The Effects of Dietary n-3/n-6 Ratio on Brain Development in the Mouse: A Dose Response Study with Long-Chain n-3 Fatty Acids

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This study examines the effects of the ratio of n-3/n-6 fatty acids (FA) on brain development in mice when long-chain n-3 FA are supplied in the diet. From conception until 12 days after birth, B6D2F$_1$ mice were fed liquid diets, each providing 10% of energy from olive oil, and a further 10% from different combinations of free FA concentrates derived from safflower oil (18:2n-6), and fish oil (20:5n-3 and 22:6n-3). The range of dietary n-3/n-6 ratios was 0, 0.25, 0.5, 1.0, 2.0 and 4.0, with an n-6 content of greater than 1.5% of energy in all diets, and similar levels of total polyunsaturated fatty acids (PUFA). In an additional group of ratio 0.5, 18:2n-6 was partially replaced by its Δ6 desaturation product, 18:3n-6. Biochemical analyses were conducted on 12-day-old pup brains, as well as on samples of maternal milk. No obvious effects on overall pup growth and development were observed, apart from a smaller litter size at ratio 1. Co-variance analysis indicated that increasing the n-3/n-6 ratio was associated with slightly smaller brains, relative to body weight. We found that 18:2n-6 and 20:5n-3 were the predominant n-6 and n-3 FA in the milk; in the brain these were 20:4n-6 and 22:6n-3, respectively. Increasing dietary n-3/n-6 ratios generally resulted in an increase in n-3 FA, with a corresponding decrease in n-6 FA. The n-3/n-6 ratio of the milk lipids showed a strong linear relationship with the diet, but in the brain the rate of increase tended to decrease beyond 0.5 (phosphatidylcholine, PC) and 0.25 (phosphatidylethanolamine, PE), such that there was a significant quadratic contribution to the relationship. The partial replacement of dietary 18:2n-6 with 18:3n-6 raised levels of 20:4n-6 in milk, brain PC, and brain PE. These results indicate that the n-3/n-6 ratio of the phospholipids in the developing mouse brain responds maximally to maternal dietary long-chain n-3/n-6 ratios of between 0.25 and 0.5.
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The membrane phospholipids of the central nervous system contain high levels of polyunsaturated fatty acids (PUFA), particularly arachidonic acid, 20:4n-6, and docosahexaenoic acid, 22:6n-3 (1). It is known that 22:6n-3, occurs at high concentrations in synaptic membranes (2) and in photoreceptor cells in the retina (3,4). These long-chain PUFA accrue rapidly in the brain during the prenatal and suckling periods (5–7), and are formed from their respective dietary precursors, 18:2n-6 and 18:3n-3, by the same series of desaturation and elongation reactions, with n-3 having the competitive advantage over n-6 compounds *in vitro* (8). Both fetal liver and brain have the metabolic capacity to synthesize long-chain PUFA (9,10). There is recent evidence suggesting that 18:3n-3 is converted in fetal and pup liver to 22:6n-3, which is then secreted into blood as lipoproteins, and incorporated selectively into nervous tissue (11). Studies in both developing rat and chick brain have demonstrated preferential uptake of 22:6n-3 over its precursors (12,13). Dose-response studies conducted by varying the amount of dietary 18:3n-3 during the developmental period (14), and in weanling animals (15–17), showed a decrease in brain 22:5n-6 and an increase in brain 22:6n-3 with increasing dietary 18:3n-3. Much of this effect appeared to be in comparison with an n-3 deficient group, with smaller differences at the higher dosage levels, suggesting regulatory limits. The relationship between brain FA composition and dietary long-chain n-3 FA may be different from that seen with dietary 18:3n-3. Feeding of fish oil to adult rats resulted in a rapid increase in levels of 22:5n-3 and 22:6n-3, as well as 20:5n-3 (which is usually present in brain only in trace amounts), with corresponding decreases in 22:5n-6, as well as 20:4n-6, suggesting that the brain may be vulnerable to an excess of long-chain n-3 PUFA (18,19). The developing brain, because of its affinity for long-chain n-3, may be particularly susceptible to such effects. There is particular concern that decreases in 20:4n-6 may be associated with adverse effects (20). Thus the present study investigated the relationship between the dietary long-chain n-3/n-6 ratio and the FA composition of the brain in developing mice. The n-3 FA were provided as the long-chain compounds found in fish oil, predominantly 20:5n-3 and 22:6n-3, and using ratios of 0, 0.25, 0.5, 1.0, 2.0 and 4.0. While the 0 group was clearly an n-3 deficient group, the values above 0.25 considerably exceeded current dietary recommendations (21), with the intent of challenging the regulatory capacity of the system. The study included two groups of ratio 0.5, one as part of the dose-response series where the n-6 was provided as 18:2n-6, and the other where a portion of the 18:2n-6 was replaced by 18:3n-6. Previous work comparing labelled dietary 18:3n-6 with 18:2n-6 showed increased incorporation into brain 20:4n-6 with 18:3n-6 (22). Therefore comparison of these two groups addressed whether levels of 20:4n-6 in the brain would increase in animals fed 18:3n-6 relative to those receiving the same amount of long-chain n-3, but only 18:2n-6 in the diet. The dietary treatments were imposed throughout gestation and lactation. The FA composition of milk lipids and pup brain phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions was determined on day 32 postconception (12 days after birth); other measurements included pup body and brain growth, as well as eye-opening score.

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Abbreviations: ANOVA, analysis of variance; EFA, essential fatty acids; FA, fatty acids; GLC, gas-liquid chromatography; GLM, general linear model; MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; SAS, Statistical Analysis Systems; SFA, saturated fatty acids.

MATERIALS AND METHODS

Animals. Parents were 28- to 32-week-old B6D2F$_1$ hybrid mice purchased from Charles River Breeding Laboratories (St. Constant, P.Q., Canada). They were maintained under a reversed 12 hr light:12 hr dark schedule at 22 ± 1°C, with tap water and lab chow (PMI Lab Diet—formerly Purina—# 5001, St. Louis, MO) available *ad libitum* until breeding. The animals were group-housed in standard plastic cages containing Beta-Chip hardwood bedding (Northeastern Products Corp., Warrensburg, NY) and toilet tissue for nesting material.

Diets. Maternal animals were fed one of six liquid diets, each of which provided 20% of the calories from oil, but which varied in their n-3/n-6 ratio. The diet, as used in our previous studies (23–25), was specifically formulated for our use by BioServ, ref. # F2187 (Frenchtown, NJ). This provided 1 kcal/mL, with 20% of the calories from protein (fat-free casein), 20% from oil, 60% from carbohydrate (maltose-dextrin), supplemented with minerals and vitamins. The dietary oil mixtures were prepared in our laboratory from olive oil (13% 18:2n-6 as triglyceride), and free fatty acid concentrates, Safflower 70, derived from safflower oil and containing 69.7% 18:2n-6, and EPA 50, derived from fish oil concentrate and containing 41.2% 20:5n-3 and 6.1% 22:6n-3 (Callanish Ltd., Breasclete, Isle of Lewis, U.K.). The use of these free fatty acid concentrates in the formulation of the diets allowed us to provide high n-3/n-6 ratios (0.0, 0.25, 0.5, 1.0, 2.0, and 4.0), while ensuring similar levels of PUFA (approximately 8% of total dietary energy) (Table 1). All groups received sufficient n-6 FA (>1.5% of total dietary energy), such that we were studying only the effect of increasing n-3, and not that of n-6 deficiency. The n-6 was supplied as 18:2n-6, and the n-3 predominantly as 20:5n-3 and 22:6n-3. This design therefore addressed the effects of increasing the dietary n-3/n-6 ratio ((20:5n-3 + 22:6n-3)/18:2n-6) while keeping the total amount of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) relatively constant; thus, as n-3 increased, n-6 decreased. The alternative of increasing n-3 while keeping n-6 constant would have had the problem of causing these other factors to vary concomitantly. A similar dietary formulation has been used in a recently published study of varying dietary n-6/n-3 ratio on the brain fatty acid (FA) composition in weanling rats (27). An additional group was fed a diet with a n-3/n-6 ratio of 0.5 and the same amount of EPA 50 as the 0.5 group. In this group, 18:2n-6 was replaced partially by 18:3n-6 (GLA 70, containing 19% 18:2n-6 and 68.7% 18:3n-6). Additionally, α-tocopherol was added to the oil mixtures, such that the prepared liquid diet contained 150 i.u. per liter.

Breeding protocol. Animals were mated daily, and checked for the presence of copulatory plugs after 7 hr. Pregnant dams were assigned randomly to each dietary condition on day 0, and all subsequent days were designated as "days post-conception." Dams were fed daily preweighed amounts of the liquid diet, based on 0.65 kcal/g body wt/day, until day 20. Fresh diet was prepared every two days, and diets and dietary oils were stored in the refrigerator under nitrogen at all times. Birth occurred on day 19 or 20, and all dams and litters were weighed on

TABLE 1

Selected Fatty Acid Composition of Dietary Oil Mixtures[a]

	0	0.25	0.5	1.0	2.0	4.0	0.5[b]
% Energy							
Olive oil	50.00	50.00	50.00	50.00	50.00	40.00	56.7
Safflower 70	50.00	35.70	26.70	16.00	5.85	1.00	
EPA 50		14.30	23.30	34.00	44.15	59.00	23.3
GLA 70							20.0
% Fatty acids							
16:0	10.8	9.8	9.1	8.4	7.7	5.9	8.3
16:1	0.7	2.5	3.6	4.3	6.3	8.0	0.8
18:0	2.4	3.1	3.5	4.0	4.5	5.2	3.8
18:1n-9	39.6	38.1	37.2	36.0	35.0	28.2	37.9
18:1n-7		0.3	0.5	0.7	0.9	1.2	0.5
18:2n-6	41.4	31.7	25.7	18.5	11.6	7.2	11.7
18:3n-6		0.2	0.3	0.4	0.5	0.7	14.0
20:4n-6		0.1	0.2	0.2	0.3	0.4	0.2
20:5n-3		5.9	9.6	14.0	18.2	24.3	9.6
22:6n-3		0.9	1.4	2.1	2.7	3.6	1.4
Other n-3[c]		1.2	2.1	3.1	4.0	5.4	2.1
Total SFA	13.2	12.9	12.6	12.4	12.2	11.1	12.1
MUFA	40.3	40.9	41.3	41.0	42.2	37.4	39.2
PUFA	41.4	40.0	39.0	38.3	37.3	41.6	39.2
n-3		8.0	13.1	19.2	24.9	33.3	13.1
n-6	41.4	32.0	26.2	19.1	12.4	8.3	25.9

[a] Calculations are based on GLC analysis of component oils (olive oil, 13% n-6; safflower 70, 70% n-6; EPA 50, 4% n-6, 56% n-3; GLA 70, 88% n-6).

[b] In this group the dietary 18:2n-6 was replaced partially by 18:3n-6.

[c] 18:4n-3, 20:4n-3, 22:5n-3.

day 20. At this time, litters were culled to six (three of each sex). From day 20 until day 32 post-conception, when the pups were tested, lactating mothers were fed the appropriate liquid diet *ad libitum* at 1.5 × strength (daily intake = 0.975 kcal/g). Differences in food intake among the groups were negligible. On day 32, two pups of each sex from each litter were assessed for eye-opening on a scale from 0 to 1 (detailed criteria available on request). One male and one female from each litter were then anaesthetized with Halothane and decapitated. The brains were extracted and trimmed by removal of the olfactory bulbs anteriorly and 2 mm below the medulla posteriorly. The two brains from each litter were pooled, weighed within two minutes to the nearest 0.1 mg, and frozen at −50°C until analysis of fatty acid composition. The hearts, livers and kidneys were also extracted, pooled and stored; these data are the subject of a separate report (26). The remaining pups were separated from the dams overnight, and on day 33 the dams were milked, following an adaptation of the procedure described by Mills *et al.* (27). The milk was also stored at −50°C. All tissue was coded so that biochemical analysis was done independently of knowledge of the treatment group.

Brain lipids. The homogenized brains were extracted according to a modified method of Bligh and Dyer (28), using chloroform/methanol (1:1, v/v), in the presence of 0.02% BHT (w/v). After separation and drying under nitrogen, the total lipids were fractionated by thin-layer chromatography, using silica gel plates (Analtech GF) and a chloroform/methanol/acetic acid/water (50/30/4/2, by vol) solvent system. The fatty acids of the resulting phospholipid fractions were methylated with 14% boron trifluoride in methanol, and analyzed on a gas chromatograph (Perkin-Elmer 8420, Norwalk, CT) equipped with a flame-ionization detector and a 15 m × 0.32 m (i.d.) capillary column (Supelco Wax 10, Bellefonte, PA). The temperature program for gas-liquid chromatography (GLC) consisted of a 2-min hold at 160°C, followed by a 2°/min increase to 190°C. After 10 min at 190°C the temperature was increased at 5°/min to 220°C, followed by a 2-min hold at 220°C. Fatty acids were identified by comparison of their retention times with those of authentic standards.

The concentration of total phospholipids, and the percent distribution of phospholipid subclasses were measured by high-performance liquid chromatography (System Gold, Beckman Canada, Mississauga, Ontario, Canada) using a Si Ultrasphere column (5 microns, 4.6 mm i.d. × 250 mm, Beckman) and a mass detector (ACS 750/14, Applied Chromatography Systems Ltd., Lutons, Bds., U.K.) as described previously (29).

Statistical analyses. The maternal and pup growth data were analyzed using the general linear model (GLM) provided by the Statistical Analysis System (SAS) to do analysis of variance (ANOVA). Individual group means were compared using Tukey's t-test; least squares means resulting from covariance analysis were compared using t-tests; the alpha level was set at 0.05. In the absence of a treatment by sex interaction, the litter mean score, collapsed across sex, was used as the unit of analysis for the data on pup growth and development. As the fatty acid determinations were conducted on the pooled brains from a litter, the litter also represents the unit of analysis for these measures. Regression analyses described the nature of the dose-response relationship between the dietary n-3/n-6 ratio and the n-3/n-6 ratio of milk and brain lipids. A linear model was used initially, and, when this proved significant, it was subsequently determined whether the inclusion of a quadratic component made a significant additional contribution. The sample sizes are shown in the respective tables.

RESULTS

Maternal variables and pup growth and development. Maternal weight gain did not differ among the groups during pregnancy, but litter size, as reflected by the number of live pups on day 20, was significantly different, $F(5,45) = 3.73$, $p < 0.01$, with ratio 1.0 having smaller litters (mean (pups) ±SEM: 5.3 ± 0.9) than ratio 0.25 (9.0 ± 0.5) and ratio 2.0 (9.1 ± 0.8). Neither pup weight on day 20 and day 32, nor eye-opening score, differed significantly. Brain weight showed a trend toward decreasing as the n-3/n-6 ratio increased, particularly at ratio 2.0. This was confirmed by a covariance analysis, where the least squares means of brain weight adjusted for body weight indicated a significant treatment effect, $F(5,41) = 3.08$, $p < 0.02$, with ratio 0.25 (mean (g) ± SEM: 0.347 ± 0.003), 1.0 (0.345 ± 0.003), and 2.0 (0.342 ± 0.003) being significantly lower than ratio 0 (0.356 ± 0.003. Ratio 4.0 (0.348 ± 0.003) was marginally lower than ratio 0, $p < 0.08$. Ratio 0.5 (3.353 ± 0.003) did not differ from any other group.

Lipid analysis. The n-3/n-6 ratio provides a summary of the outcome of the dietary treatment. There was a clear treatment effect on the milk lipids, $F(5,34) = 841.34$, $p < 0.0001$, brain phosphatidylcholine (PC), $F(5,40) = 191.6$, $p < 0.0001$, and brain phosphatidylethanolamine (PE), $F(5,41) = 164.9$, $p < 0.0001$. As is shown in Figure 1, the relationship between the n-3/n-6 ratio of the maternal diet and that of the milk was represented by a linear dose-response model. The pattern in the brain phospholipids

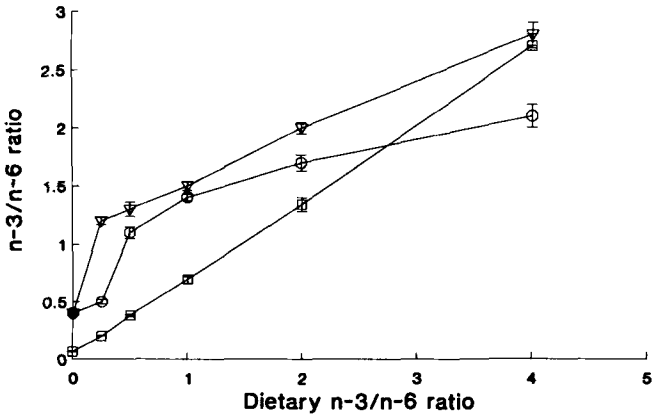

FIG. 1. Effects of maternal dietary n-3/n-6 ratio (x) on the n-3/n-6 ratio (y) of milk lipids (□) and pup brain phosphatidylcholine (PC, ○) and phosphatidylethanolamine (PE, ▽). Pregnant mice were fed diets of increasing n-3/n-6 ratio from conception to day 32 post-conception (12 days after birth). The values represent the group mean ±SEM. Regression analysis shows a linear relationship of the treatment with milk lipids ($y = 0.04 + 0.67x$, $r^2 = 0.99$) whereas in brain the relationship includes a quadratic component (PC: $y = 0.41 + 1.05x - 0.16x^2$, $r^2 = 0.90$; PE: $y = 0.70 + 0.88x - 0.09x^2$, $r^2 = 0.90$. In PC, all groups differ except 0.25 and 0.0: in PE all groups differ except 0.5 which does not differ from 0.25 and 1.0.

was somewhat different. In both the PC and PE fractions there was an initial steep increase, followed by increments which were smaller in magnitude, although still significantly different from each other. Thus the relationship with the dietary n-3/n-6 ratio included a significant quadratic component. In PC, all groups differed except 0.25 and 0.0; in PE all groups differed except 0.5 which did not differ from 0.25 and 1.0.

The values for individual FA are given in Tables 2, 3, and 4. From these it is clear that in both PC and PE the increase in the n-3/n-6 ratio was due to an increase in n-3, with a reciprocal decrease in n-6. In the milk lipids, 18:2n-6 and 20:5n-3 were the major FA. Their elongation products, 20:4n-6 and 22:6n-3, were the predominant n-6 and n-3 compounds, respectively, in the brain. Very little 18:2n-6 was observed in the brain, and 20:5n-3 was seen only in any appreciable amounts (>1%) in the PE fraction of the groups receiving n-3/n-6 ratios of 2 and 4. Conversely, 22:5n-6 was seen only at ratio 0 in both PC and PE. In contrast to other n-6 FA, levels of 20:3n-6 increased as the

TABLE 2

Effects of Maternal Dietary n-3/n-6 Ratio on Selected Fatty Acid Composition of Milk Lipids on Day 33 Postconception[a]

n-3/n-6 Ratio	0.0 n = 9	0.25 n = 7	0.5 n = 6	1.0 n = 7	2.0 n = 6	4.0 n = 6	0.5[b] n = 7
14:0	13.3 ± 0.3	14.0 ± 0.4	14.6 ± 0.3	13.6 ± 0.5	14.4 ± 0.5	15.5 ± 0.4	14.9 ± 0.3
16:0	21.0 ± 0.4	22.1 ± 0.6	22.9 ± 0.6	22.5 ± 0.8	22.0 ± 0.6	21.6 ± 1.1	19.8 ± 3.2
16:1	1.5 ± 0.07	1.9 ± 0.08	2.5 ± 0.1	2.9 ± 0.1[c]	3.1 ± 0.2[c]	3.7 ± 0.2[c]	2.3 ± 0.08
18:0	2.2 ± 0.04	2.4 ± 0.05	2.4 ± 0.07	2.4 ± 0.1	2.3 ± 0.06	2.4 ± 0.06	2.3 ± 0.07
18:1	24.2 ± 0.5	22.6 ± 0.9	22.5 ± 1.0	22.1 ± 1.2	19.6 ± 1.1	16.6 ± 1.0[c]	20.5 ± 0.8
18:2n-6	13.4 ± 0.2[c]	12.0 ± 0.2	10.4 ± 0.4[c]	7.6 ± 0.2[c,d]	5.0 ± 0.2[c,e]	3.4 ± 0.2[c,f]	4.7 ± 0.2[g]
18:3n-3	0.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.03	0.2 ± 0.01	0.3 ± 0.01	0.2 ± 0.06	0.2 ± 0.03
18:4n-3	0.9 ± 0.04[c]	0.6 ± 0.03	0.5 ± 0.03	0.5 ± 0.04	0.4 ± 0.02[c]	0.2 ± 0.06[c]	0.4 ± 0.02
20:2n-6	1.0 ± 0.04[c]	0.5 ± 0.02	0.4 ± 0.03[c]	0.2 ± 0.02[c,d]	0.1 ± 0.01[c]	0.0 ± 0.0[c]	0.1 ± 0.02[g]
20:3n-6	0.5 ± 0.07	0.5 ± 0.02	0.4 ± 0.02	0.3 ± 0.01	0.3 ± 0.01[c]	0.2 ± 0.05[c]	3.1 ± 0.8[g]
20:4n-6	0.6 ± 0.01[c]	0.4 ± 0.03	0.3 ± 0.06	0.3 ± 0.01	0.3 ± 0.01	0.2 ± 0.06[c]	1.4 ± 0.05[g]
20:5n-3	0.01 ± 0.01[c]	0.7 ± 0.02	1.5 ± 0.04[c]	2.5 ± 0.1[c,d]	3.6 ± 0.2[c,e]	5.6 ± 0.2[c,f]	1.6 ± 0.08
22:4n-6	0.3 ± 0.07[c]	0.1 ± 0.0	0.06 ± 0.03	0.2 ± 0.003	0.2 ± 0.01	0.3 ± 0.06	0.09 ± 0.02
22:5n-6	0.2 ± 0.05	0.1 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.4 ± 0.02[g]
22:5n-3	0.0 ± 0.0[c]	0.9 ± 0.02	1.5 ± 0.7[c]	2.1 ± 0.1[c,d]	2.7 ± 0.1[c,e]	3.8 ± 0.2[c,f]	1.5 ± 0.05
22:6n-3	0.06 ± 0.02[c]	0.5 ± 0.02	0.7 ± 0.02[c]	0.9 ± 0.04[c,d]	1.1 ± 0.05[c,e]	1.5 ± 0.060[c,f]	0.6 ± 0.03

[a]Values are expressed in % and represent group means ± SEM with n = number of dams.

[b]In this group the dietary 18:2n-6 was replaced partially by 18:3n-6.

Groups are significantly different by Tukey's t-test, $p < 0.05$: c, each group *vs* 0.25; d, 1.0 *vs* 0.5; e, 2.0 *vs* 1.0; f, 4.0 *vs* 2.0; g, 0.5 (18:3n-6) *vs* 0.5 (18:2n-6).

TABLE 3

Effects of Maternal Dietary n-3/n-6 Ratio on Selected Fatty Acid Composition of Brain Phosphatidylcholine in B6D2F$_2$ Mouse Pups on Day 32 Postconception[a]

	0.0 n = 9	0.25 n = 9	0.5 n = 6	1.0 n = 10	2.0 n = 6	4.0 n = 6	0.5[b] n = 9
14:0	2.3 ± 0.1[c]	3.1 ± 0.2	2.3 ± 0.2[c]	1.7 ± 0.1[c]	2.3 ± 0.2[c]	2.3 ± 0.1[c]	3.1 ± 0.3[g]
16:0	41.9 ± 0.3[c]	48.3 ± 0.4	41.1 ± 0.7[c]	38.0 ± 0.3[c,d]	40.7 ± 0.5[c,e]	41.3 ± 0.8[c]	47.1 ± 0.7[g]
16:1	4.6 ± 0.3	5.1 ± 0.2	3.8 ± 0.2[c]	3.8 ± 0.09[c]	4.4 ± 0.1	4.7 ± 0.1	5.4 ± 0.4[g]
18:0	10.5 ± 0.4[c]	7.2 ± 0.3	12.1 ± 0.4[c]	13.4 ± 0.2[c]	11.7 ± 0.2[c,e]	10.9 ± 0.3[c]	7.7 ± 0.6[g]
18:1	19.4 ± 0.3	20.2 ± 0.3	19.5 ± 0.2	20.1 ± 0.2	19.3 ± 1.8	21.5 ± 0.2	19.3 ± 0.4
18:2n-6	1.3 ± 0.05[c]	1.5 ± 0.03	1.3 ± 0.04	1.3 ± 0.03[c]	1.3 ± 0.03[c]	0.9 ± 0.1[c,f]	0.3 ± 0.05[g]
18:3n-3	0.1 ± 0.02	0.0 ± 0.0	0.3 ± 0.2[c]	0.1 ± 0.02[d]	0.1 ± 0.02	0.2 ± 0.04	0.0 ± 0.0[g]
18:4n-3	0.5 ± 0.03	0.5 ± 0.03	0.6 ± 0.02[c]	0.6 ± 0.02[c]	0.6 ± 0.02[c]	0.7 ± 0.02[c]	0.3 ± 0.07[g]
20:2n-6	0.4 ± 0.03	0.4 ± 0.04	0.5 ± 0.02	0.5 ± 0.01	0.1 ± 0.03[c,e]	0.3 ± 0.1[f]	0.0 ± 0.0[g]
20:3n-6	0.4 ± 0.02	0.5 ± 0.07	0.7 ± 0.01[c]	0.9 ± 0.01[c,d]	0.9 ± 0.06[c]	0.8 ± 0.03[c]	0.9 ± 0.05
20:4n-6	8.7 ± 0.2[c]	7.8 ± 0.2	6.5 ± 0.2[c]	6.0 ± 0.1[c]	4.7 ± 0.1[c,e]	3.8 ± 0.2[c]	9.0 ± 0.2[g]
20:5n-3	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.01[c]	0.2 ± 0.01[c,d]	0.4 ± 0.03[c,e]	0.04 ± 0.04[f]	0.7 ± 0.0[g]
22:4n-6	1.6 ± 0.07[c]	0.5 ± 0.02	0.9 ± 0.03[c]	0.7 ± 0.03[c]	0.3 ± 0.01[c,e]	0.2 ± 0.04[c]	0.0 ± 0.05
22:5n-6	2.5 ± 0.1[c]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.01[c]	0.8 ± 0.02[c,d]	1.0 ± 0.06[c]	1.1 ± 0.07[c]	0.1 ± 0.03[g]
22:6n-3	5.1 ± 0.2	5.1 ± 0.2	9.7 ± 0.7[c]	11.7 ± 0.3[c,d]	10.0 ± 0.3[c,e]	10.2 ± 0.6[c]	6.1 ± 0.3[g]

[a]Values are expressed in % and represent group means ± SEM with n = number of litters.

[b]In this group the dietary 18:2n-6 was replaced partially by 18:3n-6.

Groups are significantly different by Tukey's t-test, $p < 0.05$: c, each group *vs* 0.25; d, 1.0 *vs* 0.05; e, 2.0 *vs* 1.0; f, 4.0 *vs* 2.0; g, 0.5 (18:3n-6) *vs* 0.5 (18:2n-6).

TABLE 4

Effects of Maternal Dietary n-3/n-6 Ratio on Selected Fatty Acid Composition of Brain Phosphatidylethanolamine in B6D2F$_2$ Mouse Pups on Day 32 Postconception[a]

n-3/n-6 Ratio	0.0 n = 9	0.25 n = 9	0.5 n = 5	1.0 n = 10	2.0 n = 7	4.0 n = 7	0.5[b] n = 9
14:0	0.1 ± 0.05	0.7 ± 0.4	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.01	0.2 ± 0.1	0.1 ± 0.03
16:0	11.4 ± 0.3	10.5 ± 0.3	10.9 ± 0.4	11.6 ± 0.2	11.3 ± 0.4	11.2 ± 0.3	10.8 ± 0.1
16:1	0.4 ± 0.04	0.4 ± 0.1	0.5 ± 0.8	0.4 ± 0.03	0.4 ± 0.03	0.6 ± 0.6	0.4 ± 0.02
18:0	24.5 ± 0.5	23.7 ± 0.5	23.2 ± 0.9	25.2 ± 0.3	24.3 ± 0.6	22.0 ± 0.9	23.6 ± 0.9
18:1	8.6 ± 0.1	7.9 ± 0.3	9.2 ± 0.2[c]	9.5 ± 0.2[c]	9.5 ± 0.3[c]	10.0 ± 0.3[c]	7.9 ± 0.1[g]
18:2n-6	0.7 ± 0.03	0.6 ± 0.05	0.8 ± 0.03[c]	0.6 ± 0.03	0.6 ± 0.04	0.5 ± 0.04	0.2 ± 0.02[g]
18:3n-3	0.4 ± 0.8[c]	0.2 ± 0.03	0.4 ± 0.1	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.02	0.2 ± 0.01
18:4n-3	0.6 ± 0.1	0.4 ± 0.06	0.5 ± 0.02	0.6 ± 0.03	0.5 ± 0.03	0.6 ± 0.04	0.4 ± 0.02[g]
20:2n-6	0.6 ± 0.03[c]	0.3 ± 0.06	0.6 ± 0.05[c]	0.7 ± 0.04[c]	1.0 ± 0.07[c,e]	1.0 ± 0.04[c]	0.2 ± 0.01
20:3n-6	0.6 ± 0.02	0.5 ± 0.04	0.9 ± 0.02[c]	0.9 ± 0.03[c]	1.1 ± 0.06[c,e]	1.1 ± 0.03[c]	0.8 ± 0.03
20:4n-6	21.8 ± 0.1[c]	18.7 ± 0.7	18.0 ± 0.4	16.1 ± 0.3[c]	13.7 ± 0.2[c,e]	11.4 ± 0.6[c,f]	21.2 ± 0.4[g]
20:5n-3	0.0 ± 0.0	0.1 ± 0.03	0.3 ± 0.03	0.6 ± 0.02[c]	1.3 ± 0.07[c,e]	2.3 ± 0.2[c,f]	0.1 ± 0.01
22:4n-6	6.4 ± 0.07[c]	4.5 ± 0.1	3.3 ± 0.05[c]	2.4 ± 0.07[c,d]	1.4 ± 0.07[c,e]	0.9 ± 0.06[c,f]	5.1 ± 0.1[g]
22:5n-6	7.3 ± 0.2[c]	0.4 ± 0.06	0.2 ± 0.06	0.2 ± 0.02	0.1 ± 0.01	0.1 ± 0.02[c]	0.6 ± 0.01
22:5n-3	0.2 ± 0.04[c]	0.8 ± 0.06	1.4 ± 0.04[c]	2.0 ± 0.04[c,d]	3.0 ± 0.09[c,e]	3.8 ± 0.09[c,f]	0.8 ± 0.02[g]
22:6n-3	15.1 ± 0.5[c]	27.2 ± 0.7	28.1 ± 1.1	27.3 ± 0.5	30.1 ± 0.7	33.4 ± 0.8[c,f]	26.7 ± 0.9

[a]Values are expressed in % and represent group means ± SEM with n = number of litters.

[b]In this group the dietary 18:2n-6 was replaced partially by 18:3n-6.

Groups are significantly different by Tukey's t-test, $p < 0.05$: c, each group *vs* 0.25.; d, 1.0 *vs* 0.5; e, 2.0 *vs* 1.0; f, 4.0 *vs* 2.0; g, 0.5 (18:3n-6) *vs* 0.5 (18:2n-6).

dietary n-3/n-6 ratio increased. No consistent dose-response effects were observed on the levels of SFA in the brain. In PE and PC, levels of 18:1 increased with increasing levels of n-3; in the milk, levels of 18:1 decreased with increasing n-3, whereas those of 16:1 increased. Quantification of the individual phospholipid classes in the brain showed only a small range over the groups (wt%: PC: 30.5–34.8; PE: 26.1–28.2; PI: 2.3–2.8; PS: 7.7–8.6; SM: 3.1–4.9). Comparison of the two ratio 0.5 groups indicated that the effect of 18:3n-3 was to increase levels of 20:4n-6 in both milk and brain PC and PE. In PC, but not in PE, there was an accompanying slight decrease in levels 22:6n-3.

DISCUSSION

This study sought to examine the effects of varying maternal dietary long-chain n-3 to n-6 FA ratios on the incorporation of n-3 FA into the brains of the developing offspring. A question of particular interest was whether the brain would show the capacity to regulate its fatty acid composition at the higher dietary ratios. The results provide support for such regulation. While the brain showed continually increasing n-3/n-6 ratios with dietary increases, the maximum effect had occurred by 0.5 in PC and 0.25 in PE, with a significantly lower rate of increase beyond these values. Thus, as with the work done with 18:3n-3, the largest effect seen was in comparison with the deficient group. This is supported by the observation that 22:5n-6 was only present to any appreciable extent in the ratio 0 group.

Although 20:5n-3 was the major n-3 FA in the milk, 22:6n-3 predominated in the brain; levels of 20:5n-3 in the brain were generally low, exceeding 1% in PE only at the higher ratios. The companion study on the livers of these animals demonstrated that 22:6n-3 was also the major n-3 FA in the liver, with the maximum 22:6n-3 content seen at ratio 0.5 (26). Although only trace amounts of 20:5n-3 and 22:6n-3 were found in the milk of ratio 0, the brains of these pups showed 5.1% and 15.1% 22:6n-3 in PC and PE, respectively. These data show that there is considerable accretion of long-chain n-3 PUFA during prenatal brain growth, and that dietary n-3 deficiency during gestation may be offset partially by selective retention of 22:6n-3 by the dam, possibly in the liver (30). The absence of n-3 postnatally in the milk of these dams may be explained by depletion of these stores, or, alternatively, by mechanisms which vary in terms of promoting the availability of stored PUFA prenatally to the fetus as compared with postnatally to the milk. Increases in n-3 were generally accompanied by decreases in n-6, particularly of 20:4n-6, thereby reflecting the similar n-3/n-6 reciprocity in the diet. It was shown recently in rats that with a minimal level of 18:2n-6 (0.3% of calories) the amount of 20:4n-6 in the brain remained constant, and, beyond a minimum, was independent of levels of dietary 18:3n-3 (31). This suggests that the present effects on 20:4n-6 may be due specifically to long-chain n-3. Interestingly, levels of 20:4n-6 were increased in both milk and brain by partial replacement of dietary 18:2n-6 with 18:3n-6. There are two possible mechanisms which may account for this, both related to desaturase activity. It is known that 20:5n-3 and 22:6n-3 inhibit Δ6 and Δ5 desaturases (32,33), which reduces the formation of 20:4n-6 from 18:2n-6; the presence of large amounts of 18:2n-6 may result in substrate inhibition of the Δ6 desaturase (34). In both instances, the provision of 18:3n-6 would avoid the effects of a decline in Δ6 desaturase activity. In contrast with the other n-6, 20:3n-6 increased. This replicates our previous findings and suggests inhibition of Δ5 desaturase activity by FA derived from fish oil (23,25). The general absence of effects on the SFA is consistent with other work (35).

With respect to the increase in 18:1 seen with increasing n-3, a similar finding has been reported for chick brain (36).

The data on the milk lipids support previous work in showing an effect of maternal dietary PUFA composition (37,38); they also extend these findings by demonstrating the dose-response nature of this relationship. In contrast with the tendency of the n-3/n-6 ratio to level off in the brain, that of the milk lipids showed a strong linear increase with increasing dietary ratios. These results suggest therefore that it is the metabolism of the pup, not the dam, which is regulating either supply or incorporation into the developing brain.

No systematic effects on overall growth and development were observed, except at ratio 1.0, as seen by the smaller litter size. Also, relative to body weight, increasing the n-3/n-6 ratio resulted in a very small decrease in brain weight. We had shown previously that n-3 supplementation slightly accelerated eye-opening, and that this correlated positively with other indices of sensorymotor development (22). However, in the present study, no effect on the rate of eye-opening was observed.

The diets used in this study were clearly experimental in that they provided only long-chain n-3 FA, with no 18:3n-3, and the n-3/n-6 ratios beyond 0.25 were high. Moreover, species differences in desaturase activities (39) may limit the generalizability of these results. Nevertheless, the findings may be of relevance to questions concerning the provision of long-chain n-3 FA in human infant feeding. They suggest that provision of n-6 FA partially as 18:3n-6 may help to offset the decrease in 20:4n-6 observed with dietary long-chain n-3 FA. They also clearly support a trend toward regulatory limits on the incorporation of long-chain n-3 FA into the mouse brain, showing a maximum response to maternal dietary n-3/n-6 ratios of 0.25–0.5

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