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The hydroxylated form of docosahexaenoic acid (DHA-H) modifies the brain lipid composition in a model of Alzheimer's disease, improving behavioral motor function and survival☆

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ABSTRACT

We have compared the effect of the commonly used ω-3 fatty acid, docosahexaenoic acid ethyl ester (DHA-EE), and of its 2-hydroxylated DHA form (DHA-H), on brain lipid composition, behavior and lifespan in a new human transgenic *Drosophila melanogaster* model of Alzheimer's disease (AD). The transgenic flies expressed human Aβ42 and tau, and the overexpression of these human transgenes in the CNS of these flies produced progressive defects in motor function (antigeotaxic behavior) while reducing the animal's lifespan. Here, we demonstrate that both DHA-EE and DHA-H increase the longer chain fatty acids (≥18C) species in the heads of the flies, although only DHA-H produced an unknown chromatographic peak that corresponded to a non-hydroxylated lipid. In addition, only treatment with DHA-H prevented the abnormal climbing behavior and enhanced the lifespan of these transgenic flies. These benefits of DHA-H were confirmed in the well characterized transgenic PS1/APP mouse model of familial AD (5xFAD mice), mice that develop defects in spatial learning and in memory, as well as behavioral deficits. Hence, it appears that the modulation of brain lipid composition by DHA-H could have remedial effects on AD associated neurodegeneration. This article is part of a Special Issue entitled: Membrane Lipid Therapy: Drugs Targeting Biomembranes edited by Pablo V. Escribá.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and the main form of progressive dementia in the elderly [1]. AD is characterized by the loss of synapses and neurons, in conjunction with the formation of extracellular deposits of β-amyloid (Aβ) and the intraneuronal accumulation of neurofibrillary tangles as a result of abnormal tau protein hyperphosphorylation [2]. Cognitive deficits arise when this damage occurs in brain regions associated with learning and memory, such as the hippocampus, one of the structures that is most vulnerable in AD [3].

AD has been associated with a loss of DHA from neuronal membranes in the CNS, a modification that has been linked to the anomalous processing of the amyloid precursor protein (APP), resulting in the accumulation of the β-amyloid peptide (Aβ) and cognitive decline [4–5]. DHA is the main ω-3 polyunsaturated fatty acid (PUFA) in the brain and it provides great flexibility to the membranes of neurons, as well as influencing other biophysical properties. In fact, diets enriched in DHA appear to delay AD-associated neurodegeneration and possibly other types of dementia [6–7]. Most food supplements contain the ethyl ester form of this ω-3 fatty acid (DHA-EE), which increases the amount of DHA in tissues [8]. However, other forms of DHA can also be found in the form of triacylglycerols or phospholipids and there is evidence that their bioavailability may be better than that of DHA-EE [9–12].

Here we have evaluated the effects of a new form of DHA, 2-hydroxy-DHA (DHA-H), which was previously seen to produce promising benefits in AD [13–15], As such, we assessed the effects of DHA-H on the brain lipid composition, motor activity and behavior, and the lifespan of a transgenic *Drosophila melanogaster* fly that expresses human Aβ42 and Tau, animals that display neural dysfunction and

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that undergo neurodegeneration. As these flies are also characterized by the accumulation of human Aβ and Tau, they are considered to be a useful model to study human neurodegenerative diseases like AD [16]. Among the advantages of using *Drosophila* as a model system to study disease are: its short lifespan, allowing experiments to be carried out over the entire life of the animal; their small size makes it possible to use a large number of animals; and the wealth of genetic data available for this system. Indeed, a wide range of drugs have already been tested in *Drosophila* models of AD, including anti-inflammatory mimetics of ApoE protein [17], γ-secretase inhibitors [18], antibiotics [19] and EGFR inhibitors [20]. Here, we found that only DHA-H significantly improved the lifespan and climbing behavior of these transgenic flies, concomitantly altering their brain lipid composition.

2. Materials and methods

2.1. Fly stock maintenance and food supplementation

The flies were maintained by serial transfer in 150 ml bottles containing 30 ml of corn meal medium (water, agar, salt, sugar, yeast and corn meal), with or without a DHA-EE or DHA-H supplement (1, 3, 10, 30 or 100 μg/ml). The medium was also supplemented with a fungicide (methyl-4-hydroxybenzoate) and an antibacterial agent (propionic acid), and active yeast powder was added to the surface of the medium. The flies were kept on a 12 h day/night cycle at 25 °C (±1 °C) and 65% (±5%) relative humidity.

2.2. APP expression and Aβ accumulation in transgenic flies

The GAL4/UAS system was used to express the human APP gene in *Drosophila* [21]. In this system, the Gal 4 yeast transcriptional activator directs the transcription of the transgene under the control of an upstream activating sequence (UAS). Flies carrying the UAS-human Aβ42 and human tau 2N4R transgenes (stock 33771) were crossed with flies carrying Gal 4 predominantly expressed in neurons (*elav*-GAL4^{c155}, stock 8760 [22]). Only the F1 offspring of these parental flies expressed Aβ and tau, displaying locomotor dysfunction and a shorter lifespan. Fly stocks were kindly provided by the Bloomington Drosophila Stock Center (Indiana University, USA).

2.3. DHA-H administration to 5xFAD mice

A double transgenic PS1/APP mouse was used here that harbours five human mutations associated with familial AD (5xFAD; line Tg6799): the *Swedish* (K670N/M671L), *Florida* (I716V) and *London* (V717I) mutations in APP; and the M146L and L286V clinical mutations in the human PS1. Both transgenes are expressed under the control of the Thy-1 promoter and the mice display cognitive decline from 4 months of age [23]. Transgenic 5xFAD and wild type (WT) mice were purchased from Jackson Laboratories (USA) and they were maintained on a B6/SJL hybrid genetic background (C57BL/6 × SJL) by crossing heterozygous transgenic mice with B6/SJL WT (F1) breeders. All animals were housed at a controlled temperature of 22 °C (±2 °C) and 70% humidity on a 12 h–12 h light-dark cycle, and they were provided a standard laboratory diet *ad libitum* (Panlab A03; Barcelona, Spain).

WT and 5xFAD transgenic male mice received DHA-H orally (Lipopharma Therapeutics; Palma de Mallorca, Spain), dissolved in 5% ethanol at a dose of 5, 20, 50 and 200 mg/kg·daily, or the vehicle solution alone. These treatments started when the mice reached 3 months of age (dosed 5 days/week) and they were continued until the mice reached 7 months of age. During the last month of treatment, all the animals were submitted to a hypocaloric diet to perform the selected behavioral spatial learning and memory test (food craving test in a radial arm maze) [13]. A total of 42 animals were used in this study: 10 vehicle-treated WT, 9 vehicle-treated 5xFAD and 23 DHA-H-treated 5xFAD mice: 5 mg/kg, $n = 5$; 20 mg/kg, $n = 7$; 50 mg/kg, $n = 7$; and 200 mg/kg, $n = 4$. All the protocols employed were approved by the Bioethics Committee at the University of the Balearic Islands, and they are in accordance with national and international guidelines on animal welfare.

2.4. Protein isolation and western blotting

The heads of strain 33771 and 8760 flies, and of the F1 offspring (33771 × 8760: 150–200 heads per sample) were homogenized in Tripure Isolation Reagent (Roche) and protein samples were isolated according to the manufacturer's instructions. Briefly, fly heads were homogenized in 1 ml of Tripure using a Teflon pestle, and 0.2 ml of chloroform was added to separate the aqueous and organic phases. After centrifugation at 12000 ×*g* (15 min, 4 °C), the upper aqueous phase was removed, and stored at −80 °C, and 1.5 ml of 2-propanol was added to the remaining sample to precipitate the protein. After a 30 min incubation, the proteins and fly exoskeleton were recovered by centrifugation at 12000 ×*g* (10 min, 4 °C), and the resulting pellet was washed twice with 2 ml of 0.3 M guanidine hydrochloride in 95% ethanol and once with absolute ethanol. The final pellet containing the precipitated proteins and exoskeleton was solubilized overnight at room temperature (RT) in 0.2 ml of a buffer containing: 8 M urea; 4% (*w*/*v*) SDS; and 20 mM Tris-HCl [pH 7.4]. Finally, the sample was disrupted by ultrasound (3 pulses, 100 W and 10 s each), and the non-soluble material (exoskeleton) was removed after centrifugation at 12000 ×*g* for 10 min. The same procedure was followed to isolate the protein from the WT and 5xFAD mouse brain (hippocampus), although the last centrifugation step to remove the exoskeleton was omitted. The protein concentration in the resulting supernatants was determined by absorbance spectroscopy using a commercial kit (Bio-Rad DC protein assay).

Protein samples (120 μg per lane for fly samples or 20 μg per lane for mouse samples) were resolved on 16% (for Aβ peptide) or 12% polyacrylamide gels (for tau, APP, PS1-CTF or α-tubulin) in Tris-tricine or Tris-glycine electrophoresis buffers, respectively. To detect Aβ, proteins were transferred to methanol-activated PVDF membranes (Bio-Rad, Madrid, Spain), while nitrocellulose membranes were used for other proteins (GE Healthcare, Little Chalfont Buckinghamshire, UK). Both membranes were subsequently blocked with 5% (w:v) non-fat dry milk in TBS supplemented with 0.1% (v:v) Tween-20. The membranes were then incubated overnight at 4 °C with the corresponding primary antibody: mouse monoclonal anti-human-β-amyloid 1–16 (clone 6E10, 1:2000: Signet Labs, Dedham, MA, USA); rabbit polyclonal anti-human-tau (Tau46, 1:1000: Thermo Scientific, Pierce, Rockford, IL, USA); rabbit polyclonal anti-APP-C-terminal (1:6000: Calbiochem, Nottingham, UK); mouse monoclonal anti-PS1-C-terminal-fragment (CTF, 1:1000: Millipore, Chemicon international, Germany) or mouse monoclonal anti-α-tubulin (1:5000: Sigma). Antibody binding was detected with horseradish peroxidase-conjugated anti-mouse/rabbit IgG (1:2000: GE Healthcare, Little Chalfont Buckinghamshire, UK) and then visualized by chemiluminescence using the ECL western blot detection kit (GE Healthcare, Little Chalfont Buckinghamshire, UK). The intensity of the bands was quantified by integrated optical density using Quantity One software (Bio-rad, Madrid, Spain) and the results were normalized to the α-tubulin content.

2.5. Longevity

After hatching, we determined the longevity of the transgenic flies of the different genotypes that were treated with the vehicle or different DHA forms. For this purpose, 50 flies were placed in a vial containing food in the presence or absence of the food supplements indicated below. These flies were transferred to fresh vials and counted every 3 days, analyzing at least three vials for each experimental group.

2.6. Climbing ability

The motor activity of the flies was tested using the antigeotaxic (climbing) assay. Briefly, flies of the indicated genotypes ($n = 20$) were placed in a 100 ml graduated cylinder with a mark made 9.5 cm from the bottom. The cylinder was gently tapped to ensure all the flies were at the bottom of the tube and the number of flies above the mark was recorded after 20 s. Five trials were performed to obtain an average number for each experimental age.

2.7. Lipid extraction and gas chromatography analysis

Fly heads (approximately 20 mg) were incubated for 48 h in 3 ml of a chloroform:methanol solution (2:1, v:v) under an argon atmosphere at RT [24]. After 48 h, all the heads had sunk to the bottom of the vial and they were homogenized at 4 °C using a Polytron PT3100 homogenizer (Kinematica, Luzern, Switzerland). The samples were centrifuged at 1000 ×*g* for 10 min at 4 °C and the supernatant was recovered. The pellet was then washed for 1 h with 3 ml chloroform:methanol (2:1, v:v) and the supernatant recovered again. Both supernatants containing the lipids were combined with 0.2 volumes of 0.9% NaCl, and the mixture was vortexed and centrifuged at 1000 ×*g* for 10 min at 4 °C. The lower, organic phase was evaporated in a pre-weighed tube and it was maintained under vacuum for 12 h to eliminate traces of solvent. The mass of the extracted lipid was calculated by the weight difference, and the lipid mixture was then supplemented with 1 μmol margaric acid (C17:0) as an internal standard for GC analysis. The lipid film was resuspended in 166 μl hexane to help dissolve the more apolar lipids, and the fatty acids were transmethylated in an argon atmosphere by incubating the lipid mixture for 90 min at 100 °C in glass screw-cap tubes containing 3 ml methanol:acetylchloride (10:1, v:v).

Fatty acid methyl esters (FAMEs) were extracted with hexane by adding 3 ml H_2O and 1 ml hexane to the transmethylation reaction, then thoroughly vortexing the mixture. After centrifugation at RT (1000 ×*g*, 10 min), the upper phase containing the FAMEs was collected and the remaining volume was washed twice with 1 ml hexane. The three hexane phases collected were combined, evaporated under an argon or nitrogen flow and resuspended in 1 ml of hexane. Half of this material was analyzed directly by GC and the remainder was evaporated to derivatize the free hydroxyl groups. Hydroxy-fatty acids cannot be completely volatized in their methyl ester form but they require a second derivatization, involving the addition of a trimethylsilyl (TMS) group [25]. Accordingly, the lipid film was dissolved in N,O-bis (trimethylsilyl) acetamide (0.1–5.0 mg lipid for 200–400 μl trimethylsilylation reagent) and heated at 70 °C for 30 min in a screw-capped vial. The solvent was evaporated and the lipid film was resuspended in 500 μl hexane.

FAMEs and their trimethylsilylated counterparts were analyzed on an Agilent J&W HP-88 capillary column (30 m × 0.25 mm × 0.20 μm) in an Agilent 7890A GC system equipped with a flame ionization detector and a 7693 auto-injector (Santa Clara, CA, USA), using 1.3 ml/min of helium as a carrier and a split ratio of 5:1. Upon sample injection, the column was equilibrated at 130 °C for 5 min, and the temperature was then increased to 160 °C at a rate of 2.5 °C/min and subsequently, up to 220 °C at 2 °C/min. Finally, the column was left at 220 °C for 5 min, while the injector and detector temperatures were kept at 250 °C. Areas of the peaks were quantified using margaric acid (C17:0) as an internal standard and corrected using the calculated weight of the lipids extracted. The identification of peaks was performed using standards of the different hydroxylated and non-hydroxylated fatty acids.

2.8. Statistics

The data were expressed as the mean (±SEM) from at least three independent experiments. The data from the control and problem groups were compared using the unpaired *t*-test or the log rank test (to compare fly survival from the Kaplan-Meier plots), and the level of significance chosen was $p = 0.05$.

3. Results

3.1. Lifespan and locomotor activity of Drosophila melanogaster expressing human Aβ42 and tau

Transgenic flies expressing human Aβ42 and tau experience neural dysfunction and neurodegeneration [16]. As indicated above, flies carrying transgenes of the UAS-human Aβ42 and human tau 2N4R isoform (stock 33771) were crossed with flies carrying a Gal 4 (elav-GAL4c155) transgene (stock 8760), and the resulting F1 offspring expressed both the human tau protein and Aβ42 peptide (Fig. 1, panel A). However, it should be noted that endogenous tau-like protein also appeared to be weakly expressed in the progenitors due to a cross-reaction with the primary antibody (Fig. 1, panel A, lanes 1 and 2).

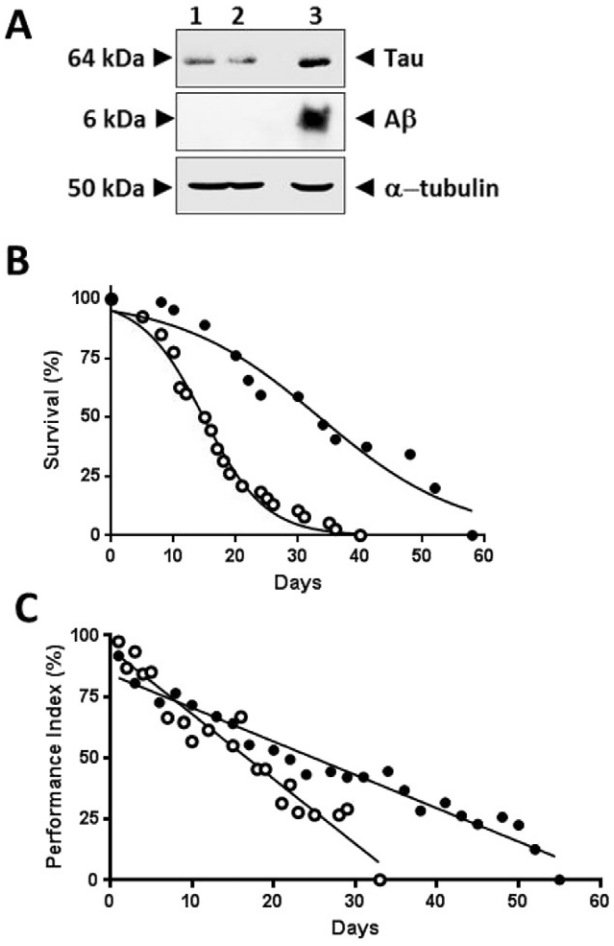

Fig. 1. Characterization of the flies. (A) Protein samples were obtained from fly heads, and used to examine the Aβ and tau expression in western blots. Flies from the F1 generation (lane 3) had more human Tau protein and Aβ peptide than their progenitors (lane 1: Gal4-expressing flies; lane 2: flies expressing Aβ and tau under the control of the UAS promoter). Quantitative analysis revealed a statistically significant increase in Tau protein in F1 flies compared to the progenitors in lane 2 (49.7 ± 5.3%). While the Aβ peptide was not observed at all in the progenitors, Tau protein was in fact present in both generations, indicating the presence of a human-Tau homolog in *Drosophila melanogaster*. (B) Representative survival curves of flies that did not express Aβ and that carried Tau (stock 33771, solid symbols), and Aβ and Tau expressing F1 flies (33771 × 8760, open symbols). The expression of both human transgenes diminished the median survival of fruit flies from 33.3 days (stock 33771) to 14.4 (F1: $n = 150$ flies per group). (C) Representative motor performance of stock 33771 (solid symbols) and of Aβ/tau expressing F1 stock 33771 × 8760 flies (open symbols). The expression of both human transgenes diminished the median locomotor performance of fruit flies from 26.2 days (stock 33771) to 17.6 (F1: $n = 60$ flies per group).

The flies expressing Aβ42 and tau had a significantly shorter lifespan (median survival 14.4 ± 1 days) than the healthy control 33771 flies (median survival 33.3 ± 2 days, $p < 0.01$: Fig. 1, panel B), and a similar negative effect on climbing behavior was also observed in these F1 flies. While the 33771 control flies exhibited the expected gradual age-related decline in locomotor activity, with a 50% loss of performance at 26.2 ± 2.6 days (Fig. 1, panel C), the decline in performance of F1 flies expressing tau and Aβ42 was accelerated with a 50% loss at 17.6 ± 1.7 days ($p < 0.01$). Thus, over-expression of Aβ42 and tau compromises fly survival and motor function, although the deficient performance in the climbing test may also reveal altered spatial orientation, particularly since superior cognitive abilities like learning are affected in flies expressing Aβ42 [26].

3.2. DHA-H augments the lifespan and motor activity of F1 Aβ42/tau expressing flies

Larval and adult fly food was supplemented with DHA-EE or DHA-H at concentrations of 1, 3, 10 and 30 μg/ml, and the survival and locomotor activity (climbing ability) of these flies was analyzed (Fig. 2, panels A and B). Supplementing larval and adult fly food with DHA-H increased the median survival of F1 flies, with a maximal effect at 30 μg/ml, whereas the DHA-EE supplement had no such effect (Fig. 3, panel A). Similarly, DHA-H but not DHA-EE significantly improved the scores achieved by F1 flies in the climbing test (Fig. 2, panels C and D), again with a maximum effect at 10–30 μg/ml (Fig. 3, panel B). Since the climbing test is used extensively to assess the locomotor capacity of flies, these results demonstrate an improved locomotor activity in DHA-H-treated flies. However, such an improvement could be due to ameliorated spatial orientation more than just improved motor function.

3.3. DHA-H improves the cognitive scores of 5xFAD mice

To assess whether the improvement in climbing provoked by DHA-H is in part due to improved spatial cognitive ability, we extended our research to a more complex mouse model of AD (5xFAD mice) [23]. We first performed a molecular characterization of 5xFAD mice relative to the WT (wild type mice). As shown in Fig. 4 western blots, APP (amyloid precursor protein) appeared as a double band (mature and immature APP) in WT mice (mouse APP) and it accumulated more strongly in the 5xFAD brain (human and mouse APP) compared with the WT brain. On the other hand, mouse PS1-CTF (γ-secretase active center) was observed as a unique band in the WT mice whereas the human PS1-CTF appeared as a second band above the mouse PS1-CTF band in 5xFAD tissue. No differences were found between WT and 5xFAD mice in the total amount of PS1-CTF detected. Finally, an Aβ load was clearly detected in the 5xFAD brain tissue but not in the WT mice. These animals were orally administered DHA-H for 4 months and cognitive scores were assessed in the radial arm maze (RAM) test. This test evaluates learning ability in terms of spatial orientation, whereas additional parameters like the speed of the mice during the test may reflect a deterioration in motor activity. Administering scaling doses of DHA-H improved the RAM performance of 5xFAD mice, which otherwise displayed altered spatial learning abilities when compared to WT control mice (Fig. 4). Indeed, DHA-H administration produced a dose-dependent decrease in total, reference and working memory errors (RME and WME, respectively) relative to the untreated 5xFAD mice [13]. Interestingly, the lowest dose of DHA-H administered (5 mg/kg·day) did not induce any significant amelioration, whereas higher doses of 20, 50 and 200 mg/kg·day did improve cognitive scores in 5xFAD mice (Fig. 4, panel A). Conversely, there were no significant changes in mouse speed between the WT and 5xFAD genotypes, in accordance with the lack of motor dysfunction at this age in 5xFAD mice shown previously [27]. In fact, DHA-H treatment did not modify the speed of 5xFAD mice, except in those mice that received the highest dose (200 mg/kg) that were significantly slower (Fig. 4, panel C). However, when doses of DHA-H as high as 200 and 500 mg/kg have been tested previously, they were seen to be very safe as single acute doses and they failed to provoke any serious deleterious effects on motor parameters [15]. Therefore, these results show that DHA-H administration improved cognition ability in terms of spatial orientation without

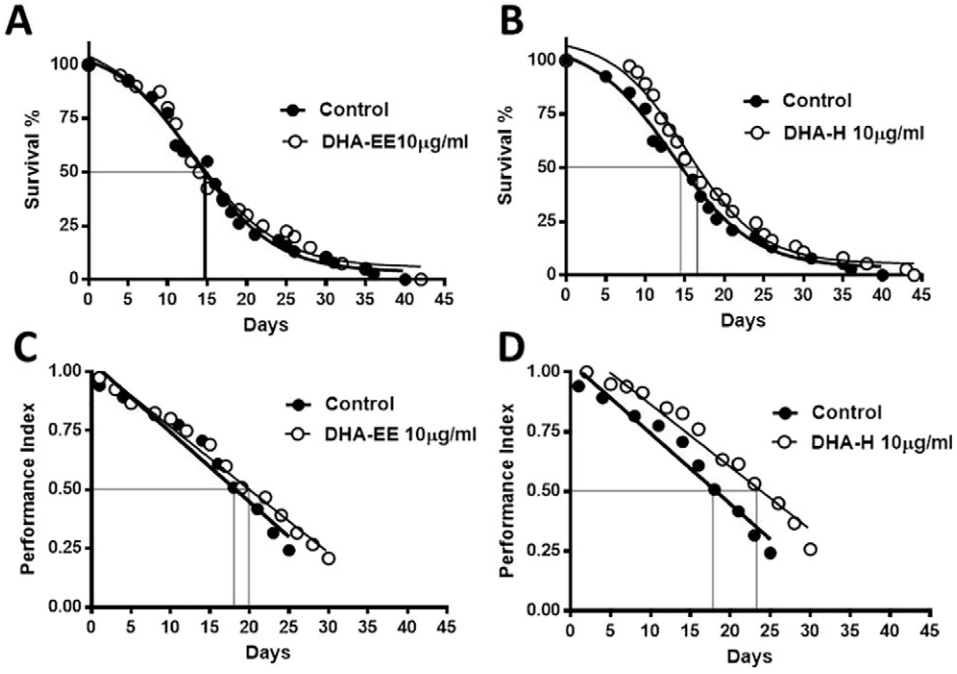

Fig. 2. A. Representative survival curve of F1 flies after food supplementation with DHA-EE (10 μg/ml, open dots) or without food supplementation (control; filled dots). B. Representative survival curve of F1 flies after food supplementation with DHA-H (10 μg/ml, open dots) or without food supplementation (control; filled dots). C. Representative motor performance of F1 flies after food supplementation with DHA-EE (10 μg/ml, open dots) or without food supplementation (control; filled dots). D. Representative motor performance of F1 flies after food supplementation with DHA-H (10 μg/ml, open dots) or without food supplementation (control; filled dots).

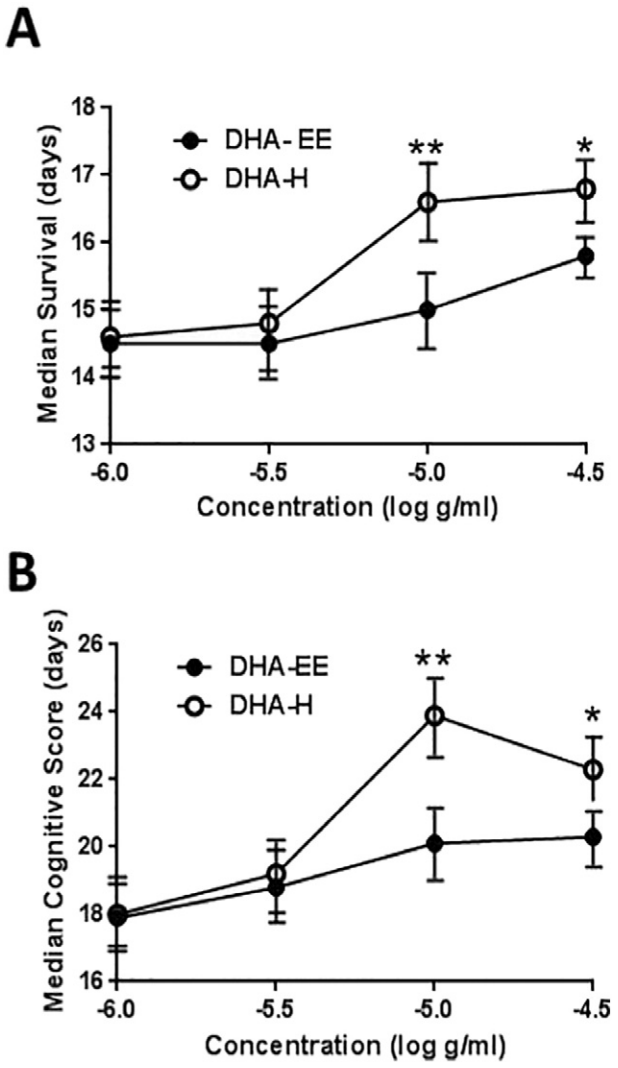

Fig. 3. Effects of DHA-H and DHA-EE on the lifespan and motor activity of Drosophila. (A) Median survival of F1 flies fed with a DHA-EE (solid symbols) or DHA-H (open symbols) supplement. As a dietary supplement, DHA-H was more effective than DHA-EE in increasing the lifespan of the flies. (B) Median locomotor performance index of F1 flies fed with a DHA-EE (solid symbols) or DHA-H (open symbols) supplement. As a dietary supplement, DHA-H was more effective than DHA-H in improving locomotor performance: $^{*}p < 0.05$ with respect to untreated control F1 flies; $^{**}p < 0.01$ with respect to untreated control F1 flies.

affecting the motor capacity of mice at the therapeutic doses of 20 and 50 mg/kg·day. Extrapolation of these results to the *Drosophila* AD model suggests that the DHA-H-mediated improvement in climbing may be explained by the ameliorated spatial orientation of treated flies compared to the F1 control flies.

3.4. DHA-EE or DHA-H induced distinct changes in the fatty acid composition of fly brains

Supplementing the diet of the F1 flies with DHA-EE and DHA-H induced a variety of changes in the fatty acid composition in their brain (Table 1). The head of control flies (those which did not receive any dietary supplement) contained approximately 56 mol% of short chain fatty acids (12–16 carbon atoms), which decreased to 49 and 51 mol% in the heads of flies that received DHA-EE and DHA-H, respectively. In terms of longer chain fatty acids (≥18C), both DHA-EE and DHA-H augmented their presence in the brain. Consistent with previous studies, control brain tissue lacked C20 and C22 polyunsaturated fatty acids (PUFAs) [28], yet omega-3 EPA (20:5n-3) and DHA (22:6n-3) but not omega-6 ARA (20:4n-6) were detected in the brains of flies that received the DHA-EE-supplement. Interestingly, the amount of DHA detected (1.6 ± 0.3 nmol /mg lipid) was much lower than that of EPA (42.6 ± 1.5 nmol/mg lipid: Table 1), indicative of the rapid conversion of the 22:6n-3 into the 20:5n-3 form (both omega-3 fatty acids).

Despite receiving a supplement of DHA-H (up to 100 μg/ml for lipidomic studies), this synthetic fatty acid was not detected in the heads of these flies, indicating that *Drosophila melanogaster* possesses the enzyme machinery necessary for rapid DHA-H metabolism. This conversion was not associated with the production of 20:4n-6 (ARA), 20:5n-3 (EPA) or the non-hydroxylated analogue of DHA-H, 22:6n-3 (DHA). An analysis of the fatty acid profile of flies that received DHA-H identified a clear chromatographic peak that was not present in the heads of control flies or in those that received DHA-EE (Fig. 5C and insert). This peak did not correspond to a hydroxylated fatty acid as it had the same retention time after trimethylsilanization. This metabolic derivative of DHA-H accounted for ca. 4 mol% of all the fatty acids in the *Drosophila* brain.

4. Discussion

Classical non-mammalian animal models to study human disease include vertebrates (zebrafish), invertebrates (the fruit fly *Drosophila*) and the nematode *Caenorhabditis elegans*. These simple models have many advantages: their small size, high fecundity, experimental tractability, homology with the human basic cellular and molecular mechanisms and cost-effective experimentation. In the past few years, new models have been introduced to study the biology of aging. These models include cnidarians (hydra), urochordates (*Botryllus schlosseri*), tunicates (Ciona) or Echinodermata (sea urchin) [29].

Drosophila melanogaster flies that express human transgenes represent model systems with several advantages for the study of AD. First, the Drosophila APP homologue, an APP-like protein (APPL), lacks the Aβ peptide region and its processing does not produce neurotoxicity [30]. Therefore, human APP expression in transgenic flies makes it possible to discriminate between the abnormal effects of exogenous human APP and secreted Aβ. In addition, the short life cycle of flies enables cognitive parameters to be assessed in different conditions over the animals' entire lifespan, and any effects on survival can also be studied. Moreover, their small size makes it possible to analyze a large number of animals. Here, we have used a model in which male flies carrying a UAS-human Aβ42 and a human tau 2N4R isoform (stock 33771) were crossed with female flies carrying a Gal 4 transgene (elav-GAL4c155; stock 8760), producing F1 offspring that express both human tau and Aβ42 peptides. In this F1 generation, the expression of both the human tau and Aβ peptide provoked worse survival and poorer performance in terms of climbing. Moreover, an additional study carried out on transgenic 5xFAD mice supported the fact that DHA-H has a beneficial effect on superior cognitive functions like spatial orientation, without affecting locomotor function, in turn suggesting that the improved climbing behavior in flies is promoted by enhanced cognitive ability. These results validate the use of *Drosophila melanogaster* models carrying human transgenes to study the effects of DHA-EE and DHA-H dietary supplementation in AD. In fact, similar models have been used elsewhere to successfully study the effect of drugs or nutraceuticals in the treatment of AD [17–20].

We compared two forms of dietary DHA supplementation in this *Drosophila melanogaster* model of AD to investigate possible differences in their efficacy as nutritional supplements to treat this neurodegenerative disease: the ethyl ester form of DHA (DHA-EE) and DHA hydroxylated at carbon 2 (DHA-H). A number of epidemiological studies have shown an inverse correlation between dietary DHA intake (or fish intake) and the incidence of AD, associating high levels of DHA with a reduced risk of impaired cognitive function [31–33]. However, elsewhere no clear effect of DHA administration was observed on cognitive decline [34,35]. Here, we show that DHA-H enhances the survival and climbing

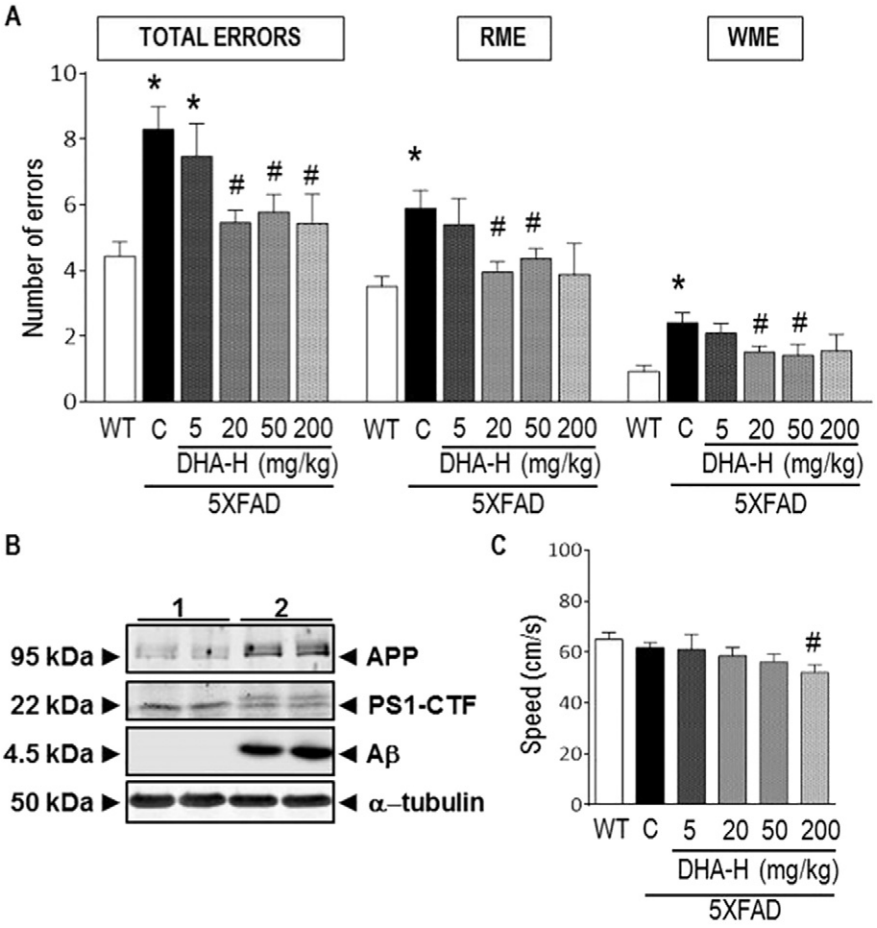

Fig. 4. Spatial learning ability of 5xFAD mice in a Radial Arm Maze (RAM) test. (A) Number of total errors, reference memory errors (RME, entrance into unbaited arms) and working memory errors (WME, re-entry into visited arms) of DHA-H-treated 5xFAD mice. A significant increase was observed in the number of errors in 5xFAD mice relative to the WT mice, as well as a dose-dependent decrease in 5xFAD mice receiving DHA-H at 20, 50 and 200 mg/kg·day relative to the 5xFAD control mice. (B) Molecular characterization of 5xFAD (lines 2) relative to the WT mice (lines 1), analyzing human APP and PS1 over-expression in western blots. APP appeared as a double band (mature and immature APP) in WT mice (mouse APP) and it accumulated more strongly in the 5xFAD brain (human and mouse APP). Mouse PS1-CTF (γ-secretase active center) was observed as a unique band in the WT mice whereas the human PS1-CTF appeared as a second band above the mouse PS1-CTF band in 5xFAD tissue. No differences were found between WT and 5xFAD mice in the total amount of PS1-CTF detected. An Aβ load was clearly detected in the 5xFAD brain tissue but not in the WT mice. (C) The speed parameter was measured during test performance in order to detect locomotor disabilities in DHA-H-treated mice. No differences in this parameter were detected, except in the animals that received 200 mg/kg·day, in which a discrete yet significant decrease in speed was evident compared with untreated 5xFAD control mice. Bars represent mean ± SEM. Statistical analysis was performed by one-way ANOVA and Tukey's post-hoc test: *, $p < 0.05$ compared to WT mice; #, $p < 0.05$ compared to untreated 5xFAD control mice.

ability of flies expressing human tau and Aβ42, whereas the DHA-EE form produced a similar tendency but that did not reach statistical significance. We previously demonstrated that DHA-H restores cognitive behavior and induces neuronal cell proliferation in a mouse model of AD based on Aβ accumulation (5xFAD mice) [13]. Moreover, DHA-H was also seen to modify the brain membrane lipid composition in these transgenic mice, enriching membranes in phosphatidylethanolamine (PE) carrying long polyunsaturated fatty acids (PUFAs) while reducing the total amyloid load and tau phosphorylation [14]. In our transgenic fly model, the expression of both Aβ42 and tau transgenes is under the control of the UAS-Gal4 system, so that neither DHA-H nor DHA-EE influence amyloid or tau expression (data not shown), unlike the transgenic mouse model where Aβ is produced via APP processing [14].

With respect to the lipid profile, we previously demonstrated that DHA-H treatment markedly increased the presence of phospholipids composed of long fatty acyl chains in the brain of 5xFAD transgenic mice relative to shorter acyl chains [14]. This is in accordance with the data presented here, showing that both DHA-EE and DHA-H modify lipid content in the head, also inducing an increase in longer chain fatty acids (≥18C). DHA and EPA do not appear to be present in the heads of flies [31] and we confirmed this in flies fed with the control diet (i.e. without a supplement of DHA-EE or DHA-H). However, DHA (22:6n-3) was found in the head of the flies supplemented with DHA-EE (100 μg/ml), demonstrating that this w-3 is readily incorporated into the organism after DHA food supplementation. Moreover, an EPA peak (20:5n-3) was also observed in the heads of *Drosophila* fed with DHA-EE (22:6n-3), and the relative levels of EPA (20:5n-3) and DHA (22:6n-3) suggested that ca. 95% of the DHA (22:6n-3) taken up by *Drosophila* was converted into EPA (20:5n-3). This metabolism of DHA (22:6n-3) into EPA (20:5n-3) is consistent with other studies where ca. 85% of DHA (22:6n-3) was metabolized into EPA (20:5n3) in flies fed with DHA [28]. By contrast, neither the hydroxylated DHA-H analogue nor DHA were found in the heads of the flies fed with DHA-H (100 μg/ml). However, we did detect a new chromatographic peak in the head of flies that received DHA-H that was not present in control flies nor in those that received DHA-EE as food supplement. Hence, DHA-H would appear to be readily metabolized and incorporated into the organism through the diet, and this novel peak may represent a metabolite derived from DHA-H. Interestingly, this metabolite was a non-hydroxylated fatty acid, which indicates the capacity of flies to dehydroxylate DHA-H.

The fact that neither DHA (22:6n-3) nor EPA (20:5n-3) were detected in DHA-H supplemented flies suggests a rapid conversion of DHA-H

Table 1
Fatty acid composition of the heads of flies fed with the vehicle alone (F1; control), DHA-EE or DHA-H (100 μg/ml). Values are the average ± SEM of 2–4 separate experiments and they are expressed as nmol/mg lipid.

FA	F1 (control)	DHA-EE	DHA-H
12:0	157.9 ± 35.7	98.1 ± 7.6*	120.5 ± 27.2*
14:0	668.3 ± 25.4	484.6 ± 4.3*	592.1 ± 64.9
14:1n-5	34.5 ± 3.0	24.7 ± 0.6*	30.7 ± 3.7
16:0	704.3 ± 16.2	709.9 ± 1.9	649.5 ± 19.6
16:1n-7	502.2 ± 4.2	516.1 ± 0.9	502.4 ± 3.0
18:0	106.8 ± 1.8	175.8 ± 0.6#	156.1 ± 4.6#
18:1n-9	571.5 ± 22.4	687.3 ± 3.1#	627.8 ± 41.6#
18:2n-6	542.2 ± 19.8	512.3 ± 3.3	428.4 ± 38.2*
18:3n-3	115.9 ± 9.2	120.3 ± 2.8	123.5 ± 3.1
20:0	12.2 ± 0.4	16.8 ± 0.2#	18.0 ± 1.7#
20:4n-6	ND	ND	ND
20:5n-3	ND	42.6 ± 1.5#	ND
22:6n-3	ND	1.6 ± 0.3#	ND
SFA	48.3 ± 1.4	43.8 ± 0.4*	47.3 ± 2.3
MUFA	32.4 ± 0.6	36.2 ± 0.1*	35.7 ± 1.1*
PUFA	18.5 ± 0.3	20.0 ± 0.3*	17.0 ± 1.2
UFA	50.4 ± 0.5	56.2 ± 0.4*	52.7 ± 2.3
SFA/MUFA	1.50 ± 0.07	1.21 ± 0.01*	1.33 ± 0.10
SFA/PUFA	2.69 ± 0.07	2.20 ± 0.05*	2.86 ± 0.34
SFA/UFA	0.99 ± 0.02	0.78 ± 0.01*	0.91 ± 0.08
MUFA/PUFA	1.72 ± 0.02	1.81 ± 0.02*	2.12 ± 0.09*,#

DHA-H, 2-hydroxydocosahexaenoic acid; DHA, docosahexaenoic acid; ND, not detected. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; UFA, unsaturated fatty acid.

* Statistically significant decrease compared to control samples ($p < 0.05$).

\# Statistically significant increase compared to control samples ($p < 0.05$).

into non-hydroxylated fatty acids other that the omega-3 DHA and EPA. However, this does not exclude the possibility that DHA-H activates the enzymatic machinery to rapidly metabolize 22:6n-3 and 20:5n-3. These data are consistent with previous studies showing that DHA-H administration could be a good strategy to treat AD [13,14]. Nevertheless, the precise mechanism by which cognitive scores are improved in animal models of AD is not fully understood. It is most likely that the neuroprotective effect of this ω-3 fatty acid is mediated by its regulation of membrane lipids [14]. Indeed, such a dual interaction of hydroxylated fatty acids with membrane lipids and proteins has been shown elsewhere [36,37].

Finally, the use of the *Drosophila* AD model allowed the effect of different doses of DHA-EE and DHA-H to be studied on cognitive/motor behavior and lifespan. Compared with the complexity of the RAM tests and with the length of the treatments necessary in mice AD models (4 months or more), the *Drosophila* model appears to be an efficient screening method to investigate the potential effects of different compounds prior to their investigation in more complex models. In this context, the effect of omega-3 fatty acids has been studied elsewhere in different mice models of Alzheimer's disease [38,39]. Although some treatments clearly prevent cognitive dysfunction in mice or in *Drosophila* models, these promising results often do not translate to humans. For this reason, the use of alternative non-human-primate models (NHPs), which are phylogenetically much closer to humans, is being developed for the study of Alzheimer's disease [40]. Further studies will be needed to identify the non-hydroxylated lipid derivative of DHA-H and to determine if it plays a role in the actions provoked by DHA-H that restore motor activity in AD-like flies.

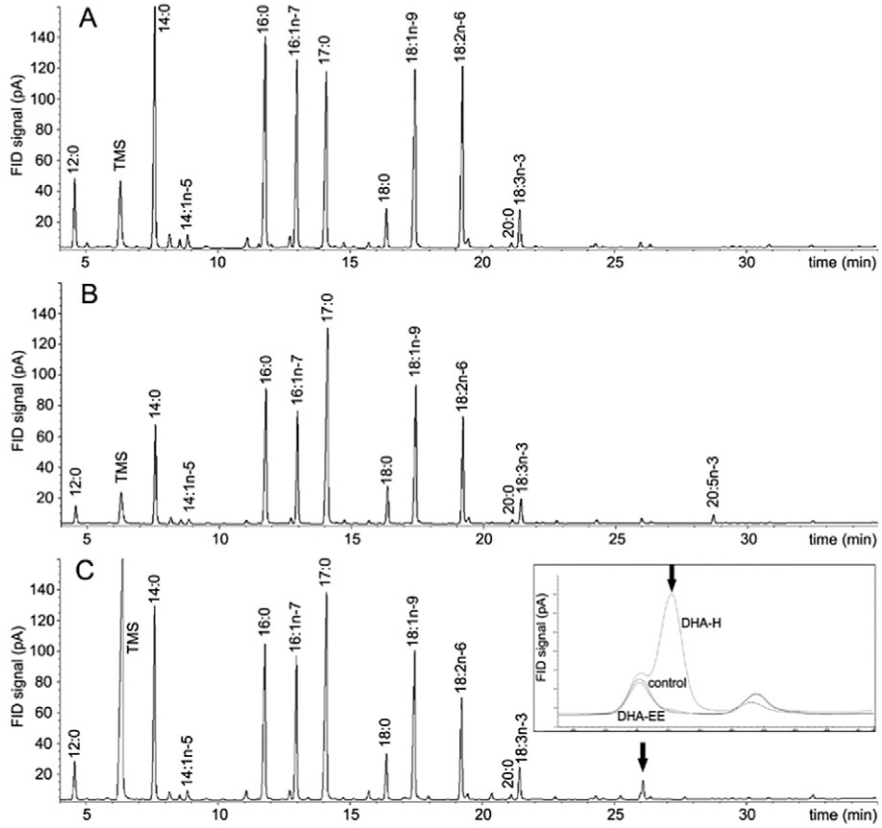

Fig. 5. Effect of DHA-H on the fatty acid profile in the brain. Representative chromatograms of fatty acid composition in *Drosophila melanogaster* fed with: (A) basal diet; (B) basal diet supplemented with DHA-EE (100 μg/ml); and (C), basal diet supplemented with DHA-H (100 μg/ml). Total lipids from the heads of flies were extracted and transmethylated with acetyl chloride in methanol. Further derivatization with N,O –bis (trimethylsilyl) acetamide was performed on half of the sample to distinguish hydroxylated from non-hydroxylated fatty acids. Fatty acids were analyzed on an Agilent 7890A GC system with a HP88 capillary column, and the peaks were identified by comparison to fatty acid standards. The arrow in panel C indicates the presence of one non-hydroxylated peak present in the DHA-H but not in the control or DHA-EE-supplemented flies. Inset in panel C shows the region of the three chromatograms where the previously mentioned peak is located superimposed and amplified.

Author contributions

Pablo V. Escribá and Xavier Busquets conceived and designed the experiments. Raheem J. Mohaibes, Manuel Torres, Maria A. Fiol-deRoque, David J. López and Margarita Ordinas performed the experiments. José A. Castro supervised the work with the flies and reviewed the paper. Pablo V. Escribá and Xavier Busquets wrote the paper.

Conflict of interests

The authors have no conflict of interests to declare.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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