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Developmental docosahexaenoic and arachidonic acid supplementation improves adult learning and increases resistance against excitotoxicity in the brain

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Through metabolic imprinting mechanisms a number of bioactive molecules including polyunsaturated fatty acids affect brain functions in the developmental age and longer-lasting beneficial effects are expected. In this study pregnant rats were offered diets either containing no docosahexaenoic acid (DHA) and arachidonic acid (AA) (Placebo diet) or an excess amount of these long chain polyunsaturated fatty acids (LC-PUFA) (Supplement diet) up to the time of weaning. Bilateral *N*-methyl-D-aspartate (NMDA) induced neurodegeneration in the entorhinal cortex of offspring in the age of 4 months was used as a tool to investigate the neuroprotective property of the developmentally supplemented DHA and AA treatments. Hippocampus-dependent spatial learning was measured in Morris water maze and the extent of neuronal lesion in the injected brain area was evaluated. Under baseline condition, in intact or sham-lesioned rats, the Morris water maze performance was superior in the supplemented group compared to the placebo controls. NMDA-lesion in the entorhinal cortex area decreased spatial learning in the supplement-treated rats while insignificantly diminished it in the placebo controls. The same supplementation attenuated the lesion size induced by the NMDA injection into the entorhinal and ventral hippocampal areas. We concluded that LC-PUFA supplementation during fetal and early postnatal development results in long-term enhancement of spatial learning ability of the offspring and offers resistance against excitotoxic brain lesion which lasts up to the adult age.

Keywords: LC-PUFA, brain development, learning, excitotoxic lesion, neuroprotection

Human and other mammals need essential fatty acids to synthesize long-chain fatty acids (LC-PUFA) including n-3 type of docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), furthermore, n-6 type of arachidonic acid (AA, 20:4n-6) (32). All of these fatty acids have ample amount of functional tasks to perform in the membranes as structural components of phospholipids, supporting membrane fluidity (41), neurotransmission through the membranes (35), initiation of intracellular molecular signaling and gene expression (10), acting as antioxidants, being metabolized towards eicosanoids from AA and EPA (27) or to neuroprotective substances like docosanoids, the DHA-derived bioactive lipids, like neuroprotectin D1 (5). For the developing brain during pregnancy both DHA and AA is needed in relatively high amount which might not be satisfied by endogenous synthesis in the mother (8, 37). Considering pathology, the developmental DHA deficiencies may be associated with foetal alcohol syndrome, attention deficit hyperactivity disorder, cystic

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fibrosis, phenylketonuria, unipolar depression (23), and suboptimal neurodevelopment, coronary heart disease and postpartum depression as discussed by others (26).

Developmental supply of long-chain polyunsaturated fatty acids (LC-PUFA's), first of all DHA, the endproduct of n-3 PUFA synthesis, is essential for brain growth and for maturation and functioning of retina in human infants (7). Membrane phospholipids of neuronal synapses and retina are especially rich in DHA (13, 36). LC-PUFA deficiency might be critical under demanding conditions like brain growth in the last three months of pregnancy and at neonatal age up to 3–4 months and a positive LC-PUFA status is associated with a better neurodevelopmental outcome (1, 29).

To prove the health promoting evidence for a supplementation of these fatty acids and especially that of DHA during not only pathological but also apparently normal early development was the aim of a number of studies carried out in human population (14, 15, 27, 39). Some of these studies confirmed the beneficial effect of supplementation, DHA alone and together with AA were effective (11, 20), while there are reports with no effect (18). Experimental studies on animals reporting beneficial effects of developmental supplementation with first of all DHA on attention, learning and memory are scarce and such treatments were mainly performed in adult age in which it improved cognition (3, 16, 19). Conversely, an inhibited cognitive function in case of DHA deficiency was more frequently reported in rats (6, 28, 40, 42).

In our previous study we have shown that excitotoxic lesion of cholinergic neurons was attenuated by developmental treatment of LC-PUFA (21). The brain lesion occurred during the end phase of developmental treatment at 2 weeks of age. The question how long the beneficial effects of the developmental treatment last in the course of further lifespan was left open. Here we investigated the adult age of 4 months which was well after finishing the treatment at the weaning age. Two questions have been asked in the present study: (1) how the capacity of spatial learning and memory appears in the latent 4 months adult age after the developmental supplementation with DHA and AA, and (2) based on the well-documented neurotrophic action of DHA during development whether or not it is reflected in resistance against excitotoxic brain lesion also in the adult age? Two synthetic types of diets were offered to the pregnant rats, one was lacking LC-PUFA's as DHA, EPA and AA, and another was supplemented with these fatty acids. For the excitotoxic lesion model we have selected the glutamate analogue NMDA-induced lesions in the entorhinal cortex (33). Entorhinal cortex is a relay structure between the hippocampus and the neocortex and plays a key role in hippocampal functions as learning and memory.

Methods

Animals and treatment

Female Harlan–Wistar rats were started feeding with one of the experimental diets about one week prior to conception. The pregnant rats were kept on feeding with the same diet throughout the entire pregnancy period and also during lactation until weaning. The mother rats were fed either by (1) Supplement formula, containing high concentrations of DHA, EPA and AA in addition to the essential fatty acids as precursors, linoleic acid (LA) and α-linolenic acid (LNA), or by (2) Placebo formula, which did not contain the end product PUFA's, only the precursor fatty acids LA and LNA but even these essential fatty acids in low range. The Supplement and Placebo foods were synthetic formulas containing appropriate amounts of proteins and carbohydrates (originated from Numico Research Laboratories, Wageningen,

The Netherlands). The fatty acid compositions of the diets are listed in Table I. In addition to saturated fatty acids (SFA) and mono-unsaturated fatty acids (MUFA), the different diets contained n-6 and n-3 PUFA's. The difference between Supplement and Placebo foods is obvious, since the Supplement food was created in a way to provide a large amount of DHA, EPA and AA, the end products of n-3 and n-6 types of fatty acid synthesis, while the Placebo food lacked LC-PUFA's end products (Table I). Both types of food were comparable to each other in providing equal amount of fat as energy. In the Placebo food mainly MUFA's replaced LC-PUFA's (21). Both diets contained the precursor fatty acids, LA and LNA, which are necessary to the endogenous synthesis of n-3 and n-6 types of LC-PUFA's. For more details about the diets consult our previous paper (21) (Table I).

Table I. Fatty acid composition of diets used in the experiment

Fatty acids	Placebo	Supplement
Total SFA	**9.3**	**20.0**
Total MUFA	**71.2**	**20.6**
18:2n6 (LA)	15.1	32.3
18:3n6	0.2	0.6
20:3n6	0.0	0.3
20:4n6 (AA)	0.0	9.4
Total PUFAn6	**15.3**	**42.6**
18:3n3 (LNA)	3.3	3.5
20:5n3 (EPA)	0.0	2.6
22:6n3 (DHA)	0.0	13.6
Total PUFAn3	**3.3**	**19.7**

Values of different fatty acids are expressed in percentages of total amount of fatty acids as 100% (see also 21).
Abbreviations: SFA – saturated fatty acids, MUFA – mono-unsaturated fatty acids, LA – linoleic acid, AA – arachidonic acid, LNA – α-linolenic acid, EPA – eicosapentaenoic acid, DHA – docosahexaenoic acid

Supplement and placebo-treated male offspring were randomly grouped together in the housing cages (3 rats per cage) after weaning. The rats were provided with free access to food and water at a standard temperature of 23 ± 1 °C in a light-controlled room (light on from 07:00 a.m. to 07:00 p.m.). Behavioral test was performed during the light phase between 9:00 a.m.to 1:00 p.m. The supplemented and placebo rats were divided into three groups: (1) left intact, (2) subjected to sham-lesion, and (3) subjected to NMDA lesion. The animal experiments were carried out in accordance with the European Community Council Directive for the care and use of laboratory animals and were approved by the Scientific Ethical Committee on Animal Experimentation, Animal Health Care Station, Lehel u. 43–47, H-1135 Budapest, Hungary (1327/003/Fov/2005).

Excitotoxic lesion in the enthorinal cortex with NMDA

Partial lesioning of the entorhinal cortex was carried out bilaterally. Rats were anaesthetized with sodium pentobarbital by intraperitoneal injection and positioned in a stereotactic frame. With a Hamilton syringe of 5 µl, 30 nmol*N*-methyl-D,L-aspartate (NMDA, Sigma, St. Louis) dissolved in phosphate-buffered saline pH7.4 (PBS) was injected bilaterally into each of three different locations of the entorhinal cortex. The volume of each injection was 0.5 µl and

the infusion time was 0.1 µl/min. At the end of each injection the needle was left in place for another 3 min to prevent loss of the toxin in the needle track. Altogether 6 injections (6 times 30 nmol NMDA) were applied to the entorhinal cortices. The stereotaxic coordinates measured from Bregma according to the atlas of Paxinos and Watson (34) were: craniocaudal –6.4, –6.7, –6.8; lateral 4.0, 2.4, 3.5; dorsoventral 8.2, 7.8, 7.4. The injection locations were approached at an angle of 15 degrees. Sham-lesioned animals received PBS solution alone. Animals were allowed to recover after surgery for a week before the behavioral testing was started.

Spatial learning in Morris water maze

The test procedure started at the 8th postoperative day and continued for another 4 days, i.e. 5 daily sessions were performed. The Morris water maze test (31) was carried out in a black round water tank (diameter 153 cm, height 63 cm) filled to a depth of 53 cm with water of 26 ± 1°C. The tank was divided into 4 (imaginary) quadrants. A black hidden platform with a diameter of 8 cm was submerged in the center of one quadrant 1.5 cm below the water surface. Four different starting positions were equally spaced around the perimeter of the tank. The animals had to learn to find the hidden platform orientating by different visual cues placed on the wall of the experimental chamber. The order of starting positions varied randomly between sessions, but was constant within a daily session. Four trials were given per daily session. Rats were placed into the water at a starting position facing the wall of the tank. Each trial lasted until the rats found the platform. If the platform was not found during 90 sec, the operator led the animal to it. Rats spent 30 sec on the platform at the end of each trial. Latency time to find the platform was measured during each trial. Spatial learning performance was reflected by the mean escape latency calculated by averaging all four trials at each session. After the conclusion of the 5-day-long learning period the probe trial was given in which the platform was abolished from the maze and the rats were subjected for an additional swimming trial. During that memory test the time spent around the removed platform, within a circle of 15 cm, was measured 30, 60 and 90 seconds after starting the trial. The results were depicted in a cumulative way.

Brain tissue analysis

a) Brain fixation and sectioning. Brain fixation was carried out in the NMDA- and sham-lesioned animals at the 13th postoperative day by transcardial perfusion of heparinized PBS pH 7.4 followed by 4% paraformaldehyde (Sigma-Aldrich, Hungary) solution containing 0.05% glutaraldehyde (Sigma-Aldrich, Hungary) in 0.1 M phosphate buffer (PB, pH 7.4) after adequate deep pentobarbital anaesthesia. After postfixation of the brains for 48 h in the same fixative the brains were stored in 0.1 M PB containing 0.1% Na-azide until histological examinations. For histological processing the brains were cryoprotected by storage in PB containing 30% sucrose and sectioned in a Leica cryostat microtome at a thickness of 30 µm to obtain horizontal sections of the entorhinal cortex and ventral hippocampus in sham-operated control and NMDA-lesioned animals.

b) Immunocytochemistry (ICC). Immunohistological staining was used for identification of neuronal loss and the degree of microglia activation around the lesion. Mouse anti-neuron-specific nuclear protein (Neuronal Nuclei, NeuN, MAB377, Chemicon) antibody recognizes most neuronal types in the central nervous system but not glial cells. Therefore, it is an adequate tool for outlining the precise area of loss of neurons in an anatomical structure after different lesions. This staining, therefore, allows a quantitative measurement of lesion size,

which reflects the area of neuronal loss. The antibody against the integrin, CD11b, was selected to label activated microglia. In the control brain baseline level activity of microglia is minimal and hardly visible after immunocytochemical staining. Microglia activation becomes markedly enhanced in and around the lesion or degeneration of the nervous tissue. Mouse anti-rat CD11b (MAB1405Z, Chemicon) as primary antibody was used in the microglia immunoassay. Both NeuN and CD11b antibodies were applied in a dilution rate of 1:1000. The biotinylated secondary horse anti-mouse antibody and the Vectastain ABC kit were obtained from Vector Labs (CA, USA) and used in 1:500 dilution rates. The staining was completed with nickel-enhanced diaminobenzidine (DAB, 3,3'-diamino benzidine, Sigma) reaction in the presence of H_2O_2.

The extent of neuronal loss and that of microglial activation around the lesion were manually delineated and quantified with the Quantimet 600 (Leica) image analysis system in the NMDA-lesioned rats. The sham lesion did not evoke neuronal loss or considerable microglia activation consequently the sham-lesioned animals were not analyzed except some examples. Two brain sections were selected (at Bregma levels of –7.6 and –6.4) per animal and the measurements were averaged.

Statistics

Statistical comparisons of behavioral data between groups were carried out using two-way ANOVA with repeated measures on factors of treatment and sessions (SigmaPlot 12.1). In cases in which the overall ANOVA indicated significant effects, post hoc analysis was applied using Fischer's LSD test. For the morphological data Student's *t*-test was applied. The statistical significance was set at $p < 0.05$. Means and standard errors of means (SEM) were presented to demonstrate the results.

Results

Spatial learning in Morris water maze

The spatial learning data are summarized in Fig. 1 which was evaluated by two-way ANOVA with repeated measures. In the intact and sham-operated groups there were differences between the supplement and placebo groups in the learning of spatial learning paradigm ($F_{1,14} = 12.91$, $p = 0.003$ and $F_{1,16} = 12.18$, $p = 0.003$, respectively). The same treatment effect could be found also in the data of probe trial: intact groups: $F_{1,14} = 24.77$, $p < 0.001$; sham-operated groups: $F_{1,16} = 8.86$, $p = 0.009$. Regarding the lesioned groups no difference could be found: spatial learning: $F_{1,25} = 2.22$, $p = 0.148$, probe trial: $F_{1,25} = 0.691$, $p = 0.414$. This means that the NMDA lesion abolished the originally existing group difference in the learning capability.

Using two-way ANOVA with repeated measures the performance of the supplement versus placebo groups were compared separately. There was a difference among the three PUFA-supplemented groups: $F_{2,27} = 7.91$, $p = 0.002$, the performance of the intact and sham groups did not differ ($p = 0.60$) but the NMDA group performed inferior compared to both the intact ($p = 0.001$) and the sham ($p = 0.006$) groups. Among the three placebo control groups no significant difference could be found ($F_{2,28} = 1.99$, $p = 0.156$), i.e. the lesion did not worsen their performance. These results showed again that the supplemented animals were sensitive to brain damage during learning and performing memory.

Both experimental groups under all the three conditions (intact, sham and NMDA) showed clear learning effect along the 5 daily sessions, i.e. the latency to find the platform

decreased significantly ($p < 0.001$ in all the three experiments). Significant interaction between the factors of treatment and sessions was only found in the experiment with intact rats ($F_{4,79} = 3.75$, $p = 0.009$) in spatial learning. The same was true in case of probe trial: $F_{2,47} = .42$, $p = 0.003$. This result means that any brain manipulation, even sham-lesioning disturbs learning progression in a detectable way. The results of post hoc analysis of differences between the groups along the sessions and minutes of observation in the probe trial are shown in Fig. 1. These analyses clearly show that in the intact and sham conditions there were differences between the supplemented and placebo control groups. In case of lesioned rats the insignificant difference with ANOVA was accepted and was not followed by post hoc *t*-test analysis (Fig. 1).

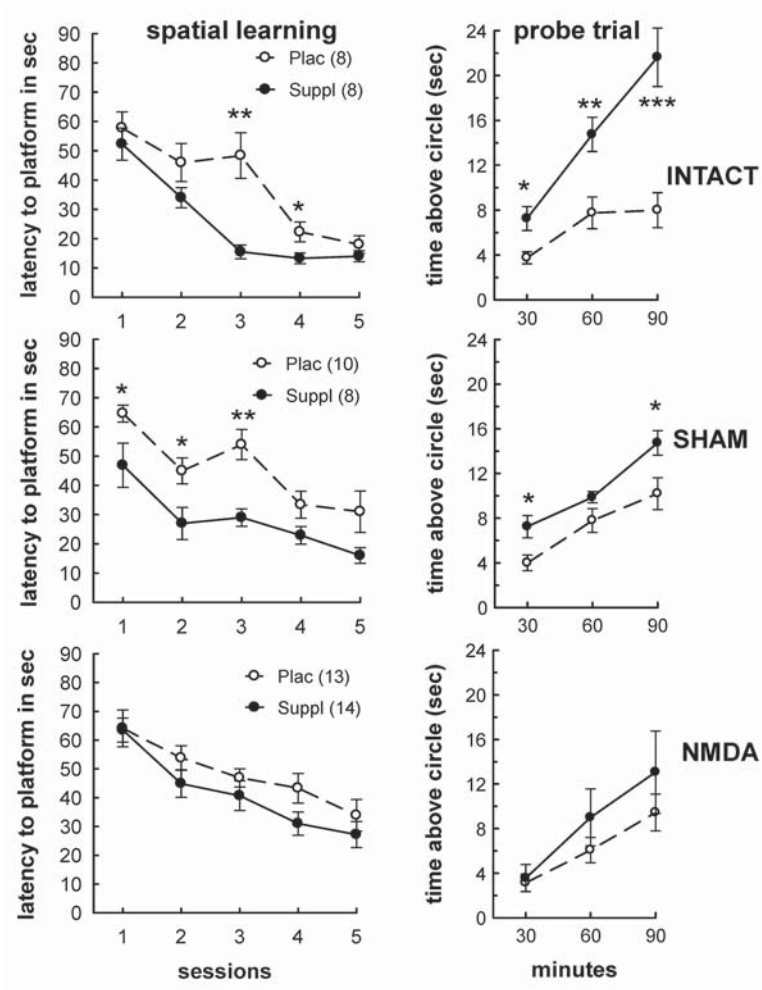

Fig. 1. Morris water maze spatial learning (left side panels) and retention of learned response during probe trial (right side panels) performed at 4 months of age. Rat offspring were subjected to either supplement (Suppl) or placebo (Plac) diets during development. Three experiments were performed with intact, sham-lesioned and NMDA-lesioned rats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between groups. The number of rats in the different groups is shown in brackets

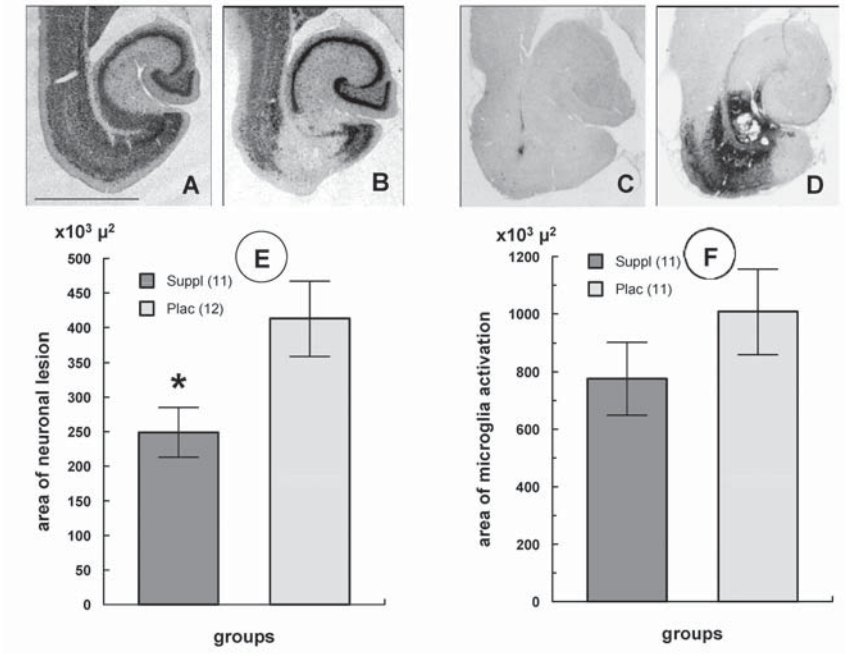

Fig. 2. Effect of NMDA lesion on the size of neuronal loss (panel E) and the extent of activated microglia invasion around the lesion (panel F) in adult offspring of dams supplemented (Suppl) and placebo-treated (Plac) during fetal and neonatal ages. The upper figures show the size of neuronal lesion (panel B, NeuN ICC staining), and the extent of microglia activation (panel D, CD11b ICC staining) against the appropriate sham-injected controls (panel A and C, respectively). The bar at panel A represents 1 mm. The number of rats in the different groups is shown in brackets

Lesion size and extent of microglia reaction

Figure 2 shows the size of neuronal lesion (panels A, B) and the extent of microglial activation (panels C, D) in the entorhinal cortex-ventral hippocampus region. The lesion largely resigned into the enthorinal cortex but also invaded the ventral hippocampus CA1 pyramidal layer (panel B). The areas of loss of NeuN positive neurons and the extent of microglia activation were counted at Bregma levels of –7.6 and –6.4 and the results were averaged. It is shown in the figure that the PUFA supplements diminished the size of neuronal lesion ($t = 2.46$, $df = 21$, $p = 0.022$) which amounted to 40% (panel E). The size of microglial invasion inside and around the lesion also decreased but insignificantly (panel F, 23% decrease, $t = 1.19$, $df = 19$, $p = 0.25$). It is obvious that the size of microglia activation was much larger than that of neuronal loss ($t = 4.20$, $df = 19$, $p < 0.001$, compare panels B and D as well as placebo groups in panels E and F), i.e. microglia reaction was intensive in the brain region surrounding the lesion core, which region corresponds to the penumbra (Fig. 2).

Discussion

Polyunsaturated fatty acids (DHA, EPA, AA) supplementation during fetal and early postnatal development of rats resulted in a long-term change in those brain mechanisms which are responsible for learning and memory. The supplement diet-exposed offspring learned better

the spatial learning task as compared to the placebo-exposed controls in the 'Intact' and 'Sham' experiments, in which animals were not disturbed or were sham-operated before the behavioral test, respectively. This effect was obvious during both the entire 5-session-long learning period and also in the probe trial testing retention of memory. However, if the supplement diet-treated rats were exposed to NMDA lesion the superior learning performance against placebo controls was abolished and also the better memory performance during the probe trial was dismissed. These behavioral results first suggest that the LC-PUFA supplementation enhanced learning and memory functions shown in the Morris task. Secondly, when the entorhinal cortex hippocampal region was damaged the learning deficit became overt in this group. Contrary to the results obtained in the supplemented offspring the decline in learning performance of the NMDA-treated placebo control rats was minimal against the sham-lesioned and intact placebo animals (statistically not significant). Thus, in this behavioral phenotype the function of entorhinal cortex-hippocampal unit worked probably on a lower efficiency level from the beginning of the post-developmental period. It may be recalled here that the placebo food did not contain end-product LC-PUFA's and in this sense it was a PUFA-deficient diet compared even to the standard rat chow. It is known from our earlier study (21) that the placebo diet contained 3.4 times less LA and 2.1 times less LNA as the standard (control) diet. In addition, the standard diet contained a minimal amount of AA and a considerable amount of EPA and DHA while as shown in Table I the placebo food contained none of them. Therefore, it may be assumed that the function supporting learning and short-term memory in the entorhinal cortex-hippocampus unit was relatively underdeveloped or disturbed in response to demand in this group.

In the other part of this study the extent of lesion was studied and compared between the two groups. This part of the study attempted to answer the question whether the developmental treatment with the supplement diet evoked a longer-term neuroprotective effect against glutamate excitotoxity or not as it had been already found earlier in the postnatal period of two weeks (21) in which age the supplementation proved to be neuroprotective on cholinergic neurons. In the present study it was found that in the adult age of 4 months the entorhinal cortex neurons showed some but significant resistance against the excitotoxic lesion evoked by injection of glutamate analogue NMDA in the supplemented offspring. Thus, the supplement diet proved to be neuroprotective well after the termination of supplying the supplementing diet and decreased the size of neuronal lesion shown on NeuN-stained sections. The brain lesion was accompanied by microglia activation and the size of area filled with activated microglia was also measured around the lesion. Although there was a tendency for a decline in the size of microglia activation in the supplemented group this decrement was not significant. It may be concluded that imprinting of microglia function during development by LC-PUFA's followed different rules.

The finding that NMDA lesion decreased learning performance of supplemented rats although the LC-PUFA supplementation evoked certain neuroprotective action, i.e. decreased the lesion size, needs some clarification. The explanation might be that the neuroprotective effect was partial and not sufficient to prevent the learning deficit after NMDA lesion in this phenotype.

The long-term effects of n-3 LC-PUFA supplementation during development may be explained by the metabolic imprinting mechanism occurring in the developing brain (38). It may be hypothesized that these PUFA's are involved in metabolic programming and cognitive functions during fetal development. Other nutrients like for example choline has also been shown to exert functional imprinting regarding learning and memory (30). Fetal

undernourishment (4) as well as feeding of a cafeteria diet (17) may predispose obesity and related metabolic diseases in the offspring and these perinatal conditions are associated with low maternal PUFA availability for the developing fetus (38). Metabolic imprinting is accompanied by epigenetic molecular changes, but results on these processes on LC-PUFA's have not been available yet. It is known that during brain development not only n-3 LC-PUFA's are necessary but also AA and not only for normal body growth but also for a proper brain development. The time-dependent equilibration between n-6 (AA) and n-3 LC-PUFA's are necessary for a normal brain growth (22). In the present study the supplement diet met this requirement. In summary, DHA stays as the primary candidate for neuronal development in relation to cognition, and in some *in vitro* experiments it has been shown that DHA promotes neurite outgrowth of PC12 cells and also growth cone behavior triggered by nerve growth factor (24).

The function of hippocampus in cooperation with the entorhinal cortex as a relay cortical area towards neocortex is central in the execution of learning and memory processes (9, 12). A number of observations show that damaging of this hippocampal-cortical connection impairs learning (2, 25, 33). These structures proved to be more sensitive to NMDA lesion in supplemented offspring as compared to placebo treated offspring. It was concluded that the supplement diet probably increased the learning potential of entorhinal cortex–hippocampal complex in performing the Morris spatial learning task. By this way one of the LC-PUFA-effected functional brain areas may be named and that is the entorhinal cortex-hippocampal area.

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