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RESEARCH ARTICLE

Cognitive recovery and restoration of cell proliferation in the dentate gyrus in the 5XFAD transgenic mice model of Alzheimer's disease following 2-hydroxy-DHA treatment

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Abstract Alzheimer's disease (AD) is the most common neurodegenerative disorder in the elderly. In the last years, abnormalities of lipid metabolism and in particular of docosahexaenoic acid (DHA) have been recently linked with the development of the disease. According to the recent studies showing how hydroxylation of fatty acids enhances their biological activity, here we show that chronic treatment with a hydroxylated derivative of DHA, the 2-hydroxy-DHA (2OHDHA) in the 5XFAD transgenic mice model of AD improves performance in the radial arm maze test and restores cell proliferation in the dentate gyrus, with no changes in the presence of beta amyloid (Aβ) plaques. These results suggest that 2OHDHA induced restoration of cell proliferation can be regarded as a major component in memory recovery that is independent of Aβ load thus, setting the starting point for the development of a new drug for the treatment of AD.

Keywords Alzheimer's disease · Lipid metabolism · 2OHDHA · 5XFAD Alzheimer model · Cognition · Neurogenesis

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and the main form of progressive dementia in the elderly (Schaeffer et al.

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2011). The pathophysiology of AD is characterized by loss of synapses and neurons accompanied with formation of extracellular deposits of fibrillar β-amyloid (Aβ), these deposits known as senile plaques, and by the intraneuronal accumulation of neurofibrillary tangles, which result from the abnormal hyperphosphorylation of tau protein (Zhang et al. 2007). Cognitive deficits arise from the damage of brain regions associated with learning and memory, such as the hippocampus, one of the most vulnerable brain regions to AD (Thompson et al. 2004). The subgranular zone (SGZ) of the granular cell layer (GCL) of the hippocampal dentate gyrus (DG) is one of the two neurogenic niches in the mammalian adult brain, along with the anterior part of the subventricular zone (Abrous et al. 2005). The role of impaired neurogenesis in the progression of AD has recently gained relevance not only in animal models but also in humans (Rodríguez and Verkhratsky 2011). In numerous studies on AD animal models the decrease of neurogenesis was described (Donovan et al. 2006; Demars et al. 2010; Rodríguez et al. 2008), although the opposite data also exist (Jin et al. 2004b; Jin et al. 2004a; López-Toledano and Shelanski 2007). Despite these contradictions, the restoration or potentiation of neurogenesis has been recently considered as a potential therapeutic strategy that may reduce or delay early cognitive symptoms of the disease (Rodríguez et al. 2011; Mu and Gage 2011).

Lipid metabolism is considered to contribute to the development of AD (Hartmann et al. 2007), with indications that alterations in cholesterol, triglycerides or omega-3 (n-3) fatty acids (FA) are associated with the cognitive decline observed in AD (Morley and Banks 2010). Furthermore, it has been suggested that Aβ production is directly affected by the lipid composition of the membrane, these being key settings for either the amyloidogenic or the non amyloidogenic pathway (Hartmann et al. 2007; Grimm et al. 2011).

The docosahexaenoic acid (DHA, 22:6 n-3) is the most abundant n-3 polyunsaturated FA (PUFA) in the brain (Hashimoto et al. 2011). DHA is involved in the development and function of the central nervous system, contributing to synaptogenesis, synaptic function, cognition and neuroprotection (Salem et al. 2001). The DHA is primarily obtained through the diet and its deficiency is related to cognitive decline in aged brains and to neurodegenerative diseases such as AD (Uauy and Dangour 2006). Several animal studies have demonstrated that oral intake of DHA or fish oil reduces AD-like brain pathology (Lim et al. 2005; Green et al. 2007). Treatment with DHA significantly improves cognitive deficits, protects from loss of synaptic proteins and lowers insoluble Aβ in transgenic AD mouse models (Calon et al. 2004; Lim et al. 2005). Moreover, numerous epidemiological studies indicate an inverse correlation between DHA intake and AD incidence, associating high levels of DHA with a reduced risk of impaired cognitive functions (Kalmijn et al. 1997; Morris et al. 2003; Johnson and Schaefer 2006). However, direct administration of DHA has no clear effect on cognitive decline (Freund-Levi et al. 2006; Quinn et al. 2010). Recently, some studies have also related DHA to neurogenesis suggesting that this n-3 PUFA could modulate the generation of new functional neurons (Su 2010). DHA is able to promote proliferation in cultured embryonic neural stem cells and neurogenesis in the hippocampus of adult and aged rats with an improvement in learning and cognition (Kawakita et al. 2006; Dyall et al. 2010). Transgenic mice for n-3 PUFA, which have a high level of DHA, have shown increased hippocampal neurogenesis and neurite outgrowth as well as improvements in spatial learning (He et al. 2009). These findings indicate a potential mechanism by which DHA improves the neuronal function in hippocampus thus facilitating or promoting learning and behavior by generating new hippocampal neurons (Su 2010). It has been suggested that the biological activity of DHA could be modulated trough neuroprotectin D1 (NPD1; 10,17S-docosatrien) (Lukiw and Bazan 2008). NPD1 is a natural hydroxylated derivative of DHA that is able to modulate an intrinsic neuroprotective, anti-inflammatory, and antiapoptotic gene-expression program that promotes cell survival and reverts Aβ induced neuronal death (Lukiw et al. 2005). It has been reported previously that hydroxylation of FA enhances their biological activity. A hydroxylated synthetic derivative of oleic acid, 2-hydroxyoleic acid (Minerval), has become a potent antitumor compound that modifies the composition and structure of lipid membranes (Lladó et al. 2009, 2010; Barceló-Coblijn et al. 2011; Terés et al. 2012).

According to these data, and the apparently enhanced power of hydroxylated FA, we propose to use synthetic PUFA 2-hydroxy-DHA (2OHDHA) as a new tool for the treatment of AD-associated cognitive deficits. 2OHDHA is a hydroxylated derivative of

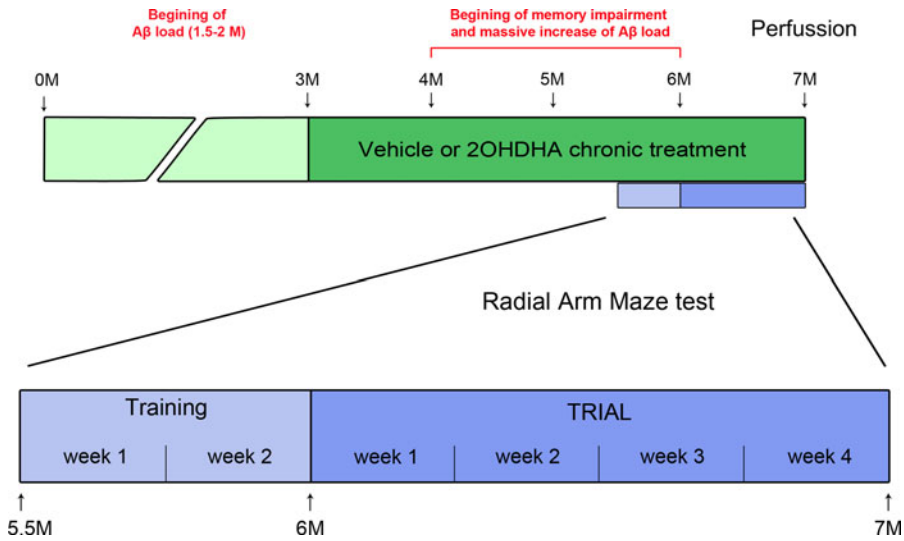

Fig. 1 Experimental paradigm. At 3 months of age animals started to be treated with either vehicle (Non-Tg and 5XFAD groups) or 2OHDHA (2OHDHA+5XFAD group) for 4 months. At 5.5 months of age the animals were trained in the RAM finishing 2 weeks later. At 6 months of age, animals were exposed to the trial phase of the RAM, finishing 4 weeks later, at 7 months of age, when all animals were perfused

DHA that has been developed by hydroxylating the alpha-carbon of the precursor lipid. Here we report the effect of 2OHDHA on memory and on cell proliferation in the dentate gyrus in the transgenic AD model mice.

Materials and methods

All the experiments were performed in agreement with the Bioethical Committee of the University of Balearic Islands (Permit Number): #XB023-2010. All efforts were made to reduce the number of animals by following the 3R's.

Animals and treatments

We used male 5XFAD double transgenic mice (line Tg6799, Jackson Laboratories®, USA). 5XFAD mice co-express Familial AD (FAD) mutant forms of human APP (the Swedish mutation: K670N, M671L; the Florida mutation: I716V; the London mutation: V717I) and presenilin-1 (M146L; L286V) transgenes under transcriptional control of the neuron-specific mouse Thy-1 promoter (Oakley et al. 2006). 5XFAD animals (B6/SJL genetic background) were maintained by crossing heterozygous transgenic mice with B6/SJL F1 breeders. All 5XFAD transgenic mice were heterozygous with respect to both transgenes, and non-transgenic (Non-Tg) wild-type littermate mice were used as controls.

Non-Tg controls ($n = 10$) and 5XFAD ($n = 11$) animals were treated with vehicle (5 % intraesophagic ethanol solution, 15 ml/kg/day), whilst the experimental group, 5XFAD+2OHDHA, ($n = 12$) was treated with 2OHDHA (15 mg/kg/day dissolved in 5 % ethanol; Lipopharma Therapeutics, Spain). The treatment was carried out according to the disease progression described by Oakley et al. 2006 (Fig. 1). The treatment (dosed 5 days/week) started at 3 months of age before the massive increase of Aβ load, (which occurs after the 4 months of age- Quinn et al. 2010). The treatment continued for the next 4 months to give enough treatment to reverse the memory impairments that begins at 4–6 months of age (Oakley et al. 2006).

All the animals were housed under controlled temperature and humidity conditions (22 ± 2 °C; 70 % humidity) and 12–12 h light–dark cycles with standard laboratory diet (Panlab A03, Spain) and water ad libitum. The body weight was measured daily for all animals.

Radial arm maze (RAM) test

RAM is a spatial learning and memory test that allows assessing both working memory (WM) and reference memory (RM) (Wirsching et al. 1984) reflecting both hippocampal and frontal function (Hasselmo and Eichenbaum 2005; Martin and Clark 2007). Prior to the experiments all the animals were isolated and submitted to food restriction until reaching 80–85 %

of the normal body weight in ad libitum feeding and were maintained in these conditions until the end of the test. After food restriction and 2 weeks before trials started, the animals were trained in the eight RAM (LE766/8, Panlab SL, Barcelona, Spain). Each mouse was placed in the centre of the maze and allowed to look for the reward, a 45 mg food pellet (Dustless Precision Pellets, Bio-Serv, US) located at the end of every arm. Each session finished when the animal either succeeded in finding the eight baited arms or failed in complete all the arms after 10 min. The movement of every animal was recorded with a digital video tracking system (LE 8300 with software SEDACOM v1.3, Panlab, SL, Spain). The training was repeated 5 days/week, during 2 weeks. After the training, when the mice reached 6 months of age, the experimental paradigm started (Fig. 1). In all the sessions just four arms were baited compared to the training protocol. Each session finished when the animals either succeeded in finding the four baited arms or failed after 10 min. The performance was assessed taking into account (1) the time to achieve the test (2) the number of WM errors (WME: re-entrances in a previous visited baited arm), (3) the number of RM errors (RME: entrances in an unbaited arm) and (4) the total number of errors (WME + RME). The test was repeated 5 days/week for 4 weeks. Once the RAM test was finished, the animals were sacrificed and the brains were used for the subsequent anatomical studies.

Fixation and tissue processing

Non-Tg ($n = 4$), 5XFAD ($n = 7$) and 5XFAD+2OHDHA ($n = 7$) males were anaesthetized, after behavioral analysis, with an intraperitoneal injection of Ketamine/Xylacine (100/10 mg/Kg) at 7 months of age and then transcardically perfused with 25 ml of 0.9 % saline solution followed by 75 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed from the cranium and bisected down the midline. The tissue was then post-fixed in the same fixative for 24 h and kept in 0.1 M PB, pH 7.4. Sagital sections of the hemibrains were cut into 40–50 μm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB pH 7.4, were collected and stored in cryoprotectant solution (25 % sucrose and 3.5 % glycerol in 0.05 M PB at pH 7.4). Sagital vibratome sections between levels L 1.20 mm to L 2.16 mm (hippocampus) were selected for immunohistochemistry (IHC) according to the mouse brain atlas of Paxinos and Franklin (2004).

Antibodies

A polyclonal affinity-purified rabbit antiserum raised against phosphorylated Histone H3 (HH3; Upstate, USA; #06-570) was used for the determination of proliferating cells. For identification of Aβ-plaques we used a monoclonal mouse antiserum against amino acid residues 1–16 of Aβ (clone 6E10) that reacts with abnormally processed isoforms, as well as precursor forms of Aβ (Covance, Emeryville, CA, USA). And for the identification of astroglia we used a monoclonal mouse antiserum generated against glial fibrillary acidic protein (GFAP) from pig spinal cord (Sigma-Aldrich Company Ltd., UK; #G3893). The specificity of the antibodies has been reported previously using IHC and western blots (Krishnamoorthy et al. 2006; Eng et al. 2000; Ohno et al. 2007). To determine the specificity of the antibodies omission of primary and/or secondary antibodies were also carried out, which resulted in total absence of target labeling (data not shown).

Immunohistochemistry

IHC experiments were performed as described previously (Rodríguez et al. 2008, 2009). Briefly, sections were incubated for 48 h at room temperature in either a cocktail of primary antibodies containing a rabbit polyclonal antiserum against HH3 (1:1,000) and a mouse monoclonal antiserum against GFAP (1:60,000) or in a sequential manner to detect first Aβ in a mouse monoclonal antiserum against Aβ (1:2,000) and secondly with a mouse monoclonal antiserum against GFAP (1:60,000). For the detection of the primary antibodies the sections were placed for 1 h in the secondary antibodies: either biotinylated donkey anti-rabbit IgG (1:400; Jackson Immunoresearch, Stratech Scientific Ltd., Soham, UK) or biotinylated horse anti-mouse IgG (1:400; Vector Laboratories Ltd., Peterborough, UK). Then the sections were incubated in a solution of biotin–avidin complex (ABC, 1:200) from the Elite kit (Vector Laboratories Ltd., Peterborough, UK) for 30 min. Finally, for the HH3 and Aβ detection and

visualization, the ABC was revealed by the incubation in a solution prepared from the SGZ kits (Vector Laboratories Ltd., Peterborough, UK) for 5 min; and for the GFAP in a solution containing 0.022 % of 3,3′ diaminobenzidine (Aldrich, Gillingham, UK) and 0.003 % H_2O_2 in 0.1 M Tris-buffered saline for 2.5 min.

HH3 and Aβ plaques determination and analysis

We determined the numerical density (Nv) of HH3-immunoreactive neurons (HH3-IR) as labeled cells per volume unit (#/mm^3) on 3–12 non-consecutive sagital sections per animal. Subsequently, we determined the Nv and size (mm^2) of Aβ plaques in the GCL of the DG on 4 non-consecutive sections of the same animals used for the proliferation analysis. Brain sections were analyzed and photomicrographs were taken using a Nikon Eclipse 80i microscope coupled with an 8001 MicroFiRE camera and all the area measurements were done using computer-assisted imaging analysis (ImageJ 1.43, NIH, USA). The entire HH3 positive cell and Aβ plaque counting as well as the IOD measurement in the DG were conducted by a single investigator (M.A.F-dR.) who was blind to the subject number and group assignment. All slides were coded prior by another lab member (R.G.) and codes were not broken until all measurements were completed.

Inverse optical density (IOD) measurement

Using computer-assisted imaging analysis (Image J 1.43) we analyzed the density of intraneuronal Aβ on the same four sections used for the Aβ plaques counting. We measured the optical density (OD) of the GCL of the DG as described previously (Noristani et al. 2010). Briefly, to exclude any experimental errors all images were taken at constant light intensity and keeping the same optical filters, used to ensure the specificity of the signal recorded by the camera.

The staining was observed throughout the thickness of the section (40 μm) using light microscopy. The OD was calculated from a relative scale of intensity ranging from 0 to 255, with readout of 255 corresponding to the area with very low Aβ-immunoreactive (Aβ-IR) and 0 corresponding to the densest area of labeling. The calibration density was kept constant for the whole procedure to avoid experimental variances. The sections background OD was determined from the *corpus callosum* (CC) that was considered as blank since Aβ labeling in the CC is virtually absent, and CC also presents a similar labeling to GFAP that DG.

To analyze the change in Aβ-IR density against constant control, the 255 was divided by control region (CC) and the obtained factor was multiplied by the region of interest (GCL) in every given section. IOD was obtained by subtracting from the obtained background level (set at 255).

$$\mathrm{IOD} = 255 - \left(\mathrm{GCL}\frac{255}{\mathrm{CC}}\right)$$

Measurements of mean density were taken and averaged, after background subtraction, from the GCL of the ML of each slice. The results are shown as inverse Aβ-IR density (IOD/pixel).

Statistical analysis

One-way analysis of variance (ANOVA) followed by either post hoc Bonferroni analysis or unpaired *t* test comparisons were used to determine differences between Non-Tg controls, 5XFAD or 5XFAD+2OH-DHA animals (implemented by GraphPad Prism 4.0; GraphPad Software, Inc., USA). All the data is presented as Mean ± SEM.

Results

Effect of 2OHDHA on radial maze learning ability

On the fourth week of trial 5XFAD showed a significant delay of 65 % in the time performance compared to Non-Tg control animals (124.4 ± 12.38 vs. 75.69 ± 4.779 s respectively; $p < 0.01$; Fig. 2a). This delay was paralleled by a significant increase in the total number of errors, close to 50 %, compared to the Non-Tg control group (12.27 ± 1.048 vs. 8.200 ± 0.5993 respectively; $p < 0.05$; Fig. 2b).

Chronic treatment with 2OHDHA showed no evident changes in the time performance and total number of errors during the first 3 weeks of trial neither in Non-Tg, nor in 5XFAD nor in 5XFAD+2OHDHA. However, on the fourth week of trial a clear performance improvement in treated animals when compared to the continuous alteration was observed in 5XFAD animals, which was similar to Non-Tg control values; as showed by the significant genotype/treatment effect in both the

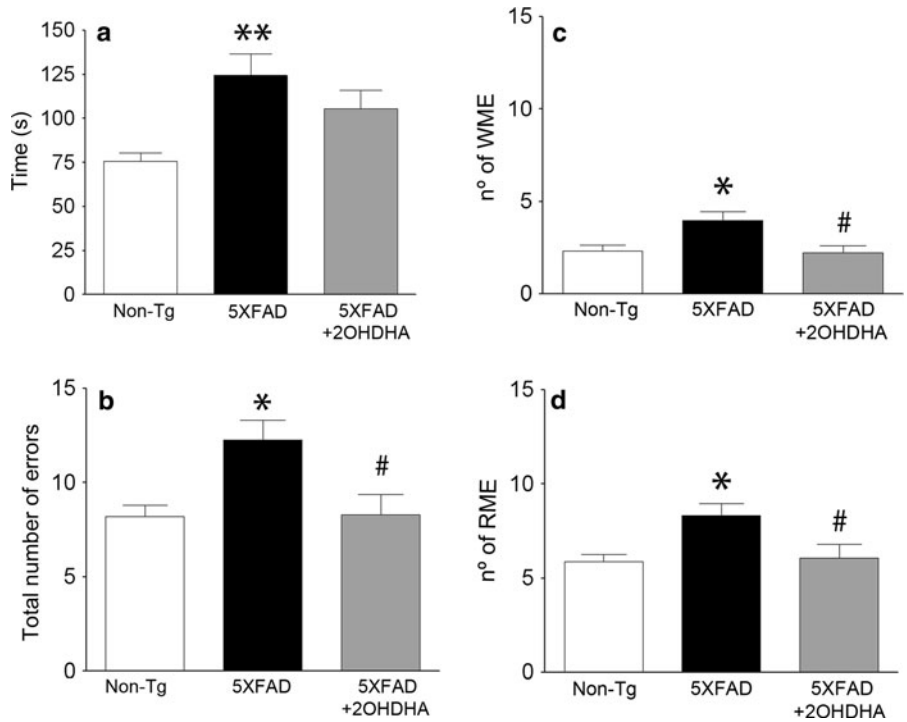

Fig. 2 Learning ability on the RAM. *Bar graphs* showing the RAM performance at the fourth week of trial (5 trials/week) at 7 months of age after 4 months of treatment (5 days/week): Non-Tg ($n = 10$), 5XFAD ($n = 11$) and 5XFAD+2OHDHA ($n = 12$). **a** Time performance, **b** total number of errors, **c** number of working memory errors (WME, re-entrance in visited arms) and **c** number of reference memory errors (RME, entrance in un-baited arms). *Bars* represent mean ± SEM. One-way ANOVA followed by Bonferroni post hoc test. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; difference from Non-Tg group; #: $p \leq 0.05$ difference from 5XFAD group

time performances ($F_{2,32} = 4.056, p = 0.0276$) and the total number of errors ($F_{2,32} = 5.771$, $p = 0.0076$; Fig. 2a, b).

Chronic 2OHDHA treatment showed an improvement in the time performance since 5XFAD treated animals complete the RAM test 15 % faster than non-treated 5XFAD animals (105.4 ± 10.34 vs. 124.4 ± 12.38 s respectively) even if this difference was not significant. Nevertheless, and more importantly, the total number of errors was massively reduced. 5XFAD+2OHDHA animals showed a significant decrease in total number of errors (Fig. 2b) by more than 30 % compared to the 5XFAD animals (8.287 ± 1.086 vs. 12.27 ± 1.048 respectively; $p < 0.05$) reaching similar levels to those seen in Non-Tg control animals.

When separately considered WM and RM, presented as WME and RME, the untreated 5XFAD animals showed a significant impairment, which was completely reverted after the chronic treatment with 2OHDHA. However, there was a more relevant improvement in WME than in RME, as revealed by the significant genotype/treatment effect ($F_{2,32} = 5.685$, $p = 0.0081$; $F_{2,32} = 4.591$, $p = 0.0182$ respectively).

5XFAD animals showed a significant increase in the number of WME (Fig. 2c) being higher than 70 % compared to Non-Tg control animals (3.955 ± 0.4770 vs. 2.320 ± 0.3200 respectively; $p < 0.05$). The number of RME (Fig. 2d) was also higher, with an increase of 40 % compared to Non-Tg controls (8.318 ± 0.6428 vs. 5.880 ± 0.3901 respectively; $p < 0.05$).

When considering 5XFAD+2OHDHA animals, they showed a significant decrease in both kinds of errors, being of 45 % in WME (Fig. 2c), compared to 5XFAD animals (2.217 ± 0.4011 vs. 3.955 ± 0.4770 respectively; $p < 0.05$), reaching normal Non-Tg values. 5XFAD+2OHDHA animals also showed a significant decrease in the number of RME (Fig. 2d), around 30 % compared to 5XFAD animals (6.071 ± 0.7387 vs. 8.318 ± 0.6428 respectively; $p < 0.05$) returning to the same level of errors seen in Non-Tg control animals. These results suggest that the memory recovery after the chronic treatment with 2OHDHA can modify both WM and RM, although there is a more important recovery in WM, as revealed by a further decrease of 15 %.

2OHDHA effects on cell proliferation in the dentate gyrus

In both, Non-Tg controls and 5XFAD animals, there was a consistent and detectable number of newly generated

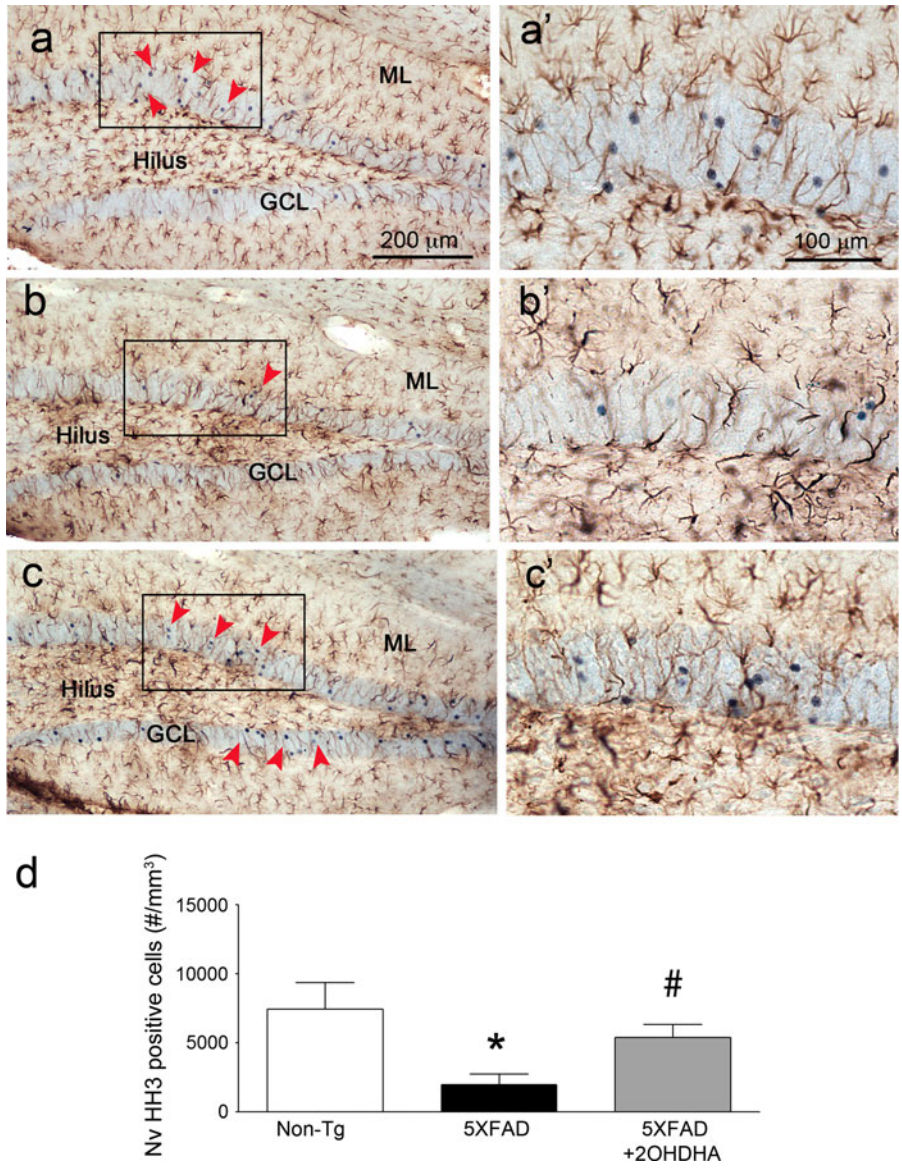

Fig. 3 Neurogenesis in the DG. Brighfield micrographs illustrating the DG cell proliferation (HH3, *blue*) in (**a**–**a**') Non-Tg, (**b**–**b**') 5XFAD and (**c**–**c**') 5XFAD+2OHDHA animals; appearing after 2OHDHA, a clear recovery in the rate of neurogenesis compared to 5XFAD animals and returning to Non-Tg control rates. The proliferative cells rarely co-localize with GFAP positive glial cells (*brown*, <2 %). (d) Bar graph illustrating HH3 Nv (#/mm^3) in the DG of the different experimental groups. Bars represent mean ± SEM, Non-Tg ($n = 4$) 5XFAD ($n = 7$), 5XFAD+2OHDHA ($n = 7$). One-way ANOVA followed by Unpaired *T* test. *$p \leq 0.05$; difference from Non-Tg group; #: $p \leq 0.05$ difference from 5XFAD group. *ML* molecular layer, *GCL* granular cell layer. (Color figure online)

cells as revealed by HH3-labeling. These newly formed cells showed typical morphology of newborn cells such as irregular shape and small size being occasionally grouped in clusters as described previously in normal animals as well in the 3xTg-AD mouse model of AD (Abrous et al. 2005; Rodríguez et al. 2008, 2011; Mu and Gage 2011). Surprisingly in the 5XFAD animals and their Non-Tg littermates, hippocampal cell proliferation was not restricted to the basal layer of the GCL of the DG but also in apical layers.

The quantitative analysis of the cell proliferation (Fig. 3) showed a significant genotype/treatment effect ($F_{2,17} = 5{,}981$, $p = 0.0123$) with a significant reduction in the Nv of HH3-IR cells in the 5XFAD animals, being less than 70 % compared to Non-Tg (1,970 ± 767.1 vs. 7,431 ± 1,918 respectively; t = 3.150, $p = 0.0117$), revealing a substantial decrease in the cell proliferation rate in the 5XFAD transgenic animal model of AD (Fig. 3d).

This major decrease in the Nv of HH3 cells observed in 5XFAD animals was restored by the chronic treatment with 2OHDHA with an increase of 270 % when compared to untreated 5XFAD animals (5,374 ± 939.7 vs. 1,970 ± 767.1 respectively; t = 2.806, $p = 0.0159$)

(Fig. 3d). HH3-IR cells in the 5XFAD+2OHDHA animals were reaching values closer to those seen in Non-Tg controls. Indeed, no statistical significant differences were found between Non-Tg and 5XFAD+2OHDHA mice (7,431 ± 1,918 vs. 5,374 ± 939.7 respectively; t = 1.092; $p = 0.3031$).

It is also remarkable that after the chronic treatment with 2OHDHA all newly generated cells appeared in both suprapyramidal and infrapyramidal blades of GCL of the DG, whilst in Non-Tg animals the majority of the newly generated cells were localized in the suprapyramidal blade of the GCL (Fig. 3a–c). When considering the phenotype of proliferating cells in the GCL of the DG we found that HH3-IR cells were rarely co-localized with GFAP. In fact, less than 2 % of HH3-positive cells showed glial phenotype, suggesting that the proliferation cells are of neural lineage as previously reports in the 3xTg-AD model (Rodríguez et al. 2008, 2009, 2011).

2OHDHA and Aβ accumulation in the GCL of the dentate gyrus

As expected, in Non-Tg control animals there was no Aβ accumulation in any of the hippocampal subfields including the DG (Fig. 4a). In contrast, in 5XFAD a massive presence of Aβ was detected throughout the whole hippocampus (Fig. 4b): including the subiculum, CA1 and CA3 as well as the layers of the DG including both upper and lower blade of de ML, together with the hilus and the GCL The Aβ load was not changed after the chronic treatment with 2OHDHA (Fig. 4c) and the GFAP-IR distribution remained the same in all the groups.

Since previous results showed that hippocampal HH3-IR is located in the DG, we also assessed and analyzed the Aβ load in this hippocampal region. No significant changes were observed in the DG of the hippocampus in the 5XFAD+2OHDHA animals when compared to the untreated 5XFAD animals neither in the Nv of Aβ plaques (3,560 ± 534.5 vs. 2,659 ± 303.3 respectively; t = 1.361 $p = 0.2157$) nor in the area size of the plaques (0.0007856 ± 7,462e−005 vs. 0.0008818 ± 9,136e−005 respectively; t = 0.8250, $p = 0.4366$). In both groups the total area of the DG occupied by Aβ plaques was similar comprising 9–11 % of the total area. The analysis of the Aβ deposit specifically in the GCL of the DG did not reveal significant changes. When the 5XFAD+2OHDHA animals where compared to the

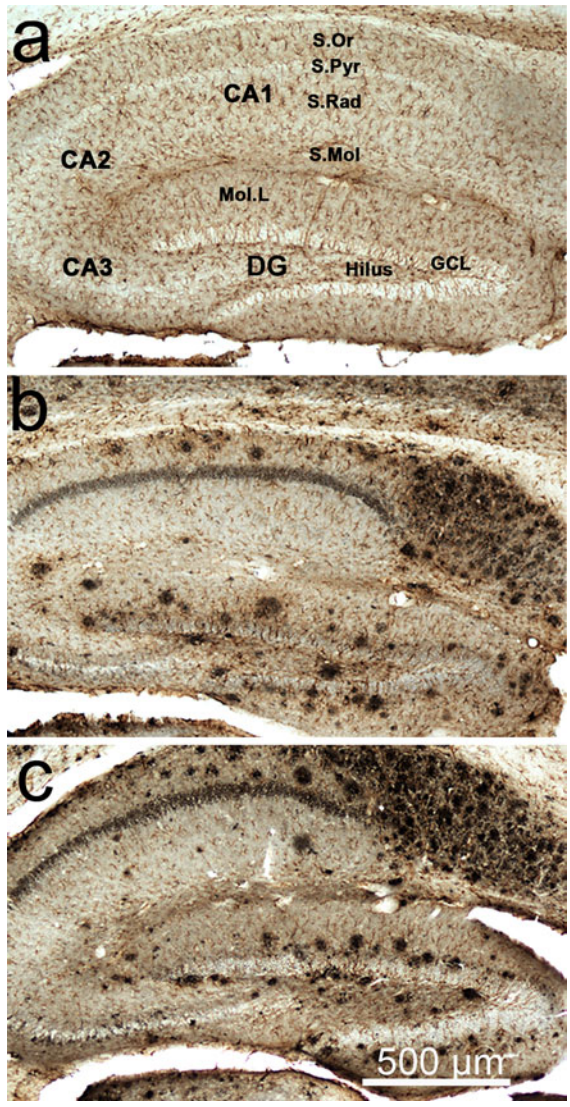

Fig. 4 Aβ load in the DG. Brightfield micrographs illustrating Aβ plaques accumulation (*blue*) and GFAP positive glial cells (*brown*) in the DG of the hippocampus of **a** Non-Tg, **b** 5XFAD and **c** 5XFAD+2OHDHA animals. In 5XFAD animals there was a massive accumulation of Aβ plaques which is not changed by the treatment (see Results). *DG* dentate gyrus, *GCL* granule cell layer, *Mol.L* molecular layer, *S.Mol* stratum lacunosum molecular, *S.Or* stratum oriens, *S.Pyr* stratum pyramidale, *S.Rad* stratum radiatum. (Color figure online)

5XFAD no changes in the Nv of Aβ plaques (2,049 ± 317.7 vs. 1,453 ± 286.5 respectively; t = 1.356, $p = 0.2172$) or in the area size of the plaques (0.0009025 ± 8.820e−005 vs. 0.0008469 ± 6.194e−005 respectively; t = 2.434, $p = 0.0591$) were detected. Again, the total area of the GCL occupied by Aβ plaques

was similar in both groups, comprising a 6–7 % of the total area. Similarly, there were no differences in the intracellular Aβ-IR in the GCL in 5XFAD+2OHDHA animals compared to 5XFAD.

Discussion

The modification of naturally active molecules, such as lipids, represents a new tool in the development of medicaments (Escribá 2006). In the past we have demonstrated that the 2-hydroxylation of a natural-occurring FA as oleic acid produced a new molecule with antitumoral activity (Lladó et al. 2009) by interfering with the sphingolipid synthesis pathway by activating the sphingomyelin synthase (Barceló-Coblijn et al. 2011).

In the present study, we used 5XFAD transgenic mice to investigate the effects of 2OHDHA chronic treatment on memory performance and neurogenesis in AD, as well as their relation to the Aβ accumulation in the GCL of the hippocampus. We found a significant memory impairment in the 5XFAD mouse model in both WM and RM by the RAM test together with a decrease in hippocampal cell proliferation at 7 months of age, when there is a massive accumulation of Aβ. Chronic treatment with 2OHDHA resulted in recovery of the spatial memory impairment, with a major improvement in WM. This improvement in spatial memory was concomitant with a significant recovery in the rate of hippocampal cell proliferation.

The observed impairment in memory in the 5XFAD mouse model of AD is in agreement with previous behavioral studies in this and other AD transgenic models, for example APP single transgenic, PS1xAPP double transgenic and 3xTg-AD (Ashe 2001; Oddo et al. 2003). In addition, we have described impairments in both RM (reflecting hippocampal function) and WM (reflecting both hippocampal and prefrontal cortex functions). Previous studies had already described a decline in various types of hippocampus-dependent memory in the 5XFAD mouse model including spatial memory in the Morris water maze and temporal memory in the auditory trace fear conditioning (Ohno et al. 2006) as well as contextual fear memory (Ohno 2009). Importantly, these memory impairments start to occur at 6 months of age in accordance with the massive increase of Aβ levels and amyloid burden, which, in the 5XFAD model, appear between 4 and 6 months of age (Oakley et al. 2006). This reported memory impairment is obviously linked with the onset of hippocampal synaptic dysfunction such as reduced levels of baseline transmission and deficient LTP in 5XFAD mice (Kimura and Ohno 2009).

Similarly to other findings (Haughey et al. 2002; Wen et al. 2002; Dong et al. 2004), which demonstrated impaired neurogenesis in transgenic mice having mutant forms of APP and/or presenilin-1 and our recent results in the 3xTg-AD mouse model of AD (Rodríguez et al. 2008, 2009, 2011); we have detected a significant decrease of cell proliferation in the 5XFAD animals at 7 months of age. The impaired cell proliferation observed in 5XFAD animals could be due to a reduced level of neurotrophic factors, like the brain-derived neurotrophic factor (BDNF), that starts early in the course of human AD (Peng et al. 2005; Devi and Ohno 2012) and observed in the 5XFAD animals from 3 months of age (Devi and Ohno 2012).

Chronic treatment with 2OHDHA (or DHA) up-regulate cell proliferation to normal values observed in the healthy brain. Concievably, this increased hippocampal cell proliferation could enhance the hippocampal network promoting the long-term potentiation (LTP, that is known to be affected in AD-Yamin 2009) thus improving in the RM. However, we have showed that the chronic treatment with 2OHDHA results in a larger improvement of WM than RM. In this sense, the recovery of the hippocampal cellular network can have an indirect effect on the WM; due to the dense projection from the hippocampus to the prefrontal cortex with strong excitatory action (Vertes et al. 2007). However, not only the prefrontal cortex but also the hippocampus subserve spatial WM in rodents (Gordon 2011) indicating that the improvement seen in the spatial WM could be a concomitant effect due to the recovery of local and hippocampus-cortex connection. Similar improvement in RM in young rats and an improvement in both WM and RM in aged rats after chronic treatments with DHA have been described previously (Gamoh et al. 2001, 1999).

The 2OHDHA being a direct derivative of DHA may act through the same pathways than DHA. In this sense, it has been shown that the n-3 deficiency reduce the levels of BDNF and signaling through the BDNF receptor TrkB proportionally to brain DHA levels (Bhatia et al. 2011) and that the n-3 PUFA supplementation enhance BDNF expression (Katsuki et al.

2009) correlating with an increase of neurogenesis in mouse hippocampus (Blondeau et al. 2009). Moreover, it has been showed that BDNF promotes LTP improvement (Pang and Lu 2004; Lu et al. 2008) setting a possible connection between DHA, BDNF and LTP. In addition, the 2OHDHA being a hydroxylated derivative of DHA, as well as NPD1, may possibly demonstrate a neuroprotective (Zhao et al. 2011) and antiapoptotic capacity (Calandria et al. 2012), enhancing the maintenance of the hippocampus, not only as an inducer of cell proliferation, but also protecting existing neurons.

Although there is some evidence indicating that DHA directs the amyloidogenic processing of APP towards the non-amyloidogenic pathway, with a consequent reduction of Aβ (Grimm et al. 2011), we failed to detect changes in the Aβ load neither in the whole DG nor in the GCL of the hippocampus after the chronic treatment with 2OHDHA. Besides not being exactly the same molecule, this difference may be due to different factors such as the dose and the treatment length, as well as the animal model of AD used. In this case, the 5XFAD is a hyperamyloidosis model that rapidly accumulates high levels of Aβ, in both plaques and intracellularly (Oakley et al. 2006), which could mask small changes in the total pool of Aβ. Nevertheless, we have reported a recovery of memory and enhanced cell proliferation in the 5XFAD+2OHDHA animals despite of absence of changes in Aβ, suggesting that cell proliferation is not directly affected by Aβ plaques in the DG of the hippocampus.

Clinical perspectives

Although evidence from this and other studies suggest that omega 3-fatty acids may protect from cognitive decline, clinical trials carried out with DHA showed no solid evidence on cognitive function benefits (Quinn et al. 2010; Sydenham et al. 2012). The cognitive recovery after treatment with 2OHDHA in 5XFAD mice and in human APP/Tau transgenic *Drosophila* models is greater than the obtained with DHA (manuscript in preparation). This opens the possibility for positive results in future clinical studies using 2OHDHA.

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