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Decrease in Neuron Size in Docosahexaenoic Acid-Deficient Brain

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Docosahexaenoic acid is an important fatty acid for neuronal function because its deficiency leads to many behavioral and functional deficits. In a previous study, we reported that docosahexaenoic acid deficiency caused a reduction in the size of neurons of the CA1 region in the hippocampus. To extend these results to other regions of the brain, the present study entailed a morphologic analysis of neuronal size in hippocampus, hypothalamus, piriform cortex, and parietal cortex in rats that were raised on docosahexaenoic acid-deficient and supplemented diets for three generations. Neuron size in these regions was measured both at weaning (21 days) and maturity (68 days), and docosahexaenoic acid content in the brain was measured on a separate set of sibling rats using fatty acid analysis. Neuron size in hippocampus, hypothalamus, and parietal cortex decreased in weanling and in piriform cortex in mature rats raised on the docosahexaenoic acid-deficient diet. The brains of these rats exhibited a nearly 90% decrease of docosahexaenoic acid. Decrease of neuron size has been linked to a loss of optimal function in neurons. In the United States, human infant-milk formulas use vegetable oils as fat sources that lack docosahexaenoic acid. If docosahexaenoic acid deficiency reduces neuron size, then human infants raised on these formulas may also have smaller neurons relative to breast-fed infants.

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Introduction

Docosahexaenoic acid (DHA, 22:6n-3) is a major component of membrane phospholipids in nerve cells [1-4]. DHA rapidly accumulates in the brain through in utero nutrition, mother's milk, and diet during perinatal and early postnatal life [5-7]. When n-3 fatty acid-deficient diets are fed to animals for two to three generations, a marked decrease in nervous system DHA results [8-12]. Loss of brain DHA results in the loss of many sensory, behavioral, and cognitive functions both in animals and humans. These include loss in visual sensitivity [10,13, 14], visual acuity [15-17], visual attention and visual recognition memory [18], dark adaptation responses [19], two-odor discriminations [20,21], and spatial navigation memory [12,20,22-25]. Supplementation of DHA is known to slowly restore the levels of brain docosahexaenoic acid in animals that were previously raised on DHA-deficient diets [26-29]. DHA-repleted animals can recover from deficits in spatial tasks [29]; however, electroretinographic parameters and mean arterial blood pressure do not normalize even after DHA is repleted [30,31].

The present understanding of the impact of a loss of brain DHA on brain anatomy and morphology is minimal. Apart from two previous morphometric studies involving DHA-deficient hippocampus [32] and DHA-supplemented cortical neurons in culture [33], insights into morphologic changes in the DHA-deficient brain comes from ultrastructural studies in which synaptic vesicle number [34,35] or receptor density [36] were analyzed after n-3 deficiency. Our previous study also examined a number of other morphologic parameters in the hippocampus, including layer volume, density, and neuron number. Although no differences were detected in these parameters [32], further studies with larger sample sizes are required to demonstrate that conclusively.

This study was conducted to investigate whether changes in the neuronal size were limited to the hippocampus or were a more general feature of brain. To address this question, the hippocampus, hypothalamus, piriform cortex (endopiriform cortex), and parietal cortex were selected for neuron size analysis. In addition, the number

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of animals in both DHA-deficient and -supplemented diets were increased so that statistical power is augmented relative to the initial study.

Materials and Methods

Animals and Diets

All experimental procedures were performed in accordance with the policies of the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee. All animals were bred and housed in the National Institute on Alcohol Abuse and Alcoholism animal facility until perfusion. The animals were kept on a 12-hour light:dark cycle with light onset at 0600 hours. The level of fluorescent luminance averaged 65 lux in the colony. Room temperature was maintained at 21°C, with average relative humidity at 50%. Food and water were provided ad libitum.

Long Evans female rats (first generation, F1) were received from a commercial source (Charles River, Portage, MI) at 3 weeks of age and were randomly divided into four groups. Each group was put on a custom pelleted diet (see below). At 8 weeks of age, these animals were mated with chow-fed (NIH-31 chow) males. Litters (second generation, F2) were culled to 10 per group, and the pups remained with their dams during the preweaning period. The dams continued their respective diets during the suckling period. At weaning, these pups were fed the same diets as their dams. At 8 weeks of age, females from the F2 generation were selected and bred to generate the third generation (F3). These animals were also withdrawn onto the same diets and were housed in cages (48 × 27 × 20 cm) in groups of 2-3. A total of 40 male rats from 20 independent litters were selected from the F3 generation for morphologic investigations. One rat from each litter for each dietary group was allocated to the 21-day (n = 10) and 68-day (n = 10) cohorts. For fatty acid analysis, only hippocampal tissue was used; this analysis was performed on a group of 12 siblings from the same litters as those used for neuroanatomy, six animals from each dietary group.

The animals were raised on two semisynthetic diets based on the AIN-93 diet [37], varying only the types of fat used (Table 1). The n-3-deficient diet was formulated by using safflower oil because it had a low concentration of alpha-linolenic acid (18:3n-3). The other diet was supplemented with flaxseed oil as a source of alpha-linolenic acid and also with DHASCO (Martek Biosciences Corporation, Columbia, MD) as a source of DHA. This supplement resulted in a high n-6/n-3 ratio in the n-3-deficient diet compared with that of the n-3 supplemented group. The two diets were labeled as the *LA* (low alpha-linolenic acid) and *+LNA/DHA* (alpha-linolenic acid and DHA) diets.

Table 1. Diet composition

Ingredient	Amount (gm/100 gm diet) LA	+LNA/DHA
Casein, vitamin free	20	20
Carbohydrate:		
Cornstarch	15	15
Sucrose	10	10
Dextrose	19.9	19.9
Maltose dextrin	15	15
Cellulose	5	5
Salt mix	3.5	3.5
Vitamin mix	1	1
L-Cystine	0.3	0.3
Choline bitratrate	0.25	0.25
TBHQ	0.002	0.002
Fat:		
Hydrogenated coconut oil	8.1	7.45
Safflower oil	1.9	1.77
Flaxseed oil	none	0.48
DHASCO*	none	0.3

n-3 fatty acid-deficient, and n-3 fatty acid-adequate plus docosahexaenoic acid diets were based on the AIN-93 diet (Reeves et al, 1993) with several modifications to obtain the extremely low n-3 fatty acid level in the deficient diet.

* A trademark of Martek Corp (Columbia, MD) containing 46% of fatty acid as DHA.

Abbreviations:
DHA = Docosahexaenoic acid
LA = Linoleic acid
LNA = Alpha-linolenic acid
TBHQ = T-butylhydroquinone

Perfusion and Tissue Preparation

At the appropriate age, each animal was deeply anesthetized and perfused through the heart with 0.1 M phosphate-buffered saline followed by buffered 4% paraformaldehyde. The brains were removed, weighed, and kept in buffered 4% paraformaldehyde for 24 hours and then stored in 0.1 M phosphate buffer with 0.02% azide until cutting. A thin block of approximately 3-4 mm was removed from the middle of each brain that included septal hippocampus, hypothalamus, piriform cortex (dorsal endopiriform cortex), and parietal cortex. This block was sliced in 40-μm-thick coronal sections on a cryostat and mounted on gelatin-coated slides. Cresyl violet was used to visualize the morphology of the neurons in different regions.

Morphologic Measurements: Neuron Area

Generally neuron soma area or neuron size measurements were made in the left brain-hemisphere of each animal, except if quality of staining was not appropriate, in which case a switch was made to the right hemisphere. Areas of the hippocampus, hypothalamus, piriform cortex, and parietal cortex were selected in coronal sections [38] lying close to the middle of the anterioposterior extent of the brain (Fig 1). Neurons in each of these areas were distinguished from glia on the basis of size and the presence of a large and relatively pale nuclei and well-defined Nissl material in their cytoplasm. Each neuron was visualized at a magnification of 100× (oil immersion objective) and focused on a plane in which at least one nucleolus could be observed; the neuronal soma was traced in this plane.

Hippocampus

In each animal, neuron size measurements were made in CA1–3 layers at the septal (approximately –2.3 mm from Bregma) eccentricity in the left hippocampus based on the maps of Paxinos and Watson [38]. Cell size measurements were made in the center of each perikaryal layer in border-to-border (dorsoventral) sweeps. A minimum of 100 or more neurons were sampled and drawn in these sweeps using the following procedure: The microscope field was swept from border-to-border in a layer, and neurons were identified and drawn. This process was continued until 100 neurons were drawn. If 100 neurons were not sampled before reaching the border of a layer, the sweep was continued until this criterion was met. This procedure was performed to avoid sampling biases created by stopping in the middle of a layer because neuron size may vary within the width of the layer.

Hypothalamus

In three hypothalamic areas, that is, dorsomedial, ventromedial, and arcuate nucleus of the hypothalamic region, 50 neurons were sampled

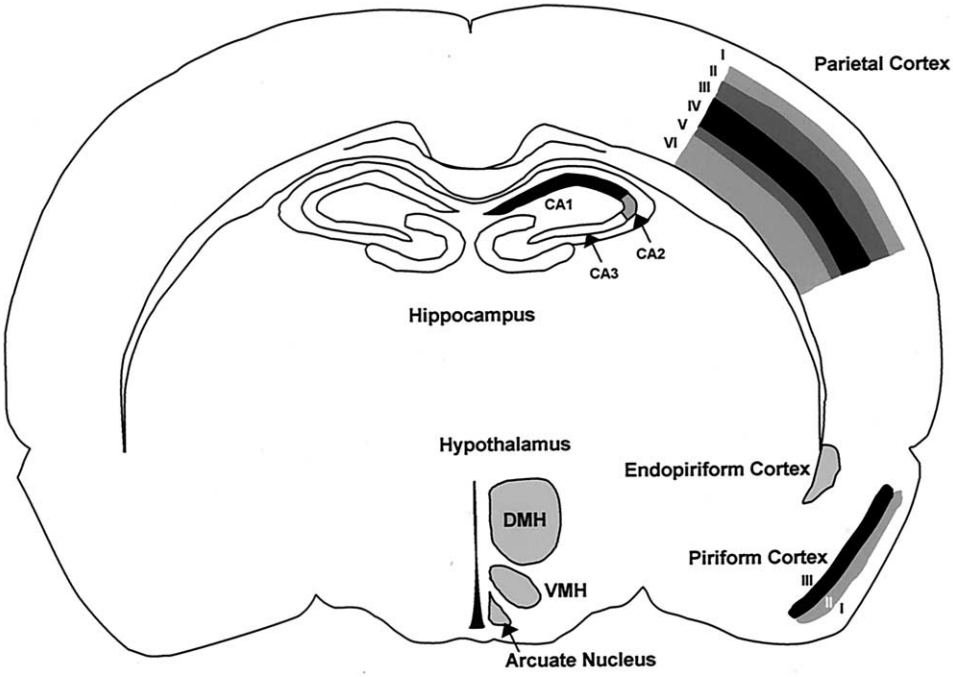

Figure 1. Camera lucida drawing of a coronal section, lying midway between anterioposterior extent of the rat brain, depicting regions of the hippocampus, hypothalamus, piriform cortex, and parietal cortex where neuron size measurements were made. Cornu Ammon's layers (CA1-3) of the hippocampus; dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), arcuate nuclei of the hypothalamus, layers I-III of the piriform cortex, endopiriform cortex, and layers I-VI of the parietal cortex are presented.

and drawn for each animal. The procedure used to sample these neurons was similar to the one used for endopiriform cortex (see below). At a low magnification (4× air objective), each of the three areas was outlined, and a square grid was superimposed to place 10 or more markers. At higher magnification (100× oil-immersion objective), the five closest neurons to each marker were drawn to sample 50 or more neurons for each area.

Piriform and Dorsal Endopiriform Corticies

Neuron-size measurements were performed in Layers II and III in the middle of the rostrocaudal extent of the piriform cortex. A single section was selected, and 50 cells in the middle of the above layers were drawn in dorsoventral sweeps. An electronic outline of each layer was first drawn at lower magnification (4× air objective) using the Neurolucida software program (MicroBrightField Inc., Colchester, VT). Electronic markers were placed in the middle of the outlines. The neurons were visualized at a higher magnification (100× oil-immersion objective) at these markers, and 50 neurons were drawn in sweeps within the width of each layer, similar to the procedure performed in the hippocampus.

In the same section, cell size measurements were also made in the left dorsal endopiriform cortex. In the coronal plane, dorsal endopiriform cortices extend ventrolateral to the corpus callosum and on average occupy a cross-sectional area of approximately 0.35 mm^2. To sample neurons in this area, a square grid (125-150 μm), at a low magnification (4× objective) was superimposed on this area using Neurolucida. For each animal, the grid was adjusted so that 10 or more grid intersections fell within the bounds of the endopiriform area. Electronic markers were placed on these intersection points, and at a higher magnification (100× oil-immersion objective) neurons were drawn around these markers by enclosing them with an electronic square box (80 × 80 μm^2). The five neurons closest to each marker were drawn and 50 or more neurons were sampled for each animal.

Parietal Cortex

For the parietal cortex, neuron size measurements were made in layers II, III, IV, V, and VI (layer I being the agranular layer), and a total of 50 or more neurons were sampled for each layer. At low magnification (4× air objective), each layer was identified, and an electronic line was drawn to represent the width of the layer. At higher magnification (100× oil-immersion objective), microscope field sweeps were made and cells were drawn parallel to the drawn line. These sweeps were carried on until all 50 neurons were sampled for a particular layer. All sweeps were completed in a border-to-border fashion so that variability in the measurement of neuron size could be avoided.

Fatty Acid Composition

Hippocampal tissue was used to establish the effect of diets on the total lipid and phospholipid fatty acid composition. Twelve animals were euthanized by decapitation, and the right and left hippocampi immediately dissected from the brain. Lipids were extracted from these hippocampi according to the method of Folch [39]. Fatty acids were analyzed by gas liquid chromatography using a Hewlett-Packard Series 5,890 II GC equipped with a flame ionization detector and fused silica capillary column (DB-FFAP; 30 m × 0.25 mm × 0.25 μm; J&W, Folsom, CA). Unknown fatty acid peaks were identified and quantified in comparison with standard mixtures using the internal standard (22:3n3) method, as previously described [29].

Data Analysis and Statistics

Student's *t* test (alpha ≤ 0.05) for independent samples used to analyze the effect of diet on the weight percent of each fatty acid. The nonparametric Mann-Whitney *U* test (alpha ≤ 0.05) was used to analyze the effects of diet (separately for two age groups) on neuron size in all the regions of the brain mentioned above.

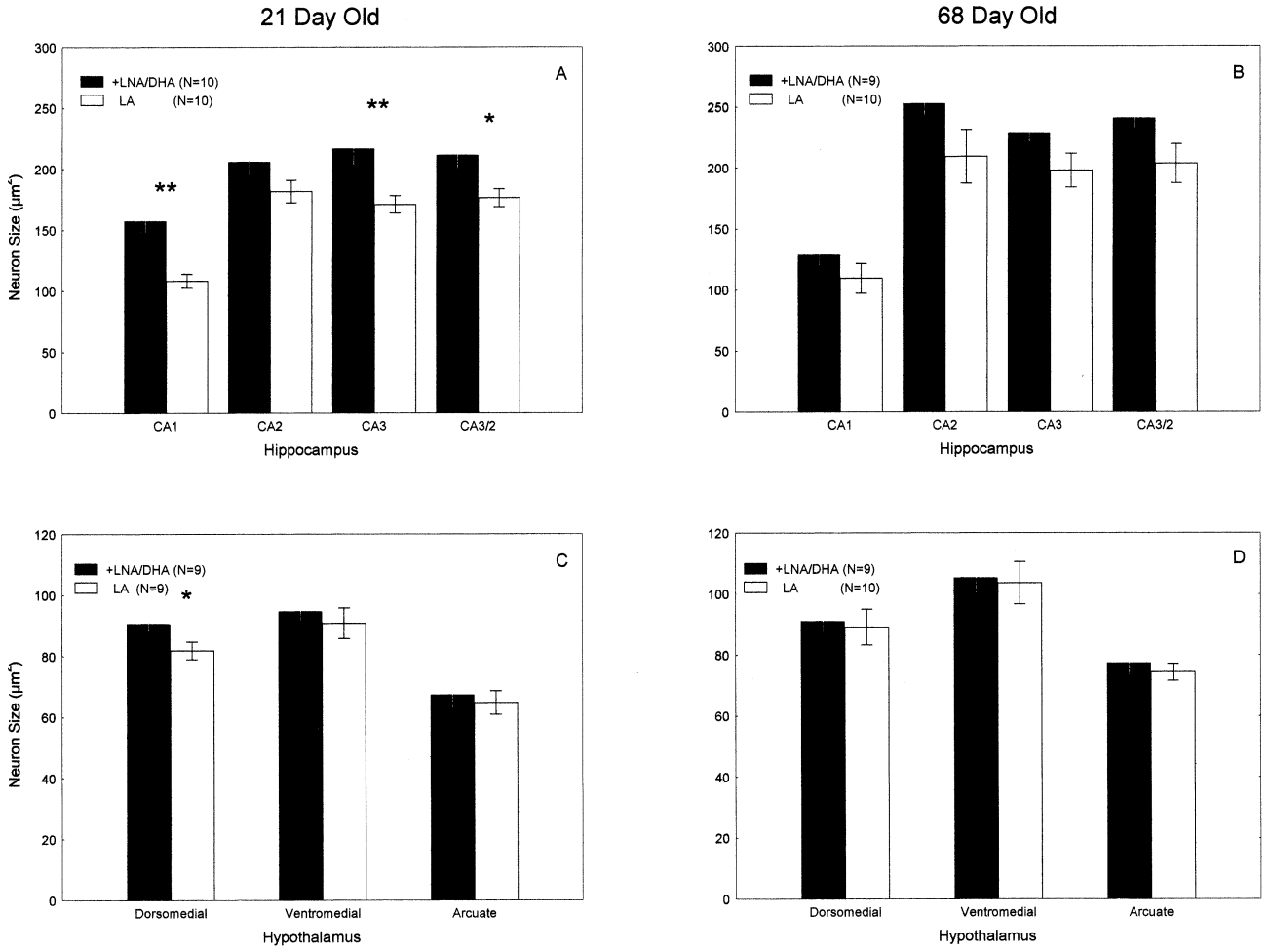

*Figure 2. Neuron size in CA1, CA3, and CA3/2 layers of the hippocampus were smaller in n-3-deficient animals. This decrease in size was significant at 21 days (*P < 0.05; **P < 0.005) and not at 68 days (A, B). In the dorsomedial, ventromedial, and arcuate nuclei of the hypothalamus, neuron size was smaller in n-3-deficient animals at 21 days and 68 days. Neuron size in the dorsomedial hypothalamus decreased significantly (*P < 0.05) at 21 days (C, D). Data were analyzed using the nonparametric Mann-Whitney test.*

Results

Body and Brain Weight

At 21 days of age the body weight of animals on the linoleic acid (LA) diet (54.8 ± 1.1 gm) was significantly ($P < 0.04$) lower than animals on the +LNA/DHA diet (58.7 ± 1.4 gm). At 68 days of age the body weight of LA animals (349.2 ± 8.4 gm) was again lower than that of the +LNA/DHA animals (419.5 ± 11.5 gm), and this difference was highly significant ($P < 0.0001$). The brain weights of rats on the LA diet, however, were not significantly different from the brain weights of animals on the +LNA/DHA diet at both ages ($P > 0.05$ and $P > 0.99$). No change in the weight of the brains was assessed at the time of histologic processing, that is, at the time of brain cutting. The shrinkage of brain tissue in these animals was not measured. Because animals from both dietary groups were perfused in the same manner, and their brains were processed similarly, shrinkage, if any, should be similar. The following data therefore are not corrected for tissue shrinkage.

Neuron Area

Hippocampus. Neuronal soma were traced in the left hippocampus of 39 brains belonging to the two diet groups. One 68-day brain in the +LNA/DHA group could not be analyzed because of difficulties in histologic processing. On average, 111 (range = 100-126), 113 (range = 101-135), and 108 (range = 100-124) neurons were drawn in the CA1, CA2, and CA3 layers at the septal eccentricity of the hippocampus in the various groups. The size of CA1 pyramidal neurons decreased by 31% in the LA group compared with the +LNA/DHA group at 21 days, which was statistically significant ($P < 0.003$); however, a 13% decrease in neuron size in the 68-day-old animals was not significantly different (Fig 2A,B). In the CA2 layer, neuron size decreased by 14% in the LA animals compared with +LNA/DHA animals for both age groups, and this difference was not significant. For the CA3 layer, a significant ($P < 0.005$) decrease in neuron size by 21% in the LA animals was observed in 21-day-old rats, but a decrease of 13% in the 68-day-old rats was not

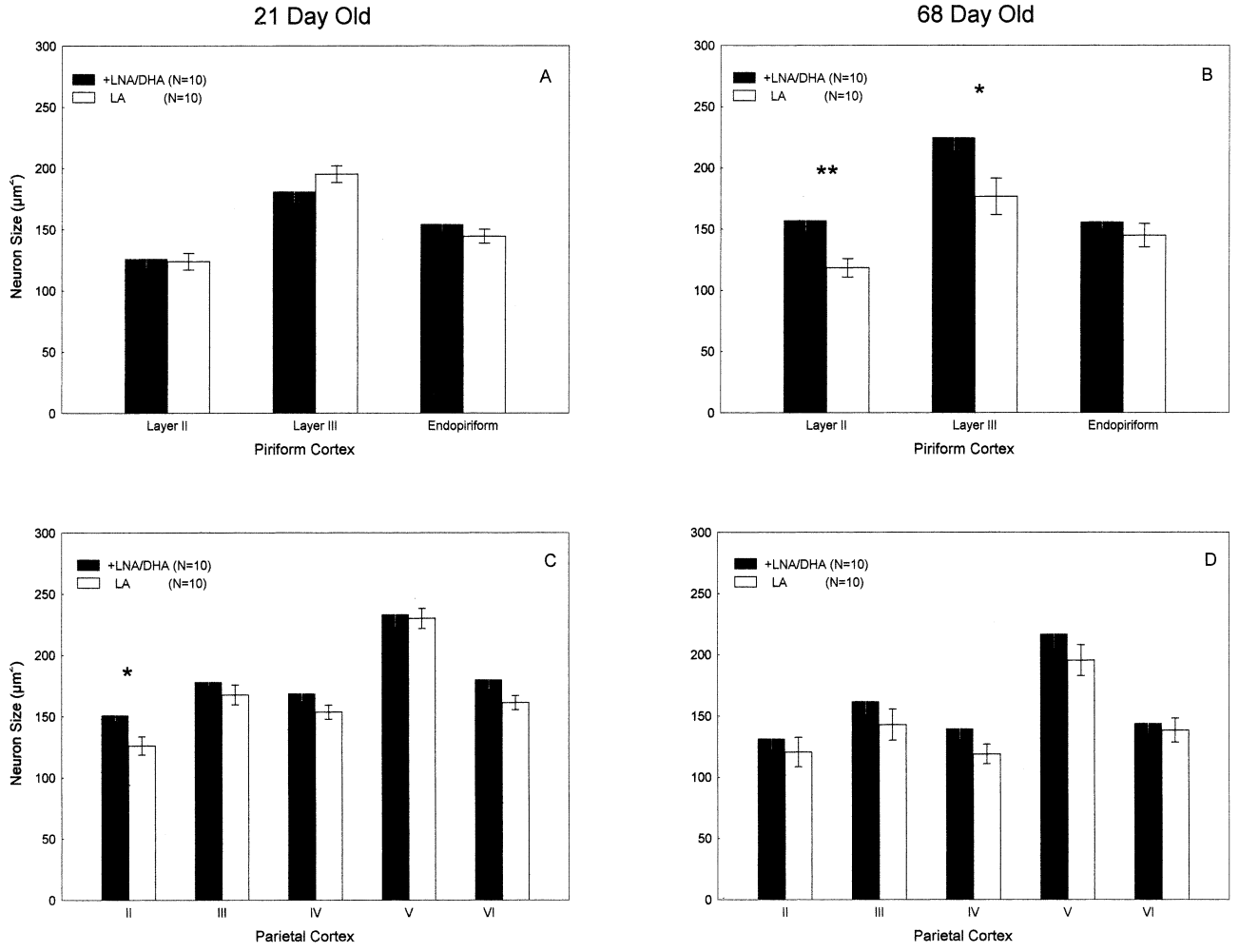

*Figure 3. No differences were detected in the size of neurons at 21 days in layer II and III in the piriform cortex. At 68 days, neurons size significantly decreased in both layer II and III (*P < 0.05; **P < 0.005) in the piriform cortex. No significant change in neuron size was observed in endopiriform cortex in either 21- or 68-day-old animals (A, B). Generally, size of the neurons was smaller in all the layers (II-VI) of the parietal cortex in 21- and 68-day-old animals that were on an n-3 fatty acid-deficient diet (C, D). Neuron size decreased significantly (P < 0.05) in layer II of 21-day-old raised on n-3 fatty acid-deficient diet.*

significantly different. Because the borders of the CA2 layer cannot be determined with Cresyl violet stain, layers CA3 and CA2 were merged (CA3/2). Neuron size decreased by 15% in the merged CA3/2 layer of 21-day-old rats on the LA diet, which was significantly ($P < 0.05$) smaller than neurons of rats on +LNA/DHA diet. Neuron size in 68-day-old animals did not quite reach statistical significance ($P = 0.06$, Fig 2B), however, because of an increase in variability in these samples.

Hypothalamus. In all animals, on average 55 (range = 50-57) neurons were sampled in the dorsomedial, 56 (range = 50-73) in the ventromedial, and 52 (range = 50-64) in the arcuate nucleus of the hypothalamus. The size of neurons in both age groups was smaller in the LA diet group (Fig 2C, D). Although this decrease was small (6%) in 21-day-old animals and even smaller (3%) in 68-day-old animals, neuron size in the dorsomedial hypothalamus decreased significantly ($P < 0.05$) in 21-day-old animals (Fig 2C).

Piriform and Dorsal Endopiriform Cortex. In layer II, 58 (range = 50-78) neurons and in layer III, 57 (range = 50-73) neurons were sampled in the piriform cortex in all animals. Changes in neuron size in 21-day-old animals were rather modest. The LA diet led to a small decrease (1%) in neuron size in layer II, although in layer III neuron size increased by 8%. These differences were not statistically significant (Fig 3A, B). In contrast, in 68-day-old rats the LA diet led to large reductions in neuron size in both layers II and III of the piriform cortex. A decrease in neuron size of 32% and 27% was observed in these layers, which was statistically significant at $P < 0.005$ and $P < 0.05$, respectively.

An average of 53 (range = 50-57) neurons was sampled in the endopiriform cortex for all animals. In 21-day-old and 68-day-old animals there was a small decrease of 7% in the LA diet group, and no statistical difference was detected from the +LNA/DHA diet group (Fig 3A, B).

Parietal Cortex. Fifty-eight neurons (range = 50-77) in layer II, 55 (range = 50-68) in layer III, 56 (range = 50-77) in layer IV, 55 (range = 50-73) in layer V, and 60 (range = 50-87) neurons in layer VI were sampled and drawn in both diet groups at 21 and 68 days of age. In both

Table 2. Fatty acyl composition of the hippocampus in 21- and 68-day-old rats fed n-3-deficient or n-3-supplemented diets

	21-day-old		68-day-old	
Fatty Acid	LA (n = 8)	+LNA/DHA (n = 6)	LA (n = 6)	+LNA/DHA (n = 6)
10:0[1]	0.1 ± 0.02	0.1 ± 0.02	0.0 ± 0.0	0.0 ± 0.0
12:0	0.04 ± 0.003	0.03 ± 0.002*	0.03 ± 0.004	0.03 ± 0.005
14:0	0.4 ± 0.02	0.3 ± 0.02*	0.2 ± 0.01	0.2 ± 0.01
16:0 DMA	1.5 ± 0.3	0.8 ± 0.3	1.2 ± 0.4	0.2 ± 0.01
16:0	22.6 ± 0.2	21.3 ± 0.2*	22.4 ± 0.3	22.6 ± 0.2
18:0 DMA	1.7 ± 0.4	0.9 ± 0.4	1.5 ± 0.5	3.1 ± 1.8
18:0	20.0 ± 0.2	20.1 ± 0.2	17.8 ± 0.3	18.2 ± 0.2
20:0	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01
22:0	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.03	0.3 ± 0.01
24:0	0.6 ± 0.02	0.6 ± 0.1	0.7 ± 0.04	0.1 ± 0.1*
Total saturated	47.5 ± 0.4	44.7 ± 0.6*	44.4 ± 1.0	47.2 ± 3.2
16:1n7	0.4 ± 0.01	0.4 ± 0.01	0.3 ± 0.01	0.3 ± 0.01*
18:1 DMA	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.5
18:1n9	11.5 ± 0.1	12.4 ± 0.2*	13.7 ± 0.4	15.5 ± 0.3*
18:1n7	2.6 ± 0.02	2.5 ± 0.02*	3.3 ± 0.1	3.0 ± 0.04*
20:1n9	0.3 ± 0.0	0.3 ± 0.02*	0.7 ± 0.03	0.8 ± 0.02*
22:1n9	0.1 ± 0.01	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.01
24:1n9	0.4 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	0.7 ± 0.1*
Total monounsaturated	15.7 ± 0.2	16.5 ± 0.2*	19.5 ± 0.5	21.1 ± 0.3*
18:2n6	0.5 ± 0.1	0.9 ± 0.02*	0.3 ± 0.04	0.6 ± 0.03*
18:3n6	0.03 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0.01 ± 0.002
20:2n6	0.1 ± 0.002	0.1 ± 0.002*	0.05 ± 0.01	0.1 ± 0.01*
20:3n6	0.3 ± 0.01	0.6 ± 0.01*	0.2 ± 0.03	0.5 ± 0.01*
20:4n6	12.8 ± 0.2	11.5 ± 0.1*	12.1 ± 0.2	10.8 ± 0.1*
22:2n6	0.03 ± 0.01	0.05 ± 0.01	0.0 ± 0.0	0.0 ± 0.0
22:4n6	3.5 ± 0.04	2.5 ± 0.03*	3.7 ± 0.04	2.9 ± 0.1*
22:5n6	11.8 ± 0.1	0.9 ± 0.1*	10.6 ± 0.2	0.3 ± 0.01*
Total n-6	29.1 ± 0.2	16.6 ± 0.1*	27.0 ± 0.3	15.1 ± 0.2*
22:5n3	0.05 ± 0.01	0.2 ± 0.004*	0.1 ± 0.02	0.2 ± 0.01*
22:6n3	1.7 ± 0.1	13.6 ± 0.02*	2.0 ± 0.1	14.0 ± 0.4*
Total n-3	1.7 ± 0.1	13.9 ± 0.02*	2.1 ± 0.1	14.3 ± 0.4*
22:5n6/22:6n3	7.1 ± 0.3	0.1 ± 0.01*	5.4 ± 0.2	0.02 ± 0.001*
22:5n6+22:6n3	13.5 ± 0.2	14.5 ± 0.1*	12.5 ± 0.2	14.3 ± 0.4*
n-6/n-3	17.1 ± 0.8	1.2 ± 0.02*	13.2 ± 0.6	1.1 ± 0.03*
n-6+n-3	30.9 ± 0.2	30.5 ± 0.3	29.0 ± 0.3	29.5 ± 0.4

[1] 12:1n9, 14:1n9, 18:3n3, 20:3n3, 20:5n3 were below detection level (i.e., <0.01 wt%) when total fatty acid methyl esters were analyzed.
Comparisons were made with the Student *t* test. Superscripts * ($P < 0.05$) compares low alpha-linolenic acid and supplemented alpha-linolenic acid plus docosahexaenoic acid (+LNA/DHA) diets at 21-day or 68-day period.

Abbreviations:
DHA = Docosahexaenoic acid
DMA = Dimethylacetal derivative
LA = Linoleic acid
LNA = Alpha-linolenic acid

age groups, neuron size decreased in the LA diet group by 17%, 6%, 10%, 3%, and 12% in layers II, III, IV, V, and VI, respectively. The decrease in neuron size, however, was statistically significant ($P < 0.05$) only in layer II of 21-day-old rats (Fig 3C, D).

Total Fatty Acid Analysis. The total fatty acid content (mean = 26.9 μg/mg fresh weight) in the hippocampus was not different between diets groups (Table 2). The LA diet groups expressed a low n-3 and high n-6 fatty acid content compared with the +LNA/DHA group. Thus the n-6/n-3 fatty acid ratio was higher in the LA group compared with the +LNA/DHA group. However, the sum of n-6 and n-3 fatty acids in all diet groups was similar (Table 2). The change in the levels of n-3 fatty acids largely reflected a change in the levels of DHA and a change in the levels of n-6 fatty acids, resulting principally from a change in docosapentaenoic acid (DPAn-6) and, to a lesser degree, in docosapentaenoic acid (22:4n-6). The levels of DHA in the LA group were reduced by approximately 88% compared with the n-3-supplemented diet group (+LNA/DHA) with a reciprocal increase in DPAn-6 (94%) and 22:4n-6 (27%). There was a slight increase in the arachidonic acid (AA, 20:4n-6) in the LA groups in 21-day-old and 68-day-old animals.

Discussion

Our data suggest that neuron size decreases in the hippocampus, hypothalamus, piriform cortex, and parietal cortex with brain DHA deficiency. In general, reduction in neuron size in the DHA-deficient diet group was of a larger magnitude in rats of weaning age (21 days) relative to those at maturity (68 days). This finding suggests that neurons in the DHA-deficient brain grow at a slower rate than rats on an n-3 supplemented diet. The absence of a significant difference in the neuron size in the 68-day-old animals in most brain areas (Figs 2, 3) suggests that some developmental adaptations may have occurred in the deficient group. This finding was, however, not true for layer II and III in the piriform cortex, in which neuron size significantly decreased in the deficient group at this age (Fig 3). Hippocampal neuron size was decreased in both the 21- and 68-day-old animals after the n-3-deficient diet, with statistical significance being reached in the younger animals where the mean differences were greater and nearly reached ($P = 0.06$) in the mature group. This result possibly was supported by the observed statistical differences in neuron size of hippocampus in the DHA-deficient and DHA-supplemented diet groups at 110 days of age [32].

The present study was designed to conduct a precise and systematic morphologic analysis of neuron size in different regions of the brain to assess the effects of n-3 dietary deficiency. The precision of unbiased experimental protocols along with the use of a large sample of animals increased sensitivity to treatment effects, that is, DHA deficiency. Our initial observation [32] that loss of brain DHA leads to a reduction in neuronal size in the CA1 region of the hippocampus has been independently confirmed in this study. In addition, this investigation reports that other areas of the brain also reveal a similar decrease in the neuron size with brain DHA deficiency.

A decrease in neuron size associated with DHA deficiency is of its own accord an important finding. Neuron size has been reported to change with steroids [40] and nerve growth factor [41]. Because hippocampus neurons synthesize nerve growth factor [42] and DHA deficiency leads to a decrease in the nerve growth factor content in the hippocampus [43], it may be proposed that a reduction in neuronal DHA could decrease nerve growth factor (or other factors) in these cells and thereby alter their size.

Whether reduction in neuron size subsequent to DHA-deficiency leads to an adverse functioning of these cells or brain areas requires further investigation. One report suggests that the amplitude of Na+, K(+)-ATPase activity in nerve terminals was reduced to 60% with a decrease in dietary alpha-linolenic acid [44]. Other studies have suggested that supplementation with DHA can abolish long-term depression by facilitating *N*-methyl-D-aspartate channel activity in the pyramidal neurons of the hippocampus [45] and cerebral cortex [46]. Also, addition of DHA was demonstrated to increase the excitatory postsynaptic potential in the hippocampus neurons, increasing the amplitude of long-term potentiation in aged rats [47]. Based on these studies, it is speculated that deficiency of DHA may retard facilitatory mechanisms in neurons, creating behavioral deficits in the organisms. These changes in neuronal morphology, biochemistry, and physiology associated with a loss of brain DHA may underlie the deficits previously observed in a variety of behaviors [11,12,20-25].

If brain cells do get smaller with DHA deficiency, then infants fed vegetable-oil based formulas devoid of DHA [48] may have smaller neurons than do breast-fed infants, because mother's milk contains DHA [49,50]. Nutritional insufficiency of DHA is not only created by vegetable oil-based infant milk formulas, but infants receiving milk from many Western mothers also receive low levels of DHA. Low levels are caused by the fact that mothers in the West consume diets that are low in n-3 fatty acid and high in n-6 rich oils [51], reducing DHA in their plasma and milk [50]. The inadequate supply of DHA in the mother's diet and subsequent lowering of DHA during pregnancy and lactation [52,53] can lead to a DHA deficiency that may simulate a two-generation animal model of n-3 deficiency. The present study uses more extreme conditions of n-3 fatty acid deficiency because both DHA and its precursor LNA were eliminated or severely limited for three generations. The reduction in brain DHA in formula-fed infants [49,54,55], although significant, is not of the magnitude observed in our n-3 deficient rats. Therefore, although changes in the neuronal size in the brains of formula-fed infants remains a distinct possibility, further studies will be required under milder conditions of n-3 limitation to determine if this is the case.

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