Summary of Ph.D. Thesis

EFFECTS OF POLYUNSATURATED FATTY ACIDS ON BRAIN DEVELOPMENT AND AGING

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2004

Introduction

There has been growing interest in long chain polyunsaturated fatty acids (LC-PUFAs) and their role in the brain in the last few decades. Mainly docosahexaenoic acid (DHA) and arachidonic acid (ARA) seem crucial for neurons. It has been revealed recently, that the brain requires these fatty acids in order to develop and function properly. The lack or low intake of given DHA and ARA causes serious problems in learning, memory, visual and auditory functions.

Brain growth and functional development also require adequate supplies of ARA and DHA in infants. Lack of both DHA and ARA disturbs neural integrity and function of fetus and neonates as well. The main DHA increments in brain parallels brain growth spurt, and synaptogenesis in rats. ARA content of brain is also increasing during the maturation of the brain. There are several pathological cases were reported to be related with inadequate DHA intake during brain development. It has also been revealed that decreased DHA concentration in the brain was accompanied with poorer performance in learning tasks in rats. Moreover, there is an age-related LC-PUFA loss in the brain, which may related to an impairment in cognitive function in aged rats. Similar decline in cognitive function is observed in spontaneously hypertensive rats (SHR), and the changes in the brain fatty acid content may play role in this impairment. The effect of the lack or decreased amount of LC-PUFAs in the brain are well known, but beneficial effect of LC-PUFA supplementation on developing and aging brain is relatively not well studied. In this present work, we try to demonstrate the positive effect of LC-PUFA treatment on brain function in newborn, aged and SHR rats.

Aims

1. We proposed that dietary manipulation of first of all DHA supplementation may be able to serve as a neuroprotector in the infant rats. Testing this theory, dams were fed with three types of

2. diet, a PUFA deficient, an n-6 PUFA sufficient with little amount of n-3 PUFA, and a both n-6 and n-3 sufficient. Pups of these dams were injected with NMDA into the nucleus basalis magnocellularis (NBM) at the age of 14 days. Cholinergic neurons of NBM project fibers to the ipsilateral neocortex, and play important role for in maintaining normal function of neurons there. Two days after the injection, a decreasing number of neurons in the NBM and, the degeneration of fibers in the neocortex were measured. Fatty acid composition of PE, PC, PS, and PI were also determined.

3. In our experiment, we aimed at showing the beneficial effect of maternal LC-PUFA supplementation on learning performance. Dams were divided into three groups and received three different types of diet during pregnancy and lactation. The three diets contained LC-PUFAs in various amount, Placebo was deficient in both n-6 and n-3 PUFAs, Control showed normal physiological state: high amount of n-6 and little amount of n-3, while Supplement group was enriched in both types of LC-PUFAs. Brain fatty acid content was followed during aging, and the rats performed spatial discrimination learning task at the age of 12 month and 26 month.

4. We aimed at demonstrating whether an increasing level of DHA in neural membranes of old rats could be associated with improved learning performance. 24-moths-old rats were fed either fish oil supplemented chow or normal chow. After 1 month of feeding, Morris water maze performance was tested, and fatty acid composition of rat brain was also determined.

5. Our goal was to find relationship between hypertension and brain fatty acid content, and their effect on learning performance in aged rats. Rats received either LC-PUFA supplemented diet or control diet through their life. The systolic blood pressure, brain fatty acid content and spatial discrimination learning task were measured, and the data were correlated to reveal beneficial effect of LC-PUFAs on hypertension and learning performance.

Materials and Methods

Excitotoxic lesion of NBM cholinergic neurones.
20 nmol of racemic mixture of NMDA (Sigma, ST. Louis, MO, USA) in 0,4 µl phosphate-buffered saline (PBS, pH 7,4) was injected unilaterally in the right hemisphere in a volume of 0,1 µl into the nucleus basalis magnocellularis (NBM). A stereotaxic frame was used for positioning the head of pups. The intact left hemisphere served as control side for each individual case.

Cholinergic cell number.
Choline acetyltransferase (ChAT) and p75 low-affinity neurotrophin receptor protein ($p75^{NTR}$) were stained with immunocytochemical methods in the NBM to assay cell
bodies and dendrites. The total number of stained cholinergic cell bodies were counted in the NBM region around the level of NMDA lesion in both injected and non-injected sides. The loss of neurones at the injected side was calculated as a percentage of the cell number at the intact side, which was considered as 100%.

Dendrite density.
Computerized image analysis system (Quantimet 600HR, Leica, Germany) was used to measure the degree of dendrite arborisation in the penumbra region. We measured both types of cholinergic markers (ChAT and $p75^{NTR}$) for the analysis.

Axon degeneration.
The degree of axon degeneration was measured from neocortex, where NBM projects fibers ipsilaterally, after 48 hours survival time. Cholinergic fiber density was assayed on superficial layers (I to IV), and deeper layers (V and VI) separately.

Fatty acid analysis.
Lipid classes were separated on TLC plates (20x20, Silica G 60) using methyl-acetate/i-propanol/chloroform/methanol/0,25% KCl (25:25:25:10:9 vol/vol) mobile phase. Lipids were transmethylated in presence of absolute methanol containing 5% HCl for 2,5 h at 80 °C. Fatty acid methyl esters were determined by gas chromatography (Hewlett-Packard Model 6890) with FFAP column (30 mX0,32

mmX0,25 μm film thickness; Supelco, Bellefonte, PA, USA). To identify peaks, Supelco fatty acid standards were used (Catalogue No. 4-7085-U, 4-7015).

Holeboard spatial learning task.
The floor of testing box contained 16 small removable pits in rows of four, and chocolate chips were placed in four of them always in the same pattern. The animals had to learn to find only the baited holes during a training of 7 days (two trials per day.) The trials were ended either after 3 minutes, or when the rats found all four baited holes. Reference memory score was calculated for each trial as: Ref. Memory = Number of visits and revisits to baited holes/ Number of visits and revisits to baited holes + Number of visits to non-baited holes. The equation reflects the learning ability.

Morris water maze learning task.
The animals performed four daily trials on five consecutive days. The escape latency reaching the hidden platform was registered at each trial. The place of the hidden platform was unaltered during the experiment. The trials were terminated when the rat climbed on the platform, or 90 s had elapsed. The daily mean escape latencies and first trial at each daily session were evaluated.

Blood-pressure measurement.
A tail-cuff method was used to measure systolic blood pressure (BP) at the ages of 53, 54, 55 and 79 weeks.

Results

Results of the neuroprotective effect of LC-PUFAs.
Due to the high amount of n-3 fatty acids in supplement diet, the ratio of DHA increased in PE (Table 1.) compared to both Control and Placebo. Competing n-3 fatty acids with n-6 for the same place in phospholipids, the increased DHA caused decreased in the level of ARA and 22:5n6 compared to Placebo and Control, while 22:4n6 remained unchanged.

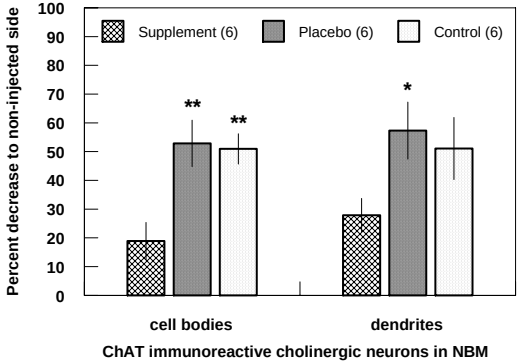
Table 1. Percent fatty acid content of phosphatidylethanolamine derived from forebrain of infant rats

Fatty acids	Supplement	Placebo	Control	Anova *P*
20:4n6 (ARA)	17,57±0,24**	18,82±0,28	17,83±0,59	0,016
22:4n6	4,70±0,13	4,78±0,11	5,13±0,27	0,233
22:5n6	1,08±0,03**§§	2,89±0,07##	2,26±0,08	0,000
22:6n3 (DHA)	25,19±0,71**§§	19,36±0,39	20,45±1,34	0,000

Means±S.E.M.s are shown; **P*<0,05; ***P*<0,01 versus Placebo; §*P*<0,05; §§*P*<0,01 versus Control; ##*P*<0,01 versus Control.

Similar results were obtained from the rest of the phospholipid classes.
The results of ChaT immunostaining demonstrate (Fig.1.) that more neurons survived in the Supplement group than in both the Placebo and Control groups (left side, *P*<0,005). Only 18,9±6,5% of neurons died in the Supplement group which was significantly lower than in the case of the other two diets (Placebo: 52,8±8,1%, *P*<0,005 versus Supplement; Control: 50,9±5,3%, *P*<0,005 versus Supplement). The dendrite arborisation of survived neurons remained more preserved in the Supplement group compared to the Placebo (*P*<0,05 versus Supplement). There was no significant difference between the Placebo and Control groups either survived neurons or dendrite arborisation.

Fig.1.



The results of the another cholinergic marker, $p75^{NTR}$, supported the results of the ChAT labeling. The degree of lost neurons was markedly lower in the Supplement group than in the Placebo and in the Control (P=0,025; post hoc t-tests: P<0,05 Placebo versus Supplement; P<0,02 Control versus Supplement). The dendrite arborisation seemed more vulnerable in the Placebo group, the degree of loss was higher, (P=0,128; P<0,05 Placebo versus Supplement). No difference was obtained between the Control and Placebo groups in any case with this method either. The loss of projecting axons to the cortex was measured at superficial layers (I-IV) and deeper layers (V-VI). The effect of diets showed significant effect (P<0,05) on the relative loss AChE fiber density. The degree of degeneration was more pronounced in the superficial layers than in the deeper layers (P<0,001,). Lower fiber loss was observed in the superficial layers in the Supplement group compared to the Placebo (P<0,02 versus Supplement) and Control (P<0,05 versus Supplement). At the deeper layers significant difference was observed only between the Supplement and the Control groups (P<0,05).

Results of the late effect of maternal LC-PUFA supplementation.

After using multivariate two-way ANOVA analysis, it has been revealed that the ratio of all fatty acids in the brain changes significantly during aging (P=0,000).

Briefly, the content of both main SFAs, 16:0 and 18:0 gradually decreased from birth till adulthood, but increased in the old rats. The level of all types of MUFAs was increasing during aging, mainly between birth and adulthood; however slight reduction was measured in the 26 months old rats. The most marked changes were obtained in the level of 18:1n9. The amount of oleic acid increased with 286%, 271% and 299% in the Supplement, Placebo and Control groups, respectively. It seemed that the brain was not able to retain the high amount of various PUFAs. ARA, which is the most important n-6 fatty acid, showed an age-dependent, dramatic decrease till weaning (34%, 39% and 34% in the Supplement, Placebo and Control groups), but only little after it (only 14%, 17% and 19% in the Supplement, Placebo and Control groups from the age of 35 days till the age of 26 months). Level of DHA was diminishing continuously during aging (47%, 34% and 32% in the Supplement, Placebo and

Control groups. DPA appeared to substitute the lost ARA and mainly DHA, the level of DPA increased in the old rats again. But the decreasing ARA and DHA were replaced by MUFAs in adulthood, and MUFAs and SFAs in old rats. However, the effect of maternal supplementation was subsided after weaning.

First, at the youngest age group (right after birth) the level of DHA increased significantly at expense of n-6 PUFAs (specifically DPA and ARA) and MUFAs (18:1n7, 18:1n9) due to the DHA-enriched diet. At the same time, the concentration of n-6 PUFAs and MUFAs reduced significantly in the Supplement group compared to Placebo or Control. The level of 16:0 decreased in the Supplement group compared to Placebo group, too. The following age group, where fatty acids were measured, was day 35, the time of weaning. Significant difference was not observed in the case of MUFAs and ARA in the Supplement group compared to the Control and the Placebo groups. DHA remained significantly higher in the Supplement group than in the Control and Placebo groups, while DPA remained significantly lower in the Supplement group than in the Control and Placebo groups. There was no difference in the fatty acid content of brain between the dietary groups in the adult (Table 2.)

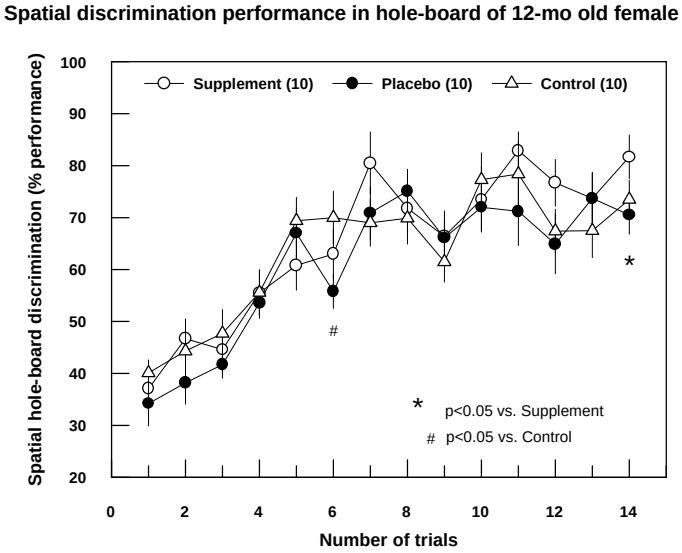
Table 2. Fatty acid content of PE from 13 months old rat brain

Fatty acids	Supplement	Placebo	Control	Anova P
SFA				
16:0	3,96±0,24	4,95±0,42	4,39±0,17	0,107
18:0	13,04±0,26	14,10±0,53	13,52±0,75	0,336
MUFA				
18:1n7	2,35±0,05	2,23±0,13	2,32±0,09	0,662
18:1n9	13,96±0,27	15,20±0,81	14,18±0,36	0,266
20:1n9	3,49±0,18	3,34±0,28	3,07±0,17	0,482
PUFA				
20:4n6 (ARA)	10,88±0,15	10,64±0,47	11,00±0,18	0,746
22:4n6	5,70±0,18	5,07±0,27	5,35±0,08	0,118
22:5n6	0,41±0,01	0,43±0,02	0,48±0,03	0,156
22:6n3 (DHA)	17,74±0,35	15,12±1,11	16,73±0,35	0,065

Significant difference was not found in the case of old rats, either.

There was no considerable difference between the dietary groups in adulthood in learning performance (Fig.2.). However, the Placebo group tended to perform worse than the other two groups, but significant differences were observed at only two trials on day 6 and 14. Supplement and Control groups did not differ from each other.

Fig. 2.



Results of the study of short term fish oil administration in old rats.
Due to the high amount of DHA in the diet, the level of DHA in the brain elevated in the Fish oil group from 15,97% to 17,21% (P<0,05). This was the only significant alteration. (Table 3.)

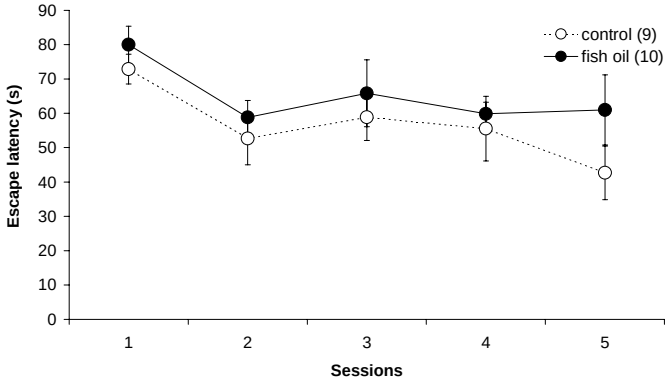
Table 3. Fatty acid composition of PE in old rat brain

Fatty acid	Control	Fish oil
16:0	5,75±1,56	5,52±0,13
16:1n7	0,73±0,29	0,63±0,15
18:0	13,81±0,74	13,83±0,81
18:1n9	13,22±0,76	13,72±0,56
18:1n7	5,44±0,53	5,59±1,28
18:2n-6	0,83±0,22	0,61±0,20
20:1n-9	3,39±0,33	3,69±0,62
20:4n-6	10,92±1,05	9,32±0,57
22:4n-6	5,60±0,38	5,05±0,35
22:6n-3	15,97±0,56*	17,21±0,54

Means±S.E.M.s are shown; *P<0,05.

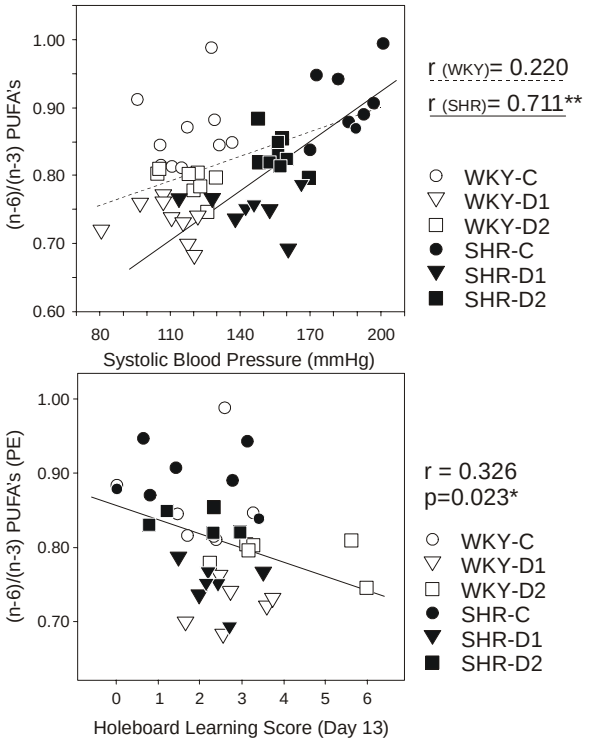
Fig. 3. shows that there was no difference in Morris water maze task between the two groups. It seemed that despite increased level of DHA in PE, the learning performance was not improved.

Fig.3. Morris water maze performance in old rats.



Results of the long term LC-PUFA feeding in SHR rats.
The (n-6)/(n-3) ratio seemed the most sensitive to both diet and hypertension. Both experimental diets reduced the (n-6)/(n-3) ratio, while blood pressure had opposite effect on this ratio.. Fig 4. represents the correlation between blood pressure and (n-6)/(n-3) in PE. A positive correlation can be observed between the two parameters. Furthermore, (n-6)/(n-3) ratio correlated directly with learning score in the holeboard test. We found strong negative correlations (Fig. 54.)

Fig. 4. Correlation of systolic blood systolic blood press on week 53 and (n-6)/(n-3) ratio in PE, and of (n-6)/(n-3) ratio in PE and learning score.



Summary

Dietary DHA supplementation was used in different ages to prove the beneficial effect of DHA on the brain function in rats. There are two main susceptible ages for the DHA supplementation: fetal age, the time of brain development and aging, when the brain looses higher amount of DHA. We focused on these two periods in our experiments.

1. Using different types of semi-synthetic diets we showed that LC-PUFAs, mainly DHA had neuroprotective effect on NMDA excitotoxicity in 14 days old rats. We found more neurons survived in the nucleus basalis magnocellularis (NBM) and less axonal degeneration in the cortex after NMDA injection into the NBM, in the pups whose mother received LC-PUFA supplementation during pregnancy and lactation, compared to control (enriched in n-6 PUFAs, but poor in n-3 PUFAs) and placebo (lack of both types of LC-PUFAs) groups. The level of DHA was elevated in all phospholipid classes in the supplemented group compared to the control and placebo groups. In conclusion, developmental DHA treatment increased the viability of cholinergic neurons.

2. Furthermore in studying the latent functional consequences, we have found that DHA deficiency during brain development had long-term effect on cognitive function of rats. The dams received the same diet as above during pregnancy and lactation. After weaning the pups received the same control diet. The placebo group performed worse in spatial discrimination learning task at the age of 1 year, than control and supplemented groups. However, the brain fatty acid profiles in the three groups have been restored by this age.

3. Related to aging, short-term fish oil supplementation did not improve learning performance in 24 moths old rats. After 1 month of fish oil treatment, the level of DHA in the brain increased in old rats, compared to controls. However, the fish oil group did not perform better in the Morris water maze task. It indicated, that restoration of the level of DHA was not enough to improve learning performance.

4. LC-PUFA administration from 4 to 80 weeks of age, and hypertension had huge impact on brain LC-PUFA status and on

learning performance (hole-board spatial discrimination test) in old spontaneous hypertensive rats (SHR). Hypertension decreased the level of LC-PUFAs and the ratio of n-3/n-6 PUFAs in the brain, while long term LC-PUFA feeding increased the level of LC-PUFAs and the ratio of n-3/n-6 PUFAs. We found strong positive correlation between blood pressure and the ratio of n-6/n-3 PUFAs, and strong negative correlation between learning performance and the ratio of n-6/n-3 PUFAs.

Acknowledgement

I am very grateful to the following persons for contributing in my Ph.D. project during the last couple of years:

Prof. Dr. Tibor Farkas my supervisor, who introduced me to the word of lipids. He was the most influential person in my life. He made huge impact on me as a scientist and a man as well. It is very hard to sustain his inheritance.
Prof. Dr. Csaba Nyakas professor at University of Semmelweiss, was my main co-author in my papers. He taught me the immunostaining methods in Groningen. He was the best collaborating partner who I have ever worked with.
Prof. Dr. Penke Botond my tutor, who got my career started. He rescued me from the College of Food Industry and always supported me during my Ph.D. period and after it.
Prof. Dr. László Vígh the head Institute of Biochemistry, who let me work in his institute and helped me after Tibor Farkas's death.
Dr. Eszter Farkas at the Department of Anatomy, made the linguistic correction of my thesis. She was also co-author in one of my paper. I learnt so much from her. Last, but not least she cooked for me in Groningen.
Dr. Zsuzsa Penke at the Department of Animal Physiology, made the learning tasks for us.
And my dearest collegians I have ever met: Judit Baunoch, Gwendolyn Barcelo, and Erika Zukic. They created that friendly environment in which it was fantastic to work.
Thank you all!

List of publications

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