30–0.40 g/100 g of total fatty acids; Table 2). The 20:4*n*−6 and 22:6n−3 were from single-celled organism triacylglycerols and were added to formula at the Nestlé Product Technology Centre (Konolfingen, Switzerland). The formulas contained 57.9 g of fat/liter, 4.143 MJ/liter, and a macro- and micronutrient composition similar to that used (14). The formula-fed piglets were bottle-fed by hand until 18 d of age (14). Littermates were not assigned to the same diet. Piglet procedures were approved by the University of British Columbia Animal Care Committee and conformed to Canadian Council on Animal Care guidelines.

Sample Collection. Piglets were anesthetized by intramuscular injection of 37.5 mg/kg ketamin hydrochloride (MTC Pharmaceuticals, Cambridge, Canada) and 3.75 mg/kg xylazine hydrochloride (Bayvet Division, Chenango, Etobicoke, Canada) at 18 d of age, 3–4 h after the last feeding (14). Blood samples were

Abbreviations: 2-AG, *sn*-2-arachidonoylglycerol; anandamide, *N*-arachidonoylethanolamine; CB, cannabinoid; MAG, monoacylglycerol; LCPUFA, long-chain polyunsaturated fatty acids; NAE, *N*-acylethanolamine.

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Table 1. Fat components of formulas varying in 20:4*n*−6 and 22:6*n*−3 content

Oil	Content in formula, g/100 g of oil	
	C−	C+
Milk fat	1.70	1.70
MCT	8.70	8.70
Palm olein	4.90	4.40
Trisun oil	8.00	8.30
Soybean oil	6.50	6.30
Single-cell AA	0.00	0.30
Single-cell DHA	0.00	0.20

C−, control diet without 20:4*n*−6 and 22:6*n*−3; C+, control diet with added 20:4*n*−6 and 22:6*n*−3; AA, arachidonic acid; DHA, docosahexaenoic acid; MCT, medium-chain; triacylglycerol; single cell, refers to a single-celled organism triacylglycerol source; Trisun oil is a commercial source of sunflower oil.

drawn by intracardiac puncture, and animals were killed by intracardiac injection of 200 mg of pentobarbital per kg of body weight. Brains were removed rapidly and weighed, and the entire frontal cortex, striatum, hippocampus, and hypothalamus were removed for neurotransmitter analysis (15, 16). Remaining brain tissue was homogenized in buffer (0.32 M sucrose/15 mM Tris·HCl/1 mM EDTA/1 mM $MgCl_2$/1.5 mM glutathione, pH 7.4), frozen in liquid N_2, stored in aliquots at −80°C, and later analyzed for phospholipids (ref. 15; S.I., G. Crozier, M. Turini, S. de la Presa Owens, and R. Dyer, unpublished data) and primary amides, NAEs, and MAGs, as reported herein. In another set of experiments, six distinct brain regions were dissected rapidly from piglets fed with sow's milk or diets with and without 20:4*n*−6 and 22:6*n*−3, and were analyzed for 20:4*n*−6 and 22:6*n*−3 NAEs.

NAE, Primary Amide, and MAG Analysis. Brain samples were extracted with $CHCl_3$/MeOH 2:1 (vol/vol) containing [$^2H_8$]-20:4*n*−6-NAE, and [$^2H_8$]-2-AG as internal standards. To purify and characterize NAEs and MAGs, the organic phase was dried and then was submitted to chromatographic steps, including SiO_2 open-bed chromatography and normal-phase HPLC. For normal-phase HPLC, column fractions containing NAEs and MAGs were eluted with increasing concentrations of 2-propanol in hexane (17). Quantification of NAEs and MAGs was by GC–electron impact mass spectrometry of corresponding trimethylsilyl ether derivatives. Briefly, HPLC fractions with the same retention time as synthetic NAEs (23–29 min) and 2-AG and 1(3)-AG (16–22 min) were derivatized with 20 ml of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane for 2 h at room temperature and were analyzed by GC–electron impact mass spectrometry in selected-ion monitoring mode (18, 19). Ions were selected at m/z = 427, 419, 412, and 404 for 20:4*n*−6 NAE (corresponding to molecular ions and −15 mass unit fragment ions of [$^2H_8$]- and nondeuterated 20:4*n*−6 NAE, respectively) and at m/z = 530, 522, 515, and 507 for 2-AG (corresponding to the molecular ions and the −15 mass unit fragment ions of [$^2H_8$]- and nondeuterated 2-AG, respectively). Sensitivity of measurement was 1 pmol. NAE and 2-AG amounts were quantified by comparison to coeluting deuterated standards, calculating peak area ratios at m/z − 15 mass units fragment ions. For quantification of 22:4*n*−6 NAE/MAG, 20:5n−3 NAE, 22:5*n*−3 NAE, and 22:6*n*−3 NAE/MAG, standard curves were constructed with 0.1–5.0 nmol of derivatized synthetic compounds and were run immediately after sample HPLC fractions containing these compounds.

Statistical Analyses. Pairwise comparisons were made by using one-sided Student *t* tests to evaluate whether dietary 20:4*n*−6 and 22:6*n*−3 can increase corresponding NAEs. Statistical significance was evaluated at $P < 0.05$ and $0.05 < P < 0.10$. For whole-brain homogenate and brain section NAE analyses, n = 3 analyses per group.

Table 2. Major fatty acid components of formulas varying in 20:4*n*−6 and 22:6*n*−3 content

FA	Content, g of FA/100 g of total FA		
	Formula		
	C−	C+	Sow's milk
8:0	17.20	14.90	ND
10:0	13.50	13.00	ND
12:0	1.00	0.30	0.10
14:0	0.80	0.60	2.40
16:0	11.30	10.90	28.10
18:0	3.20	3.30	5.60
16:1*n*−7	0.10	0.20	4.70
18:1*n*−9	33.30	35.10	32.60
18:2*n*−6	15.60	16.40	20.40
20:2*n*−6	ND	ND	0.40
18:3*n*−3	1.50	1.60	ND
18:3*n*−6	ND	0.10	0.20
20:3*n*−6	ND	ND	0.20
20:4*n*−6	ND	0.40	0.70
22:4*n*−6	ND	ND	0.10
20:5*n*−3	ND	ND	0.10
22:5*n*−3	ND	ND	0.60
22:6*n*−3	ND	0.30	0.10

C−, control diet without 20:4*n*−6 and 22:6*n*−3; C+, control diet with added 20:4*n*−6 and 22:6*n*−3. FA, fatty acid; ND, not detected.

Results and Discussion

Polyunsaturated NAEs. Supplementation of control diets with 20:4n−6 and 22:6n−3 led to brain homogenate increases in 20:4*n*−6 (3.9-fold), 22:4*n*−6 (1.6-fold), 20:5*n*−3 (5.2-fold), 22:5*n*−3 (9.4-fold), and 22:6*n*−3 NAEs (9.5-fold; Table 3), and to statistically significant increases for the combined measurements of 20:4*n*−6 and 22:4*n*−6 NAEs (n = 6; $P < 0.03$) and for 20:5*n*−3, 22:5*n*−3, and 22:6*n*−3 NAEs (n = 9; $P < 0.02$, unpaired one-sided Student's *t* tests). The levels of NAEs were statistically similar between piglets fed diets supplemented with 20:4*n*−6 and 22:6*n*−3 and those fed sow's milk. The increases in 22:4*n*−6, 20:5*n*−3, and 22:5*n*−3 can be accounted for in that 22:4*n*−6 is the two-carbon elongation product of 20:4*n*−6, and 20:5*n*−3 and 22:5*n*−3 are retroconversion products of 22:6*n*−3 (20). The consistent ability of dietary 20:4*n*−6 to increase NAEs of the *n*−6 family (20:4*n*−6 and 22:4*n*−6) and of dietary 22:6*n*−3 to increase NAEs of the *n*−3 family (20:5*n*−3 22:5*n*−3, and 22:6*n*−3) is remarkable, and, despite the small sample size used herein, adds credibility to the observation that dietary LCPUFA can increase corresponding whole-brain homogenate NAEs in a manner consistent with known fatty acid metabolism. The increase in all of the above NAEs could be biologically important, because NAEs having fatty acids with at least 20 carbons and three double bonds bind CB1 receptors (21–24). The 22:6*n*−3 NAE molecule is of particular interest, because 22:6*n*−3 is abundant in the brain and important for infant brain development (25). Dietary 22:6*n*−3 has been shown to be associated with infant mental development in some studies (26) but not others (27) and is thought to be limiting in infants not fed adequate *n*−3 fatty acids (28). Fride *et al.* (22) showed 20:4*n*−6 NAE and synthetic 22:6*n*−3 NAE have similar cannabimimetic

Table 3. Diet-induced changes in polyunsaturated brain MAGs and NAEs

	Content, pmol/mg of brain lipid extract		
	Formula		
LCPUFA	C−	C+	Sow's milk
MAG, 4–6 double bonds			
20:4*n*−6	66.00 ± 9.38*†	44.40 ± 16.13	44.10 ± 21.73
22:4*n*−6	3.53 ± 0.65*	6.23 ± 1.82	6.13 ± 1.42
22:6*n*−3	3.87 ± 0.03*‡	5.93 ± 1.37	6.67 ± 1.92
NAE, 4–6 double bonds			
20:4*n*−6	1.02 ± 0.34*‡	3.96 ± 0.91	3.33 ± 0.80
22:4*n*−6	0.74 ± 0.03*‡	1.15 ± 0.02	1.46 ± 0.48
20:5*n*−3	0.33 ± 0.12*‡	1.74 ± 0.47	1.66 ± 0.13
22:5*n*−3	0.18 ± 0.09*‡	1.69 ± 0.49	1.40 ± 0.52
22:6*n*−3	0.95 ± 0.21*‡	9.03 ± 3.60	3.94 ± 1.24

Values represent means ± standard errors. Pairwise comparisons using an unpaired, one-sided Student *t* test ($P < 0.05$, $n = 3$ per group) are indicated.
*C− different from sow-fed group. C+ group was not statistically significantly different from sow-fed group ($P > 0.10$).
†Statistically different at $0.05 < P < 0.10$. C−, control diet without 20:4*n*−6 and 22:6*n*−3; C+, control diet with added 20:4*n*−6 and 22:6*n*−3. 18:3*n*−3 NAE was not detected and was omitted.
‡C− different from C+.

Table 4. Diet-induced changes in NAEs from specific brain regions

		Content, pmol/mg of brain lipid extract		
		Formula		
Brain section	NAEs	C−	C+	Sow's milk
Brainstem	20:4*n*−6	1.10 ± 0.09*	1.60 ± 0.23	2.50 ± 0.35
	22:6*n*−3	0.70 ± 0.06*†‡	4.90 ± 1.79	9.90 ± 1.39
Auditory cortex	20:4*n*−6	3.00 ± 0.23*	4.00 ± 0.69	7.30 ± 1.33
	22:6*n*−3	0.80 ± 0.23*†	4.40 ± 0.87	8.00 ± 1.62
Visual cortex	20:4*n*−6	3.50 ± 0.35	—	4.00 ± 0.58
	22:6*n*−3	3.30 ± 0.17	—	2.10 ± 0.35
Cerebellum	20:4*n*−6	0.50 ± 0.06*	—	0.80 ± 0.06
	22:6*n*−3	0.50 ± 0.17	—	0.40 ± 0.06
Striatum	20:4*n*−6	1.80 ± 0.02*‡	1.50 ± 0.06	1.10 ± 0.17
	22:6*n*−3	1.00 ± 0.12*†	2.00 ± 0.29	3.20 ± 0.17
Hippocampus	20:4*n*−6	2.20 ± 0.46	2.20 ± 0.17	2.00 ± 0.17
	22:6*n*−3	1.20 ± 0.12	0.90 ± 0.06	1.40 ± 0.17

Values represent means ± standard errors. Pairwise comparisons using an unpaired, one-sided Student *t* test ($P < 0.05$, $n = 3$ per group) are indicated.
*C− different from sow-fed group. C+ group was not statistically significantly different from sow-fed group ($P > 0.10$).
†C− different from C+.
‡Statistically different at $0.05 < P < 0.10$. C−, control diet without 20:4*n*−6 and 22:6*n*−3; C+, control diet with added 20:4*n*−6 and 22:6*n*−3. —, not determined for technical reasons.

activities when injected in mice; although *in vitro*, there is discrepant data showing a poor affinity of 22:6*n*−3 NAE for CB1 receptors (29, 30).

Substrate pools of 22:6*n*−3 in specific organs can be elevated by diet and selective transport and metabolism. Supporting the concept that higher substrate levels of 22:6*n*−3 in specific organs can then lead to higher levels of 22:6*n*−3 NAE is the observation that bovine retina [containing phosphatidylethanolamine and phosphatidylcholine with 22:6*n*−3 on both *sn*-1 and *sn*-2 positions, and *N*-docosahexaenoyl phosphatidylethanolamine (all of which are 22:6*n*−3 NAE precursors)] also contains appreciable 22:6*n*−3 NAE (31).

Although there were notable differences in the amount of 20:4*n*−6 and 22:6*n*−3 between sow's milk and our 20:4*n*−6- and 22:6*n*−3-supplemented diet (Table 2; refs. 32–34), there were similar levels of unsaturated (20:4*n*−6, 22:4*n*−6, 20:5*n*−3, 22:5*n*−3, and 22:6*n*−3) NAEs in these two groups. There may be a critical threshold level of dietary 20:4*n*−6 and 22:6*n*−3 needed to maintain "normal" NAE levels, but without normal dietary levels of 20:4*n*−6 and 22:6*n*−3 (as in our control diet), there is a relative deficiency of brain NAEs. It is important to recognize that there are other factors in sow's milk besides dietary fatty acids that could influence NAE levels, such as the triacylglycerol positional distribution of fatty acids (which affects fatty acid absorption), cholesterol, and hormones (34).

Dietary 18:2*n*−6 and 18:3*n*−3 are essential fatty acids and respective precursors of 20:4*n*−6 and 22:6*n*−3. We have shown also that dietary 20:4*n*−6 and 22:6*n*−3 can effectively increase levels of the corresponding NAEs in piglets only when the diet contains adequate levels of 18:2*n*−6 and 18:3*n*−3 (data not shown). Thus, in the essential fatty acid-deficient state, NAE metabolism is perturbed fundamentally. Further, we have preliminary indications that supplementation with dietary 18:2*n*−6 and 18:3*n*−3 does not lead to the same magnitude of increase in 20:4*n*−6 and 22:6*n*−3 NAEs as was seen with the down-stream precursors 20:4*n*−6 and 22:6*n*−3 (data not shown). Therefore, to elevate brain NAEs, it could be necessary to supplement infant formulations with additional dietary 20:4*n*−6 and 22:6*n*−3, rather than 18:2*n*−6 and 18:3*n*−3 (although the former are more expensive and oxidatively less stable).

In a separate preliminary feeding experiment, 20:4*n*−6 and 22:6*n*−3 NAEs also were measured in distinct piglet brain regions, with focus on those regions having higher amounts of CB receptors and NAEs (Table 4). Differences in NAEs among the three groups were region-specific. For example, levels of NAEs in hippocampus were resistant to dietary modulation. Relative to piglets fed control diet, in piglets fed diets supplemented with 20:4*n*−6 and 22:6*n*−3, there were increases in 22:6*n*−3 in auditory cortex (5.5-fold), and brainstem (7.0-fold, statistical trend), and striatum (2-fold). In contrast to results with brain homogenates, there were no statistical differences in 20:4*n*−6 NAE between supplemented- and control-diet groups for the specific brain regions examined. However, supporting the observation that both 20:4*n*−6 and 22:6*n*−3 can increase corresponding NAEs is that, relative to sow's milk-fed piglets, piglets fed diets lacking 20:4*n*−6 and 22:6*n*−3 had reduced amounts of 20:4*n*−6 NAE in brainstem, auditory cortex, and cerebellum, and they also had reduced amounts of 22:6*n*−3 NAE in brainstem, auditory cortex, and striatum. In these same regions, there were not significant differences in NAEs between sow's milk- and supplemented diet-fed piglets.

Finally, in a separate feeding experiment with mice, male Rj:NMRI mice (Elevage Janvier, Le Genest-Saint-Isle, France) weighing 43 g on d 58 (housed 10 per cage) received ad libitum quantities of powdered diets containing 90% fat-free AIN93G rodent diet (Dyets, Bethlehem, PA), 0.4% milk fat (all percentages are wt/wt), 1.2% palm olein, 1.9% Trisun sunflower oil (Oleificio SABO, Manno, Switzerland), 1.5% soybean oil, and 5.1% medium-chain triacylglycerol (all the above ingredients except AIN93G were from Nestlé affiliated companies) for 58 d. Part of the medium-chain triacylglycerol in the above control diet was replaced with 1.1% single-celled algal oil providing 0.5% 20:4*n*−6 in a 20:4*n*−6-supplemented diet. In mice fed diets supplemented with 20:4*n*−6, relative to control, there was no significant difference in body weight, food intake, or 16:0 NAE, but there was a 5.8-fold statistically significant increase in 20:4*n*−6 NAE from 21.8 ± 8.7 to 125.8 ± 8.7 pmol/mg of brain lipid extract (means ± standard errors, $n = 5$ per group, $P < 0.01$, one-sided Student's *t* test). Complete data from this experiment will be published separately. Thus, similarly to the pig, these data

show that dietary 20:4$n-6$ can increase levels of corresponding 20:4$n-6$ NAE in whole mouse brain.

How Dietary LCPUFA Can Modulate Brain NAE Levels. Dietary 20:4$n-6$ and 22:6$n-3$ can modulate NAE levels by influencing levels of NAE precursors such as *N*-acylphosphatidylethanolamines in neuronal membranes (35) or by providing direct fatty acid substrate for *de novo* synthesis (36). Additionally, the phospholipid acyl donor during *N*-acylphosphatidylethanolamine synthesis is unusual in being an *sn*-1-arachidonoyl phospholipid species (37, 38). The extent to which the *sn*-1-polyunsaturated phospholipid pool is modifiable by diet is not known (see below). Dietary LCPUFA could affect NAE metabolism by a number of other means including physical effects on membranes such as the blood–brain barrier (39), which can influence receptor number and kinetics (40); modulation of intracellular calcium levels in some cell types (41); and modulation of protein kinase C (42) and hormones.

Physiological Effects of Increasing Brain Levels of Polyunsaturated NAEs. On binding to CB1 and CB2 receptors, the activated receptors mediate inhibition of adenylate cyclase and activation of mitogen-activated protein kinase. CB1 receptors also mediate inhibition of N- and P/Q-type calcium channels and stimulation of potassium channels. NAEs also can have non-CB1/non-CB2 receptor-mediated activities (6, 9). High doses of injected anandamide or anandamide analogues in mice (10–100 mg/kg of body weight) cause typical *in vivo* cannabimimetic inhibitory effects (motor activity, rearing activity, ring catalepsy, hypothermia, analgesia, and agonistic behavior) and inhibition of leukocyte phagocytosis. In contrast, low doses of injected anandamide (0.01 mg/kg of body weight) stimulated activities in open field and ring, increased aggressive behavior, and stimulated phagocytosis (43). Thus, in the present nutritional studies, it is difficult to predict whether LCPUFA-induced changes in NAEs will increase or decrease such downstream behaviors. NAEs also have roles in vocalizations (44); appetite (45); intestinal motility (46); slow-wave sleep, rapid-eye movement, wakefulness, and memory consolidation (47); and buffering dopamine overproduction (48), to name a few.

Monoarachidonoylglycerols. 2-AG resembles 20:4$n-6$ NAE in molecular conformation, and *sn*-1-, *sn*-2-, and *sn*-3-arachidonoyl MAGs have affinity for CB1 receptors (49, 50). Arachidonoyl-containing MAGs could be more important biologically than 20:4$n-6$ NAE, because 2-AG concentration is known to be 200-to 800-fold greater than 20:4$n-6$ NAE in some rodent brain regions (51). 2-AG is also found in spleen, pancreas, and intestinal cells of rats and inhibits 20:4$n-6$ NAE breakdown (52). MAGs such as *sn*-2-linoleoylglycerol and *sn*-2-palmitoylglycerol (not examined presently) could also be important, because they can potentiate CB receptor binding of 2-AG (53). Free 20:4$n-6$ and *sn*-2-palmitoylglycerol, 2-oleoylglycerol, 2-linoleylglycerol, and 2-docosahexaenoylglycerol are inactive or weakly active on CB1 receptors in neuroblastoma cells (50). In the present study, levels of 20:4$n-6$ MAG (1- and 2-isomers combined) did not increase in response to supplementation with dietary 20:4$n-6$ and 22:6$n-3$, whereas levels of 22:6$n-3$ MAG did increase. Kinetic studies with labeled fatty acids, with particular focus on *sn*-2-arachidonoyl-containing phospholipids (54, 55) and triacylglycerols, are needed to elucidate the above finding.

Diet-Induced Changes in Phospholipids Compared with NAEs. After feeding of the experimental diets and preparation of an identical brain homogenate, relative to control, supplementation with 20:4$n-6$ and 22:6$n-3$ led to a small increase in brain 22:6$n-3$ in only phosphatidylcholine, and no change in 20:4$n-6$ in any phospholipid classes examined (ref. 15; S.I., G. Crozier, M. Turini, S. de la Presa Owens, and R. Dyer, unpublished data). In these studies, no distinction was made, however, between the *sn*-1 and *sn*-2 position of phospholipids, and 20:4$n-6$ and 22:6$n-3$ NAEs are derived from LCPUFA esterified to the *sn*-1 position of phosphoglycerides (6). The *sn*-1 LCPUFA phospholipid pool represents only a minor fraction of the overall LCPUFA measured in such previous studies. Overall, the lack of change in 20:4$n-6$ in brain phospholipids after different diets exemplifies the importance of examining additional nonphospholipid lipid pools, such as the NAE pool, to predict possible functional sequelae and follow-up experimentation in human and animal fatty acid feeding trials.

Conclusions. We have demonstrated for the first time, to our knowledge, that dietary 20:4$n-6$ and 22:6$n-3$ can modulate levels of bioactive brain NAEs. This intriguing observation has raised a number of important questions. What is the optimal dietary dose of LCPUFA (arachidonic acid and docosahexaenoic acid, added separately and together) for increasing levels of brain NAEs and what are the physiologic and behavioral implications? What are the pathways and kinetics for formation of brain NAEs and MAGs from dietary LCPUFA? Does the same increase in 20:4$n-6$ NAE and 22:6$n-3$ NAE observed in piglets occur in brain and other organs from other animals, human infants, clinical populations, or elderly persons after 20:4$n-6$ and 22:6$n-3$ supplementation? What could be the clinical applications of these findings?

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