



# A specific multi-nutrient enriched diet enhances hippocampal cholinergic transmission in aged rats<sup>☆</sup>



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## ABSTRACT

Fortasyn Connect (FC) is a specific nutrient combination designed to target synaptic dysfunction in Alzheimer's disease by providing neuronal membrane precursors and other supportive nutrients. The aim of the present study was to investigate the effects of FC on hippocampal cholinergic neurotransmission in association with its effects on synaptic membrane formation in aged rats. Eighteen-month-old male Wistar rats were randomized to receive a control diet for 4 weeks or an FC-enriched diet for 4 or 6 weeks. At the end of the dietary treatments, acetylcholine (ACh) release was investigated by in vivo microdialysis in the right hippocampi. On completion of microdialysis studies, the rats were sacrificed, and the left hippocampi were obtained to determine the levels of choline, ACh, membrane phospholipids, synaptic proteins, and choline acetyltransferase. Our results revealed that supplementation with FC diet for 4 or 6 weeks, significantly enhanced basal and stimulated hippocampal ACh release and ACh tissue levels, along with levels of phospholipids. Feeding rats the FC diet for 6 weeks significantly increased the levels of choline acetyltransferase, the presynaptic marker Synapsin-1, and the post-synaptic marker PSD-95, but decreased levels of Nogo-A, a neurite outgrowth inhibitor. These data show that the FC diet enhances hippocampal cholinergic neurotransmission in aged rats and suggest that this effect is mediated by enhanced synaptic membrane formation. These data provide further insight into cellular and molecular mechanisms by which FC may support memory processes in Alzheimer's disease.

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## 1. Introduction

Synapse loss and membrane-related pathology play a central role in the pathogenesis of Alzheimer's disease (AD) (Arendt, 2009; Selkoe, 2002) and consequently provide viable intervention targets. In early AD, the increased synapse loss is associated with memory decline (Selkoe, 2002; Sperling et al., 2011; Terry et al., 1991). Reducing synapse loss and membrane-related pathology may preserve or improve neurotransmission and, thereby, positively affect memory and other cognitive functions.

Fortasyn Connect (FC) is a specific nutrient combination designed to ameliorate synapse loss and synaptic dysfunction in AD (Van Wijk et al., 2014) by addressing nutritional needs believed to exist in these patients (Lopes da Silva et al., 2013; Mi et al., 2013). FC comprises precursors and cofactors required for the formation and

maintenance of neuronal membranes, that is, uridine (as uridine monophosphate, UMP), the omega-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid, choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium.

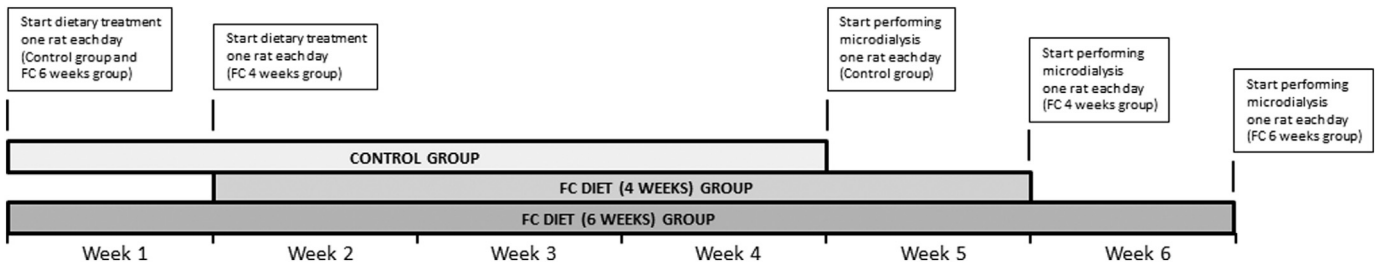
FC is present in Souvenaid, a medical food intended for early AD patients. This medical food significantly improved memory performance in drug-naïve patients with mild AD in 2 recent clinical trials (Scheltens et al., 2010, 2012). In addition, electroencephalography measures suggested that the medical food preserved functional brain network organization, counteracting the pattern usually seen in AD patients (de Waal et al., 2014; Scheltens et al., 2012).

The aim of the present study was to further investigate the physiological mechanism underlying the cognitive enhancing effect of FC by supplementing aged rats with a diet enriched with this specific nutrient combination. It is well established that improved learning and memory in various tasks is associated with augmented cholinergic neurotransmission in the hippocampus (Fadda et al., 2000; Nail-Boucherie et al., 2000). Therefore, we assessed the effect of an FC-enriched diet on acetylcholine (ACh) release, indicative of hippocampal cholinergic neurotransmission, by in vivo microdialysis

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**Fig. 1.** Timeline of the experimental protocol. To perform microdialysis on each rat on separate days, termination of the experimental procedures for each group was arranged to 3 consecutive weeks by arranging the onset of dietary treatments accordingly: dietary treatment for control and FC diet 6 weeks groups was initiated on the same day whereas dietary treatment for FC diet 4 weeks group was initiated 1 week later. Abbreviation: FC, Fortasyn connect.

in the right hippocampi of aged rats. This was followed by an investigation of the effects of FC on synaptic membrane formation, to further investigate its mode of action. This is the first study to determine the effect of chronic treatment with this specific nutritional combination on hippocampal ACh release in association with its effects on synaptic membranes in aged rats.

We found that dietary supplementation with FC diet for 4 or 6 weeks significantly enhanced hippocampal ACh release, both under basal conditions and after atropine stimulation. This observation was accompanied by enhanced levels of tissue ACh and membrane phospholipids. In addition, FC diet treatment for 6 weeks significantly increased the levels of choline acetyltransferase (ChAT) and the presynaptic and postsynaptic markers Synapsin-1 and PSD-95, respectively, while decreasing levels of Nogo-A, a neurite outgrowth inhibitor. Hence, the present data show that consumption of the FC-enriched diet enhances hippocampal cholinergic neurotransmission, which probably can be ascribed to the concurrently observed enhanced synaptic membrane formation.

## 2. Methods

### 2.1. Animals

Eighteen-month-old male Wistar rats (Experimental Animals Breeding and Research Center, Uludag University Medical School, Bursa, Turkey) were group housed in a temperature controlled room with free access to standard rat chow and water under a 12/12 hour light-dark cycle. The experimental protocol was approved by the Animal Care and Use Committee of Uludag University, Bursa, Turkey (Approval ID: 2012-03/03), and all experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. All efforts were made to minimize the number of animals used and the individual level of discomfort.

### 2.2. Dietary supplementation

Rats were randomized to control and FC diet groups. Rats in the control group ( $n = 4$ ) received a regular rat chow for 4 weeks, whereas rats in the FC group were fed the FC diet for either 4 ( $n = 5$ ) or 6 ( $n = 5$ ) weeks.

Dietary treatment for control and FC diet 6 weeks groups was initiated on the same day, whereas dietary treatment for FC diet 4 weeks group was initiated 1 week later. With this set-up, we were able to perform microdialysis on each rat on separate days. Timeline of the experimental protocol is provided in Fig. 1.

Both the control diet and the FC-enriched diet were AIN-93 M based (Reeves et al., 1993), isoenergetic, and fulfilled all dietary requirements. Both diets contained the standard vitamin mix (AIN-93-VX) and mineral mix (AIN-93M-MX). The diets differed in

composition with regard to the fat blends used, as well as a number of supplemented nutrients, including choline, B-vitamins, antioxidants, UMP, and lecithin. A detailed overview of the contents of diets is presented in Table 1. The diets were formulated by Nutricia Research, Nutricia Advanced Medical Nutrition (Utrecht, the Netherlands), manufactured by Ssniff Spezialdiäten (Soest, Germany) and presented to the animals as pellets. All diets were stored at  $-20^{\circ}\text{C}$  until use, to prevent lipid oxidation. Reanalysis of the diets at the end of the study confirmed that all fatty acids were still present in the original amounts.

No significant difference was observed between treatment groups in terms of mean daily amount of food consumed and weight gain (data not shown) during the treatment.

### 2.3. Surgical procedures

One day before the completion of dietary treatment, a probe was inserted in the right hippocampus of rats in each group to perform in vivo microdialysis. Rats were anesthetized with ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively) and then placed in a stereotaxic frame. Subsequently, skulls were exposed, and a small hole was drilled over the right hippocampus. A hand-made probe

**Table 1**  
Detailed compositions of the 2 experimental diets

Ingredients (g/100 g diet)	Diets	
	Control	Fortasyn
Cornstarch, pregelatinized	35.6	32.5
Caseine	14.0	14.0
Maltodextrin, 10 DE	15.5	15.5
Sucrose	10.0	10.0
Dextrose	10.0	10.0
Soy oil	1.900	
Coconut oil	0.900	0.100
Corn oil	2.200	1.700
Fish oil		3.200
Cellulose powder	5.0	5.0
Mineral mix (AIN-93M-MX)	3.5	3.5
Vitamin mix (AIN-93-VX)	1.0	1.0
L-cystine	0.180	0.180
Choline chloride (50%)	0.230	0.922
Tert-butylhydroquinone	0.0008	0.0008
UMP (UMP disodium salt)		1.0
Soy lecithin		0.755
Vitamin B6 (pyridoxin hydrochloride, 100%)		0.00328
Folic acid (100%)		0.00060
Vitamin B12 (cyanocobalamin, 0.1%)		0.00350
Ascorbic acid (100%)		0.160
Vitamin E (tocopherol acetate, 50%)		0.4650
Selenium (sodium selenite pentahydrate, 100%)		0.00034
Total	100.0	100.0

All amounts of nutrients are indicated in g/100 g of diet.

Key: UMP, uridine monophosphate.

(molecular weight cutoff of dialysis membrane was 13,000 Da and length was 1 mm) was implanted in the CA1 region of the hippocampus by using Dura at Bregma as the reference point (coordinates were: AP  $-4.0$  mm; ML  $-2.2$  mm; DV  $2.6$  mm; Paxinos and Watson, 2004) and then fixed to the skull using acrylic cement. After surgery the rats were placed in individual cages and allowed to recover from anesthesia for 24 hours. During this period, they remained calm and showed no signs of overt discomfort or pain.

#### 2.4. *In vivo* microdialysis

*In vivo* microdialysis experiments were carried out 24 hours after surgery to avoid effects of anesthesia. The dialysis probe inserted in CA1 region of the right hippocampus of freely-moving rats was perfused at a rate of  $2 \mu\text{L}/\text{min}$  with artificial cerebrospinal fluid (pH = 7.4) of the following composition: 148 mM NaCl, 3.0 mM KCl, 1.4 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgCl}_2$ , 1.3 mM  $\text{NaH}_2\text{PO}_4$ , 0.2 mM  $\text{Na}_2\text{HPO}_4$ , and neostigmine ( $1 \mu\text{M}$ ) to block enzymatic degradation of ACh.

Each microdialysis sample was collected with 30 minutes intervals. Samples collected during the first hour of microdialysis were discarded. After collection of the first 3 microdialysates, which were referred to as baseline samples, the perfusion medium was replaced with another which contained atropine sulfate ( $10 \mu\text{M}$ ). This perfusion was continued for 1 hour, and then atropine-containing medium was replaced with the initial atropine-lacking medium.

Microdialysis was terminated 6 hours after the start of collecting the first baseline sample, and rats were sacrificed under ketamine and xylazine ( $80 \text{ mg}/\text{kg}$  and  $10 \text{ mg}/\text{kg}$ , respectively) anesthesia. Brains were immediately taken out and the left hippocampi were obtained, weighed, and homogenized in 50 volumes of ice-cold deionized water with 10 up-and-down strokes, using a teflon-glass homogenizer (Wheaton, Milville, NJ, USA) to assay levels of tissue choline and ACh, as well as phospholipids, synaptic proteins, and ChAT. The placement of microdialysis probes were confirmed in the right hippocampi of randomly selected rats.

#### 2.5. Analyses of ACh and choline

Levels of choline and ACh in microdialysis samples and hippocampal homogenates were analyzed by high performance liquid chromatography (Hitachi, Tokyo, Japan) equipped with an electrochemical detector (Antec BV, Zoeterwoude, the Netherlands). A BAS ACh/choline analytical column cartridge and post-column enzyme reactor (MF-6150 and MF-6151) were used at  $37^\circ\text{C}$  to separate ACh and choline. The mobile phase contained 40 mM  $\text{Na}_2\text{HPO}_4$ , 0.5 mM  $\text{Na}_2\text{EDTA}$ , and 0.5 mL/L Kathon CG at pH 8.4, and the flow rate was  $1 \text{ mL}/\text{min}$ .

#### 2.6. Phospholipid assay

Hippocampal phospholipids were extracted according to the Folch method (Folch et al., 1957) and measured as described previously (Cansev and Wurtman, 2007). One mL aliquots of the hippocampal homogenates were mixed with 3 mL of a chloroform plus methanol mixture (2:1 vol/vol) and vortexed for 30 seconds. After cooling for about 1 hour on ice, each mixture was sequentially combined with 3 mL of the chloroform plus methanol mixture, and 1 mL of ice-cold deionized water. It was then vortexed vigorously and allowed to stand overnight at  $+4^\circ\text{C}$  (18–20 hours). The organic (lower) and aqueous (upper) phases were separated by centrifugation (10 minutes at  $+4^\circ\text{C}$ ; 1000 g) and the upper phase was discarded. Aliquots (0.1 and 0.4 mL) of the lower (organic) phase were

dried under vacuum for phospholipid analyses. Residues of 0.1 mL aliquots of the lower phase were assayed for total phospholipids by measuring phosphorus content (Svanborg and Svennerholm, 1961). Residues of 0.4 mL aliquots of the lower phase were reconstituted in 40  $\mu\text{L}$  methanol and subjected to thin-layer chromatography using silica G plates (Adsorbosil Plus-1, Alltech, USA), and a solvent system consisting of chloroform/ethanol/triethylamine/water (30:34:30:8) as the mobile phase. Phospholipid standards (Sigma, St. Louis, MO, USA) were used to identify the corresponding bands under UV light after the plates were sprayed with 0.1% diphenylhexatriene in petroleum ether. Bands for individual phospholipid classes (phosphatidylcholine [PC], phosphatidylethanolamine [PE], phosphatidylserine [PS], phosphatidylinositol [PI], and sphingomyelin [SM]) were scraped off the plates and extracted into 1 mL of methanol, dried under vacuum, and assayed for phosphorus content (Svanborg and Svennerholm, 1961).

Aliquots of hippocampal homogenates were assayed for total protein using a bicinchoninic acid reagent (Perkin Elmer, Norwalk, CT, USA). Phospholipid levels were expressed as nmol/mg protein.

#### 2.7. Western blot analysis

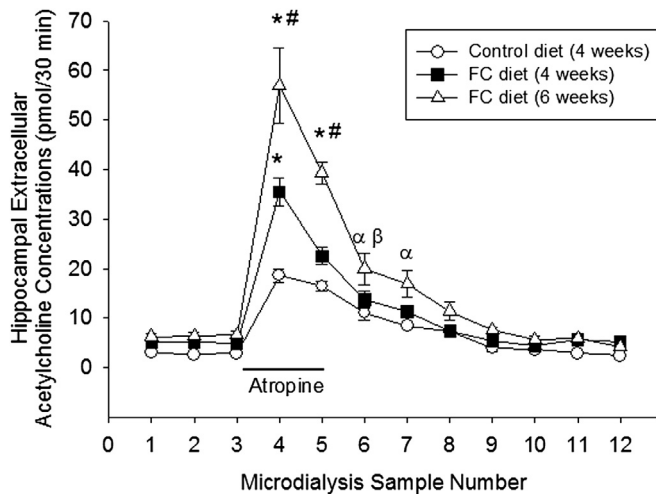
Synaptic proteins were assayed by Western blot as previously described (Sakamoto et al., 2007; Wurtman et al., 2006). Briefly, aliquots of hippocampal homogenates were mixed with equal volumes of Laemmli loading buffer and boiled before gel electrophoresis. Equal amounts of protein were loaded and separated using SDS-PAGE (4%–20%; Bio-Rad, Hercules, CA, USA). Proteins were then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The remaining binding sites were blocked with 4% nonfat dry milk (Carnation, Glendale, CA, USA) for 30 minutes in Tris-Buffered Saline and Tween 20 (TBST). Membranes were then rinsed 5 times in TBST buffer and incubated overnight in TBST solution containing the primary antibody of interest (goat anti-PSD-95 [Abcam, Cambridge, MA, USA; ab90426], rabbit anti-synapsin-1 [Abcam; ab18814], and rabbit anti-Nogo-A [Abcam; ab62024]). Next day, blots were incubated for 1 hour with the appropriate peroxidase-linked secondary antibody followed by detection and visualization of protein-antibody complexes using the enhanced chemiluminescence system (GE Healthcare Biosciences, Pittsburgh, PA, USA) and Kodak X-AR films which were digitized using a Supravista S-12 scanner (UMAX Technologies, Freemont, CA, USA). Immunoreactive bands were compared densitometrically using the Public Domain NIH Image program available at <http://rsb.info.nih.gov/ij/> according to the instructions of the software. Membranes were stripped using a stripping buffer (Thermo Fisher Scientific, Rockford, IL, USA) and then incubated with  $\beta$ -tubulin antibody (mouse anti- $\beta$ -tubulin [Sigma; T5076]) used as the loading control.

#### 2.8. ChAT level assay

ChAT levels were determined using an enzyme-linked immunosorbent assay kit (Cusabio, Wuhan, Hubei, China) with a detection range of 0.156–10 ng/mL according to the manufacturer's instructions.

#### 2.9. Statistics

Statistical analyses were performed using Sigma Plot software version 12.0. Data were expressed as mean  $\pm$  standard error of means. All data were analyzed using 1-way analysis of variance (ANOVA) followed by post hoc Tukey test, except for repetitive data which were analyzed by 2-way ANOVA with repeated measures followed by Tukey test.  $p$ -values  $< 0.05$  were considered significant.



**Fig. 2.** Hippocampal extracellular ACh concentrations. Following collection of 3 basal samples, the perfusion medium was replaced with another medium which contained atropine sulfate (10  $\mu$ M) for 1 hour, and then the hippocampus was perfused with the original medium which did not contain atropine. Microdialysates were collected every 30 minutes for a total of 6 hours. Data are expressed as mean  $\pm$  SEM. \*  $p < 0.001$  and  $^{\alpha} p < 0.05$  compared with respective time points in control-diet group. #  $p < 0.001$  and  $^{\beta} p < 0.05$  compared with respective time points in 4 weeks FC diet group using. Abbreviations: ACh, acetylcholine; FC, Fortasyn connect; SEM, standard error of the mean.

### 3. Results

#### 3.1. ACh release

Average levels of extracellular ACh in 3 baseline samples were  $2.9 \pm 0.1$ ,  $5.1 \pm 0.4$ , and  $6.5 \pm 0.4$  pmol/30 minutes in rats supplemented with control, FC (4 weeks), and FC (6 weeks) diets, respectively. Compared with the control group, supplementation with FC diet for 4 or 6 weeks significantly enhanced (by 1.75- and 2.24-fold, respectively;  $p < 0.001$  for each) average baseline extracellular ACh concentrations.

Atropine stimulation enhanced hippocampal extracellular ACh concentrations by a maximum of 6.5-fold (from mean baseline concentration of 2.9–18.6 pmol/30 minutes) at 30 minutes in control diet-treated rats although it enhanced hippocampal extracellular ACh concentrations by a maximum of 7-fold (from mean baseline concentration of 5.1–35.5 pmol/30 minutes) or 8.8-fold (from mean baseline concentration of 6.5–56.9 pmol/30 minutes) in rats consuming the FC diet for 4 or 6 weeks, respectively (Fig. 2). The enhancements in ACh concentrations in FC 4 weeks and FC 6 weeks groups were significantly larger compared with those in control group, and significantly higher extracellular ACh concentrations were measured in FC 6 weeks group compared with the FC 4 weeks group up to 2 hours after the onset of atropine stimulation (Fig. 2). Two-way ANOVA revealed a significant effect of dose ( $F[2, 11] = 32.965$ ;  $p < 0.001$ ), time ( $F[2, 11] = 97.666$ ;  $p < 0.001$ ), and a significant interaction of dose and time ( $F[2, 22] = 9.276$ ;  $p < 0.001$ ).

#### 3.2. Tissue ACh and choline content

ACh levels in left hippocampi of rats in the control, FC 4 weeks and FC 6 weeks groups were  $194 \pm 12$ ,  $249 \pm 11$ , and  $290 \pm 17$  pmol/mg protein, respectively (Fig. 3A). Compared with control group, tissue ACh content was significantly greater in FC 4 weeks ( $p < 0.05$ ) and FC 6 weeks ( $p < 0.001$ ) groups (Fig. 3A). On the other hand, tissue choline content was not statistically different in the control

( $331 \pm 18$  pmol/mg protein), FC 4 weeks ( $334 \pm 16$  pmol/mg protein), and FC 6 weeks groups ( $353 \pm 24$  pmol/mg protein) (Fig. 3B).

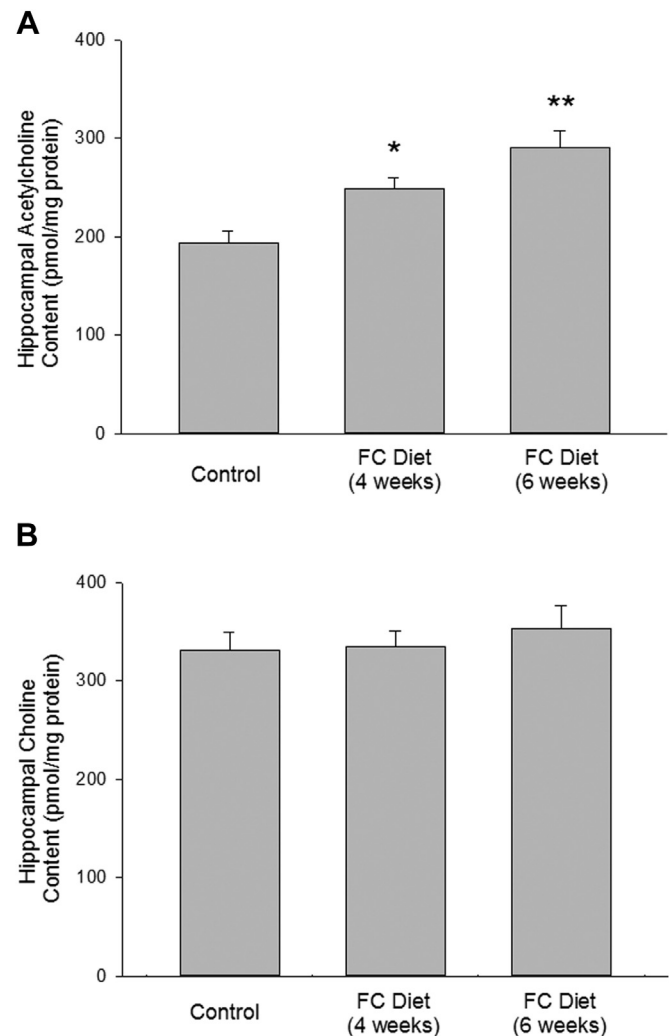
#### 3.3. Tissue phospholipid levels

Treatment with FC diet increased the contents of total and individual phospholipids in hippocampi of aged rats. Compared with the control group ( $321 \pm 15$  nmol/mg protein), FC diet for 4 or 6 weeks enhanced levels of total phospholipids in the hippocampus significantly by 1.2- ( $388 \pm 11$  nmol/mg protein;  $p < 0.05$ ) or 1.4- ( $447 \pm 13$  nmol/mg protein;  $p < 0.001$ ) fold, respectively (Table 2).

Levels of PC, PE, and PI were enhanced by supplementation with FC diet for 4 weeks by 1.3-, 1.4-, and 1.3-fold ( $p < 0.05$  for each), respectively (Table 2). Supplementation with FC diet for 6 weeks enhanced levels of PC, PE, PI, PS, and SM by 1.5- ( $p < 0.001$ ), 1.5- ( $p < 0.001$ ), 1.4- ( $p < 0.05$ ), 1.3- ( $p < 0.05$ ), and 1.3- ( $p < 0.05$ ) fold, respectively (Table 2).

#### 3.4. Levels of synaptic proteins

Dietary enrichment with FC diet for 6 weeks significantly enhanced levels of presynaptic and postsynaptic proteins while decreasing those of Nogo-A, a neurite outgrowth inhibitor.



**Fig. 3.** Hippocampal tissue ACh (A) and choline (B) content. Rats were sacrificed immediately after microdialysis experiments, and left hippocampi were obtained and homogenized to measure tissue ACh and choline content. Data are expressed as mean  $\pm$  SEM. \*  $p < 0.05$  and \*\*  $p < 0.001$  compared with control group. Abbreviations: ACh, acetylcholine; SEM, standard error of the mean.



**Table 2**  
Hippocampal phospholipid levels

Groups	Phospholipids (nmol/mg protein)					
	Total phospholipids	PC	PE	PI	PS	SM
Control	321 ± 15	139 ± 8	124 ± 5	9.8 ± 0.7	24.3 ± 1.2	12.3 ± 0.6
FC diet (4 wk)	388 ± 11*	187 ± 8*	171 ± 6*	13.2 ± 0.9*	28.9 ± 1.1	14.5 ± 0.9
FC diet (6 wk)	447 ± 13** <sup>†</sup>	202 ± 9**	190 ± 8**	13.7 ± 0.8*	30.9 ± 1.4*	15.8 ± 0.9*

\*  $p < 0.05$  and \*\*  $p < 0.001$  compared with control and <sup>†</sup>  $p < 0.05$  compared with FC diet (4 weeks).

Key: FC, Fortasyn connect; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

Compared with the control group, levels of Synapsin-1 (Fig. 4A) and PSD-95 (Fig. 4B) increased by 78% ( $p < 0.001$ ) and 75% ( $p < 0.05$ ) in rats receiving the FC diet for 6 weeks, respectively. On the contrary, levels of Nogo-A were decreased by 66% ( $p < 0.05$ ) in FC 6 weeks group (Fig. 4C). Although treatment with FC diet for 4 weeks tended to enhance levels of presynaptic and postsynaptic proteins and decrease those of Nogo-A, the effect did not reach statistical significance (Fig. 4).

### 3.5. ChAT levels

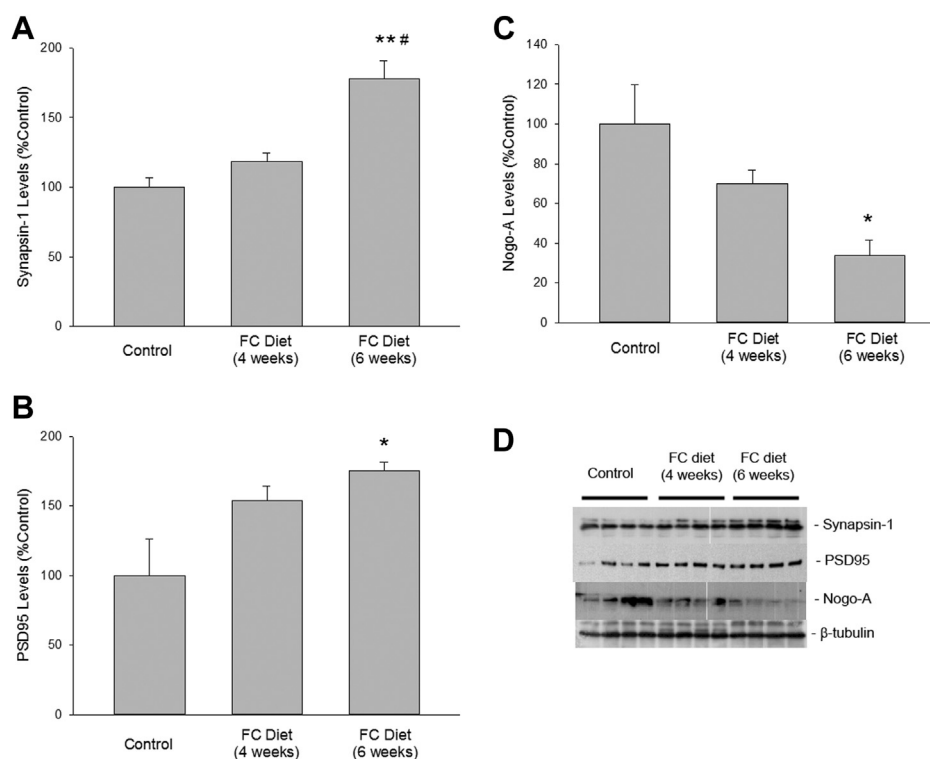
Treatment with FC diet for 6 weeks significantly enhanced ChAT levels compared with the control group ( $1.049 \pm 0.02$  vs  $0.868 \pm 0.06$  ng/mg protein;  $p < 0.05$ ). Although treatment with FC diet for 4 weeks tended to enhance ChAT levels, the effect did not reach statistical significance (Fig. 5).

## 4. Discussion

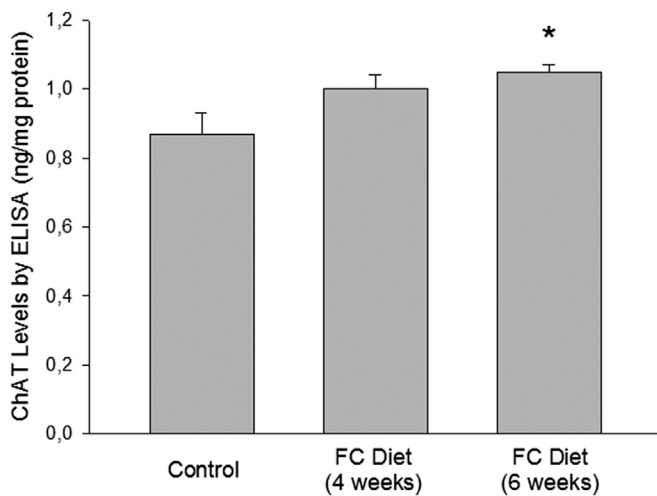
The present data show that dietary enrichment with FC of aged rats significantly enhances hippocampal ACh levels and release,

indicating enhanced cholinergic neurotransmission in the hippocampus, which was accompanied by increased levels of total and major individual phospholipids, ChAT, and the synaptic proteins Synapsin-1 and PSD-95, but decreased levels of the neurite outgrowth inhibitor, Nogo-A. These data suggest that previously observed positive effects of dietary supplementation with FC on learning and memory (Schelten et al., 2010, 2012) may at least in part be ascribed to the enhancement of hippocampal cholinergic neurotransmission.

FC is a specific nutrient combination containing nutritional precursors and cofactors that act together to support neuronal membrane formation and function. In this way, FC hypothetically improves processes relevant in AD that are partly or largely dependent on membrane formation and function, for example, synapse formation, neurotransmission, and memory performance (Van Wijk et al., 2014). The precursors for membrane synthesis provided by FC (DHA, eicosapentaenoic acid, uridine, and choline) act by enhancing the substrate-saturation of the enzymes that catalyze the rate-limiting steps in the synthesis of phospholipids, the main constituents of neuronal membranes (Wurtman et al., 2009). Other nutrients in FC (B-vitamins, vitamin C, vitamin E,



**Fig. 4.** Levels of synaptic proteins in the hippocampus. Synapsin-1 (A), PSD-95 (B) and Nogo-A (C) were assayed by Western blot using homogenates of left hippocampi. β-tubulin, a structural protein, levels of which did not differ between treatment groups, was used as protein loading control. Protein bands were given in panel D. Areas under the absorbance curve were expressed as arbitrary units and normalized as percentages of those generated in the same blot using samples from hippocampi of control animals. \*  $p < 0.05$ , \*\*  $p < 0.001$  compared with control group and #  $p < 0.05$  compared with 4 weeks FC diet group. Abbreviation: FC, Fortasyn connect.



**Fig. 5.** Hippocampal ChAT levels. Levels of ChAT in left hippocampi were assayed by ELISA method using a commercial rat ChAT kit. ChAT levels were expressed as ng/mg protein. Data are expressed as mean  $\pm$  SEM. \*  $p < 0.05$  compared with control group. Abbreviations: ChAT, choline acetyltransferase; ELISA, enzyme-linked immunosorbent assay; FC, Fortasyn connect; SEM, standard error of the mean.

selenium, and phospholipids) act as cofactors by increasing the availability of membrane precursors or by directly affecting the neuronal membrane or membrane synthesis (Van Wijk et al., 2014). Various in vitro and in vivo studies have previously shown effects of supplementation with combinations of membrane precursors and cofactors on phospholipid synthesis (e.g., Wurtman et al., 2006), neurite outgrowth (e.g., Pooler et al., 2005), levels of specific presynaptic and postsynaptic proteins (e.g., Cansev and Wurtman, 2007), number of dendritic spines (e.g., Sakamoto et al., 2007), neurotransmitter levels and release (e.g., Cansev et al., 2008; Wang et al., 2007), receptor signaling (e.g., Savelkoul et al., 2012), and cognitive performance (e.g., de Wilde et al., 2011; Holguin et al., 2008a, 2008b; Koivisto et al., 2014; Wiesmann et al., 2013). The clinical studies to date show that an FC-containing medical food improves memory function in early AD patients (Scheltens et al., 2010, 2012) and preserves functional brain network organization as measured by electroencephalography (de Waal et al., 2014). In the current in vivo study, we further explored the physiological mechanism underlying the cognitive enhancing effects of membrane precursors and cofactors by using the specific nutritional composition tested in these clinical trials.

Because improved learning and memory in various tasks is associated with enhanced cholinergic neurotransmission in the hippocampus (Fadda et al., 2000; Nail-Boucherie et al., 2000), we investigated the effect of FC diet supplementation on ACh release by in vivo microdialysis in aged rats. We found that supplementation with FC diet for 4 or 6 weeks significantly enhanced basal release of ACh in the hippocampus by 1.75- and 2.24-fold as compared with the control group, respectively. In addition, atropine-stimulated release of ACh in the hippocampus was enhanced by feeding the FC diet for 4 or 6 weeks by a maximum of 7- and 8.8-fold, respectively, as opposed to 6.5-fold in control group. Hence, the effect of FC supplementation was time dependent, and treatment for more than 6 weeks may further increase the basal and stimulated ACh release.

As discussed later, the enhanced ACh release under baseline and atropine-stimulated conditions is probably because of the increased levels of presynaptic membrane-dependent elements including presynaptic boutons and vesicles along with an increased ACh synthesis. The enhancements are probably not because of a decrease in ACh catabolism as was shown in a previous study with UMP treatment (Wang et al., 2007) and our unpublished observations

where we found no effects on acetylcholinesterase activity when feeding 21- to 24-month-old rats an FC-enriched diet (N. van Wijk, M.C. de Wilde and L. M. Broersen, unpublished results). Additionally, the enhancement in atropine-stimulated ACh release could be explained by autoregulation of presynaptic muscarinic receptors. The release of ACh from central cholinergic neurons is believed to be subject to autoregulation, whereby the neurotransmitter released interacts with presynaptic muscarinic receptors to inhibit the further release of neurotransmitter (Chesselet, 1984). It is therefore possible that the greater hippocampal extracellular ACh concentrations under baseline conditions in FC-treated rats provided a greater inhibition in ACh release through stimulation of presynaptic muscarinic autoreceptors, which in turn led to the enhanced release of ACh compared with control rats upon blockade of these receptors by atropine.

Our findings on enhanced ACh release with FC dietary enrichment are in line with previous reports which also tested effects of membrane precursors (choline, DHA, and uridine) on cholinergic transmission. For example, a choline enriched diet fed to rats was previously shown to increase basal and/or stimulated cortical and hippocampal release of ACh in rats (Beninger et al., 1984; Köppen et al., 1997). Dietary supplementation with DHA-containing phospholipids has been demonstrated to restore aging-induced reductions in hippocampal basal and stimulated ACh release (Favrelière et al., 2003). While basal ACh release was enhanced by 29%–59%, high-potassium-stimulated ACh release was enhanced by about 32% in 18-month-old rats fed the diet with DHA-containing phospholipids for 3 months (Favrelière et al., 2003). Furthermore, dietary enrichment with omega-3 PUFAs (including DHA) or DHA-containing phospholipids normalized omega-3 PUFA deficiency-induced changes in hippocampal basal and stimulated ACh release (Aid et al., 2003, 2005). In support of our present data, consumption of a diet containing UMP and standard levels of choline by young and aged rats led to augmented striatal ACh levels and increased basal and stimulated release of ACh in a previous study (Wang et al., 2007). The authors reported that supplementing aged rats for 1 or 6 weeks with 2.5% (wt/wt) UMP enhanced basal ACh release in striatum by, respectively, 2.0- and 2.7-fold versus controls (Wang et al., 2007). Consumption of a diet with lower levels (0.5%; wt/wt) of UMP for 1 week increased baseline ACh levels by 1.2-fold along with an enhancement in atropine-stimulated ACh release by 5.0-fold (Wang et al., 2007). Our present data are in line with previous findings reporting an enhancement in cholinergic neurotransmission after nutritional supplementation. On the other hand, a comparison with regard to the extent of the increase in ACh release and levels reported in these previous studies and our present results may not be reasonable because of the differences in experimental setup (e.g., brain region, age of animals, duration, nutrient levels). Further investigation should therefore focus on a comparison of efficacy of different combinations of nutrients.

The enhancement in hippocampal ACh release by dietary intervention with FC was accompanied by increased tissue content of ACh in the hippocampus. Moreover, levels of ChAT, the enzyme which mediates the synthesis of ACh from choline and acetyl-CoA, were enhanced by the FC diet, suggesting that synthesis of ACh was elevated. This suggestion is further supported by the present finding that the tissue content of choline did not change. This indicates that the additional choline in the FC diet made available for the brain was directly or indirectly used for ACh formation. Indirectly, FC-induced increased brain levels of PC and SM (as discussed in the following), for which choline is used, also could be a source of additional choline for ACh synthesis. Because brain levels of PC (and SM) are orders of magnitude greater than those of ACh, the 45% increase in PC in the present study could readily provide sufficient choline for the observed increases in ACh.

The present data on ChAT levels add up to the results of a previous study which showed that the reduction of ChAT immunoreactivity in A $\beta$ 42-infused rats was prevented in those receiving an FC-enriched diet (de Wilde et al., 2011). The increased tissue ACh levels found in the present study are also in line with previous publications (Cohen and Wurtman, 1976; Wang et al., 2007), reporting increased ACh tissue levels after supplementation with membrane precursors.

Although we found a nonsignificant 15% increase in ChAT by 4 weeks FC diet treatment compared with the control group, we observed a significant 29% increase in hippocampal ACh content and a significant 1.9-fold increase in ACh release following atropine stimulation. In addition, compared with the control group, 6 weeks FC diet treatment increased the levels in the hippocampus of ChAT with 21%. This discrepancy between the magnitude of effects on ACh levels and release and ChAT levels might be explained by an increase in the activity of ChAT enzyme. Further studies are needed to investigate the effect of FC diet on ChAT enzyme activity.

The FC-induced increase in hippocampal ACh content and release might involve enhanced synaptic membrane formation and consequently enhanced synapse formation. We therefore analyzed levels of phospholipids and synaptic proteins in hippocampal tissue samples of aged rats. We found that consumption of the FC diet for 4 or 6 weeks significantly enhanced the levels of total and individual phospholipids, including PC, the major membrane phospholipid. The cellular loci of the increased membrane phospholipids probably include synaptic vesicles and synaptic membranes, because the FC diet also raised the levels of the presynaptic vesicular protein Synapsin-1 and the postsynaptic protein PSD-95. Hence, supplementation with FC may produce more or larger synapses or synaptic vesicles which may contribute to increasing hippocampal ACh content and release. In accordance with our findings, the increase in amounts of membrane phospholipids after oral supplementation of DHA and/or UMP reported in previous studies (Cansev and Wurtman, 2007; Cansev et al., 2008, 2009; Sakamoto et al., 2007; Wurtman et al., 2006) were also shown to be associated with increased levels of specific presynaptic and postsynaptic proteins.

Interestingly, in contrast to the enhancement in synaptic proteins, levels of Nogo-A, a myelin-associated protein which inhibits neurite growth (Chen et al., 2000), were reduced significantly after 6 weeks of FC diet treatment. Nogo-A is expressed by both oligodendrocytes and neurons in the aged hippocampal formation and over-expressed by hippocampal neurons in AD (Gil et al., 2006). In addition, Nogo-A and its receptor have been localized in synaptic contacts (Liu et al., 2003) and the blockade of Nogo-A enhances axonal sprouting and synapse formation in neurons (Blochliger et al., 2001). Therefore, our present data suggest that the FC diet not only reduced the Nogo-A-induced inhibition of neurite outgrowth but may also help reduce the inhibitory effects of Nogo-A on neuronal plasticity and the formation of new synaptic contacts, as supported by the present evidence on synaptic proteins.

As observed in the current experiment, the increased formation of synaptic membranes and/or an increased ACh synthesis may enhance hippocampal neurotransmission, and collectively this may partly underlie the cognitive enhancing effects of FC. Nevertheless, the effects of the FC diet are probably not specific for cholinergic synapses and the effects are likely not only confined to the hippocampus. For example, supplementation of rodents with phospholipid precursors has previously been shown to enhance levels of hippocampal mGluR1 receptors (Sakamoto et al., 2007), along with those of Synapsin-1 and PSD-95, indicating an involvement of glutamatergic neurotransmission. Additionally, dietary supplementation with UMP has previously also been shown to increase striatal levels of dopamine in aged rats (Wang et al., 2005).

Therefore, it is highly possible that changes in other neurotransmitter systems may have contributed to the previously observed improvements with the FC nutrient combination in experimental and clinical studies. Further studies will be conducted to investigate the possible involvement of other systems that may underlie the cognitive enhancing effects of FC.

In conclusion, chronic consumption of the combination of the membrane precursors and cofactors contained within FC by aged rats enhances hippocampal cholinergic neurotransmission which is paralleled by increased synthesis of synaptic membranes. This is the first study to present a combined data set which provides further insight into cellular and molecular mechanisms by which combined supplementation of nutrients that are deficient in AD may support mnemonic processes.

## Disclosure statement

The present research has been partly funded by Nutricia Research. Mehmet Cansev is a scientific consultant to Nutricia Research. Nick van Wijk, John W.C. Sijben, and Laus M. Broersen are employees of Nutricia Research. The experimental protocol was approved by the Animal Care and Use Committee of Uludag University, Bursa, Turkey (Approval ID: 2012-03/03).

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