

Neuroprotective Effects of a Specific Multi-Nutrient Intervention Against A β ₄₂-Induced Toxicity in Rats

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Abstract. Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia in the elderly. Substantial evidence suggests a role for nutrition in the management of AD and especially suggests that interventions with combinations of nutrients are more effective than single-nutrient interventions. The specific multi-nutrient combination Fortasyn™Connect (FC), shown to improve memory in AD, provides phosphatide precursors and cofactors and is designed to stimulate the formation of phospholipids, neuronal membranes, and synapses. The composition comprises nucleotides, omega-3 polyunsaturated fatty acids (n3 PUFA), choline, B-vitamins, phospholipids, and antioxidants. The current study explored the protective properties of FC in a membrane toxicity model of AD, the amyloid- β 1–42 (A β ₄₂) infused rat, which shows reduced exploratory behavior in an Open Field and impaired cholinergic functioning. To this end, rats were fed an FC enriched diet or a control diet and five weeks later infused with vehicle or A β ₄₂ into the lateral ventricle. Ten weeks post-infusion A β ₄₂-rats fed the FC diet showed increased membrane n3 PUFA and phosphatidylcholine content while they did not show the reductions in exploratory behavior or in choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT) immunoreactivity that were seen in A β ₄₂-rats fed the control diet. We conclude that FC protects the cholinergic system against A β ₄₂-induced toxicity and speculate that the effects of FC on membrane formation and composition might be supportive for this protective effect. Based on these data a long-term intervention study was started in the prodromal stages of AD (NTR1705, LipiDiDiet, EU FP7).

Keywords: Alzheimer's disease, amyloid- β , cholinergic markers, dietary intervention, dietary precursors, membrane, nutrition, protection

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of progressive cognitive impairment and dementia in the elderly [1, 2]. Traditionally, AD is characterized by the presence of amyloid plaques and neurofibrillary tangles in the brain. However, several lines of evidence suggest that the progression of AD better

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correlates with the loss of synaptic membranes [3–6] and synapses [7–9]. Although the exact cause of AD is unknown, ample evidence indicates that nutrition can reduce AD risk factors and suggests that nutrition can play a role in the management of AD [10–14]. We [13, 15] and others [16–18] propose that specific nutrients should be combined in order to be effective in the management of AD. Since AD progression was shown to correlate with synaptic membrane and synapse loss, a nutritional composition was designed to stimulate the formation of membranes, neurites, and synapses. The fundamental building blocks of membranes are phospholipids [19] and can be synthesized from the dietary precursors docosahexaenoic acid (DHA), uridine, and choline by the CDP-choline cycle or Kennedy cycle [20]. Providing additional nutrients, such as the B-vitamins which facilitate the biosynthesis of phosphatidylcholine (PChol) from hepatic phosphatidylethanolamine (PEth), can add to the stimulation of phospholipid synthesis [11, 21]. The specific combination of dietary precursors and cofactors, FortasynTMConnect (FC), designed to stimulate the formation of phospholipids, neuronal membranes, and synapses, consists of DHA, eicosapentaenoic acid (EPA), uridine monophosphate (UMP), choline, folic acid, vitamin B6, vitamin B12, ascorbic acid, α -tocopherol, selenium, and phospholipids (PChol and phosphatidylserine). Recently, this nutritional intervention was tested in a randomized double-blind controlled clinical study and was shown to improve memory in drug-naive mild AD patients [22]. Now, we focus on whether this specific FC enriched diet can offer protection against the membrane disturbing [23, 24] effects of amyloid- β (A β).

A key factor in the development of AD is the formation of dense plaques in the patient's brain which primarily consist of A β [25]. To date, the mechanisms of A β -induced neural dysfunction and subsequent memory impairment are only partly understood and subject to extensive study. However, a specific A β species, A β _{1–42} (A β ₄₂) in its oligomeric conformation, is often considered as the primary pathogenic agent in the onset of the amyloid cascade, eventually resulting in a loss of synapses, neurites, and neuronal membranes [26, 27]. It has been suggested that the neurotoxic cascade of A β is initiated at neuronal cell membranes [28] and that membrane composition influences the level of A β toxicity [29–31]. Adding to this, an interesting and early characteristic [32] in the development of AD that may be linked to neuronal membrane degeneration is the profound reduction in acetylcholine synthesis by choline acetyltransferase

(ChAT) [33–35]. The toxic effects of A β ₄₂ on cholinergic parameters are well-studied through direct infusion of A β ₄₂ into the cerebrovascular system of rats or mice. In the course of days to weeks after A β ₄₂ infusion, reductions can be observed in ChAT immunoreactive neurons [36, 37], acetylcholine transport capacity as visualized by reduced vesicular acetylcholine transporter (VACHT) immunoreactivity [38], and reduced exploratory behavior in a novel environment [39]. Based on these studies, we suggest that the A β ₄₂ infusion model may offer a method to study the effects of preventive intervention on membrane-related damage induced by A β ₄₂ infusion.

Recently, we postulated that multi-nutritional intervention may provide therapeutic potential in delaying cognitive decline and AD [15]. Since intervention with FC is intended to stimulate membrane formation and to alter membrane composition, we speculate that this may prevent the toxic effects of A β ₄₂ on membrane parameters as outlined above. To this end, rats fed the FC enriched diet received an intracerebroventricular A β ₄₂ infusion and several weeks later the effect on membrane characteristics and cholinergic parameters in the magnocellular basal nucleus (NBM) was measured. In addition, exploratory behavior was assessed in the Open Field as a quick behavioral read-out parameter.

MATERIALS & METHODS

Animals

Male Sprague Dawley rats (Charles River Netherland, The Netherlands) of 8 weeks of age at arrival (4 groups of $n = 8$, 4 rats per cage) were housed in a temperature- and light-controlled room (dark period: 7:00 h–19:00 h), with *ad libitum* access to food and tap water throughout the experiment. All animal experimental protocols were conducted in accordance with international and national laws and institutional guidelines and approved by the local ethics committee (DEC Consult, Bilthoven, The Netherlands).

Experimental staging

Figure 1 provides an overview of the staging of the experiment. Before dietary intervention started, the rats were housed until the age of 4½ months to become adult. After 4 weeks of dietary intervention, surgery was performed and 1 week later the rats were infused. Ten weeks later the rats were tested in the Open Field

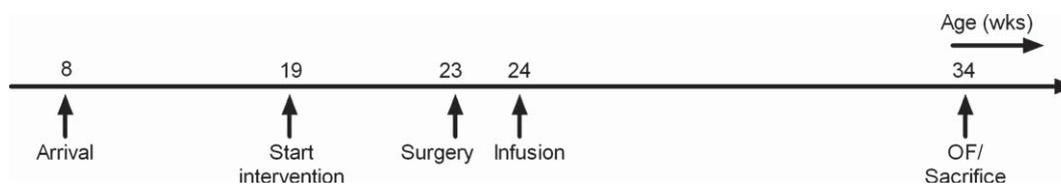


Fig. 1. Overview of experimental staging. Animals arrived at the age of 8 weeks and were housed in a temperature- and light-controlled room throughout the experiment. Start of dietary intervention was at the age of 19 weeks. Surgery and infusions were performed at respectively 23 and 24 weeks of age and the Open Field (OF) test was performed at 34 weeks of age where after the rats were sacrificed.

and thereafter sacrificed for brain collection. All procedures are described in more detail below.

Diets

Four weeks before surgery, rats were switched to either the FC diet or the control diet. As indicated in Table 1, the FC diet (Research Diet Services BV, Wijk bij Duurstede, The Netherlands) was enriched in omega-3 polyunsaturated fatty acids (n3 PUFAs) (Loders Croklaan BV, Wormerveer, The Netherlands), UMP (Mitsubishi International GmbH, Dusseldorf,

Germany), B-vitamins (Research Diet Services BV), phospholipids (Cargill Texturant Solutions, Zaandam, The Netherlands), choline (Research Diet Services BV), and antioxidants (Research Diet Services BV) compared to the control diet (Teklad standard lab chow) (Harlan, Horst, The Netherlands). All diets were prepared starting from the control diet. Barley was partially replaced by the non-fat nutrients (e.g., UMP, choline) while the PUFAs and phospholipids were exchanged within the fat fraction of the diet using specific oil blends. All diets were iso-caloric and had the same total fat content and the same level of PUFAs. The diets were visually indistinguishable from each other to secure blindness to treatment conditions.

Table 1
Compositions of the experimental diets (g/100 g)

Nutrient	Control	FC diet
Weat	25.9	25.9
Barley	25.0	23.0
Semolina	25.0	25.0
Soybean meal	8.5	8.5
Whey	5.0	5.0
Bentonite (binder)	1.0	1.0
CaCO ₃	1.40	1.40
Dicalciumphosphate	0.30	0.30
NaCl	0.50	0.50
L-lysine HCl	0.18	0.18
DL-methionine	0.05	0.05
Oil	5.0	5.0
C-18 : 1n9	1.13	0.83
C-18 : 2n6 (LA)	2.22	1.42
C-20 : 4n6 (AA)	0.00	0.04
total n6*	2.22	1.46
C-18 : 3n3 (ALA)	0.23	0.04
C-22 : 6n3 (DHA)	0.00	0.40
C-20 : 5n3 (EPA)	0.00	0.40
total n3*	0.23	0.96
UMP	–	1.000
Soy lecithin	–	0.400
Vitamin/mineral premix	2.20	2.20
Choline	–	0.313
Folic acid	–	0.00070
Vitamin B6	–	0.003
Vitamin B12	–	0.0000011
Vitamin C	–	0.160
Vitamin E	–	0.157
Selenium	–	0.0001110
*n3/n6 ratio	0.10	0.65

Surgery

Four weeks after onset of dietary intervention surgery was performed on all rats for placement of a guide cannula above the right lateral ventricle. Rats were anesthetized using an O₂/N₂O mixture with 2–3% isoflurane (InstruVet CV & AUV Dierencooperatie, Cuijk, The Netherlands) and were placed in a stereotactic device (Kopf instruments, Tujunga, CA). The skin on the head was exposed, cleaned with alcohol and treated with lidocaine (MP Biomedicals BV, Eindhoven, The Netherlands) as a local anesthetic. A longitudinal incision was made to expose the skull and a small hole was drilled above the right lateral ventricle (1.1 mm posterior and 1.6 mm lateral to bregma). A stainless steel guide cannula (Ø 0.6 mm, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands) was placed in the hole projecting 2.5 mm into the brain and was secured to the skull using cyanoacrylate. Three stainless steel screws were inserted around the cannula to provide extra grip for the dental cement. Cannula and screws were secured with dental cement (Simplex Rapid, Dental Union, Nieuwegein, The Netherlands) and the skin was sutured to fit nicely around the dental cement cap.

Once the animals recovered from the anesthetic, they were transferred to their home cage.

A β ₄₂ infusions

Peptide preparation

A β ₄₂ peptides were synthesized at the Department of Medical Chemistry, University of Szeged, as it was previously described [40] by a solid-phase procedure involving Wang-resin and Fmoc chemistry. Crude peptide was purified on a C-4 RP-HPLC column; pure fractions were pooled and lyophilized. Purity control was achieved by amino acid analysis (HP Amino Quant amino acid analyzer) and mass spectrometry (ESI MS, FinniganMat TSQ 7000).

Solution preparation

A β ₄₂ solution was prepared by dissolving 200 μ g in 100 μ l ultra-pure water. This solution was aged for 2 h at room temperature before being used for the infusions. This protocol results in an A β solution containing a mixture of A β oligomers, protofibrils, and fibrils. The aggregation grade of the colloidal A β solution was controlled by dynamic light scattering, atomic force microscopy, and transmission electron microscopy. All species were seen in the sample. Freshly prepared A β ₄₂ solutions were used for each day of infusions.

Infusion paradigm

One week following surgery on three consecutive days, an inner cannula was inserted into the guide cannula projecting into the lateral ventricle (3.5 mm below the dura) and connected to a 10 μ l syringe using PEEK-tubing (PK005-020, Western Analytical Products, Murrieta, CA). Over a 10 min period, 3 μ l of A β ₄₂ solution was infused using a precision syringe pump (PHD 2000, Harvard Apparatus Inc., Holliston, MA) and afterward a 5 min pause was applied before removal of the cannula from the guide. The animals from each dietary intervention group were divided into 2 groups of 8 rats, one group receiving A β ₄₂ infusions and the other saline (Sham). Rats in the A β ₄₂ group received a total of 20 μ g A β ₄₂ during the three infusions.

No complications were seen as a consequence of surgery, although 4 Sham animals in the control diet group and 1 in the FC diet group died 1 day after the last infusion was administered. Section was performed on these animals but cause of death remains unknown. The remainder of the animals (27 in total) did not show any complications.

Exploratory behavior

To measure exploratory behavior of the rats, we subjected them to a 15 min Open Field (OF) session; longer sessions would be more representative for baseline activity [41]. Ten weeks post-infusion the rats were placed in the OF during the dark period. The OF consisted of a black, circular polyethylene arena (140 cm in diameter) with a 50 cm high wall and was illuminated with a 15 watt light bulb. Rats were placed in the middle of the arena and were allowed to explore the OF freely for 15 min. Walking patterns were observed with a camera coupled to an automated tracking system (EthoVision 3.1, Noldus, Wageningen, The Netherlands). As a second measure for exploratory behavior, rearing frequency was also scored and was defined as the number of times the rat stood erect on its hind-legs. The OF was cleaned with 70% alcohol and allowed to air-dry between successive animals.

Brain collection and processing

Fifteen weeks after onset of dietary intervention and thus 10 weeks after A β ₄₂ infusions, the animals were sacrificed at the age of 34 weeks. Rats were anesthetized with an overdose of pentobarbital and transcardially perfused with ice-cold saline (9 g/l NaCl) containing 5.8 mM EDTA (VWR International, Roden, The Netherlands). Brains were carefully removed and split into the left and the right hemisphere. The left hemisphere was snap frozen in liquid nitrogen and stored at -80°C for fatty acid analyses. The right hemisphere was placed in 4% paraformaldehyde (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) for 24–30 h before storage in PBS with 0.1% azide at 4°C (Sigma). Two weeks later, 30 μ m sections were cut using a vibratome and collected in 10 alternating series. The distance between two successive slices within one series was approximately 300 μ m. Slices were stored in PBS with 0.1% azide at 4°C until immunohistochemistry was performed.

Fatty acid analysis

Left hemispheres were used for phospholipid and fatty acid analysis. Hemispheres were cut into small pieces and homogenized with 2 ml water in a Potter tube. The homogenate was vortexed for 5 min with 2 ml methanol, 1 ml EDTA, and 1 ml dichloromethane. After 5 min, 1 ml EDTA and 1 ml dichloromethane were added and vortexed for 5 min then centrifuged at 3000 rpm for 30 min. The dichloromethane

layer (bottom layer) was transferred to a new tube and 1 ml methanol was added, vortexed and centrifuged for 10 min at 3000 rpm. Thirty μ l of the dichloromethane/methanol sample was injected into the HPLC and the phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine fractions were collected. The phospholipid fractions were evaporated and after addition of 0.3 ml 20% boron trifluoride-methanol complex (VWR International) in methanol, they were placed under a nitrogen atmosphere at 100°C for 60 min (methylation). After cooling, the samples were vortexed with 2 ml hexane and 1 ml 2.5 M NaOH for 2 min. The top layer (hexane fraction) was transferred to a new tube and evaporated under a nitrogen flow. The residue was dissolved in 80 μ l iso-octane, transferred to a glass insert and fatty acid composition was analyzed in a gas chromatograph (column: CP-SIL88 (for FAME analysis) 50 m \times 0.25 mm id. 0.22 μ m film thickness (Varian) Cat. no. 7488). Areas under the curve were taken as relative measures of brain fatty acid and phospholipid content.

Immunohistochemistry

Free-floating sections from 2 successive series were stained for ChAT (1 : 500, Chemicon International, Temecula, CA, Cat. no. AB144P) or VACHT (1 : 4000, Chemicon International, Cat. no. AB1578). Antigen retrieval was necessary for VACHT immunohistochemistry. To this end, sections were incubated in 0.01 M sodium citrate buffer in a water bath at 80°C. After 20 min, sections were allowed to cool to room temperature. The next steps were identical for ChAT and VACHT staining. Briefly, sections were incubated with 0.3% hydrogen peroxide for 10 min to eliminate

endogenous peroxidase activity. Sections were incubated overnight at room temperature with primary antibodies for ChAT or VACHT. The sections were subsequently incubated with biotinylated rabbit anti-goat IgG (Bio-Connect B.V. - Vector Laboratories, Huissen, The Netherlands) for 2 h followed by 1.5 h incubation with ABC (avidin-biotin complex, ABC Elite, Vector, Burlingame, CA). Sections were rinsed between all steps with TBS three times for 10 min each. The peroxidase reaction was developed in 0.05% diaminobenzidine (DAB) (Sigma) and 0.01% H₂O₂ until a dark brown reaction product was evident. Sections were rinsed in TB and mounted on 1% gelatinized slides, dehydrated and cover slipped in Entellan (VWR International).

Image collection

VACHT sections were matched to those of one animal to locate comparable series of sections in all animals. ChAT stained sections of the adjacent series were matched with their counterparts from the VACHT staining. Digital grayscale overview images of the NBM at 5 \times magnification were taken at a standard background of 32 on a scale of 0 to 255 (0 being white and 255 being black) followed by 20 \times magnification image collection for the analyses using Scion Image (Scion Corporation, MD). Slices with VACHT staining required an additional step to increase contrast between background and stained cells and fibers using a DAB filter. The NBM was identified in 8 slices in the series of which five successive images were selected for analyses starting approximately around the first appearance of the hippocampus at bregma -0.80 and then at -1.30, -1.60, -1.88, and -2.30 (see Fig. 2) [42]. The surface areas of the NBM in these slices did

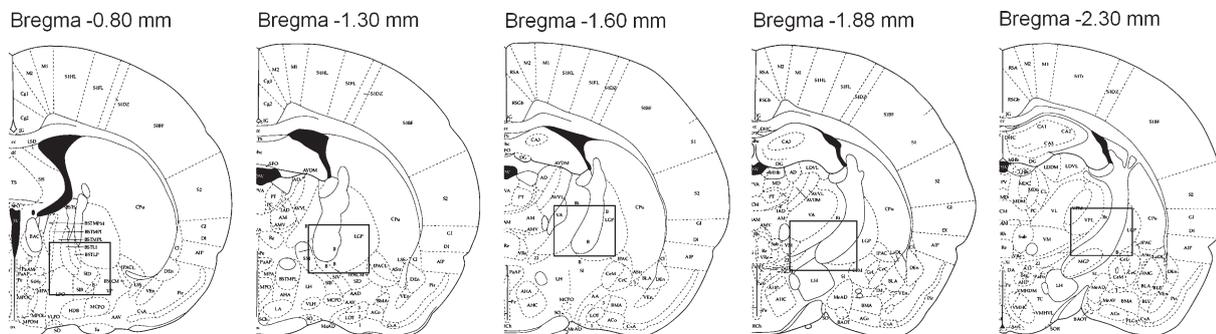


Fig. 2. Localization of the region of interest for ChAT and VACHT analyses. Location of the 5 successive images of the NBM used for the analyses of the ChAT and VACHT staining according to the Paxinos brain atlas [42]. The region of the NBM measured is indicated by the square. Images for each animal were related to these coordinates in order to match the images between animals.

not differ between groups. All images were filed to perform image analyses at a later stage.

Image analysis

The numbers of ChAT and VAcHT immunoreactive neurons were counted in the NBM using an automated routine in Scion Image. To identify the cells, a threshold of gray value was determined. All pixel groups above this threshold were counted as cells. Because ChAT and VAcHT stainings were very different in intensity, two different threshold values were employed. The thresholds were calculated using a reference area within each section lacking stained cell bodies. The average grayscale of these areas plus three times the standard deviation was used as threshold value. Using this method the threshold was calculated to be 150 for ChAT and 70 for VAcHT on a scale of 0 to 255. Structures exceeding this threshold and larger than 3 pixels for ChAT sections or 15 pixels for VAcHT sections were counted as cells. The number of cells per animal was determined by counting the numbers of cells of all five images per animal.

Statistical analyses

Throughout the experiment the animals and diets were coded to make the observers blind to the treatment of the rats. The codes were unblinded after analyses had been completed. All data were analyzed using SPSS 15.0 (SPSS Inc., Chicago, IL). The current study

tested the influence of two factors and their interaction on brain phospholipid and fatty acid levels, on exploratory behavior and on cholinergic markers. With this respect, dietary intervention is taken as Factor 1 and A β_{42} infusion as Factor 2. All statistical analyses tested for Factor 1 (Diet), Factor 2 (A β_{42}) and for Factor 1 * Factor 2 interaction effects (Diet * A β_{42}) using ANOVA wherein p values <0.05 were considered significant. When ANOVA revealed a significant Factor 1 * Factor 2 interaction effect post-hoc analyses were performed to detect between group differences.

Specifically for the analyses of the effects on brain phospholipid and fatty acid content it was expected that FC intervention would lead to increased brain phospholipid levels, increased DHA, decreased AA and increased n3/n6 PUFA ratio, while no effect of A β_{42} infusion was expected. Therefore, ANOVA p -values for Factor 1 and for Factor 2 effects were divided by 2 to allow for one-tailed analysis of the effects on brain phospholipid and fatty acid contents.

RESULTS

Statistics

The overall statistical analysis results are presented in Table 2. Post-hoc analyses were performed for the exploratory and cholinergic markers and are presented in Table 3.

Table 2
Statistical analyses results

		Main effects		Interaction effects
		Group 1 (Diet)	Group 2 (A β_{42})	Diet * A β_{42}
Phospholipids [§]	Total PL	F = 1.60, $p = 0.109$	F = 0.049, $p = 0.41$	F = 0.019, $p = 0.892$
	PChol	F = 3.07, $p \leq 0.05$ *	F = 0.048, $p = 0.42$	F = 0.060, $p = 0.809$
	PEth	F = 0.718, $p = 0.204$	F = 0.50, $p = 0.44$	F = 0.035, $p = 0.854$
	PSer	F = 1.529, $p = 0.115$	F = 0.43, $p = 0.26$	F = 0.050, $p = 0.826$
Fatty acids [§]	DHA	F = 27.15, $p \leq 0.001$ *	F = 0.10, $p = 0.38$	F = 1.09, $p = 0.31$
	AA	F = 90.70, $p \leq 0.001$ *	F = 1.22, $p = 0.14$	F = 0.069, $p = 0.79$
	n3/n6 ratio	F = 250.53, $p \leq 0.001$ *	F = 0.62, $p = 0.22$	F = 0.14, $p = 0.71$
Exploration	Distance	F = 1.68, $p = 0.208$	F = 0.64, $p = 0.433$	F = 3.56, $p \leq 0.03$ *
	Rearing	F = 1.64, $p = 0.213$	F = 5.12, $p \leq 0.04$ *	F = 3.11, $p \leq 0.05$ *
Cholinergic markers	ChAT	F = 6.41, $p \leq 0.02$ *	F = 0.88, $p = 0.358$	F = 4.71, $p \leq 0.02$ *
	VAcHT	F = 2.58, $p = 0.123$	F = 2.33, $p = 0.141$	F = 2.85, $p = 0.062$ #

Statistical analyses of all results with ANOVA showing the main factor 1 effects (diet intervention), main factor 2 effects (A β_{42} infusion) and factor 1 * factor 2 interaction effects (diet * A β_{42}). [§]Factor 1 and factor 2 effects for the brain phospholipid and fatty acid content are showing the one-tailed p -values (values out of ANOVA analyses divided by 2) based on the clear expectation that FC intervention would increase phospholipid and fatty acid content. Total PL: total phospholipids; PChol: phosphatidylcholine; PEth: phosphatidylethanolamine; PSer: phosphatidylserine; DHA: docosahexaenoic acid; AA: arachidonic acid. $p \leq 0.05$. #: nearly significant.

Table 3
Post-hoc analyses results for exploratory and cholinergic markers.

	Exploration		Cholinergic markers	
	Distance	Rearing	ChAT	VAcHT
Control Sham versus Control A β ₄₂	0.018*	0.052#	0.032*	0.037\$
FC Sham versus FC A β ₄₂	0.633	0.248	0.485	0.918
Control Sham versus FC Sham	0.821	0.514	0.987	0.846
Control A β ₄₂ versus FC A β ₄₂	0.028*	0.008*	0.002*	0.033\$
Control Sham versus FC A β ₄₂	0.526	0.748	0.524	0.771
Control A β ₄₂ versus FC Sham	0.012*	0.119	0.016*	0.032\$

Post-hoc analyses showing group differences for exploratory and cholinergic markers. * $p \leq 0.05$; #: nearly significant; \$ significance based upon nearly significant interaction effect.

Table 4
Relative brain phospholipid contents

Diet	Total PL		PChol		PEth		PSer	
	Sham	A β ₄₂	Sham	A β ₄₂	Sham	A β ₄₂	Sham	A β ₄₂
Control	19.5 \pm 1.6	20.2 \pm 1.5	5.8 \pm 0.5	6.0 \pm 0.2	11.8 \pm 1.0	12.8 \pm 1.2	1.9 \pm 0.3	1.6 \pm 0.1
FC	22.9 \pm 4.3	24.5 \pm 3.2	7.6 \pm 1.4*	7.5 \pm 0.9*	13.0 \pm 2.2	14.8 \pm 2.0	2.3 \pm 0.7	2.1 \pm 0.3

A main effect of FC diet Intervention on neuronal PChol level was observed. No effect of A β ₄₂ infusion and no interaction effect were found on neuronal membrane phospholipid levels. Values represent areas under the curve after HPLC analysis (arbitrary units). Statistical analyses results are shown in Table 2. Total PL: Total phospholipids; PChol: Phosphatidylcholine; PEth: Phosphatidylethanolamine; PSer: Phosphatidylserine. * $p \leq 0.05$.

Table 5
Relative brain PUFA contents.

Diet	DHA		AA		n3/n6 PUFA ratio	
	Sham	A β ₄₂	Sham	A β ₄₂	Sham	A β ₄₂
Control	16.6 \pm 0.26	16.1 \pm 0.37	9.0 \pm 0.13	9.2 \pm 0.09	1.03 \pm 0.022	1.00 \pm 0.032
FC	18.4 \pm 0.60*	19.0 \pm 0.43*	7.6 \pm 0.23*	7.7 \pm 0.09*	1.46 \pm 0.020*	1.45 \pm 0.029*

Intervention with the FC diet increased DHA, decreased AA and increased the n3/n6 PUFA ratio. A β ₄₂ infusion had no effect on these parameters and no interaction effect with dietary intervention was found. DHA: Docosahexaenoic acid; AA: Arachidonic acid. Fatty acid content is presented as a percentage of total fatty acid content. Statistical analyses results are shown in Table 2. Values represent means \pm standard errors. * $p \leq 0.05$.

Phospholipid content

Table 4 lists the relative levels of brain phospholipids of Sham and A β ₄₂ rats. ANOVA analyses of phospholipid levels showed a Factor 1 effect of FC intervention on phosphatidylcholine (PChol) ($F = 3.07$, $p \leq 0.05$). No Factor 1 effects were found for levels of total phospholipid ($F = 1.60$, $p = 0.109$), phosphatidylethanolamine (PEth) ($F = 0.718$, $p = 0.204$) and phosphatidylserine (PSer) ($F = 1.529$, $p = 0.115$). Infusion with A β ₄₂ (Factor 2) had no effect on total phospholipid, PChol, PEth and PSer levels (respectively $F = 0.049$, $p = 0.41$; $F = 0.048$, $p = 0.42$; $F = 0.50$, $p = 0.44$ and $F = 0.43$, $p = 0.26$). No interaction effects between dietary intervention and A β ₄₂ infusion were observed (respectively $F = 0.019$, $p = 0.892$; $F = 0.060$, $p = 0.809$; $F = 0.035$, $p = 0.854$ and $F = 0.050$, $p = 0.826$).

Fatty acid profile

Table 5 summarizes the effect of FC intervention on fatty acid content of brain phospholipids. ANOVA analyses revealed Factor 1 effects on DHA, arachidonic acid (AA), and n3/n6 PUFA ratio. FC intervention resulted in increased levels of DHA ($F = 27.15$, $p \leq 0.001$) decreased AA level ($F = 90.70$, $p \leq 0.001$) and an increased n3/n6 PUFA ratio ($F = 250.53$, $p \leq 0.001$). Brain phospholipid levels of EPA, the other n3 PUFA added to the FC diet, were not detectable. No effects of A β ₄₂ infusions (Factor 2) on the levels of DHA, AA, and the n3/n6 PUFA ratio were detected (respectively $F = 0.10$, $p = 0.38$; $F = 1.22$, $p = 0.14$ and $F = 0.62$, $p = 0.22$). No interaction effects between with dietary intervention and A β ₄₂ infusions were observed ($F = 1.09$, $p = 0.31$; $F = 0.069$, $p = 0.79$; $F = 0.14$, $p = 0.71$, respectively).

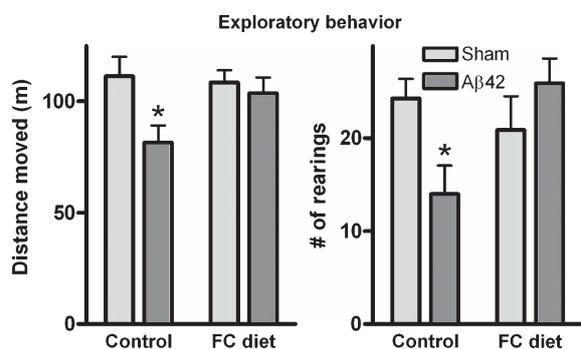


Fig. 3. Exploratory behavior in the Open Field. ANOVA analyses revealed that exploratory behavior as illustrated by distance moved and rearing frequency was influenced in the study ($F=3.56$, $p \leq 0.03$ and $F=3.11$, $p \leq 0.05$, respectively). A β ₄₂ induced a decrease in exploratory activity in rats fed the control diet as visualized by a decrease in the distance moved (post-hoc: $p=0.018$) and a decrease in the total number of rearings (post hoc: $p=0.052$). In contrast, no difference between A β ₄₂ and Sham rats fed the FC diet was observed (post-hoc: $p=0.63$ and $p=0.25$, respectively). Compared to Sham rats on the control diet no differences were found in Sham FC rats (post-hoc: respectively $p=0.82$ and $p=0.51$) or A β ₄₂ FC rats (post-hoc: respectively $p=0.53$ and $p=0.75$) were found. Bars represent means \pm standard errors. *: $p < 0.05$.

Exploratory behavior

The effect of A β ₄₂ infusion and FC intervention on exploratory behavior is plotted in Fig. 3. No main effect of diet intervention (Factor 1) was observed on exploratory markers (distance moved: $F=1.68$, $p=0.208$; rearing: $F=1.64$, $p=0.213$). A main Factor 2 effect (A β ₄₂ infusion) on rearing was detected ($F=5.12$, $p \leq 0.04$) while no effect on distance moved was found ($F=0.64$, $p=0.433$). Factor 1 * Factor 2 interactions were found for both distance moved and the number of rearings. Post-hoc analyses revealed that A β ₄₂ infused rats fed the control diet were the only group differing from all the other groups showing reduced exploratory behavior. See Table 3 for all individual group comparisons.

Cholinergic markers

Figure 4 shows the effects of A β ₄₂ infusion and FC intervention on cholinergic markers. Representative pictures for the ChAT and VACHT staining are shown in Fig. 5. ANOVA analyses revealed a main effect of diet intervention (Factor 1) on the cholinergic marker ChAT ($F=6.41$, $p \leq 0.02$) but not on VACHT ($F=2.58$, $p=0.123$). No main Factor 2 effects (A β ₄₂ infusion) on ChAT and VACHT were detected (respectively $F=0.88$, $p=0.358$ and $F=2.33$, $p=0.141$). Factor 1

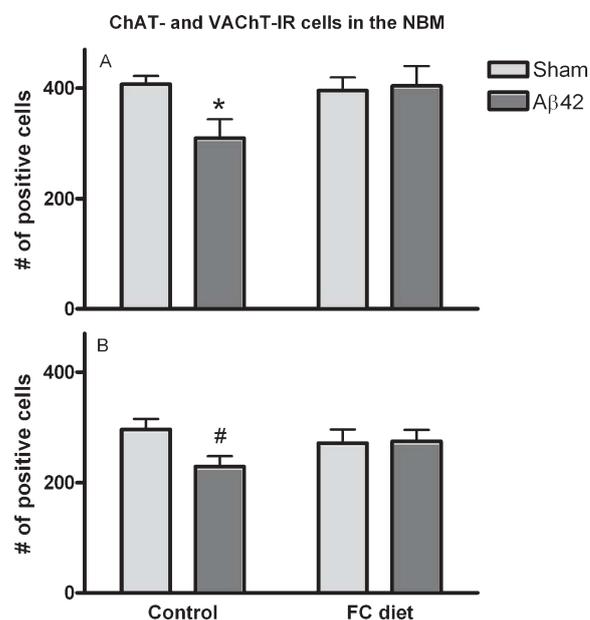


Fig. 4. ChAT and VACHT immunoreactivity in the NBM. Feeding with the FC diet prevented the A β ₄₂-induced decline in ChAT and VACHT immunoreactive cells in the NBM. Factor 1 * Factor 2 interactions were found for ChAT ($F=4.71$, $p \leq 0.02$) and nearly for VACHT ($F=2.85$, $p=0.062$). Post-hoc analyses revealed that A β ₄₂ infused rats fed the control diet showed reduced ChAT and VACHT positive cells compared to the other groups. See Table 3 for all individual group comparisons. Bars represent means \pm standard errors.

* Factor 2 interactions were found for ChAT ($F=4.71$, $p \leq 0.02$) and nearly for VACHT ($F=2.85$, $p=0.062$). Post-hoc analyses revealed that A β ₄₂ infused rats fed the control diet showed reductions in cholinergic markers compared to all other groups. See Table 3 for all individual group comparisons.

DISCUSSION

The current study clearly shows that intracerebroventricular injections of A β ₄₂ result in reduced exploratory activity and in reduced ChAT and VACHT immunoreactivity in the NBM. Intervention with FC prevented the A β ₄₂-induced decline in exploratory activity and the reductions in ChAT and VACHT immunoreactivity while no effects of FC on these parameters were seen in Sham animals. Furthermore, FC intervention changed neuronal membranes by increasing phospholipid content and increased the total level of DHA, decreased AA and increased n3/n6 PUFA ratio.

The current effects of A β ₄₂ confirm and extend earlier findings with A β infusions on exploratory activity

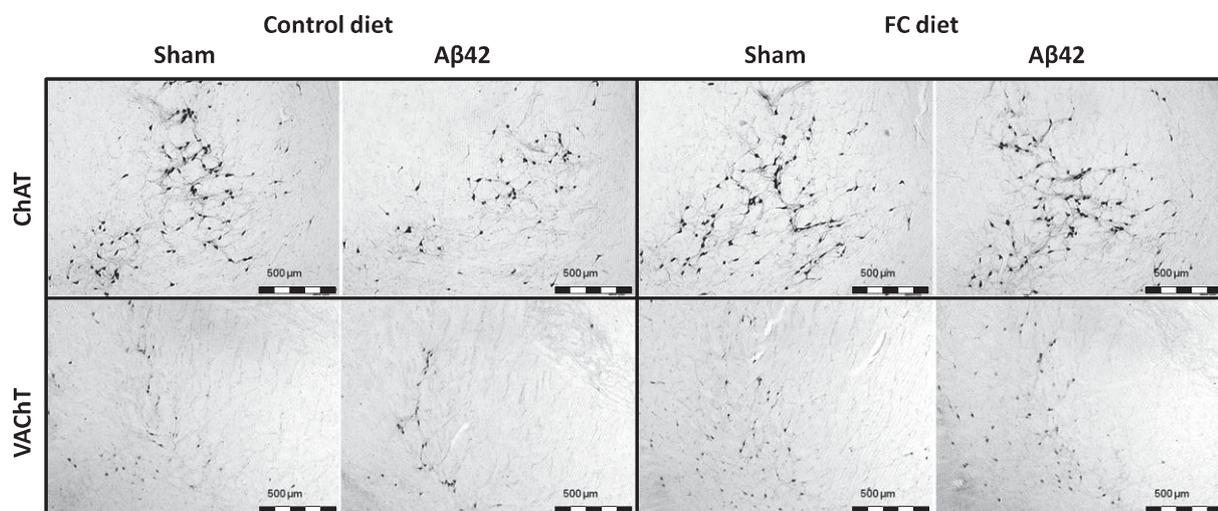


Fig. 5. ChAT and VACHT staining in rat brain slices. Representative pictures of ChAT (top row) and VACHT (bottom row) immunoreactivity in the NBM at 20x magnification are shown. The pictures clearly show that the NBM of A β ₄₂ infused rats fed the control diet (A β ₄₂ Control) show fewer stained cells compared to the other groups for both ChAT as VACHT staining.

and the cholinergic system. Others have shown that, irrespective of the route of administration or the fragment used, A β reduced the number of ChAT positive neurons in the medial septum or NBM [36, 37, 43–47]. This reduction was also reflected in reduced ChAT enzyme activity [47] and reduced acetylcholine transport capacity [38], leading to reduced acetylcholine synthesis and release [48]. The current findings provide additional support for these effects of A β on cholinergic neurons. The toxic effect of A β is further illustrated by the reduction in exploratory activity shown in the Open Field, which has also been observed by others [39, 49, 50]. It would be interesting to study the effects of FC on A β ₄₂ plaque formation especially since we and others have shown A β reductions as a result of DHA intervention [51–55]. However, no detectable A β ₄₂ plaques were found in infused rat brains and therefore FC effects on plaque formation could not be studied in this model.

Dietary intervention with FC prevented A β ₄₂-induced cholinergic and behavioral decline. To date, only a limited number of nutritional intervention studies have shown beneficial effects of individual nutrients on the cholinergic system but these studies were restricted to ChAT activity and immunoreactivity. In aged rats, ChAT activity could be partly restored by supplementation with α -tocopherol [56]. In addition, supplementation with choline alfoscerate restored ChAT activity levels [57] and ChAT immunoreactivity in aged rats [58]. Conversely, ChAT activity was reduced in aged mice supplemented with choline [59],

while phosphatidylcholine supplementation to Dull mice had no effect on ChAT activity in the cortex [60]. Furthermore, fatty acid enriched diets like a soybean-oil diet or diets enrichment with alpha-linolenic or linoleic acid had no effect on ChAT activity levels in adult rats [61, 62]. Together, these studies do not show clear beneficial effects of single nutrient intervention on cholinergic system integrity. We postulated that multi-nutritional intervention may prevent the toxic effects of A β ₄₂ on membrane parameters and indeed we show in this paper that a multi-nutrient approach with FC prevented A β ₄₂-induced decline of the cholinergic system markers ChAT and VACHT. Moreover, FC intervention prevented the decline in exploratory behavior induced by A β ₄₂ infusions. These results indicate that multi-nutrient intervention with FC can protect the cholinergic system against the toxic effects of A β ₄₂ and in addition prevent the behavioral decline that is associated with cholinergic cell damage.

A β ₄₂ treated rats provided with FC displayed similar levels of exploratory behavior and ChAT and VACHT immunoreactivity compared to Sham rats. Two possible mechanisms may explain the effect of FC in A β ₄₂ rats. First, the formation of ChAT and VACHT may be increased and thereby counteract the toxic effects of A β ₄₂. Second, FC may protect cholinergic cells from A β ₄₂-induced toxicity by maintaining the membrane's functional integrity. The FC composition was designed to enhance the formation of membrane phospholipids and indeed, intervention with FC increased relative levels of neuronal membrane PChol confirming the

effects of DHA and UMP on phospholipid formation [63, 64]. Both the Sham and the A β ₄₂ infused rats showed comparable effects of FC intervention on membrane formation and fatty acid composition. Because of these comparable effects on neuronal membranes and the fact that no differences in ChAT and VAcHT immunoreactivity were found between Sham rats receiving the control diet or the FC diet, it appears unlikely that the production of ChAT and VAcHT is directly influenced by FC. Rather, these data suggest that the changes in membrane integrity induce properties that protect the cholinergic system against A β ₄₂-induced toxicity. This is underscored by the fact that A β ₄₂ has membrane disrupting properties [65] by binding to the membrane [30, 66] leading to membrane fluidity reductions [67, 68] and membrane perforations [69–71]. The role of the membrane in A β ₄₂ toxicity is even more emphasized by the differential sensitivity to A β ₄₂ between subpopulations of neuronal cells either in cultures or in animal brains being explained by distinctive differences in membrane binding of A β ₄₂ [72]. In addition, inhibiting membrane binding capacity of A β ₄₂ itself demonstrated a lack of toxicity in primary neuronal cultures [66]. Therefore, we conclude that FC protects the cholinergic system against A β ₄₂-induced toxicity and speculate that the effects of FC on membrane formation and composition might be supportive for this protective effect by reducing the potential binding capacity of A β ₄₂ to the membrane thereby limiting the membrane disrupting properties. These data support the suggestion that early intervention with these specific brain-supportive nutrients, in addition to improving memory in diagnosed AD patients [22] may also prevent or delay cognitive decline and AD [15].

The cholinergic system is affected in AD, with decreases in acetylcholine as one of the more prominent hallmarks, caused by a decrease in production by the enzyme ChAT [73, 74]. The majority of currently available medical treatments for AD focus on increasing levels of acetylcholine by preventing its breakdown by acetylcholine esterase (AChE). Indeed, studies show that treating AD patients with AChE-inhibitors can increase acetylcholine levels and give patients some relief from their symptoms [75, 76]. The present data indicate that nutritional intervention may add value to current drug treatments by reducing the toxic effects of A β ₄₂. Furthermore, based (amongst others) on these data a long-term intervention study was started in the early, prodromal stages of AD (NTR1705, within the LipiDiDiet project, EU FP7 program) [77]. Primary outcome measure of the study

is cognitive performance but also focuses on development of plasma biomarkers like A β ₄₀ and A β ₄₂.

In summary, our experiments show that intervention with the FC enriched diet protected cholinergic neurons in the basal forebrain against A β ₄₂ toxicity, affected neuronal membrane fatty acid composition and phospholipid levels and in addition prevented the decline in exploratory behavior seen in A β ₄₂ infused animals on a control diet. It is speculated that the protective effects of FC may depend on improved membrane integrity thereby limiting the membrane binding and membrane disrupting properties of A β ₄₂. The data suggest that multi-nutrient intervention with specific precursors and cofactors (Fortasyn™Connect) may benefit AD patients by protecting cholinergic neurons against A β ₄₂-induced toxicity.

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