The low density lipoprotein receptor is not necessary for maintaining mouse brain polyunsaturated fatty acid concentrations

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Abstract The brain cannot synthesize n-6 or n-3 PUFAs de novo and requires their transport from the blood. Two models of brain fatty acid uptake have been proposed. One requires the passive diffusion of unesterified fatty acids through endothelial cells of the blood-brain barrier, and the other requires the uptake of lipoproteins via a lipoprotein receptor on the luminal membrane of endothelial cells. This study tested whether the low density lipoprotein receptor (LDLr) is necessary for maintaining brain PUFA concentrations. Because the cortex has a low basal expression of LDLr and the anterior brain stem has a relatively high expression, we analyzed these regions separately. LDLr knockout (LDLr$^{-/-}$) and wild-type mice consumed an AIN-93G diet ad libitum until 7 weeks of age. After microwaving, the cortex and anterior brain stem (pons and medulla) were isolated for phospholipid fatty acid analyses. There were no differences in phosphatidylserine, phosphatidylinositol, ethanolamine, or choline glycerophospholipid esterified PUFA or saturated or monounsaturated fatty acid concentrations in the cortex or brain stem between LDLr$^{-/-}$ and wild-type mice. These findings demonstrate that the LDLr is not necessary for maintaining brain PUFA concentrations and suggest that other mechanisms to transport PUFAs into the brain must exist.—Chen, C. T., D. W. L. Ma, J. H. Kim, H. T. J. Mount, and R. P. Bazinet. **The low density lipoprotein receptor is not necessary for maintaining mouse brain polyunsaturated fatty acid concentrations. *J. Lipid Res.* 2008.** 49: **147–152.**

Supplementary key words arachidonic acid • docosahexaenoic acid • transport • knockout

The brain is particularly rich in two PUFAs, arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) (1). Within the brain, these PUFAs regulate membrane fluidity, neuronal survival (2–4), and signal transduction, as they are coupled to multiple neuroreceptors via phospholipase A_2 (5). Deficits or altered metabolism of 20:4n-6 and 22:6n-3 are associated with impaired neurodevelopment (6, 7) and several neurological disorders (8–10). Scott and Bazan (11) were the first to demonstrate that the liver synthesizes 22:6n-3 from its nutritionally essential 18 carbon precursor α-linolenic acid (18:3n-3) and then secretes the newly synthesized 22:6n-3 into the plasma, where it is available for uptake by the brain. It has since been observed that adipose can serve as a 22:6n-3 reserve for the brain, especially during dietary 22:6n-3 deprivation (12, 13). Although cell culture studies demonstrate that the brain can desaturate and elongate linoleic acid (18:2n-6) and 18:3n-3 to 20:4n-6 and 22:6n-3, respectively (14), the in vivo rate of this reaction within the brain is very slow (15, 16). Unlike the liver, when dietary intake is restricted, the brain does not upregulate its capacity to synthesize 20:4n-6 or 22:6n-3 (17–19), further emphasizing the importance of a plasma supply of these PUFAs to the brain.

The mechanism by which PUFAs enter the brain is not agreed upon (20–22). Two general models involving either *a*) passive diffusion of unesterified plasma fatty acids (23–26) or *b*) uptake of lipoproteins via a receptor-mediated process, likely the low density lipoprotein receptor (LDLr), have been proposed (26, 27). Studies examining unesterified fatty acids in artificial membrane systems (28, 29) and the intravenous infusion of radiolabeled albumin-bound unesterified fatty acids (30) are commonly thought to support a passive diffusion mechanism. The selective uptake of PUFAs but not saturated fatty acids into the brain (31–33), the high concentration of 22:6n-3 esterified in lipoproteins (26, 34), and the pres-

Manuscript received 27 August 2007 and in revised form 10 September 2007.

Published, JLR Papers in Press, October 11, 2007.
DOI 10.1194/jlr.M700386-JLR200

Abbreviations: 16:0, palmitic acid; 18:2n-6, linoleic acid; 18:3n-3, α-linolenic acid; 20:4n-6, arachidonic acid; 22:6n-3, docosahexaenoic acid; CerPCho, sphingomyelin; ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; LDLr, low density lipoprotein receptor; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.

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ence of the LDLr on the brain endothelium (35–37) have been proposed to support a lipoprotein-mediated transport system. Because cholesterol is not transported from LDL into the brain (38), it was further suggested that the LDLr is expressed on the luminal membrane of endothelial cells and is absent from the abluminal membrane (27). Thus, LDL would be taken up by the LDLr on the luminal membrane and hydrolyzed within endothelial cells, allowing unesterified fatty acids, but not intact, cholesterol-containing lipoproteins, to enter the brain parenchyma (27).

With the availability of LDLr knockout ($LDLr^{-/-}$) mice (39), it is possible to test whether the LDLr is necessary for maintaining normal brain PUFA concentrations. Brain LDLr mRNA expression is much lower than that in liver, and within the brain, LDLr expression is relatively higher in brain stem than in cortex (35, 40). Brain LDLr expression does not change with age (35, 37). Thus, we completed our analyses in adult (7 week old) mice and analyzed the cortex and brain stem separately. If the LDLr is necessary for PUFA transport into the brain, we anticipated finding that $LDLr^{-/-}$ mice would have decreased brain PUFA concentrations and that this decrease would be larger in the anterior brain stem, where there is relatively higher expression of LDLr.

MATERIALS AND METHODS

All procedures were carried out in accordance with the policies set out by the Canadian Council on Animal Care (41) and were approved by the Animal Ethics Committee at the University of Toronto (protocol 20006703). Male C57BL/6J wild-type and B6.129S7-Ldlrtm1Her/J ($LDLr^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 3 weeks of age and were kept until 7 weeks of age in an animal facility in which temperature, humidity, and light cycle were controlled; the animals had ad libitum access to food (AIN-93-G, D10012G; Research Diets, New Brunswick, NJ) and water. The diet consisted of 200 g/kg casein, 3 g/kg L-cystine, 397 g/kg corn starch, 132 g/kg maltodextrin-10, 100 g/kg sucrose, 50 g/kg cellulose, 50 g/kg soybean oil, 0.014 g/kg *t*-butylhydroquinone, 35 g/kg mineral mix (S10022G), 10 g/kg vitamin mix (V10037), and 2.5 g/kg choline bitartrate. The 18:2n-6 and 18:3n-3% composition of the diet (measured by gas chromatography) was 40.5% and 5.4%, respectively, and longer-chain PUFAs were not detected. We analyzed 7 week old mice because in them the blood-brain barrier is intact (42), the brain has reached its adult size (43), brain PUFA concentrations have plateaued (44, 45), and the brain half-lives of PUFAs in rodents are ~1 month (46, 47). At 7 weeks of age, mice were subjected to head-focused high-intensity microwave irradiation (6 kW, 0.88–0.91 s; Muromachi brain fixation system; Stoelting Co., Chicago, IL) to stop brain fatty acid metabolism (48, 49). The brain was excised and the cortex and anterior brain stem (pons and medulla) were removed and placed on dry ice before being stored at −80°C for further analysis.

Genotyping

We independently confirmed the genotypes of all mice supplied by the Jackson Laboratory. DNA was extracted from ~0.5 cm of mouse tail using phenol and purified by ethanol precipitation. The extracted DNA was kept in 25–50 μl of trishydroxymethylaminomethane ethylenediamine tetraacetic acid buffer (pH 8.0), out of which 0.5 μl was used for PCR amplification. Amplified products were analyzed on a 1.5% agarose gel (10 μl/lane) containing ethidium bromide and visualized using the Fluorchem image system (Packard model 8000). The PCR primers used to probe for the LDLr gene were 5′-CCATATGCATCCCCAGTCTT-3′ and 5′-GCGATGGATACACTCACTGC-3′ (Integrated DNA Technologies, Coralville, IA). The PCR mixture (25 μl) contained 10 mM deoxynucleoside triphosphate mixture, 25 mM $MgCl_2$, 10 μM of each primer set, and 0.25 μl of Platinum Taq polymerase. The PCR was carried out under the following conditions: preincubation at 94°C for 3 min, followed by amplification for 30 cycles. Each amplification cycle consisted of denaturation at 94°C for 20 s, annealing at 68°C for 20 s, and extension at 72°C for 1.5 min. Cycling was followed by a final extension step at 72°C for 7 min and then cooling to 4°C.

Brain lipid extraction and chromatography

Total lipids were extracted from the cortex and brain stem according to the method of Folch, Lees, and Sloane Stanley (50). TLC plates (catalog number 10011; Analtech, Newark, DE) were activated by heating at 100°C for 1 h. Phospholipid fractions were separated along with authentic standards in chloroform/methanol/2-propanol/KCl (0.25%, w/v)/triethylamine (30:9:25:6:18, v/v). Bands corresponding to choline glycerophospholipid (ChoGpl), ethanolamine glycerophospholipid (EtnGpl), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns), and sphingomyelin (CerPCho) were visualized under ultraviolet light after lightly spraying with 8-anilino-1-naphthalene sulfonic acid (0.1%, w/v). Bands were scraped into a test tube containing a known amount of heptadecanoic acid and converted to fatty acid methyl esters with 14% boron trifluoride/methanol at 100°C for 1 h or 1.5 h for CerPCho. Fatty acid methyl esters were quantified on an Agilent 6890N gas chromatograph (Agilent, Palo Alto, CA) equipped with an flame ionization detector and separated on an Agilent J&W fused-silica capillary column (DB-23; 30 m, 0.25 μm film thickness, 0.25 mm inner diameter). Samples were injected in splitless mode. The injector and detector ports were set at 250°C. Fatty acid methyl esters were eluted using a temperature program set initially at 50°C and held for 2 min, increased at 20°C/min and held at 170°C for 1 min, and increased at 3°C/min and held at 212°C for 10 min to complete the run. The carrier gas was helium, set to a 0.7 ml/min constant flow rate. Peaks were identified by retention times of fatty acid methyl ester standards (Nu-Chek-Prep, Elysian, MN). Fatty acid concentrations (nmol/g brain) were calculated by proportional comparison of gas chromatography peak areas with that of the heptadecanoic acid internal standard. The AIN-93G diet was measured in quadruplicate according to the method described above.

Statistics

Results are expressed as means ± SD. Means were compared by unpaired, two-tailed *t*-tests, and statistical significance was set at $P < 0.05$.

RESULTS

Mouse body and brain region weights

$LDLr^{-/-}$ mice weighed slightly less than wild-type controls (21 ± 2 vs. 22 ± 1 g for $LDLr^{-/-}$ and wild-type mice, respectively; $P = 0.003$). Similar to a report on whole brain (38), there were no statistically significant differences in microwaved cortex (160 ± 19 vs. 172 ± 20 mg for $LDLr^{-/-}$ and wild-type mice, respectively) or brain stem (50 ± 5 vs.

TABLE 1. Cerebral cortex fatty acid concentrations of major phospholipid classes from WT and LDLr$^{-/-}$ mice

	ChoGpl		EtnGpl		PtdSer		PtdIns	
Fatty Acid	WT (n = 9)	LDLr$^{-/-}$ (n = 8)	WT (n = 9)	LDLr$^{-/-}$ (n = 8)	WT (n = 9)	LDLr$^{-/-}$ (n = 8)	WT (n = 9)	LDLr$^{-/-}$ (n = 8)
14:0	111 ± 14	116 ± 7	ND	ND	ND	ND	13 ± 9	10 ± 2
16:0	16,218 ± 1,285	16,932 ± 845	1,663 ± 106	1,674 ± 69	218 ± 44	195 ± 48	297 ± 30	309 ± 40
16:1n-9	209 ± 18	225 ± 13	54 ± 24	48 ± 4	ND	ND	11 ± 10	9 ± 1
18:0	3,862 ± 325	3,989 ± 179	5,353 ± 290	5,441 ± 366	4,035 ± 319	4,075 ± 301	1,076 ± 89	1,113 ± 115
18:1n-9	6,215 ± 517	6,481 ± 297	2,231 ± 148	2,369 ± 216	860 ± 74	851 ± 82	239 ± 20	249 ± 13
18:1n-7	1,788 ± 139	1,860 ± 85	545 ± 110	647 ± 115	70 ± 7	69 ± 7	61 ± 5	64 ± 3
18:2n-6	249 ± 30	271 ± 13	85 ± 11	92 ± 9	22 ± 4	22 ± 4	16 ± 9	14 ± 3
18:3n-3	ND	ND	ND	ND	ND	ND	ND	ND
20:0	62 ± 11	57 ± 10	82 ± 9	96 ± 15	42 ± 12	47 ± 12	17 ± 11	19 ± 14
20:1n-9	190 ± 15	198 ± 9	264 ± 16	278 ± 24	52 ± 5	54 ± 3	26 ± 11	23 ± 2
20:2n-6	72 ± 8	75 ± 6	56 ± 9	54 ± 7	20 ± 4	20 ± 4	13 ± 7	10 ± 4
20:3n-6	112 ± 13	110 ± 6	121 ± 9	122 ± 8	36 ± 5	38 ± 5	17 ± 5	16 ± 3
20:3n-3	36 ± 7	35 ± 10	31 ± 13	31 ± 20	ND	ND	21 ± 10	21 ± 6
20:4n-6	1,904 ± 158	1,983 ± 60	3,033 ± 144	3,040 ± 245	184 ± 20	185 ± 13	925 ± 66	939 ± 91
20:5n-3	ND	ND	25 ± 6	24 ± 8	ND	ND	ND	ND
22:0	86 ± 17	78 ± 11	43 ± 15	43 ± 16	66 ± 20	82 ± 50	40 ± 15	53 ± 28
22:1n-9	36 ± 7	36 ± 7	41 ± 11	35 ± 12	24 ± 10	29 ± 15	16 ± 11	17 ± 6
22:2n-6	ND	ND	58 ± 34	37 ± 21	ND	ND	49 ± 39	44 ± 33
22:3n-6	ND	ND	ND	ND	ND	ND	ND	ND
22:4n-6	138 ± 11	136 ± 8	995 ± 91	994 ± 83	215 ± 25	241 ± 83	53 ± 12	55 ± 20
22:5n-6	36 ± 8	31 ± 7	147 ± 38	139 ± 33	69 ± 13	86 ± 45	21 ± 11	20 ± 12
22:5n-3	36 ± 12	34 ± 9	106 ± 17	104 ± 20	30 ± 10	42 ± 22	13 ± 7	12 ± 5
22:6n-3	1,135 ± 142	1,218 ± 67	5,819 ± 392	6,038 ± 678	2,868 ± 309	2,939 ± 226	147 ± 39	175 ± 67
24:0	52 ± 21	54 ± 19	76 ± 56	85 ± 50	47 ± 26	99 ± 82	29 ± 13	22 ± 6
24:1n-9	ND	ND	ND	ND	ND	ND	ND	ND

Data shown are nmol/g and are means ± SD. ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; LDLr, low density lipoprotein receptor; LDLr$^{-/-}$, LDLr knockout; ND, not detected; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; WT, wild-type.

53 ± 6 mg for LDLr$^{-/-}$ and wild-type mice, respectively) weights between LDLr$^{-/-}$ and wild-type mice.

Cortex and brain stem esterified fatty acid concentrations

There were no statistical differences in cortical esterified fatty acids from the ChoGpl, EtnGpl, PtdIns, PtdSer, or CerPCho (data not shown for CerPCho) fractions between wild-type and LDLr$^{-/-}$ mice (**Table 1**). There were no statistical differences in brain stem esterified fatty acids from the ChoGpl, EtnGpl, PtdIns, PtdSer, or CerPCho (data not shown for CerPCho) fractions between wild-type and LDLr$^{-/-}$ mice (**Table 2**).

TABLE 2. Brain stem fatty acid concentrations of major phospholipid classes from WT and LDLr$^{-/-}$ mice

	ChoGpl		EtnGpl		PtdSer		PtdIns	
Fatty Acid	WT (n = 9)	LDLr$^{-/-}$ (n = 8)	WT (n = 9)	LDLr$^{-/-}$ (n = 8)	WT (n = 9)	LDLr$^{-/-}$ (n = 8)	WT (n = 9)	LDLr$^{-/-}$ (n = 8)
14:0	125 ± 21	113 ± 14	ND	ND	ND	ND	16 ± 3	20 ± 4
16:0	11,837 ± 1,891	11,235 ± 1,493	2,424 ± 1,074	2,354 ± 1,057	276 ± 52	301 ± 67	762 ± 106	744 ± 133
16:1n-9	176 ± 30	168 ± 22	306 ± 202	445 ± 392	ND	ND	24 ± 4	25 ± 6
18:0	4,232 ± 686	4,072 ± 534	6,525 ± 2,909	6,430 ± 2,588	4,051 ± 647	3,853 ± 406	1,390 ± 165	1,441 ± 230
18:1n-9	7,358 ± 1,245	6,898 ± 786	9,917 ± 4,587	8,488 ± 3,129	2,924 ± 508	2,673 ± 218	961 ± 141	911 ± 143
18:1n-7	2,220 ± 366	2,106 ± 227	3,022 ± 1,583	2,341 ± 1,089	222 ± 41	205 ± 20	224 ± 31	210 ± 31
18:2n-6	255 ± 57	249 ± 35	232 ± 122	208 ± 93	23 ± 3	31 ± 12	40 ± 6	41 ± 6
18:3n-3	ND	ND	ND	ND	ND	ND	ND	ND
20:0	186 ± 33	175 ± 15	579 ± 288	473 ± 175	125 ± 17	118 ± 11	93 ± 66	78 ± 12
20:1n-9	705 ± 114	623 ± 55	2,977 ± 1,347	2,526 ± 852	475 ± 72	427 ± 43	190 ± 27	177 ± 28
20:2n-6	97 ± 18	90 ± 9	317 ± 147	269 ± 98	41 ± 7	41 ± 4	21 ± 4	19 ± 3
20:3n-6	78 ± 15	73 ± 9	395 ± 184	359 ± 128	69 ± 13	67 ± 6	42 ± 7	41 ± 6
20:3n-3	ND	ND	50 ± 19	65 ± 33	ND	ND	13 ± 3	15 ± 5
20:4n-6	717 ± 119	717 ± 116	3,219 ± 1,396	3,099 ± 998	241 ± 42	238 ± 20	864 ± 113	884 ± 138
20:5n-3	ND	ND	43 ± 17	54 ± 34	ND	ND	ND	ND
22:0	114 ± 21	119 ± 27	78 ± 33	93 ± 54	142 ± 24	141 ± 19	71 ± 19	80 ± 17
22:1n-9	74 ± 13	69 ± 7	230 ± 98	209 ± 83	58 ± 11	53 ± 5	31 ± 5	30 ± 5
22:2n-6	ND	ND	85 ± 34	105 ± 81	22 ± 9	25 ± 11	18 ± 8	23 ± 7
22:3n-6	ND	ND	ND	ND	ND	ND	ND	ND
22:4n-6	85 ± 14	82 ± 14	1,771 ± 751	1,721 ± 568	191 ± 35	190 ± 24	64 ± 13	64 ± 13
22:5n-6	ND	ND	74 ± 25	97 ± 70	17 ± 3	22 ± 7	8 ± 2	11 ± 4
22:5n-3	ND	ND	147 ± 68	147 ± 61	25 ± 7	25 ± 7	11 ± 3	15 ± 4
22:6n-3	1,263 ± 225	1,187 ± 126	5,493 ± 2,431	5,345 ± 1,774	1,027 ± 168	1,029 ± 135	203 ± 23	198 ± 26
24:0	98 ± 19	92 ± 15	72 ± 33	74 ± 43	58 ± 12	64 ± 14	53 ± 9	56 ± 22
24:1n-9	ND	ND	ND	ND	ND	ND	ND	ND

Data shown are nmol/g and are means ± SD.

DISCUSSION

The cortex and brain stem phospholipid esterified fatty acid concentrations from wild-type mice in this study are similar to literature values (45, 51), and slight discrepancies are likely attributable to differences in diet and brain region analyses (1, 52). 20:4n-6 from the plasma unesterified pool is rapidly esterified to ChoGpl and PtdIns (51, 53), whereas 22:6n-3 is rapidly esterified to EtnGpl and ChoGpl (54, 55). Subsequent remodeling and de novo phospholipid synthesis likely explain relative mass differences between phospholipid species (56, 57). In the microwaved brain, >99.9% of 20:4n-6 or 22:6n-3 is esterified to phospholipids (15, 58). In the current study, we measured the esterified fatty acid concentration of individual phospholipid fractions, as there may be subtle differences in a fatty acid species that could be masked when examining them collectively. Furthermore, because of regional differences in LDLr expression, we examined the cortex and brain stem separately. Despite a life-long deficiency of the LDLr receptor in these brain regions, this study did not detect a significant difference in any measured brain fatty acid concentration at 7 weeks of age. Whether or not this lack of difference also occurs in younger or older mice is not known and warrants further study.

Because mammalian tissues, including the brain, cannot synthesize n-6 or n-3 PUFAs de novo, these PUFAs in cortex and brain stem of $LDLr^{-/-}$ mice in the current study must have entered the brain via an LDLr-independent mechanism. One possibility is that another lipoprotein receptor, such as the class B scavenger receptor (59), the LDLr-related protein (60), or the very low density lipoprotein receptor (61), which are responsible for HDL, LDL, and VLDL uptake, respectively, and are present in the brain (40), could contribute to brain PUFA uptake. Alternatively, proteins such as lipoprotein lipase (26) or endothelial lipase (62–64) could cleave PUFAs esterified within lipoproteins at the blood-brain barrier, allowing for entry of the unesterified PUFAs into the brain. It is also possible that fatty acids only enter the brain, to a quantitatively significant extent, from the plasma unesterified pool. Studies in artificial membranes, not containing LDLr, support a passive diffusion-mediated uptake of unesterified fatty acids (23, 28, 65), and infusion of physiologically relevant doses of albumin-bound radiolabeled fatty acids [22:6n-3, erucic acid (22:1n-9), 20:4n-6, 18:3n-3, 18:2n-6, palmitic acid (16:0)] into the rat femoral vein results in rapid brain uptake of the intact (confirmed by HPLC) tracer (15, 16, 30, 51, 66–68). On this note, the net rate of 22:6n-3 uptake from the plasma unesterified pool as measured over a 5 min intravenous infusion, when extrapolated over 1 day, approximates the rate of loss of 22:6n-3 administered intracerebroventricularly (0.26 μmol/g brain/day) (69). Furthermore, 2 h after oral [^{3}H]16:0 administration to rats, resulting in plasma esterified and unesterified [^{3}H]16:0, uptake from the plasma unesterified [^{3}H]16:0 pool approximates total brain [^{3}H]16:0 uptake (70). Collectively, these latter two studies suggest that uptake from the plasma unesterified pool is sufficient to explain brain total 22:6n-3 and 16:0 loss and uptake, respectively.

The LDLr regulates LDL-cholesterol transport in several tissues (71, 72), and it would be of interest to determine whether LDLr deletion resulted in lower PUFA levels in these tissues. However, one notable exception appears to be the brain, where despite its presence at the endothelium, it does not appear to be involved in LDL-cholesterol transport (73, 74), and deletion of the LDLr has no measurable effect on brain cholesterol concentration (38). In the current study, deletion of the LDLr had no effect on the concentration of saturated or monounsaturated fatty acids, which can be synthesized de novo within the brain, nor did it alter the concentration of n-6 or n-3 PUFAs, which require transport into the brain. In conclusion, the LDLr is not necessary for maintaining the PUFA concentration of the brain, and other mechanisms to transport PUFAs into the brain must exist.

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to D.W.L.M. and R.P.B.

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