Brain astrocyte synthesis of docosahexaenoic acid from n-3 fatty acids is limited at the elongation of docosapentaenoic acid

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Abstract The phospholipids, particularly phosphatidylethanolamine, of brain gray matter are enriched with docosahexaenoic acid (22:6n-3). The importance of uptake of preformed 22:6n-3 from plasma compared with synthesis from the α-linolenic acid (18:3n-3) precursor in brain is not known. Deficiency of 18:3n-3 results in a compensatory increase in the n-6 docosapentaenoic acid (22:5n-6) in brain, which could be formed from the precursor linoleic acid (18:2n-6) in liver or brain. We studied n-3 and n-6 fatty acid incorporation in brain astrocytes cultured in chemically defined medium using delipidated serum supplemented with specific fatty acids. High performance liquid chromatography with evaporative light scattering detection and gas liquid chromatography were used to separate and quantify cell and media lipids and fatty acids. Although astrocytes are able to form 22:6n-3, incubation with 18:3n-3 or eicosapentaenoic acid (20:5n-3) resulted in a time and concentration dependent accumulation of 22:5n-3 and decrease in 22:6n-3 g/g cell fatty acids. Astrocytes cultured with 18:2n-6 failed to accumulate 22:5n-6. Astrocytes secreted cholesterol esters (CE) and phosphatidylethanolamine containing saturated and monounsaturated fatty acids, and arachidonic acid (20:4n-6) and 22:6n-3. These studies suggest conversion of 22:5n-3 limits 22:6n-3 synthesis, and show astrocytes release fatty acids in CE.—Innis, S. M., and R. A. Dyer. **Brain astrocyte synthesis of docosahexaenoic acid from n-3 fatty acids is limited at the elongation of docosapentaenoic acid. *J. Lipid Res.* 2002.** 43: **1529–1536.**

Supplementary key words astrocyte • docosahexaenoic acid • docosapentaenoic acid • alpha linolenic acid • desaturation • elongation • cholesterol ester • phosphatidylethanolamine

Brain gray matter is highly enriched in the long chain polyunsaturated fatty acids docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6) (1). Docosahexaenoic acid is formed from 18:3n-3 by Δ6 and Δ5 desaturation and elongation which gives rise to 20:5n-3 and 22:5n-3 in endoplasmic reticulum; 22:5n-3 is then elongated to 24:5n-3 and undergoes a second Δ6 desaturation to 24:6n-3 and is translocated to the peroxisomes where it undergoes one cycle of β-oxidation to form 22:6n-3 (**Fig. 1**) (2–4). Whether the Δ6 desaturase responsible for the desaturation of 18:3n-3 and 24:5n-3 are the same or different enzymes, and the steps involved in the intracellular movement of 24:6n-3 are not completely understood (5, 6). Synthesis of 20:4n-6 from 18:2n-6 is believed to involve the same Δ6 and Δ5 desaturases involved in the metabolism of 18:3n-3. Further metabolism of 20:4n-6 leads to formation of 22:5n-6 in an analogous pathway to that used for formation of 22:6n-3 (6). Dietary deficiency of 18:3n-3 results in a characteristic increase in 22:5n-6 in brain phospholipids, such that the total amount of carbon chain n-6 plus n-3 fatty acids is maintained (7–10). Despite this, decreased 22:6n-3 in the developing brain and retina results in decreased visual and neural function, and altered monoaminergic neurotransmitter metabolism (9–13). Uptake and conversion of ^{14}C-labeled 18:3n-3 to n-3 products by brain and isolated brain cells has been shown (14–16). However, developing brain is also able to take up 22:6n-3 and 20:4n-6 from plasma (12, 17, 18). The importance of uptake of 22:6n-3 from plasma compared with synthesis of 22:6n-3 in the brain following uptake of n-3 fatty acid precursors is not known (5). However, studies with preterm infants have shown that the dietary intake and blood lipid level of 22:6n-3 is positively related to visual and neural development (19–22).

Previous in vitro studies using ^{14}C precursors have shown brain astrocytes, but not neurons, are capable of forming 22:6n-3 and have suggested that astrocytes may be important in supplying 22:6n-3 to other brain cells (15,

Abbreviations: CE, cholesterol ester; dbc-AMP, dibutyryl cyclic AMP; ELSD, evaporative light scattering detection; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triglyceride.

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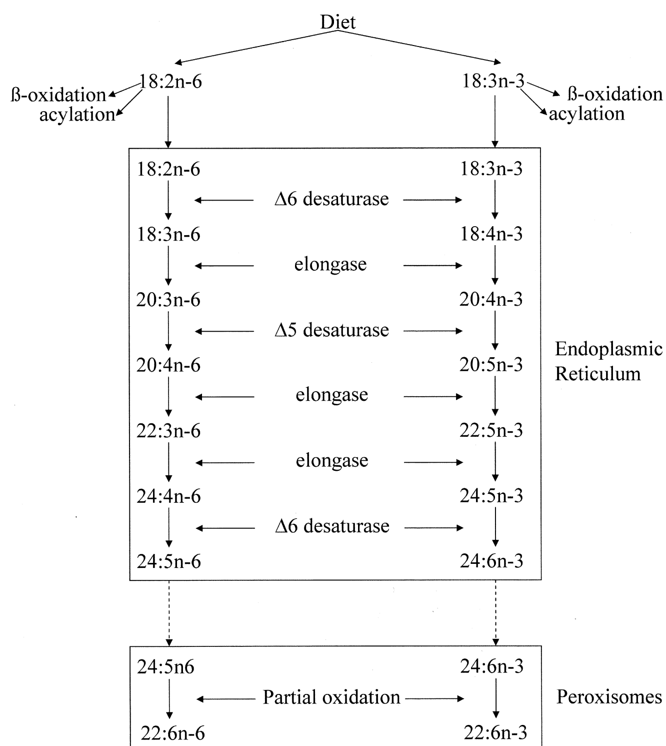

Fig. 1. Schematic of the desaturation and elongation of 18:2n-6 and 18:3n-3.

16). In this study, we extend the understanding of n-3 fatty acid metabolism in the brain by demonstrating that the elongation of 22:5n-3 limits the formation of 22:6n-3 from n-3 fatty acid precursors in neonatal brain astrocytes. We also show that astrocytes fail to accumulate 22:5n-6 when cultured in the presence of 18:2n-6 without 18:3n-3. Because serum contains both phospholipids and n-6 and n-3 fatty acids, we used chemically defined media with delipidated serum either unsupplemented or supplemented with specific fatty acids. By use of high performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) we also show that brain astrocytes secrete cholesterol esters (CE) and phosphatidylethanolamine (PE). Our findings suggest that elongation, and subsequent Δ6 desaturation of 24:6n-3 and 24:5n-6, limits the formation of 22:6n-3 and 22:5n-6 in brain astrocytes, and suggest astrocytes may play an important role in providing fatty acids to other cells through secretion in CE.

MATERIALS AND METHODS

Materials

Fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Nuchek Prep (Elysion, MN). Rat glial fibrillary acidic protein (GFAP) and its conjugate, rabbit anti-rat IgG, was from DAKO (Santa Barbara, CA). Dibutyryl cAMP and fatty acid free BSA were from Sigma (St Louis, MO). Fetal calf serum (FCS), DMEM/F12, trypsin, streptomycin, penicillin, and Versene were from Canadian Life Technologies (Burlington, Canada).

Cell culture

Primary cultures were prepared from forebrain gray matter of 1-day-old rat pups after dissecting away meningeal tissues using the procedures of McCarthy and de Vellis (23). The resulting mixed glial culture enriched in astrocytes, but containing oligodendrocytes and neurons was seeded at a density of 1×10^6 in 75 cm^2 tissue culture flasks and maintained in DMEM/F12 supplemented with 10% FCS, 50 μg/ml streptomycin, and 50 U/ml penicillin. The media was changed 24 h after seeding, and thereafter every 48 h. Neurons fail to survive the initial phase of these culture conditions and were eliminated by the third day of culture. Contaminating oligodendrocytes, which form a top layer of process bearing cells, were removed between days 8 and 10 by dissociation (23). The astrocytes formed a confluent monolayer within about 4 days and were ≥95% astrocytes when characterized by GFAP staining and light microscopy as described by Bock et al. (24). Astrocytes from neonatal rat brain, termed type-A astrocytes, undergo morphological and histochemical changes similar to in vivo differentiation when treated with dibutyryl cAMP (25). Within 3 to 4 h of addition of dibutyryl cAMP to the culture, astrocytes transform from a polygonal morphology to a process bearing morphology similar to that found in 10-day-old rat pup astrocytes (25). Whether these morphological and histochemical changes include changes in n-6 and n-3 fatty acid uptake is not known. In initial studies, we therefore compared n-3 and n-6 fatty acid uptake and incorporation in neonatal type A astrocytes and in astrocytes transformed with dibutyryl cAMP. Time and substrate concentration studies with 18:3n-3 supplemented media found no differences in the accumulation of n-3 fatty acids between neonatal astrocytes and astrocytes transformed with dibutyryl cAMP. We note, however, that transformed cell lines of neural origin are known to lose their ability to desaturate and elongate fatty acids (26, 27), and dibutyryl cAMP may not induce biochemical development analogous to that in vivo. Only results for neonatal astrocytes without treatment with dibutyryl cAMP are presented.

Experimental procedures

The uptake and incorporation of n-3 and n-6 fatty acids into astrocyte lipids was studied in 6-well tissue culture plates. The cells were maintained at 37°C in a 5% CO_2 incubator during all experiments, in 2.5 ml DMEM/F12 with 5% delipidated FCS, with media changed every 24 h. Delipidated FCS was prepared according to the method of Rothblat et al. (28), and the absence of n-6 and n-3 fatty acid checked by gas liquid chromatography (GLC) (21, 29). Fatty acids, 0–50 μm, as their sodium salts, were complexed with fatty acid free bovine serum albumen at a molar ratio of 2:1. For each experiment, a minimum of three parallel cell cultures were established.

Lipid analysis

Total lipids were extracted from the cells and media (30), the organic phase evaporated under nitrogen, and the lipids solubilized in chloroform-methanol-acetone-hexane (2.0:3.0:0.5:0.5, v/v/v/v). Separation of polar and non-polar lipids, including individual classes, was achieved using a HPLC (Waters 2690 Alliance HPLC, (Milford MA), equipped with an auto-sampler and column heater. The sample chamber was kept at 18°C and the column heater at 35°C. The column was a Waters YMC-Pack Diol 120NP, 25 cm × 4.6 mm id, 5 μm particle size and 12 nm pore size. We used a quaternary solvent system of hexane-petroleum ether, 97:3 (v,v); methanol-triethylamine-acetic acid, 765:15:13 (v/v/v); acetone-triethylamine-acetic acid, 765:15:13 (v/v/v); isopropanol-acetic acid, 800:40 (v/v) in a linear gradient with a flow rate of 2 ml/min. The column eluant was split 10:90 to an ELSD (Alltech, model 2000; Mandel Scientific, Guelph, Canada)

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and a fraction collector (Gilson FC204, Mandel Scientific). ELSD detection and quantitation of the separated lipid classes was performed with a nitrogen flow rate of 1.8 ml/min, a drift tube temperature of 60°C, and the impactor OFF. Calibration curves to determine the linear range of the analysis were established using authentic standards for each lipid class, and samples were quantified using the external standard method. The identification of cholesterol ester recovered in the media was confirmed by GCL-mass spectrometry (Varian Saturn II; Varian Canada, Mississagua, Canada). Fatty acids were quantified as their respective methyl esters using heptadecanoic acid (17:0) as the internal standard, with a Varian 3400 GLC equipped with a flame ionization detector, Varian Star data system and a 30 m × 0.25 mm id glass capillary SP 2330 columns (21, 29).

RESULTS

Although brain astrocytes as well as neurons are enriched in 22:6n-3 (31), this fatty acid must be obtained either by uptake and further desaturation and elongation of 18:3n-3 or other intermediary metabolites or by uptake of 22:6n-3 itself from plasma. Astrocytes cultured in defined media with delipidated serum with and without 18:3n-3 had similar concentrations of 22:6n-3 in individual phospholipids and triglycerides (**Fig. 2**). As is characteristic of brain gray matter (1), 22:6n-3 was enriched in cultured neonatal astrocyte phosphatidylethanolamine. Neural cells typically contain very low 22:5n-3. Supplementation with 18:3n-3, however, resulted in accumulation of 22:5n-3 in all lipid classes, such that 22:5n-3 became the major n-3 fatty acid in triglycerides, phosphatidylserine, and phosphatidylcholine. The major product of 18:3n-3 incorporated into the cell lipids of astrocytes cultured with 18:3n-3 was 22:5n-3 (**Fig. 3**). Increasing concentrations of the 18:3n-3 substrate resulted in increased incorporation of 22:5n-3. No accumulation of 24 carbon chain n-3 fatty acids was found (<0.01% total cell fatty acids).

Consistent with the limiting step in 22:6n-3 formation occurring at the level of elongation of 22:5n-3 to 24:5n-3 followed by a second Δ6 desaturation, the major n-3 fatty acid in astrocytes cultured with 20:5n-3 was 22:5n-3 (**Fig. 4**). Astrocytes readily took up and esterified 22:6n-3 from the media into cellular lipids. This was accompanied by a significant ($P < 0.05$) increase in the astrocyte 20:5n-3 and 22:5n-3 compared with cells cultured without 22:6n-3, indicating active chain shortening (partial oxidation) and retro-conversion of 22:6n-3. Astrocytes cultured in n-3 fatty acid deficient media with 50 μM 18:2n-6 did not accumulate 22:5n-6 for reciprocal replacement of 22:6n-3 (**Fig. 5**). Rather, astrocytes cultured with 18:2n-6 accumulated 20:4n-6 and 22:4n-6, but not 22:5n-6.

Culture in the presence of lipid-free serum allowed investigation of the lipids secreted by the astrocytes. This was facilitated by our development of sensitive HPLC lipid separation with quantification with ELSD which avoids losses, and allows analysis of much lower lipid concentrations than methods based on thin layer chromatography and inorganic phosphorous or enzymatic assays. Specific secretion of CE into the media is evident from the enrichment of CE in the media when compared with the astro-

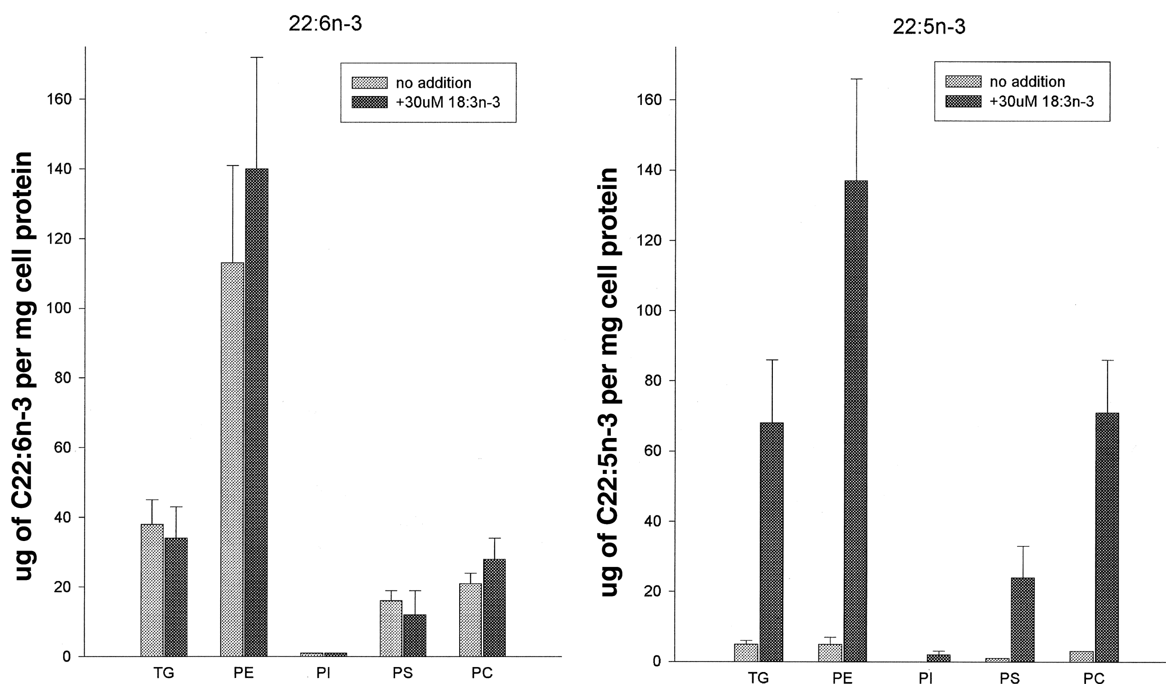

Fig. 2. Concentrations of 22:6n-3 and 22:5n-3 in neonatal astrocytes cultured with delipidated serum with 30 μM 18:3n-3 for 72 h. The cell lipids, triglycerides (TG), phophatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC) were separated by HPLC and detected and quantified by ELSD, fractionated, and the fatty acids determined as their methyl esters using GLC as described in Materials and Methods. The data are means and standard error from a minimum of three separate cultures. Small standard errors do not signify in the plot.

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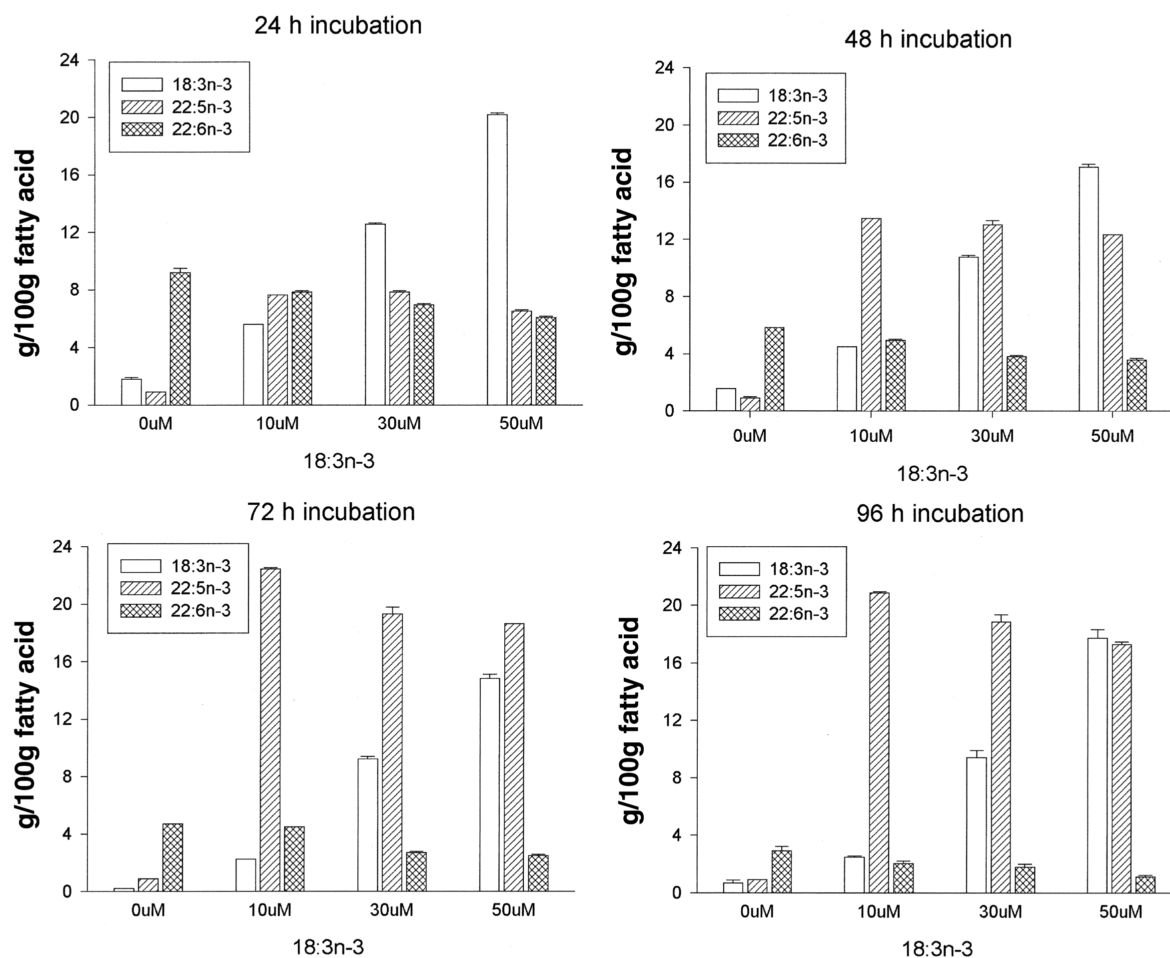

Fig. 3. Concentrations of n-3 fatty acids in cell lipids of neonatal astrocytes cultured with delipidated serum with 0, 10, 30 or 50 μM 18:3n-3 for 24, 48, 72 or 96 h. The cell lipids were extracted and analyzed by capillary GLC as described in Materials and Methods. The data are means and standard error from a minimum of three separate cultures. Small standard errors do not signify in the plot.

cytes in which phosphatidylcholine, phosphatidylethanolamine, and unesterfied cholesterol were the major lipids (Fig. 6). CE secreted into the media was high in 18:0 (60.5 ± 0.4% fatty acids) with smaller amounts of 16:0 (8.1 ± 3.0%), 18:1 (8.8 ± 1.0%), 20:4n-6 (0.4 ± 0.1%), and 22:6n-3 (0.7 ± 0.2%); 18:3n-3 was not secreted in cholesterol esters even in astrocytes cultured with this fatty acid. However, astrocytes cultured with 18:3n-3 did secret 22:6n-3 in CE.

DISCUSSION

The neonatal brain accumulates large amounts of 22:6n-3 in the period before the onset of myelination, which in the rat occurs 7–14 d after birth (32). Decreased 22:6n-3 in brain membrane phospholipids, secondary to inadequate dietary 18:3n-3, results in a reciprocal increase in 22:5n-6 in brain (7–10). The results of these studies show that the major product of 18:3n-3 and 20:5n-3 incorporated into astrocytes prepared from 1-day-old rat brain is 22:5n-3. Culture in n-3 fatty acid deficient media with 18:2n-6 similarly resulted in accumulation of 20:4n-6 and 22:4n-6, but not 22:5n-6. The pathway for synthesis of 22:6n-3 from 18:3n-3 involves Δ6 desaturation and elongation and Δ5 desaturation to 20:5n-3, followed by elongation to 22:5n-3 then to 24:5n-6, and a second Δ6 desaturation to 24:6n-3 (Fig. 1). The 24:6n-3 is then shuttled to the peroxisomes and converted to 22:6n-3 through one cycle of β-oxidation (2–4). Synthesis of 22:5n-6 from 18:2n-6 occurs through an analogous pathway (6). Analysis of lipids from cells cultured with 18:3n-3 or 18:2n-6 found no evidence of accumulation of carbon-chain-24 n-3 or n-6 fatty acids, despite high concentrations of 22:5n-3 or 22:4n-6 in the cells. Concentrations of 18:3n-3, 20:5n-3, and 22:5n-3 are usually exceedingly low in neural cells in vivo (1). Our results suggest that the limiting step in the metabolism of n-3 fatty acid precursors to 22:6n-3 is at the level of elongation to the 24-carbon-chain metabolites and the subsequent Δ6 desaturation. Elongation does not limit the initial steps of 18:3n-3 metabolism, thus it is possible that the elongation of 22-carbon-chain fatty acids is coordinately regulated with the capacity for further Δ6 desaturation and peroxisomal chain shortening. Should the desaturation of 18:3n-3 and 24:5n-3 involve the same Δ6 desaturase enzyme, 18:3n-3 could competitively inhibit further metabolism of 22:5n-3.

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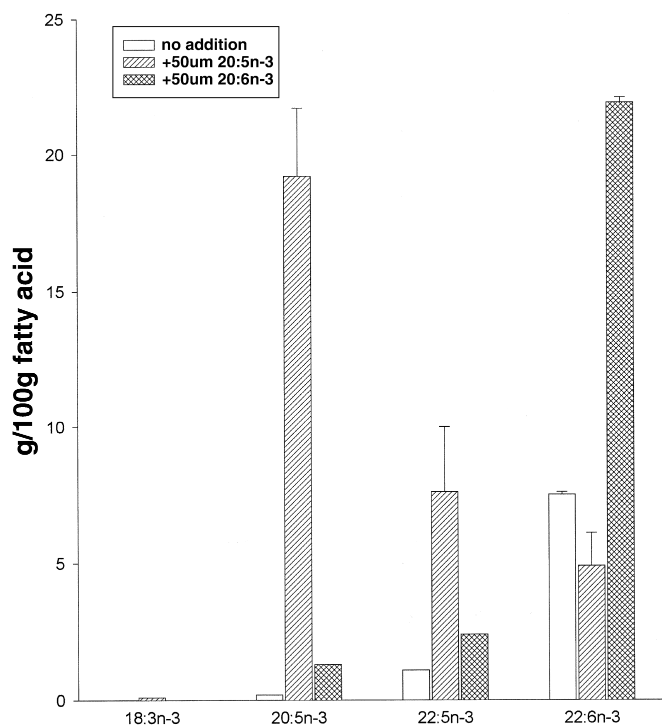

Fig. 4. Concentrations of n-3 fatty acids in cell lipids of neonatal astrocytes cultured in delipidated serum supplemented with 50 μM 20:5n-3 or 22:6n-3 for 72 h. The cell lipids were extracted and analyzed by capillary GLC as described in Materials and Methods. The data are means and standard error from a minimum of three separate cultures. Small standard errors do not signify in the plot.

The accumulation of 22:5n-3 in astrocytes cultured with 20:5n-3, and no 18:3n-3, however, further suggests that astrocyte metabolism of n-3 fatty acids is limited by the further metabolism of 22:5n-3.

Previous studies have provided evidence of 22:6n-3 synthesis in brain and brain astrocytes based on the recovery of ^{14}C from [1-^{14}C]18:3n-3 in 22:6n-3 (14–16, 33). Consistent with our results, others have shown the most abundant radio-labeled product in astrocytes cultured with [1-^{14}C]18:3n-3 is 22:5n-3, not 22:6n-3 (33), although this does not exclude the capacity for 22:6n-3 synthesis (15, 16). We suggest the current evidence indicates astrocyte synthesis of 22:6n-3 from n-3 precursors, or of 22:5n-6 from 18:2n-6 under conditions of n-3 fatty acid deficiency, is not the major route through which developing brain accumulates high concentrations of 22:6n-3 or 22:5n-6. However, it is possible that primary cultures of neonatal brain astrocytes loose a capacity for further metabolism of 22:4n-6 and 22:5n-3, which is expressed in vivo.

Rapoport et al. (17) have estimated that 2–8% of rat brain phospholipid 22:6n-3 is replaced daily with 22:6n-3 from the plasma unesterified fatty acid pool. Turnover of 22:6n-3 involves de-esterification and re-esterification, and appears to be related to phospholipase-A_2 activity and receptor-dependent signal transduction involving GTP proteins (34–37). These pathways are also critical to the functional roles of 22:6n-3 in neural tissues. Efficient recycling and reacylation is likely to conserve a large proportion of 22:6n-3 released during phospholipid deacyla-

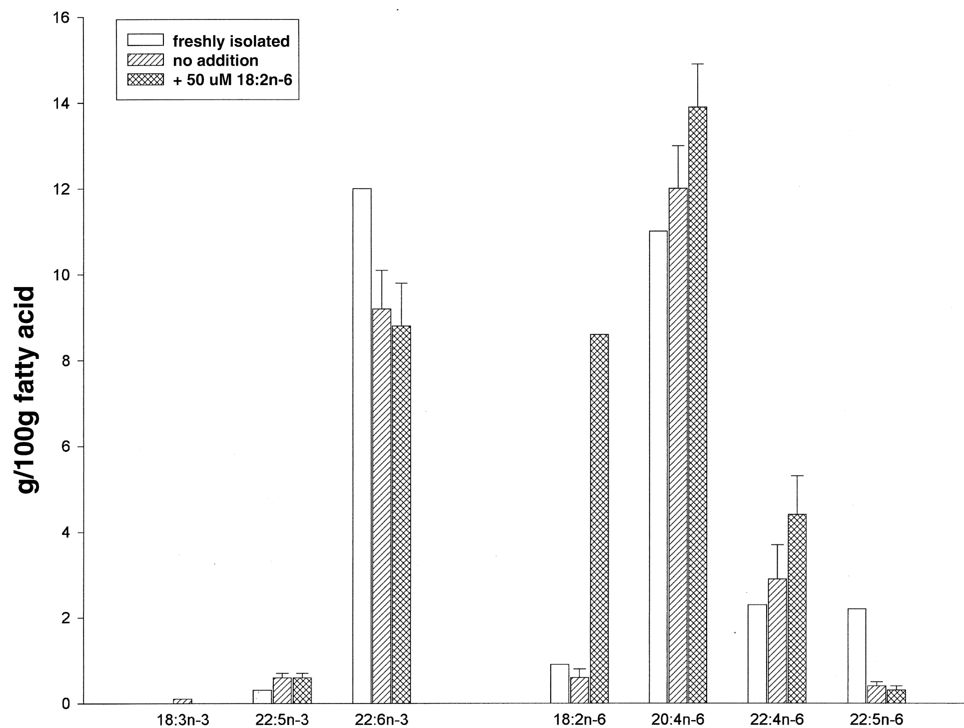

Fig. 5. Concentrations of n-3 fatty acids in cell lipids of freshly isolated neonatal astrocytes and astrocytes cultured with delipidated serum without or with 50 μM 18:2n-6 and no n-3 fatty acids. The cell lipids were extracted and analyzed by capillary GLC as described in Materials and Methods. The data are means and standard error from a minimum of three separate cultures. Small standard errors do not signify in the plot.

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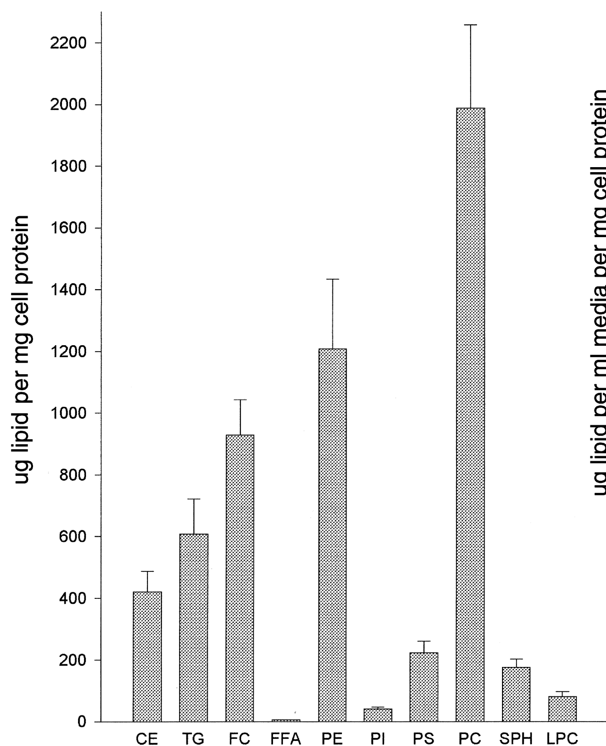

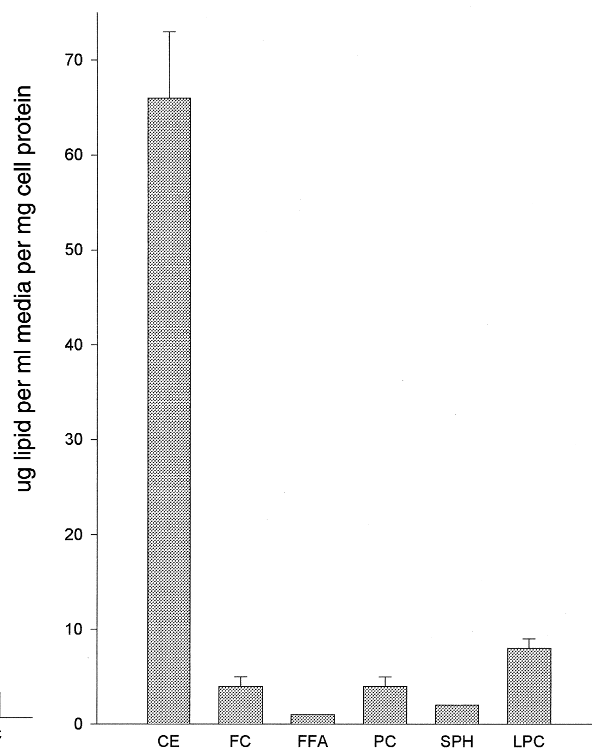

Fig. 6. Concentrations of individual lipids in neonatal brain astrocytes and media following culture for 12 h with lipid-free serum. TG, free cholesterol (FC), esterified cholesterol (CE), unesterified fatty acids (FFA), PE, PC, sphingomyelin (SpH), PS, PI, and lysophophatidylcholine (LPC) were separated by HPLC, then detected and quantified by ELSD as described in Materials and Methods. The data are means and standard error from a minimum of three separate cultures. Small standard errors do not signify in the plot. The concentrations of TG, PE, PI and PS in the culture media were <1 μg/ml media/mg cell protein.

tion (37) consistent with the ability of the adult brain to retain 22:6n-3 even during severe dietary n-3 fatty acid deficiency (38). Net accumulation of 22:6n-3, however, is essential to support the rapid structural lipid growth that occurs during brain development (9), and failure to do so is accompanied by reduced visual and neural function (9–13). Consequently, an understanding of the importance of the plasma supply of 22:6n-3 in maintaining optimal neural concentrations of 22:6n-3 is important in resolving the significance of low plasma 22:6n-3 in infants fed formulas without this fatty acid (19–22), and in some psychiatric diseases (39). It is clear that the liver is able to desaturate 18:3n-3 to 22:6n-3 (3) and many studies have provided evidence that plasma 22:6n-3, which could be derived from synthesis in liver or from the diet, is taken up and esterified into brain lipids (12, 17, 18, 40). It is also clear that the ability of the brain (12, 18) and astrocytes in culture to take up 22:6n-3 (Fig. 3) (33) far exceeds the efficiency with which 18:3n-3 can be converted into 22:6n-3 and incorporated into membrane lipids. The capacity for chain shortening and retro-conversion of 22:6n-3 to 22:5n-3 and 20:5n-3 in astrocytes cultured with 22:6n-3 suggests that the ability for peroxisomal β-oxidation is not likely to limit 22:6n-3 synthesis. The cumulative evidence thus supports the view that the main source of brain 22:6n-3 is the plasma, being derived from the liver or diet.

Neonatal brain astrocytes cultured in the presence of lipid-free serum preferentially secreted CE, and small amounts of lysophosphosphatidylcholine, phosphatidylcholine, sphingomyelin, and unesterfied fatty acids. This is significant because cholesterol and non-essential saturated and monounsaturated fatty acids are preferentially synthesized de novo in the developing brain (41, 42), probably from acetyl-CoA in astrocytes. Oxidation of 18:3n-3 may contribute acetyl-CoA for de novo fatty acid and cholesterol synthesis in the brain (41, 43–45). Synthesis of specific lipoproteins in brain (46) and of apolipoproteins by astrocytes (47, 48) has been reported. Further, astrocytes play an important role in supplying 22:6n-3 to neurons and cerebromicrovascular cells (15, 16, 49). Our results suggest CE and other lipids, potentially secreted with apolipoproteins could be important in the intracellular trafficking of cholesterol and non-essential and essential n-6 and n-3 fatty acids from astrocytes to other neural cells.

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