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ORIGINAL ARTICLE

Reduced blood-brain barrier expression of fatty acid-binding protein 5 is associated with increased vulnerability of APP/PS1 mice to cognitive deficits from low omega-3 fatty acid diets

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Abstract

Lower levels of the cognitively beneficial docosahexaenoic acid (DHA) are often observed in Alzheimer's disease (AD) brains. Brain DHA levels are regulated by the blood-brain barrier (BBB) transport of plasma-derived DHA, a process facilitated by fatty acid-binding protein 5 (FABP5). This study reports a 42.1 ± 12.6% decrease in the BBB transport of ^{14}C-DHA in 8-month-old AD transgenic mice (APPswe, PSEN1ΔE9) relative to wild-type mice, associated with a 34.5 ± 6.7% reduction in FABP5 expression in isolated brain capillaries of AD mice. Furthermore, short-term spatial and recognition memory deficits were observed in AD mice on a 6-month n-3 fatty acid-depleted diet, but not in AD mice on control diet. This intervention led to a dramatic reduction (41.5 ± 11.9%) of brain DHA levels in AD mice. This study demonstrates FABP5 deficiency and impaired DHA transport at the BBB are associated with increased vulnerability to cognitive deficits in mice fed an n-3 fatty acid-depleted diet, in line with our previous studies demonstrating a crucial role of FABP5 in BBB transport of DHA and cognitive function.

Keywords: Alzheimer's disease, blood-brain barrier, cognitive function, docosahexaenoic acid, fatty acid-binding protein, omega-3 fatty acids.

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by the progressive deterioration of cognitive function and behavior that significantly impairs the activities of daily living (Imbimbo *et al.* 2005). Lower docosahexaenoic acid (DHA) levels have been identified in the frontal cortex as well as in the hippocampus and parahippocampus of post-mortem AD brains, relative to age-matched healthy brains (Söderberg *et al.* 1991; Lukiw *et al.*

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Abbreviations used: AD, Alzheimer's disease; BBB, blood-brain barrier; CNS, central nervous system; DHA, docosahexaenoic acid; FABP, fatty acid-binding protein; n-3, omega-3; NOR, novel object recognition.

2005). These brain regions are important for learning and memory (Lukiw *et al.* 2005; Kim *et al.* 2010), suggesting a correlation between cognitive impairment in AD and lower brain DHA levels.

Given that brain DHA is primarily derived from the plasma (Rapoport *et al.* 2001, 2007), one likely reason for the lower brain DHA observed in AD is reduced blood-brain barrier (BBB) transport of this fatty acid, a suggestion that is supported by the findings of Calon *et al.* (2011) reporting that BBB transport of DHA is decreased in 3xTg AD mice (with *Psen1* mutation, APPswe and tauP301L transgenes). Using a different AD mouse model (Tg2576, over-expres sing APPswe), dietary n-3 fatty acid restriction for 5 months lead to decreased cortical DHA content in AD mice but not wild-type (WT) mice (Calon *et al.* 2005). Together, these findings suggest that an AD-related impairment in the BBB reduces its ability to transport adequate DHA from the plasma, with dietary intervention further augmenting this dysfunction.

The mechanism of fatty acid transport into the brain has been the subject of much attention with both passive diffusion and protein-mediated transport being implicated (Chen *et al.* 2008; Hamilton *et al.* 2012; Nguyen *et al.* 2014; Ochiai *et al.* 2017). Regardless of the mechanism by which fatty acids cross the apical membrane of the brain endothelial cell, it is likely that once they enter the aqueous environment of the cytosol, they require assistance from an intracellular lipid-binding protein to facilitate trafficking to the abluminal membrane given their poor aqueous solubility. We have recently demonstrated that the intracellular fatty acid-binding protein (FABP5) plays a significant role in the BBB transport of DHA (Pan *et al.* 2015, 2016), facilitating uptake of DHA into brain endothelial cells *in vitro* and transport across the BBB *in vivo*.

It has been reported that the levels of another isoform of FABP, FABP3, are decreased in post-mortem AD brains (Cheon *et al.* 2003), and so it was postulated that a reduction in BBB transport of DHA in AD may be associated with decreased expression of BBB-relevant FABPs. As FABP5 is the predominant FABP isoform expressed at the BBB, a reduction in FABP5 may lead to reduced DHA uptake into the CNS in AD, rendering AD mice more sensitive to the cognitive-declining effects of DHA deficiency. Therefore, in the present study, the expression and function of FABP5 at the BBB was measured in an animal model of AD (APP/PS1 mice). The APP/PS1 mice harbour chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe/Swedish mutations K595N/M596L) and mutant human presenilin-1 (PS1/ΔE9). The BBB transport of ^{14}C-DHA was assessed in 8-month-old WT and APP/PS1 mice using an *in situ* transcardiac perfusion technique, with brain capillary-enriched fractions being isolated from both genotypes to compare expression of FABP5. WT and APP/PS1 mice (from 8 months of age) were fed either a control or an n-3 fatty acid-deficient diet (n-3 depleted diet) for 6 months, after which the Y maze spatial recognition, novel object recognition (NOR) and water maze tests were performed to assess cognitive function. Brain fatty acid levels were measured in these mice post-mortem, using gas chromatography with flame ionization detection (GC-FID) to associate cognitive function with brain DHA levels. To our knowledge, this is the first study showing decreased BBB expression of FABP5 in AD and a relationship between reduced cognitive function from DHA depletion to BBB transport phenomena.

Materials and methods

Materials

Bovine serum albumin, Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline, Supelco® 37-component FAME mix, methyl nonadecanoate, calcium chloride, glucose, sodium chloride, potassium chloride, magnesium sulfate, sodium bicarbonate and monosodium dihydrogen orthophosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). ^{14}C-DHA and DHA were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA) and Cayman Chemicals (Ann Arbor, MI, USA) respectively.

Animals

The study was not pre-registered. All animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee (MIPS.2012.46) and performed in accordance with the National Health and Medical Research Council guidelines for the care and use of animals for scientific purposes at Monash University. Double transgenic C57BL/6J mice (APP/PS1) expressing chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe/Swedish mutations K595N/M596L) and mutant human presenilin-1 (PS1/ΔE9) and their WT littermates (RRID: MGI: 5702670) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed as a group of 3–5 in a tinted open top mouse cage at 21 ± 2°C, kept under a 12 : 12-h reverse light-dark cycle (lights on at 7 pm), with food and water provided *ad libitum* from 8 weeks of age. Welfare-related assessments were performed daily, as required by the approved animal ethics protocol. All mice were fed standard chow (WEHI irradiated mouse breeder cubes, Ridley Agri Products, Melbourne, Victoria, Australia), which contains approximately 8.5% fat, primarily from canola oil (Table 1), unless specified for dietary intervention (Section Impact of DHA deficiency on cognitive function). A total of 8 APP/PS1 and 8 WT mice (male, 8–9 months old) were used for *in situ* transcardiac perfusion, 23–24 mice from each genotype (female, 8–9 months) were used for brain capillary-enriched fraction isolation, and 25 APP/PS1 and 22 WT mice were employed for behavioral assessments (female, 14–15 months). Sample size calculations were performed using a calculation program available from http://www.openepi.com/SampleSize, with 95% confidence interval and 80% power for studies comparing two groups. For behavioral studies, the number of animals used was determined from a previous study, where an effect on behavioral outcomes was detected (Pan *et al.* 2016).

Table 1 Fatty acid composition of rodent diets

	Standard chow	Control diet	n-3 depleted diet
Myristic acid 14:0	0.50	Trace	0.16
Palmitic acid 16:0	13.69	4.30	5.76
Stearic acid 18:0	6.02	2.01	2.49
Palmitoleic acid 16:1	0.49	0.29	0.16
Oleic acid 18:1	55.06	55.73	67.60
Gadoleic acid 20:1	0.99	1.00	1.71
Linoleic acid 18:2 n-6	21.37	21.63	16.98
α-Linolenic acid 18:3 n-3	n.d.	14.04	0.62
Arachidonic acid 20:4 n-6	n.d.	n.d.	Trace
EPA 20:5 n-3	n.d.	n.d.	n.d.
DHA 22:6 n-3	0.40	n.d.	n.d.

Data are presented as mg/100 mg total fatty acid. n.d. = not detectable.

Measurement of ^{14}C-DHA transport across the BBB

To assess ^{14}C-DHA transport across the BBB, an *in situ* transcardiac perfusion was performed as previously described (Pan *et al.* 2015). Mice were anaesthetized using intraperitoneal ketamine/xylazine, a method of anaesthesia used previously for this technique (Pan *et al.* 2015). Briefly, the left and right jugular veins of anaesthetized mice were severed and the descending thoracic aorta was clamped, followed by perfusion into the left ventricle of the heart at a rate of 10 mL/min. The mice were first perfused with Krebs carbonate-buffered physiological saline (KBR) for 1 min at 37°C to remove residual blood (to avoid potential binding of DHA to plasma proteins) and then with KBR containing 0.09 mM ^{14}C-DHA for 1 min. The concentration of ^{14}C-DHA is similar to free plasma DHA concentrations reported previously (Abdelmagid *et al.* 2015). The perfusion rate was controlled by a Harvard infusion pump (Harvard Apparatus, Holliston, MA, USA). At the end of the perfusion, mice were decapitated and the whole brain was harvested. Sample preparation, radioactivity detection and calculation of brain-to-perfusate ratio (with vascular volume subtraction) were performed as previously described (Pan *et al.* 2015).

Measurement of FABP5 expression in mouse brain capillary-enriched fractions

Mouse brain capillary-enriched fractions were isolated from 8-month-old female APP/PS1 mice and their WT littermates as described previously (Triguero *et al.* 1990). Briefly, brain cortices were homogenized in ice-cold DMEM using a Dounce homogenizer (Tissue Grinder, Potter-ELV, Millville, NJ, USA). The capillary-enriched fractions were separated from brain parenchyma using 15% w/v bovine serum albumin with centrifugation at 2000 xg for 30 min at 4°C. Brain cortices of four mice of the same genotype were randomly pooled to obtain ample capillary-enriched fractions. Proteins were extracted from the capillary-enriched fractions using two freeze-thaw cycles and sonication, with the total protein concentration estimated by absorption at 280 nm using a NanoDrop® 1000 Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). The expression of FABP5 was measured via ELISA (DL Sci&Tech Development, Wuxi, Jiangsu, China), as detailed previously (Pan *et al.* 2015). FABP5 protein expression was normalized to total protein concentration and expressed as pg/mg.

Impact of DHA deficiency on cognitive function

At 8 months of age, female APP/PS1 and WT mice were allocated into two groups by an independent researcher using simple randomization (i.e. mouse cages were labelled with a number randomly generated by computer, and mice in even numbered cages were fed a control diet and mice in odd numbered cages were fed an n-3 depleted diet). Mice were fed with these diets for 6 months. The control diet was standard AIN93G rodent chow and the n-3 depleted diet was a low n-3 fatty acid modified version of AIN93G (Specialty Feeds, Glen Forrest, Western Australia, Australia), with both diets containing approximately 7% fat (Table 1). In the n-3 depleted diet, canola oil from the control diet was replaced with peanut oil and sunflower oil to maintain the same level of n-6 fatty acids but reduced n-3 fatty acids (Table 1). The only source of n-3 fatty acid in the control diet and n-3 depleted diet is α-linolenic acid. The n-3 depleted diet has a much lower α-linolenic acid composition. As DHA can only be converted from its n-3 fatty acid precursor in the body, the lower α-linolenic acid in the diet would warrant lower amounts of DHA available for the mouse. Prior to any behavioral experiments, mice were individually housed for 2 weeks and to reduce handling stress, mice were regularly handled by the experimenter for 3–5 min at a fixed time daily, 1 week prior to commencing the behavioral assessments. Before each session, mice were allowed to acclimatize in the behavioral assessment room for 1 h. A unique number was assigned to each mouse, and the experimenters were blinded to the genotype of the mice when performing behavioral assessments. The timeframe for the behavioral assessments are summarized in Fig. 1. The genotype and treatment of mice, which had previously been allocated an identification number, were unknown to the experimenter at the time of behavioral studies.

The Y-maze was used to assess short-term spatial memory, using a protocol slightly modified from Dellu *et al.* (1997). The Y-maze is composed of three arms (50 cm L × 16 cm W × 31 cm H) in a shape of capital Y, with each arm decorated with different shapes as visual cues on the inner wall of the maze. Each animal was allowed to explore the Y-maze (with the novel arm closed) for 5 min in the training session. After 2 h, animals were placed back to the same Y-maze with all three arms accessible for 5 min. Mouse activity in the Y-maze was recorded and the time spent in each arm and the total distance travelled were analyzed using video tracking (Biobserve® Viewer 3, Bonn, Germany). Data were presented as time spent in the novel and familiar arm, and the total distance travelled was used to assess the activity level of the mice. Animals which spend more than 45% of time in the starting arm were removed from the study.

The novel object recognition (NOR) task was used to measure recognition memory function (Antunes and Biala 2012). On day 1 and 2, the animals were introduced into an open arena (40 cm L × 40 cm W × 30 cm H) for a 5-min acclimatization session. On day 3, the animals were allowed to explore the arena for 5 min, with two identical objects (250 mL Schott duran bottles, with their surfaces covered by masking tape) placed in the middle of the arena. Animals showing bias (> 60% of total exploration time) to either object were removed from the study. After 2 h, the trained animals were tested in the arena with one of the familiar objects

Fig. 1 Timeframe for the behavioral assessments. Following 6 months of dietary intervention, the wild-type mice and APP/PS1 mice underwent a series behavioral studies, such as Y maze, NOR and water maze, prior to the end point studies., i.e. quantification of brain DHA levels, and transcardiac in situ perfusion.

replaced with a novel object (a can of Pepsi®). The distance travelled by the animal within the arena on day 1 and 2 (to measure locomotor activity), and the time spent exploring each object on day 3 was determined by video tracking (Biobserve®). To assess any anxiety in mice, the percentage of time that mice displayed thigmotaxis (the tendency to remain close to vertical surfaces) was calculated for the day 1 acclimatization session as detailed previously (Simon *et al.* 1994) and this data was presented as percentage of time that mice spent in the centre zone of the open arena. The ability of the animal to recognize the novel object was determined by the preference index, calculated using eqn 1.

$$\text{preference index} = \frac{\text{novel object exploration time}}{\text{total objects exploration time}} \quad (1)$$

The water maze was used to assess spatial learning, with a protocol similar to the method previously described by Gulinello *et al.* (2009) and adapted in our laboratory (Pan *et al.* 2016). The mice underwent a series of trials on two consecutive days. Consistent and distinct spatial cues were placed externally in the room and on the internal wall of the pool (120 cm in diameter and 20 cm in depth), dividing the pool into four quadrants. An escape platform (12 cm in diameter) was placed in a fixed position diagonally opposite the starting position. On day 1 (cued training), the animals were placed into the pool at a fixed starting position and allowed to find the visible escape platform (0.5 cm above water, marked with a flag) in four trials with an inter-trial interval of 30 ± 10 min. Mice failing to find the platform by 60 s were manually guided to the platform on which they were left for 10–20 s. On day 2 (spatial trial), the mice underwent three hidden platform trials (submerged 0.5 cm below the water, without a flag), with an inter-trial interval of 30 ± 10 min. Swimming behavior was recorded and the position of the mouse was determined by video tracking. The platform visit latency (escape latency) and average swimming velocity was determined by video tracking. Mice which failed to find the escape platform in all four trials on day 1 were excluded from study.

Analysis of brain fatty acids

Following behavioral studies, total lipids were extracted from the brain cortex of mice and prepared as methylesters (FAME) for gas chromatography (GC) according to a previously published method (Fraser *et al.* 2008), with methyl nonadecanoate (C19:0ME) as the internal standard. Analysis of FAME was conducted using a 6850 Agilent GC-FID (Agilent Technologies, Mulgrave, Vic., Australia), equipped with a 30 m × 0.25 mm id column with a 0.25 m thick film (df) of DB-5 ms phase. A quantity of 1 μL of Supelco® 37-component FAME mix or sample solution was injected into the GC inlet at a split ratio of 20 : 1. The oven was programmed to change from 60°C (0.1 min) to 280°C at 20°C/min and held for 5 min. Hydrogen (99.999% pure) was used as carrier gas at a constant flow rate of 1.5 mL/min and the detector temperature was 250°C. Data were processed using Chemstation software (Agilent, Santa Clara, CA, USA). The FAME peak of stearic acid, palmitic acid, arachidonic acid and DHA in the samples was identified according to the retention time and elution order of the FAME mix. The peak area of these fatty acids was normalized to the peak area of the internal standard and the weight of the cortical tissue. The absolute amount of DHA in the sample was calculated by comparison of peak areas to a calibration curve created by spiking DHA into brain homogenate, and expressed as mg of DHA per g of brain cortex.

Statistical analysis

All data are expressed as mean ± SEM. Data analyses were performed using SPSS (IBM, North Castle, NY, USA) or Prism software (GraphPad, La Jolla, CA, USA). The comparisons between experimental and control groups were evaluated by Student *t*-tests when only two groups were compared. ANOVAs were performed for analyses where genotype and diet effects were investigated, with repeated measures in the water maze. For the Y-maze spatial recognition test, a two-way ANOVA with repeated measures was performed followed by *post hoc* pairwise ANOVA comparisons if an interaction or trend of interaction ($p < 0.1$) was identified. Statistical significance was set at a value of $p < 0.05$.

Results

BBB transport of DHA and FABP5 expression are reduced in 8-month-old APP/PS1 mice

A transcardiac *in situ* brain perfusion study was performed in 8-month-old male APP/PS1 and their WT littermates, and a significant 42.1 ± 12.6% decrease in ^{14}C-DHA transport across the BBB was observed in APP/PS1 mice (Fig. 2a; $n = 8$, unpaired Student t-test, $^{**}p = 0.0024$). Given female APP/PS1 mice displayed more robust pathology (Wang *et al.* 2003), female mice were used for all the follow-up studies. As reduced BBB transport of DHA could be attributed to decreased BBB expression of FABP5 (Pan *et al.* 2015), FABP5 expression at the BBB in 8-month-old female APP/PS1 and WT controls was examined. A 34.5 ± 6.7% lower expression of FABP5 in brain capillary-enriched fractions of female APP/PS1 mice compared to WT littermates was revealed (Fig. 2b; $n = 7$–8, unpaired Student *t*-test, $^{***}p = 0.0002$).

Mice fed an n-3 depleted diet exhibited reduced body weight but unaffected activity levels

After 6 months of dietary intervention, the body weight of mice in all groups were measured (Table 2). A two-way ANOVA revealed a main effect of diet ($F_{1,43} = 11.9$, $p = 0.0018$, $n = 11$–13), where mice fed an n-3 depleted diet demonstrated a 12.9 ± 4.0% lower body weight than mice fed a control diet, regardless of genotype. Changes in body weight could influence the activity levels of mice, and subsequently any changes in behavior may be interpreted as altered cognitive function, rather than a diet-induced change in weight and mobility. Therefore, the motor activity of the mice was assessed in the behavioral paradigms used in this study. There was no significant impact of diet, genotype or any interactions between the two factors identified in the distance travelled in the Y-maze paradigm (Fig. 3a), in NOR acclimatization sessions (Fig. 3b), or in the average swimming velocity measured in the water maze (Fig. 3c).

Table 2 Body weight (gram) of WT and APP/PS1 mice fed a control or n-3 depleted diet

Control diet		n-3 depleted diet	
WT ($n = 13$)	APP/PS1 ($n = 11$)	WT ($n = 12$)	APP/PS1 ($n = 11$)
37.6 ± 1.5	38.7 ± 1.5	32.8 ± 1.2	33.2 ± 1.7

APP/PS1 mice fed an n-3 depleted diet demonstrated short-term memory deficits in both Y-maze and NOR

At 14–15 months of age, assessments of learning and memory were performed in WT and APP/PS1 mice fed either a control or an n-3 depleted diet. In the Y-maze spatial recognition test (Fig. 4a), the time mice spent in the novel and familiar arms was analyzed using a two-way ANOVA with repeated measures, revealing an overall arm effect ($F_{1,36} = 56.6$, $p < 0.001$), a main genotype effect ($F_{1,36} = 7.2$, $p = 0.011$) and a trend for genotype x diet interaction ($F_{1,36} = 3.0$, $p = 0.09$). Further *post hoc* pairwise ANOVA comparisons indicated that all mice except APP/PS1 mice fed an n-3 depleted diet spent more time in the novel arm, an indication of normal memory function. In APP/PS1 mice fed an n-3 depleted diet, the inability to distinguish between the familiar and novel arm was indicative of impaired short-term spatial memory.

In the NOR task (Fig. 4b), a two-way ANOVA of the preference index revealed a significant effect of genotype ($F_{1,24} = 9.0$, $p = 0.0063$), no main effect of diet, but an interaction between genotype and diet ($F_{1,24} = 7.3$, $p = 0.0126$). Furthermore, Tukey multicomparisons revealed that the preference index for APP/PS1 mice fed an n-3 depleted diet was significantly lower than WT mice regardless of diet (control, $p < 0.05$; n-3 depleted diet, $p < 0.01$) and lower than APP/PS1 mice fed the control diet ($p < 0.05$). These data suggest that APP/PS1 mice fed an n-3 depleted diet were unable to differentiate between the novel and previously explored familiar object, as these mice spent an equivalent amount of time exploring both objects in the final testing session (Fig. 4c). The percentage of time spent proximal to the perimeter walls (an indicator of thigmotaxis) that each cohort of mice displayed in the first habituation session was analyzed and a two-way ANOVA did not reveal any effect of genotype or diet, nor an interaction between the two factors. All animals spent a comparable time in the centre zone of the open arena (Fig. 4d).

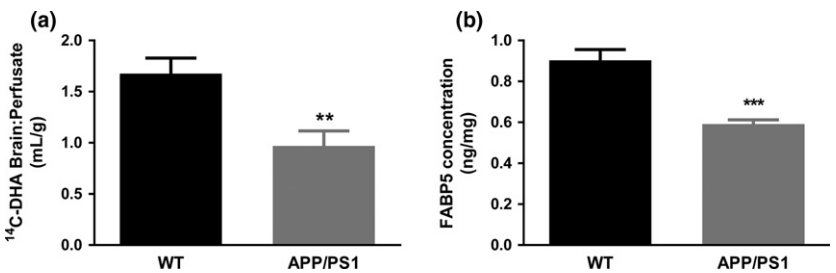

Fig. 2 (a) The ^{14}C-DHA brain:perfusate ratio in 8-month-old male WT and APP/PS1 mice, demonstrating a reduced ^{14}C-DHA transport across the blood-brain barrier (BBB) of APP/PS1 mice compared to WT littermates ($n = 8$ animals, unpaired Student *t*-test, $^{**}p = 0.0024$). N represents number of animals used. (b) The expression of fatty acid-binding protein 5 (FABP5) in brain capillary-enriched fractions (isolated from 8 to 9 months old WT and APP/PS1 female mice) assessed by ELISA, with the expression of FABP5 normalized to total microvascular protein. A significant reduction in the expression of FABP5 in cerebral microvessels isolated from APP/PS1 mice was identified ($n = 7$–8, unpaired Student *t*-test, $^{***}p = 0.0002$), where n represents the number of microvessel fractions, with each fraction isolated from 2 to 3 animals of the same genotype.

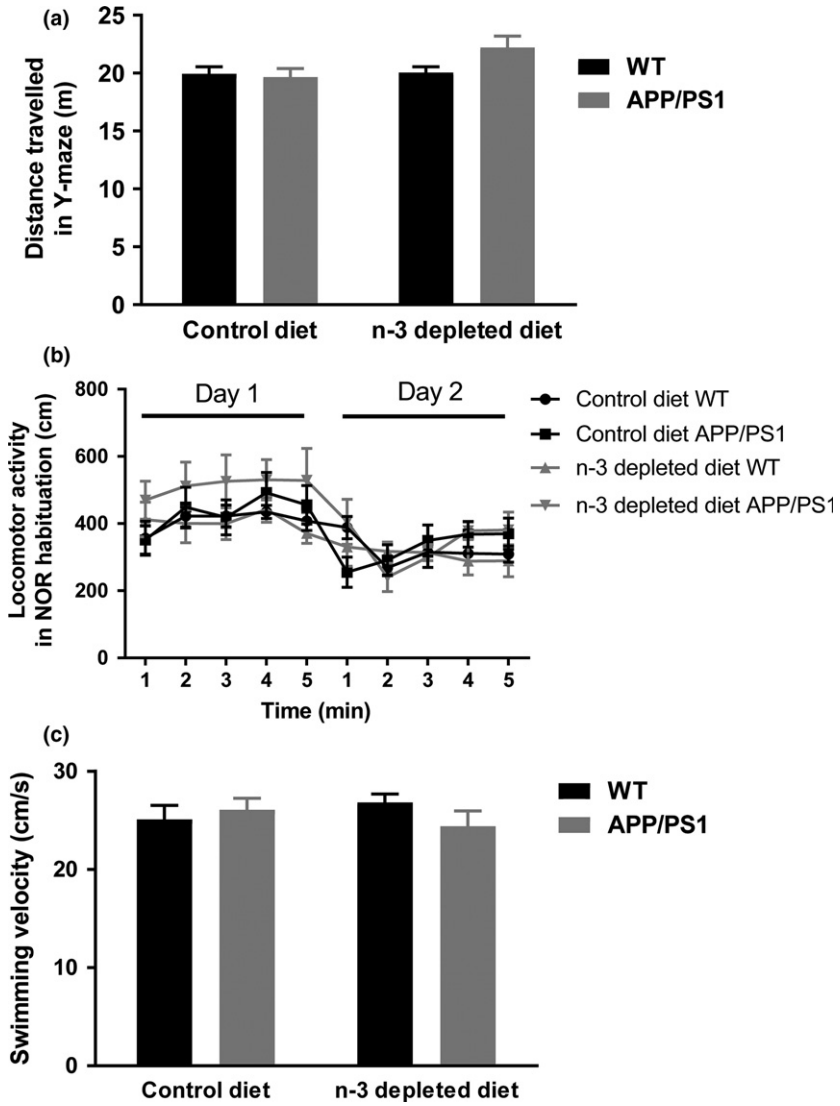

Fig. 3 (a) The distance travelled by the mice in the Y-maze, where no main effect of genotype or diet was identified, nor an interaction between the two factors (two-way ANOVA; $n = 8$–12). (b) The locomotor activity of the mice in the NOR habituation session on day 1 and day 2, with data plotted as distance travelled by the mice in the NOR arena (cm) versus time (min). No main effect of genotype, diet or session was identified nor an interaction between these factors (two-way ANOVA with repeated measures; $n = 5$–8). (c) Swimming velocity (cm/s) of WT and APP/PS1 mice fed a control or an n-3 depleted diet, with no main effects of genotype or diet, nor an interaction between the two factors (two-way ANOVA; $n = 11$–13). *N* represents number of animals used.

APP/PS1 mice displayed impaired memory retention over 24 h in the water maze task, with no effect of the n-3 depleted diet

A significant effect of session was observed during the cued training sessions (two-way repeated measures ANOVA; $F_{3,35} = 15.6$, $p < 0.001$) and the spatial trials (two-way repeated measures ANOVA; $F_{2,36} = 10.6$, $p < 0.001$), indicating that over successive trainings, all mice, regardless of genotype or diet, exhibited a decreased latency to find the platform (Fig. 5). At the completion of the cued training sessions, all mice were able to swim to the platform with no significant differences in latency (two-way ANOVA, $n = 9$–13); however, in the first spatial trial, it took APP/PS1 mice significantly longer to locate the platform than the WT mice regardless of diet (two-way ANOVA, $F_{1,40} = 5.1$, $p = 0.03$, $n = 9$–13), indicating impaired memory retention over 24 h.

Brain DHA levels are reduced from APP/PS1 transgenes and DHA dietary deficiency

Total brain DHA levels were measured using GC in WT and APP/PS1 mice fed either a control or an n-3 depleted diet (Fig. 6). A main effect of genotype ($F_{1,12} = 7.1$, $p = 0.0203$) and diet ($F_{1,12}$=5.0, $p = 0.0449$) was identified, suggesting that total brain DHA levels are lower in APP/PS1 mice regardless of diet, and an n-3 depleted diet could lower the brain DHA levels in mice of both genotype. It appears that APP/PS1 mice fed the n-3 depleted diet displayed the lowest brain DHA levels. No significant alteration to the brain levels of stearic, palmitic and arachidonic acids has been observed.

Discussion

A correlation between diminished plasma/brain DHA levels and reduced cognitive function in AD has been reported

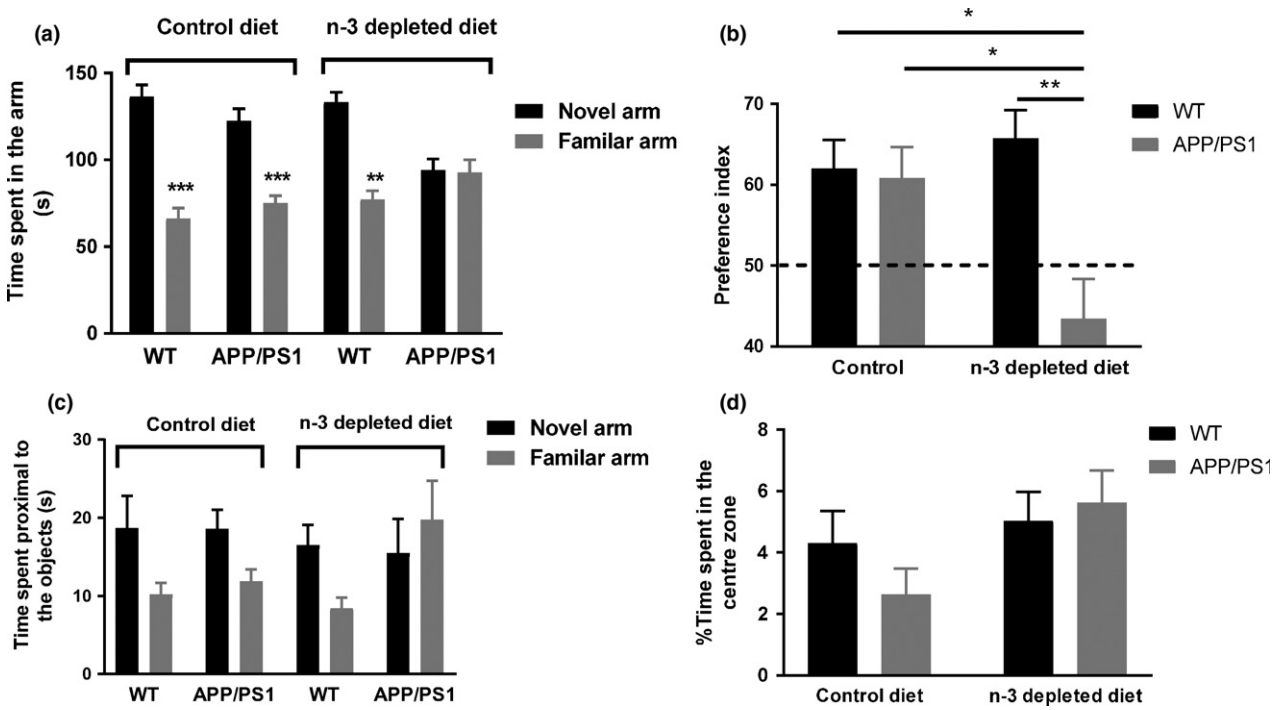

Fig. 4 (a) The time mice spent in the novel and familiar arm of the Y-maze over 5 min, with all mice except APP/PS1 mice fed an n-3 depleted diet spending significantly more time in the novel arm than the familiar arm (pairwise ANOVA comparison, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 8$–12). (b) The preference index calculated from the NOR task ($n = 5$–8), with APP/PS1 mice fed an n-3 depleted diet exhibiting a significantly lower preference for the novel object compared to the familiar object relative to WT mice fed a control diet ($^{*}p < 0.05$) or an n-3 depleted diet ($^{**}p < 0.01$), and APP/PS1 mice fed a control diet ($^{*}p < 0.05$). The dash line at 50 refers to an equal exploration of the two objects by chance, i.e. no preference. (c) The time (sec) mice spent in proximity to the novel and familiar object in the NOR test. (d) The percentage of time mice spent in the centre zone of the open arena in the first habituation session in NOR, with no effect of genotype or diet observed. N represents number of animals used.

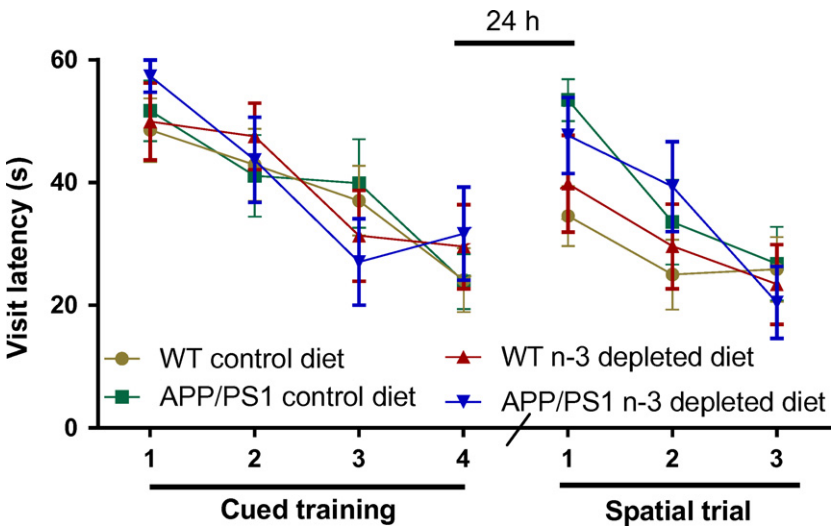

Fig. 5 Performance of WT and APP/PS1 mice fed a control diet or an n-3 depleted diet ($n = 9$–13) in the cued training and spatial trials of the water maze paradigm, assessed as absolute escape latency, with APP/PS1 mice showing impaired memory retention over 24 h (two-way ANOVA, $^{*}p = 0.03$). N represents number of animals used.

(Cunnane *et al.* 2012; Yassine *et al.* 2016), suggesting that lower brain DHA levels could potentially contribute to the cognitive impairment observed in AD. As brain DHA is primarily plasma-derived, reduced brain DHA levels could be attributed to a deficiency in BBB transport of DHA in AD, though other mechanisms may affect brain DHA levels. Consistent with this suggestion, Calon (2011) has demonstrated lower BBB transport of DHA in 3xTg AD mice, however, no mechanism was proposed. We have recently demonstrated that FABP5 plays a significant role in the BBB

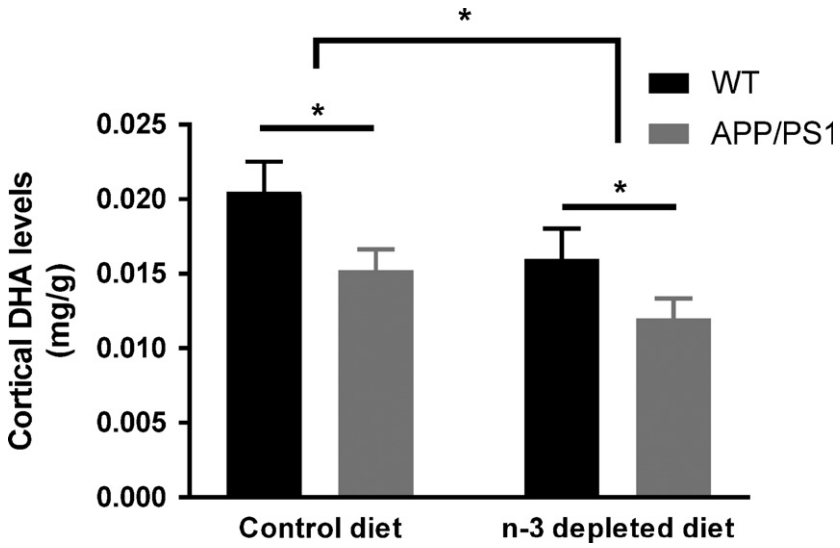

Fig. 6 Total brain cortical DHA levels in WT and APP/PS1 mice fed either a control or an n-3 depleted diet (n=4), with a two-way ANOVA revealing a main effect of genotype ($F_{1,12}$ = 7.1, *p = 0.0203), and a diet effect ($F_{1,12}$ = 5.0, *p = 0.0449). Lower brain DHA levels were observed in APP/PS1 mice relative to WT littermates, regardless of diet, and the n-3 depleted diet significantly reduced the brain DHA levels in both WT and APP/PS1 mice. *N* represents number of animals used.

transport of ^{14}C-DHA (Pan *et al.* 2015), raising the possibility that changes to FABP5 levels in AD may mediate altered BBB transport of DHA and alter cognitive function. In the current study, therefore, the BBB transport of ^{14}C-DHA and the levels of FABP5 in APP/PS1 mice have been evaluated, alongside an investigation assessing whether these AD mice are more vulnerable to cognitive decline following a DHA (n-3)-deficient diet.

In male APP/PS1 mice, we demonstrated a significant (42.1 ± 12.6%) reduction in BBB transport of ^{14}C-DHA. Although not an intact physiological system, the transcardiac perfusion technique employed is a commonly used method to assess BBB transport of probe compounds (Banks *et al.* 2008; Urayama *et al.* 2015), and a concentration of DHA was perfused which was similar to that observed in plasma (Abdelmagid *et al.*, 2015). Therefore, it is expected that the results generated using this technique would be representative of those observed in an intact physiological system. Given that female APP/PS1 mice display an advanced AD pathology earlier than male APP/PS1 mice (Wang *et al.* 2003), an even lower BBB transport of DHA in female APP/PS1 mice could be postulated. Reduced BBB expression of FABP5 in the female APP/PS1 mice demonstrated in the present study lends support to this postulation, as decreased BBB expression of FABP5 could compromise BBB transport of DHA (Pan *et al.* 2015). One of the mechanisms responsible for this reduction in FABP5 expression could be altered Peroxisome proliferator-activated receptor (PPAR) activity in AD, given that at least in prostate cells, PPAR agonists have been shown to regulate FABP5 (Morgan *et al.* 2010) and decreased PPAR activity is associated with an increased risk of AD (Heneka *et al.* 2011). However, whether PPAR agonism regulates FABP5 at the BBB, and whether reduced PPAR activity leads to lower FABP5 expression in AD requires further investigation. Given that FABP5 has been shown to be up-regulated by DHA (Figueroa *et al.* 2016), the reduced FABP5 at the BBB in APP/PS1 mice could also be secondary to lower plasma levels of DHA. Indeed, lower DHA levels in the liver have been reported in 3-month-old APP/PS1 mice compared to WT littermates (Perez *et al.* 2010), which suggests a possible lower plasma DHA level in the APP/PS1 mice, as liver fatty acid levels correlate well with plasma fatty acid levels (Carpentier *et al.* 2008). The lower plasma/liver DHA levels could be as a result of altered lipid homoeostasis in the periphery, similar to the alteration in lipid homoeostasis reported in AD brains (Xu and Huang 2006).

FABP5 (Pan *et al.* 2015), fatty acid transport protein (FATP-1) (Ochiai *et al.* 2017) and Mfsd2a (Nguyen *et al.* 2014) have been demonstrated to be involved in the BBB transport of DHA, with Msfd2a specifically transporting only esterified DHA (Nguyen *et al.* 2014). It is also possible that FATP-4 is involved in the cellular uptake of DHA (Murphy 2017). Given un-esterified DHA is the main source of brain DHA (Chen *et al.* 2015), FABP5 could retain plasma-derived un-esterified DHA in the brain endothelial cells, allowing abluminal FATP-1 to transport DHA into the brain (Ochiai *et al.* 2017). Of particular importance, we reported a dramatic reduction in the brain microvascular expression of FABP5 in APP/PS1 mice, to which we attribute the reduced BBB transport of ^{14}C-DHA observed in this mouse model of AD. However, given the roles of FATP-1 and FATP-4 in the trafficking of DHA, both of which are expressed at the BBB (Mitchell *et al.* 2011), we also assessed whether their expression was affected in brain homogenates of APP/PS1 mice. Interestingly, we demonstrated down-regulation of FATP-1 (35.2 ± 9.0%) and FATP-4 (40.6 ± 16.5%) in the brain homogenates of 14–15 months old APP/PS1 mice compared to their WT littermates (Supporting Information). Given the expression of FATP-1 and FATP-4 was examined

in brain homogenates (which contains non-endothelial components also expressing these membrane transporters (Mitchell *et al.* 2011)), it cannot be confirmed that the reduction in these membrane transporters is specific to the microvasculature, and further studies assessing the expression of these membrane transporters in isolated cerebral capillaries is required. Nevertheless, taken together with the reduced brain microvascular expression of FABP5, we have demonstrated that processes important in the overall blood-to-brain trafficking of DHA are affected in APP/PS1 mice. Other alterations at the BBB may also contribute to decreased DHA transport across the BBB, for example, general membrane thickening as demonstrated previously in 3xTg AD mice (Mehta *et al.* 2013a,b).

Having established that APP/PS1 mice exhibit lower DHA transport across the BBB, and that this is associated with reduced FABP5 expression, dietary intervention studies were conducted to further elucidate how APP/PS1 mice respond to an n-3 depleted diet. The duration of the dietary intervention used in the present study (6 months) was comparable to other published studies manipulating dietary n-3 fatty acids (Calon *et al.* 2005; Ouellet *et al.* 2009). A battery of cognitive assessments was performed at the end of the 6-month dietary intervention. Although the dietary intervention affected the body weight of the animals, the activity levels of the mice were unaffected. Hence, the body weight reduction observed after feeding the n-3 depleted diet did not appear to be a contributing factor in the performance of mice in subsequent assessments of cognitive function. Such reduction in body weight is in line with previous reports (Pachikian *et al.* 2008).

In the Y-maze spatial recognition test, all mice except the APP/PS1 mice fed an n-3 depleted diet were able to distinguish the novel arm from the familiar arm and spent more time in the novel arm. This observed impairment in short-term spatial memory was not found in APP/PS1 mice fed the control diet, nor in WT mice fed an n-3 depleted diet, suggesting a combined effect of the APP/PS1 transgenes and diet. Similarly, in the NOR task, APP/PS1 mice fed an n-3 depleted diet failed to differentiate the novel object from the familiar object, suggesting impaired recognition memory under the influence of both APP/PS1 transgenes and dietary intervention. In the present study, APP/PS1 mice fed the n-3 depleted diet displayed comparable percentages of thigmotaxis (wall hugging behavior) in the first habituation session of the NOR paradigm compared to other mice, and therefore any observed differences in novel arm/object exploration time is unlikely to be a result of diet-induced anxiety. The cognitive decline in the Y-maze spatial recognition and NOR tests in APP/PS1 mice fed the n-3-deficient diet suggests that the low dietary DHA/n-3 fatty acid intake may have escalated the progression of cognitive impairment in mice possessing APP/PS1 genes. Deficits in memory retention of APP/PS1 mice have been demonstrated previously in the water maze (Lok *et al.* 2013). Interestingly, in our water maze paradigm, APP/PS1 mice displayed impaired memory retention regardless of diet, indicating that n-3 dietary depletion could not further deteriorate the long-term spatial memory of the APP/PS1 mice.

The APP/PS1 mice fed a control diet displayed normal short-term memory function in both Y-maze spatial recognition and NOR tasks, but impaired long-term memory (24 h) in the water maze. Learning tasks involving short-term memory tasks are likely to employ different brain circuitry and learning strategies to spatial memory tasks relying on long-term memory (e.g. water maze). Both the Y-maze spatial recognition test and NOR paradigm involve one training session, and require only a relatively short period of information retention (2–4 h). This may have allowed the APP/PS1 mice which were fed the control diet to perform normally. In contrast, in the water maze, where memory retention over longer periods was required, the APP/PS1 mice performed more poorly than WT animals even when fed the control diet.

Having demonstrated that consumption of an n-3 depleted diet could deteriorate the cognitive function of APP/PS1 mice (as assessed in the Y-maze spatial recognition and NOR tests), we measured total brain DHA levels in order to establish an association between brain DHA levels and cognitive impairment. No significant alteration to brain levels of stearic, palmitic and arachidonic acids was observed in APP/PS1 mice, suggesting that the impaired cognitive function is more likely to be a consequence of reduced brain DHA levels. Total brain DHA levels were lower in APP/PS1 mice in general, which is in line with the reduction in BBB transport of ^{14}C-DHA. A decrease (39%) in brain DHA levels has also been previously observed in 3xTg AD mice (12–13 months of age) (Zhao *et al.* 2011) and a 3.7% reduction has been reported in the same mouse strain at 16 months old (Phivilay *et al.* 2009), a mouse model where reduced BBB transport of ^{14}C-DHA was reported (Calon 2011). Therefore, the findings in the APP/PS1 mouse model reflect those previously observed in the 3xTg AD mice, lending support to the contention that BBB dynamics are important in AD for maintenance of brain DHA levels. However, the current study did not rule out other mechanisms which may contribute to the lower brain DHA levels in APP/PS1 mice which underwent dietary intervention. For example, it is possible that there was reduced conversion of dietary DHA precursors to DHA in the liver of APP/PS1 mice, leading to reduced plasma levels of DHA subsequently available for brain uptake, given that no pre-formed DHA was available from the diets used in the current study. Indeed, lower liver DHA levels have been demonstrated in 3-month-old APP/PS1 mice fed a diet without pre-formed DHA compared to WT littermates on the same diet (Perez *et al.* 2010). It is also possible that the synthesis and metabolism of DHA was altered in the brain of APP/PS1

mice, leading to lower brain DHA levels, which is supported by the fact that lipid homoeostasis is altered in AD brains (Xu *et al.*, 2016).

Notably, in the current studies, APP/PS1 mice fed the n-3 depleted diet had the lowest brain DHA levels, which is due to the transgenes and n-3 dietary depletion. Similar findings have also been observed in Tg2576 mice (an AD mouse model over-expressing the human AD gene APPswe) that underwent 5-month-old n-3 fatty acid dietary restriction (Calon *et al.* 2005). The increased vulnerability of APP/PS1 mice to cognitive deficits from an n-3 depleted diet could be explained by FABP5 deficiency at the BBB, in addition to a potential reduction in brain microvascular FATP-1 and FATP-4. In animals with normal brain microvascular FABP5 and membrane transporter, transport of DHA across the BBB may be sufficiently effective to extract DHA from the limited available supply of plasma DHA. In contrast, in APP/PS1 mice, where FABP5 levels are diminished, the effectiveness of such a mechanism may be significantly reduced. Interestingly, it has been demonstrated that DHA dietary supplements are more effective if administered early enough in AD (Thomas *et al.* 2015). It is possible that in early AD, sufficient BBB FABP5 is available to facilitate DHA transport, while such a mechanism may become dysfunctional in late stage AD, despite supplementation with DHA.

The impaired BBB transport of DHA resulting from reduced brain microvascular FABP5 may not be the only mechanism responsible for the increased vulnerability of APP/PS1 mice to cognitive deficits from an n-3 depleted diet. Brain parenchymal FABP5 has been previously demonstrated to be important for cognitive function (Yu *et al.* 2014), albeit the mechanisms proposed by the authors in that study were independent of the critical role of FABP5 in CNS access of DHA which we have postulated (Pan *et al.* 2016). Furthermore, it remains unknown whether brain parenchymal FABP5 is also reduced in AD, as our current studies only assessed the impact of AD on brain microvascular FABP5. It is known that FABP5 can shuttle a variety of fatty acids into the nucleus, including DHA. DHA binds to PPARα, PPARβ/δ and PPARγ, non-selectively (Xu *et al.* 1999). The activation of these PPARs could restore cognitive deficits induced by drugs (Plaza-Zabala *et al.* 2010), enhance hippocampal neurogenesis and spatial memory in mice (Yu *et al.* 2014), and actively improve hippocampal-based cognitive function in AD (Denner *et al.* 2012). Therefore, if brain parenchymal FABP5 is also down-regulated in AD, it is possible that DHA (n-3) dietary depletion could further diminish PPAR activation and subsequently deteriorate cognitive impairment.

In summary, the present study demonstrated that the BBB transport of ^{14}C-DHA is reduced in APP/PS1 mice, an effect postulated to involve reduced FABP5 expression at the BBB. Other mechanisms (e.g. FATP-1, FATP-4) that are involved in the BBB transport of DHA also appear to be affected in AD, albeit their levels were only measured in total brain homogenate samples. The deficiency of FABP5 at the BBB, and perhaps FATP-1 and FATP-4, is thought to affect APP/PS1 mice such that they are more vulnerable to DHA deficiency, with reduced DHA access into the CNS and impairments in short-term spatial memory and recognition memory. FABP5 might therefore represent a therapeutic target that can be pharmacologically up-regulated in AD, to help restore the BBB transport of DHA and re-establish normal brain DHA levels, and ultimately halt the development of cognitive impairment.

Acknowledgements

The authors declare that the results from this study have not been previously published in any language anywhere and are not under simultaneous consideration by another journal, and that its publication is approved by all authors and tacitly or explicitly by Monash University. If accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. This work was supported by The Judith Jane Mason and Harold Stannett Williams Memorial Foundation and The William Buckland Foundation. There are no competing financial interests.

All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1: Expression of (a) FATP-1 and (b) FATP-4 in the brain homogenate of 15-month-old WT and APP/PS1 mice as assessed by ELISA (DL Sci&Tech Development, Wuxi, Jiangsu, China), with down-regulation of both FATP-1 and FATP-4 observed in the APP/PS1 mice compared to controls (**p = 0.0018, *p = 0.0285, n = 7–8). Data are presented as mean ± SEM.

Table S1: Statistics sheet.

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