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Alterations in Brain Function After Loss of Docosahexaenoate Due to Dietary Restriction of n-3 Fatty Acids

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Abstract

The concentration of the major polyunsaturated fatty acid (PUFA) in brain, docosahexaenoate, may be markedly reduced by two or more generations of dietary restriction of sources of n-3 fatty acids in the diet. Such a deficiency was induced through the feeding of safflower oil as the principal source of essential fatty acids. The reference point for this diet was an n-3 adequate diet to which alpha-linoleate and docosahexaenoate were added through the addition of a small quantity of flax seed or algael oils, respectively. The loss of brain DHA was associated with poorer performance in spatial tasks and an olfactory-cued reversal learning task. No difference could be observed in the hippocampal gross morphology. This study demonstrates the importance of providing a source of n-3 fatty acids during mammalian growth and development.

Index Entries: Docosahexaenoic acid; brain development; essential fatty acids; behavior; spatial task; Morris water maze; olfactory discrimination; n-3 fatty acid deficiency; hippocampus; neuro-anatomy.

Introduction

In the last two decades, several randomized, controlled trials have been conducted wherein docosahexaenoate (DHA) has been added as a supplement to infant formulas for premature (Uauy et al., 1990; Carlson et al., 1993, 1994; Werkman et al., 1996) or term infants (Birch et al., 1992; Innis et al., 1994; Agostoni et al., 1995; Carlson et al., 1996, Jorgensen et al., 1996; Innis et al., 1996; Jensen et al., 1997; Auestad et al., 1997; Scott et al., 1998; Birch et al., 1998, 2000; Willatts et al., 1998; Makrides et al., 2000). In many of the recent trials, both arachidonic acid (AA) and DHA have been added simultaneously in order to better mimic the fatty acid composition of human milk (Jensen et al., 1999). In these trials, measures related to neural function or development including visual acuity, cognitive tests, problem-solving tasks, and motor development have been assessed (for reviews, *see* Hamosh and Salem, 1998; Gibson and Makrides, 1998). Many of these trials have shown a benefit related to the nutritional value of the long-chain polyunsaturates, particularly for premature infants (Uauy et al., 1990; Carlson et al., 1993, 1994; Werkman, 1996) but also for term infants (Birch et al., 1992, 1998, 2000; Agostoni et al., 1995; Carlson et al., 1996; Willatts et al., 1998). Although other trials have not shown a benefit of formula supplementation, a meta-analysis of the preterm (SanGiovanni et al., 2000a) and term (SanGiovanni et al., 2000b) infant studies has indicated a significant benefit for visual acuity. It has also been demonstrated that the infant brain has a loss in DHA when formula is consumed with essential fats supplied only by vegetable

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oils (Farquharson et al., 1992; Makrides et al., 1994; Jamieson et al., 1999). This has led to the introduction of infant formulas containing the long-chain polyunsaturates (LCP), AA and DHA, in most countries. However, despite evidence of the nutritional importance of these LCPs in the early diet, formulas with LCPs are not permitted in North America at this time.

In addition to the public-health issues raised concerning the addition of DHA/AA in infant formula, there is the more basic problem as to what the essential role of DHA is in the nervous system. Indeed, one of the most remarkable conjectures in modern biology is the working hypothesis in this field, i.e., that the change in physiological function and behavior can be attributed to the loss of a single double bond on a highly unsaturated lipid. It is well-known now that when the fatty acyl composition of the adult mammalian nervous system is analyzed after n-3 fat deficiency during early development, there is a reciprocal replacement of DHA with the n-6 docosapentaenoic acid (DPAn6) (Mohrhauer and Holman, 1963; Galli et al., 1971). It is noteworthy that the only difference in structure is the double bond in the n-3 position on DHA!

In order to examine the nature of the alteration in neural function induced by loss of DHA, animal models of n-3 fatty acid deficiency are required. Both the Bourre (Bourre et al., 1984, 1989) and Okuyama (Okuyama et al., 1997) groups have developed rat models of n-3 fatty acid deficiency using n-6 rich oils such as safflower or peanut oil over two generations or more based on earlier work (Lamptey and Walker, 1976). This multi-generational treatment has been found to be necessary in order to cause significant depletion of DHA in the nervous system (Tinoco et al., 1979) as the brain appears to have a high priority for any n-3 fat sources in the body (Salem et al., 1986; Salem, 1989). Greiner et al. have recently shown that if the all of the nutrients in the diet are carefully controlled so that n-3 fats are not inadvertently introduced, the reduction in the second generation can be as much as 82% in the olfactory bulb (Greiner et al., 1999) and 81% in the whole brain (Greiner et al., 2000).

Now that reliable methods are available for the inducement of brain DHA deficiency, it is important that facile and reproducible assays for nervous system function be developed. In this article, second- and third-generation rats with a marked loss of brain DHA are examined using two different behavioral approaches. In the first, spatial tasks, i.e., the Morris water maze, is employed; in the second approach, olfactory-cued go, no-go tasks involving learning reversals are used.

Methods

Study Design

The overall design of this series of experiments is presented in Fig. 1. Weanling, female, Long Evans rats were obtained from Charles River (Portage, MI). They were split into two groups (randomly except that their mean body weights were equalized) and fed either an n-3 Adequate (n-3 Adq) or an n-3 Deficient (n-3 Def) diet. The nutrient composition of these two diets, based on the AIN-93 formulation (Reeves et al., 1993) is presented in detail in Table 1. The dependent variable was the addition of the n-3 fats, alpha-linolenate (LNA), and DHA to the n-3 Adq diet. The females were mated at 11 wk of age and the offspring weaned to the same diets as their mothers. The litters were culled to 10. Males from these litters (F2 generation) were used for the water maze using only one animal per litter ($n = 15$).

In a separate experiment, a similar dietary regime was carried out as that described earlier and the F2 females maintained on their respective diets until 8 wk of age, mated, and their offspring weaned to the same diets as their mothers ($n = 15$ litters per diet group). The F3 males were used for the olfactory learning experiments. Again, only one male was used from each litter for an $n = 11$ (Def) and $n = 9$ (Adq).

All animals were maintained in a conventional animal facility with controlled temperature (23 ± 1°C), humidity, and illumination (12 h, 0700–1900). Food and water was provided on an *ad libitum* basis. All animal experiments were performed under protocols approved by the NIAAA Animal Care and Use Committee and met NIH Guidelines for the use of animals in research.

Lipid Analysis

After the behavioral experiments (22 wk of age), F2 generation animals were sacrificed and the brains rapidly removed. The brains were cut saggitally and half of the brain frozen at –80°C until lipid analysis. Lipids were extracted using the method of Folch et al. (1957) and transmethylated using BF3-methanol after the method of Morrison and Smith (1964). Gas chromatographic analysis of fatty acid methyl esters on capillary columns was as described by Salem et al. (1996).

Olfactory-Based Discriminations

F3 generation rats were tested using a reversal-learning paradigm in an olfactory-based behavioral task in which water served as the reward (Slotnick, 1990). At 7 wk of age, rats began a partial water-

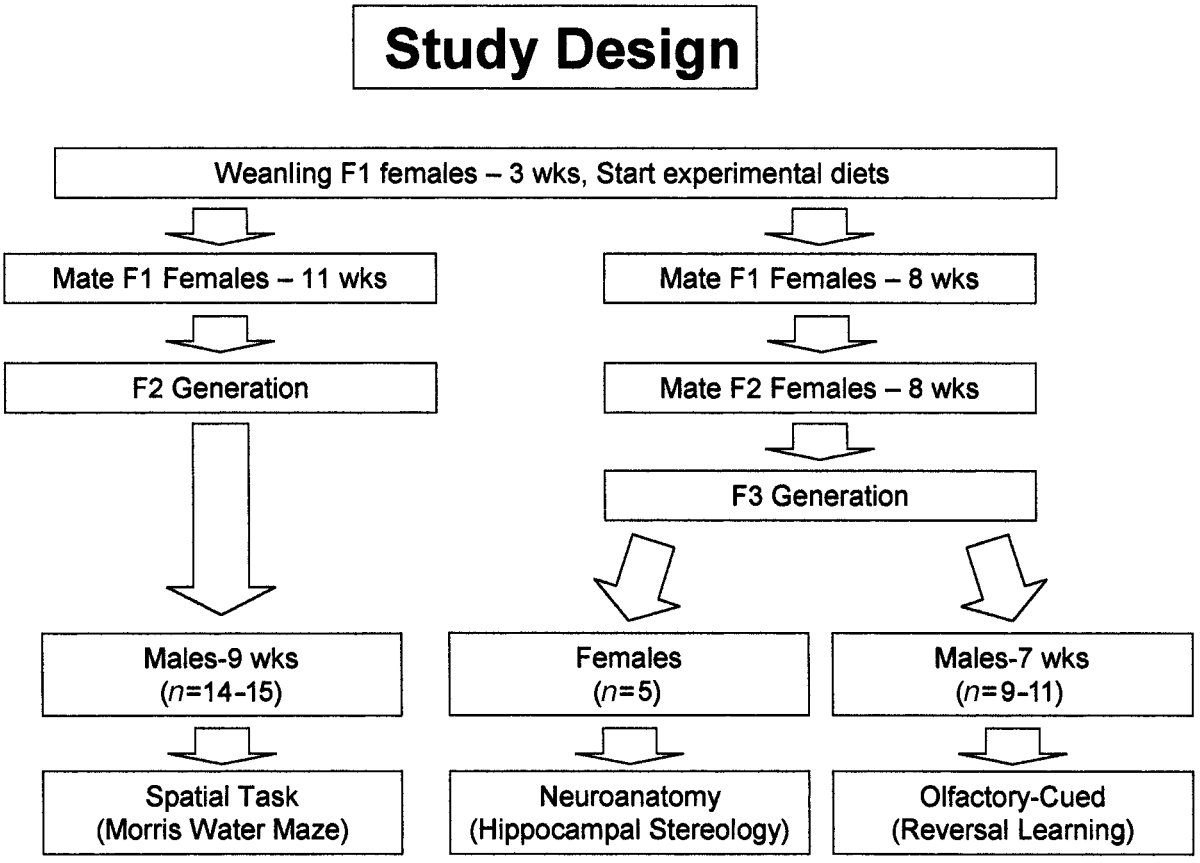

Fig. 1. The flow diagram of the experiment showing the study design, multi-generational dietary feeding regime, the biological endpoints, and numbers of litters for each component.

deprivation schedule in which they received one-third of their normal daily intake to increase their motivation for the task. At 9 wk of age, rats (n = 11 for n-3 Def group, n = 9 for n-3 Adq group) were trained to attend to odors in the olfactometer and obtain a water reward (Slotnick, 1994). Upon completion of the training sessions (approx 1 wk), rats had 1 d in which they were introduced to the S– concept and then they began the reversal-learning task. During the first day of testing (reversal 0, original learning), ethyl acetate and isopropyl acetate were presented in a random order to each rat as either the S+ or S– odor. Water was available after the S+ odor presentation, but not after the S– odor presentation. As previously described, rats rapidly learn to respond at the water tube only after the S+ odor is encountered (Lu et al., 1993). Responses at the water tube after the S– odor are scored as errors. After each rat reached a criterion of 85% correct responses in a block of 20 trials, the session was terminated. The following day (Reversal 1), the S+ and S– designations were reversed and the task was repeated. This procedure was followed for a total of five reversals (days). Total errors (responses after S– odors and no responses after S+ odors) were scored.

Morris Water Maze

Performance in spatial tasks was determined using the Morris water maze (Morris et al., 1982; Morris, 1984) as described in detail by Moriguchi et al. (2000). Briefly, a circular pool, 4 feet in diameter, was filled with water at 20°C up to two feet in depth. The pool was divided into four quadrants and rats were given two trials per day with starting points at the two corners of a quadrant, located diagonally across from the platform. The rats were acclimated to the water for 3 min and allowed a visible trial on the next day. On four subsequent days, they were allowed up to 90 s/trial to find the hidden platform. The total number of seconds spent in reaching the platform (sum of the two trials) is the escape latency. On the day following the last session, the platform was removed and the number of crossings of each quadrant was recorded (the retention trial).

Table 1
Composition of n-3 Deficient and n-3 Adequate Diets

	g/100 g diet	
Casein (vitamin free)[a]	20	
Carbohydrate:	60	
Cornstarch	15	
Sucrose	10	
Dextrose	20	
Maltose-dextrin	15	
Cellulose	5	
Salt mix	3.5	
Vitamin mix	1.0	
L-cystine	0.3	
Choline bitartrate	0.25	
TBHQ	0.002	
Fat Sources:	10	
	n-3 Def	n-3 Adq
Coconut oil (hydrogenated)	8.1	7.75
Safflower oil	1.9	1.77
Flaxseed oil	0	0.48
DHASCO[b]	0	0.3
	% of Total fatty acids[c]	
Saturates	80.9	75.6
Monounsaturates	3.9	4.8
18:2n-6	15.1	15.7
18:3n-3	0.04	2.6
22:6n-3	0	1.3

[a]Delipidated casein was obtained from Research Organics (Cleveland, OH).

[b]DHASCO is from Martek Biosciences and contains about 40% of fatty acids as DHA.

[c]Other long-chain polyunsaturates such as 20:4n6, 20:5n3, 22:5n3 were below 0.01% of total fatty acids or not detected; 20:2n6 was detected at the 0.05–0.06 level in both diets.

Morphology

A separate group of n-3 Adq (n = 4) and n-3 Def (n = 5) F3 animals, were used to conduct morphological analysis of the hippocampus. The animals were perfused transcardially with physiological saline and 4% para-formaldehyde at 110 d of age, and their brains were fixed in 4% para-formaldehyde for 48 h at 4°C before cutting. Frozen sections 40-µm thick were cut on a cryostat and stained with Cresyl Violet to visualize perikaryal layers (CA1, CA3/2, granular, and hilar layers) in these animals. Unbiased stereological methods (Cavalieri's point-counting; *see* West and Gundersen, 1990) were employed to estimate the volume of all of the above layers. Briefly, 10–12 equally spaced sections through the entire anterior-posterior extent of the hippocampus were selected using systematic-random sampling (West, 1999). On each section a point counting grid of a preset size was overlaid onto the image using Stereo Investigator (MicroBrightField, Inc, Colchester, VT) and the number of points that hit the layer were counted to estimate the area of each layer in that section. Estimates of areas of all the layers in all the sections were averaged individually and multiplied with the anterior-posterior length of the layer to calculate its volume. (West and Gundersen, 1990; West, 1999).

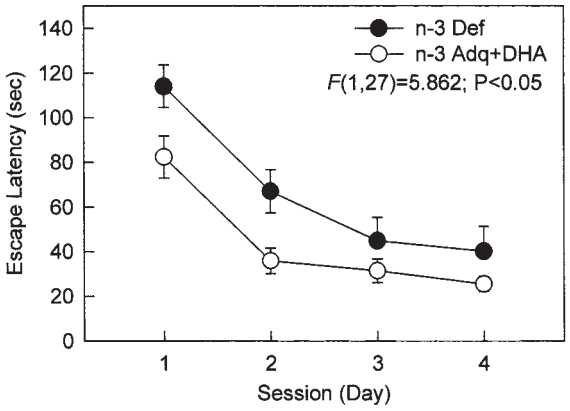

Fig. 2. The escape latencies in the Morris water maze for the n-3 adequate (n-3 Adq) and n-3 deficient (n-3 Def) rats of the second generation (n = 15/group).

Statistical Analyses

Data were analyzed using Statistica (Statsoft, Tulsa, OK). One-way (memory-retention data) and two-way analysis of variance (ANOVA) (escape latency and the olfactory reversal data) statistical tests were used for data analysis.

Results

Brain Lipid Analysis

Analysis of the rat brain total lipid extracts demonstrated the success of the dietary technique for the profound induction of n-3 fatty acid deficiency. The brain total lipid extract DHA (n-3 Adq was 12.7%) was reduced by 76% in the n-3 Def animals while the DPAn6 had increased by 47-fold.

Spatial Tasks

The escape latency of the n-3 Def rats was significantly increased with respect to that of the n-3 Adq animals (Fig. 2). The mean value was greater at all time points over the 4 d of testing. Fractional analy-

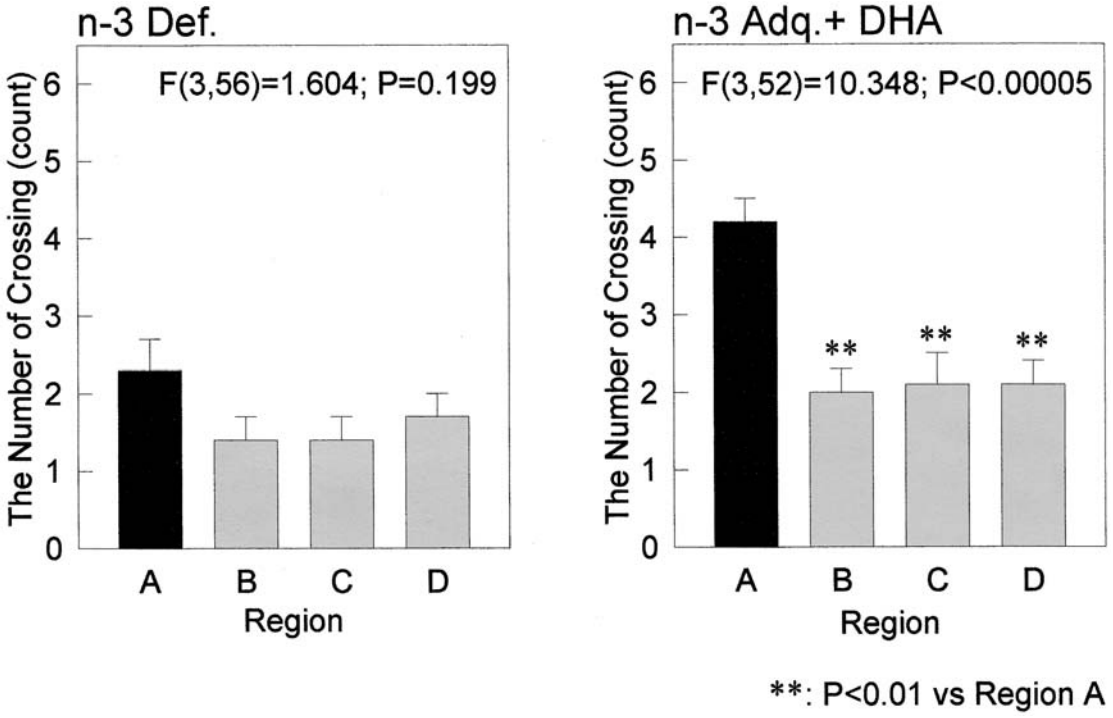

Fig. 3. The memory retention trial results for the F2 generation rats in the n-3 deficient (n-3 Def) and n-3 adequate (n-3 Adq) groups (n = 15/group).

sis of swimming indicated that the n-3 Def animals spent significantly more time swimming than the n-3 Adq and the amount of resting time was the same in the two groups.

In the memory-retention trial, the number of crossings of region A, the former position of the platform, was approximately twice that of the other three quadrants in the n-3 Adq group (Fig. 3). This difference was highly significant ($p < 0.00005$). In contrast, there was only a slight increment in the mean number of quadrant A crossings in the n-3 Def group, and this difference was not significant ($p < 0.2$).

Olfactory-Based Reversal Learning

Both groups of rats were tested using a reversal learning paradigm (Fig. 4). The data are shown expressed as the number of errors per day vs the number of reversals, starting with the introduction to the S– trial, the initial learning trial, and progressing through the fifth reversal (R5). N-3 Def rats made significantly more total errors over the entire experiment compared to n-3 Adq rats (n-3 Def, 500 ± 155; n-3 Adq, 303 ± 55; $p = 0.002$). A two-way ANOVA analysis with reversal number as the repeated measure indicated a significant diet effect ($F(1,18) = 5.06$, $p < 0.04$) as well as a reversal effect ($F(1,108) = 6.25$, $p < 0.001$).

Hippocampal Neuroanatomy

Volumetric measurement of each perikaryal layer was carried out in the left hippocampus of each animal. The mean volume (mm^3) of CA1 (2.29 ± 0.06), CA3/2 (3.46 ± 0.07), granular (2.13 ± 0.08) and hilar (1.42 ± 0.14) layers in n-3 Adq group were not significantly different from the mean volume of CA1 (2.22 ± 0.08), CA3/2 (3.29 ± 0.10), granular (1.76 ± 0.07), and hilar (1.06 ± 0.11) layers in the n-3 Def group.

Discussion

Recently, work from our laboratory has demonstrated that spatial-task performance, including escape latency and memory retention in the Morris water maze, are poorer in rats with low brain DHA (Moriguchi et al., 2000). This work, which is an entirely independent experiment to that previously reported, confirms those findings as well as extends them to a different dietary formulation. In this experiment, the n-3 Adq diet contained both LNA and DHA. In the experiments performed by Moriguchi et al. (2000), the n-3 Adq diet contained only LNA. The brain levels of DHA achieved with this diet indicated a slightly higher (e.g., 7% in the hippocampus)

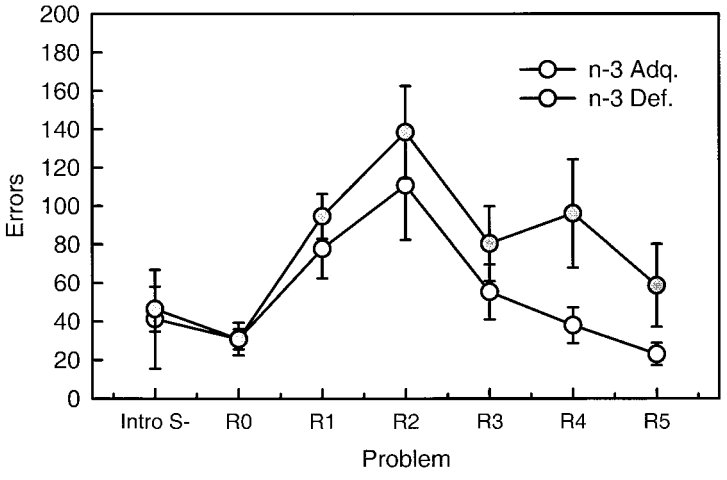

Fig. 4. Reversal learning in a two-odor olfactory-discrimination task for n-3 deficient (n-3 Def) and n-3 adequate (n-3 Adq) rats of the F3 generation. The n-3 Def rats made significantly more errors (responses after S– odor presentation and no responses after S+ odor presentation) than the n-3 Adq group on a series of five reversals in a reversal learning task; diet effect $F(1,18) = 5.06$, $p < 0.04$. The repeated measure effect (reversal) was also significant, $F(1,108) = 6.25$, $p < 0.001$.

overall brain level due to the presence of preformed dietary DHA.

One of the key goals of this work was to establish reliable methods in which to assess brain function. It is now clear that the Morris water-maze tasks, when defined in the manner performed herein, provide an appropriate task in this regard. It must be emphasized that this task can be made more or less difficult by altering the size of the pool, the age and thus the size of the rat, and the sensory cues available, including both those of the olfactory and visual modalities. It is not necessarily better to make it more difficult by using more subtle cues. Also, overtraining of animals, for example, extending the number of escape latency trials from 8 to 12, results in the disappearance of the difference observed in F2 n-3 Def vs n-3 Adq rats in the memory-retention trial (Moriguchi et al., 2000). What is critical is that there is a match between the level of difficulty of the task and the degree of impairment produced by the experimental intervention. In this case, the intervention is the reduction in brain DHA due to multigenerational deprivation of n-3 fat sources in the diet. This is a very subtle intervention in relation to those often used in the study of neuroscience, e.g., ablation of a brain nuclei or a chemical/pharmacological insult that may drastically alter neurotransmitter function. A task must be properly defined so that it has the correct sensitivity so that the differences in brain function between n-3 Adq and Def animals can be detected. The Morris water maze, as defined by Moriguchi et al. (2000), has the proper degree of difficulty so that it is a reliable and sensitive test for brain DHA deficiency. Some have succeeded at observing differences in the Morris water maze in n-3 deficient rats (Coscina et al., 1985; Frances et al., 1996; Jensen et al., 1996; Nakashima et al., 1993; Moriguchi et al., 2000) while in other experiments, no differences were observed (Wainwright et al., 1994, 1998). It is suggested that this is most likely attributable to the variations in the degree of difficulty of the task.

In this set of experiments, reversal learning using a two-odor olfactory-discrimination task was used for the first time on n-3 deficient animals. Tasks of this nature are well-known to the neuroscience and the sensory-behavior communities as a means of measuring set learning ability (Nigrosh et al., 1975; McBride and Slotnick, 1997). Here again, it is important to define tasks that have the proper degree of difficulty so that they are sensitive to the subtle permutation made by substituting DPAn6 for DHA in membrane phospholipids. Higher-level tasks such as those involving cognitive function are ones that may be more likely to differentiate between n-3 adequate and deficient brain states. Reversal learning provides an avenue for exploration of losses in cognitive function due to low brain DHA. The experi-

ment presented here was our first attempt at such an application and must be considered preliminary. In the future, it will be important to demonstrate that control animals continue to make fewer errors as the number of reversals increases in order to demonstrate the validity of this technique.

No differences were detected in the mean volumes of CA1, CA3/2, granular, and hilar layers in n-3 Adq and n-3 Def diet groups. Additional morphological parameters of neuronal density, total number and cell body size were subsequently collected to see if n-3 deficiency affected them. No differences were detected in the density or total number of neurons or cell body size of neurons in these dietary groups except that neurons in the CA1 region had significantly smaller cell bodies in animals on n-3 Def diet compared to animals on n-3 Adq diet (Ahmad et al., 2000).

Once it is established that there are changes in behavior that reflect a diminution in brain function in n-3 Def rats, the question arises as to what variable is responsible for the loss in function. The only difference between the two experimental diets was the addition of a small amount of flaxseed and algael oil to the n-3 Adq diet. The flaxseed oil supplied LNA and the algael oil supplied DHA. Of course, it cannot be ruled out that there were nonlipid substances in those oils that led to improved neural performance. However, this is highly unlikely. It is clear that the manipulation led to verification of the *a priori* hypothesis that a diet that led to decreased levels of brain DHA would also lead to losses in neural function as reflected by poorer behavioral performance. A more specific hypothesis is that the crucial dependent variable is the loss of neuronal aminophospholipid DHA (Salem and Niebylski, 1995) because these are the fractions where the DHA is most highly concentrated (Salem et al., 1986; Salem, 1989).

However, an alternate hypothesis may be that the increase in DPAn6 is responsible for the loss of function in the n-3 deficient state. This may be unlikely, but it is possible that DPAn6 would adequately substitute for DHA if preformed DPAn6 is fed in its place during neurodevelopment. That is, the brain-membrane requirement may be for any 22-carbon highly unsaturated fat, but these fats must be available to the brain at an adequate level during the rapid brain-growth spurt. The supply of linoleate alone may not be adequate as a source of DPAn6 because metabolism may be too slow to support optimal neural development (Lands, 1991). This hypothesis must be directly tested if the claim that the nervous system has a specific requirement for DHA is to be established.

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