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Assessment of diets containing curcumin, epigallocatechin-3-gallate, docosahexaenoic acid and α-lipoic acid on amyloid load and inflammation in a male transgenic mouse model of Alzheimer's disease: Are combinations more effective?

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ABSTRACT

Increasingly, evidence is accumulating pointing at a protective role of a healthy diet at decreasing the risk of Alzheimer's disease. To test the effectiveness of nutritional components, the following food-derived compounds: curcumin alone *(curcumin)*, curcumin combined with (−)epigallocatechin-3-gallate (EGCG), docosahexaenoic acid (DHA) and α-lipoic acid (ALA) (*curcumin + EDA*), or a combination of EGCG, DHA and ALA (*EDA)* were assessed in male Tg2576 transgenic mice on amyloid plaque load, amyloid levels (Aβ40/Aβ42, but not oligomers due to tissue limitations), microglial activation and memory using the contextual and cued fear conditioning test. The combination diet EDA, resulted in the strongest reduction of amyloid plaque load in both the cortical ($p < .0001$) and hippocampal ($p < .0001$) areas of the Tg2576 mouse brain, along with lower $A\beta_{40}/A\beta_{42}$ levels in the frontal cortex ($p = .000129$ and $p = .000039$, respectively) and $A\beta_{42}$ levels in the temporal lobe ($p = .000082$). A curcumin only diet was shown to lower amyloid plaque load ($p = .028$), but when combined with EGCG, DHA and ALA did not result in further decreases in amyloid plaque load. The EDA combination group showed the most prominent decrease in microglial activation (number of microglia around plaques: $p < .05$ and $p < .0001$, respectively, for the cortex and hippocampus). Analysing the hippocampal associated contextual fear conditioning revealed that both the curcumin + EDA ($p < .0001$) and EDA groups ($p = .001$) spent increased time on *freezing* compared to the control group. In addition, the curcumin + EDA group showed a significant increase in time spent *freezing* compared with the curcumin only group. In the amygdala associated cued test, all mice demonstrated the ability to associate the conditioned stimulus with the unconditioned stimulus as evidenced by a significant increase in *freezing* behaviour in response to the presentation of the cue ($p < .0001$). Post-hoc analysis showed that only curcumin + EDA ($p < .0001$) and EDA groups ($p < .0001$) developed a significant increase in *freezing* during the cue presentation. The results from this study show that the combination of EGCG, DHA and ALA (EDA) appeared to have the most potent anti-inflammatory and neuroprotective effect. Our results also demonstrate that interactions between nutraceutical products might result in

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counterproductive outcomes, highlighting the fact that manufacturers of nutraceuticals containing multiple compounds should be careful not to claim additive or synergistic effects of their combination products in vivo without having tested it in animal models and/or human clinical trials.

1. Introduction

Alzheimer's disease (AD) is the most common form of cerebral degeneration leading to dementia. It is characterized by the presence of intracellular neurofibrillary tangles (composed of the microtubule associated protein tau) and extracellular amyloid deposits (composed of beta amyloid-Aβ peptide) (Ittner and Gotz, 2011), cholinergic deficit accompanied by glucose deficit, chronic microglial activation and oxidative stress (Retz et al., 1998). The major protein component of the extracellular amyloid deposits is a small 4 kDa spontaneously aggregating peptide of 39–43 amino acids termed beta amyloid (Aβ). Aβ is derived proteolytically from a larger parent molecule termed the amyloid precursor protein (APP) and is insufficiently cleared from the brain (Tarasoff-Conway et al., 2015). As a result, it accumulates to form oligomers as well as larger aggregates - the senile plaques. Though Aβ is thought to be a one of the major contributors to the pathogenesis of AD, current symptomatic treatments only target symptoms such as the cholinergic deficit and do not prevent disease progression (Lane et al., 2004). Further common features of AD are oxidative stress and chronic neuroinflammation (Halliwell, 2006; Perry et al., 1998; Smith et al., 1996). Nitric oxide (NO) and its reaction product with superoxide, peroxynitrite, have also been shown to be elevated in AD and mild cognitive impairment (MCI) patients (Ahmed et al., 2005; Luth et al., 2002). A deficiency in the brain levels of reduced glutathione (GSH), a major antioxidant and co-substrate for enzymes such as glutathione peroxidase and glyoxalase I, has been demonstrated in AD patients (Saharan and Mandal, 2014).

Pathological changes in the brain of a person at risk for developing AD may commence as early as 30 years prior to clinical dementia symptoms (Villemagne et al., 2013). Therefore, the earlier that pharmacological interventions can be initiated, the greater the potential to prevent or delay AD. Diet is now considered to be an important factor in the development of sporadic AD (Solfrizzi et al., 2003), as many epidemiological studies have reported that consumption of fruit and vegetables, and omega-3 fatty acids reduce AD incidence (Rossi et al., 2008). Given that plant foods are derived from biological systems, they contain many compounds in addition to traditional nutrients that can elicit biological responses, and are therefore termed phytonutrients (Gupta and Prakash, 2014). Many individual compounds have now been identified, which could be the "active" neuroprotective ingredients in healthy foods. As many clinical trials aiming at amyloid reduction or clearance have not been successful in patients with already established disease (Castello et al., 2014), prevention is now considered a more promising approach for AD than treatment.

The ideal drug candidates could be the phytonutrients in fruits and vegetables; able to inhibit plaque formation, plaque associated neuroinflammation as well as oxidative and carbonyl stress in the pre-symptomatic stages of the disease. One of the largest groups of phytonutrients that may confer beneficial health effects are the polyphenols. Over the past decade, polyphenols, which are abundant in fruits and vegetables, have gained recognition for their antioxidant properties and their putative roles in protecting against chronic diseases such as cancer and cardiovascular diseases (Fernandez-Arroyo et al., 2015; Kwan et al., 2015). Increasingly, evidence is accumulating pointing at a protective role of a healthy diet. For example, one epidemiological study conducted over a ten-year period, reported that consumption of fruit and vegetable juices (high in polyphenols) greater than 3 times a week resulted in a 76% reduction in the risk of developing probable AD over a nine year period (Dai et al., 2006). Another recent epidemiological study of 1010 subjects aged 60–93 reported that individuals who consumed curry (containing curcumin) "often" and "very often" had significantly better cognitive test scores as measured via the mini-mental state exam (Ng et al., 2006). To test the effectiveness of nutritional supplements on the reduction of Aβ plaque formation and associated neuroinflammation in a mouse model of familial AD, we have chosen the following food-derived compounds: curcumin alone *(curcumin)*, curcumin combined with (−)epigallocatechin-3-gallate (EGCG), docosahexaenoic acid (DHA) and α-lipoic acid (ALA) (*curcumin + EDA*), or a combination of EGCG, DHA and ALA (*EDA)*.

Curcumin is an ingredient of the Indian spice turmeric (*Curcuma longa Linn*) and is also used to treat a variety of ailments in traditional Indian medicine (Gupta et al., 2012). Curcumin has been shown to have antioxidant, anti-inflammatory and cholesterol lowering properties (Ammon et al., 1993; Venigalla et al., 2015). There is also increasing evidence showing that curcumin can inhibit Aβ aggregation (Thapa et al., 2015; Yang et al., 2005) and oligomer formation (Feng et al., 2014). Polyphenols from green tea (*Camellia sinensis*) have been shown to be powerful hydrogen-donating antioxidants, and scavengers of ROS and RNS in vitro (Halliwell and Gutteridge, 2015; Salah et al., 1995). Among the green tea polyphenols, a subclass termed catechins has received the greatest attention, of which green tea is one of the best-known dietary sources. Of the four major tea catechins, (−)epigallocatechin-3-gallate (EGCG) is the major constituent (~60%), followed by (−)-epigallocatechin (EGC), then (−)-epicatechin (EC), and then lastly (−)-epicatechin-3-gallate (ECG) (Nagle et al., 2006). EGCG has previously been shown to prevent neuronal death caused by Aβ neurotoxicity (Rezai-Zadeh et al., 2008a). EGCG also modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in AD mouse models (Rezai-Zadeh et al., 2005; Walker et al., 2015).

Docosahexaenoic acid (DHA) is an essential omega-3 (n-3) polyunsaturated fatty acid (PUFA) that is found abundantly in marine fish. DHA is associated with learning-memory and is also required for the structure and function of brain cell membranes. DHA is known to be the most important n-3 PUFA in the brain, accounting for roughly 15% of total fatty acids in the grey matter where it is enriched at synapses. The interest in dietary DHA supplementation has arisen from the view of helping to protect from neuronal degeneration and therefore prevent neurological diseases such as AD. Converging epidemiological data suggest that a low dietary intake of n-3 PUFA is a risk factor for dementia (Cole et al., 2009). In AD patients, DHA is known to be decreased (Conquer et al., 2000), while people who ingest higher levels of DHA are less likely to develop dementia or AD (Lopez et al., 2011; Schaefer et al., 2006). A diet enriched with the omega-3 fatty acid docosahexaenoic acid reduces the amyloid burden and improves cognition in AD mouse models and patients (Fiol-deRoque Fiol-deRoque et al., 2013; Lim et al., 2005; Perez et al., 2010; Shinto et al., 2014).

α-lipoic acid (ALA) is a naturally occurring precursor of an essential co-factor for mitochondrial enzymes, including pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase. ALA has been shown to have a variety of properties which interfere with pathogenesis of AD. For instance, lipoic acid (LA) increases acetylcholine (ACh) production by activating choline acetyltransferase and increases glucose uptake, thus supplying more acetyl-CoA for the production of ACh. ALA chelates redox-active transition metals, inhibits the formation of hydroxyl radicals and ROS, increases the levels of reduced glutathione and down-regulates redox-sensitive inflammatory processes (Maczurek et al., 2008). In addition, ALA can scavenge lipid peroxidation products such as 4-hydroxynonenal (4-HNE) and acrolein (Maczurek et al., 2008). In vitro studies have shown that ALA protects cholinergic neurons from Aβ induced neurotoxicity (Bielarczyk et al., 2006), and

inhibits the formation of Aβ fibrils (Ono et al., 2006). Chronic dietary ALA administration has been shown to significantly improve metabolic activity and cognitive function and to reduce brain oxidative stress in aged mice, rats, and dogs (Quinn et al., 2007). A study showed that chronic dietary supplementation with ALA in Tg2576 mice enhanced both learning and memory retention and reduced nitrotyrosine levels (Quinn et al., 2007). However, there were no changes in Aβ levels, plaque deposition or markers of oxidative stress (Quinn et al., 2007). Therefore, dietary supplementation with lipoic acid may provide a potential treatment for preventing memory deficits in AD, independent of Aβ levels.

Previous studies have demonstrated that many of these compounds when examined separately have shown the ability to reduce amyloid plaque pathology in transgenic mice, most notably in aged mice with varying characteristics of advancing pathology. Therefore, the present study aimed to evaluate the efficacy of combinations of these nutritional compounds (EGCG, DHA and ALA) with or without curcumin in suppressing amyloid plaque, amyloid levels, plaque associated neuroinflammation, as well as decreasing cognitive deficits in male Tg2576 mice compared with using them alone. We were also interested in examining the long-term preventative effect of these compounds, commencing at 6 months of age in Tg2576 mice, that are yet to develop amyloid pathology and then feeding the nutritional supplements for 12 months, until they were 18 months old. Thus, the overall aim of the study was to determine whether these nutritional supplement compounds could provide preventative effects for AD mice when used in combination, that if positive, could be useful for informing future human trials.

2. Materials and methods

2.1. Animals

Male Tg2576 (APPswe) transgenic mice were used as the experimental model, which is well characterized for familial AD (McGowan et al., 2006; Sankaranarayanan, 2006; Sasaki et al., 2002). The Tg2576 mouse model expresses the Swedish mutation of APP (APPK670N, M671 L) at a high level under the control of the hamster prion protein (PrP) promoter (Hsiao et al., 1996). This mutation leads to overexpression of APP, with concomitant increase in secreted Aβ40 and Aβ42 [28, 31]. As Tg2576 mice age, they develop diffuse neuritic plaques, starting at around 12 months of age similar to those seen in AD. By 18–21 months of age Tg2576 mice have diffuse and cored amyloid plaques similar to those observed in human AD (Kawarabayashi et al., 2001). They also show evidence of oxidative damage in the brain, preceding plaque formation at approximately 8 months of age, measured via F2-iPs (Pratico et al., 2001). In addition, Tg2576 mice have been shown to develop age-dependent behavioural memory deficits measured by Y maze, T-maze, Morris water maze, and fear conditioning tests (Chapman et al., 1999; Hsiao et al., 1996; Quinn et al., 2007). Animals were obtained from a colony of Tg2576 mice (n = 20–30/group, male) maintained at the Animal Resources Centre (ARC), Perth, Western Australia. All animals were housed in cages in a controlled environment at 22 °C on a 12 h day/night cycle. Diet and water were consumed ad libitum. Principles of laboratory animal care (NIH publication No. 86–23, revised 1985) and the Australian code of practice for the care and use of animals for scientific purposes [National Health and Medical Research Council (NHMRC) 2004] were followed, and the experimental protocols were approved by the Animal Ethics Committee of University of Western Australia. Animals were randomly assigned to one of the four experimental groups at six months of age, 1) control diet, n = 26; 2) curcumin only diet, n = 30; 3) EGCG + DHA + ALA (EDA) diet, n = 30; and 4) Curcumin + EDA diet, n = 27; for approximately 12 months. Mice were weighed during each week of the study to monitor their health and consumption of the nutritional supplement containing diets.

Table 1
Dietary composition for the four experimental diet groups.

	Control	Curcumin	Curcumin + EDA	EDA
Ingredient	g/1 kg diet			
Sucrose	150	149.97	149.99	150
Casein	140	139.97	139.99	140
Canola Oil	40	39.99	40	40
Cellulose	50	49.99	50	50
Starch	422.20	421.71	420.89	421.29
Dextrinised Starch	155	154.97	154.99	155
DL-Methionine	1.80	1.80	1.80	1.80
Lime (Fine Calcium Carbonate)	13.13	13.12	13.12	13.12
Salt (Fine Sodium Chloride)	2.59	2.59	2.59	2.59
Potassium Dihydrogen Phosphate	8.75	8.75	8.75	8.75
Potassium Sulphate	1.63	1.63	1.63	1.63
Potassium Citrate	1.00	1.00	1.00	1.00
AIN_93_G_Vitamins	1.00	1.00	1.00	1.00
AIN_93_G_Trace Minerals	1.40	1.40	1.40	1.40
Choline Chloride 60% *w/w*	2.50	2.50	2.50	2.50
Curcumin	–	0.59	0.59	–
EGCG	–	–	0.36	0.36
DHA	–	–	0.36	0.36
ALA		1	1	1

1) control diet, 2) curcumin only diet, 3) Curcumin + EDA diet, and 4) EDA diet. All diets were based on the standard AIN-93 M rodent diet (Reeves et al., 1993), modified slightly with an increased sucrose content (15%) to improve the diet palatability.

2.2. Diets

The research diets were prepared and pelleted by Specialty Feeds (Glen Forrest, WA, Australia) (Table 1). All diets were based on the standard AIN-93 M rodent diet (Reeves et al., 1993). The concentrations of all nutritional compounds investigated in the current study were selected from previously published studies. EGCG (#E4143 Sigma-Aldrich, Castle Hill, NSW, Australia) was incorporated in the diet at a dose of 50 mg/kg body weight (BW) (around 0.0357% = 357 ppm) (Rezai-Zadeh et al., 2008b). Curcumin (#C1386, Sigma-Aldrich) was incorporated in the diet at a dose of approximately 500 ppm (83 mg/kg BW, around 0.0593%) (Begum et al., 2008). DHA (#D2534, Sigma-Aldrich) was incorporated in the diet at a dose of 50 mg/kg BW (around 0.0357%) (Giunta et al., 2010). ALA (#T5625, Sigma-Aldrich) was incorporated in the diet at a dose of 140 mg/kg BW (around 0.1%) (Quinn et al., 2007). The nutritional compounds were mixed with the oil of the diet, canola oil, before being added to the other ingredients of the AIN-93 M diet, to ensure homogenous mixing of the nutritional compounds within the diet. Diets were stored at 4 °C and changed daily to prevent peroxidation. In order to monitor that the nutritional supplements were consumed at the desired dose, the weight of the animals was followed weekly throughout the feeding period of 53 weeks. In a pilot experiment, it was discovered that the animals disliked the taste of the diets containing curcumin, and therefore an additional 5% sucrose was included in the diet, compared to the usual 10% sucrose content of the AIN-93 M rodent diet, to improve the palatability and ensure that the calculated nutritional compound dose was consumed. For this study, all the diets were prepared from the same modified AIN-93 M rodent semi-pure diet with 15% sucrose and then the various nutritional compounds were added. Thus, all the groups had the 15% sucrose added, including the controls.

2.3. Tissue collection for immunohistochemistry

At 18 months of age, animals were euthanized by an overdose of anaesthetic (80 mg/kg ketamine and 15 mg/kg xylazine), and transcardially perfused with phosphate-buffered saline (PBS), followed by

4% paraformaldehyde in 0.1 M PBS. Their brains were removed and post-fixed in the same fixative overnight at 4 °C before being immersed in 30% sucrose for 2 days. Brains were sectioned at 50 μm with a Zeiss cryostat and 3 series of sagittal sections of the right hemispheres were collected. Four brains were collected from the non-treated Tg2576 mice, and 5 from each of the treated groups.

2.4. Nissl staining

This staining was used to identify structures of the hippocampus and the cortex. The 1st series of sections were dehydrated in gradient ethanol (50%, 70%, 95%, 100%, 30 s each) before they were de-fatted in 95% ethanol with 5% acetic acid pre-warmed to 55-60 °C for 15–20 min. They were then incubated in 0.2% cresyl violet solution pre-warmed to 55-60 °C for 2–3 min. After rinse with 70% ethanol for 30 s and 95% ethanol for 1 min, sections were then differentiated in 95% ethanol with 0.5% acetic acid for 10–15 min until the optimal colour was observed. Sections were then dehydrated in the gradient ethanol (70%, 95%, 100%, 5 min each) and cleared in xylene (10 min) before they were coverslipped with the DPX mounting medium.

2.5. Immunohistochemistry

Detection of amyloid plaques with an Aβ antibody: the 2nd series of sections were incubated with 1% normal goat serum for 2 h to block non-specific binding sites before they were sequentially incubated in a mouse anti-1E8 antibody solution (1:500 for 48 h at 4 °C), biotinylated goat anti-mouse IgG (1:200 for 2 h at room temperature). This Aβ antibody is known to bind to amino acids 17–22 of Aβ and detects multiple Aβ peptides including Aβ[1–40], Aβ[1–42] and Aβ[1–43] (Tammer et al., 2002). At the end of the incubation with the secondary antibody, vectastain peroxidase ABC (1:500 in PBS, 2 h) was employed. The peroxidase reaction was carried out using a developer solution containing 0.4 mg/ml DAB and 0.0006% hydrogen peroxide dissolved in TBS that resulted in the development of brown deposits in amyloid plaques.

Double fluorescent immunostaining of amyloid plaques and Iba-1 positive microglia: the 3rd series of sections were immersed into primary antibody mixture of rabbit-anti-Iba-1 (1:1000, Wako, #019-19741) and mouse-anti-1E8 (1:500) with 0.1% Triton-X for 48 h at 4 °C, followed by incubation in the fluorescent secondary antibodies (goat-anti-rabbit AlexaFluoro594-LifeTechnologies, A11008 for the visualisation of anti-Iba-1, and goat-anti-mouse AlexaFluor488-Life Technologies, A11005 for anti-IE8, in a dilution of 1:200 for 2 h at room temperature). Sections were mounted and cover slipped with Vectashield mounting medium hard set with DAPI (Vectorlabs).

2.6. Measurement of total plaque area/load using stereology

Every third section was chosen and unbiased stereological estimates of plaque load were obtained with a commercially available software Stereo Investigator (MBF Bioscience, Williston, VT, USA) using a Zeiss Axio Imager.M2 microscope (Carl Zeiss AG, Oberkochen, Germany). To obtain the area fraction covered by plaques, the following areas of interest were chosen: the hippocampus, including the subiculum, CA1–3, and the dentate gyrus and the neocortex and outlined based on their cytoarchitecture described by Franklin and Paxinos on sagittal sections (Franklin and Paxinos, 2013). The area and volume were measured using the Area Fraction Fractionator probe (Heggland et al., 2015). The Cavalieri estimator uses a point grid to estimate the volume of three-dimensional structures based on two-dimensional slices of the object. The volume/area fraction approach of stereological methods provides information about volumetric relations of the components of structures. The area and the area fraction of both the hippocampus and the neocortex containing the senile plaques were estimated on the immunostained sections using a 20× objective. The size of the counting frame was 200 × 200 μm, with a sampling grid area 500 × 500 for the hippocampus, and 500 × 500 μm counting frame with a sampling grid area 1250 × 1250 for the cortex. The grid spacing was set to 50 μm for all cases. All points associated with either dense or diffuse plaques were marked. In our analysis we calculated the fraction (%) of area occupied by plaques. To ensure the precision of our stereological estimates, the Gunderson coefficient error (CE) was calculated and kept smaller than 0.1 in all cases (m = 1).

2.7. Determination of Iba-1 positive microglia around amyloid plaques

To determine the number of microglia around amyloid plaques in cortex and hippocampus, a non-stereology method was used as described by (Serrano-Pozo et al., 2013). In detail, 10 sagittal sections (sections from cortex or hippocampus) containing amyloid plaques were selected from all sagittal sections of each animal. Using a 20× objective, one field (size: 448 × 336 μm), containing one amyloid plaque, was selected from each section. These 10 fields from the cortex and 10 fields from the hippocampus of each mouse brain were selected for counting under a 63× objective. The microglia surrounding the plaques was plotted along the z axis of the section, which ensured that all microglia from the top to the bottom of the whole section (50 μm) were counted.

2.8. Calculation of perimeter (circumference) of plaques

10 sagittal sections (the same sections from cortex or hippocampus as used for microglia counting) containing amyloid plaques were selected from each mouse brain. Using a 20× objective, the perimeter of the plaque was determined using Image J after capturing an image in comparison to an internal scale bar (Serrano-Pozo et al., 2012).

2.9. Determination of overall microglia numbers using stereology

The estimation of the absolute microglial cell number in the neocortex and hippocampus of above mice was also counted on the fluorescent series using the Optical Fractionator probe (Bastide et al., 2014). The contour of the neocortex and hippocampus was first drawn on every 6 sections under 2.5× objective. The size of the counting grid was 1000 × 1000 μm and the size of the counting frame was 100 × 100 μm for both the neocortex and the hippocampus. The guard zones were set up to be 2 μm at the top and the bottom of the sections. Microglia was plotted on the screen using a marker as the focus moved from the top to the bottom of the sections using a 63× oil objective. This has led to the Gunderson coefficient error less than 0.1 in all cases (m = 1).

2.10. Tissue collection and measurement of Aβ by ELISA

A separate group of animals, at 18 months of age, were euthanized by an overdose of anaesthetic (80 mg/kg ketamine and 15 mg/kg xylazine), the brains removed and snap frozen in liquid nitrogen and stored at −80 °C for subsequent analysis of brain Aβ levels. The dissected brain regions (frontal cortex, temporal lobe and cerebellum) were homogenized in 5 M guanidine HCl, 50 mM Tris HCl, and then analysed for $A\beta_{40}$ and $A\beta_{42}$ levels using commercially available enzyme-linked immunosorbent assays (ELISAs) specific for $A\beta_{40}$ and $A\beta_{42}$ (Invitrogen, Carlsbad, CA, USA). Samples were analysed in duplicates and the average value reported per wet weight brain tissue.

2.11. Cued and contextual fear conditioning

The cognitive effects of the nutritional supplement diets were evaluated with the contextual and cued fear avoidance test. Fear conditioning tasks measure an animal's ability to learn and remember an association between an auditory tone and footshock (cued fear), or

between an environment and footshock (contextual fear). Both tasks are sensitive to lesions of the amygdala, but contextual fear conditioning is also disrupted by hippocampal lesions (Holland and Bouton, 1999; Phillips and LeDoux, 1992). This test has been used in numerous studies evaluating memory in transgenic models of AD (Comery et al., 2005; Jacobsen et al., 2006; Quinn et al., 2007), and also requires much less training time for mice than other learning and memory tasks (Shoji et al., 2014).

A total of 26 animals (control), 30 animals (curcumin), 27 animals (curcumin + EDA) and 30 animals (EDA) from each treatment group underwent behavioural testing at 18 months of age using the *'Video Freeze'* fear conditioning system (Med Associates, St. Albans, VT, USA). The test chamber (26 cm × 22 cm × 18 cm high) had clear Plexiglas sides and a grid floor that was used to deliver a mild foot shock. The chamber was placed inside a sound-attenuated chamber (internal dimensions: 56 cm × 38 cm × 36 cm) that was equipped with an infrared camera to observe mice without disturbance. The shock grid floor and the Plexiglas chamber were cleaned thoroughly with 70% aqueous ethanol before each test.

On the training day, mice were placed into the test chamber and allowed to explore for 2 min. The conditioned stimulus (CS) (a white noise 80 dB sound) was presented for 30 s and followed immediately by a mild foot shock (2 s, 0.7 mA) that served as the unconditioned stimulus (US). After 30 s, the mice received a second CS–US pairing, followed by another 30 s rest interval and a third CS-US paring. The Video Freeze system was used to control the timing of the CS and US presentations. Mice were tested for contextual and cued fear conditioning 24 h and 48 h after the conditioning training. For the context test, mice were placed back into the original test chamber for 5 min and freezing behaviour was recorded. Freezing behaviour was defined as a mouse remaining immobile for an entire second to register as a freeze. This was measured by the 'Video Freeze' system, which been previously validated (Anagnostaras et al., 2000). Freezing during this context test reflects hippocampal-dependent memory of the association between the contextual cues and the foot shock stimulus (Phillips and LeDoux, 1992).

On day 3, mice were tested for responses to the auditory CS in a new novel environment. For the CS test, white Plexiglas inserts were placed over the sides and floor of the chamber to alter the shape, texture and colour of the chamber. Mice were placed into this new chamber and freezing was recorded for 3 min during this 'pre-CS' phase. The auditory CS was then presented for another 3 min and freezing behaviour was recorded as described. Freezing in response to the tone presented in the new novel environment reflects amygdala-dependent memory of the association between the tone CS and the foot shock US formed during conditioning (Phillips and LeDoux, 1992).

2.12. Data analysis and statistics

Fluorescent and bright field images were acquired through a Zeiss AxioImager.M2 research microscope equipped with ApoTome (Carl Zeiss AG, Germany). When it was necessary, contrast and brightness were adjusted using Adobe Photoshop. One-way ANOVA with Bonferroni post-hoc test for multiple comparison was performed of the fractional area between groups using GraphPad Prism 6. Results were considered statistically significant when $p < .05$ (GraphPad Software, San Diego, CA). Values are expressed in forms of the mean and standard deviation (± SD). $A\beta_{40}/A\beta_{42}$ means and standard deviations were calculated using conventional methods. A one-way ANOVA was used to evaluate significant differences between the dietary interventions. Significant main effects were further analysed using a Tukey's post hoc test. A criterion alpha level of $P < .05$ was used for all statistical comparisons. $A\beta_{40}/A\beta_{42}$ levels were analysed using SPSS version 18.0 (SPSS, Chicago, IL, USA). The total number of microglia in the neocortex and the hippocampus, the number of microglia around plaques in selected sections as well as the number of microglia/100 μm plaque perimeter were compared using one-way ANOVA in Graphpad Prism 6. For the fear conditioning test, total time of freezing was analysed using one-way ANOVA, but for freezing in 1 min blocks, the pre-CS vs post-CS 3 min, the repeated measures two-way ANOVA was applied in Graphpad Prism 6. The difference was considered statistically significant when $p < .05$.

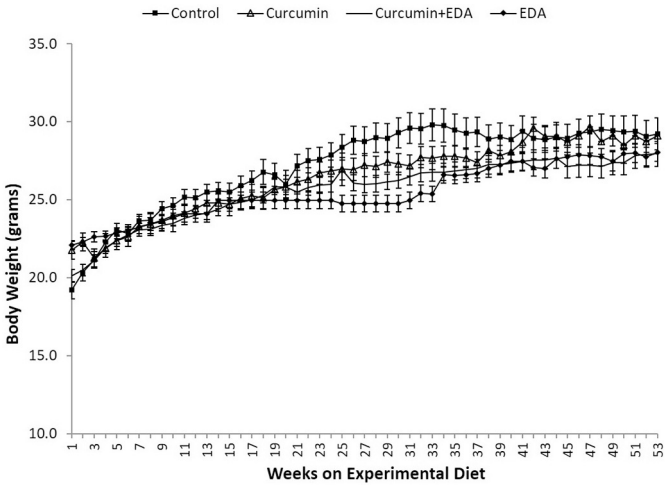

Fig. 1. Body weight of the animals during the experimental period.
The weight of the animals per group was monitored every week starting from week 1 to week 53 of the experimental diet period. The body weight of all four groups of experimental animals increased in a similar manner throughout the experiment, suggesting that all animals consumed the food pellets containing the bioactive nutrient in a similar amount. There were no statistical differences in body weight between the four groups at the end of the diet period at 18 months of age. Values are mean ± SEM of 26 animals (control), 30 animals (curcumin), 27 animals (curcumin + EDA) and 30 animals (EDA) per group.

3. Results

3.1. Influence of experimental foods on body weight

In the present study, we set out to compare the therapeutic effects of specific bioactive nutrients with anti-amyloidogenic and anti-inflammatory activities and their combinations on amyloid level, amyloid plaque load, neuroinflammation and cognition after 12 months of feeding. As some of the compounds might have an unpleasant or strong taste, it is possible that our experimental foods would not be consumed at the same rate as the normal chow, and therefore the animals would be under dosed. Therefore, the body weight of the animals was followed once a week for one year (Fig. 1). At the end of the study, no significant differences in body weight were observed between the 4 different groups, suggesting that the diets were consumed at equal levels and the dose of drug(s) consumed by each experimental group was as predicted.

3.2. Effect of bioactive nutrients on plaque load - determination of plaque load in the cortex and the hippocampus by stereological analysis

In order to compare the effect of the different bioactive nutrients on amyloid plaque load, we performed un-biased stereological sampling of the area covered by plaques using immunohistochemical detection of beta-amyloid in all four groups of animals (4 mice for the unfed controls, 5 mice for each treated group) at 18-months of age (after 12 months of feeding) (Table 2). Since the majority of the amyloid plaques were found in the cortical and hippocampal areas, we focused our measurements on these structures (Fig. 2). Areas of interest were delineated on sagittal sections of the right hemisphere using the "Mouse Brain in Stereotaxic Coordinates" Atlas (Paxinos and Watson, 2014) (Fig. 2). The cortical areas measured included the piriform, insular,

Table 2
Stereological parameters and results of the plaque load analysis in the cortex and the hippocampal regions of Tg2576 mice on the four experimental diets.

	Cortex, control	Cortex, curcumin	Cortex, curcumin + EDA	Cortex, EDA	Hippocampus, control	Hippocampus, curcumin	Hippocampus, curcumin + EDA	Hippocampus, EDA
Number of animals	4	5	5	5	4	5	5	5
SRS grid size	1250 × 1250	1250 × 1250	1250 × 1250	1250 × 1250	500 × 500	500 × 500	500 × 500	500 × 500
Counting frame size	500 × 500	500 × 500	500 × 500	500 × 500	200 × 200	200 × 200	200 × 200	200 × 200
Grid spacing (um)	50	50	50	50	30	30	30	30
Number of sections	23.5 ± 2.52	30 ± 0.7	28.2 ± 2.17	28.8 ± 2.59	20.3 ± 3.40	24.2 ± 2.28	27.2 ± 3.70	25.6 ± 3.05
Number of sampling sites	329 ± 66.6	399 ± 96.5	385 ± 41	381 ± 39.5	345 ± 34.2	432 ± 90.4	405 ± 174	424 ± 48
Total plaque points counted	1098 ± 205	1077 ± 283	872 ± 325	393 ± 81	377 ± 51.6	279 ± 119	350 ± 178	99 ± 62
Plaque fraction area (%)	5.56 ± 0.529	4.25 ± 0.985	3.89 ± 1.34	1.67 ± 0.44	3.19 ± 0.303	1.86 ± 0.481	2.31 ± 1.09	0.662 ± 0.404
CV (SD/mean)	0.095	0.23	0.34	0.26	0.094	0.25	0.47	0.61
$2CE/CV^2$	0.01	0.001	0.001	0.004	0.04	0.01	0.003	0.01
Reduction in % compared to control	N/A	23.6	40.1	70	N/A	41.6	27.5	79.3
Plaque fraction CE	0.01	0.01	0.012	0.018	0.021	0.026	0.027	0.061

The number of animals examined per group, SRS grid and counting frame size, average number of sections examined, average number of sampling sites per brain, total number of points counted for plaque area, average plaque fraction area within the groups, coefficient of variance (CV = SD/mean), 2CE/CV2 ratio, which describes the contribution of stereological variance to the total variance, reduction of area fraction measured in percentage compared to control group and plaque fraction CE are displayed. The CE of the stereological estimates was lower than 0.1 (10%) in all groups.

primary and secondary somatosensory and motor, visual, auditory, and the hippocampal areas included the hippocampal formation, the subiculum, the CA1-3 regions, the dental gyrus and the entorhinal cortex. All cell layers within the areas of interests were included. An example of delineation of the sagittal sections stained with the $A\beta_{41}/A\beta_{42}/A\beta_{43}$-specific 1E8 antibody in a mouse fed with the control diet was shown in Fig. 2.

In the control diet fed Tg2576 group, the densest plaque loads were observed in all cortical areas, especially in the piriform and visual cortices, with more modest expression in the somatosensory and motor areas. In the hippocampus, the subiculum showed the highest levels of plaques, followed by the CA1-3 and the entorhinal cortical areas. In the control diet fed Tg2576 group, the highest percentage of areas occupied by amyloid plaques was found in the cortex (5.56 ± 0.53%, $n = 4$), and slightly less in the hippocampus (3.19 ± 0.3%, n = 4).

In the cortex, we found a significant effect of treatment [$F(3,15) = 14.20$, $p = .0001$] throughout the groups, when analysed by one-way ANOVA. Significant main effect was further analysed using Tukey's post-hoc test. We found that curcumin and curcumin + EDA led to a slight (non-significant) decrease of amyloid plaque load compared to the non-fed Tg2576 group, from 5.56 ± 0.53% (control) to 4.25 ± 0.98% (curcumin) and 3.89 ± 1.34% (curcumin + EDA),

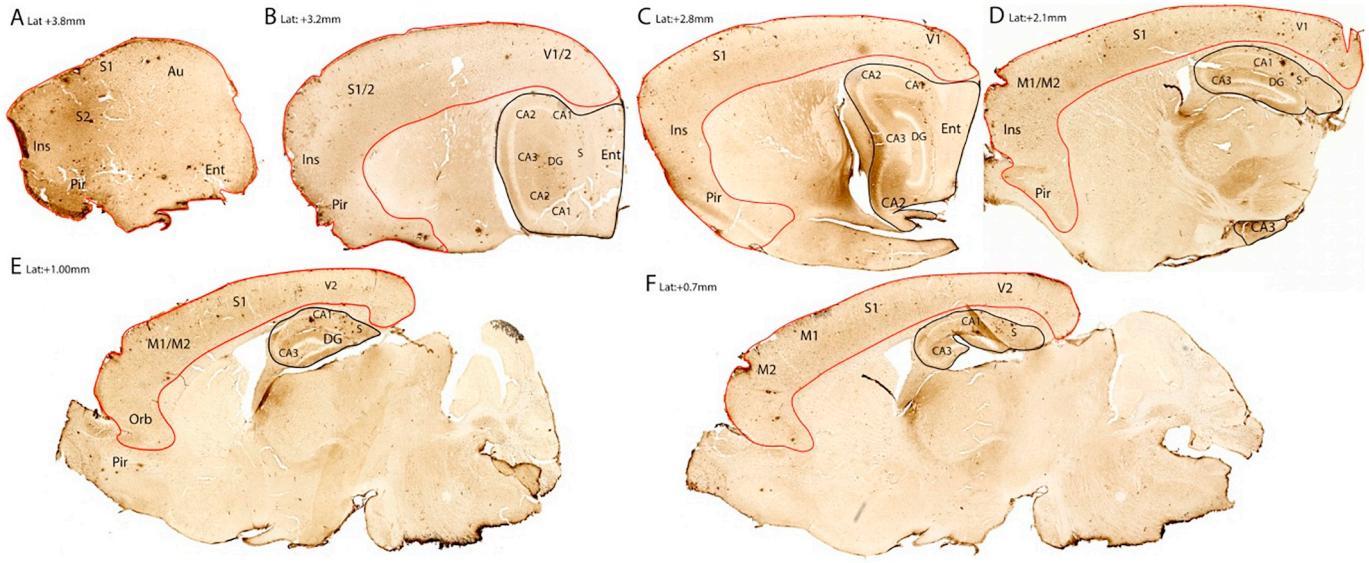

Fig. 2. A representative example of delineations of the areas included in the stereological estimation of plaque load on sagittal sections in the cortex (red outline) and the hippocampus (black outline).
Amyloid plaques were labelled using immunohistochemistry with an Aβ-specific antibody (1E8), here was an example from a 18-month-old male Tg2576 (APPswe) mouse on a curcumin supplemented diet. Sections were arranged from lateral to medial (L-M). For the delineation and identification of borders, the "Mouse Brain in Stereotaxic Coordinates Atlas" of Paxinos and Franklin (Fourth edition) was used. (Pir, piriform cortex; Ins, insular cortex; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; M1, primary motor cortex; M2, secondary motor cortex; V1, primary visual cortex; V2, secondary visual cortex; Orb, orbital cortex; Ent, entorhinal cortex; Au, auditory cortex; S, subiculum (including the dorsal-, post-, para-, pre-subiculum); CA1, field of CA1 of the hippocampus; CA2, field of CA2 of the hippocampus; CA3, field of CA3 of the hippocampus; DG, dentate gyrus (including the lacunosum moleculare, molecular and granule cell layers). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

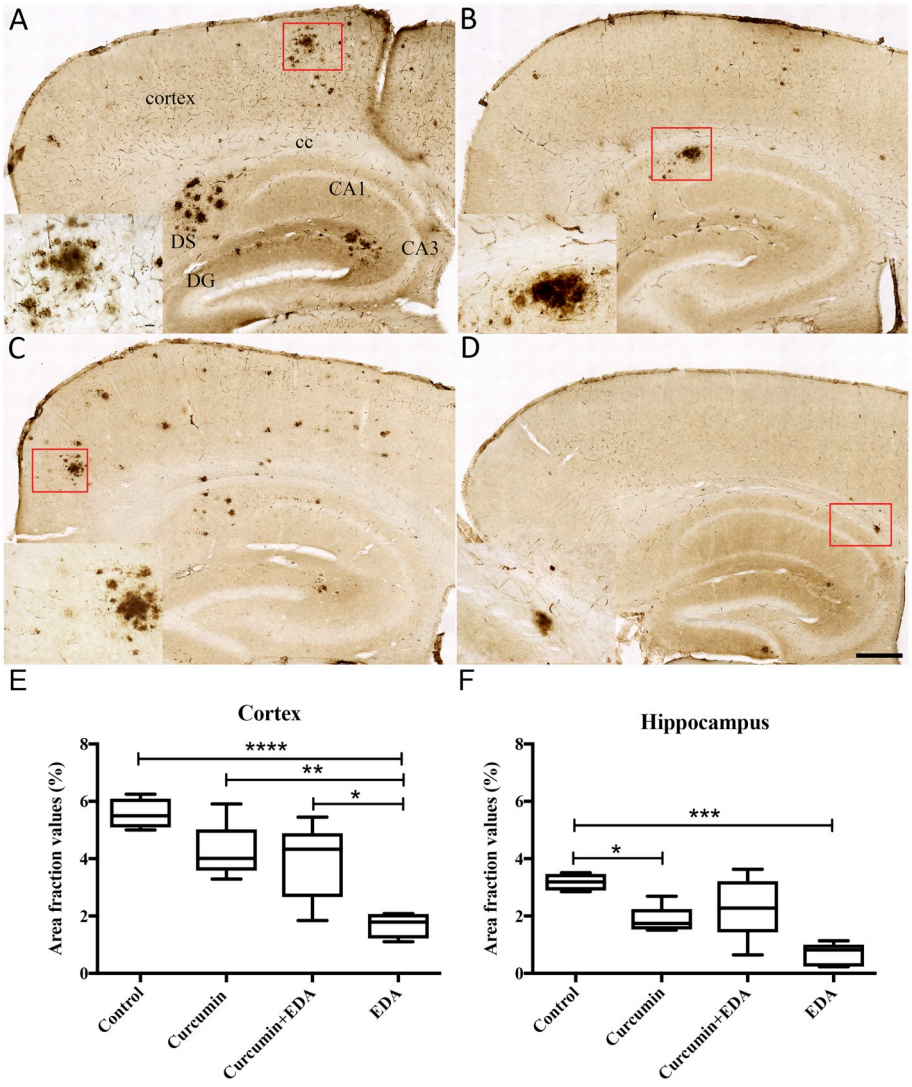

Fig. 3. Plaque distribution in the hippocampal and cortical areas of Tg2576 mouse brains on four different experimental diets.
A) The Tg2576 mice on control diet exhibit a high plaque load in the hippocampus, in particular in the subicular region (DS) and the dentate gyrus (DG). B) Curcumin fed animals showed significant reduction of plaque formation in the hippocampus, and moderate, but non-significant reduction in the cortical areas. C) The curcumin + EDA diet did not significantly reduce plaque formation compared to the control group. EDA diet alone (D) reduced plaque formation significantly in both the cortical and hippocampal areas of the brain. Scale bar represents 500 μm. Estimated plaque load (area fraction) in the cortical (E) and hippocampal (F) regions in the right hemisphere of 18-month-old male Tg2576 (APPswe) mice. Values are mean ± SEM of 5 animals per group. **** P < .0001; *** *P* < .001; ** P < .01; * P < .05.

respectively (Fig. 3 B–C). The reduction of plaque load in EDA fed Tg2576 mice was much more pronounced ($p < .0001$, from 5.56 ± 0.53% to 1.67 ± 0.44%, reduction by 70%) (Fig. 3 D-E). In the hippocampus, we also found a significant effect of treatment [$F(3,15) = 11.20$, $p = .0004$] throughout the groups, when analysed by one-way ANOVA. Tukey's post-hoc test showed that curcumin treatment led to significant decrease of amyloid plaque load compared to the normal diet fed group (1.86 ± 0.48%) ($p = .028$). However, the combination of curcumin + EDA did not lead to a significant reduction of plaque load (2.31 ± 1.09%, n = 4; $p = .2$), while there was a tendency of reduction (Fig. 3 F). As in the cortex, mice fed with EDA showed the most significant decrease of amyloid plaque load when compared to the normal diet fed group, showing reduction from 3.19 ± 0.30% to 0.662 ± 0.18% ($p < .0001$). In summary, EDA was the most potent combination of bioactive nutrients tested in our study in terms of plaque lowering effects.

3.3. Effect of the three experimental diets on brain Aβ $_{40/42}$ levels

Biochemical analyses were performed to determine the effects of the combination of the nutritional supplements on total $A\beta_{40}/A\beta_{42}$ levels (not amyloid oligomers) in the frontal cortex, temporal lobe (including hippocampus) and the cerebellum. In the frontal cortex, we found a significant reduction in $A\beta_{42}$ in the EDA group compared to the control and curcumin groups [$F(3,36) = 10.60$, $p = .000039$]. The EDA group also demonstrated a significant reduction in $A\beta_{40}$ compared to the curcumin + EDA group [$F(3,36) = 9.10$, $p = .000129$]. When looking at the temporal lobe, a significant reduction in $A\beta_{42}$ levels was observed in the EDA group compared to the other diet groups [$F(3,36) = 9.65$, $p = .000082$]. Neither the curcumin group, nor the curcumin + EDA group showed reductions in $A\beta_{42}$ compared to the control group in the temporal lobe. All three nutritional experimental diets (curcumin, curcumin + EDA, EDA) showed significant reductions in $A\beta_{40}$ in the temporal lobe compared to the control group [$F(3,36) = 35.44$, $p = .000007$]. In the cerebellum, a significant reduction in $A\beta_{40}$ levels was observed in the EDA group compared to all other diet groups [$F(3,36) = 19.51$, $p = .000001$]. No changes were found in $A\beta_{42}$ levels in the cerebellum [$F(3,36) = 0.15$, $p = .929$] (Fig. 4).

3.4. Effect of experimental diets on microglial pathology in plaque rich brain areas

Iba-1 positive microglia surrounding the plaques in both the hippocampal and cortical areas could be detected, with the Iba-1 positive microglia located in close proximity to the plaques (Fig. 5A-D).

3.5. Determination of microglia numbers around plaques

The number of microglia around amyloid plaques (as a marker of neuroinflammation) was determined by a non-stereological method. In

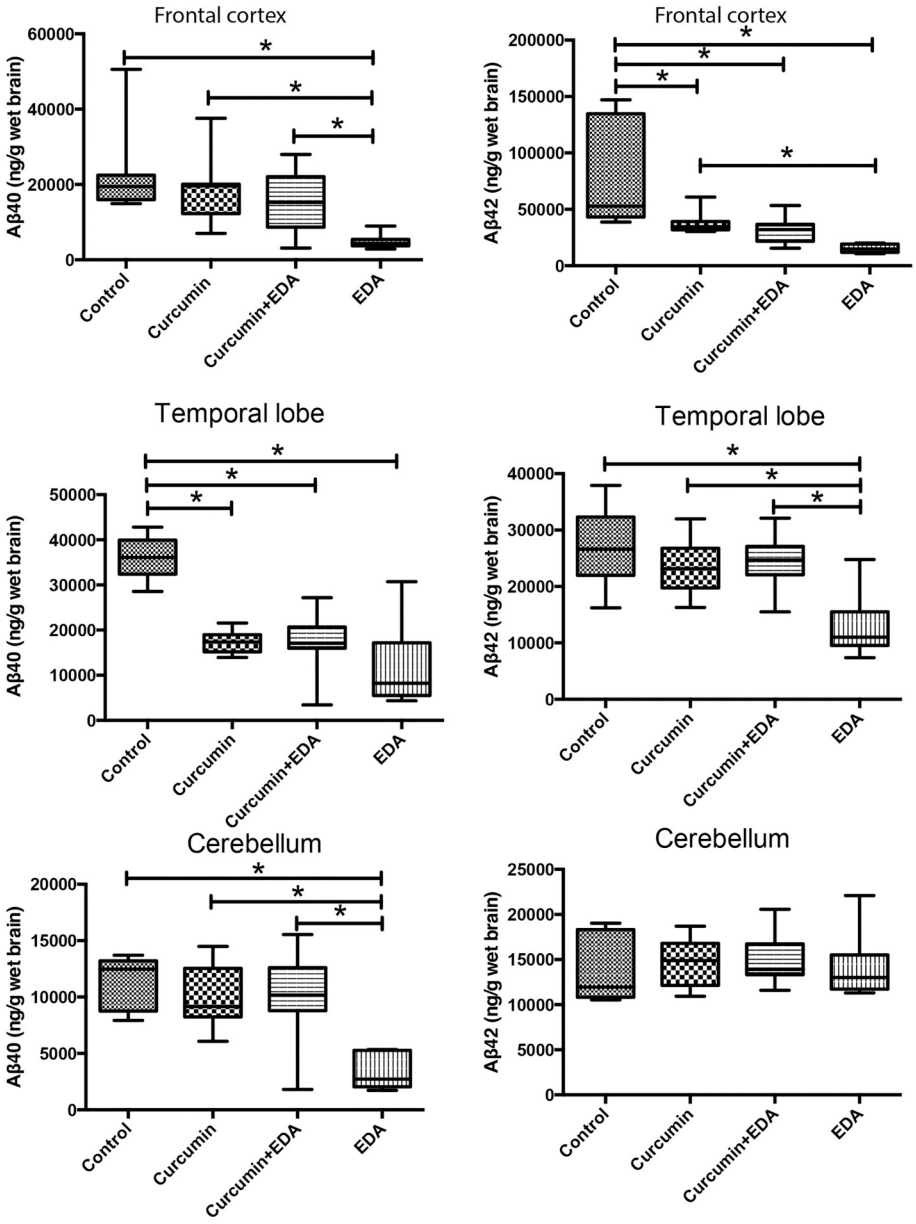

Fig. 4. Amyloid beta levels in different brain areas of Tg2576 mice on four different experimental diets. Brain Aβ40 and Aβ42 levels measured in frontal cortex (A-B), Temporal Lobe (C-D), and Cerebellum (*E*-F) of Tg2576 mice fed with nutritional compounds for 12 months. Boxes represent 1st quartile to 3rd quartile, the middle horizontal line represents the median of each group. The whiskers represent the minimum and maximum values for each group, $n = 10$ animals per group. * $P < .05$.

detail, 10 fields of the cortex and hippocampus which contain amyloid plaques, respectively, were selected from each mouse brain using a 20× objective. The selected neocortical areas included the frontal, motor, sensory, visual and auditory cortical areas. The selected hippocampal areas included CA1-3 and the dentate gyrus. Iba-1 positive microglia were counted in a field of 448 × 336 μm using a 63× objective.

In the cortex, we found significant [$F(3,186) = 6.155$, $p = .0005$] differences across the groups using one-way ANOVA. The control diet fed Tg2576 mice exhibited 320 ± 64 Iba-1 positive microglia around the plaques. When using multiple comparisons, the curcumin and curcumin + EDA fed mice did not show any significant decrease in microglia numbers around plaques compared to the control fed Tg2576 mice. In contrast, the EDA group had only 222 ± 46 microglia, which is a 25% decrease compared with the control group ($p < .05$); 28% compared to the curcumin fed group ($p = .006$) and 32% compared to the curcumin + EDA fed group ($p = .0006$) (Fig. 6A, Table 3). Similar results were obtained in the hippocampus, one-way ANOVA showing significant [$F(3,126) = 10.8$, $p = .0001$] differences across the groups. Again, when using multiple comparisons, EDA treatment alone significantly decreased the number of microglia compared to all the other groups by 60% compared to the control fed animals ($p < .0001$); by 53% compared to the curcumin fed animals ($p = .0002$); and by 52.5% compared to the curcumin + EDA fed animals ($p = .0005$) (Fig. 6B, Table 3). Taken together, these results confirmed that in both the neocortex and the hippocampus, consumption of EDA led to a statistically significant reduction in plaque area, accompanied by overall less Iba-1 positive microglia around the plaques compared to the control group.

3.6. Determination of total microglia numbers in cortex and hippocampus (stereological)

The total number of Iba-1 positive microglia in the entire cortex was

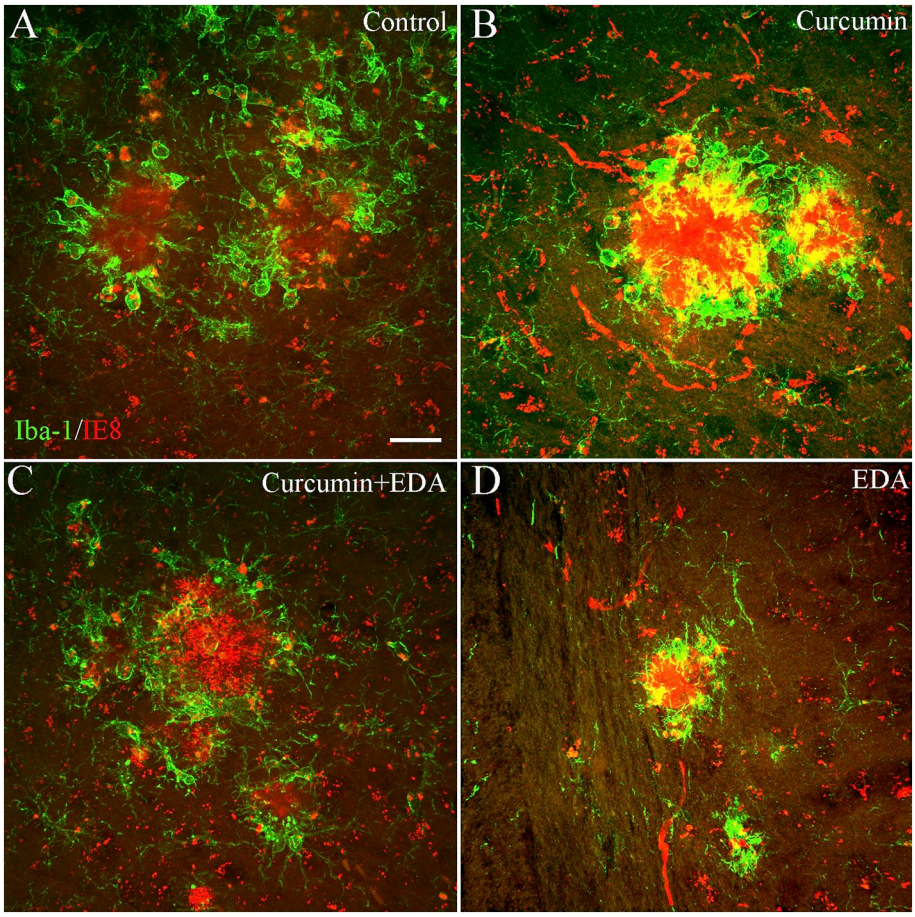

Fig. 5. Immunohistochemical visualisation of Iba-1$^+$ microglia (red) around amyloid plaques (green) in mice on four different experimental diets.
A-D Plaque pathology in the cortical areas of 18-month-old male Tg2576 (APPswe) mice was co-localized with increased density of Iba-1 labelled microglia around the plaques. A) Control fed Tg2576 mice showed high density of microglia numbers around the plaques, with large cell bodies. B) Curcumin fed animals showed a somewhat denser accumulation of microglia around the plaques, also overlapping (yellow) with the plaque density, indicating possible phagocytic activity. C) Curcumin + EDA fed animals showed similar patter to the curcumin fed animals, however, overlapping between the two markers was not observed. D) EDA fed animals showed decreased number of microglia around the plaques, together with some overlapping between the plaque and the microglia density. Green represents Iba-1 positive microglia cells, and red represents amyloid plaques. Scale bar is 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly different across all groups [$F(3,8) = 5.288$, $p = .02$], with the EDA (176,697 ± 8610) group showed a significant difference compared to the curcumin + EDA treated group only ($p = .03$). When compared with the other groups, there was a trend of decreased number of microglia (control group: 220845 ± 19,171, curcumin group: 225180 ± 41,802, curcumin + EDA group: 217234 ± 50,519) (Fig. 7A, Table 4). In the hippocampus, we also found significant differences across the groups in the total number of Iba-1 positive microglia [$F(3,8) = 7.604$, $p < .01$]. We found that the total number of microglia significantly decreased in the EDA group (33,719 ± 8170) compared to both the control group (70,704 ± 7082, $p = .01$) and the curcumin + EDA fed group as well (68,064 ± 4434, $p = .01$) (Fig. 7B, Table 4). These results indicate that EDA was the most potent anti-inflammatory treatment among all tested supplements.

3.7. Effect of experimental diets on memory deficits in cued and contextual fear conditioning

All mice responded to the electric foot shocks delivered during the conditioning *(training)* phase (i.e. vocalisation). Repeated measures ANOVA detected a significant effect of 'time' [$F(4, 448) = 101.5$, $p < .0001$] indicating that mice in all groups showed freezing as a response to the unconditioned stimulus. There was also a 'treatment' by 'time' interaction [$F(12, 448) = 7.945$, $p < .0001$] indicating that the

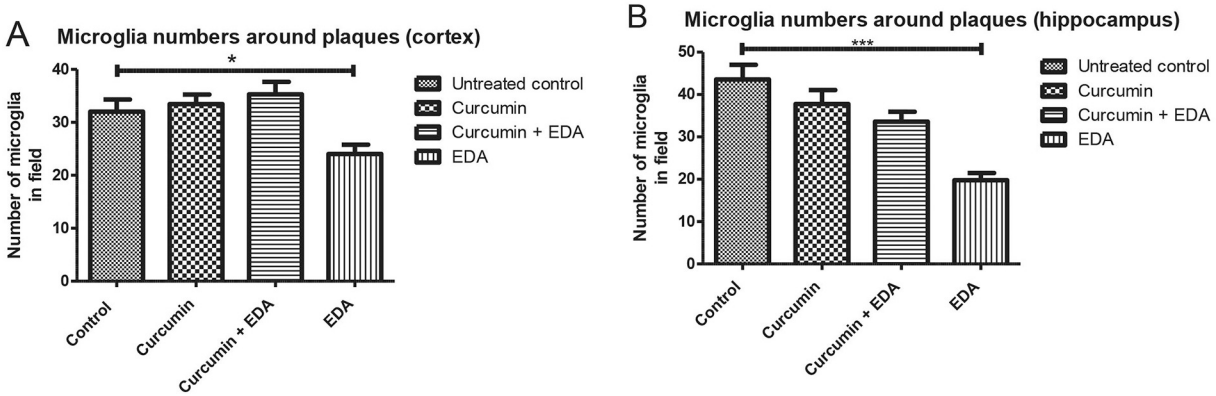

Fig. 6. Iba-1 labelled microglia around amyloid plaques in mice on the four different experimental diets using a non-stereology method. A. The number of Iba-1 positive microglia around plaques in the neocortex. B. The number of Iba-1 positive microglia around plaques in the hippocampus.

Table 3
Parameters and results about the number of microglia cells located around the amyloid plaques.

	Cortex, control	Cortex curcumin	Cortex, curcumin + EDA	Cortex, EDA	Hippocampus, control	Hippocampus, curcumin	Hippocampus, curcumin + EDA	Hippocampus EDA
Number of animals	4	5	5	5	4	5	5	5
Number of sections	10	10	10	10	10	10	10	10
Number of sampling sites	10	10	10	10	10	10	10	10
Size of the counting field (μm X μm)	448 × 336	448 × 336	448 × 336	448 × 336	448 × 336	448 × 336	448 × 336	448 × 336
Perimeter of plaques (μm)	237.53 ± 115.52	247.61 + ~139.92	268.58 + 160.82	163.68 + 73.55	300.14 + 178.36	240.48 + 140.96	236.75 + 165.88	147.30 + 80.20
Total number of microglia around plaques	320 ± 64	337 ± 56	358 ± 119	222 ± 46	435 ± 33	391 ± 123	365 ± 43	218 ± 68
Average number of microglia around plaques	32 ± 6.4	33.7 ± 5.6	35.8 ± 11.9	22.2 ± 4.6	43.5 ± 3.3	39.1 ± 12.3	36.5 ± 4.3	21.8 ± 6.8

conditioning was different across treatment groups. EDA-treated mice exhibited increased *freezing* by the end of the conditioning compared to all other groups (Fig. 8A). Analysing the hippocampal associated contextual fear conditioning revealed a significant treatment effect between groups [$F(3, 109) = 7.156$, $p = .0002$)]. Post-hoc analysis showed that both the curcumin + EDA ($p < .0001$) and EDA groups ($p = .001$) spent increased time on *freezing* compared to the control group. In addition, the curcumin + EDA group showed a significant increase in time spent *freezing* compared with the curcumin only group (Fig. 8B). Similar treatment effects were found when data were analysed across 1-min blocks (data not shown).

In the amygdala associated cue test, all mice demonstrated the ability to associate the CS with the US as evidenced by a significant increase in *freezing* behaviour in response to the presentation of the cue [RM ANOVA for '1 min block': $F(3, 109) = 23.18$, $p < .0001$]. There was also a significant 'treatment' by "time" interaction [$F(15, 545) = 8.407$, $p < .0001$] as all treatment groups showed a stronger *freezing* response than control mice across time (Fig. 8C). To understand this further, we analysed percentage of time *freezing* pre-cue and post-cue. This analysis revealed a significant effect of 'treatment' [$F(3, 109) = 23.18$, $p < .0001$], 'time' [$F(1, 109) = 78.6$, $p < .0001$], and a 'treatment' by 'time' interaction [$F(3, 109) = 12.32$, $p < .0001$] indicating that the treatments modulated the conditioned *freezing* response of mice to the cue. Post-hoc analysis clarified that only curcumin + EDA ($p < .0001$) and EDA groups ($p < .0001$) developed a significant increase in *freezing* during the cue presentation (Fig. 8D).

4. Discussion

The current study presented a comprehensive investigation of the therapeutic effects of a number of food-derived compounds in an Alzheimer's disease mouse model. Our results highlight that the nutritional compounds curcumin, EGCG, DHA and ALA do result in reductions in amyloid plaque load, microglial activation, $A\beta_{40}/A\beta_{42}$ levels and memory deficits in male Tg2576 mice, with the EDA group showing the most prominent changes; wherein only EDA treatment significantly reduced Aβ42 in both the frontal and temporal cortex as well as improved memory compared to the other diets that were tested within the current study. Additionally, our results show that the EDA did not negatively impact the potential therapeutic efficacy of curcumin. Furthermore, curcumin treatment was not seen to influence Aβ42 levels and memory, which is consistent with clinical trials investigating curcumin for Aβ clearance.

The EDA treatment led to a significant reduction in amyloid plaque load in both the cortex and the hippocampus after 12 months feeding, whereas curcumin treatment significantly decreased the amyloid plaque load only in the hippocampus. The EDA group also significantly decreased the level of $A\beta_{40}/A\beta_{42}$ in the frontal cortex and the level of $A\beta_{42}$ in the temporal cortex, whereas both curcumin, curcumin + EDA, and EDA significantly decreased the level of $A\beta_{40}$ in the temporal cortex. Consistent with amyloid reduction, the EDA group significantly decreased the number of microglia around the amyloid plaques in both the cortex and hippocampus as well as the total number of microglia in both the cortex and the hippocampus. In the cortex and hippocampus, we found a decrease of plaque load on the curcumin or EDA diet, but the combination of curcumin with the three other dietary compounds (curcumin + EDA) did not decrease further the amount of amyloid plaque load.

To visualize the amyloid plaque load in the Tg2576 mice, we used a highly specific recombinant Fab antibody, designated 1E8, which reacts with several Aβ peptides, $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{43}$ in ELISA and immunoblotting experiments (Tammer et al., 2002). Different forms of Aβ are present in the brain during the lifetime of Tg2576 mice, including Aβ monomers, soluble Aβ oligomers and insoluble Aβ. In our present study, we only measured total Aβ levels, as the primary focus was on the measurement of amyloid plaque formation in the cortical and

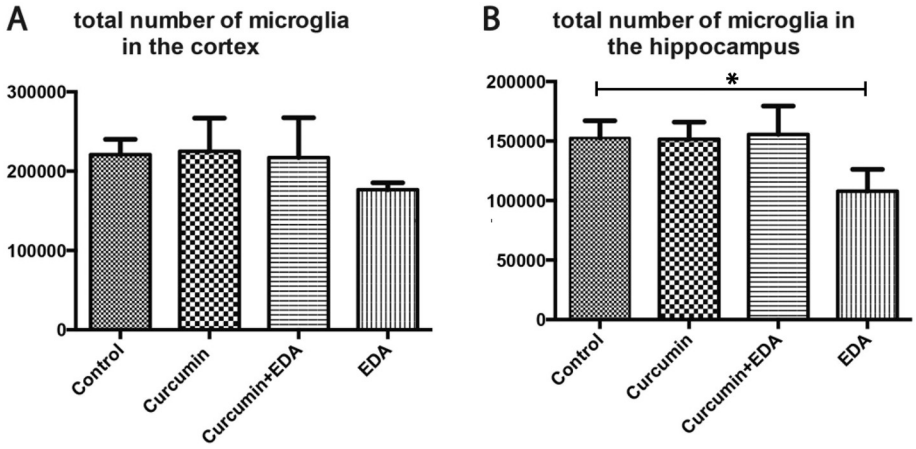

Fig. 7. Total Iba-1 labelled microglia in four different experimental diet groups using stereology. A. The number of Iba-1 positive microglia in the cortex. B. The number of Iba-1 positive microglia in the hippocampus.

hippocampal areas in mice fed the experimental diets. The calculated cortical plaque area in our findings is in accordance with previous studies (Callahan et al., 2001; Kumar-Singh et al., 2005).

There is increasing evidence showing that curcumin alone can inhibit Aβ aggregation, inflammation and oxidative damage, e.g. in PC12 cell cultures (Thapa et al., 2015) and Tg2576 transgenic mice (Lim et al., 2001). In this study Tg2576 mice aged 10 months old were fed a curcumin diet (160 ppm) for 6 months. Lim et al., (2001) reported that both soluble and insoluble amyloid beta levels in the Tg2576 transgenic mice were significantly reduced after 160 ppm (low dose) and 5000 ppm (high dose) of dietary curcumin feeding after 6 months. The curcumin dose used in this study (500 ppm) stands between those used by Lim et al., (2001) although, our feeding protocol was double the time. Our results showed a 41.6% decrease of plaque load in the hippocampus and 23.6% decrease in the cortical areas by curcumin alone, which is comparable to that shown in the Lim study (Lim et al., 2001). While curcumin has been shown to correct aberrant inflammation and promote resolution (He et al., 2014), interestingly, within the present study, although the total number of microglia in either the neocortex or the hippocampus of the curcumin treated group did not significantly decrease compared to the control group (Fig. 6), dividing the number of microglia by the perimeter of the amyloid plaques, showed that the number of microglia immediate to the plaques, were in fact higher in the curcumin treated group. This observation is similar to a previous report where, on curcumin treatment, the number of microglia in neuronal layers (not adjacent to the amyloid plaques) were reported to decrease, but increased within (and in close proximity to) plaques, indicating amyloid induced microglial phagocytosis (Lim et al., 2001). In another study, curcumin and melatonin were combined to treat the APP/PS mouse (Gerenu et al., 2015). Like our results, curcumin/melatonin did not decrease the total number of microglia using Iba-1 antibody, but did decrease the type IV activated microglia by approximately 50% (Gerenu et al., 2015). These suggest that curcumin is involved in different pathways which are regulating the amyloid production, clearance, and microglia activation separately.

Though it has been shown that both ALA and curcumin inhibit amyloid fibril formation in vitro (Landau et al., 2011; Ono et al., 2006), they may have slightly different effects in vivo. For example, ALA and DHA decreased hyperactivation of the tau C-Jun N-terminal kinases (JNKs) and increased MAP1B, dephosphorylated (active) MAP2, and acetylated α-tubulin. These suggest that they can increase microtubule stability and maintenance of active compensatory MAPs (Ma et al., 2014), which may account for the decreased number of microglia around the plaques of reduced size. Frautschy et al., (1998) investigated in more detail the number and morphology of plaque associated microglia in the Tg2576 mice, compared to the non-transgenic counterpart. They found 2–5 fold increase in microglia numbers in the amyloid expressing areas of the brain, as well as increased size of microglia (enlarged microglia in and around the plaques were described) (Frautschy et al., 1998). Many studies have also shown that DHA protects from Aβ accumulation and ameliorates cognitive impairment in rodent models of AD (Calon et al., 2005; Cole and Frautschy, 2006). A more recent study by Teng et al. (2015) demonstrated that DHA supplementation in an APP/PS1 Tg rat model reduced Aβ plaque deposition and modestly improved behavioural deficits.

We also demonstrated that nutritional supplementation with curcumin, curcumin + EDA and EDA showed reduced memory deficits compared to the control group mice, when looking at hippocampal-dependent memory. Interestingly, during the altered context (pre-CS) test condition only the curcumin + EDA and EDA groups had a significant increase in the percentage of time spent freezing, compared to the control group, with the EDA group also demonstrating reduced memory deficits compared to the curcumin only group. The curcumin only group for this altered context test demonstrated no significant difference from the control animals. It is worth noting that there was no clear cue response in the control-treated mice during the cued fear test. A possible explanation for this maybe that given the control mice are not wild type control mice, but rather that they are an AD mouse used as a control. Thus, it would be assumed based on previous literature that they should have an impairment, which is demonstrated with the cued response. Additionally, when looking at the cued response in the experimental animals, the combination treatment groups rescued this impairment, when compared to the control group. As mentioned, the curcumin only diet was the only treatment group that did not demonstrate a significant difference from the control animals.

Consistent with previous results, we also found that the EDA group significantly decreased the amyloid load and the number of microglia around the amyloid plaques in the hippocampus. This might be partly due to the fact that dietary DHA is able to inhibit prostaglandin formation in peripheral tissues and in the brain, which may lead to decreased microglia activation (Strokin et al., 2004). A study by Rezai-Zadeh et al.,. reported that EGCG reduced Aβ generation in vitro in neuron-like cells and primary neuronal cultures from Tg2576 mice, along with promotion of the non-amyloidogenic α-secretase proteolytic pathway (Rezai-Zadeh et al., 2008a). To validate these findings, they treated 12-month-old Tg2576 mice with 20 mg/kg EGCG via intraperitoneal injections for 60 days, and showed decreased Aβ levels and plaque load in the brain, along with promotion of the α-secretase pathway (Rezai-Zadeh et al., 2008a). These previous data raise the possibility that dietary supplementation with EGCG may decrease Aβ levels and plaque load via promotion of the non-amyloidogenic α-secretase pathway. Quinn et al. (2007) showed that chronic dietary

Table 4
Stereological parameters and results about the absolute number of microglial cells in the cortex and the hippocampus of the Tg2576 mice on the four experimental diets.

	Cortex, control	Cortex, curcumin	Cortex, curcumin + EDA	Cortex, EDA	Hippocampus, control	Hippocampus, curcumin	Hippocampus, curcumin + EDA	Hippocampus, EDA
Number of animals	4	5	5	5	4	5	5	5
Distance between Sections (um)	300	300	300	300	300	300	300	300
Disector height (um)2	16	16	16	16	16	16	16	16
Guard zones (lm)2	2	2	2	2	2	2	2	2
SRS grid size	1000 × 1000	1000 × 1000	1000 × 1000	1000 × 1000	1000 × 1000	1000 × 1000	1000 × 1000	1000 × 1000
Counting frame size	100 × 100	100 × 100	100 × 100	100 × 100	100 × 100	100 × 100	100 × 100	100 × 100
Grid spacing (um)	50	50	50	50	30	30	30	30
Number of sections	11 ± 0.82	13.6 ± 0.55	13.6 ± 1.52	13.4 ± 0.89	12.25 ± 1.26	15 ± 0.71	13.8 ± 1.64	14.2 ± 0.45
Number of sampling sites	106.5 ± 12.26	137.8 ± 11.01	117.8 ± 21.67	117.6 ± 8.17	57.5 ± 10.02	65.4 ± 9.50	60.4 ± 6.35	58.8 ± 7.05
Total number of microglia counted	315.25 ± 48.09	346.6 ± 49.95	332.8 ± 87.90	226.2 ± 34.07	189.25 ± 9.29	198.4 ± 10.45	167 ± 28.85	146.8 ± 24.95
Estimated number of microglia	220,845 ± 19,171.86	225,180.6 ± 41,802.51	217,234.8 ± 50,159.03	176,697.6 ± 8610.15	152,538.5 ± 14,585.39	151,543.2 ± 14,407.31	155,512 ± 23,897.44	107,909.2 ± 18,408.95

supplementation with α-lipoic acid (ALA) in Tg2576 mice improved both learning and memory retention and reduced nitrotyrosine levels. However, there were no changes in Aβ levels, plaque deposition or markers of oxidative stress. ALA has been shown to re-solubilize Aβ in a dose-dependent manner and enhance the extraction of Aβ from the frontal cortex of APP transgenic mice (Fonte et al., 2001).

The combination of ALA and DHA has also been shown to be neuroprotective in a tau knockout model (Ma et al., 2014). Hippocampal synaptic deficits were partially corrected with dietary supplementation with DHA and both behavioural and synaptic deficits were fully corrected by combining DHA with ALA. DHA or DHA/ALA also restored phosphorylated and total GSK3β and attenuated hyperactivation of the tau C-Jun N-terminal kinases (JNKs) while increasing MAP1B, dephosphorylated (active) MAP2, and acetylated α-tubulin, suggesting improved microtubule stability and maintenance of active compensatory MAPs (Ma et al., 2014). In line with these findings, we found that EDA significantly decreased the total number of microglia in the hippocampus compared with that of the other groups, which is in contrast to findings from the curcumin treated group. Furthermore, EDA decreased the number of microglia around the amyloid plaques and even the density of microglia. This suggests that EDA might play multiple roles in protecting the mouse from cognitive decline by ameliorating the amyloid pathology and the subsequent microglia activation. Different from our expectations, combination of curcumin and EDA did not result in a synergistic effect on decreasing the number of microglia in either the neocortex or the hippocampus. This again suggests that each of these compounds may take their effect through different signal pathways and some of them are possibly counteracting with each other to different extents. Ma et al. (2009) observed a synergism of curcumin with DHA in a triple transgenic Aβ mouse model. Treatment of 3xTg-AD mice on high-fat diets with fish oil or curcumin, or a combination of both for 4 months reduced phosphorylated JNK, IRS-1, and tau and prevented the degradation of total IRS-1. This was accompanied by improvement in Y-maze performance (Ma et al., 2009). The results from their study suggested that curcumin and DHA may be a viable treatment strategy, with another study by Wu et al. (2014) also showing that curcumin and DHA could counteract neuronal dysfunction after traumatic brain injury. Further to this, a study by Giunta et al. (2010) showed that fish oil may enhance the oral bioavailability of EGCG in Tg2576 mice, and had a synergistic effect on decreasing cerebral Aβ levels. The results from our present study do not appear to show any synergistic advantage of the addition of curcumin to EGCG, DHA and ALA, although the EDA group did demonstrate significantly greater reductions in both Aβ plaque load and levels, compared to the controls and other nutritional supplement groups. The reason for the greater reductions observed in the EDA group, over the curcumin + EDA group is unknown, and warrants further investigation into the interactions between these nutritional compounds in this particular combination.

A study by Parachikova et al. (2010) combined a large number of dietary supplements including several components used in our current study (curcumin, EGCG and ALA). They observed that the combined nutritional supplementation had a positive effect on decreasing cognitive impairment and whole brain levels of $A\beta_{40}$ and $A\beta_{42}$ in Tg2576 mice, with the most notable reduction in whole brain $A\beta_{40}$ levels (Parachikova et al., 2010). They found that the combined diet had no effect on reducing the insoluble $A\beta_{42}$ brain levels, whilst in our study we observed significant reductions in $A\beta_{42}$ levels in the frontal cortex for all dietary groups compared to control mice, and also in the temporal lobe, but only in the EDA group. In our study we evaluated total $A\beta_{40}$ and $A\beta_{42}$ brain levels within different brain regions (frontal cortex, temporal lobe and cerebellum), while Parachikova et al. (2010) only measured whole brain Aβ levels. This study may have found that their dietary intervention did have some potential in reducing insoluble $A\beta_{42}$ brain levels, if they had analysed separate regions of the brain tissue.

A recent study by Parrott et al. (2015) examined a combination

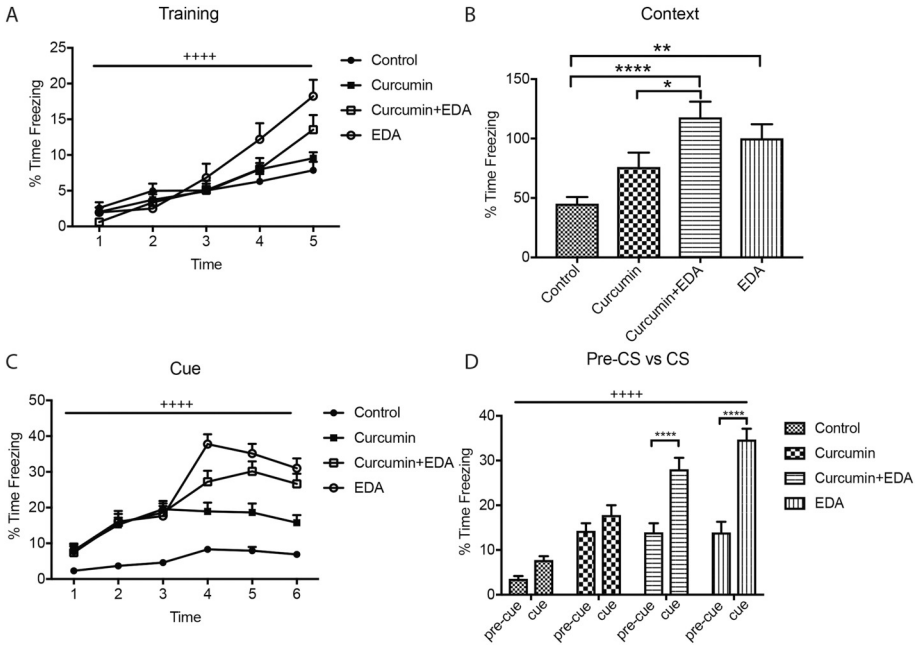

Fig. 8. Contextual and cued fear conditioning behavioural testing in mice on four different experimental diets. Contextual and cue freezing in the fear conditioning test. Time spent *freezing* in conditioning (A) and cue test (C) across 1-min blocks as well as total time freezing in the context test (B) and percentage of time spent *freezing* [%] prior to and during cue presentation (D). Data are presented as mean ± SEM for 26 animals (control), 30 animals (curcumin), 27 animals (curcumin + EDA) and 30 animals (EDA) per group. All mice exhibited an increased *freezing* response. A. Repeated measures ANOVA detected a significant effect of 'treatment' by 'time' interaction ($p < .0001$) indicated by ++++. C. Freezing in the cue test. Repeated measures ANOVA detected a significant effect of 'treatment' by 'time' interaction ($p < .0001$) indicated by ++++. D. Freezing pre-cue and post-cue. ANOVA showed a significant effect of 'treatment' by 'time' interaction ($p < .0001$) indicated by +++++.

whole-food diet containing freeze-dried fish, vegetables, and fruits to investigate if it would improve cognitive function in TgCRND8 mice. This study unexpectedly demonstrated that a whole-food diet containing freeze-dried fish, vegetables, and fruits resulted in worse cognitive function on tests of spatial memory than transgenic animals fed a control diet (Parrott et al., 2015). The authors suggested that normally adaptive cellular responses to the dietary phytochemicals were impaired by amyloid-beta deposition leading to increased oxidative stress, neuroinflammation, and behavioural deficits (Parrott et al., 2015). Although, as all of the dietary components were given in the one diet, they could not determine whether it was the composite effect of the entire diet or related to the relative abundance of a particular dietary component. These unexpected results highlight the potential complexity of food-food interactions. Parrott et al. (2015) cautioned against the use of high supplemental doses of phytochemicals and the need to better assess these compounds.

Further, evidence from previous studies in the literature suggest that the compounds employed within the current study, namely curcumin, DHA, EGCG and ALA, are not indicative of reducing transgene expression, given that no significant difference in APP levels were observed between control vs compound supplementation in tg2576 mice or primary neuronal cells derived from the tg2576 mouse (Lim et al., 2005; Lim et al., 2001; Obregon et al., 2006; Quinn et al., 2007). These reports therefore imply that the compounds investigated within the study do not influence transgene expression but rather target the APP processing pathway and AD associated pathology.

The current study observed differential $A\beta_{40/42}$ concentrations within different regions of the tg2576 mouse brain and these differences could be attributed to regional differences in neuronal activity (Bero et al., 2011). Interestingly, Aβ concentrations in the frontal cortex and temporal lobe observed within the various treatment groups, followed similar trends to the pathological data in the cortex, wherein EDA diet lowered Aβ to the greatest extent. Not surprisingly, Aβ concentrations in the cerebellum were inconsistent with the pathology data observed in the frontal cortex and hippocampus as it should be noted that clinically the cerebellum is the last region to be affected by amyloid pathology and thus the effect of treatment in the cerebellum will be minimal.

Additionally, previous studies provide insight into the bioavailability of the nutritional compounds investigated within the current study. The dietary administration of the doses of curcumin (83 mg/kg BW) and EGCG (50 mg/kg BW) to tg2576 mice, as employed within the current study, resulted in plasma concentrations of 0.035 μM and ~0.7 nM, respectively and brain concentrations of 0.469 μM and ~0.55 nM, respectively (Begum et al., 2008; Giunta et al., 2010). Further, the oral administration of 300 mg/kg BW DHA to Wistar rats (6× the current study), was seen to result in plasma and brain DHA concentrations of 0.547 umol/L (170% increase from vehicle) and 161 nmol/mg protein (10% increase from vehicle) in the cortex (Hashimoto et al., 2005), while the oral administration of 600 mg/day lipoic acid in human subjects, has been reported to result in a serum lipoic acid concentration of 2.17 μg/ml (where placebo showed 0.03 μg/ml) (Shinto et al., 2014). Given the importance of clinical translation, dose translation from animal studies to clinical trials is vital. Previously, the US FDA (2005) and Reagan-Shaw et al. (2008) have published tables utilizing the body surface area scaling method to calculate an appropriate human drug dosage from animal studies. Using these tables, the concentrations required to achieve benefits similar to those observed in the current study in human subjects would approximately equate to 6.7 mg/kg, 4 mg/kg, 4 mg/kg, 11.38 mg/kg, for curcumin, EGCG, DHA and ALA, respectively. However, recent research has called into question the accuracy of using the body surface area scaling method and it is more appropriate to use pharmacokinetic modeling to accurately calculate human dosing from animal trials (Blanchard and Smoliga, 2015). Therefore, further studies are warranted to verify these doses in a clinical setting.

It should be noted that there were several limitations with our study. One limitation was that we did not measure soluble Aβ oligomers in this study due to tissue constraints. Future studies looking at the efficacy of these compounds should also look at measuring the different pools of Aβ species. A second limitation was that our study was conducted using male Tg2576 mice. It is known that the prevalence of AD is higher in women (Snyder et al., 2016), than men with a recent Alzheimer's Association report estimating that at age 65, women have about a 1 in 6 chance of developing Alzheimer's compared with a 1 in 11 chance for men. Therefore, our current study is not able to establish whether there are gender effects with these nutritional compounds. Future studies will be required to demonstrate the effects of these compounds in female mouse models of AD. It should also be noted that memory was only assessed using the cued and contextual fear conditioning test. The contextual and cued fear conditioning test was used as the primary assessment of memory in this study as it requires

minimal training, less handling of the mice (Shoji et al., 2014), and therefore less potential of affecting outcomes based on training times and increased handling that multiple memory tests would involve. Although, this memory test has been used in numerous studies evaluating memory in transgenic models of AD (Comery et al., 2005; Jacobsen et al., 2006; Quinn et al., 2007), and requires much less training time for mice than other learning and memory tasks (Shoji et al., 2014), future studies should also assess the efficacy of the identified nutritional compounds using additional memory tests.

5. Conclusions

While many studies suggested the effectiveness of EGCG, DHA and ALA alone on Aβ accumulation and cognitive impairment associated with this process, this is the first report that investigates the combined effects of these compounds, with or without curcumin in male mice. Based on our results, the combination of these three compounds would require further investigation, because not only their effect was found to be more significant than curcumin and curcumin combined with EGCG, DHA and ALA, but also only males were used. These results should raise awareness that interactions between these nutritional compounds and other complementary medicine/nutraceutical products might result in counterproductive outcomes. It also highlights the fact that manufacturers of nutraceutical combination products should be careful not to claim additive or synergistic effects of their combination products in vivo without having tested it in animal models and/or human clinical trials.

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Conflict of interest

The authors declare no conflict of interest.

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