Chronic dietary n-6 PUFA deprivation leads to conservation of arachidonic acid and more rapid loss of DHA in rat brain phospholipids.

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Abstract  To determine how the level of dietary n-6 PUFA affects the rate of loss of arachidonic acid (ARA) and DHA in brain phospholipids, male rats were fed either a deprived or adequate n-6 PUFA diet for 15 weeks postweaning, and then subjected to an intracerebroventricular infusion of [3H]-ARA or [3H]-DHA. Brains were collected at fixed times over 128 days to determine half-lives and the rates of loss from brain phospholipids (Jout). Compared with the adequate n-6 PUFA rats, the deprived n-6 PUFA rats had a 15% lower concentration of ARA and an 18% higher concentration of DHA in their brain total phospholipids. Loss half-lives of ARA in brain total phospholipids and fractions (except phosphatidylinositol; PtdIns, phosphatidylserine. PtdSer, phosphatidylserine). Thus, a low n-6 PUFA diet can be used to target brain ARA and DHA metabolism.—Lin, L. E., C. T. Chen, K. D. Hildebrand, Z. Liu, K. E. Hopperton, and R. P. Bazinet. Chronic dietary n-6 PUFA deprivation leads to conservation of arachidonic acid and more rapid loss of DHA in rat brain phospholipids. J. Lipid Res. 2015. 56: 390–402.

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The brain is specifically enriched with the two PUFAs arachidonic acid (20:4n-6; ARA) and DHA (22:6n-3), which are considered important for normal brain function. Bioactive mediators from ARA and DHA, the eicosanoids and docosanoids, respectively, regulate many processes including neuroinflammation, pain perception, and blood flow (1–6). As a result, altered levels or metabolism of these PUFAs have been implicated in many neurological and psychiatric disorders, including Alzheimer’s disease, Parkinson’s disease, bipolar disorder, and major depression (7–9).

ARA and DHA can either be obtained directly from the diet or synthesized from their nutritionally essential precursor fatty acids linoleic acid (18:2n-6; LA) and α-linolenic acid (18:3n-3; ALA) respectively, which are the main dietary n-6 and n-3 PUFAs. Thus, it is of interest to determine whether changes in the level of dietary n-6 or n-3 PUFAs can alter the concentrations of ARA and/or DHA in the brain, and more importantly, whether these dietary changes affect their metabolism. In fact, clinical trials in humans are underway, with the assumption that lowering n-6 PUFAs may decrease brain ARA and its metabolism (1).

Several studies have investigated the effects of dietary n-3 PUFA deprivation on brain ARA and DHA concentrations and metabolism in rats. Feeding rats an n-3 PUFA-deprived diet that contains ALA at a concentration of 0.04% versus an n-3 PUFA-adequate diet containing ALA at 4.4% of all fatty acids decreased the concentration of DHA by 37% and increased docosapentaenoic acid (DPA) n-6 concentration by 95% in brain total phospholipids, but did not change the concentration of ARA (10). This study also reported that n-3 PUFA deprivation conserves DHA in rat brain phospholipids extending the half-life from 33 to 90 days. This conservation response was selective for DHA, as n-3 PUFA deprivation did not alter the half-life or rate of ARA metabolic consumption (11). Alterations in brain DHA concentration and metabolism appear to occur below a threshold of 0.8% ALA in the diet,

Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; ChoGpl, choline glycerophospholipid; COX, cyclooxygenase; cPLA2, cytosolic phospholipase A2; DPA, docosapentaenoic acid; EnnGpl, ethanolamine glycerophospholipid; FAME, fatty acid methyl esters; GC-FID, gas chromatography with flame-ionization detection; H-ARA, 5,6,8,9,11,12,14,15- [3H]-arachidonic acid; H-DHA, 4,7,10,13,16,19-[3H]-docosahexaenoic acid; iPLA2, calcium-independent phospholipase A2; LA, linoleic acid; LSC, liquid scintillation counting; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.

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where there is a decrease in the activity of the DHA-metabolizing enzymes, calcium-independent phospholipase A₂ (iPLA₂) and cyclooxygenase (COX) 1, and an increase in the activity of the ARA-selective enzymes calcium-dependent cytosolic phospholipase A₂ (cPLA₂) and secretory phospholipase A₂ as well as COX-2 (12, 13). Lower concentrations of DHA in the brains of n-3 PUFA-deprived rats are likely due to a reduction in the incorporation of DHA into brain phospholipids, as well as a reduction in DHA recycling (14).

Compared with dietary n-3 PUFA manipulation, few studies have examined dietary n-6 PUFAs and the brain. Reducing the percentage of total fatty acid from 28% LA to 2% LA decreases brain ARA by 28% and increases DHA by 11% (15). Dietary n-6 PUFA deprivation also reduces expression of ARA-selective enzymes (cPLA₂ and COX-2) and increases expression of DHA-selective enzymes [iPLA₂ and 15-lipoxygenase (LOX)] (16). There was a corresponding decrease in the protein levels of activator protein (AP)-2α and nuclear factor κB p65 (transcription factors for cPLA₂ and COX-2), as well as an increase in the levels of sterol-regulatory element binding protein 1 (an iPLA₂ transcription factor) (16). Furthermore, the DHA uptake rate into brain phospholipids is increased by 45% in rats fed an n-6 PUFA-deprived diet, which could account for the increase in phospholipid DHA (17). In the same study, the turnover of DHA in brain total phospholipids was increased 30–84% in the choline glycerophospholipid (ChoGpl), phosphatidylinositol (PtdIns), and phosphatidylserine (PtdSer) fractions, but not the ethanolamine glycerophospholipid (EthGpl) fraction.

As of yet, no study has investigated the effect of n-6 PUFA deprivation on the rate of loss of ARA and DHA in brain phospholipids. The objective of this study was to determine the rate of loss of both ARA and DHA from brain phospholipids in rats that are fed either the adequate or deprived n-6 PUFA diet as used in previous studies (15–17), the n-6 PUFA-adequate diet contained safflower oil (32.3 g/kg), hydrogenated soybean oil (5 g/kg), and coconut oil (55 g/kg). The n-6 PUFA-deprived diet did not contain safflower oil, a significant source of LA. Instead, it contained hydrogenated coconut oil (87.3 g/kg) and olive oil (5 g/kg). Both diets had equal amounts of flaxseed oil (7.7 g/kg).

Total lipids were extracted from ~0.5 g of each diet (n = 3) and analyzed by gas chromatography with flame-ionization detection (GC-FID) as described below. Resultant fatty acid concentrations are shown in Table 2. LA accounted for ~20% of total fatty acids.

### MATERIALS AND METHODS

#### Animals

All procedures were approved by the Animal Ethics Committee at the University of Toronto (Protocol # 20010100), in accordance with policy statements of the Canadian Council on Animal Care. One hundred and twelve male Fischer (CDF) rats were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and arrived at the Division of Comparative Medicine animal facility at 21 days of age. Rats were housed in a 22°C environment with a 12 h light-dark cycle, and they received ad libitum access to water and food throughout the study. Upon arrival, they were randomized to receive either the n-6 PUFA-deprived (n = 56) or n-6 PUFA-adequate diet (n = 56) (supplementary Fig. 1). Measurements of body weight and food intake were carried out on a weekly basis for 15 weeks. Following 15 weeks of feeding, 8 rats from each dietary group were euthanized by high-energy, head-focused microwave irradiation (13.5 kW for 1.6 s; Cober Electronics Inc., Norwalk, CT) as previously described (18–21). Their brains were removed, dissected sagittally, and stored at −80°C for measurements of eicosanoid levels, docosanoids levels, and baseline brain phospholipid fatty acid concentrations.

#### n-6 PUFA-adequate and n-6 PUFA-deprived diets

The n-6 PUFA-adequate and n-6 PUFA-deprived rodent diets were based on the AIN-93G formulation with a 10% fat composition (22, 23) as used by others (15–17). The diets were purchased from Dyets Inc. (Bethlehem, PA), under the following product names: Revised Modified n-6 PUFA Adequate Diet (Dyet #180780) and Custom Modified n-6 PUFA Deficient Diet (Dyet #180784). In order to be consistent with the literature (15–17), we chose to use the terms “adequate” and “deprived” to describe the 24% and 2% of total fatty acids LA levels in the diets. However, we recognize that LA requirements are controversial and point the reader to the methods of Igarashi et al. (17) as well as other papers for more details (24, 25). The compositions of both diets are shown in Table 1. As in previous studies (15–17), the n-6 PUFA-adequate diet contained safflower oil (32.3 g/kg), hydrogenated soybean oil (5 g/kg), and coconut oil (55 g/kg). The n-6 PUFA-deprived diet did not contain safflower oil, a significant source of LA. Instead, it contained hydrogenated coconut oil (87.3 g/kg) and olive oil (5 g/kg). Both diets had equal amounts of flaxseed oil (7.7 g/kg).

Total lipids were extracted from ~0.5 g of each diet (n = 3) and analyzed by gas chromatography with flame-ionization detection (GC-FID) as described below. Resultant fatty acid concentrations are shown in Table 2. LA accounted for ~24% of total fatty acids.

| Ingredient | n-6 PUFA-Adequate Diet (g/kg of Diet) | n-6 PUFA-Deprived Diet (g/kg of Diet) |
|------------|---------------------------------------|---------------------------------------|
| Casein     | 200                                   | 200                                   |
| t-Cystine  | 3                                     | 3                                     |
| Sucrose    | 99.98                                 | 99.98                                 |
| Cornstarch | 150                                   | 150                                   |
| Maltose dextrin | 150                                 | 150                                   |
| Dextrose   | 200                                   | 200                                   |
| Hydrogenated coconut oil | 55 | 87.3 |
| Olive oil | 0                                      | 5                                     |
| Flaxseed oil | 7.7                                 | 7.7                                   |
| Safflower oil | 32.3                                | 0                                     |
| Hydrogenated soybean oil | 5 | 0 |
| t-Butylhydroquinone | 0.02 | 0.02 |
| Cellulose  | 49.5                                  | 49.5                                  |
| Mineral Mix #210025 | 35 | 35 |
| Vitamin Mix #310025 | 10 | 10 |
| Choline bitartrate | 2.5 | 2.5 |

The only difference between the two diets is the amount of hydrogenated coconut oil, olive oil, safflower oil, and hydrogenated soybean oil. The safflower oil and soybean oil in the n-6 PUFA-adequate diet were replaced by hydrogenated coconut oil and olive oil.
Intracerebroventricular infusion of 3H-ARA and 3H-DHA

Rats were euthanized by microwave irradiation (13 kW for 1.6 s) at the following time points: 4, 16, 32, 64, and 128 days post intracerebroventricular infusion (supplementary Fig. 1). Four n-6 PUFA-deprived rats and 4 n-6 PUFA-adequate rats were euthanized at each time point from both the 3H-ARA-infused and 3H-DHA-infused groups. Brains were removed and stored at −80°C.

Extraction and isolation of brain phospholipids

Brains were homogenized, and their total lipids were extracted by the chloroforom-methanol-0.88% KCl (2:1.0.75) Folch, Lecs, and Stanley method (27). TLC was used to isolate the total phospholipids, as well as the phospholipid classes from the total lipid extract as previously described (11, 26). TLC plates were washed in chlororomethanol (2:1 by volume) and were activated for 1 h at 100°C. TLC G-plates (EMD Chemical, Gibbstown, NJ) in a heptane-diethyl ether-glacial acetic acid solution system (60:40:2 ml by volume) were used for neutral lipid separation. TLC H-plates (Analtech, Newark, DE) in a chloroform-methanol-2-propanol-0.25% KC triethyamine (30:9:2:5:6:18 by volume) system were used to separate the phospholipid classes. The plates were sprayed with 0.1% 8-anilino-1-naphthalene sulfonic acid for UV visualization of the fatty acid bands. The bands containing total phospholipids, and the phospholipid fractions (CholGpl, EtnGpl, PtdSer, and Ptdlns) were collected into tubes. For GC-FID analysis, a known amount of heptadecanoic acid (17:0) standard was added. In preparation for both GC-FID and HPLC analysis, the fatty acids were converted into fatty acid methyl esters (FAMEs) by treatment with 14% boron trifluoride-methanol at 100°C for 1 h.

Quantitation of baseline brain phospholipid fatty acids by GC-FID

FAMES were analyzed by a Varian-430 gas chromatograph (Varian, Lake Forest, CA) with an FID and a Varian FactorFour capillary column (VF-23ms; 30 m × 0.25 mm inner diameter × 0.25 μm film thickness). The FAMES were dissolved in hexane and injected in splitless mode. The injector and detector ports were set at 250°C. The FAMES were eluted with increasing temperatures. The temperature program started at 50°C for 2 min, increased 20°C/min, held at 170°C for 1 min, increased 3°C/min, and finally, held at 212°C for 5 min. The helium carrier gas had a flow rate of 0.7 ml/min. Output peaks were identified using known retention times of authentic FAME standards (Nu-Chek Prep Inc., Elysian, MN). Fatty acid concentrations were calculated by comparison of the GC fatty acid peak areas to the internal 17:0 standard peak area (11, 26, 28).

Quantitation of baseline brain eicosanoids and docosanoids

As described previously (29), composite standards of lipid metabolites (natural or deuterated; Cayman Chemicals Co., Ann Arbor, MI) were diluted in ethanol from stock solutions to perform an eight-point calibration curve (0.05 to 5 ng). Internal standard...
mixtures in ethanol were added to both the composite standards and the samples prior to extraction. Extraction and sample preparations were performed in siliconized glassware. To minimize auto-oxidation, fatty acids were extracted on ice, in a reduced light condition, using solvents that contained 0.1% butyalted hydroxytoluene. The frozen brain halves were homogenized in methanol. One nanogram of internal standard mixture was added to a 250 mg aliquot of each homogenized brain. Externarl ARA, EPA (20:5n-3), and DHA standards were prepared in a similar way. The samples were mixed for 1 min, incubated on ice for 30 min, and centrifuged at 1,000 g for 10 min. The supernatants were collected. The pellet was resuspended in ethanol for 1 min and centrifuged again for a second extraction. The resultant ethanolic supernatants were combined with the methanolic supernatants, previously extracted. After evaporation with nitrogen gas, the supernatants were suspended in 10% ethanol, acidified to pH 3 with 1 N HCl, and triply extracted with ethyl acetate. The ethyl acetate layer was washed to neutrality with water and dried under nitrogen gas. The residues from the brain and external standard samples were reconstituted in acetonitrile-water (1:1 by volume) and transferred into the inserts of amber vials for immediate LC/MS/MS analysis. LC/MS/MS was performed using a 1290 UHPLC System (Agilent Technologies, Santa Clara, CA) and a QTRAP5500 Mass Spectrometer (ABSciex, Framingham, MA). The chromatography was done at a 600 μL/min flow rate on a Zorbax SB-Phenyl column (Agilent Technologies; 3.0 × 50 mm, 3.5 μm). The gradient started at 80% water and, over 9 min, ramped up to 100% acetonitrile. The mass spectrometer was operated in negative electrospray ionization mode with a source temperature setting of 600°C and a voltage setting of 4,500 V. The precursors to product ion mass transitions were obtained through scheduled multiple reaction monitoring. Quantitative analysis was performed by Analyst 1.5.2 Software (AB-Sciex). The area ratios of the integrated peaks (natural to deuterated standard) were plotted against the standard curves for quantification. The limit of quantification was 0.025 ng per sample, and values between 0.005 ng and 0.025 ng were considered semiquantitative.

Confirmation of radiotracer identity by HPLC

Total phospholipids were extracted from brain homogenate and methylated as described above. Samples were reconstituted in acetonitrile. As described in previous studies (11, 26, 28, 30), FAMEs were separated by HPLC (Waters 2690, Boston, MA) with a Luna C18 reverse column (4.6 × 250 mm, 100 Å; Phenomenex, Torrance, CA) and an in-line UV photodiode array detector (Waters 996) set at a 242 nm wavelength. The system was first stabilized at a 1 ml/min flow rate with a gradient system consisting of i) 100% water and ii) 100% acetonitrile. The gradient was then set to 85% (i) for 30 min, and then increased to 100% (ii) over 10 min. It was held there for 20 min before returning back to 85% (ii) over a 5 min period. Fractions were collected at 1 min intervals for 35 min, and each of the 55 fractions was measured for radioactivity by LSC. Similar to what has previously been reported, ARA and DHA had elution times of 35 and 31 min, respectively (11, 31). Quantification of radioactivity by LSC

LSC was used to measure the radioactivity of the total phospholipids, fractions, as well as 4day brain phospholipid HPLC fractions. Samples were put into scintillation vials, and 5 ml of scintillation cocktail (GE Healthcare, Life Sciences, Baie d’Urfe, QC, Canada) was added. Radioactivity was quantified using a Packard TRI-CARB9200TR liquid scintillation analyzer (Packer, Meriden, CT) with a detector efficiency of 61.07% for tritium. The measurements were given in disintegrations per minute and were converted to nCi/brain (11, 26).

Calculations and statistics

The data were expressed as means ± SEM. Differences in body weights and food intake between the two dietary groups were assessed using repeated-measures ANOVA (SigmaPlot 12.5; SigmaPlot Software, San Jose, CA). Curves depicting the loss of radioactivity over time post intracerebroventricular infusion were logarithmically transformed and fit with linear regression. The slopes of these linear regressions were tested using an ANOVA to determine whether they were significantly different from zero as well as if they differed between the adequate and deprived n-6 PUFA groups (GraphPad Prism 5; GraphPad Software, La Jolla, CA). Statistical significance was taken at P < 0.05. Loss half-lives of 3 H-ARA and 3 H-DHA were calculated from the slopes of the linear regressions using equation 1, and the rate of loss (Jout) in nmol/g brain/day was calculated using equation 2 (10, 11, 32):

\[
J_{\text{out}} = 0.693 \cdot C_{\text{i}} / t_{1/2}
\]

where \( C_{\text{i}} \) is the baseline brain phospholipid concentration of the fatty acid of interest (ARA or DHA). For the purpose of calculating \( J_{\text{out}} \), the half-life that was calculated from the slope of the linear regression was treated as a constant and applied to all measurements of baseline brain phospholipid concentrations (n = 8 baseline measures per dietary group). This produced a distribution of \( J_{\text{out}} \) values, for which we calculated the mean and SEM. Differences in \( J_{\text{out}} \) between the deprived and adequate n-6 PUFA rats were assessed using the Student’s t-test.

RESULTS

Body weights and food intake

Body weights increased over time as expected (supplementary Fig. 2). Although there were some significant differences between body weights of adequate and n-6 PUFA-deprived rats at certain points in time (P < 0.05), the magnitude of these differences were small as shown previously by others (15). After 15 weeks of feeding, in the 3 H-ARA infusion group, the n-6 PUFA-adequate and n-6 PUFA-deprived rats had a mean weight of 363 ± 2.9 g and 356 ± 2.2 g, respectively. In the 3 H-DHA infusion group, the n-6 PUFA-adequate rats had a mean weight of 347 ± 3.7 g and the n-6PUFA-deprived rats had a mean weight of 344 ± 3.1 g.

Food intake, like body weight, was largely the same between both dietary groups (supplementary Fig. 3). The few significant differences at specific time points were small, and the pattern of food intake differences did not match the pattern of body weight differences. The mean weights of the adequate and deprived n-6 PUFA rat brains after 15 weeks of feeding were 1.7 ± 0.006 g and 1.7 ± 0.02 g, respectively (P > 0.05).

Radiotracer identification

Brain samples (4 days postinfusion) were analyzed by HPLC and LSC to confirm radiotracer identity. ARA elutes at 35 min (11). All the radioactivity in the 3 H-ARA-infused rat
brain phospholipids eluted at 35 min and was identified as ARA in both the adequate and deprived n-6 PUFA rats (Fig. 1A). DHA elutes at 31 min. All of the radioactivity in the ³H-DHA-infused rat brain phospholipids eluted at 31 min and was identified as DHA in both dietary groups (33) (Fig. 1B).

### Baseline brain eicosanoid and docosanoid concentrations

After 15 weeks of feeding, the n-6 PUFA-deprived rats had significantly lower levels ($P < 0.05$) of several ARA-derived eicosanoids (prostaglandin [PG]F2α, 5-hydroxyeicosatetraenoic acid [HETE], 8-HETE, 11-HETE, 12-HETE, and 15-HETE), but not PGF2, 6-keto-PGF1α, 9-HETE, 5,6-epoxyeicosatrienoic acid [EET], 8,9-EET, 11,12-EET, and 14,15-EET (Fig. 2A). The largest difference was seen in PGF2α, which was about 86% lower in the n-6 PUFA-deprived rats than the n-6 PUFA-adequate rats. In contrast, nearly all detectable EPA-derived eicosanoids were found at higher concentrations in the deprived n-6 PUFA rats (5-hydroxyeicosapentaenoic acid [HEPE], 8-HEPE, 9-HEPE, 11-HEPE, 15-HEPE, and 18-HEPE, but not 12-HEPE) (Fig. 2B). The magnitude of these differences ranged from 1.8-fold (5-HEPE) to 9.9-fold (15-HEPE). Interestingly, there were no significant differences in docosanoid levels between the two dietary groups (Fig. 2C). The following eicosanoids and docosanoids were also assayed, but their levels were below the detection limit: Thromboxane (TX)B2, 8-isoprostane, PGD2, 11-HPETE, 8-HEPE, 9-HEPE, 11-HEPE, 15-HEPE, and 18-HEPE, but not 12-HEPE) (Fig. 2B). The changes in total phospholipid concentrations were reflected by changes in the phospholipid fractions, although some fractions changed more than others for certain fatty acids. ARA was ~20% lower ($P < 0.05$) in the CholGpl, EthGpl, and PtdSer fractions but not the PtdIns fraction of the n-6 PUFA-deprived rats (Table 4). DPAn-6

### Baseline brain phospholipid fatty acid concentrations

Table 3 depicts the concentrations of esterified fatty acids found in the total brain phospholipids of both adequate and n-6 PUFA-deprived rats after 15 weeks of feeding. There was a 15% lower concentration of ARA in the deprived versus n-6 PUFA adequate rats (5,191 ± 158 vs. 6,077 ± 103 nmol/g of brain, $P < 0.05$). This was accompanied by a 50% lower concentration of DPAn-6 (22:5n-6) (79 ± 2.7 vs. 158 ± 3.7 nmol/g of brain, $P < 0.05$). Conversely, there was an 18% higher concentration of DHA and a 164% higher concentration of DPAn-3 (22:5n-3) in the n-6 PUFA-deprived rats (7,323 ± 200 vs. 6,232 ± 97 and 267 ± 8.5 vs. 101 ± 2.4 nmol/g of brain, respectively, $P < 0.05$). Interestingly, the largest percentage difference between the dietary groups was seen in EPA. The n-6 PUFA-deprived rats had a >10-fold higher level of EPA (20:5n-3) than the adequate n-6 PUFA rats (9.2 ± 1.5 vs. 94 ± 3.4 nmol/g of brain).

The changes in total phospholipid concentrations were reflected by changes in the phospholipid fractions, although some fractions changed more than others for certain fatty acids. ARA was ~20% lower ($P < 0.05$) in the CholGpl, EthGpl, and PtdSer fractions but not the PtdIns fraction of the n-6 PUFA-deprived rats (Table 4). DPAn-6

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**Fig. 1.** HPLC separation of radioactivity in brain total phospholipids of ³H-ARA- or ³H-DHA-infused rats. HPLC separation of radioactivity in brain total phospholipids from ³H-ARA-infused (A) and ³H-DHA-infused (B) deprived and adequate n-6 PUFA rat brains, 4 days postinfusion (n = 4 in each dietary group). A: Radioactivity elutes at 35 min, the elution time of ARA. B: Radioactivity elutes at 31 min, the elution time of DHA.
was 50–56% lower ($P < 0.05$) in all four fractions. A 9% and 11% higher concentration of DHA was found in the n-6 PUFA-deprived rat PtdSer and ChoGpl fractions, respectively (1,940 ± 38 vs. 1,776 ± 13 and 1,137 ± 19 nmol/g of brain, $P < 0.05$). DPAn-3 was higher in the n-6 PUFA-deprived rats by 105, 159, 145, and 167% in the ChoGpl, EtnGpl, PtdSer, and PtdIns fractions, respectively ($P < 0.05$). EPA was >10-fold higher in the ChoGpl and EtnGpl fractions of the n-6 PUFA-deprived rats and was >1.5-fold higher in the PtdSer and PtdIns fractions compared with the n-6 PUFA-adequate rats.

### Brain phospholipid ARA and DHA rate of loss

The loss of $^3$H-ARA and $^3$H-DHA from brain phospholipids was plotted from 4 to 128 days postinfusion and then logarithmically transformed for linear regression analysis (Figs. 3, 4). All slopes were negative and significantly different from zero ($P < 0.0001$) (Table 5). Linear regression analysis of the slopes for $^3$H-ARA showed significant differences between n-6 PUFA-adequate and n-6 PUFA-deprived rats in the total phospholipid pool as well as all phospholipid fractions, except for PtdSer ($P < 0.05$). The slopes were more negative in the adequate n-6 PUFA group than in the n-6 PUFA-deprived group, reflective of a shorter loss half-life and a more rapid loss of $^3$H-ARA over time ($P < 0.05$). To take into account the differences in baseline concentrations of ARA between the two dietary groups, the number of ARA molecules lost per gram of brain each day ($J_{\text{out}}$) was calculated. The difference in $J_{\text{out}}$ between dietary groups was generally even larger than the difference in the $t_{1/2}$, except in the PtdIns fraction; the n-6 PUFA-deprived group had a significantly lower $J_{\text{out}}$ than the n-6 PUFA-adequate group ($P < 0.001$) in the total phospholipid, ChoGpl, EtnGpl, and PtdSer fractions. $J_{\text{out}}$ in the total phospholipid pool was 134.7 ± 2.3 nmol/g of brain/day in the n-6 PUFA-adequate group versus 85.4 ± 2.6 nmol/g of brain/day in the n-6 PUFA-deprived group.
of brain/day in the n-6 PUFA-deprived group \((P < 0.05)\). This corresponds to a daily fractional loss of 2.2\% versus 1.6\% in the adequate versus deprived groups. The \(J_{\text{out}}\) (nmol/g of brain/day) in the phospholipid fractions ranged from 6.9 ± 0.06 (PtdSer) to 43 ± 0.6 (ChoGpl) in the n-6 PUFA-adequate group, and from 4.0 ± 0.1 (PtdSer) to 24 ± 0.3 (ChoGpl) in the n-6 PUFA-deprived group. Overall, ARA was lost from brain phospholipids at a slower rate in the n-6 PUFA-deprived group.

There were no differences in the slopes and thus, no difference in the loss half-lives for \(^{3}H\)-DHA between the adequate and deprived n-6 PUFA rats. However, due to the higher baseline DHA concentrations in the deprived rats, the n-6 PUFA-deprived group appeared to have a higher \(J_{\text{out}}\) for DHA than the adequate n-6 PUFA group. This difference was only significant in the total phospholipid pool and the ChoGpl fraction. The \(J_{\text{out}}\) for total phospholipids in the adequate and n-6 PUFA-deprived groups was 129.1 ± 11.4 nmol/g of brain/day and 145.7 ± 15.5 nmol/g of brain/day, respectively \((P < 0.05)\). The \(J_{\text{out}}\) (nmol/g of brain/day) for the phospholipid fractions ranged from 2.3 ± 0.3 (PtdIns) to 113.5 ± 7.8 (EmGpl) in the n-6 PUFA-adequate group, and from 2.4 ± 0.3 (PtdIns) to 121 ± 10.2 (EmGpl) in the n-6 PUFA-deprived group. Overall, DHA seemed to be lost, when measured in terms of \(J_{\text{out}}\) from brain phospholipids at a more rapid rate in the n-6 PUFA-deprived rats.

### DISCUSSION

This study investigated the effect of an adequate (24\% LA) versus deprived (2\% LA) n-6 PUFA diet on the rate of loss of ARA and DHA from rat brain phospholipids. The diets used in this study were similar to those used in previous studies examining body and organ weights, enzyme expression, and brain DHA uptake and turnover (15–17). In brain total phospholipids, there was a 15\% reduction in ARA concentration and an 18\% increase in DHA concentration with the n-6 PUFA-deprived diet. These changes are comparable to the 28\% reduction in ARA and the 11\% increase in DHA found previously in brain total lipids (15). For the first time, we show that lowering the amount of n-6 PUFA in the diet leads to longer ARA loss half-lives in brain total phospholipids, ChoGpl, EtnGpl, and PtdIns pools. After factoring in the concentration of ARA in the baseline, uninfused rats, the n-6 PUFA-deprived rats had a slower net rate of loss (a lower \(J_{\text{out}}\)) of ARA in the total phospholipids, ChoGpl, EtnGpl, and PtdIns pools. This may have been caused by a decrease in cPLA\(_2\) and COX-2 activity, which has been reported in rats upon 15 weeks consumption of a low n-6 PUFA diet (16). It would have also been interesting to calculate the half-lives of ARA and DHA within brain neutral lipids as fatty acid turnover within these lipids is PL\(_{A_{2}}\) independent, and future studies should consider this. Decreased cPLA\(_{2}\) activity likely reflects a decreased ARA turnover and less opportunity for ARA to be lost through eicosanoid production or \(\beta\)-oxidation. The decrease in eicosanoid production shown here may not be enough to account for the decreased loss of ARA, unless the eicosanoids have very short half-lives. Thus, there is also likely a decreased amount of \(\beta\)-oxidation or other catabolic processes, which is an area requiring further research.

In contrast to our observations with ARA, there was a more rapid net loss (a higher \(J_{\text{out}}\)) of DHA from the brain total phospholipids and the ChoGpl pool in the n-6 PUFA-deprived rats, but not from the other phospholipid fractions. This difference in the rate of loss of DHA was a function of the higher brain DHA concentrations in the deprived versus adequate n-6 PUFA rats because the fractional losses and the loss half-lives for DHA were not significantly different between the two dietary groups in any of the phospholipid pools. In the n-6 PUFA-deprived rats, the \(J_{\text{out}}\) of DHA for total phospholipids was smaller than the \(J_{\text{in}}\) (net rate of incorporation) of DHA found previously, whereas the \(J_{\text{in}}\) approximately matched the \(J_{\text{out}}\) in the n-6 PUFA-adequate rats (17). A more rapid daily uptake rate compared with loss rate of DHA may account for the higher concentration of DHA in the n-6 PUFA-deprived rats than in the n-6 PUFA-adequate rats upon 15 weeks of feeding. However, caution should be taken when comparing and combining results for kinetic analyses from two different studies, and future experiments should be completed under similar conditions. One important distinction between our intracerebroventricular method to calculate phospholipid half-lives and the use of a pulse intravenous infusion can be seen in glycerophospholipid species. While the half-life or the net rate of entry \(J_{\text{in}}\), as calculated upon an intravenous pulse infusion, approximates the half-life or \(J_{\text{out}}\) for brain total phospholipids, this does not hold true for measured glycerophospholipid species. One reason for the discrepancy in glycerophospholipid...
species half-lives is likely due to remodeling/exchange of radiolabeled fatty acids between phospholipid species that occurs over time, which does not occur upon acute pulse labeling (34). However, it is also possible that fatty acids other than the plasma unesterified pool enter the brain, which would be captured in our study.

Similar to previous studies, one limitation of this study was that measuring the radioactivity of the rat brains required euthanization of the rats and removal of their brains. Thus, different rats had to be used at each time point postinfusion, and the actual loss of radioactivity in the individual rat brain could not be tracked over 128 days. This posed a problem when trying to calculate the SEM of \( J_{\text{out}} \) because the \( J_{\text{out}} \) calculation was the quotient of two measurements: the baseline ARA or DHA concentration and the loss half-life of ARA or DHA. Only patient for two measurements: the baseline ARA or DHA concentration and the loss half-life of ARA or DHA. Thus, consistent with past literature, the calculated loss half-lives were treated as constants and were applied to all baseline concentration measurements of PGF\(_2\alpha\) and COX-2, which are both enzymes required for the synthesis of PGF\(_2\alpha\) and COX-2. As seen in previous studies, an n-6 PUFA-deprived diet causes a reduction in the activity of cPLA\(_2\) and COX-2, which are both enzymes required for the synthesis of PGF\(_2\alpha\) (16). However, this effect was selective for PGF\(_2\alpha\) because there were no significant reductions in the levels of the other nonautooxidative eicosanoids that were detected (PGE\(_2\) and PGL\(_2\)). This suggests that the reductions in enzyme activity can selectively reduce certain eicosanoids but not others. The mechanism behind this is unknown. Furthermore, it could be that the differences in the concentration of other eicosanoids may only appear when the brain is responding to stress. For example, in a neuroinflammatory state, there is an increased production of PGE\(_2\) (5). Perhaps, in such a state, the n-6 PUFA-deprived rats would have a larger increase in eicosanoid production than the n-6 PUFA-deprived rats. The remaining eicosanoids that changed are thought to be autooxidative, meaning that they are capable of being

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**TABLE 4. Fatty acid concentrations in brain glycerophospholipid fractions**

| Fatty Acid | Adequate | Deprived | Adequate | Deprived | Adequate | Deprived | Adequate | Deprived |
|------------|----------|----------|----------|----------|----------|----------|----------|----------|
| 14:0       | 103.9 ± 1.7 | 124.8 ± 2.5 \(^a\) | 9.4 ± 0.4 | 11.2 ± 0.6 \(^a\) | 2.3 ± 0.3 | 5.1 ± 0.4 | 4.5 ± 0.6 | 4.5 ± 0.2 |
| 14:1n-7    | ND ± ND | ND ± ND | ND ± ND | ND ± ND | ND ± ND | ND ± ND | ND ± ND | ND ± ND |
| 16:0       | 13,546.5 ± 113.2 | 13,764.7 ± 203.2 |
| 16:1n-7    | 147.2 ± 1.9 | 182.1 ± 4.6 | 228.3 ± 118.8 | 218.7 ± 86.6 |
| 18:0       | 3,573.1 ± 22.4 | 3,639.3 ± 44.5 | 5,059.1 ± 148.8 | 4,800.2 ± 146.1 |
| 18:1n-9    | 5,987.7 ± 71.3 | 6,617.8 ± 88.7 \(^a\) | 2,975.5 ± 144.9 | 3,307.7 ± 192.1 |
| 18:1n-7    | 1,448.1 ± 8.2 | 1,394.1 ± 65.8 | 754.7 ± 9.8 | 838.3 ± 29.4 |
| 18:2n-6    | 192.9 ± 2.3 | 126.8 ± 1.9 | 92.4 ± 3.6 | 66.0 ± 2.4 \(^a\) |
| 18:3n-6    | 9.1 ± 0.3 | 9.2 ± 0.2 | 10.4 ± 0.6 | 9.3 ± 0.6 |
| 18:3n-3    | 8.5 ± 0.4 | 9.4 ± 1.0 | 17.4 ± 1.7 | 17.2 ± 1.9 |
| 20:0       | 55.6 ± 1.0 | 54.4 ± 1.1 | 78.9 ± 1.1 | 61.6 ± 2.5 \(^a\) |
| 20:1n-9    | 286.6 ± 5.8 | 289.6 ± 5.5 | 755.6 ± 104.8 | 838.3 ± 29.4 |
| 20:1n-7    | 5,987.7 ± 71.3 | 6,617.8 ± 88.7 \(^a\) | 2,975.5 ± 144.9 | 3,307.7 ± 192.1 |
| 20:2n-6    | 1,441.0 ± 20.7 | 1,147.8 ± 65.8 | 754.7 ± 9.8 | 838.3 ± 29.4 |
| 20:3n-6    | 9.1 ± 0.3 | 9.2 ± 0.2 | 10.4 ± 0.6 | 9.3 ± 0.6 |
| 20:3n-3    | 8.5 ± 0.4 | 9.4 ± 1.0 | 17.4 ± 1.7 | 17.2 ± 1.9 |
| 20:0       | 55.6 ± 1.0 | 54.4 ± 1.1 | 78.9 ± 1.1 | 61.6 ± 2.5 \(^a\) |
| 20:1n-9    | 286.6 ± 5.8 | 289.6 ± 5.5 | 755.6 ± 104.8 | 838.3 ± 29.4 |
| 20:1n-7    | 5,987.7 ± 71.3 | 6,617.8 ± 88.7 \(^a\) | 2,975.5 ± 144.9 | 3,307.7 ± 192.1 |
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| 20:3n-6    | 9.1 ± 0.3 | 9.2 ± 0.2 | 10.4 ± 0.6 | 9.3 ± 0.6 |
| 20:3n-3    | 8.5 ± 0.4 | 9.4 ± 1.0 | 17.4 ± 1.7 | 17.2 ± 1.9 |
| 20:0       | 55.6 ± 1.0 | 54.4 ± 1.1 | 78.9 ± 1.1 | 61.6 ± 2.5 \(^a\) |
| 20:1n-9    | 286.6 ± 5.8 | 289.6 ± 5.5 | 755.6 ± 104.8 | 838.3 ± 29.4 |
| 20:1n-7    | 5,987.7 ± 71.3 | 6,617.8 ± 88.7 \(^a\) | 2,975.5 ± 144.9 | 3,307.7 ± 192.1 |
| 20:2n-6    | 1,441.0 ± 20.7 | 1,147.8 ± 65.8 | 754.7 ± 9.8 | 838.3 ± 29.4 |
| 20:3n-6    | 9.1 ± 0.3 | 9.2 ± 0.2 | 10.4 ± 0.6 | 9.3 ± 0.6 |
| 20:3n-3    | 8.5 ± 0.4 | 9.4 ± 1.0 | 17.4 ± 1.7 | 17.2 ± 1.9 |
| 20:0       | 55.6 ± 1.0 | 54.4 ± 1.1 | 78.9 ± 1.1 | 61.6 ± 2.5 \(^a\) |

**Brain fatty acid concentrations in phospholipid fractions of unfasted rats after 15 weeks of feeding (n = 8 per dietary group). Data are means ± SEM.**

*Indicates significant difference from the adequate n-6 PUFA group (P < 0.05).
the n-6 PUFA-deprived rats. Brain phospholipid ARA concentrations were 6,077 ± 103 nmol/g of brain and 5,191 ± 158 nmol/g of brain, respectively. This raises an interesting question: why does a reduction to 5,191 nmol/g of brain lead to a 0.0006 nmol/g of brain reduction in 5-HETE when the amount of ARA available is still >2 million times the amount needed to supply the 0.0019 nmol of 5-HETE in the n-6 PUFA-adequate rats? Perhaps this highlights how important phospholipid ARA is in its other roles aside from eicosanoid production or that ARA is regulating the enzymatic synthesis of the eicosanoids. It may also be nonenzymatically derived (29). This suggests that these eicosanoid reductions could be due to the lower ARA concentration in the brain phospholipids of n-6 PUFA-deprived rats.

One thing to note is that in the brain, eicosanoids are present in the femtomole to picomole per gram range, whereas concentrations of ARA in brain phospholipids are just over a micromole per gram. The most concentrated eicosanoid detected was 5-HETE, which had a concentration of 0.0019 ± 0.00001 nmol/g of brain in the n-6 PUFA-adequate rats and 0.0013 ± 0.000007 nmol/g of brain in the n-6 PUFA-deprived rats. Brain phospholipid ARA concentrations were 6,077 ± 103 nmol/g of brain and 5,191 ± 158 nmol/g of brain, respectively. This raises an interesting question: why does a reduction to 5,191 nmol/g of brain lead to an ~0.0006 nmol/g of brain reduction in 5-HETE when the amount of ARA available is still >2 million times the amount needed to supply the 0.0019 nmol of 5-HETE in the n-6 PUFA-adequate rats? Perhaps this highlights how important phospholipid ARA is in its other roles aside from eicosanoid production or that ARA is regulating the enzymatic synthesis of the eicosanoids. It may also be nonenzymatically derived (29). This suggests that these eicosanoid reductions could be due to the lower ARA concentration in the brain phospholipids of n-6 PUFA-deprived rats.

Fig. 3. Loss of ³H-ARA from brain total phospholipids and fractions over time. Logarithmically transformed curves of the loss of ³H-ARA from brain total phospholipids and fractions over time with linear regression analysis (untransformed curves are inset). A: Total phospholipid. B: ChoGpl. C: EtnGpl. D: PtdIns. E: PtdSer. Data are mean ± SEM (n = 4 independent samples per dietary group per time point, except for the 4 day time point of the n-6 PUFA-adequate rats where n = 3). All slopes are significantly different from zero (P < 0.0001).
Fig. 4. Loss of $^3$H-DHA from brain total phospholipids and fractions over time. Logarithmically transformed curves of the loss of $^3$H-DHA from brain total phospholipids and fractions over time with linear regression analysis (untransformed curves are inset). A: Total phospholipids. B: ChoGpl. C: EtnGpl. D: PtdIns. E: PtdSer. Data are mean ± SEM (n = 4 independent samples per dietary group per time point). All slopes are significantly different from zero ($P < 0.0001$).
that there is a set rate at which ARA autooxidation occurs, which cannot be altered to compensate for alterations in brain phospholipid ARA concentration.

There was a significantly higher level of all detectable EPA-derived eicosanoids in the n-6 PUFA-deprived rats, except for 12-HEPE. These EPA-derived eicosanoids were all autooxidation products, and the increase we observed may be due to the 10-fold increase in brain total phospholipid EPA concentration in the n-6 PUFA-deprived rats. In our study, and as reported by others, the brain maintains very low levels of EPA (26, 36–38). Even supplementation of EPA in the diet does not create such large increases in EPA. Perhaps the extra DHA is largely being shunted toward increasing the levels of enzymatically derived docosanoids, which, similar to others, we were unable to detect in microwave-fixed rat brains (29). Previous studies using similar diets have reported that an n-6 PUFA-deprived diet increases the activity of iPLA2 and expression of 15-LOX, which increases the enzyme level of EPA derived eicosanoids in the n-6 PUFA-deprived rats, similar to others (39).

In rats, the level of LA at which tissue ARA concentrations plateau is 1,200 mg of LA per 100 g of diet, and this intake is considered the recommended minimum LA intake (25). This level is comparable to the recommended level of LA for humans: 1,000–1,500 mg of LA per 100 g of food, which equates to ~2–3% of energy (42–44). However, more recent studies suggest that these are overestimations of the actual LA requirement, and there is a concern that the amount of LA in the average human diet is too high (45). For rats, the level at which tissue ARA levels plateau cannot necessarily be substituted for the LA requirement level, as there is no evidence that ARA at this peak level is critical for health. For instance, the n-6 PUFA-deprived diet used in our study and in previous studies had a LA level that was 10% of the suggested requirement, and rats did not show significant signs of LA deficiency (15). One of the problems with earlier experiments that formed the basis of LA requirements is that many of them performed the basis of LA requirements is that many of them did not ensure an adequate level of ALA (45–49). The effects of an inadequate level of ALA were attributed to having an inadequate level of LA, and thus may have led to an overestimation of LA requirement. The n-6 PUFA-deprived diet in our study contained an adequate amount of ALA, allowing for the effects here to be attributed to changes in dietary n-6 PUFA intake, without the influence of dietary n-3 PUFA intake.

**CONCLUSION**

In summary, rats fed an n-6 PUFA-deprived diet (2% LA) or an n-6 PUFA-adequate diet (24% LA) for 15 weeks were infused with [3H]ARA or ^1^H-DHA, which allowed for the determination of the rate of loss of ARA and DHA.
from their brain phospholipids.ARA had a longer half-life and was lost at a slower rate in the total phospholipids, ChoGpl, EtnGpl, and PtdSer pools of the n-6 PUFAdriven rats, illustrating the brain’s ability to conserve ARA in response to lower dietary LA. In contrast, DHA was lost more rapidly in the total phospholipids and ChoGpl pools of the n-6 PUFAdriven rats, but not the other phospholipids fractions. These effects are approximately opposite of what was observed with the low versus high n-3 PUFAdiets in previous studies where low n-3 PUFALeads to conservation of DHA but not ARA (10, 11). Recently, the Nurses’ Health Study reported that an elevated intake of LA was associated with an increased risk of developing major depression (50), and preclinical studies suggest that drugs used to manage bipolar disorder decrease brain arachidonic acid metabolism (51, 52). While much interest in dietary LA levels have focused around coronary heart disease risk, understanding how dietary n-6 PUFAs regulate the metabolism of ARA and DHA in the brain may also be important for determining dietary requirements.

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