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Title:

Reduction in DHA transport to the brain of mice expressing human *APOE4* compared to *APOE2*.

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Short title : APOE4 reduces brain DHA uptake.

Abstract:

Benefits on cognition from docosahexaenoic acid (DHA, 22:6 n-3) intake are absent in humans carrying apolipoprotein E ε4 allele (*APOE4*), the most important genetic risk factor for Alzheimer's Disease (AD). To test the hypothesis that carrying *APOE4* impairs DHA distribution, we evaluated plasma and brain fatty acid profiles and uptake of [^{14}C]-DHA using in situ cerebral perfusion through the blood-brain barrier in 4 and 13-month-old male and female *APOE*-targeted replacement mice (*APOE2*, *APOE3* and *APOE4*), fed with a DHA-depleted diet. Cortical and plasma DHA were 9% lower and 34% higher in *APOE4* compared to *APOE2* mice, respectively. Brain uptake of [^{14}C]-DHA was 24% lower in *APOE4* versus *APOE2* mice. A significant relationship was established between DHA and apoE concentrations in the cortex of mice (r^2 = 0.21) and AD patients (r^2 = 0.32). Altogether, our results suggest that lower brain uptake of DHA in *APOE4* than in *APOE2* mice may limit the accumulation of DHA in cerebral tissues. These data provide a mechanistic explanation for the lack of benefit of DHA in *APOE4* carriers on cognitive function and the risk of AD.

Key words: apolipoprotein E, docosahexaenoic acid, blood brain barrier, long chain omega-3 polyunsaturated fatty acids, Alzheimer.

Introduction

Expression of the ε4 allele of apolipoprotein E (*APOE*) is associated with an increased risk for late onset familial as well as sporadic Alzheimer's disease (AD) (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993). The *APOE* gene has three major isoforms in humans, namely apoE2, apoE3 and apoE4. ApoE4 contains two arginine residues at position 112 and 158, instead of an arginine and a cysteine in apoE3 and a cysteine at both positions for apoE2 (Weisgraber et al., 1981). The frequency for *APOE4* allele is 13.6% in the general Caucasian population and reaches 36.7% in AD patients (Farrer et al., 1997). There is epidemiological evidence supporting lower risk of developing AD in *APOE2* carriers but higher risk in *APOE4* carriers (Corder et al., 1994; Hyman et al., 1996; Berlau et al., 2009; Small et al., 2004). Moreover, carrying two *APOE4* alleles increases the risk of developing AD by nearly fifteen times, compared to *APOE3* (Farrer et al., 1997; Holtzman et al., 2012). Magnetic resonance imaging shows that *APOE4* carriage accelerates the rate of hippocampal volume loss in non-demented older adults (Jak et al., 2007). Importantly, the alteration of apoE function caused by the *APOE4* allele has been consistently associated with a reduction of total brain apoE concentrations (Bales et al., 1997, Beffert et al., 1999).

ApoE protein transports cholesterol within the CNS and thus has an important role in brain lipid homeostasis (Boyles et al., 1989; Hauser et al., 2011). During aging, the brain loses approximately 20% of its weight, mainly in the form of lipids (Svennerholm et al., 1997). Among key brain lipids, long chain n-3 polyunsaturated fatty acids (PUFA), in particular docosahexaenoic acid (DHA, 22:6 n-3), are enriched in cerebral tissues. Most mammals must obtain preformed DHA from their diet because of their limited capacity to synthesize DHA from short chain n-3 PUFA (Plourde and Cunnane, 2007). The importance of maintaining high DHA concentrations for optimal brain function has been highlighted by series of studies in animals (Calon et al., 2004; Hooijmans et al., 2009; Arsenault et al., 2011) and clinical trials (Yurko-Mauro et al., 2010). For instance, decreasing brain DHA has been shown to be deleterious on synaptic function, processing of amyloid precursor protein, and brain glucose uptake (Bowen and Clandinin, 2002; Calon et al., 2004; Lim et al., 2005; Pifferi et al., 2005; Holguin et al., 2008; Arsenault et al., 2011), all of which have been demonstrated to be modulated by

APOE genotype (Reiman et al., 2004; Wang et al., 2005; Reiman et al., 2009; Klein et al., 2010; Arold et al., 2012; Hashimoto et al., 2012; Koffie et al., 2012; Li et al., 2012; Sen et al., 2012; Youmans et al., 2012; Zhu et al., 2012). Therefore, speculating that *APOE4*-induced depletion in DHA may underlie some of its known detrimental effects in the aging brain becomes a plausible hypothesis that needs further attention.

Most of the data from epidemiological and animal studies converge to suggest that diets enriched in n-3 PUFA, such as DHA, may protect against AD (Morris et al., 2003; Calon et al., 2004; Cunnane et al., 2009; Arsenault et al., 2011; Barberger-Gateau et al., 2011), although other studies do not support such an association (Devore et al., 2009; Engelhart et al., 2002; Mazereeuw et al., 2012). However, when detected, the association between n-3 PUFA consumption and lower incidence of cognitive decline is lost in *APOE4* carriers (Huang et al., 2005; Samieri et al., 2011). Large randomized controlled trial (RCT) performed in AD patients consuming 1.7-2.0 g per day of DHA found no improvement in cognition after 12-18 months (Freund-Levi et al., 2006, Quinn et al., 2010). However, after stratification of the study population by *APOE4* genotype, non-carriers of *APOE4* did improve their cognitive scores on Alzheimer Disease Assessment Scale-Cognitive and Mini–Mental State Examination after DHA treatment (Quinn et al., 2010). Sub-optimal n-3 PUFA bioavailability in *APOE4* carriers is a possible explanation since 1) postprandial DHA β-oxidation is higher (Chouinard-Watkins et al., 2013), 2) whole body half-life of DHA is 77% lower (Chouinard-Watkins et al., 2013), 3) plasma DHA increase in response to fish oil supplementation (3 g/day for 6 weeks) is 165% lower in *APOE4* carriers compared to non-carriers (Plourde et al., 2009) and 4) unlike *APOE2* and *APOE3* carriers, n-3 PUFA concentrations in erythrocytes are not correlated with better cognitive scores in both young (11-y-old) and older participants (65-y-old) expressing the *APOE4* allele (Whalley et al., 2008). Taken together, these results suggest that *APOE4* individuals might benefit less from dietary n-3 PUFA intake, possibly due to limited bioavailability in target tissue.

To determine whether the *APOE* genotype disrupts the accumulation of n-3 PUFA in the brain, we used human *APOE2*, *3* and *4* isoform-specific transgenic mice lacking a functional mouse apoE protein. First, we compared plasma and brain fatty acid profiles in mice in relation to expression of human *APOE2*, *3* or *4* in animals of 4 and 13 months

of age. To further investigate whether *APOE* genotype lowers uptake of DHA through the blood-brain barrier (BBB) into the brain, we performed *in situ* brain perfusion of [^{14}C]-DHA comparing *APOE2*, *3* and *4* mice at the same ages. Finally, we confirmed the association between DHA relative content and apoE concentrations using cortex samples from AD patients and control subjects.

Materials and methods

Animals

Male and female *APOE*-targeted replacement mice (*APOE2*, *3* and *4*) and C57BL6 littermate mice were used for the study (Sullivan et al., 1997). Those mice were created by gene targeting and replacing mouse *APOE* with human *APOE2*, *3* or *4*. *APOE* and C57BL6 control mice were purchased from Taconic (Hudson, NY, USA) and then reproduced at our animal facilities to complete the experimental groups. Animals were either sacrificed at the age of 4.1 ± 0.5 months (young mice) or at 13.4 ± 1.2 months (middle-aged mice). Before the age of 4 months, animals were fed with a commercial chow diet (Teklad 2018, Harlan Laboratories, Indianapolis, IN). From 4 to 13 months, animals were fed with a low n-3 PUFA, high n-6 PUFA diet (20.3% w/w carbohydrate, 66% w/w protein and 3.9 kcal/g), as described previously (Bousquet et al., 2011) and detailed in Supplementary Table 1. The final pelleted diet was produced by Research Diets Inc. (D04042202, New Brunswick, NJ, USA) using a formula precisely determined to avoid any batch to batch variations. The low n-3 PUFA, high n-6 PUFA diet was chosen to mimic the n-3 PUFA-deprived dietary environment of modern American society (Layé, 2010) and to avoid potential confounding long-term effects of circulating DHA (Ouellet et al., 2009). The diet started at 4 months of age to avoid neurodevelopmental artifacts. At the end of the experimental time, mice were perfused in the heart after deep anesthesia with ketamine/xylazine for collecting organs and tissues for Western immunoblotting and fatty acid profile determination, or were submitted to *in situ* cerebral perfusion (see below). After sacrifice, the brain was rapidly removed and the parieto-temporal cortex was dissected, frozen, powderized and kept at -80°C until lipid extraction and Western blot. All experiments were performed in accordance with the Canadian Council on Animal Care and were approved by the Institutional Committee of the Centre Hospitalier de l'Université Laval (CHUL).

Patients and handling of brain tissue

Brain samples from AD patients were obtained from the Douglas Hospital Research Center Brain Bank (Montreal, Canada) and were previously described (Julien et al., 2009). Written informed consent was obtained from all participants or their family for collecting brain samples after death. The diagnosis of AD was based on the

neuropathologic examination and was classified into definite AD or probable AD according to the Consortium to Establish a Registry for AD (CERAD 1/2) diagnostic criteria; controls were rated as possible AD or no AD (CERAD 3/4) (Mirra et al., 1994).

Brain and plasma fatty acid profile

For mouse samples, total lipids were extracted from ~30 mg parieto-temporal cortex or 0.5 ml plasma samples by the method described by Folch et al. (Folch et al., 1957). Total lipids were saponified and then transmethylated using 14% methanolic boron trifluoride. Fatty acid profiles were determined by gas chromatography equipped with a flame ionization detector (model 6890, Agilent, Palo Alto, CA, USA) and a 50-m BPX-70 fused capillary column (SGE, Melbourne, Australia; 0.25 mm inner diameter, 0.25 μm film thickness) as previously described (Plourde et al., 2009). For human brain samples, details on the methodology for fatty acid profile determination are reported elsewhere (Julien et al., 2009).

In situ cerebral perfusion

In situ cerebral perfusion technique was performed as previously described (Dagenais et al., 2000; Ouellet et al., 2009). This method allows the quantification of the transport of labeled molecules such as fatty acids through the BBB (Ouellet et al., 2009) and is performed by rigorously controlling perfusate composition, flow and volume, in the absence of peripheral metabolism (Dagenais et al., 2000). Briefly, after being anesthetized with a mixture of ketamine and xylazine (140/8 mg/kg), the right common carotid artery was catheterized after a ligation of the external branch. Then, the thorax was opened, the heart was removed and the perfusion started. The perfusion solution (bicarbonate buffered physiological saline, 128 mM NaCl, 24 mM $NaHCO_3$, 4.2 mM KCl, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 0.9 mM $MgCl_2$ and 9 mM D-glucose) was gassed with 95% O_2 and 5% CO_2 to reach a pH of 7.4 and then kept at 37°C. The perfusion flow rate was set at 1.68 ml/min for a total of 60 seconds after which the animal was decapitated. [^{14}C]-DHA (0.3 μCi/ml, 5 μM, Moravek Biochemicals, Brea, CA, USA) radiolabeled tracer was used to assess DHA transfer across the BBB. To quantify the vascular volume, [^{14}C]-sucrose (0.3 μci/ml) was perfused to another set of mice. After the perfusion, the brain was removed, the right hemisphere was digested in 1 ml of Solvable (Perkin-Elmer Life Sciences, Boston, MA, USA) overnight at 50°C. Nine ml of

HighSafe scintillation cocktail (Perkin-Elmer Life Sciences, Boston, MA, USA) was added to the samples. A Wallac scintillation counter (Perkin-Elmer Life Sciences, Boston, MA, USA) was used to quantify ^{14}C in the digested brain hemisphere. The volume of distribution (V_D) of [^{14}C]-DHA was expressed in μl/g and was calculated by dividing the concentration of [^{14}C]-DHA in the brain (μCi/g) with the concentration of [^{14}C]-DHA in the perfusate (μCi/μl). The volume of distribution (V_D) of [^{14}C]-DHA was corrected by subtracting the vascular space (V_{vasc}) also expressed in μl/g and determined by co-perfusing [^{3}H]-inulin (0.3 μCi/ml). The brain [^{14}C]-DHA transport coefficient (Clup; ml $g^{-1}s^{-1}$) was calculated by dividing the corrected apparent volume of distribution of [^{14}C]-DHA with the perfusion duration.

Western immunoblotting

Mouse parieto-temporal cortex and human parietal cortex were homogenized in 8 volumes and 5 volumes, respectively, of TBS solution containing a cocktail of proteases inhibitor (Complete™, Roche Diagnostics, Indianapolis, IN, USA) and phosphatases inhibitor (Phostop (Roche Diagnostics, Indianapolis, IN, USA), sodium orthovanadate and sodium fluoride). Samples were sonicated and centrifuged at 100,000 g for 20 min at 4°C to generate a homogenate containing TBS-soluble proteins. Protein concentration of the resulting TBS-soluble supernatant was assessed by Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA). Twenty μg of proteins were loaded and separated by sodium dodecyl sulfate-polyacrylamide electrophoresis gel and then electroblotted onto a PVDF membrane (Immobilon, Millipore, MA, USA). Membranes were blocked in 5% milk with 0.5% bovine serum albumin for 1 hour and immunoblotted with primary and then secondary antibodies followed by chemiluminescence reagents (LumiGLO Reserve Chemiluminescent Substrate Kit, KPL, Gaithersburg, MD, USA). Band intensities were determined using a KODAK Image Station 4000 MM (Molecular Imaging software version 4.0.5f7, KODAK, New Havem, CT, USA) Human apoE (1:20000, Novus Biological, Littleton, CO, USA), mouse apoE (1:2000, Novus Biological, Littleton, CO, USA) and actin (1:10000, Applied Biological Materials, Richmond, BC, Canada) antibody were used as primary antibody.

Statistical analysis

Data are presented as the Mean ± SEM. As no statistical difference has been found

between males and females, both gender were combined in each graph to enhance the statistical power. To achieve a probability higher than 90% of not committing a Type II error (power analysis), with expected changes of cortical DHA concentrations and brain DHA uptake estimated at 20%, a standard deviation of 20% and an α value set at 0.05, the number of mice per group was thus set at 10. For the human study, the magnitude of change in the measured endpoints was estimated at 20% according to published data (Bertrand et al., 1995). With an estimated standard deviation of 40% for Western Blot, and α set at 0.05, the number of human participants required per group was 20 to achieve 88% power. *APOE2*, *APOE3* and *APOE4* mice were compared by performing one-way ANOVA followed by Tukey-Kramer post hoc test and correlations were done using Pearson's correlation. No statistical comparison was made between young and middle-aged *APOE* mice because experiments were done separately. The number of mice per group is specified in each graph. Statistical significance was set at $p<0.05$. All statistical analyses were performed with Prism 4 (GraphPad software, San Diego, CA, USA) software.

Results

APOE4 genotype was associated to a lower cortex DHA relative content

Fatty acid profiles were first determined in samples from the parieto-temporal cortex to assess whether the *APOE4* genotype was associated with lower relative levels of brain DHA. In the young group of mice, the arachidonic acid (ARA; 20:4 n-6) percentage was 6% higher in *APOE3* cortex than *APOE2* mice of the same age (Fig. 1A. $p<0.05$). In separate experiments performed in 13-month-old mice, we noticed a higher proportion of n-6 docosapentaenoic acid (n-6 DPA, 22:5 n-6) in *APOE3* mice (Fig. 1B. +10%, $p<0.05$) compared to *APOE2* mice. Interestingly, among PUFAs, DHA was particularly sensitive to *APOE4* genotype since DHA proportion was 9% lower in 13-month-old *APOE4* mice compared to *APOE2* mice of the same age (Fig. 1B.).

APOE genotype modulates plasma fatty acid profile in young and old mice

N-6 and n-3 PUFA plasma composition was similar between groups in young mice (Fig. 2A.); however, significant changes were detected in 13-month-old mice. The relative content of total n-6 PUFA was 15% higher in *APOE4* mice compared to other genotypes ($p<0.05$) whereas total n-3 PUFA were nearly 40% higher in both *APOE3* and *APOE4* mice (Fig. 2B. $p<0.05$) compared to *APOE2* mice. More specifically, *APOE3* and *APOE4* expression led to 64% and 77% higher percentage of ARA in plasma total lipids compared to *APOE2* mice, respectively (Fig. 2B.). In addition, 13-month-old *APOE4* mice had 34% higher plasma DHA than *APOE2* mice (Fig. 2B. $p<0.05$).

Reduced brain DHA uptake in *APOE4* mice

The cerebrovascular volume and physical integrity of the BBB was assessed by perfusing the vascular space markers [^{14}C]-sucrose or [^{3}H]-inulin, which have a very poor BBB permeability under normal conditions. No difference of vascular volume was detected (Fig. 3A. and 3B.), suggesting that no major BBB leakage occurred during the experiments and ruling out the presence of a major cerebrovascular anomaly in *APOE4* mice. The Clup for [^{14}C]-DHA was 18% lower in 4-month-old ($p<0.05$) and 24% lower in 13-month-old ($p<0.01$) *APOE4* mice (Fig. 3A. and 3B. $p<0.05$ and $p<0.01$), compared to the *APOE2* mice, indicating that the brain transport of [^{14}C]-DHA across the BBB was reduced by *APOE4* expression in mice.

Reduced cortex human apoE content in *APOE4* mice.

As previously found in the same model (Riddell et al., 2008; Sullivan et al., 2011), the concentrations of human apoE was 56% and 43% lower in *APOE4* compared to *APOE2* mice at 4 and 13 months of age (Fig. 4A. 1) $p<0.001$ and 2) $p<0.05$, respectively). There was a significant difference in the TBS-soluble fraction and a similar trend was found in detergent-soluble fraction (Supplementary Fig. 2). These results differ from data previously published by Youmans et al., showing a reduction in apoE concentration in *APOE4* mice *but* only in detergent-soluble fractions (Youmans et al., 2012). In addition, we found that brain [^{14}C]-DHA uptake correlated with cortical apoE concentrations in young animals (Fig. 4B. 1) $r^2 = 0.34$, $p<0.01$) and with the percentage of DHA in cortical fatty acids from young and older animals (Fig. 4C. 1) $r^2 = 0.21$, $p<0.01$ and 2) $r^2 = 0.15$, $p<0.01$, respectively).

Cortex DHA correlated with apoE concentration in AD patients.

In line with previous studies, post mortem concentrations of apoE in the parietal cortex from AD patients were 61% lower compared to control donors (Fig. 5A. $p<0.01$) (Bertrand et al., 1995; Beffert et al., 1999). In addition, cortical concentrations of apoE were correlated with cortex DHA composition in AD patients (Fig. 5B. $r^2 = 0.32$, $p<0.01$) but not in control subjects (Fig. 5B. $r^2 = 0.003$, $p = 0.81$).

Discussion

The present study shows that plasma and brain fatty acid profiles as well as DHA transport across the BBB are influenced by human *APOE* genotype. Expression of human *APOE4* led to a lower proportion of DHA among brain fatty acids when compared to *APOE2* mice. The in situ demonstration of a significantly lower transfer of DHA across the BBB in *APOE4* mice suggests that impaired uptake mechanisms of DHA may have led to lower brain DHA over time. Most of the significant differences highlighted by this study were found between *APOE2* and *APOE4* mice whereas almost no difference was observed between *APOE3* and *APOE4* mice. Therefore, our results could be equally attributed to a protective effect of *APOE2* than to a deleterious effect of *APOE4*, in line with the meta-analysis of multiple epidemiological studies yielding AD risk odds ratio of 3.68 and 0.62 for *APOE4* and *APOE2,* respectively, compared to *APOE3* carriers (www.alzgene.org).

Lower brain uptake of DHA in *APOE4* compared to *APOE2* mice : possible mechanisms and consequences.

One of the most striking observations reported here is the effect of *APOE* genotype on brain of uptake of [^{14}C]-DHA. As evidenced by in situ cerebral perfusion, expression of *APOE4* led to a lower brain DHA uptake compared to APOE3 and APOE2 genotype in 4-month-old mice, but only compared to *APOE2* mice at 13 month. Limited diffusion of DHA in the brain can obviously have important long-term consequences. However, to understand how apoE influences brain DHA transport, one must first know how blood-borne DHA normally accesses the brain. Unfortunately, mechanisms by which DHA is transported through the BBB in the brain parenchyma are subject to controversies. It has been shown experimentally that free unbound DHA readily crosses the BBB through simple non-saturable diffusion (Hamilton and Brunaldi, 2007; Ouellet et al., 2009). Albeit in low concentrations at equilibrium, it is expected that enough free unbound DHA is available for the brain at any time to ensure supply to the brain (Hamilton and Brunaldi, 2007; Ouellet et al., 2009). Supporting this view, post-mortem plasma and brain DHA concentrations correlate well in animal models (Huang et al., 2007; Tu et al., 2012) and humans (Cunnane et al., 2012). On the other hand, other reports suggest that the exact plasma component to which DHA is bound can influence

its brain uptake or that fatty acid transporters may regulate DHA transport across endothelial cell membranes (Edmond, 2001; Mitchel and Hatch, 2011). Data gathered after intravenous injections of radiolabeled lipids suggests that DHA transport through the BBB is enhanced after binding to certain type of lipoproteins (Lagarde et al., 2001; Polozova and Salem, 2007), supporting a lipoprotein-mediated DHA transport to the brain. Despite the limited knowledge available about DHA transport across the BBB, several lines of thoughts can still be proposed to explain how apoE altered brain DHA uptake. First, if binding of DHA to transporters or lipoproteins is involved, then changes in apoE conformation modifying its binding properties would limit or enhance DHA transport to organs. As an example, changes in the interaction between the apoE-DHA complex and its receptors located at the BBB may therefore enhance or lower DHA transport through the BBB. One BBB receptor for which apoE affinity is known to differ between isoforms (*APOE4* and *APOE3* > *APOE2*) is the Low Density Lipoprotein receptor (LDL-R) (Malhey et al. 2009; Weisgraber et al., 1982). Secondly, the lower concentration of the apoE protein in the plasma of *APOE4* mice and humans (Fitz et al., 2012; Yasuno et al., 2012, Riddell et al., 2008) might result in less binding sites between lipoproteins and receptors, thereby reducing brain DHA uptake. On the other hand, however, results from the present in situ experiments, performed with a buffer devoid of circulating lipoproteins, strongly argue for a direct long-term effect of apoE on BBB function. The fact that DHA can cross the BBB by simple diffusion does not rule out the importance of regulatory mechanisms. Indeed, we have previously shown that chronic n-3 PUFA deprivation reduces DHA uptake into the brain, arguing that BBB permeability of DHA depend on brain needs (Ouellet et al., 2009). Such a view is also in agreement with recent reports on major BBB dysfunction in *APOE4* mice (Bell et al., 2012; Hawkes et al., 2012). Accordingly, the decreased passage of DHA across the BBB could results from a reduction of the cerebral vascularization in *APOE4* mice, as previously demonstrated (Bell et al.; 2012).

Impaired brain DHA uptake is associated with lower brain DHA concentrations in 13-month-old *APOE4*.

A change in DHA transport would be of limited consequence *per se* if not translated into altered concentrations in cerebral tissue. Replacing the coding sequence of the mouse

APOE gene with any of the 3 human *APOE* coding regions had little consequence for cortex fatty acid profiles at 4 month. Only a slightly higher relative level of ARA in young *APOE3* mice was observed. This finding is consistent with a previous study reporting no difference in the relative levels of hippocampal DHA between ~5 month-old *APOE3* and *APOE4* mice (Kariv-Inbal et al., 2012). In contrast, lower DHA relative ratio in 13-month-old *APOE4* mice indicates that long-term term expression of human *APOE4* has consequences on the brain membrane composition. Lower brain DHA uptake in *APOE4* mice over 13 months of life provides a simple explanation for observing lower cortex DHA content at this time point. Yet, the reason why lower brain DHA uptake in 4-month-old *APOE4* mice does not translate into lower cortex DHA content is unclear. Perhaps, it is simply because reduced supply of DHA needs to last over several months to translate into a significant decrease in brain DHA levels. DHA turnover in the brain is estimated to last up to 33 days in mice and 2.5 y in humans (Umhau et al., 2009; Rapoport et al., 2011). However, brain DHA half-life in *APOE4* is likely to be shorter since it was recently reported that plasma DHA half-life was 31% lower than in non-carriers (Chouinard-Watkins et al., 2013). Therefore, it is possible that DHA is β-oxidized faster in older animals (Plourde et al., 2011) leading to impaired capacity to maintain brain DHA composition, and failure to compensate the lower transport across the BBB of DHA observed in mice expressing *APOE4*. In support to this interpretation is the loss of the correlation between DHA uptake and apoE content in old mice. In any case, these data suggest that the effect of *APOE* genotype on DHA brain uptake is present early, whereas its consequence on DHA accumulation is age-dependent.

Is the rise in plasma DHA in 13-month-old *APOE4 mice* the result of reduced distribution in the brain?

Old *APOE4* mice had relatively higher plasma DHA, a result in line with observations in human *APOE4* carriers showing higher DHA relative content in plasma triglycerides than non-carriers (Plourde et al., 2009). This result might reflect a key role of apoE protein in plasma lipid transport from the liver to target tissues, including the brain (Mahley and Rall, 2000; Anil, 2007; Hooijmans and Kiliaan, 2008). Indeed, the main supply source of DHA for the brain is from the plasma (Diau et al., 2005; Umhau et al., 2009). On the one hand, the higher plasma composition in DHA observed here in

APOE4 compared to *APOE2* mice might be explained by an enhanced DHA carrying capacity in the blood. However, this should have been associated with increased brain DHA concentrations, which was not the case. On the other hand, such plasma data may also reflect a failure of DHA transport and distribution in target tissues, including the brain. Obviously, the lower relative DHA content found in the brain favors the second explanation, which is also supported by *in situ* perfusion data confirming a lower capacity of DHA to cross the BBB in 4-month-old as well as in 13-month-old *APOE4* mice. Therefore, the combination of the data generated here suggests that a lower brain DHA uptake in 13-month-old *APOE4* mice is associated with an accumulation of DHA in the blood and ultimately lower concentrations in cerebral tissue, in comparison to *APOE2* animals.

Brain DHA composition is linked to apoE content.

The data presented hitherto is significant only if representative of what is occurring in humans. Since the present experimental paradigm cannot be replicated in human volunteers, we performed linear regression analyses on brain samples from AD patients. Indeed, a significant post mortem correlation was found between DHA and apoE concentrations in the cortex of both transgenic mice and AD patients. This last observation in both species is consistent with a dose-dependent role of apoE protein content for maintaining brain DHA composition. In line with the assumption that *APOE4* allele on AD pathogenesis is deleterious and might partly be attributed to lower apoE tissue concentrations (Farrer et al., 1997, Beffert et al., 1999). The question that now has to be investigated is whether giving a high DHA diet can reverse this association and restore brain DHA in *APOE4* carriers to a level similar to non-carriers. If this hypothesis is confirmed, there is a large potential for translational research to the clinic. Therefore, the next step would be to determine adequate DHA intake for *APOE4* carriers for preventing cognitive decline.

In summary, our results indicate that cerebral uptake and homeostasis of DHA is largely influenced by the *APOE* genotype where *APOE4* carriers displayed lower brain uptake of DHA than non-carriers. Therefore, restoring n-3 PUFA homeostasis by providing a DHA-enriched diet should be considered in future trial studying *APOE4* carriers, ideally before the first symptoms of cognitive decline (Calon 2011). In addition, given the fact

that *APOE4* carrier represents 36.7% of AD patients, these results may have important nutrigenetic consequences that need to be taken into account in future DHA supplementation trials (Barberger-Gateau et al., 2011). Finally, since maintaining optimal brain DHA levels is essential for cognitive health, by showing that *APOE4* expression leads to reduced uptake and accumulation of DHA in the brain, our results also suggest a possible new mechanism by which *APOE4* aggravates the risk of developing AD.

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NS works for Nutritional Lipids, DSM Nutritional Products Inc.

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Figure legends

Figure 1 : Cerebral cortex fatty acid composition (%) in 4-month-old (A) and 13-month-old mice (B), according to *APOE* genotype. Each fatty acid is presented as % composition within total fatty acids. Cortex arachidonic acid (1), n-6 docosapentaenoic acid (2), n-6 polyunsaturated fatty acids (3) and docosahexaenoic acid (4) were analyzed by gas chromatography. Data are presented as mean ± SEM (n = 11 to 20 mice/group). One-Way ANOVA followed by post hoc Tukey-Kramer test was used to compare data. *p<0.05.

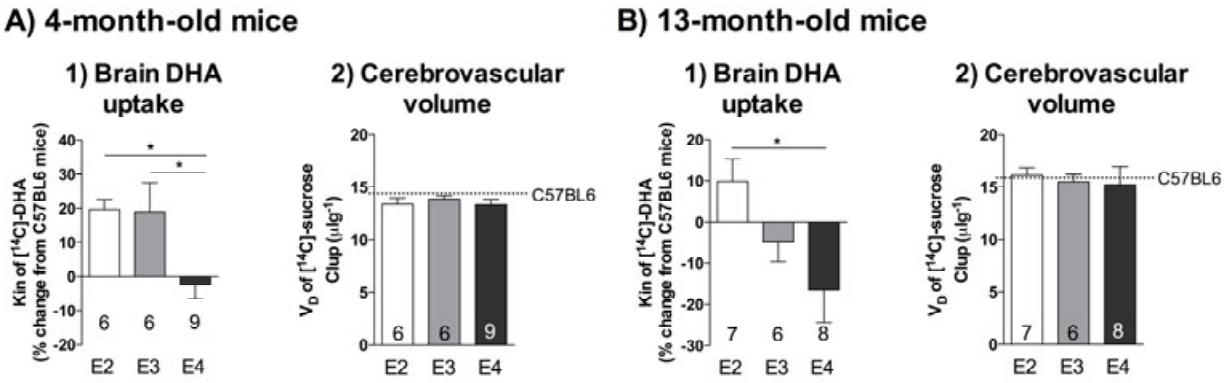
Figure 2 : Plasma fatty acid profile (%) is modulated by *APOE* genotype in 4-month-old (A) and 13-month-old mice (B). Fatty acid composition is presented as % composition within total fatty acids. Plasma arachidonic acid (1), total n-6 polyunsaturated fatty acids (2), docosahexaenoic acid (3) and total n-3 polyunsaturated fatty acids (4) were analyzed by gas chromatography. Data are presented as mean ± SEM (n = 6 to 11 mice/group). One-Way ANOVA, post hoc Tukey-Kramer was used to compare data. *p<0.05; ***p<0.0001.

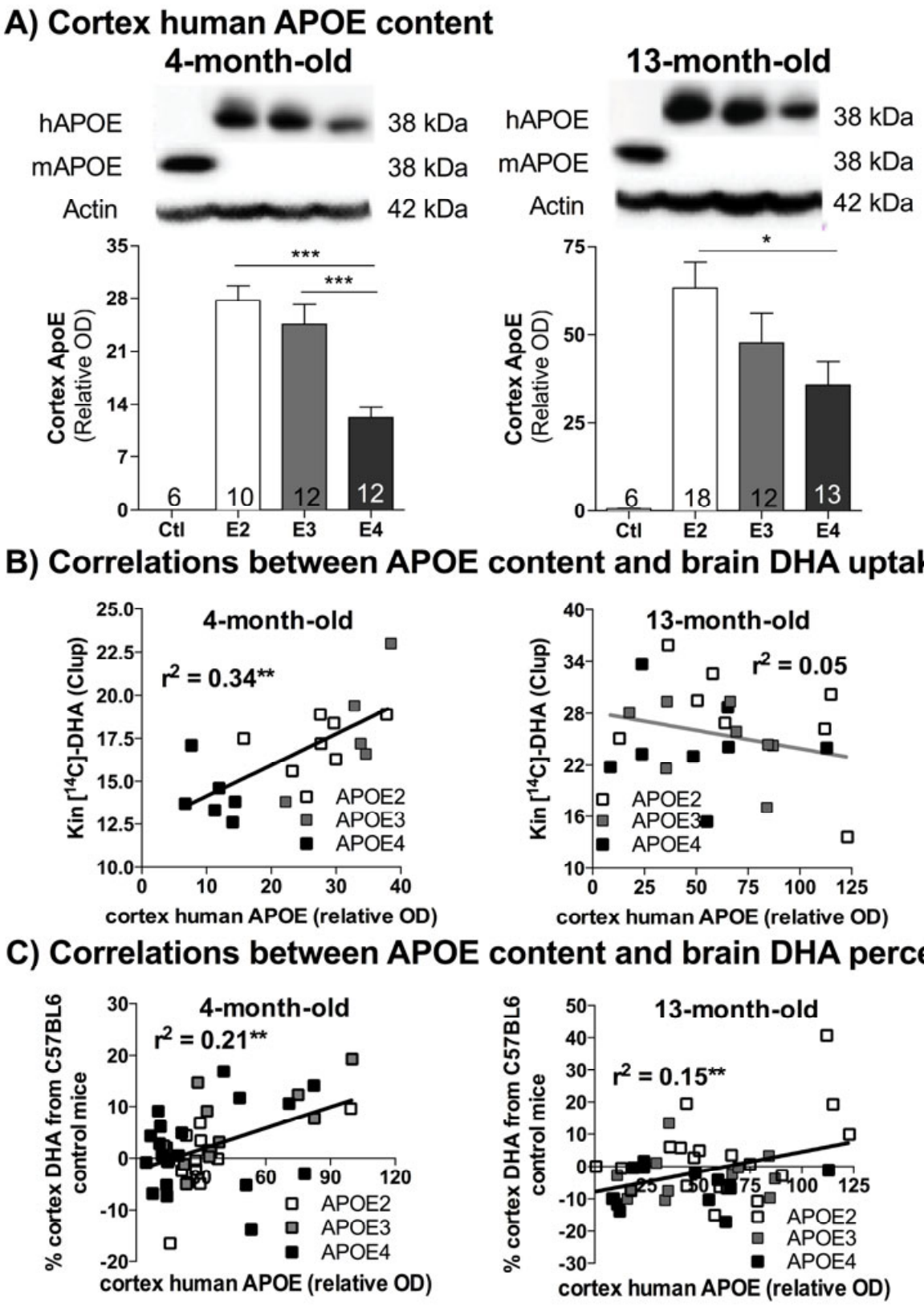
Figure 3 : Brain docosahexaenoic acid (DHA, 22:6 n-3) uptake of 4-month-old and 13-month-old APOE4 mice is lowered. Brain uptake of [^{14}C]-DHA (1) and cerebrovascular volume (2) in 4-month-old (A) and 13-month-old (B) mice determined with *in situ* cerebral perfusion technique. Data are presented as mean ± SEM (n = 5 to 9 mice/group). One-Way ANOVA, post hoc Tukey-Kramer was used to compare data. *p<0.05; **p<0.01.

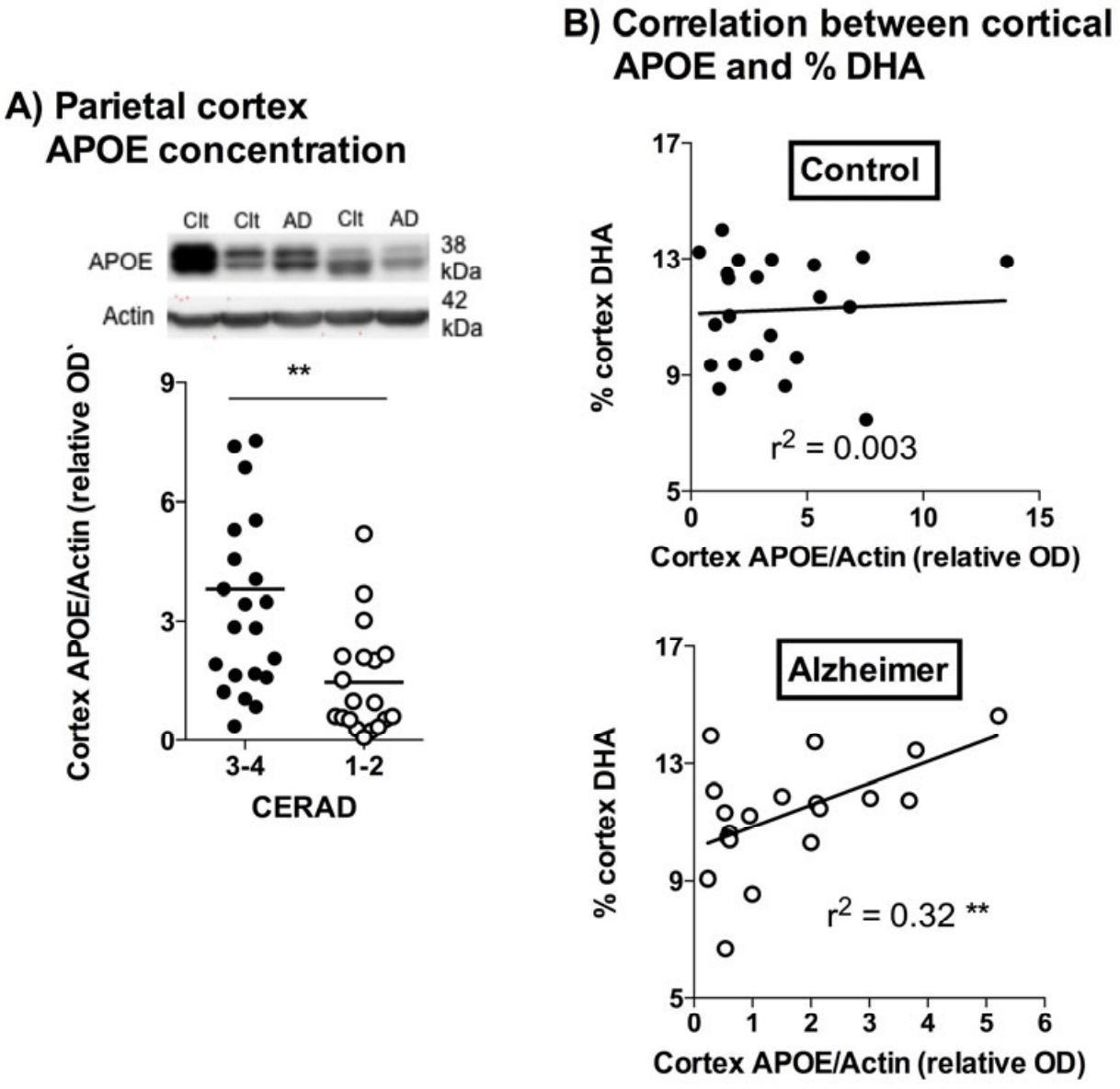
Figure 4 : Cortex human APOE content (A) correlates with docosahexaenoic acid (DHA, 22:6 n-3) passage across the blood brain barrier (B) and with % cortex DHA change compared to C57BL6 mice (C) in 4-month-old (1) and 13-month-old (2) APOE2, 3 and 4 mice. TBS-soluble APOE content in cerebral cortex was determined by Western blot. Data are presented as mean ± SEM (n = 6 to 18 mice/group). One-Way ANOVA, post hoc Tukey-Kramer was used to compare groups. Correlations between variables were evaluated using Pearson's correlation test. *p<0.05; **p<0.01; ***p<0.0001.

Figure 5 : Brain APOE concentrations correlate with docosahexaenoic acid (DHA, 22:6 n-3) relative content in AD patients. APOE concentration in TBS-soluble fraction of the parietal cortex samples was measured by Western Blot. Brain DHA is presented as %

composition within total fatty acids. Proportion of DHA within brain fatty acids was determined by gas chromatography. Data are presented as mean ± SEM (n = 20 to 22 subject/group). Welch's unpaired t-test was used to compare groups. Correlations between variables were evaluated using Pearson's correlation test **$p<0.01$.

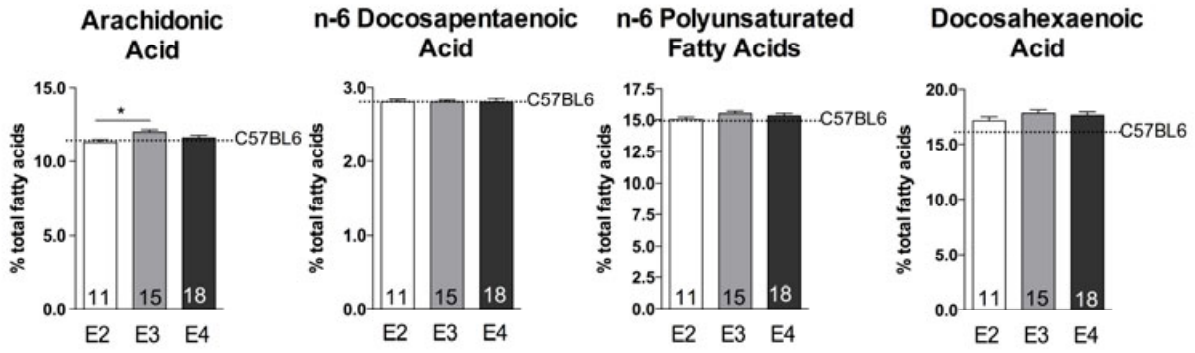

A) 4-month-old mice
1) Brain DHA uptake
2) Cerebrovascular volume
B) 13-month-old mice
1) Brain DHA uptake
2) Cerebrovascular volume
Kin of [14C]-DHA (% change from C57BL6 mice)
VD of [14C]-sucrose Ctup (µlg-1)
C57BL6
E2
E3
E4
6
6
9
7
6
8
*


A) Cortex human APOE content
4-month-old
13-month-old
hAPOE
mAPOE
Actin
38 kDa
38 kDa
42 kDa
Cortex ApoE (Relative OD)
***
***
*
6
10
12
12
18
13
Ctl
E2
E3
E4
B) Correlations between APOE content and brain DHA uptake
4-month-old
13-month-old
Kin [14C]-DHA (Clup)
r2 = 0.34**
r2 = 0.05
APOE2
APOE3
APOE4
cortex human APOE (relative OD)
C) Correlations between APOE content and brain DHA percent
% cortex DHA from C57BL6 control mice
r2 = 0.21**
r2 = 0.15**

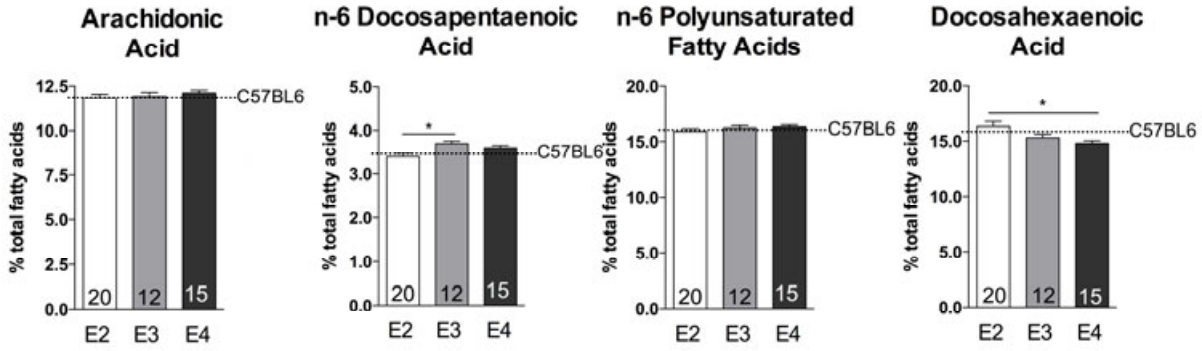

A) Parietal cortex
APOE concentration
Clt Clt AD Clt AD
APOE
38 kDa
Actin
42 kDa
**
Cortex APOE/Actin (relative OD`
9
6
3
0
3-4
1-2
CERAD
B) Correlation between cortical
APOE and % DHA
Control
% cortex DHA
17
13
9
5
r2 = 0.003
0
5
10
15
Cortex APOE/Actin (relative OD)
Alzheimer
% cortex DHA
17
13
9
5
r2 = 0.32 **
0
1
2
3
4
5
6
Cortex APOE/Actin (relative OD)

Cortex

A) 4-month-old mice

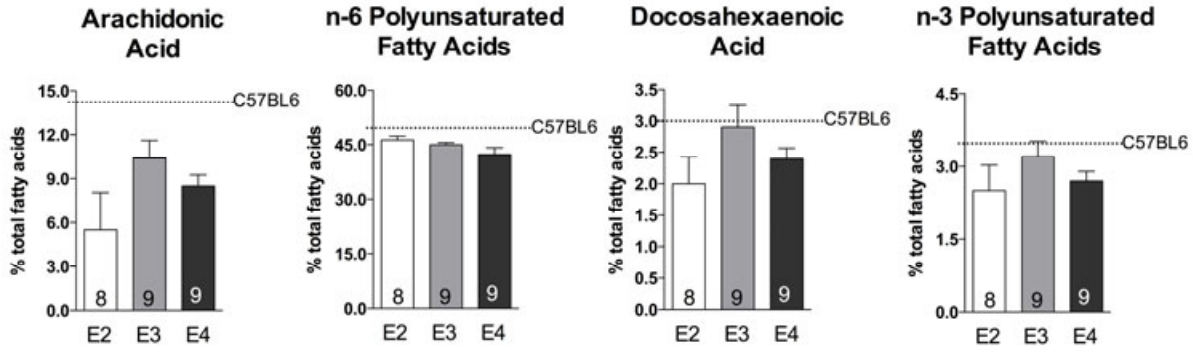


B) 13-month-old mice



Plasma

A) 4-month-old mice



B) 13-month-old mice