The level of linoleic acid in neural cardiolipin is linearly correlated to the amount of essential fatty acids in the diet of the weanling rat

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We have reported that an unidentified characteristic of the dietary fatty acid composition can greatly alter the percentage of linoleic acid (18:2n6) in the brain. Neural 18:2n6 is concentrated in cardiolipin (CL), which occurs almost entirely in mitochondria and is specifically bound by several enzymes. The purpose of the work reported here was to identify the dietary fat characteristic that is responsible for altering the 18:2n6 levels in neural CL and to establish the dose-response curve for the two variables. Weanling rats were fed for three weeks on diets designed to separate the neural effects of the amount of dietary saturated, monounsaturated, and essential fatty acids (EFA). The results revealed that the level of 18:2n6 in neural CL was very strongly correlated ($r^2 = 0.851$; $P < 0.0001$) with the amount of EFA in the diet. This relationship was linear (slope = 0.793, y-intercept = 6.53%) over a wide range of dietary EFA levels (4–15% wt/wt of diet) with the level of 18:2n6 in CL varying by two-fold. Neural 18:2n6 levels were not affected by the ratio of dietary EFA (18:2n6/18:3n3) when the total amount of EFA was held constant. These results indicate that: 1) the amount of dietary EFA substantially alters the level of 18:2n6 in CL from the brain of the weanling rat in a relatively short time; and 2) this relationship is linear over a wide range of EFA levels that are well above the accepted EFA requirements for the rat and within levels that are typical of human diets.

Keywords: dietary fat; dietary EFA; neural linoleic acid; cardiolipin; correlation; weanling rat

Introduction

The brain is very rich in lipids, with about a tenth of its wet weight and half its dry weight consisting of a large variety of lipids, many of which contain fatty acids. The neural fatty acid composition is well known to be influenced by the fatty acid composition of the diet. The effects on the brain of a deficiency of the essential fatty acids (EFA)—linoleic acid (18:2n6) and α-linolenic acid (18:3n3)—and of altering the dietary ratio of the EFA both have been studied extensively. Almost without exception, the most responsive neural fatty acids have been the highly unsaturated, 20- and 22-carbon fatty acids that are characteristic of the nervous system (for reviews see references 1–3).

Recently, we and others[4] reported that dietary fat composition also influences the level of 18:2n6 in the brain, with the changes being most predominant in mitochondrial cardiolipin (CL; also called diphosphatidyl glycerol). Importantly, these alterations in CL 18:2n6 content were large (up to 2 fold) and occurred rapidly (within one week of feeding the experimental diets to weaned rats). This novel finding was interesting because 18:2n6 in the brain is concentrated in the CL, which is almost exclusively found in the mitochondria[5] where it is associated specifically with several enzymes involved in oxidative respiration[6-11] and

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the transport of fatty acids across the inner mitochondrial membrane.[12-15] Unfortunately, due to the experimental designs neither of these reports was able to attribute the changes in CL 18:2n6 content to a specific characteristic of the dietary fat fed.

In the present paper, we report on experiments conducted to determine which dietary fat characteristic (i.e., the amount of saturated (SFA), monounsaturated (MUFA), or EFA) alters the level of 18:2n6 in neural CL. In achieving this purpose, it was also possible to establish the dose-response curve between the fatty acid composition of the diet and the level of 18:2n6 in CL from the brain.

Materials and methods

Materials

All non-fat dietary ingredients were purchased from Teklad Diets Co. (Madison, WI), except cornstarch (St. Lawrence Starch Co. Ltd., Mississauga, ONT, Canada). The beef tallow was generously donated by Canada Packers Co. (Toronto, ONT, Canada). The dietary oils were all cold-pressed and were bought from several local outlets. All chemicals were purchased from Sigma Chemicals (St. Louis, MO), except fatty acid methyl ester standards (NuChek Prep Inc., Elysian, MN) and sucrose (Analar, BDH Chemicals Canada, Toronto, ONT, Canada). Chloroform, methanol, and hexane were double-distilled and obtained from the University of Toronto's Solvent Distillation Service. Iso-octane (HPLC grade) was purchased from Fisher Chemicals (Fair Lawn, NJ). Thin-layer chromatography plates and gas chromatography capillary column were obtained through Chromatographic Specialties Inc. (Brockville, ONT, Canda).

Experimental design

Two experiments were performed. The purpose of the first experiment was to resolve which of several dietary fat characteristics was responsible for altering the level of neural 18:2n6. Rats were divided into seven groups based on body weight (n = 6/group). Each group was fed a different semi-purified diet for three weeks. The diets contained wide ranges of SFA (2.2%–7.7% wt/wt of diet), MUFA (2.7%–10.4%), and EFA (4.2%–15%), but a constant ratio of 18:2n6/18:3n3 (approximately 30) (*Table 1*). Five of the diets were formulated with SFA to MUFA ratios of one, but amounts of combined SFA plus MUFA varied in a reciprocal manner to the amount of EFA. The purpose of this group of diets was to establish a dose-response curve between the dietary and mitochondrial fatty acids. However, from these diets alone it would be impossible to identify which dietary fat characteristic caused the membrane fatty acid changes. Therefore, two other diets were made, in which the SFA and MUFA varied independently of the EFA. That is, diet 6 has a comparable EFA content to diet 2 (7%), yet an SFA/MUFA ratio of 0.5, while diet 7 also has 7% EFA, yet an SFA/MUFA ratio of 0.2. Linear correlation using all seven diets was performed on each dietary fat characteristic to determine which was the best predictor of the levels of 18:2n6 in CL. It was expected that the points representing these last two diets would only fall on the regression line of the characteristic that was responsible for the effect on 18:2n6. The total EFA in the diets accounted for approximately 99% of the polyunsaturated fatty acids (PUFA) and the amount of each EFA was always a fixed proportion of the total EFA. Consequently, it was impossible to distinguish between the effects of the total EFA, PUFA, 18:2n6, and 18:3n3 in the diet.

Table 1 Dietary fatty acid composition

	Weight % of diet						
Diet	SFA	MUFA	EFA	P/S	18:2n6	18:3n3	EFA ratio
1	7.7	7.7	4.2	0.54	4.1	0.12	34.2
2	6.7	6.1	6.9	1.03	6.7	0.21	31.9
3	5.0	4.9	9.9	1.99	9.6	0.32	30.0
4	3.6	3.5	12.5	3.43	12.1	0.44	27.5
5	2.2	2.7	15.0	6.69	14.5	0.52	27.9
6	3.7	9.6	6.5	1.77	6.3	0.25	25.2
7	2.3	10.4	7.0	2.71	6.7	0.24	27.9

Note: Diets contained 20% (wt/wt) fat. 18:2n6 and 18:3n3 were the only n6 and n3 fatty acids present in significant amounts. Fatty acid composition was determined by gas chromatography. EFA ratio = 18:2n6/18:3n3.

The purpose of the second experiment was to assess the effect of changing the dietary EFA ratio, at a constant amount of EFA, on the level of 18:2n6 in CL from neural mitochondria. Rats were divided into four groups based on body weight (n = 9/group) and each group fed a different diet for eight weeks. The diets had EFA ratios (18:2n6/18:3n3) of 1.8, 9.4, 36, and 165 (*Table 2*), but a constant amount of EFA (15% of diet), SFA (2%), and MUFA (3%).

At the end of the experimental periods, animals were decapitated (900–1000 hr), their brains quickly removed, wrapped in foil, and covered with ice until the membrane fractionation was carried out. Membrane fractionation was conducted within 30 min of sacrifice of the first animal.

Diets

Semipurified diets were made, each containing (wt/wt): protein as casein (23%), cornstarch (40%), mineral mixture (5.1%, Bernhart-Tomarelli[16]), fiber (5%, alpha-cellulose), vitamin mixture (2.5%, TD67231[17]), and supplemental L-methionine (0.25%). All diets contained 20% (wt/wt) fat, but each diet contained different proportions of beef tallow, safflower oil, olive oil, soybean oil, and/or linseed oil. All diets were adequate in EFA. The fatty acid compositions of the fat mixtures were determined by gas chromatography as described below.

Table 2 The effects of EFA ratio and 18:2n6 level in the diet on the level of 18:2n6 in neural CL

Source	Fatty acid	Experimental diet 1	2	3	4
Diet	Total EFA[a]	14.5	14.7	15.0	15.0
	18:2n6	9.4	13.3	14.7	14.9
	18:3n3	5.2	1.4	0.41	0.09
	EFA ratio[b]	1.8	9.4	36	165
	Total SFA	2.2	2.3	1.9	2.0
	Total MUFA	3.2	2.9	2.9	2.8
CL	18:2n6[c]	18.4 ± 0.5	18.3 ± 0.3	17.7 ± 0.6	18.1 ± 0.5

[a] Dietary fatty acids as percent (wt/wt) of diet.
[b] EFA ratio = 18:2n/18:3n3.
[c] CL 18:2n6 expressed as percent (wt/wt) of total fatty acids; mean ± SEM (n = 9/diet). No significant effect of diet ($P > 0.02$ by ANOVA).

Animals

Male, weanling Wistar rats (Charles River Breeding Labs, St. Constant, QUE, Canada), initially weighing 50–70 g, were used. All animals were housed individually in a room lit daily for 12 hr (700–1900 hr). They were allowed free access to both water and food. Body weights and food intakes were monitored throughout the experimental period.

Membrane fractionation and lipid analysis

Membrane fractionation on sucrose density gradients was carried out immediately following the sacrifice using the method of Whittaker and Barker.[18] Mitochondria were collected and stored overnight at −70° C until lipids were extracted the following morning. Lipid was extracted from each membrane fraction by the method of Folch et al.[19] CL was separated by thin-layer chromatography using a modified version of the method of Kennerly et al.[20] Discrete bands corresponding to CL (Rf = 0.85), phosphatidylethanolamine (Rf = 0.70), phosphatidylcholine (Rf = 0.50), phosphatidylserine (Rf = 0.30), and phosphatidylinositol (Rf = 0.05) were reliably obtained. Fatty acid methyl esters were prepared from CL by the method of Morrison and Smith,[21], and analyzed by gas chromatography. 18:1n9 and 18:1n7 did not separate reliably and are reported as the total 18:1 isomers. Details of these methods have been given previously.[22]

Statistical analysis

Statistical analysis was done using SAS 6.03 (SAS Institute Inc., Cary, NC) for the microcomputer. The relationships between several dietary fat characteristics (amounts of SFA, MUFA, EFA, and 18:2n6, and the ratio of PUFA to SFA (P/S) and PUFA to MUFA (P/M)) in the first experiment and the levels of fatty acids in CL were analyzed using linear correlation. In the second experiment, the effect of diet on the level of 18:2n6 in neural CL was analyzed by ANOVA.

Results

The energy intake, body weight gain, and brain weight were similar for animals on each of the seven diets in experiment 1 (*Table 3*).

The fatty acid profile of CL is given in *Table 4*. The most prominent effect of dietary fatty acid composition was on 18:2n6, with a two-fold difference occurring between diets 1 and 5. Other fatty acids were affected not at all or to a much lesser extent and in the opposite direction by dietary fat. Since the large change in the level of 18:2n6 was compensated for

Table 3 Total intakes of energy, SFA, MUFA, EFA, and 18:2n6, body weight gain, and brain weight

Diet	Energy intake (kcal)	SFA intake	MUFA intake	EFA intake	18:2n6 intake	Body weight gain	Brain weight
1	1346 ± 49[1]	23.6	23.6	12.8	12.5	160 ± 5	1.81 ± 0.02
2	1315 ± 45	20.0	18.2	20.6	20.0	165 ± 6	1.74 ± 0.03
3	1319 ± 27	15.0	14.7	29.7	28.8	163 ± 6	1.80 ± 0.03
4	1311 ± 29	10.7	10.4	37.2	36.1	167 ± 7	1.83 ± 0.02
5	1244 ± 24	6.2	7.6	42.4	41.0	151 ± 3	1.78 ± 0.03
6	1283 ± 20	10.8	28.0	19.0	18.4	155 ± 4	1.75 ± 0.03
7	1353 ± 46	7.1	32.0	21.5	20.6	164 ± 8	1.80 ± 0.02

Note. Values are expressed in grams (unless otherwise indicated) as mean ± SEM. Mean SFA, MUFA, EFA, and 18:2n6 intakes were calculated by multiplying the mean total food intake (in grams) by the weight % of the fatty acid(s) in the diet.

Table 4 The effects of dietary fatty acid composition on the fatty acid profile of cardiolipin from neural mitochondria

Fatty acid	Experimental diet[a]						
	1	2	3	4	5	6	7
16:0	10.8 ± 0.5[b]	9.9 ± 0.5	9.5 ± 0.5	9.8 ± 0.6	9.5 ± 0.5	10.3 ± 0.6	9.4 ± 0.4
16:1n7	4.4 ± 0.2	4.4 ± 0.2	4.8 ± 0.3	4.5 ± 0.4	4.3 ± 0.3	4.6 ± 0.4	4.3 ± 0.2
18:0	5.7 ± 0.3	5.7 ± 0.6	5.2 ± 0.3	5.4 ± 0.3	5.3 ± 0.4	5.7 ± 0.5	4.6 ± 0.2
18:1[c]	34.8 ± 1.5	33.6 ± 1.1	33.3 ± 0.5	31.2 ± 0.5	30.6 ± 0.5	35.0 ± 1.1	35.3 ± 0.7
18:2n6	9.4 ± 0.5	12.3 ± 0.5	14.1 ± 0.5	16.4 ± 0.7	18.4 ± 0.5	11. ± 0.4	12.8 ± 0.3
20:4n6	14.3 ± 0.9	15.1 ± 0.7	15.3 ± 0.7	15.0 ± 0.6	15.1 ± 0.6	15. ± 0.5	15.5 ± 0.5
22:4n6	0.53 ± 0.04	0.50 ± 0.03	0.54 ± 0.01	0.53 ± 0.03	0.55 ± 0.04	0.5 ± 0.04	0.52 ± 0.03
22:5n6	0.80 ± 0.16	0.71 ± 0.18	0.64 ± 0.31	0.92 ± 0.25	0.82 ± 0.29	1.1 ± 0.42	0.78 ± 0.29
22:6n3	6.8 ± 0.7	6.9 ± 0.7	6.7 ± 0.5	6.4 ± 0.4	6.1 ± 0.4	7.2 ± 0.4	7.0 ± 0.4

[a] Dietary fatty acid compositions given in *Table 1*.
[b] Values represent the weight percent of total fatty acids: means ± SEM (n = 6/diet). When present, 20:1n9, 20:2n6, 20:3n6 were detected at levels of less than 2% each of total fatty acids. 22:5n3 was not detected. Unidentified peak areas accounted for approximately 9–13% of total fatty acids. Weight percent values in the table have not been corrected for unidentified peak areas.
[c] Sum of 18:1 isomers.

by smaller changes in the percentages of several fatty acids, it is likely that only 18:2n6 is being influenced directly by the dietary fatty acid composition, and that the other fatty acids in CL are simply being diluted by a large increase in the amount of 18:2n6 in the phospholipid.

The relationships between the percentage of 18:2n6 in CL and several dietary fat characteristics are displayed in *Figure 1*, and the results of their linear correlation analyses are given in *Table 5*. It is evident from inspection of the graphs and the table that the amount of dietary EFA and the level of 18:2n6 in CL have a very strong linear relationship (r^2 = 0.851). The same association exists between the amount of 18:2n6 in the diet and in CL, since 18:2n6 was a constant proportion of EFA in all of the diets. There are poorer correlations between dietary SFA (r^2 = 0.405) or

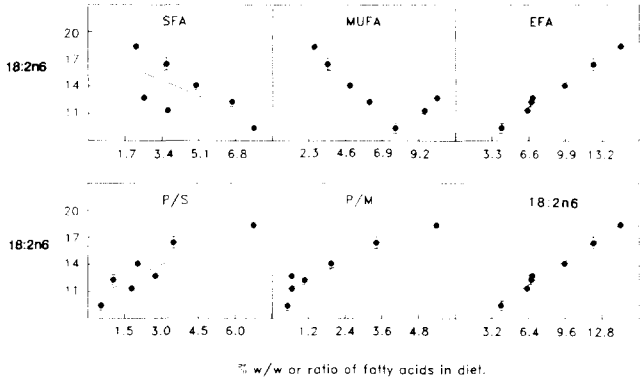

Figure 1 Several dietary fat characteristics were correlated with the linoleic acid (18:2n6) level in neural cardiolipin obtained from weanling rats fed one of seven diets for three weeks. Linoleic acid values are expressed as percent (wt/wt) of total fatty acids: mean ± SEM (n = 6/diet). The line in each graph was fitted using linear regression on individual animals (n = 42). The coefficients of determination (r^2) for linear correlation analysis are given in *Table 4*. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; EFA = essential fatty acids; P/S = polyunsaturated to saturated fatty acid ratio; P/M = polyunsaturated to monounsaturated fatty acid ratio.

MUFA (r^2 = 0.534) and the percentage of 18:2n6 in CL. Although these correlations are statistically significant, much of this is probably due to the covariation of both SFA and MUFA with EFA in five of the seven diets. In fact, it is the remaining two diets (diets 6 and 7), which were formulated to vary the SFA and MUFA content independently of the EFA, that are the farthest removed from the regression lines of 18:2n6 in CL against SFA or MUFA, but which fall on the dietary EFA line.

The regression analyses of 18:2n6 from neural CL against the dietary ratio of PUFA to SFA (P/S) or PUFA to MUFA (P/M) also yield fairly high coefficients of determination (r^2 = 0.710 and 0.770, respectively), but not as high as for dietary EFA. As well, since dietary PUFA is the numerator in both of these ratios, and is essentially equivalent to dietary EFA in this experiment, a large part of the dietary ratios' apparently good correlations with neural 18:2n6 is likely due to the tight association between dietary EFA and neural 18:2n6. Furthermore, dividing the amount of dietary PUFA by either the amount of SFA or MUFA decreases the coefficient of determination

Table 5 Linear correlation analyses of dietary fat characteristics with level of 18:2n6 in neural cardiolipin

Dietary fat characteristic	Slope	Y-intercept	r^2	P value
SFA	−1.01	18.1	0.405	0.0001
MUFA	−0.07	18.7	0.534	0.0001
EFA	0.793	6.53	0.851	0.0001
P/S	1.35	10.1	0.710	0.0001
P/M	1.49	10.5	0.770	0.0001
18:2n6	0.822	6.53	0.851	0.0001

Note: The dietary fat characteristics were the X-variable and the level of 18:2n6 in CL (% wt/wt of total fatty acids) was the Y variable. The data from individual animals was used in the analysis (n = 42). The strength of the correlation is expressed as the coefficient of determination (r^2).

compared to dietary EFA alone. Therefore, it is clear that these ratios are not the primary force behind the neural fatty acid effect.

The regression line of dietary EFA against 18:2n6 in CL is the dose-response curve for these two parameters. It has a slope of 0.793 and a y-intercept of 6.52% with no indication of any curvature. From the slope it is apparent that over the range of dietary EFA values tested, the level of 18:2n6 in CL changes by 0.8% of the total fatty acids for every 1% by weight that EFA represents in the diet.

The coefficients of determination and their associated P values for the relationships between other fatty acids in CL and dietary SFA, MUFA, EFA, and 18:2n6 were determined. The percentage of total 18:1 isomers in CL also significantly correlated ($P < 0.0001$) to the amount of dietary MUFA ($r^2 = 0.366$) and EFA ($r^2 = 0.347$), although not as strongly as 18:2n6 in CL. The relationships of 18:1 and 18:2n6 with dietary MUFA and EFA were probably the basis for the significant correlations that were found between several dietary fat characteristics and the total MUFA and PUFA in CL. No other individual fatty acid was significantly correlated to any dietary fat characteristic.

When four diets containing the same amount of EFA, but different amounts of 18:2n6 and different EFA ratios (18:2n6/18:3n3), were fed to weanling rats for 8 weeks, no significant differences were found in the level of 18:2n6 in CL from the brain (*Table 2*). It is noteworthy that the percentage of 18:2n6 in CL in these groups was similar to that present in the group fed diet 5 in the first experiment, which contained the same total amounts of EFA, SFA, and MUFA.

Discussion

The results show that the level of 18:2n6 in neural CL is better predicted by the amount of dietary EFA than dietary SFA, MUFA, P/S, or P/M. The relationship is very strong and is linear over the dietary range tested, which is well above the animals' requirement for the EFA.[23] No other fatty acids in CL were significantly correlated with any dietary fat characteristic, except the 18:1 isomers which had a weaker relationship than 18:2n6 with dietary MUFA and EFA. In addition, the increased incorporation of 18:2n6 into CL was not accompanied by increases in the levels of the elongated and desaturated n6 fatty acids. This suggests that 18:2n6 is distinguished from the other fatty acids in neural mitochondria by its sensitivity to dietary EFA. In contrast, the dietary EFA ratio did not affect 18:2n6 in CL when the amount of EFA in the diet was held constant. These results confirm our previous findings that dietary fat markedly affects neural 18:2n6 levels in CL (Dyer and Greenwood, submitted).

This work is unique among studies of dietary fat and the brain because of the analysis of neural CL and the design of its diets. In the brain, CL is the only phospholipid that contains substantial amounts of 18:2n6 and it occurs almost exclusively in the inner mitochondrial membrane.[5] Very little work has been done on CL in the brain, with only one study having investigated the effects of dietary fat on neural CL.[4] The results of this other study, which used corn oil and a fish oil, are consistent with those reported here, but the authors paid little attention to the fatty acid changes in the brain and instead focused on peripheral organs where they attributed the compositional changes to the high 20:5n3 and 22:6n3 content of fish oil. However, the use of only two diets, in which all fatty acids varied, did not allow for a definitive interpretation that the long chain polyenoic n3 fatty acids were indeed the mediator of the alterations in CL fatty acid profile observed. Dietary fat studies that have analyzed other phospholipids in the brain have yielded results which suggested that dietary fat does affect 18:2n6 levels,[24-26] but due to the low concentration of 18:2n6 in other neural phospholipids little significance was given to this observation. In addition, the 18:2n6 content of the heart and liver can be modified greatly by dietary fat, with high 18:2n6 levels being found in animals fed diets with high EFA contents.[4,27] In none of these studies, however, was the amount of 18:2n6 in the membrane associated specifically with the amount of EFA in the diet.

The amount of EFA in the diet was the best predictor of the level of 18:2n6 in CL from the brain. However, dietary EFA itself is not a single factor; rather it is the sum of 18:2n6 and 18:3n3. In this experiment, dietary 18:2n6, 18:3n3, and total EFA varied together in all of the diets. Therefore, variation in any of these three characteristics could have been the cause of the differences observed in the neural 18:2n6 levels. Dietary 18:2n6 is the most likely candidate, since this relationship could be explained simply on the basis of the diet largely determining the amount of 18:2n6 available for incorporation into neural CL.

On the other hand, such a simple explanation would not account for why membrane 18:2n6 did not vary with dietary 18:2n6 in the EFA ratio experiment (*Table 2*), in which the amount of dietary EFA was held constant, but there was a wide range of dietary 18:2n6 levels (9.4–14.9% wt/wt of diet). It is unlikely that the differences in results between these two experiments could be accounted for by the differences in the feeding period used (3 weeks for the first experiment and 8 weeks for the second), since our previous work suggests that alterations in CL 18:2n6 content in response to dietary fat composition are maximal within 3 weeks and remain constant for up to 12 weeks of feeding (Dyer and Greenwood, submitted). Thus results of the EFA ratio experiment suggest that if variation in dietary 18:2n6 is responsible for the neural 18:2n6 changes, then 18:2n6 in CL must also be affected by the amount of dietary 18:3n3 or the dietary EFA ratio.

The possibility that both of the EFA in the diet affect neural 18:2n6 is consistent with our understanding of the substrate competition between the two EFA for the same elongation/desaturation enzymes.[28] For example, if the total EFA content is fixed, the amount

of 18:3n3 must increase as the amount of 18:2n6 decreases, which will suppress the elongation and desaturation of 18:2n6, thereby making more 18:2n6 available for incorporation into CL and compensating for the lower dietary 18:2n6 intakes. This suggests that the levels of both EFA in the diet contribute to determining the level of 18:2n6 in the brain, which nicely reconciles the observation that the total amount of dietary EFA is the most consistent predictor of neural 18:2n6 levels, with the more intuitive explanation that neural 18:2n6 is responding to the level of intraneuronal 18:2n6 available for incorporation into phospholipids.

It must be borne in mind that the experimental design used in these studies does not allow us to distinguish between the effect of the absolute amount of EFA consumed (i.e., total EFA intake) versus the ratio of EFA to the sum of all other fatty acids in the diet (i.e., EFA/(MUFA + SFA)). That is, since the total amount of fat in the diet was held constant at 20% wt/wt fat in all experiments, the EFA/(MUFA + SFA) ratio and EFA content are highly correlated and hence the same coefficients of determination will be obtained when regressing these two values against CL 18:2n6 levels. It is impossible to separate these two diet fat characteristics without changing the overall fat content in the diet. For example, if fat blends comparable to those used in the first experiment were fed to rats, but at the level of 10% rather than 20% wt/wt, this would lower the absolute amount of EFA consumed but would have no impact on the EFA/(SFA + MUFA) ratio. However, this approach would add the complication that the proportion of endogenous to exogenous fatty acids available for incorporation into membranes would also vary between the 10% and 20% wt/wt fat diets.

The almost exclusive presence of CL in the inner mitochondrial membrane suggests that any functional consequences of altering neural 18:2n6 levels will occur in the mitochondria. This type of work has not been done on the brain. However, in peripheral tissues, several enzymes involved in oxidative respiration, cytochrome C oxidase,[6-9] F_1-F_0 (oligomycin-sensitive) ATPase,[10] and the ADP/ATP carrier protein,[11] associate specifically with CL. Cytochrome C oxidase is the most extensively studied of these enzymes and has an absolute requirement for two or three molecules of CL.[7] It also requires the presence of long-chain, unsaturated fatty acids in its membrane environment for optimal activity.[29]

There is also evidence in peripheral tissues to support a link between dietary fat and mitochondrial oxidative respiration. In the heart, the activities of cytochrome C oxidase and F_1-F_0 ATPase respond to the degree of saturation of dietary fat.[4,30-32] The level of 18:2n6 in mitochondrial lipids varied concurrently with these enzymatic changes, and in CL the difference in the percentage of 18:2n6 was of a magnitude comparable to that reported here.[4]

In addition to respiratory enzymes, CL has been reported to be involved in the function of carnitine palmitoyltransferase and carnitine acylcarnitine translocase which control the transport of fatty acids across the inner mitochondrial membrane for β-oxidation.[33] Carnitine palmitoyltransferase, which utilizes acyl-CoA with chain lengths of up to 20 carbons,[12] binds approximately 20 molecules of phospholipid, of which CL is the major type.[13] As well, the presence of CL has been shown to affect the activity of this enzyme in vitro, possibly by binding the products and preventing them from inhibiting the reaction.[14] Purified carnitine acylcarnitine translocase must be reconstituted into liposomes containing CL for its activity to be maximal, and in intact mitochondria from rat liver and heart, the activity of the translocase is decreased by the specific CL-binding agent, doxorubicin.[15]

These findings show that CL is involved in several mitochondrial processes which, combined with the fact that CL contains the highest 18:2n6 concentrations of any phospholipid, leads to the hypothesis that the 18:2n6 content of CL influences the interaction between CL and the enzymes that it is associated with. Furthermore, the very high degree of linearity over a wide range of dietary EFA levels makes the 18:2n6 content of CL a very precise indicator of the amount of EFA in the diet. It follows then, that the CL 18:2n6 level could be acting as the "sensor" for a mitochondrial process that must respond to the level of dietary EFA rather than just be influenced passively by the diet.

Finally, while all of the work supporting a functional role for CL in the mitochondria has been done using peripheral tissue, it has been demonstrated that neural monoamine oxidase, an outer mitochondrial membrane-bound enzyme, is influenced by feeding lard versus soybean oil diets.[34] This same dietary paradigm causes alterations in the levels of 18:2n6 from neural CL that are of the same magnitude as reported here (Dyer and Greenwood, submitted). This shows that at least one mitochondrial protein in the brain is susceptible to a modification in dietary fat that is capable of influencing neural 18:2n6.

Investigation of the functional consequences of neural 18:2n6 changes would be worthwhile because this work has implications for humans. These diets were all adequate in the EFA, which is more relevant to humans than diets deficient in EFA because EFA deficiency is rare in people, unless they have a defect in lipid absorption or are receiving one of the older parenteral formulations.[35] Like the diets used in this work, the amount of EFA consumed by the populations of different countries varies tremendously.[36] Similar variations between individuals within countries would be expected as the result of differences in wealth, customs, religious beliefs, the availability of food, and personal preferences. In Western nations, decreasing fat consumption and increasing the P/S have been recommended to lower the risk of heart disease, diabetes, and cancer, which is leading to an increase in the proportion of EFA in dietary fat. Based on the results reported here, it is reasonable to suggest that a change in dietary fat such as this will alter the

neural fatty acid profile. Unfortunately, the potential functional consequences for the brain—either good or bad—are predicted less easily.

Abbreviations used

CL cardiolipin
EFA essential fatty acids
MI mitochondria
MUFA monounsaturated fatty acids
PUFA polyunsaturated fatty acids
P/M polyunsaturated to monounsaturated fatty acid ratio
P/S polyunsaturated to saturated fatty acid ratio
SFA saturated fatty acids

References

1 Johnston, P.V. (1979). Nutrition and neural lipids. In *Advances in Nutritional Research, Vol. 2* (H.H. Draper, ed.), pp. 149–180, Plenum Press, New York
2 Menon, N.K. and Dhopeshwarkar, G.A. (1982). Essential fatty acid deficiency and brain development. *Prog. Lipid Res.* **21,** 309–326
3 Neuringer, M., Anderson, G.J., and Connor, W.E. (1988). The essentiality of n-3 fatty acids for the development and function of the retina and brain. *Ann. Rev. Nutr.* **8,** 517–541
4 Yamaoka, S., Urade, R., and Kito, M. (1988). Mitochondrial function in rats is affected by modification of membrane phospholipids with dietary sardine oil. *J. Nutr.* **118,** 290–296
5 Sastry, P.S. (1985). Lipids of nervous tissue: composition and metabolism. *Prog. Lipid Res.* **24,** 69–176
6 Aswathi, Y.C., Chuang, T.F., Keenan, T.W., and Crane, F.L. (1971). Tightly bound cardiolipin in cytochrome oxidase. *Biochim. Biophys. Acta* **226,** 42–52
7 Vik, S.B., Georgevich, G., and Capaldi, R.A. (1981). Diphosphatidylglycerol is required for optimal activity of beef heart cytochrome c oxidase. *Proc. Natl. Acad. Sci.* **78,** 1456–1460
8 Robinson, N.C., Strey, F., and Talbert, L. (1980). Investigation of the essential boundary layer phospholipids of cytochrome c oxidase using Triton X-100 delipidation. *Biochem.* **19,** 3656–3661
9 Fry, M. and Green, D.E. (1980). Cardiolipin requirement by cytochrome oxidase and the catalytic role of phospholipid. *Biochem. Biophys. Res. Comm.* **93,** 1238–1246
10 Santiago, E., Lopez-Moratalla, N., and Segovia, J.L. (1973). Correlation between losses of mitochondrial ATPase activity and cardiolipin degradation. *Biochem. Biophys. Res. Comm.* **53,** 439–445
11 Beyer, K. and Klingenberg, M. (1985). ADP/ATP carrier protein from beef heart mitochondria has high amounts of tightly bound cardiolipin, as revealed by ^{31}P nuclear magnetic resonance. *Biochem.* **24,** 3821–3826
12 Bieber, L.L. and Farrell, S. (1983). Carnitine acyltransferases. In *The Enzymes, Vol. XVI* (P.D. Boyer, ed.), pp. 627–644, Academic Press, Ltd., New York
13 Fiol, C.J. and Bieber, L.L. (1984). Sigmoid kinetics of purified beef heart mitochondrial carnitine palmitoyltransferase. *J. Biol. Chem.* **259,** 13084–13088
14 Pande, S.V., Murthy, M.S.R. and Noel, H. (1986). Differential effects of phosphatidylcholine and cardiolipin on carnitine palmitoyltransferase activity. *Biochim. Biophys. Acta* **877,** 223–230
15 Noel, H. and Pande, S.V. (1986). An essential requirement of cardiolipin for mitochondrial carnitine acylcarnitine translocase activity. *Eur. J. Biochem.* **155,** 99–102
16 Bernhart, F.W. and Tomarelli, R.M. (1969). A salt mixture supplying the National Research Council estimates of the mineral requirement of the rat. *J. Nutr.* **89,** 495–500
17 Musten, B., Peace, D., and Anderson, G.H. (1974). Food intake regulation in the weanling rat. *J. Nutr.* **104,** 563–572
18 Whittaker, V.P. and Barker, L.A. (1972). The subcellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. In *Methods of Neurochemistry, Vol. 2* (R. Fried, ed.), pp. 1–52, Marcel Dekker, NY
19 Folch, J., Lees, M., and Stanley, G.H.S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226,** 497–509
20 Kennerly, D.A., Sullivan, T.J., and Parker, C.W. (1979). Activation of phospholipid metabolism during mediator release from stimulated rat mast cells. *J. Immunol.* **122,** 152–159
21 Morrison, W.R. and Smith, L.M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron trifluoride-methanol. *J. Lipid Res.* **5,** 600–608
22 Dyer, J.R. and Greenwood, C.E. (1991). Neural 22-carbon fatty acids in the weanling rat respond rapidly and specifically to a range of dietary linoleic to α-linolenic fatty acid ratios. *J. Neurochem.* **56,** 1921–1931
23 Rivers, J.P.W. and Frankel, T.L. (1981). Essential fatty acid deficiency. *Br. Med. Bull.* **37,** 59–64
24 Lamptey, M.S. and Walker, B.L. (1978). Learning behavior and brain lipid composition in rats subjected to essential fatty acid deficiency during gestation, lactation and growth. *J. Nutr.* **108,** 358–367
25 Brenneman, D.E. and Rutledge, C.O. (1979). Alteration of catecholamine uptake in cerebral cortex from rats fed a saturated fat diet. *Brain Res.* **179,** 205–304
26 Tahin, Q.S., Blum, M., and Carafoli, E. (1981). The fatty acid composition of subcellular membranes of rat liver, heart, and brain: diet-induced modifications. *Eur. J. Biochem* **121,** 5–13
27 Innis, S.M. and Clandinin, M.T. (1981). Dynamic modulation of mitochondrial inner-membrane lipids in rat heart by dietary fat. *Biochem. J.* **193,** 155–167
28 Brenner, R.R. (1981). Nutritional and hormonal factors influencing desaturation of essential fatty acids. *Prog. Lipid Res.* **20,** 41–47
29 Vik, S.B. and Capaldi, R.A. (1977). Lipid requirements for cytochrome c oxidase activity. *Biochem.* **16,** 5755–5759
30 Abuirmeileh, N.M. and Elson, C.E. (1980). The influence of linoleic acid intake on membrane-bound respiratory activities. *Lipids* **15,** 918–924
31 Innis, S.M. and Clandinin, M.T. (1981). Dynamic modulation of mitochondrial membrane physical properties and ATPase activity by diet lipid. *Biochem. J.* **198,** 167–175
32 McMurchie, E.J., Abeywardena, M.Y., Charnock, J.S., and Gibson, R.A. (1983). Differential modulation of rat heart mitochondrial membrane-associated enzymes by dietary lipid. *Biochim. Biophys. Acta* **760,** 13–24
33 Mayes, P.A. (1983). Lipids. In *Harper's Review of Biochemistry. 19th ed.* (D.W. Martin, Jr., P.A. Mayes, V.W. Rodwell, eds., pp. 188–201, Lange Medical Publications, Los Altos, CA
34 Crane, S.B. and Greenwood, C.E. (1987). Dietary fat source influences neuronal mitochondrial monoamine oxidase activity and macronutrient selection in rats. *Pharmacol. Biochem. Behav.* **27,** 1–6
35 Yamanaka, W.K., Clemens, G.W., and Hutchinson, M.L. (1981). Essential fatty acid deficiency in humans. *Prog. Lipid Res.* **19,** 187–215
36 *FAO Monthly Bull. Stat.* (1982). **5,** 11–20

Update: At Proof

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