UNIVERSITAT DE BARCELONA

Efecto del síndrome metabólico provocado por una dieta rica en grasa en ratones APPswe/PS1dE9, modelo experimental de la enfermedad de Alzheimer, y posibles terapias farmacológicas

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RESULTADOS

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PUBLICACIÓN I

Evaluation of Neuropathological Effects of a High-Fat Diet in a Presymptomatic Alzheimer's Disease Stage in APP/PS1 Mice

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Resumen

La EA se define actualmente como un desorden neurodegerativo relacionado con la edad e incurable. Estudios recientes han apoyado la hipótesis que defiende que la EA debería ser considerada como una enfermedad metabólica.

El objetivo del presente estudio es explorar la relación entre la formación neuropatológica de placas βA en el hipocampo y la obesidad en un estadio presintomático temprano de la enfermedad (a 3 meses de edad).

Para ello, se han utilizado los ratones trangénicos APP/PS1, alimentados con dieta grasa con el propósito de investigar los posibles mecanismos moleculares involucrados en ambas patologías. Los resultados mostraron que los hipocampos de los ratones APP/PS1 alimentados con dieta grasa presentaban una disminución significativa de la vía de señalización βA, especialmente de los niveles proteicos de la EDI y la α-secretasa respecto a los alimentados con

dieta normal. Estos cambios fueron acompañados de un significativo incremento en la formación de placas en el hipocampo de estos ratones. Asimismo, se observó una disminución significativa de los niveles de PGC1α (*Peroxisome proliferator activated receptor gamma coactivator 1 α)*, cofactor involucrado en la biogénesis mitocondrial en los ratones alimentados con dieta grasa. Sin embargo, la dieta no provocó cambios ni en la expresión génica de los receptores de la insulina ni en enzimas implicadas en esta vía. Además, no se observaron cambios en cinasas involucradas en la fosforilación de TAU, tales como CDK5 (Cinasa dependiente de ciclina 5), ni tampoco en la producción de estrés oxidativo en el cerebro.

Los resultados obtenidos sugieren que los cambios tempranos en el cerebro de los ratones APP/PS1 alimentados con dieta en grasa se producen mediante el incremento del $A\beta_{1\text{-}42,}$ el cual induce una disminución de los niveles proteicos de PKA (proteína cinasa A), además de alteraciones en la vía de p-CREB (elemento de unión en respuesta al AMPc)/NMDAR2B/PGC-1α, favoreciendo la temprana neuropatología de la EA en ratones.

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Evaluation of Neuropathological Effects of a High-Fat Diet in a Presymptomatic Alzheimer's Disease Stage in APP/PS1 Mice

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Abstract. Alzheimer's disease (AD) is currently an incurable aging-related neurodegenerative disorder. Recent studies give support to the hypotheses that AD should be considered as a metabolic disease. The present study aimed to explore the relationship between hippocampal neuropathological amyloid-β (Aβ) plaque formation and obesity at an early presymptomatic disease stage (3 months of age). For this purpose, we used APPswe/PS1dE9 (APP/PS1) transgenic mice, fed with a high-fat diet (HFD) in order to investigate the potential molecular mechanisms involved in both disorders. The results showed that the hippocampus from APP/PS1 mice fed with a HFD had an early significant decrease in Aβ signaling pathway specifically in the insulin degrading enzyme protein levels, an enzyme involved in (Aβ) metabolism, and α-secretase. These changes were accompanied by a significant increase in the occurrence of plaques in the hippocampus of these mice. Furthermore, APP/PS1 mice showed a significant hippocampal decrease in PGC-1α levels, a cofactor involved in mitochondrial biogenesis. However, HFD does not provoke changes in neither insulin receptors gene expression nor enzymes involved in the signaling pathway. Moreover, there are no changes in any enzymes (kinases) involved in tau phosphorylation, such as CDK5, and neither in brain oxidative stress production. These results suggest that early changes in brains of APP/PS1 mice fed with a HFD are mediated by an increase in $A\beta_{1-42}$, which induces a decrease in PKA levels and alterations in the p-CREB/ NMDA2B /PGC1-α pathway, favoring early AD neuropathology in mice.

Keywords: Alzheimer's disease, APPSwe/PS1dE9, hippocampus, insulin receptor, mitochondria, tau

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INTRODUCTION

After over a hundred years of intense research into Alzheimer's disease (AD), the causes of this pathology's development are yet to be elucidated [1–5]. What is obvious is that the pathogenesis of AD is a complex and long-lived process [3–5]. In addition, the current drugs for AD therapy only provide a temporary improvement on cognition [5]. Due to the lack of an effective treatment to halt the progression of AD, it is necessary to investigate the potential pathways involved in this disease onset. Over the last 25 years, different hypotheses have been formulated to explain the causes of the onset of this disease. What seems to be clear is that aging is probably the main key factor in the development of late sporadic AD. In addition to aging, in the early 1990 s John Hardy proposed the "amyloid cascade" hypothesis [6–8]. This hypothesis states that depositions of the amyloid-β peptide (Aβ) produce senile plaques, that lead to the formation of neurofibrillary tangles, which in turn cause neuronal death that lead to a cognitive decline [6–10]. In the same time period, the "oxidative stress" hypothesis emerged and several research groups demonstrated the prominent role of oxidative stress in the brain of AD in the brain of the affected patients [11–13]. Likewise, Swerdlow and Khan, proposed the so-called "mitochondrial cascade" hypothesis, suggesting that mitochondria damage occurs prior to brain Aβ accumulation [14–17].

More recently, research in AD has established a clear relationship between obesity, insulin resistance, diabetes, and dementia [18–24]. In addition, Balakrishnan and colleagues demonstrated that obesity increases the plasma levels of $A\beta_{1\text{-}42}$ [20]. Therefore, the control of body weight could be an additional factor for the development of the late sporadic form of AD. Interestingly, the term "Brain Insulin Resistance", which is defined as reduced tissue responsiveness to the action of insulin, could probably fit better as a potential cause of sporadic AD [25–41]. An anomaly in the insulin response pathway could affect multiple brain cellular mechanisms such as mitochondria, Aβ metabolism, Aβ production, neuroinflammation, tau phosphorylation, and also learning and memory capacities [33–44]. Thus, some researchers denominate AD as type III diabetes [22]. Although many preclinical and clinical studies point out to this close relationship between AD and insulin resistance, few preclinical studies have evaluated it in an early stage of the disease known as presymptomatic stage.

The majority of AD preclinical research is carried out using transgenic animal models that have increased Aβ levels compared to wild-type (WT) mice. While the Aβ pathology is mimicked in these models, many other factors associated with the AD pathology are not. The APP/PS1 double transgenic mouse is a genetically modified model that has been generated to try to mimic human familial AD pathology. In the APP/PS1 line, two strategies are combined to reach elevated Aβ levels: overexpression of the mutant human amyloid precursor protein (APP) encoding gene, together with the mutant presenilin-1 (PS1) gene, which additionally impairs amyloid protein processing, leading to elevated $A\beta_{42}$ levels [45–47]. The APP/PS1 mouse is a good model to study the early onset of pathological changes, while the Tg2576, APP23, and 3xtg strains are approximate better models for the study of a late onset form of the disease (reviewed by Bilkei-Gorzo [47]). Although none of these models show, in general, brain neuronal loss from cortex and hippocampus, the APP/PS1 strain shows amyloid plaque formation along with tau protein hyperphosphorylation. Furthermore, it has been demonstrated that APP/PS1 mice show early memory loss that becomes evident at the age of 6 months [39–42].

The aims of the present study were to evaluate the molecular pathways involved in the AD pathology process in APP/PS1 mice fed with a high-fat diet (HFD) [32–37, 43, 44]. We focus on the evaluation of HFD, together with $A\beta_{1\text{-}42}$ peptide, are cofactors that trigger the AD pathology in a presymptomatic stage of the disease, at three months of age. Moreover, we tried to detect very early biochemical and molecular neuropathology clues that may allow a better understanding of a link between AD and obesity.

MATERIALS AND METHODS

Animals

APPswe/PS1dE9 (APP/PS1) and C57BL/6 (WT) male mice were used in this study. APP/PS1 animals co-express a Swedish (K594M/N595L) mutation of a chimeric mouse/human APP (Mo/HuAPP695swe), together with the human exon-9-deleted variant of PS1 (PS1-dE9), allowing these mice to secrete elevated amounts of human Aβ peptide. The generation of mice expressing the human mutated forms APPswe and PS1dE9 has already been described [40–43]. Identification of transgenic mice was carried out from tail clips, using the polymerase chain reaction (PCR)

Table 1
Description of the caloric content of the standard diet versus high fat diet

	standard diet	high fat diet
	Kcal %	Kcal %
Protein	24.0	16.4
Carbohydrate	58.0	38.6
Fat	18.0	45.0
Total	100.0	100.0

technique with the PCR conditions proposed by the Jackson Laboratory.

Sixty animals were used, divided into four groups: a) WT mice fed with a standard (CT) diet; b) WT mice fed with a high fat diet (HFD), consisting of 25% fat (45 kcal %), mainly from hydrogenated coconut oil, 21% protein (16 kcal %), and 49% carbohydrate (39 kcal %); Cat# D08061110 (Research Diets Inc., New Brunswick, USA); c) APP/PS1 mice fed with a CT diet and d) APP/PS1 mice fed with a HFD (Fig. 1; Table 1). Following *in vivo* testing, 3-month-old animals were sacrificed and at least 6 mice of each group were used for RNA and protein extract isolation, with an additional 4 mice used for immunofluorescence. The animals were kept under controlled temperature, humidity and light conditions with food and water provided *ad libitum*. Mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the *Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya*. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Novel Object Recognition test

The Novel Object Recognition test was used for testing the hippocampal-dependent recognition memory of mice. The task procedure consisted of three phases: habituation, familiarization and test phase. In habituation phase, mice explored individually a circular open-field arena of 40 cm of diameter without object for three consecutive days, 10 min for each session. On the fourth day (familiarization phase), each mouse was placed in the arena containing two identical objects (A+A) in the middle of the field for 10 min. To perform the test phase, mice were returned 24 h later to open-field arena with two objects, one was identical to the day before and the other was a novel object (A+B) for 10 min. The light intensity in the middle of the field was 30 Ix in all phases and the arena and objects were cleaned with 96% ethanol between animals, so as to eliminate olfactory cues. Exploration was defined as the orientation of snout of the animals toward the object, sniffing or touching [49]. The data were measured by discrimination index (DI), which indicates the difference in exploration time between familiar and novel object. Therefore, the exploration time of each object was divided by the total time of exploration, measured in seconds and indicated in percentage [43, 44].

Serum insulin ELISA

Heart puncture was used to collect whole blood samples from 3-month-old WT and APP/PS1 mice at the moment of death after a 5-h morning fast. Blood samples were transferred to Serum-Gel Z microcentrifuge tubes (Sarstedt, Numbrecht, Germany), for serum separation. The samples were collected and kept at room temperature, and the serum was separated by centrifugation for 10 min at 5000× g. Serum insulin levels were measured with Rat/Mouse Insulin ELISA kits (Cat #: EZRMI-13K; EMD Millipore; St. Charles, MO, USA), according to the manufacturer's instructions, utilizing 10 μl of mouse serum.

Total blood cholesterol and triglycerides measurements

Total cholesterol and triglyceride blood levels were measured following 4-h-long fast at the point of sacrifice with Accutrend Plus meter (Roche Diagnostics, Switzerland).

Glucose and insulin tolerance tests

Intraperitoneal glucose tolerance tests (IP-GTT) were performed in accordance with the previously published guidelines [50]. For IP-GTT, mice were fasted overnight for 16 h. The test was performed in a quiet room, preheated to +30°C. The tip of the tail was cut with the heparin-soaked (Heparina Rovi, 5000 IU/ml; Rovi S.A.; Madrid, Spain) scissors, 30 min prior to intraperitoneal glucose injection. Blood glucose levels in the tail vein were measured at –30, 0, 5, 15, 30, 60, and 120 min after the glucose injection with the Ascensia ELITE blood glucose meter (Bayer Diagnostics Europe Ltd.; Dublin, Ireland).

Immunofluorescence staining

Mice used for immunofluorescence studies were anesthetized by intraperitoneal injection of sodium

pentobarbital (80 mg/kg) and perfused with 4% paraformaldehyde (PFA) diluted in 0.1M phosphate buffer (PB). Brains were removed and stored in the same solution overnight (O/N) at 4°C, and then they were cryoprotected in 30% sucrose-PFA-PB solution. Samples were frozen at –80°C and coronal sections of 20 μm of thickness were obtained by a cryostat (Leica Microsystems, Wetzlar, Germany).

Free-floating sections were first washed three times with 0.1 mol/l PBS pH 7.35 and after five times with PBST (PBS 0.1 M, 0.2% Triton X-100). Then, they were incubated in a blocking solution containing 10% fetal bovine serum (FBS), 1% Triton X-100, and PBS 0.1 M- 0.2% gelatin for 2 h at room temperature. After that, slices were washed with PBST (PBS 0.1 M, 0.5% Triton X-100) five times for 5 min each and incubated with polyclonal rabbit anti-GFAP (1:1000; Dako, Glostrup, Denmark), rabbit anti-IBA1 (1:1000; Wako Chemical USA) and monoclonal anti-βA 1-42 (12F4, which detects the c-terminus of βA) (1:1000; Covance, USA) primary antibodies at 4°C O/N. Sequentially, sections were washed with PBST (PBS 0.1 M, 0.5% Triton X-100) 5 times for 5 min and incubated with Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 donkey anti-rabbit antibodies (1:500; Invitrogen, Eugene, OR, USA) for 2 h at room temperature. The staining for Aβ plaques was performed using S-Thioflavin (ThS 0.002%, Sigma-Aldrich). Slices were incubated for 8 min in the dark at room temperature, washed with 50% ethanol twice for 1 min, and rinsed with PBS 0.1 M. Later, sections were co-stained with 0.1 μg/ml Hoechst 33258 (Sigma-Aldrich, St Louis, MO, USA) for 15 min in the dark at room temperature and washed with PBS 0.1M. Finally, the slides were mounted using Fluoromount G (EMS), and image acquisition was performed with an epifluorescence microscope fluorescence filter (BX41 Laboratory Microscope, Melville, NY-Olympus America Inc.). For plaque quantification, similar and comparable histological areas were selected, particularly with the hippocampus and the whole cortical area positioned adjacently.

Immunoblot analysis

To perform western blot analysis, first hippocampi were dissected, frozen, and stored at –80°C until use. After, samples were homogenized in lysis buffer (50 mM Tris-HCl 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) and protease inhibitor mixture (Complete, Roche Diagnostics, Barcelona, Spain). The homogenates were centrifuged at 12,000 g for 5 min at 4°C, and the protein quantity of the supernatant was determined using a Pierce BCA Protein Assay Kit (Pierce Company, Rockford, MI, USA). Sequentially, 10 μg of protein per sample were analyzed using the western blot method. For that, they were denatured at 95°C for 5 min in sample buffer (0.5M Tris–HCl, pH 6.8, 10% glycerol, 2%(w/v) SDS, 5%(v/v) 2-mercaptoethanol, 0.05% bromophenol blue). Samples were separated by electrophoresis on 10% acrylamide gels (100 V cte) and they were transferred to polyvinylidene difluoride (PVDF) sheets (Immobilon-P; Millipore Corp., Bedford, MA, USA) (200 mA cte). Then, membranes were blocked for 1 h with 5% non-fat milk dissolved in TBS-T buffer (mM Tris; 1.5% NaCl, 0.05% Tween 20, pH 7.5), washed with TBS-T without containing milk 3 times for 5 min and incubated with primary antibodies O/N at 4°C, as detailed in Table 2. Subsequently, blots were washed thoroughly in TBS-T buffer and incubated at room temperature for 1 h with a horseradish peroxidase-conjugated IgG secondary antibody (Table 2), followed by enhanced chemiluminescence detection (Immobilon Western, Chemiluminescent HRP Substrate, Millipore) according to the supplier's instructions. Protein levels were determined using Chemidoc XRS+ Molecular Imager detection system (Bio-Rad), with ImageLab image analysis software. Measurements were expressed as arbitrary units and all results were normalized to the corresponding GAPDH.

RNA extraction and quantification

The hippocampi of mice were homogenized in Trizol reagent (Life Technologies Corporation) for RNA isolation, as described previously [48]. Sequentially, chloroform was added and RNA was precipitated from the aqueous phase with isopropanol at 4°C. The RNA pellet was reconstituted in RNAse-free water, with the RNA integrity determined by Agilent 2100 Bioanalyzer.

Semi-quantitative Real-time-PCR

First-strand cDNA was reverse transcribed from 2 μg of total RNA using the High Capacity cDNA Reverse Transcription kit, according to manufacturer's protocol (Applied Biosystems). Equivalent amounts of cDNA were used for qRT-PCR and each sample was analyzed in triplicate for each gene. TaqMan gene expression essays (Applied Biosystems) were used, as detailed in Table 3, and they were

Table 2
A list of antibodies used for immunoblotting and immunofluorescence

Protein	Antibody	Dilution
ADAM 10	Ab 39117 (Abcam)	1/1000
AKT	#9272 (Cell Signaling)	1/1000
p-AKT (S473)	#4060 (Cell signaling)	1/1000
APP	SIG-39152 (Covance)	1/500
BACE	#5606 (Cell signaling)	1/1000
CDK5	Sc 173 (Santa Cruz)	1/1000
p-CDK5 (Y15)	Ab63550 (Abcam)	1/500
CREB	#9197 (Cell signaling)	1/1000
pCREB (S133)	#9198 (Cell signaling)	1/1000
ERK 1/2	#9102 (Cell signaling)	1/1000
p-ERK1/2 (T202/T204)	#9101 (Cell signaling)	1/1000
GAPDH	MAB374 (Millipore)	1/2000
GSK3β	#9315 (Cell signaling)	1/1000
p-GSK3β (S9)	#9336 (Cell signaling)	1/1000
IDE	Ab 32216 (Abcam)	1/1000
NMDA R1	MAB1586 (Millipore)	1/1000
NMDA R2B	Ab 1557P (Millipore)	1/1000
pNMDA R2B (T1472)	#4208 (Cell signaling)	1/1000
NRF1	Sc 28379 (Santa Cruz)	1/1000
PGC 1α	Sc 13067 (Santa Cruz)	1/1000
PKA	Sc 28315 (Santa Cruz)	1/1000
PPARα	Ab8934 (Abcam)	1/1000
Tau	AHB0042 (Biosource)	1/1000
pTau (S199)	44734G (Life Technologies)	1/1000
pTau (S396)	44752G (Life Technologies)	1/1000
pTau (S404)	44748G (Life Technologies)	1/1000
pTau (T205)	44738G (Life Technologies)	1/1000
TFAM	DR1071b (Calbiochem)	1/1000

Table 3
A list of TaqMan probes used for Real-Time PCR analysis

Gene	TaqMan probe
App	Mm01344172_m1
Arc	Mm00479619_g1
Bdnf	Mm04230607_s1
Fos	Mm00487425_m1
Ins1	Mm01950294_s1
Insr	Mm01211875_m1
Irs1	Mm01278327_m1
Irs2	Mm03038438_m1
Igf1	Mm01228180_m1
Igf2	Mm00439564_m1
Igf1r	Mm00802831_m1
Igfbp2	Mm00492632_m1
Igfbp3	Mm01187817_m1
Gapdh	Mm99999915_g1

performed on StepOnePlus Real Time PCR system (Applied Biosystems). The values were normalized to gapdh and tbp using the delta-delta Ct method.

Antioxidant assays

One fraction of the cortex samples was homogenized manually by means of a Teflon bar after the addition of Radio Immune Precipitation Assay buffer (RIPA) containing (in mM) 50 TrisHCl, 150 NaCl, 5 NaF and 0.1% of sodium dodecyl sulphate (SDS), 1% Triton X-100, 1% Sodium deoxycholate (final pH 7.4). A cocktail of protein-inhibitors was mixed with RIPA buffer before homogenation (Leupeptin 2 ug/mL, Pepstatine, 2 ug/mL, PMSF 1 mM, NaVOn 1 mM, Aprotinin, 1.7 mg/mL). The homogenates were kept on ice for 15 min and then centrifuged at 2,000 g for 10 min and the precipitated fraction was discarded. Supernatants of cortical extracts were aliquoted and stored at –80°C for TBARS determination. Malondialdehyde (MDA) (which is an end-product of lipid peroxidation), was measured by the thiobarbituric reactive substances (TBARs) assay following the Uchiyama and Mihara method [51]. The formation of MDA-TBA adducts was fluorometrically quantified at an excitation wavelength of 515 nm and an emission wavelength of 550 nm. The calibration curve was determined using tetraethoxypropane. Values were expressed in nmol/mg protein.

The total brain proteins were determined using the Bradford protein assay [52].

Other fractions of the cortex samples were homogenized in cold phosphate buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.5) and centrifuged at 10,000 g for 10 min (4°C). The resulting supernatant was aliquoted and stored at –80°C for posterior determination of advanced oxidation protein products (AOPP) and antioxidant enzymatic activities.

AOPP content in cortex homogenates was assayed by a modification of Witko–Sarsat's method [53, 54]. The formation of AOPP was spectrophotometrically measured at 340 nm and results were obtained through a standard calibration curve using 100 μL of chloramine-T solution. AOPP concentration was expressed as μmol of chloramine-T equivalents per mg of protein.

Cellular levels of oxidant molecules are controlled by enzymatic and non-enzymatic antioxidants. The major antioxidant enzyme is superoxide dismutase (SOD), which plays a critical role in scavenging superoxide radical. In the same line, another antioxidant enzyme, glutathione reductase (GR) has the role of maintaining the reduced state of the main non-enzymatic antioxidant molecule, glutathione (GSH), by catalyzing the NADPH-dependent reduction from its oxidized form GSSG.

SOD (EC 1.15.1.1) activity was determined by using Arbor Assay Superoxide dismutase

colorimetric activity kit (Arbor Assay, Ann Arbor, MI, USA) by measuring the decrease in superoxide products (generated by xanthine oxidase system). Bovine SOD was provided by manufacturers, and, after adding increasing amounts of SOD, a sigmoideal standard curve was obtained. Enzyme's activity in samples was calculated by carrying out a four-parameter logistic curve (4PLC), fitting and values were expressed as U/mg protein.

GR (EC 1.6.4.2) activity was measured with Cayman Chemical Glutathione Reductase Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) by measuring the rate of NADPH oxidation. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Results of GR activity were expressed as mU/mg protein.

Measurement of Aβ peptides in brain tissues by ELISA

Soluble and insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ were measured in cortical extracts, according to a previously published procedure [44]. In brief, the samples were homogenized in an 8× volume of PBS with an AEBSF protease inhibitor cocktail set (Cat # 539131; Calbiochem; La Jolla, CA, USA). The soluble fraction was separated by centrifuging the samples for 10 min at 4000× g. In order to obtain the insoluble fraction, pellets containing insoluble Aβ peptides were solubilized in a 5 M guanidine HCl/50 mM Tris solution by incubating them for 3.5 h in an orbital shaker at room temperature. The levels of soluble and insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ were determined using the commercially available human ELISA kits (Cat # KHB3481 and KHB3441; Invitrogen, Camarillo, CA, USA). The data obtained from the cortical homogenates are expressed as picograms of Aβ content per milligrams of total protein (pg/mg).

Statistical analysis

All data are presented as means ± SEM, and differences are considered significant at $p < 0.05$. Differences between samples/animals were evaluated using Student's *t*-test, and 2-way ANOVA, with Tukey's *post hoc* test. Both the statistical analysis and the graphs presented here were created with the GraphPad InStat software V5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Evaluation of peripheral metabolic parameters in wild type and APPswe/PS1dE9 mice fed with a HFD

Dietary administration of control and HFD started at the time of weaning (21 days) until the age of 3 months. As expected, the intake of HFD produced a progressive obesity ($F_{(1,50)} = 120$, $p < 0.0001$). At the day of sacrifice, body weight reached a 124% increase in WT HFD versus WT CT ($p < 0.0001$), and a 139% increase in APP/PS1 fed HFD versus APP/PS1 CT group ($p < 0.0001$) (Fig. 2A, B).

Likewise, two-way ANOVA statistical test indicated a significant effect of the diet ($F_{(1,28)} = 5.681$, $p < 0.05$) and diet-strain interaction ($F_{(1,28)} = 5.681$, $p < 0.05$). HFD administration resulted in a significant increase in blood glucose levels in APP/PS1 mice fed with HFD versus APP/PS1 CT 104.6 versus 80.43 mg/dl ($p < 0.05$), but not in WT HFD versus WT CT (85 versus 85 mg/dl) (Fig. 2D). Although two-way ANOVA showed a significant effect of the diet ($F_{(1,33)} = 4.998$, $p < 0.05$) and strain ($F_{(1,33)} = 7.944$, $p < 0.01$), we found no detectable differences in glucose utilization by IGTT assay between 3-month-old APP/PS1 HFD versus WT HFD, however, we detected a significant increase in APP HFD versus WT CT (Fig. 2E). Moreover, diet-induced obesity increased plasmatic insulin levels ($F_{(1,15)} = 28.20$ $p < 0.0001$) with concentrations of 4.46 pM/ml in WT HFD (0.94 pM/ml in WT CT; $p < 0.05$) and 5.9 pM/ml in APP/PS1 HFD (1.01 in APP/PS1 CT; $p < 0.005$) (Fig. 2F).

We also analyzed triglyceride levels, and two-way ANOVA showed a significant effect of the diet ($F_{(1,16)} = 29.35$, $p < 0.0001$) but none of the strain ($F_{(1,15)} = 0.9289$, $p > 0.05$). Higher blood triglyceride concentrations were found in HFD fed mice, with 169.8 mg/dl in WT HFD (102.5 in WT CT; $p < 0.05$) and 199.8 mg/dl in APP/PS1 HFD (106 in APP/PS1 CT group; $p < 0.005$) (Fig. 2G).

Taken together, our data indicated a possible acceleration of an HFD-induced peripheral metabolic phenotype in APP/PS1 animals compared to control mice in a presymptomatic stage of experimental AD. Based on the evidence, APP/PS1 mice maintained on a standard laboratory diet present normal baseline glucose metabolism. We continued exploring the potential relationship between diet-induced obesity and the AD-type amyloidosis in the brain of APP/PS1 mice. Thus, in the following steps, we proceeded to

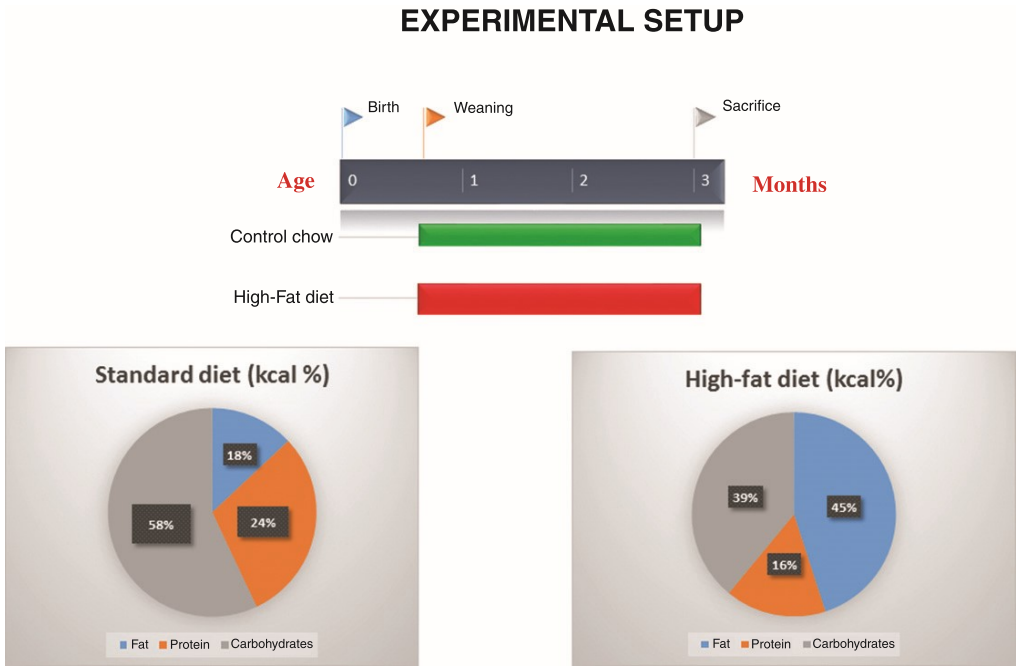

Fig. 1. Treatment schedule. Fifty animals were used, divided into 4 groups: WT mice fed with a standard (CT) diet; WT mice fed with a high fat diet (HFD) consisting of 25% fat (45 kcal %), mainly from hydrogenated coconut oil, 21% protein (16 kcal %), and 49% carbohydrate (39 kcal %); APP/PS1 mice fed with a CT diet and APP/PS1 mice fed with a HFD. Following in vivo testing, animals were sacrificed at 3 months of age.

study the effects of HFD on the brain and attempted to identify molecular pathways related to hippocampal metabolic signaling.

Evaluation of the effects of a high-fat diet on cognitive impairment in APPswe/PS1dE9 mice

We explored the possibility that HFD might accelerate cognitive impairment in 3-month-old WT and APP/PS1 mice which are not behaviorally impaired at this age [40–44]. Two-way ANOVA indicated a significant effect of the diet ($F_{(1,17)} = 11.15$, $p < 0.01$) and strain ($F_{(1,17)} = 7.849$, $p < 0.05$). Our data showed that APP/PS1 mice fed with HFD did not have an impaired capacity to learn. It was evaluated by a Novel Object Recognition test, comparing APP/PS1 HFD fed mice versus mice fed with CT diet. However, the intake of HFD significantly increased the memory loss compared with WT mice (Fig. 3A, $p < 0.05$). The total exploration time was analyzed using two-way ANOVA and did not show significant changes ($p > 0.05$)

Moreover, we studied changes in molecules directly involved in the early stages of memory consolidation processes. To this end, we analyzed the transcriptional activity of genes related to synaptic plasticity and memory, such as *Arc*, *Fos,* and *Bdnf*. They showed no significant alterations in APP/PS1 mice fed with HFD compared with APP/PS1 mice fed with CT diet (Fig. 3A, $p > 0.05$).

Furthermore, our western blot data demonstrated no differences in the protein levels of protein kinase A (PKA), in contrast to data obtained from the phosphorylation at serine 133 of cyclic AMP response element-binding protein (CREB). CREB protein levels showed a significant decrease in the hippocampus from APP/PS1 HFD fed mice compared with APP/PS1 fed with CT diet (Fig. 3B, $p < 0.05$).

Impaired NMDA receptor (NMDA-R) function may contribute to the cognitive deficit observed in AD. Thus, its alteration could be linked to AD pathogenesis. For this reason, we studied changes via western blot analysis in the NMDA-R total protein levels (NMDAR1 and NMDAR2B, and their phosphorylated form). Our results show a significant decrease in the phosphorylation of NMDAR2B in hippocampal homogenates from APP/PS1 mice fed with HFD (Fig. 3B, $p < 0.01$). However, no significant differences were detected in the protein levels of NMDAR1 between the groups (Fig. 3B).

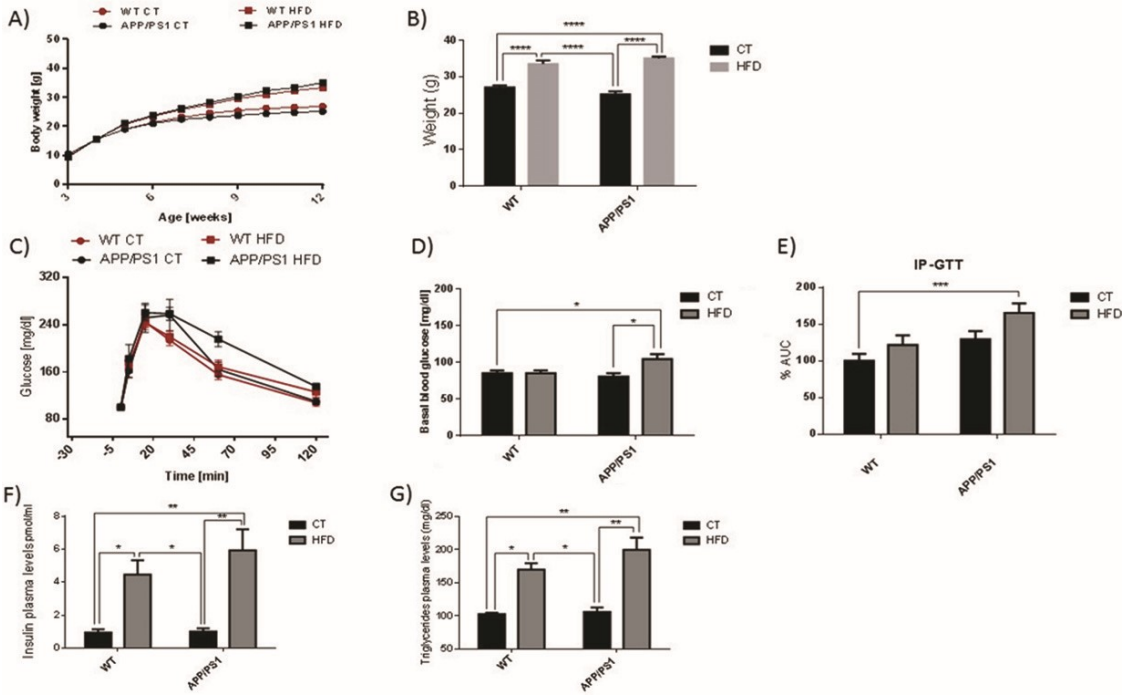

Fig. 2. Peripheral metabolic parameters in HFD-fed WT and APP/PS1 mice. A, B) Representative curve of body weight and its corresponding area under curve (AUC). C) Intraperitoneal glucose (16 h fast) tolerance tests in 3-month-old mice ($n = 5$–10 independent samples per group). D) For the IP-GTT, AUC data were calculated from the time point 0 until the end of the experiment. E) Fasting blood glucose. F) Fasting serum insulin levels ELISA. G) Total blood triglycerides in 3-month-old animals ($n = 5$–10 independent samples per group). (Statistical analysis was performed with the two-way ANOVA, with Tukey's *post hoc* test where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

High-fat diet does not enhance oxidative stress in APP/PS1 mice

Given the crucial role of oxidative stress in the pathogenesis of AD, several biomarkers were assessed in the brain homogenates from APP/PS1 and WT mice (fed HFD and CT diet). As shown in Fig. 4, there were no significant differences in lipid peroxidation, protein oxidation, and antioxidant enzymes (i.e., SOD, GSH-px, MDA, and protein carbonyls) ($p > 0.05$, respectively).

High-fat diet promotes AD-type brain amyloid deposition in APP/PS1 mice

Previous studies have demonstrated that the increase of APP was correlated with insulin resistance and pro-inflammatory gene expression [40]. In agreement with them, we demonstrated that animals fed with HFD show a significant increase in mRNA expression and protein levels of APP in APP/PS1 mice (Fig. 5A, B, $p < 0.05$, $p < 0.01$ respectively). Furthermore, while HFD did not increase β-secretase protein levels, ADAM10 (non-amyloidogenic pathway) was significantly decreased in APP/PS1 fed with HFD (Fig. 5A, $p < 0.05$). In addition, the protein levels of IDE (insulin degrading enzyme), an enzyme involved in the degradation of βA, showed a significant decrease in this group (Fig. 5C, $p < 0.05$).

We also evaluated the effect of the HFD on βA deposits in the brain of APP/PS1 mice using ThS to detect the fibrillar plaques, and 12F4 antibody to detect $A\beta_{1\text{-}42}$ diffuse plaques (Fig. 6A, B).

Two-way ANOVA showed a significant effect of the strain ($F_{(1,8)} = 23.33$, $p < 0.01$). Besides, our immunofluorescence data demonstrated a significant early accumulation of cortical βA diffuse and fibrillar plaques in APP/PS1 fed with HFD during 2 months (Fig. 6A, $p < 0.05$). These data were associated with an increase in the levels of brain $A\beta_{1\text{-}40}$ soluble and insoluble and $A\beta_{1\text{-}42}$ insoluble peptides (Fig. 6C, $p < 0.05$).

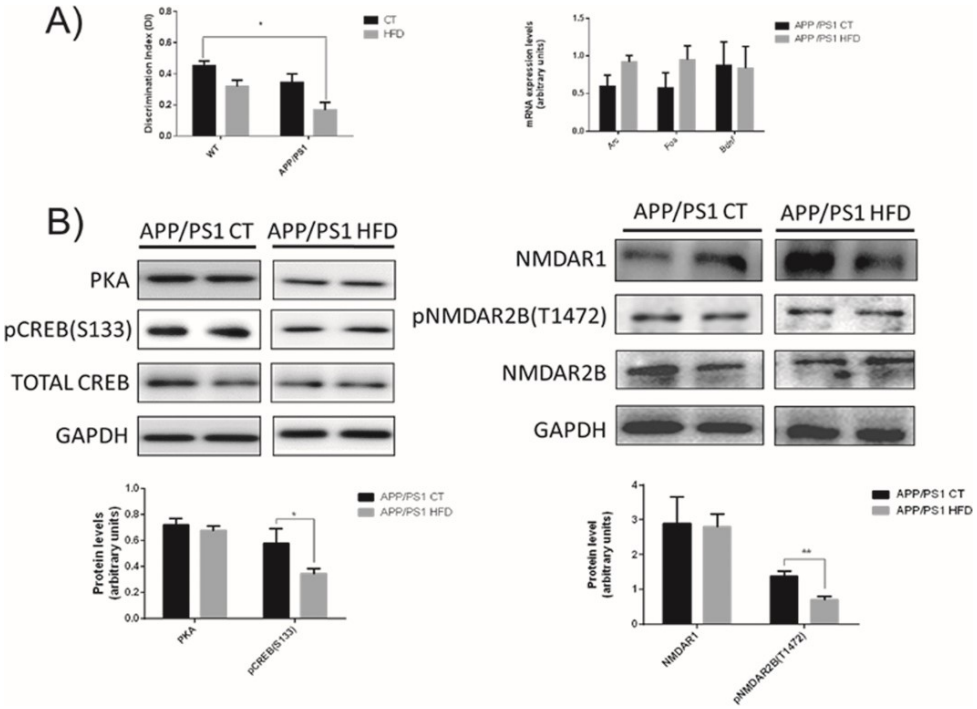

Fig. 3. A) The results of the Novel Object Recognition Test (NOR), demonstrating a significant memory loss in APP/PS1 mice fed with HFD versus WT CT group ($n = 6$–10 independent samples per group) (Statistical analysis was performed with the two-way ANOVA, with Tukey's *post hoc* test, where $^{*}p < 0.05$). Total exploration time was analyzed using two-way ANOVA, no differences were demonstrated (WT CT: 7.95 ± 0.56; WT HFD: 8.85 ± 1.18; APP CT: 7.5 ± 0.87; APP HFD: 8.85 ± 2.28). mRNA expression of early hippocampal memory genes ($n = 5$–8 independent samples per group, with 3 technical replicates per sample). B) Representative GAPDH-normalized immunoblot images and quantification ($n = 4$–6 independent samples per group) of PKA, p-CREB, CREB, and NMDA receptor subunits (Statistical analysis was performed with the student's *t*-test, where $^{*}p < 0.05$, $^{**}p < 0.01$).

Evaluation of the effects of HFD on insulin signaling and tau in the hippocampus of APPswe/PS1dE9 mice

Previous studies have already demonstrated alterations in insulin signaling pathways in brains of AD patients; however, the exact time of the onset of these alterations in experimental models of AD remains unclear. Thus, in a new series of experiments we investigated the mechanisms through which HFD could promote an AD-like amyloidosis in APP/PS1 animals compared to APP/PS1 fed with a CT diet. The results were obtained measuring mRNA levels of gene expression involved in IR signaling in the brain. For this reason, we evaluated mRNA expression of preproinsulin 1 *(Ins1)*, insulin receptor (Insr), insulin receptor substrates 1 *(Irs1)* and 2 *(Irs2)*, insulin-like growth factors I *(Igf1)* and II *(Igf2)*, IGF receptor *(Igfr)* as well as insulin-like growth factor-binding protein 2 *(igfbp2)*, at 3 month of age (Fig. 7). Our data showed no changes in mRNA levels in early stages of AD ($p > 0.05$).

Since tau phosphorylation is a hallmark of AD, we continued to study the potential molecular mechanisms leading to the AD neuropathology. We evaluated the phosphorylation of tau, downstream targets of the insulin signaling pathway, the protein kinase AKT/PKB pathway and the regulation of GSK3β downstream signaling in the brain of HFD and CT APP/PS1 mice. Our western blot data analysis of total and phosphorylated forms of AKT indicated no changes in the levels of this protein, and when we analyzed downstream substrates of this pathway, they remained unaltered in the hippocampus of both APP/PS1 mice fed with HFD and APP/PS1 mice fed with CT diet at 3 months of age (Fig. 8A, $p > 0.05$). Moreover, our data also revealed that HFD did not

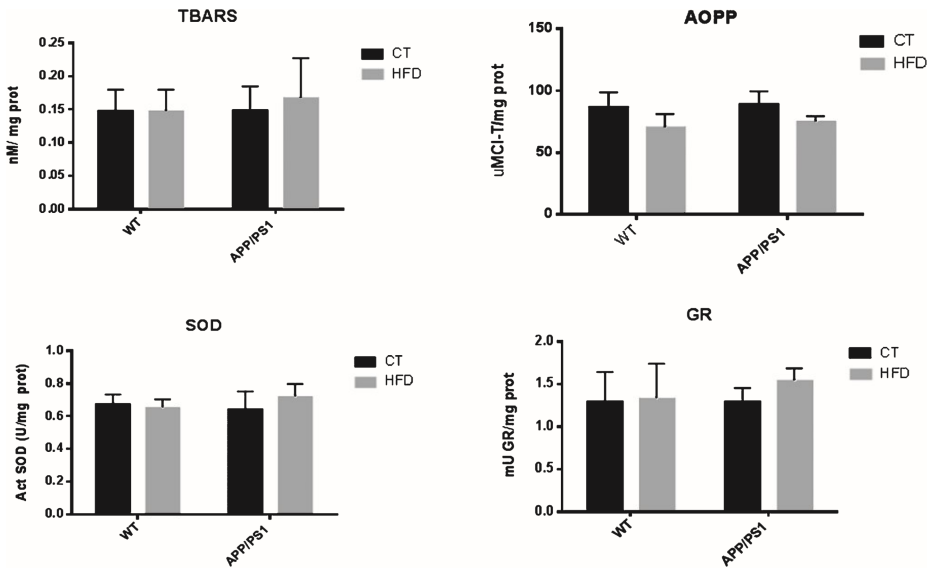

Fig. 4. The results of the oxidative stress assessment. The brain levels of malondialdehyde (MDA) (which is a product of lipid peroxidation) measured by the thiobarbituric reactive substances (TBARS), advanced oxidation protein products (AOPP), superoxide dismutase (SOD), and glutathione Reductase (GR).

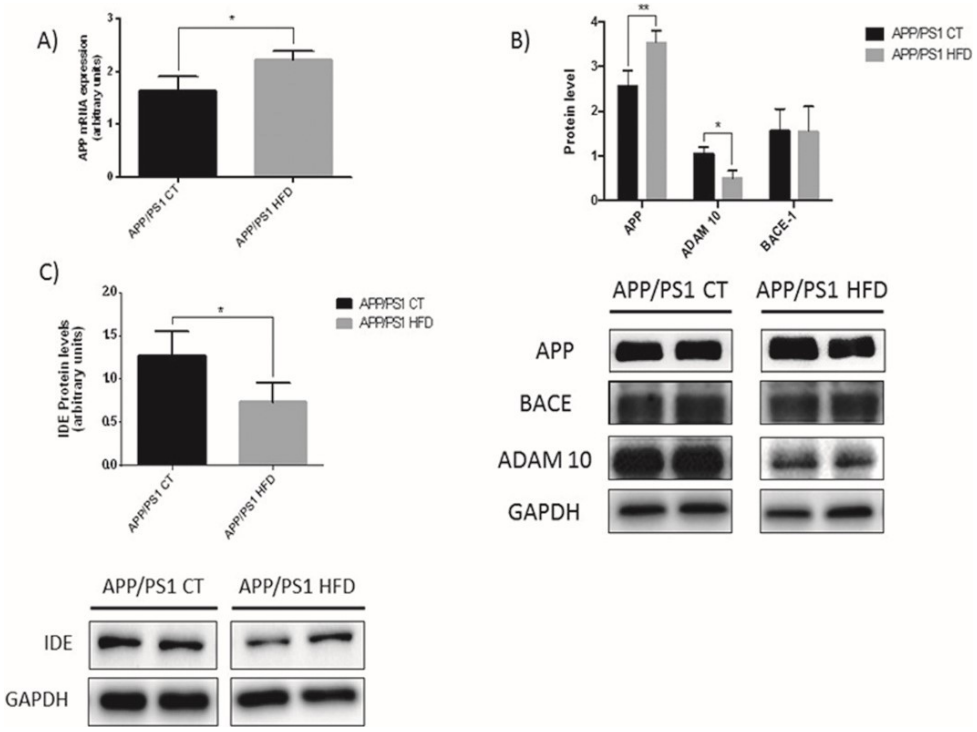

Fig. 5. A) APP mRNA expression profile ($n = 4$–6 independent samples per group, with 3 technical replicates per sample). B, C) Representative GAPDH-normalized immunoblotting images and quantification ($n = 4$–6 independent samples per group) of APP, BACE, and ADAM10 (B) and IDE (C) in the hippocampal extracts of CT-fed and HFD-fed in 3-month-old APP/PS1 mice. (Statistical analysis was performed with the student's *t*-test, where $^{*}p < 0.05$, $^{**}p < 0.01$).

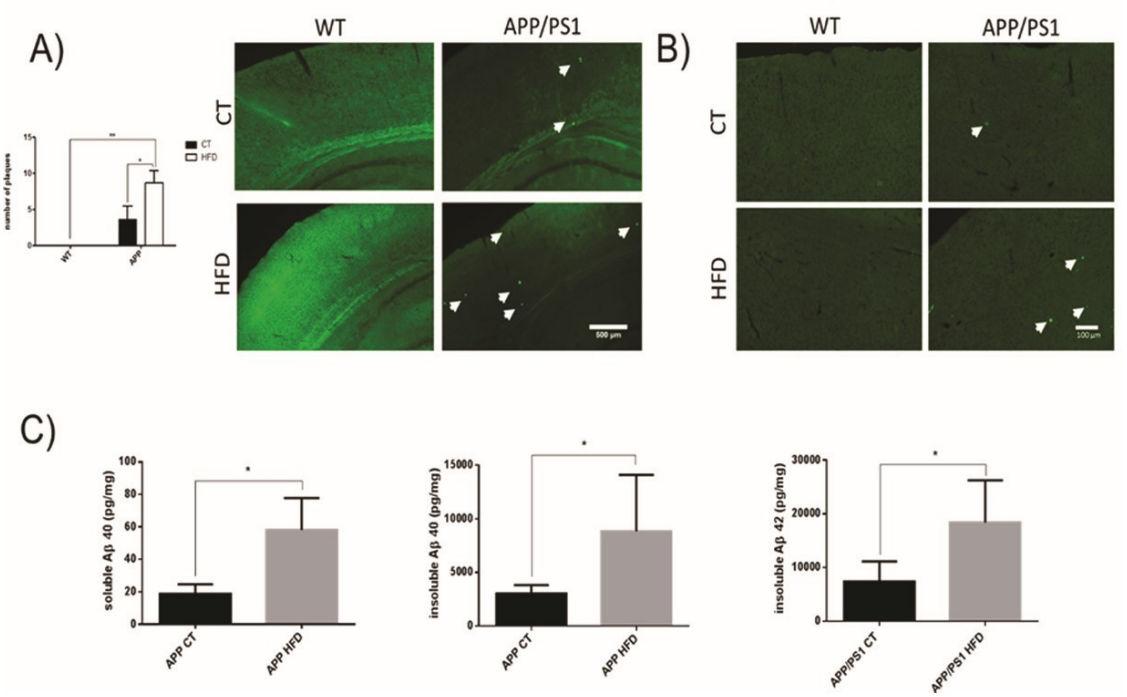

Fig. 6. A) ThS stain analysis of Aβ plaque numbers in the brains of 3-month-old WT and APP/PS1 mice ($n = 4$–6 independent samples per group, with at least 5 slices analyzed per sample) detected in green (Statistical analysis was performed with the two-way ANOVA, with Tukey's *post hoc* test, where $^{*}p < 0.05$, $^{**}p < 0.01$). B) Similar results were obtained using 12F4 (reactive to the C-terminus of Aβ) antibody that detects $A\beta_{1\text{-}42}$ diffuse plaques. C) Concentrations of soluble and insoluble human $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides in the cortical extracts in CT- and HFD-fed 3-month-old APP/PS1 mice, expressed as pg/mg of total protein as determined by ELISA ($n = 4$–6 independent samples per group, with 3 technical replicates per sample) (Statistical analysis was performed with the student's *t*-test, where $^{*}p < 0.05$).

affect major kinases (GSK3β, ERK1/2, and CDK5) involved in tau phosphorylation in the hippocampus at this age (Fig. 8A, $p > 0.05$). In addition, no significant changes in several tau phosphorylation sites were detected (Fig. 8B, $p > 0.05$).

PGC-1α is altered in the hippocampus of APPswe/PS1dE9 mice fed with HFD

Previous reported data suggest that PGC-1α expression is decreased in the brain of AD patients and that this decrease could be accompanied by mitochondrial alterations [55–58]. Our western blot data analyses demonstrated that HFD significantly decreases PGC1α protein levels, suggesting that PGC-1α could be involved in an initial step leading to alterations in mitochondrial biogenesis and metabolic function (Fig. 9, $p < 0.05$). However, downstream effectors of PGC-1α, such as nuclear respiratory factor 1 (NRF 1) and nuclear respiratory factor 2 (NRF 2), which control the nuclear genes that encode for mitochondrial proteins, and mitochondrial transcription factor A (TFAM), were not altered in the

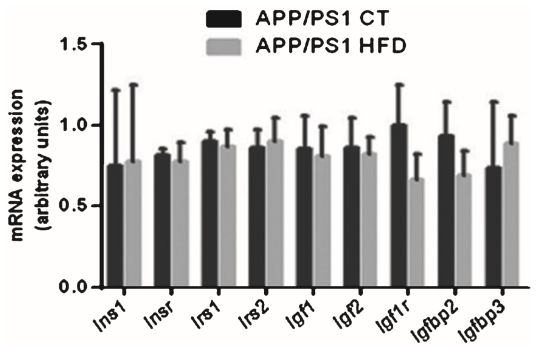

Fig. 7. mRNA expression profile ($n = 4$–6 independent samples per group, with 3 technical replicates per sample) of genes related to insulin signaling in the hippocampal extracts of CT-fed and HFD-fed 3-month-old APP/PS1 mice.

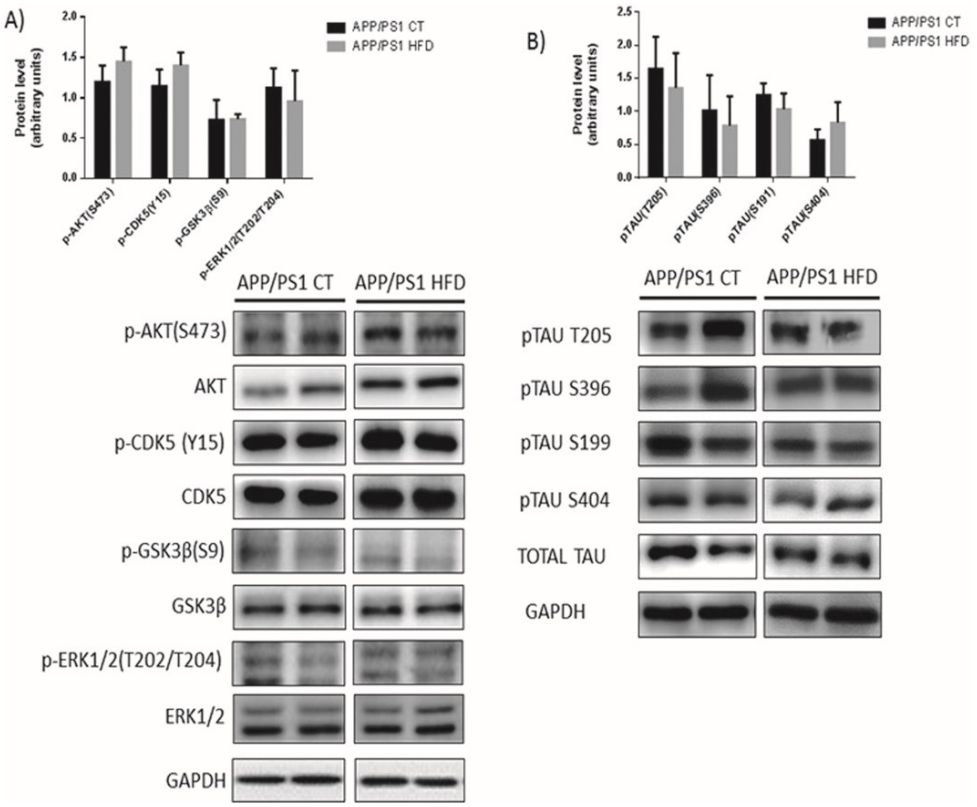

Fig. 8. Representative GAPDH-normalized immunoblotting images and quantification ($n = 4–6$ independent samples per group) of proteins related to AKT and tau signaling in the hippocampal extracts of CT-fed and HFD-fed in 3-month-old APP/PS1 mice.

hippocampus of 3-month-old APP/PS1 mice fed with HFD.

Effects of HFD on glial cells in the hippocampus of APPswe/PS1dE9 mice

Increasing evidence has demonstrated that the activation of glial cells may play an important role in the development of AD [58–60]. Furthermore, it may lead to neurotoxic damage through the generation of inflammatory responses. The analysis of our immunofluorescence images of glial cells in 3-month-old APP/PS1 mice fed with CT diet and APP/PS1 mice fed with HFD was done using an antibody that detects the glial acidic fibrillar protein (GFAP), which detects astrocytes, and the Iba1 antibody (ionized calcium binding adaptor molecule 1), which targets microglial cells. The results obtained revealed an accumulation of GFAP and Iba 1-positive activated cells around amyloid deposits/aggregates in both groups (Figs. 10 and 11). Also, the effect of HFD in the astrocytes and microglia reactivity was analyzed in different areas of the hippocampus such as cornu ammonis 1 (CA1), cornu ammonis 3 (CA3) and gyrus dentatus (GD). We did not detect changes in glial activation between dietary groups (Figs. 10 and 11).

DISCUSSION

Currently, one of the most important challenges of the 21st century is to develop drugs that can slow and modify the evolution and progression of AD. It is clear that to achieve this goal, it is necessary to determine which biochemical pathways are modulated by the Aβ neurotoxin. Presently, the underlying mechanisms through which Aβ exerts its neurotoxic effects, responsible for cognitive dysfunction at the early stage of AD, are not well understood. However, recent preclinical studies strongly suggest that obesity and type 2 diabetes are potential risk factors for AD development [61–64]. The intake of HFD, an experimental model of diet-induced obesity, may

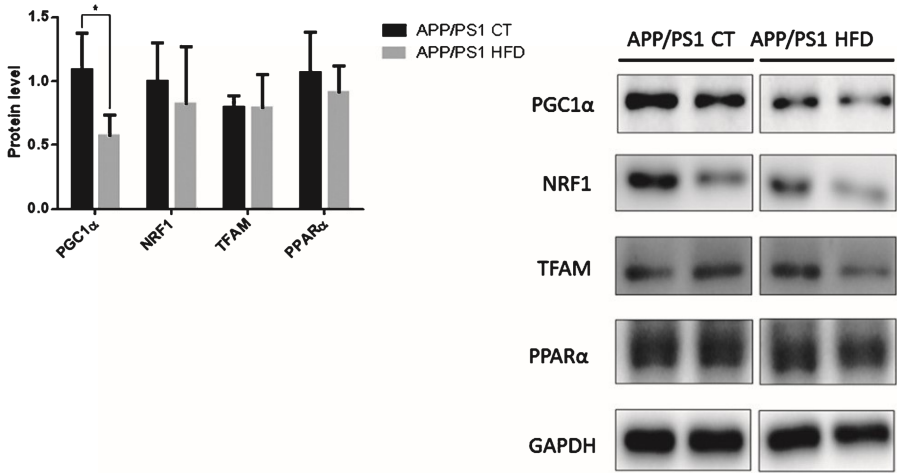

Fig. 9. Representative GAPDH-normalized immunoblotting images and quantification ($n = 4$–6 independent samples per group) of proteins related to mitochondrial biogenesis in the hippocampal extracts of CT-fed and HFD-fed in 3-month-old APP/PS1mice. (Statistical analysis was performed with the student's t-test, where $^{*}p < 0.05$).

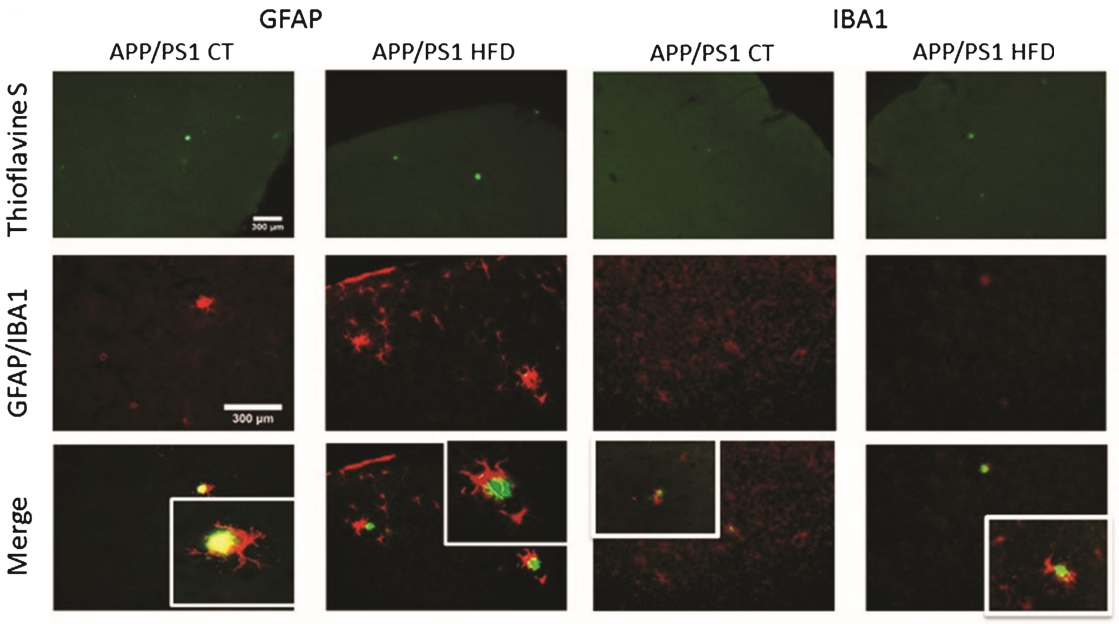

Fig. 10. Immunofluorescence against GFAP and IBA-1 in CT- fed and HFD-fed 3-month-old APP/PS1mice co-stained with ThS. In APP/PS1 mice reactive astrocytes and microglia (in red) are located around β-amyloid plaques (Aβ depositions) (in green).

lead to type 2 diabetes, and may have an effect on the biochemical regulation of the central nervous system. The HFD model had previously been used to show that diet plays an important role as a regulator of brain function, the content of lipids in the brain and processes affecting neuronal plasticity. Moreover, since myelin membranes have a very high lipid composition in cholesterol and fatty acids, the choice of diet can affect its integrity. In addition, it has been reported that alterations in the insulin/IGF-1-AKT and PGC-1α signaling pathways are involved in the process of myelinogenesis [65, 66].

Willete and colleagues demonstrated that insulin resistance in humans is associated with an increase

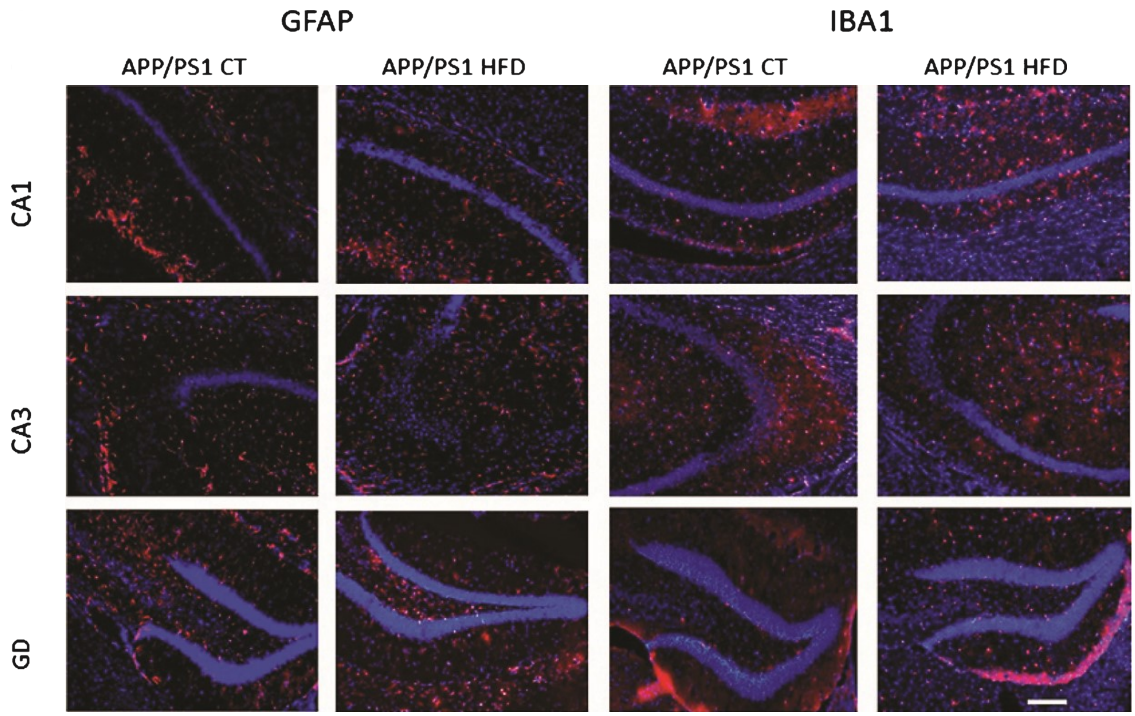

Fig. 11. Effects of HFD in glial reactivity in CA1, CA3, and GD in CT-fed and HFD-fed in 3-month-old APP/PS1mice. No changes in glial activation between both groups were detected.

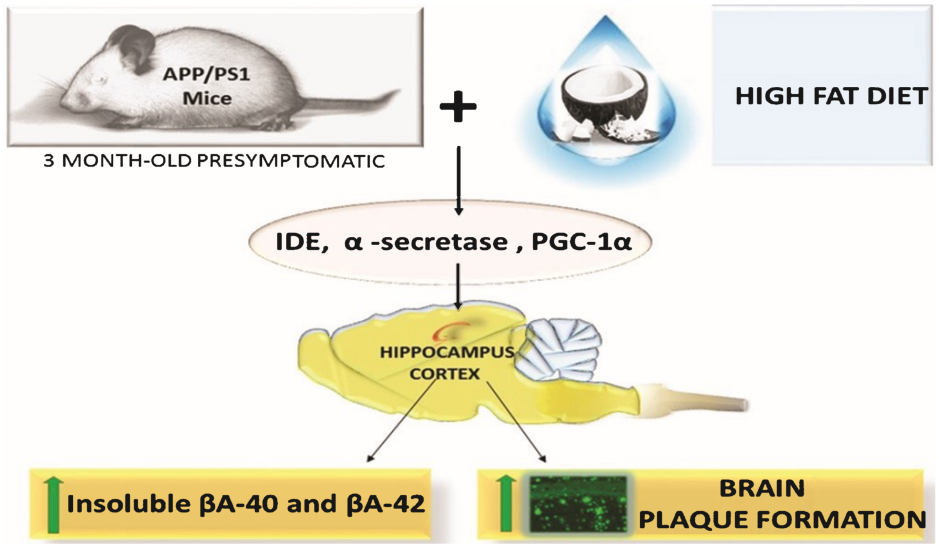

Fig. 12. A proposed mechanism whereby a HFD could elevate Aβ levels and plaque formation in brain APP/PS1 mice. HFD causes a reduction in three key molecules, PGC1α, IDE, and the α-secretase enzyme, which are directly involved in the production and metabolism of Aβ. These three proteins may constitute an early target for the treatment of AD in a presymptomatic stage.

in brain Aβ deposition [67, 68]. Preclinical reports have shown that HFD intake, in AD transgenic mice models, increases insulin resistance, Aβ deposition, tau phosphorylation and favors cognitive impairment [69–71]. In contrast, some studies argue that a HFD does not increase Aβ deposition and phosphorylation

of tau protein [72, 73]. It is important to point out that this controversy could be due to the fact that all these preclinical studies have been carried out with aged mice, in which plaques are already established and memory loss has already occurred.

Therefore, it is necessary to take action at the beginning of the disease's process, in a presymptomatic stage, and investigate how the HFD modifies those biological parameters responsible to trigger AD. For this reason, we used APP/PS1 mice, a model of familial AD that produces high levels of $A\beta_{1\text{-}42}$, one of the key factors responsible for the onset of this illness, although probably not the only one [39–44, 63].

Thus, we demonstrated that HFD results in a decrease in PGC-1α and IDE levels, an increase in plaque formation, as well as alterations in the non-amyloidogenic pathway in the hippocampus of 3-month-old mice. Unexpectedly, there was no increase in oxidative stress.

IDE is a protease involved in the degradation of Aβ and insulin [74]. Recent studies suggest that peripheral IDE could be a useful biomarker for the detection of AD [74–76]. The analysis of western blot data in the hippocampus suggests that the decrease in the protein levels of this enzyme may be associated with a reduced Aβ clearance. It is widely known that both insulin and Aβ compete for the availability of IDE, but the enzyme is much more selective for insulin than Aβ. Taking this into account, it could be possible that its reduction could favor a decrease in Aβ degradation that may lead to an increase in plaque formation [75]. In addition, our results suggest that HFD could increase plaque formation through an increase in the activity of the non-amyloidogenic pathway due to a significant reduction of ADAM10 protein level in HFD-fed APP/PS1 mice. [77–79]. In contrast, we did not find alterations in the protein level of BACE1 by western blot analysis. Likewise, in agreement with previous studies, the mRNA expression and protein levels of APP were significantly increased by HFD, suggesting that monitoring of APP protein levels may be an additional marker of both obesity and AD [27, 43]. Interestingly, our data are in line with previous studies from Pandini and colleagues, where insulin in neuroblastoma cells reduced Aβ production through the non-amyloidogenic pathway, decreased mRNA levels of APP (the precursor of Aβ peptides) and increased IDE activity and expression [28].

Previous reported data suggest that oxidative stress is present in several areas of the AD brain [76]. Those studies showed that oxidative stress causes damage to proteins, lipids, DNA, RNA, and carbohydrates in AD brain [11, 12, 80, 81]. Zhang and colleagues reported that at 3.5 months, APP/PS1 mice showed an increase in reactive oxygen species (ROS), which was associated with memory loss [42]. Studzinski and colleagues demonstrated that a mouse model of AD fed with a western diet allows for the observation of cerebral oxidative stress and the initiation of the development of Aβ brain pathogenesis [81]. In our study, APP/PS1 mice fed with a HFD did not show any significant increase of the ROS production in the brain. Therefore, our data do not verify that ROS participates in the early stages of experimental AD neuropathology promoted by HFD.

Another key question asked in this study was whether 3-month-old APP/PS1 mice fed with a HFD showed defects in insulin receptor substrate (IRS) mRNA expression and insulin signaling pathway as it was previously shown in experimental models of AD [82, 83]. This work demonstrated that HFD-feeding does not induce significant alterations in molecules involved in the IRS pathway in the hippocampus at 3 months of age. Besides, the insulin signaling pathway was not altered, since our western blot data indicated that the levels of hyperphosphorylated tau in the hippocampus were not modified as well as the activation of GSK3β. Therefore, we could conclude that the early brain abnormalities produced by obesity in APP/PS1 mice were not associated with alterations in the insulin receptor signaling pathway. Similarly, other kinases, such as ERK and CDK5, which are involved in tau phosphorylation, were not activated by the HFD.

Qin and colleagues were the first to report a reduction in the expression of PGC-1α in AD brains, suggesting that its decrease may represent a risk factor for onset and progression of dementia [55]. Thus, the preservation of its levels might be a molecular mechanism, which could confer protection against AD dementia [55–58]. Furthermore, downregulation of PGC1α has been associated with in associated with the development of skeletal muscle insulin resistance and it might be inferred that this protein could be a common link between the two diseases [85–90]. Recent evidence suggests that PGC1α and other transcription factors have a very important function in the regulation of mitochondrial biogenesis in the brain [57, 88–90]. In addition, it participates in the formation and maintenance of synapses in hippocampal neurons and in the regulation of NMDA activity [90]. Likewise, PGC1α has a powerful suppressive

effect on ROS production [88]. Moreover, it has been demonstrated that experimental inhibition of PGC1α expression correlates with the elevation of Aβ peptide generation through mechanisms involving the regulation of the non-amyloidogenic (α-secretase) and amyloidogenic (β-secretase) processing of APP [88].

Transcriptional regulation of PGC1α expression is known to be regulated by CREB [91–94]. A direct link between CREB phosphorylation and transcriptional regulation at the PGC-1α promoter has been observed in neuronal cells [92].

Our results indicated that the APP/PS1 mice fed a HFD show significant memory loss compared to WT mice fed a CT diet. This suggests a synergistic effect between diet and levels of Aβ that favors cognitive loss. Likewise, at the molecular level, our data revealed that the hippocampal protein levels of p-CREB were significantly decreased in APP/PS1 mice as a consequence of a HFD intake. In addition, it has been reported that CREB plays an essential role in hippocampal-based memory formation [92–98]. However, while p-CREB is downregulated, probably due to a decrease in PKA levels, HFD did not induce changes in the transcription of early genes involved in the memory process, such as Arc and Fos, or changes in Bdnf. Similarly, the upregulation of NMDAR2B subunit phosphorylation might be a compensatory mechanism to overcome their defective activity underlying the memory impairment that occurs in APP/PS1 mice at 6 months of age.

We showed that the hippocampal expression levels of proteins downstream of PGC1α signaling such as NRF-1 and TFAM were not modified by HFD. These data could indicate that at 3 months of age, not enough time has elapsed to detect changes in the mitochondrial biogenesis [43]. Thus, an increase in APP, associated with impairment of ADAM-10 jointly with an IDE decrease, probably contributes to the increase of hippocampal Aβ levels in APP/PS1 mice fed with a HFD. In addition, Aβ could alter the levels of PKA, which inhibits p-CREB, and decreases hippocampal PGC-1α levels, which is involved in α- and β-secretase modulation. In conclusion, our findings suggest that early alterations in Aβ/PKA, which decrease p-CREB/ PGC1α mediated by HFD in APP/PS1 mice in a presymptomatic stage, may represent a potential risk factor for the onset and progression of AD dementia (Fig. 12). Therefore, their preservation in the brain might be a molecular drug target mechanism conferring protection against metabolic AD.

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REFERENCES

[1] Vishal S, Sourabh A, Harkirat S (2011) Alois Alzheimer (1864-1915) and the Alzheimer syndrome. *J Med Biogr* **19**, 32-33.

[2] Alzheimer A, Stelzmann RA, Schnitzlein HN, Murtagh FR (1995) An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkrankung der Hirnrinde". *Clin Anat* **8**, 429-431.

[3] Wilson RS, Segawa E, Boyle PA, Anagnos SE, Hizel LP, Bennett DA (2012) The natural history of cognitive decline in Alzheimer's disease. *Psychol Aging* **27**, 1008-1017.

[4] Barker WW, Luis CA, Kashuba A, Luis M, Harwood DG, Loewenstein D (2002) Relative frequencies of Alzheimer's disease, Lewy body, vascular and frontotemporal dementia, and hippocampal sclerosis in the State of Florida Brain Bank. *Alzheimer Dis Assoc Disord* **16**, 203-212.

[5] Alzheimer's Association (2015) Alzheimer's disease facts and figures. *Alzheimers Dement* **11**, 332-384.

[6] Hardy JA, Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol Sci* **12**, 383-388.

[7] Hardy JA, Higgins GA (1992) Alzheimer's disease: The amyloid cascade hypothesis. *Science* **256**, 184-185.

[8] Hardy JA, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science* **297**, 353-356.

[9] Walsh DM, Selkoe DJ (2007) A beta oligomers—a decade of discovery. *J Neurochem* **101**, 1172-1184.

[10] Schneider LS, Mangialasche F, Andreasen N, Feldman H, Giacobini E, Jones R, Mantua V, Mecocci P, Pani L, Winblad B, Kivipelto M (2014) Clinical trials and late-stage drug development for Alzheimer's disease: An appraisal from 1984 to 2014. *J Intern Med* **275**, 251-283.

[11] Markesbery WR (1997) Oxidative stress hypothesis in Alzheimer's disease. *Free Radic Biol Med* **23**, 134-147.

[12] Bonda DJ, Wang X, Lee HG, Smith MA, Perry G, Zhu X (2014) Neuronal failure in Alzheimer's disease: A view through the oxidative stress looking-glass. *Neurosci Bull* **30**, 243-252.

[13] Swerdlow RH (2012) Mitochondria and cell bioenergetics: Increasingly recognized components and a possible etiologic cause of Alzheimer's disease. *Antioxid Redox Signal* **16**, 1434-1455.

[14] Swerdlow RH, Burns JM, Khan SM (2010) The Alzheimer's disease mitochondrial cascade hypothesis. *J Alzheimers Dis* **20**, S265-S279.

[15] Swerdlow RH, Khan SM (2015) A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. *Med Hypotheses* **63**, 8-20.

[16] Demetrius LA, Driver J (2013) Alzheimer's as a metabolic disease. *Biogerontology* **14**, 641-649.
[17] Demetrius LA, Driver J (2015) Preventing Alzheimer's disease by means of natural selection. *J R Soc Interfac* **12**, 20140919.
[18] Arnold SE, Lucki I, Brookshire BR, Carlson GC, Browne C, Kazi, H, Kim SF (2014) High fat diet produces brain insulin resistance, synaptodendritic abnormalities and altered behavior in mice. *Neurobiol Dis* **67**, 79-87.
[19] De Felice FG (2013) Alzheimer's disease and insulin resistance: Translating basic science into clinical applications. *J Clin Inves* **123**, 531-539.
[20] Balakrishnan K, Verdile G, Mehta PD, Beilby J, Nolan D, Galvão DA, Newton R, Gandy SE, Martins RN (2005) Plasma Abeta42 correlates positively with increased body fat in healthy individuals. *J Alzheimers Dis* **8**, 269-282.
[21] De Felice FG, Ferreira ST (2014) Inflammation, defective insulin signaling, and mitochondrial dysfunction as common molecular denominators connecting type 2 diabetes to Alzheimer disease. *Diabetes* **63**, 2262-2272.
[22] De la Monte SM, Wands JR (2008) Alzheimer's disease is type 3 diabetes–evidence reviewed. *J Diabetes Sci Technol* **2**, 1101-1113.
[23] Dineley KT, Jahrling JB, Denner L (2014) Insulin resistance in Alzheimer's disease. *Neurobiol Dis* **72**, 92-103.
[24] Townsend M, Mehta T, Selkoe DJ (2007) Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway. *J Biol Chem* **282**, 33305-33312.
[25] Xie L, Helmerhorst E, Taddei K, Plewright B, Van Bronswijk W, Martins R (2002) Alzheimer's β-amyloid peptides compete for insulin binding to the insulin receptor. *J Neurosci* **22**, RC221.
[26] Zhao WQ, De Felice FG, Fernandez S, Chen H, Lambert MP, Quon MJ, Krafft GA, Klein WL (2008) Amyloid β oligomers induce impairment of neuronal insulin receptors. *FASEB J* **22**, 246-260.
[27] Puig KL, Floden AM, Adhikari R, Golovko MY, Combs CK (2012) Amyloid precursor protein and proinflammatory changes are regulated in brain and adipose tissue in a murine model of high fat diet-induced obesity. *PLoS One* **7**, e30378.
[28] Pandini G, Pace V, Copani A, Squatrito S, Milardi D, Vigneri R (2013) Insulin has multiple antiamyloidogenic effects on human neuronal cells. *Endocrinology* **154**, 375-387.
[29] Beydoun MA, Beydoun HA, Wang Y (2008) Obesity and central obesity as risk factors for incident dementia and its subtypes: A systematic review and meta-analysis. *Obesity Rev* **9**, 204-218.
[30] Boitard C, Etchamendy N, Sauvant J, Aubert A, Tronel S, Marighetto A, Ferreira G (2012) Juvenile, but not adult exposure to high-fat diet impairs relational memory and hippocampal neurogenesis in mice. *Hippocampus* **22**, 2095-2100.
[31] Bruehl H, Wolf OT, Sweat V, Tirsi A, Richardson S, Convit A (2009) Modifiers of cognitive function and brain structure in middle-aged and elderly individuals with type 2 diabetes mellitus. *Brain Res* **1280**, 186-194.
[32] Oh H, Boghossian S, York D, Park-York M (2013) The effect of high fat diet and saturated fatty acids on insulin signaling in the amygdala and hypothalamus of rats. *Brain Res* **1537**, 191-200.
[33] Pessin JE, Saltiel AR (2000) Signaling pathways in insulin action: Molecular targets of insulin resistance. *J Clin Inves* **106**, 165-169.
[34] Ramos-Rodriguez JJ, Ortiz-Barajas O, Gamero-Carrasco C, de la Rosa PR, Infante-Garcia C, Zopeque-Garcia N, Lechuga-Sancho AM, Garcia-Alloza M (2014) Prediabetes-induced vascular alterations exacerbate central pathology in APPswe/PS1dE9 mice. *Psychoneuroendocrinology* **48**, 123-135.
[35] Ramos-Rodriguez JJ, Jimenez-Palomares M, Murillo-Carretero MI, Infante-Garcia C, Berrocoso E, Hernandez-Pacho F, Lechuga-Sancho AM, Cozar-Castellano I, Garcia-Alloza M (2015) Central vascular disease and exacerbated pathology in a mixed model of type 2 diabetes and Alzheimer's disease. *Psychoneuroendocrinology* **62**, 69-79.
[36] Knight EM, Martins IV, Gümüsgöz S, Allan SM, Lawrence CB (2014) High-fat diet-induced memory impairment in triple-transgenic Alzheimer's disease (3xTgAD) mice is independent of changes in amyloid and tau pathology. *Neurobiol Aging* **35**, 1821-1832.
[37] Mehla J, Chauhan BC, Chauhan NB (2014) Experimental induction of type 2 diabetes in aging-accelerated mice triggered Alzheimer-like pathology and memory deficits. *J Alzheimers Dis* **39**, 145-162.
[38] Ho L, Qin W, Pompl PN, Xiang Z, Wang J, Zhao Z, Peng Y, Cambareri G, Rocher A, Mobbs CV, Hof PR, Pasinetti GM (2004) Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *FASEB J* **18**, 902-904.
[39] Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA, Copeland NG, Lee MK, Younkin LH, Wagner SL, Younkin SG, Borchelt DR (2004) Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide *in vivo*: Evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet* **13**, 159-170.
[40] Zhang W, Bai M, Xi Y, Hao J, Liu L, Mao N, Su C, Miao J, Li Z (2012) Early memory deficits precede plaque deposition in APPswe/PS1dE9 mice: Involvement of oxidative stress and cholinergic dysfunction. *Free Radic Biol Med* **52**, 1443-1452.
[41] Zhang W, Bai M, Xi Y, Hao J, Zhang Z, Su C, Lei G, Miao J, Li Z (2012) Multiple inflammatory pathways are involved in the development and progression of cognitive deficits in APPswe/PS1dE9 mice. *Neurobiol Aging* **33**, 2661-2277.
[42] Zhang W, Hao J, Liu R, Zhang Z, Lei G, Su C, Miao J, Li Z (2011) Soluble Aβ levels correlate with cognitive deficits in the 12-month-old APPswe/PS1dE9 mouse model of Alzheimer's disease. *Behav Brain Res* **222**, 342-350.
[43] Petrov D, Pedrós I, Artiach G, Sureda FX, Barroso E, Pallàs M, Casadesús G, Beas-Zarate C, Carro E, Ferrer I, Vazquez-Carrera M, Folch J, Camins A (2015) High-fat diet-induced deregulation of hippocampal insulin signaling and mitochondrial homeostasis deficiences contribute to Alzheimer disease pathology in rodents. *Biochim Biophys Acta* **1852**, 1687-1699.
[44] Pedrós I, Petrov D, Allgaier M, Sureda F, Barroso E, Beas-Zarate C, Auladell C, Pallàs M, Vázquez-Carrera M, Casadesús G, Folch J, Camins A (2014) Early alterations in energy metabolism in the hippocampus of APPswe/PS1dE9 mouse model of Alzheimer's disease. *Biochim Biophys Acta* **1842**, 1556-1566.
[45] Kurt MA, Davies DC, Kidd M, Duff K, Rolph SC, Jennings KH, Howlett DR (2001) Neurodegenerative changes associated with beta-amyloid deposition in the brains of mice carrying mutant amyloid precursor protein and mutant presenilin-1 transgenes. *Exp Neurol* **171**, 59-71.

[46] Radde R, Bolmont T, Kaeser SA, Coomaraswamy J, Lindau D, Stoltze L, Calhoun ME, Jäggi F, Wolburg H, Gengler S, Haass C, Ghetti B, Czech C, Hölscher C, Mathews PM, Jucker M (2006) Abeta42-driven cerebral amyloidosis in transgenic mice reveals early and robust pathology. *EMBO Rep* **7**, 940-946.

[47] Bilkei-Gorzo A (2014) Genetic mouse models of brain ageing and Alzheimer's disease. *Pharmacol Ther* **142**, 244-257.

[48] Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156-159.

[49] Antunes M, Biala G (2012) The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cogn Process* **13**, 93-110.

[50] Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, Wasserman DH (2010) Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Dis Model Mech* **3**, 525-534.

[51] Uchiyama M, Mihara M (1978) Determination of malondialdehide precursor in tissues by thiobarbituric acid test. *Anal Biochem* **86**, 271-278.

[52] Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.

[53] Witko-Sarsat V, Gausson V, Nguyen AT (2003) AOPP-induced activation of human neutrophil and monocyte oxidative metabolism: A potential target for N-acetylcysteine treatment in dialysis patients. *Kidney Int* **64**, 82-91.

[54] Aydin S, Atukeren P, Çakatay U, Uzun H, Altuğ T (2010) Gender-dependent oxidative variations in liver of aged rats. *Biogerontology* **11**, 335-346.

[55] Qin W, Haroutunian V, Katsel P, Cardozo CP, Ho L, Buxbaum JD, Pasinetti GM (2009) PGC-1alpha expression decreases in the Alzheimer disease brain as a function of dementia. *Arch Neurol* **66**, 352-361.

[56] Sheng B, Wang X, Su B, Lee HG, Casadesus G, Perry G, Zhu X (2012) Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in Alzheimer's disease. *J Neurochem* **120**, 419-429.

[57] St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jäger S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM (2006) Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* **127**, 397-408.

[58] Austin S, St-Pierre J (2012) PGC1α and mitochondrial metabolism–emerging concepts and relevance in ageing and neurodegenerative disorders. *J Cell Sci* **125**, 4963-4971.

[59] Kamphuis W, Middeldorp J, Kooijman L, Sluijs JA, Kooi EJ, Moeton M, Freriks M, Mizee MR, Hol EM (2014) Glial fibrillary acidic protein isoform expression in plaque related astrogliosis in Alzheimer's disease. *Neurobiol Aging* **35**, 492-510.

[60] Mosher KI, Wyss-Coray T (2014) Microglial dysfunction in brain aging and Alzheimer's disease. *Biochem Pharmacol* **88**, 594-604.

[61] Knight EM, Martins IV, Gümüsgöz S, Allan SM, Lawrence CB (2014) High-fat diet-induced memory impairment in triple-transgenic Alzheimer's disease (3xTgAD) mice is independent of changes in amyloid and tau pathology. *Neurobiol Aging* **35**, 1821-1832.

[62] Leboucher A, Laurent C, Fernandez-Gomez FJ, Burnouf S, Troquier L, Eddarkaoui S, Demeyer D, Caillierez R, Zommer N, Vallez E, Bantubungi K, Breton C, Pigny P, Buée-Scherrer V, Staels B, Hamdane M, Tailleux A, Buée L, Blum D (2013) Detrimental effects of diet-induced obesity on τ pathology are independent of insulin resistance in τ transgenic mice. *Diabetes* **62**, 1681-1688.

[63] Haque R, Nazir A (2014) Insulin-degrading enzyme: A link between Alzheimer's and type 2 diabetes mellitus. *CNS Neurol Disord Drug Targets* **13**, 259-256.

[64] Stargardt A, Gillis J, Kamphuis W, Wiemhoefer A, Kooijman L, Raspe M, Benckhuijsen W, Drijfhout JW, Hol EM, Reits E (2013) Reduced amyloid-β degradation in early Alzheimer's disease but not in the APPswePS1dE9 and 3xTg-AD mouse models. *Aging Cell* **12**, 499-507.

[65] Nave KA (2010) Myelination and the trophic support of long axons. *Nat Rev Neurosci* **11**, 275-283.

[66] Yoon H, Kleven A, Paulsen A, Kleppe L, Wu J, Ying Z, Gomez-Pinilla F, Scarisbrick IA (2016) Interplay between exercise and dietary fat modulates myelinogenesis in the central nervous system. *Biochim Biophys Acta* **1862**, 545-555.

[67] Willette AA, Bendlin BB, Starks EJ, Birdsill AC, Johnson SC, Christian BT, Okonkwo OC, La Rue A, Hermann BP, Koscik RL, Jonaitis EM, Sager MA, Asthana S (2015) Association of insulin resistance with cerebral glucose uptake in late middle-aged adults at risk for Alzheimer disease. *JAMA Neurol* **72**, 1013-1020.

[68] Willette AA, Johnson SC, Birdsill AC, Sager MA, Christian B, Baker LD, Craft S, Oh J, Statz E, Hermann BP, Jonaitis EM, Koscik RL, La Rue A, Asthana S, Bendlin BB (2015) Insulin resistance predicts brain amyloid deposition in late middle-aged adults. *Alzheimers Dement* **11**, 504-510.

[69] Takeda S, Sato N, Rakugi H, Morishita R (2011) Molecular mechanisms linking diabetes mellitus and Alzheimer disease: Beta-amyloid peptide, insulin signaling, and neuronal function. *Mol Biosyst* **7**, 1822-1827.

[70] Takalo M, Haapasalo A, Martiskainen H, Kurkinen KM, Koivisto H, Miettinen P, Khandelwal VK, Kemppainen S, Kaminska D, Mäkinen P, Leinonen V, Pihlajamäki J, Soininen H, Laakso M, Tanila H, Hiltunen M (2014) High-fat diet increases tau expression in the brain of T2DM and AD mice independently of peripheral metabolic status. *J Nutr Biochem* **25**, 634-641.

[71] Calvo-Ochoa E, Hernández-Ortega K, Ferrera P, Morimoto S, Arias C (2014) Short-term high-fat-and-fructose feeding produces insulin signaling alterations accompanied by neurite and synaptic reduction and astroglial activation in the rat hippocampus. *J Cereb Blood Flow Metab* **34**, 1001-1008.

[72] Knight EM, Martins IV, Gümüsgöz S, Allan SM, Lawrence CB (2014) High-fat diet-induced memory impairment in triple-transgenic Alzheimer's disease (3xTgAD) mice is independent of changes in amyloid and tau pathology. *Neurobiol Aging* **35**, 1821-1832.

[73] Leboucher A, Laurent C, Fernandez-Gomez FJ, Burnouf S, Troquier L, Eddarkaoui S, Demeyer D, Caillierez R, Zommer N, Vallez E, Bantubungi K, Breton C, Pigny P, Buée-Scherrer V, Staels B, Hamdane M, Tailleux A, Buée L, Blum D (2013) Detrimental effects of diet-induced obesity on τ pathology are independent of insulin resistance in τ transgenic mice. *Diabetes* **62**, 1681-1688.

[74] Jha NK, Jha SK, Kumar D, Kejriwal N, Sharma R, Ambasta RK, Kumar P (2015) Impact of insulin degrading enzyme and neprilysin in Alzheimer's disease biology: Characterization of putative cognates for therapeutic applications. *J Alzheimers Dis* **48**, 891-917.

[75] Haque R, Nazir A (2014) Insulin-degrading enzyme: A link between Alzheimer's and type 2 diabetes mellitus. *CNS Neurol Disord Drug Targets* **13**, 259-264.
[76] Yang Y, Song W (2013) Molecular links between Alzheimer's disease and diabetes mellitus. *Neuroscience* **250**, 140-150.
[77] Endres K, Fahrenholz F (2010) Upregulation of the alpha-secretase ADAM10–risk or reason for hope? *FEBS J* **277**, 1585-1596.
[78] Musardo S, Marcello E, Gardoni F, Di Luca M (2014) ADAM10 in synaptic physiology and pathology. *Neurodegener Dis* **13**, 72-74.
[79] Saftig P, Lichtenthaler SF (2015) The alpha secretase ADAM10: A metalloprotease with multiple functions in the brain. *Prog Neurobiol* **135**, 1-20.
[80] Butterfield DA, Di Domenico F, Barone E (2014) Elevated risk of type 2 diabetes for development of Alzheimer disease: A key role for oxidative stress in brain. *Biochim Biophys Acta* **1842**, 1693-1706.
[81] Studzinski CM, Li F, Bruce-Keller AJ, Fernandez-Kim SO, Zhang L, Weidner AM, Markesbery WR, Murphy MP, Keller JN (2009) Effects of short-term Western diet on cerebral oxidative stress and diabetes related factors in APP x PS1 knock-in mice. *J Neurochem* **108**, 860-866.
[82] Chua LM, Lim ML, Chong PR, Hu ZP, Cheung NS, Wong BS (2012) Impaired neuronal insulin signaling precedes Aβ42 accumulation in female AβPPsw/PS1ΔE9 mice. *J Alzheimers Dis* **29**, 783-791.
[83] Wang X, Zheng W, Xie JW, Wang T, Wang SL, Teng WP, Wang ZY (2010) Insulin deficiency exacerbates cerebral amyloidosis and behavioral deficits in an Alzheimer transgenic mouse model. *Mol Neurodegener* **5**, 46.
[84] Peng D, Pan X, Cui J, Ren Y, Zhang J (2013) Hyperphosphorylation of tau protein in hippocampus of central insulin-resistant rats is associated with cognitive impairment. *Cell Physiol Biochem* **32**, 1417-1425.
[85] Stranahan AM (2015) Models and mechanisms for hippocampal dysfunction in obesity and diabetes. *Neuroscience* **309**, 125-139.
[86] Besseiche A, Riveline JP, Gautier JF, Bréant B, Blondeau B (2015) Metabolic roles of PGC-1α and its implications for type 2 diabetes. *Diabetes Metab* **41**, 347-357.
[87] Łukaszuk B, Kurek K, MikŁosz A, Żendzian-Piotrowska M, Chabowski A (2015) The role of PGC-1α in the development of insulin resistance in skeletal muscle - revisited. *Cell Physiol Biochem* **37**, 2288-2296.
[88] Katsouri L, Parr C, Bogdanovic N, Willem M, Sastre M (2011) PPARγ co-activator-1α (PGC-1α) reduces amyloid-β generation through a PPARγ-dependent mechanism. *J Alzheimers Dis* **25**, 151-162.
[89] Puddifoot C, Martel MA, Soriano FX, Camacho A, Vidal-Puig A, Wyllie DJ, Hardingham GE (2012) PGC-1α negatively regulates extrasynaptic NMDAR activity and excitotoxicity. *J Neurosci* **32**, 6995-7000.
[90] Cheng A, Wan R, Yang JL, Kamimura N, Son TG, Ouyang X, Luo Y, Okun E, Mattson MP (2012) Involvement of PGC-1α in the formation and maintenance of neuronal dendritic spines. *Nat Commun* **3**, 1250.
[91] Ashabi G, Ramin M, Azizi P, Taslimi Z, Alamdary SZ, Haghparast A, Ansari N, Motamedi F, Khodagholi F (2012) ERK and p38 inhibitors attenuate memory deficits and increase CREB phosphorylation and PGC-1α levels in Aβ-injected rats. *Behav Brain Res* **232**, 165-173.
[92] Sardari M, Rezayof A, Khodagholi F (2015) Hippocampal signaling pathways are involved in stress-induced impairment of memory formation in rats. *Brain Res* **1625**, 54-63.
[93] Swerdlow RH (2012) β-Apptists and Tauists, it is time for a sermon from the book of biogenesis. *J Neurochem* **120**, 347-349.
[94] Teich AF, Nicholls RE, Puzzo D, Fiorito J, Purgatorio R, Fa' M, Arancio O (2015) Synaptic therapy in Alzheimer's disease: A CREB-centric approach. *Neurotherapeutics* **12**, 29-41.
[95] Roy A, Pahan K (2015) PPARα signaling in the hippocampus: Crosstalk between fat and memory. *J Neuroimmune Pharmacol* **10**, 30-34.
[96] Kida S, Serita T (2014) Functional roles of CREB as a positive regulator in the formation and enhancement of memory. *Brain Res Bull* **105**, 17-24.
[97] Hansen RT, 3rd, Zhang HT (2013) Senescent-induced dysregulation of cAMP/CREB signaling and correlations with cognitive decline. *Brain Res* **1516**, 93-109.
[98] Kandel ER (2012) The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Mol Brain* **5**, 14.

PUBLICACIÓN II

Dexibuprofen prevents neurodegeneration and cognitive decline in APPswe/ PS1dE9 through multiple signaling pathways

Miren Ettcheto, Elena Sánchez-López, Laura Pons, Oriol Busquets, Jordi Olloquequi, Carlos Beas-Zarate, Merce Pallas, Maria Luisa García, Carme Auladell, Jaume Folch, Antoni Camins (2017). *Redox Biology 13 (2017) 345–352*

Resumen

El objetivo del presente estudio es esclarecer la vía neuronal asociada a la reducción del riesgo y progresión de la EA, observada tras la administración de AINES.

La investigación fue desarrollada mediante la administración del enantiomero activo del ibuprofeno, el dexibuprofeno DXI, con el propósito de reducir la toxicidad gástrica asociada al ibuprofeno. El DXI fue administrado en ratones hembra APPswe/PS1dE9 como modelo de EA familiar desde los 3 hasta los 6 meses de edad.

El tratamiento de DXI redujo la activación de células gliales y liberación de citocinas involucradas en procesos neurodegenerativos, especialmente TNFα. Además, en los ratones tratados se observó una disminución significativa del $\beta A_{1\text{-}42}$ soluble y la formación de placas a través de, por un lado, la reducción significativa de los niveles proteicos de APP y BACE1, y por otro, a través de potenciar la degradación del βA, mediante el incremento de la enzima degradadora de insulina. Los resultados observados demostraron que el DXI también disminuye la hiperfosforilación de TAU a través de la inhibición de la

vía de señalización de c-Abl/CABLES/p-CDK5 y previene de las alteraciones en memoria y aprendizaje espacial en los ratones APP/PS1.

Por todo ello, el tratamiento crónico del DXI podría constituir un potencial fármaco para la EA, tanto recuperando las funciones cognitivas como revertiendo las características neuropatológicas principales de la enfermedad.

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Research paper

Dexibuprofen prevents neurodegeneration and cognitive decline in *APPswe/PS1dE9* through multiple signaling pathways

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ABSTRACT

The aim of the present study is to elucidate the neuronal pathways associated to NSAIDs causing a reduction of the risk and progression of Alzheimer's disease. The research was developed administering the active enantiomer of ibuprofen, dexibuprofen (DXI), in order to reduce associated gastric toxicity. DXI was administered from three to six-month-old female APPswe/PS1dE9 mice as a model of familial Alzheimer's disease. DXI treatment reduced the activation of glial cells and the cytokine release involved in the neurodegenerative process, especially TNFα. Moreover, DXI reduced soluble β-amyloid (Aβ1-42) plaque deposition by decreasing APP, BACE1 and facilitating Aβ degradation by enhancing insulin-degrading enzyme. DXI also decreased TAU hyperphosphorylation inhibiting c-Abl/CABLES/p-CDK5 activation signal pathway and prevented spatial learning and memory impairment in transgenic mice. Therefore, chronic DXI treatment could constitute a potential AD-modifying drug, both restoring cognitive functions and reversing multiple brain neuropathological hallmarks.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia affecting elderly people [1]. AD is clinically characterized by loss of memory and cognitive functions. Moreover, histopathological hallmarks include extracellular amyloid peptide (Aβ) deposition in neuritic plaques, and intracellular deposits of hyperphosphorylated TAU (pTAU), causing the formation of neurofibrillary tangles (NFTs) and, finally, neuronal loss [2].

Despite its high prevalence and mortality, the molecular mechanisms associated with neuronal loss are still unknown. However, Aβ peptides, especially Aβ(1-42), are currently considered the main neurotoxins responsible for AD neuronal loss. According to the amyloidogenic cascade hypothesis, Aβ peptides are generated via the amyloid precursor protein (APP) through two proteolytic enzymes, the β-secretase 1 (BACE1) and the γ-secretase [3,4].

Since there are no effective treatments to prevent or cure AD, great efforts are currently directed towards identifying disease-modifying

Abbreviations: AD, Alzheimer's disease; Aβ, Amyloid beta; APP, Amyloid precursor protein; NFTs, Neurofibrillary tangles; BACE1, β-secretase 1; TNF-α, Tumor necrosis factor; iNOS, Inducible nitric oxide synthase; NSAIDs, Non-steroidal anti-inflammatory drugs; PPARγ, Peroxisome proliferator-activated receptor-γ; COX-1, Cyclooxygenase-1; COX-2, Cyclooxygenase-2; NFκβ, Nuclear factor kappa beta; IBU, Ibuprofen; pTAU, Phospho-TAU; DXI, Dexibuprofen; APP/PS1,, *APPswe/PS1dE9*; WT, Wild type; MWM, Morris water maze; NORT, Novel object recognition test; DI, Discrimination index; PFA, Paraformaldehide; nNOS, Neuronal Nitric Oxide Synthase; GFAP, Glial Fibrillary Acidic Protein; ADAM10, Disintegrin and metalloproteinase domain-containing protein 10; IDE, Insulin-degrading enzyme; CDK5, Cyclin-dependent kinase 5; GSK3β, Glycogen synthase kinase 3β; c-ABL, Abelson non-receptor tyrosine kinase; MAPK, Mitogen-activated protein kinase; PKA, Protein kinase A; SYP, Synaptophysin

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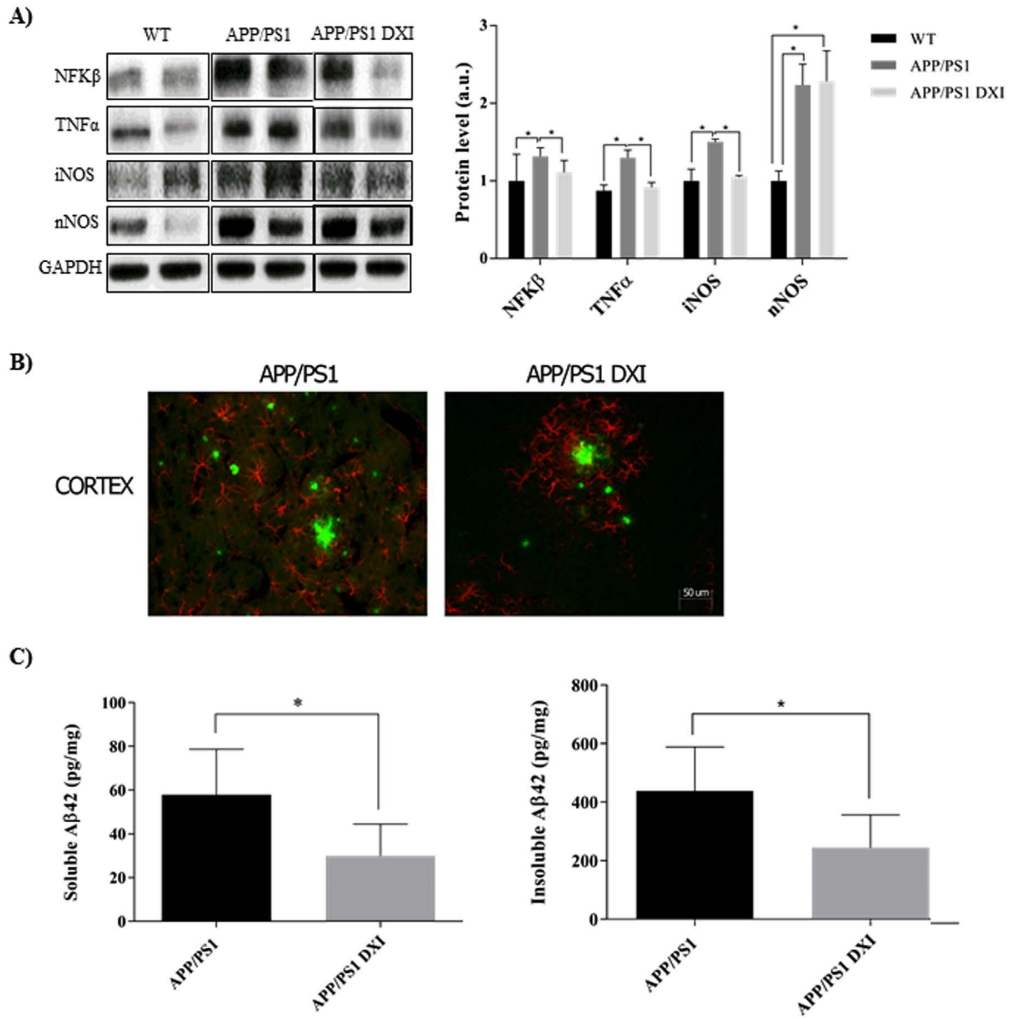

Fig. 1. A) Representative GAPDH-normalized immunoblotting images and quantification (n = 4–6 independent samples per group) of proteins related in neuroinflammation processes in the hippocampal extracts of WT, APP/PS1 and APP/PS1-DXI treated 6-month-old mice. **B)** Immunohistochemistry of ThS of Aβ plaque and GFAP in the brains of 6-month-old APP/PS1 and APP/PS1-DXI treated mice (n = 4–6 independent samples per group, with at least 5 slices analyzed per sample). **C)** Concentrations of soluble and insoluble human Aβ(1 − 42) peptides in the cortical extracts in untreated and DXI-treated APP/PS1 mice, expressed as pg/mg of total protein as determined by ELISA; n = 4–6 independent samples per group, with 3 technical replicates per sample (* denotes $p < 0.05$).

therapies, involving several compounds in different phases of development [5–7]. However, due to the complex nature of AD, one of the main concerns in drug development relies on the ability to obtain compounds capable of acting in more than a single specific disease target. Thereby, some researchers propose that the popular amyloid cascade hypothesis should be slightly modified to incorporate additional cellular and physiological components contributing to the neurodegenerative process, such as neuroinflammatory glial cells [5,7]. This hypothesis suggests that glial activation, mainly microglia, would lead to an upregulation of brain cytokines and chemokines such as IL-1β, IL-12, tumor necrosis factor (TNFα), and inducible nitric oxide synthase activation (iNOS), thus causing neuronal loss in different brain areas, especially the hippocampus [6,8].

Nevertheless, the dual effects of the inflammatory response add complexity to the process. At the beginning of the disease, inflammation constitutes a neuroprotective response intended to reduce Aβ accumulation. However, as the disease progresses, the excessive or chronic neuroinflammatory response contributes to AD development [7]. For this reason, non-steroidal anti-inflammatory drugs (NSAIDs) are being on the research focus towards AD treatment over the last years [9]. These drugs might influence the inflammatory response by activating the peroxisome proliferator-activated receptor-γ (PPARγ) and inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). In addition, some studies have revealed other interesting NSAIDs targets, such as nuclear factor kappa beta (NFκβ) and β and γ-secretases [8]. In addition, some authors reported that these benefits may only be observed in early phases of the disease [6]. Although there are still some discrepancies between epidemiological results, some studies indicate that patients under long-term treatment with NSAIDs for other conditions such as rheumatoid arthritis, present a reduced incidence of AD, suggesting that these drugs could restore some pathways altered in the disease [10,11].

Ibuprofen (IBU) is one of the most commonly used NSAIDs. Preclinical studies with IBU showed a reduction in the levels of pTAU and Aβ deposition, as well as a decrease in COX-2 expression and activated microglia [12]. Moreover, a reduction in oxidative stress

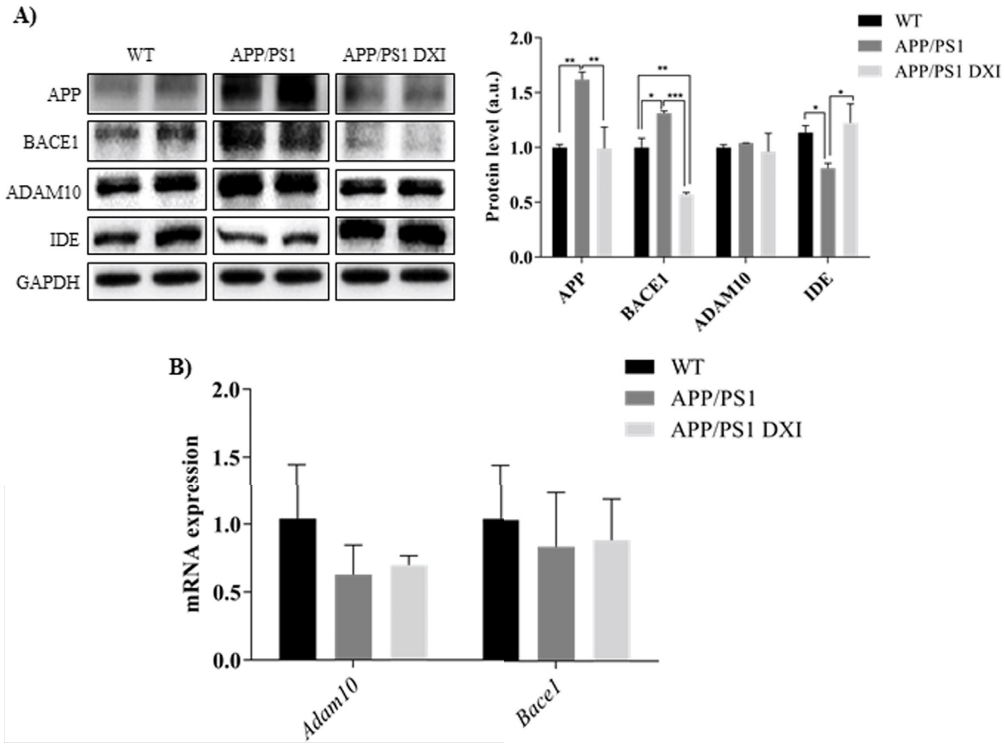

Fig. 2. A) Representative GAPDH-normalized immunoblotting images and quantification (n = 4–6 independent samples per group) of key molecules involved in APP processing APP, BACE, ADAM10 and IDE in the hippocampal extracts of WT, APP/PS1 and APP/PS1-DXI treated 6-month-old mice (* denotes $p < 0.05$, ** denotes $p < 0.01$). **B)** Quantitative analysis of mRNA expression of ADAM10 and BACE1 determined by RT-PCR.

through a decrease in NOX2 and 4HNE expression was also reported [12]. Bearing in mind these promising findings, in the present study we investigated the effects of dexibuprofen (DXI), the active enantiomer of IBU [13]. DXI provides an improvement for the treatment of several inflammatory diseases, since lower doses than its racemic counterpart are necessary to obtain identical therapeutic efficacy [14]. Furthermore, the higher solubility in water and increased absorption of DXI allow to reduce undesirable ulcerogenic side effects associated with IBU or other NSAIDs [14–16]. Additionally, preclinical data have demonstrated that DXI have a more powerful anti-inflammatory and analgesic effect than IBU [17]. Taking into account the advantages of this compound, the aim of the present study was to investigate the effects of DXI on AD-associated neuropathology markers, as well as the underlying mechanisms related to memory loss, in female *APPswe/PS1dE9* mice, a model of familial AD [18,19].

2. Materials and methods

2.1. Animals and treatment

In this study, six-month-old female *APPswe/PS1dE9* (APP/PS1) and C57BL/6 wild-type (WT) mice were used. This animal model was chosen according to previous studies reporting that female mice develop higher progressive memory impairment and AD-like neuropathology compared to male mice [20]. These transgenic mice express a Swedish (K594M/N595L) mutation of a chimeric mouse/human APP (mo/huAPP695swe), together with the human exon-9-deleted variant of PS1 (PS1-dE9).

Animals were divided in three groups (WT, APP/PS1 and APP/PS1 DXI) and, at least, 10 animals per group were used. APP/PS1 transgenic mice were non-treated (APP/PS1) or treated with water supplemented with DXI (APP/PS1 DXI), containing 50 $mg_{DXI}\ kg^{-1}\ day^{-1}$ for 3 months before sacrifice. WT mice did not receive any pharmacological treatment. All animals were given access to food or water *ad libitum* and kept under controlled temperature, humidity and light conditions. Every effort was made to reduce the number of animals and minimize animal suffering. Mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

2.2. Cognitive function test: novel object recognition

Before sacrifice, the hippocampal-dependent recognition memory of treated and non-treated mice was assessed by a novel object recognition test (NORT). The first three days, each mouse was left to get used to the open field box, without any objects (10 min/session). On the fourth day, mice were left for 10 min to explore two identical objects (A + A). On the fifth day, each mouse was exposed for 10 min to a familiar object A and a novel object, namely B. After this, the objects and the open field box were cleaned with soap and water in order to avoid the presence of olfactory signs. Recorded videos were analyzed and the discrimination index (DI) was calculated dividing the exploration time of the novel object by the total exploration time [21] Exploration was defined as sniffing or touching an object. Mice with a total exploration time of < 5 s for an object were removed from analyses [22].

2.3. Immunofluorescence

Mice were sacrificed at 6 months and, at least, 5 animals of each group were perfused before brain extraction using 4% paraformaldehyde (PFA). Tissues were processed and stained as previously described

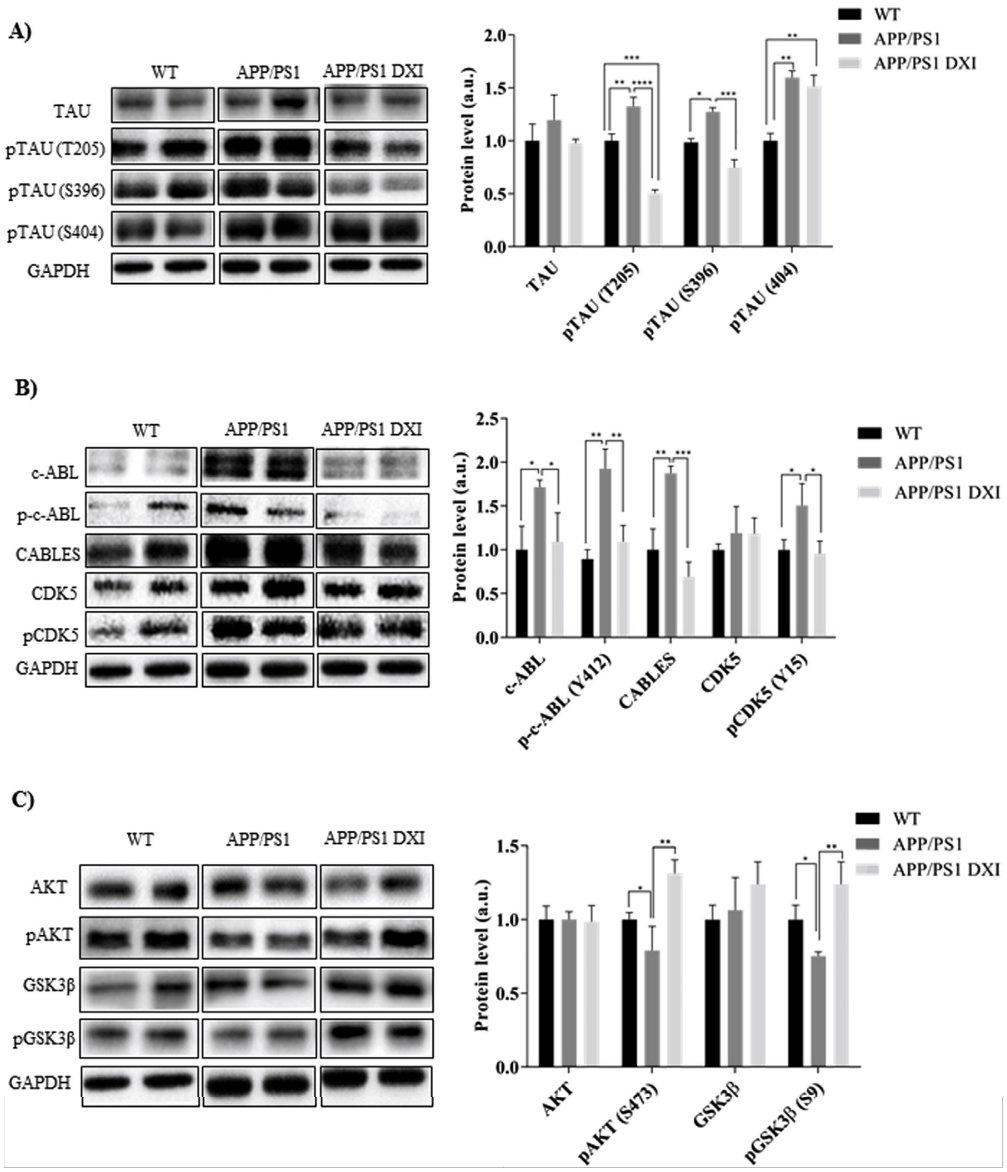

Fig. 3. Representative GAPDH-normalized immunoblotting images and quantification (n = 4–6 independent samples per group) of key molecules involved in TAU hyperphosphorilation **A)** Protein levels of TAU, pTAU (T205), pTAU(S396) and pTAU (S404) of WT, APP/PS1 and APP/PS1 treated with DXI, **B)** Protein levels of c-ABL, p-c-ABL (Y412), CABLES, CDK5 and pCDK5(Y15) of WT, APP/PS1 and APP/PS1 treated with DXI and **C)** Protein levels of AKT, pAKT, GSK3β AND PGSK3β of WT, APP/PS1 and APP/PS1 treated with DXI (* denotes $p < 0.05$, ** denotes $p < 0.01$).

by our group [23,24]. The antibodies used in this study are supplied as Table 1 of Supplementary material.

2.4. Measurement of β-amyloid peptides in cortical tissues by ELISA

Soluble and insoluble Aβ (1−42) were measured in cortical extracts using commercially available human ELISA kits (Cat # KHB3441; Invitrogen, Camarillo, CA, USA) according to manufacturer's guidelines. Data obtained from the cortical homogenates was expressed as picograms of Aβ content per milligrams of total protein (pg/mg) [25].

2.5. Sample preparation for Western blotting and Real-Time PCR

At 6 months, 5–8 animals of each group were sacrificed by cervical dislocation prior to brain dissection. For protein extraction, hippocampi were homogenized in lysis buffer (50 mM TrisHCl pH: 7.4, 150 mM NaCl, 5 mM EDTA, 1%Triton X-100) and protease inhibitor mixture (Complete, Roche Diagnostics, Barcelona, Spain). Samples were stored at −80 °C until use. Protein concentration was determined using Pierce BCA Protein Assay kit (Pierce Company, Rockford, MI, USA) [26].

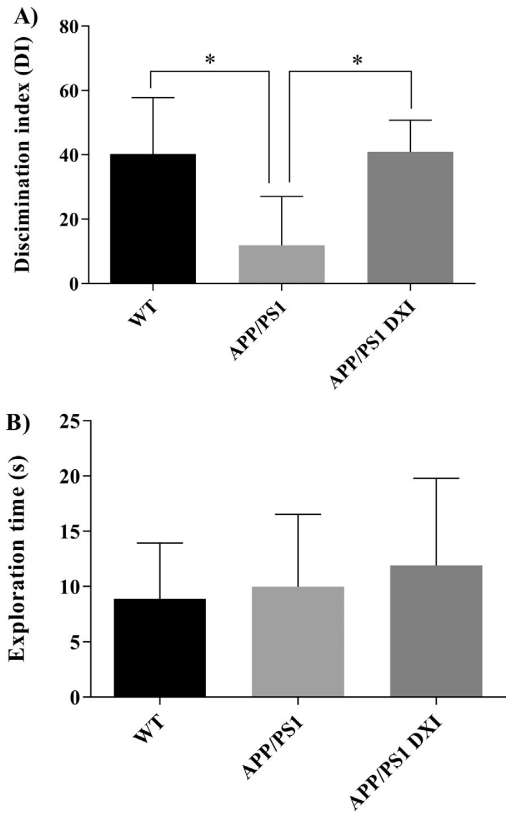

Fig. 4. Novel object recognition test. **A)** Discrimination index (DI) expressed as a percentage comparison between WT, APP/PS1 and APP/PS1 DXI. **B)** No significant effect on total exploration time during the test was found between the assessed groups (* denotes $p < 0.05$).

2.6. Western blot analysis

Aliquots of samples containing 10 μg of protein were analyzed by Western Blotting. The samples were placed in sample buffer (0.5 M Tris-HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue, pH 6.8) and denatured by boiling at 95 °C for 5 min. Samples were separated by electrophoresis on 10% acrylamide gels, transferred to PVDF (Polyvinylidene difluoride) membranes using transblot apparatus and blocked in 5% skim milk powder in TBS-T buffer (50 mM Tris, 1.5% NaCl, 0.05% Tween 20, pH 7.5) for 1 h at room temperature. They were incubated overnight at 4 °C with primary antibodies (Supplementary material, Table 2). After that, membranes were washed in TBS-T buffer and incubated with IgG secondary antibody for 1 h at room temperature. Bands were detected by chemiluminescence detection kit and using Chemi doc XRS + Molecular Imager detection system (Bio-Rad). The quantification was performed by Image Lab image analysis software. All results were normalized to GAPDH [26].

2.7. Real-Time PCR

For RNA extraction, hippocampi were homogenized by Trizol reagent (Life Technologies Corporation) and samples were stored at −80 °C until use. Equal concentrations of cDNA of each animal were used for q-PCR and each sample was analyzed by triplicate. The assays were performed on a StepOnePlus Real-Time PCR system (Applied Biosystems). The PCR reaction contained 2 μg of reverse-transcribed RNA, 2SYBRGreen qPCR Master Mix (K0253, Pierce, Thermo Fisher Scientific), and 100 mM of each primer. Analyzed genes are detailed in Table 3 of Supplementary material. Results were normalized to actin [26].

2.8. Gastric damage

After treatment, mice were sacrificed and stomachs were removed, cut and rinsed with ice-cold distilled water. The ulcer index (UI) was determined by calculating the severity of the lesions as previously

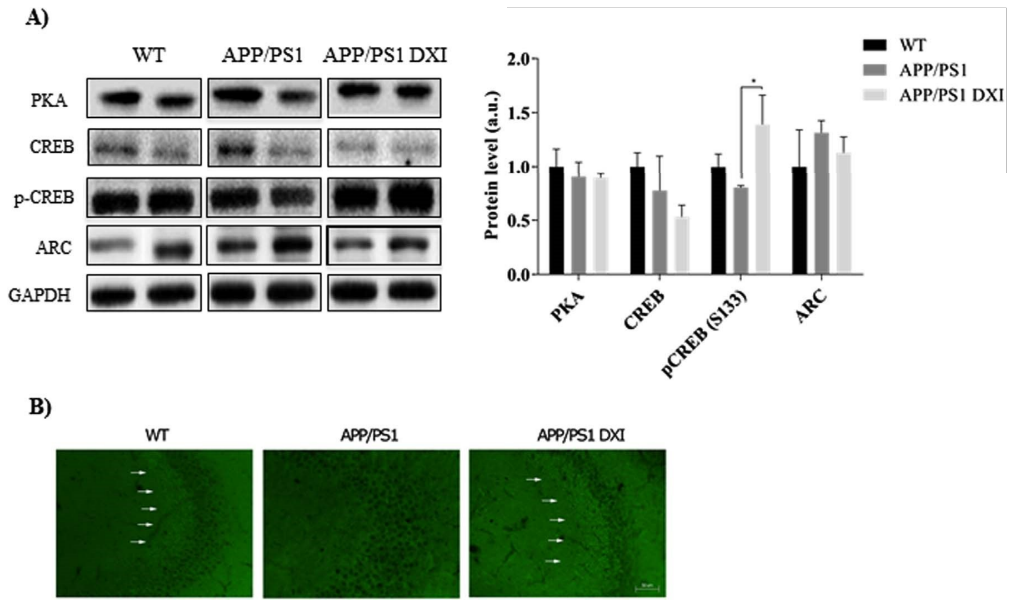

Fig. 5. A) Representative GAPDH-normalized immunoblotting images and quantification (n = 4–6 independent samples per group) of proteins related to memory processes in the hippocampal extracts of WT, APP/PS1 and APP/PS1 treated with DXI in 6-month-old mice. (* denotes $p < 0.05$). **B)** Immunohistochemistry of synaptophysin and Hoestch in the hippocampal CA3 region.

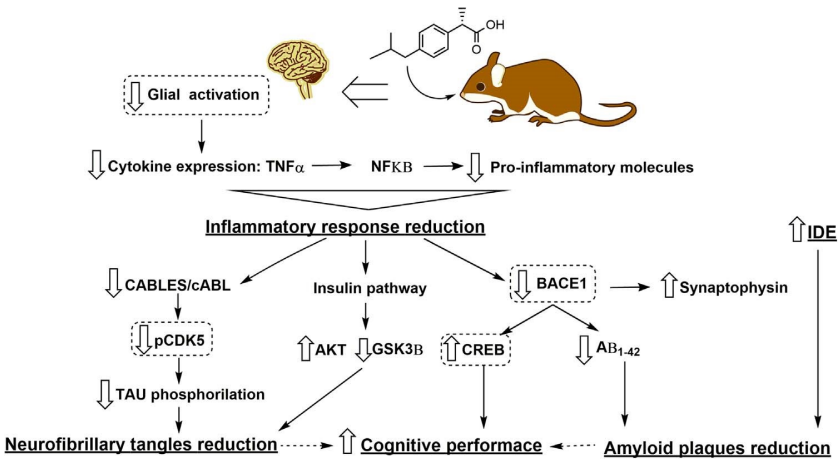

Fig. 6. A proposed mechanism where Dexibuprofen could constitute a potential treatment for AD. DXI causes a reduction in neuroinflammatory process which decreases three key signaling pathways, CABLES/c-ABL, hippocampal insulin pathway and the β-secretase enzyme, which are directly involved in the production and metabolism of Aβ, TAU phosphorylation and memory improvement. These signaling pathways constitute an early target for the treatment of AD with DXI, probably in a pre-symptomatic stage which can explain the potential use of this drug in neurodegenerative diseases.

reported by other authors [27].

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6. Data were expressed as mean ± standard deviation. Significant differences were determined by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multigroup comparisons to compare data from the experimental groups. The level of significance for the acceptance was $p < 0.05$.

3. Results

3.1. Dexibuprofen reduces neuroinflammation in APPswe/PS1dE9 mice brain

It is well known that activated astrocytes and microglia are usually localized in the brain area where Aβ deposition occurs in APP/PS1 mice [28]. We found a significant increase in NFκβ, TNFα, iNOS and neuronal Nitric Oxide Synthase (nNOS) protein level in APP/PS1 mice compared to WT ($p < 0.05$; Fig. 1A). In addition, DXI treatment significantly decreased the levels of all these proteins, with the exception of nNOS (no significant differences were observed).

In order to study the astrocytes activation, brain sections were immunostained with an anti-GFAP (Glial Fibrillary Acidic Protein) antibody. The staining against GFAP was markedly higher in the brain of untreated APP/PS1 mice compared to DXI treated mice (Fig. 1B).

Additionally, potential gastric damage of DXI was compared using the same dose of it's racemic counterpart, IBU, in WT mice for three months. In agreement with other authors, DXI induced significantly less gastric damage than IBU (Supplementary material Fig. 1).

3.2. Dexibuprofen reduces plaque burden and inhibits APP processing toward the amyloidogenic pathway in APPswe/PS1dE9 mice

APP/PS1 mice exhibit a time-dependent Aβ production and deposition in the brain with increasing age [29]. To determine the effect of DXI treatment on this process, Thioflavin-S (ThS) was used for detection of fibrillary plaques (Fig. 1B). The analysis revealed that long-term administration of DXI significantly reduced the area occupied by fibrillary plaques in the cerebral cortex of APP/PS1 mice. Additionally, as shown in Fig. 1C, DXI treatment caused a significant decrease in the levels of soluble and insoluble Aβ (1-42) ($p < 0.05$).

Western Blot analyses were carried out to investigate the mechanisms involved in APP processing that contribute to Aβ soluble production. Quantitative analysis (Fig. 2A) indicated that the levels of full-length APP were significantly increased in the hippocampus of non-treated transgenic group ($p < 0.01$), whereas a significantly decrease in the levels of this protein was found in DXI-treated mice ($p < 0.01$). In addition, DXI significantly decreased protein levels of BACE1 in the hippocampus of transgenic mice compared to non-treated APP/PS1 mice ($p < 0.001$). Moreover, DXI treatment significantly increased IDE protein levels in APP/PS1 mice hippocampus ($p < 0.05$). However, changes in ADAM10 in APP/PS1 mice after DXI treatment were not observed. As it is shown in Fig. 2B, no significant differences between mRNA expression of ADAM10 nor BACE1 were observed after DXI treatment. Therefore, these results indicate that DXI inhibits APP processing toward amyloidogenic pathway, thus suggesting that DXI lowers the plaque burden by promoting Aβ degradation through the inhibition of BACE1 and the synthesis of APP in the brain hippocampus of transgenic mice.

3.3. Dexibuprofen attenuates TAU phosphorylation in APPswe/ PS1dE9 mice

The effects of DXI on TAU hyperphosphorylation were assessed by Western blot. Data analysis showed that the levels of pTAU at Thr205 and Ser 396 were significantly decreased in the hippocampus of DXI APP/PS1 mice compared with non-treated transgenic mice ($p < 0.0001$; $p < 0.001$ respectively; Fig. 3A).

The process of TAU hyperphosphorylation is mediated mainly by two kinases: the cyclin-dependent kinase 5 (CDK5) and the glycogen synthase kinase 3β (GSK3β). In addition, upstream regulation of both kinases is mediated by c-ABL. Thus, analysis of the c-ABL signaling pathway in the hippocampus at 6 months revealed an increase in the protein levels of total and phosphorylated c-ABL on Tyr412 in APP/PS1 compared with WT mice ($p < 0.05$; $p < 0.01$; Fig. 3B). Moreover, consequently with the c-ABL-dependent CDK5 activation, CDK5Tyr15 phosphorylation was also markedly elevated ($p < 0.05$). Furthermore, in this signaling pathway, CABLES mediates the interaction between c-ABL and CDK5, and regulates CDK5 tyrosine phosphorylation by c-ABL. Western blot analyses showed a significant increase in CABLES protein levels in APP/PS1 mice compared with WT mice ($p < 0.01$). DXI-treated transgenic group showed a significant decrease in the expression of activated proteins involved in c-ABL pathway ($p^{<}0.05$). Likewise, DXI prevented the inhibition of brain insulin signaling pathway measured by p-AKT and p-GSK3β analyses, which showed a significant increased phosphorylation compared with non-treated APP/PS1 mice ($p < 0.05$; $p < 0.01$; Fig. 3C).

3.4. Dexibuprofen improves the cognitive process in APPswe/PS1dE9 mice

In the NOR test, APP/PS1 DXI mice presented a significant increase of DI compared to non-treated transgenic mice, thus indicating drug enhanced memory effects ($p < 0.05$; Fig. 4A). In addition, no differences in total exploration time were observed among the experimental groups (Fig. 4B).

At the molecular level, APP/PS1 treated with DXI for three months showed a significant increase in CREB phosphorylation at serine 133 (Ser 133) in the hippocampus compared to non-treated APP/PS1 mice ($p < 0.05$). This phosphorylation leads to the transcription of memory associated genes. Moreover, no significant hippocampal alterations in the levels of protein kinase A (PKA) nor ARC were observed in APP/PS1 mice compared to WT groups (Fig. 5A).

Finally, in order to evaluate synaptic integrity, we investigated whether the protein levels of the synaptic marker synaptophysin (SYP) in transgenic mice after a chronic DXI-treatment were modified. DXI treatment induced a consistent increase in SYP protein levels of DXI APP/PS1 mice hippocampus compared with vehicle-treated APP/PS1 mice (Fig. 5B). Thus, these data indicate that DXI treatment could improve the memory process through the p-CREB.

4. Discussion

Several epidemiological studies have reported that chronic use of NSAIDs reduces the risk for AD [10]. In the same line, the present research demonstrates that a chronic DXI treatment ameliorates learning and memory deficits in APP/PS1 mice, thus suggesting that DXI could be an AD modifying drug. DXI exerts significant effects on the reduction of Aβ production, both enhancing Aβ clearance and inhibiting aggregation of Aβ into amyloid plaques, as well as inhibiting TAU phosphorylation.

Brain gliosis activation is an important pathological feature of all neurodegenerative disorders, also AD [30]. Previous preclinical research has reported that activation of microglia and astrocytes is markedly enhanced and probably correlates with cognitive deficits and amyloid plaques in the brain of APP/PS1 [31,32]. The results of the present investigation show that DXI treatment markedly reduces the activated astrocytes as well as the cytokine expression -mainly TNFα- in the hippocampus of APP/PS1 mice. Microglia activation and cytokine increase in brain are important in the onset of AD, since it has been demonstrated that drugs against TNFα improve AD neuropathology [17,33]. Therefore, the significant reduction of inflammatory mediators observed in DXI treated APP/PS1 mice could partially explain the neuroprotective properties of this NSAID.

The DXI effect on decreasing brain Aβ accumulation might be attributed to two main factors: 1) the direct APP inhibition and 2) the inhibition of hippocampal BACE1 expression, thus regulating Aβ processing towards amyloidogenic pathways [34]. Furthermore, our results show that DXI treatment markedly increased IDE expression in the hippocampus, suggesting that the effect of DXI on lowering Aβ deposition might also be attributable to its role in promoting Aβ degradation [35].

Besides Aβ pathology, the abnormal increase of the hyperphosphorylated TAU protein, the major component of intracellular neurofibrillary tangles (NFTs), contributes to the development of AD [36]. The results of the current study show that DXI treatment significantly reduced pTAU at multiple sites in the hippocampus of APP/PS1 mice, a matter of importance given that hyperphosphorylated TAU is directly implicated in memory dysfunction in AD [23,37]. However, Kitazawa and colleagues demonstrated that lipopolysaccharide treatment, a compound which activates brain microglia, significantly exacerbated the TAU pathology in the 3xTg-AD mice without affecting APP processing [37–39]. Thus, microglia activation could directly affect TAU phosphorylation, independently of Aβ modulation.

To further elucidate the mechanism for the DXI-dependent reduction in TAU phosphorylation, we assessed the changes in activation of CDK5 and GSK3β -the two major kinases that phosphorylate TAU- by using specific antibodies [40]. It has been demonstrated that CDK5 activity is regulated by its phosphorylation at Tyr15 site, exhibiting an increased CDK5 activation in APP/PS1 mice [40,41]. Moreover, in vitro and in vivo studies have shown that active c-ABL, a kinase triggered by Aβ, promotes CDK5 phosphorylation at Tyr15 site and increases its activity leading to TAU phosphorylation [41]. We found that DXI treatment reduced the elevated levels of pCDK5 at Tyr15 site in the brain of APP/PS1 mice, suggesting that DXI attenuates TAU hyperphosphorylation through its inhibitory effect on CDK5 signal pathway. Moreover, it is known that c-ABL phosphorylates CDK5 on Y15 through the adaptor protein CABLES, thus stimulating the CDK5 kinase activity [41]. Furthermore, it has been reported that c-ABL can be activated in response to oxidative stress, DNA damage and inflammation. Our results showing a significant reduction of c-ABL, CABLES, pCDK5 (Y15) and some TAU phosphoepitopes in the DXI treated group are in accordance to this data. These findings might be explained by the reduction in the levels of cytokines and interleukins -especifically TNFα- involved in the activation of c-ABL. Moreover, since our results indicate that DXI could alter the CDK5 pathway by decreasing the inflammatory response, CDK5 could be the link between glial activation and TAU phosphorylation. Therefore, c-ABL might be upstream of CDK5 activation and DXI might attenuate TAU phosphorylation through inhibiting the CDK5 signal pathway TNFα/c-ABL/CABLES/CDK5.

The role of impaired brain insulin signaling in the pathogenesis of AD is also supported by several recent preclinical studies showing improvements in cognition, memory and TAU phosphorylation by treatment with intranasal insulin. Our results have shown that DXI treatment increased both pGSK3β and pAKT activity in APP/PS1.

Our results also demonstrate that DXI reverses cognitive deficits in the APP/PS1 mice, since the performance of the NOR test was improved in the DXI treated group against the non-treated APP/PS1. In previous studies, Trinchese and colleagues reported that synaptic plasticity and memory loss in APP/PS1 mice occur as early as 3–4 months of age and it is independent of plaque formation [42,43]. Thus, targeting synapses and molecular mechanisms of memory, mainly CREB phosphorylation, could be a suitable target for AD improvement, since synaptic dysfunction occurs independently of plaque formation.

Previous studies reported that loss of synapses is one of the main neuropathological hallmarks of AD, which strongly correlates with the cognitive decline [14]. The beneficial effects of DXI on cognitive improvement in the APP/PS1 mice are likely attributable to the combined effects of the reduction in pro-inflammatory molecules and BACE1, as well as the increase in p-CREB expression and synaptic integrity [44].

PKA is the main kinase involved in CREB phosphorylation and contributes to the process of memory formation by regulating the classical cAMP/PKA/CREB pathway [44]. However, no-changes on PKA expression were observed in the hippocampus of APP/PS1 mice. Since BACE 1 is involved in the regulation of CREB phosphorylation, we suggest that DXI treatment could increase pCREB levels by targeting BACE1 [45].

We considered APP/PS1 transgenic mice the best model to study DXI effects and mechanisms involved in AD, since these animals show amyloid plaques deposits and TAU hyperphosphorylation. Notwithsantding, no animal model reproduces all the features involved in the human AD. Moreover, only female mice were used in this study, since it has been demonstrated that they present higher vulnerability to AD in comparison with age-matched males.

5. Conclusions

In conclusion, this study shows that DXI could exert multiple beneficial effects against AD, both decreasing the inflammatory response and the levels of amyloid plaques and neurofibrillary tangles, while

enhancing the memory. Thus, our results provide more evidence showing that NSAIDs could reduce AD risk through multiple mechanisms. In addition, this is the first study reporting new targets that could clarify the mechanisms involved in DXI enhancement of memory and cognitive performance in a familial AD animal model. These mechanisms are TNFα/c-ABL/CDK5/TAU inhibition, insulin signaling activation and BACE1/SYP/p-CREB modulation (Fig. 6).

Competing interests

None declared.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.06.003.

References

[1] G. Bedse, F. Di Domenico, G. Serviddio, et al., Aberrant insulin signaling in Alzheimer's disease: current knowledge, Front. Neurosci. 9 (2015) 1–13.
[2] B. Winblad, P. Amouyel, S. Andrieu, et al., Defeating Alzheimer's disease and other dementias: a priority for European science and society, Lancet Neurol. 15 (2016) 455–532.
[3] T. Suzuki, A. Kimura, S. Hata, Alternative cleavage mechanism of human APP by BACE1, Alzheimer's Dement. (2016) 12.
[4] R. Anand, K.D. Gill, A.A. Mahdi, Therapeutics of Alzheimer's disease: past, present and future, Neuropharmacology 76 (2014) 27–50.
[5] D.J. Selkoe, J. Hardy, The amyloid hypothesis of Alzheimer's disease at 25 years, EMBO Mol. Med. 8 (2016) 1–14.
[6] V. Calsolaro, P. Edison, Neuroinflammation in Alzheimer's disease: current evidence and future directions, Alzheimers Dement. 12 (2016) 719–732.
[7] K.L. Viola, W.L. Klein, Amyloid β oligomers in Alzheimer's disease pathogenesis, treatment, and diagnosis, Acta Neuropathol. 129 (2015) 183–206.
[8] P.L. McGeer, J. Rogers, E.G. McGeer, Inflammation, antiinflammatory agents, and Alzheimer's disease: the last 22 years, J. Alzheimer's Dis. 54 (2016) 853–857.
[9] S. Weggen, J.L. Eriksen, P. Das, et al., A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity, Nature 414 (2001) 212–216.
[10] S. Côté, P.-H. Carmichael, R. Verreault, et al., Nonsteroidal anti-inflammatory drug use and the risk of cognitive impairment and Alzheimer's disease, Alzheimer's Dement. 8 (2012) 219–226.
[11] P.L. McGeer, M. Schulzer, G.M. Edith, Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease, Neurology 47 (1996) 425–432.
[12] J.L. Eriksen, S. a. Sagi, T.E. Smith, et al., NSAIDs and enantiomers of flurbiprofen target gamma-secretase and lower Abeta 42 in vivo, J. Clin. Investig. 112 (2003) 440–449.
[13] E. Sánchez-López, M.A. Egea, A. Cano, et al., PEGylated PLGA nanospheres optimized by design of experiments for ocular administration of dexibuprofen–in vitro, ex vivo and in vivo characterization, Colloids Surf. B 145 (2016) 241–250.
[14] M. Ankarcrona, B. Winblad, C. Monteiro, et al., Current and future treatment of amyloid diseases, J. Intern. Med. 280 (2016) 177–202.
[15] X. Zhang, X. Liu, T. Gong, et al., In vitro and in vivo investigation of dexibuprofen derivatives for CNS delivery, Acta Pharmacol. Sin. 33 (2012) 279–288.
[16] D.Q. Jin, J.Y. Sung, Y.K. Hwang, et al., Dexibuprofen (S(+)-isomer ibuprofen) reduces microglial activation and impairments of spatial working memory induced by chronic lipopolysaccharide infusion, Pharmacol. Biochem. Behav. 89 (2008) 404–411.
[17] S.T. Ferreira, J.R. Clarke, T.R. Bomfim, et al., Inflammation, defective insulin signaling, and neuronal dysfunction in Alzheimer's disease, Alzheimer's Dement. 10 (2014) S76–S83.
[18] D. Puzzo, W. Gulisano, A. Palmeri, et al., Rodent models for Alzheimer's disease drug discovery, Expert Opin. Drug Discov. 10 (2015) 703–711.
[19] E. Trushina, L. Zhang, S. Zhang, et al., Restoration of axonal trafficking of mitochondria averts cognitive decline in mouse models of familial Alzheimer's disease, Alzheimer's Dement. 10 (4) (2014) 238.
[20] S.S. Jiao, X. Le Bu, Y.H. Liu, et al., Sex dimorphism profile of Alzheimer's disease-type pathologies in an APP/PS1 mouse model, Neurotox. Res. 29 (2016) 256–266.
[21] S. Abad, J. Camarasa, D. Pubill, et al., Adaptive plasticity in the hippocampus of young mice intermittently exposed to MDMA could be the origin of memory deficits, Mol Neurobiol. 53 (2016) 7271–7283.
[22] M. Ettcheto, D. Petrov, I. Pedros, et al., Evaluation of neuropathological effects of a high-fat diet in a presymptomatic Alzheimer's disease stage in APP/PS1 mice, J. Alzheimer's Dis. 54 (2016) 233–251.
[23] D. Porquet, P. Andrés-Benito, C. Griñán-Ferré, et al., Amyloid and tau pathology of familial Alzheimer's disease APP/PS1 mouse model in a senescence phenotype background (SAMP8), Age 37 (2015) 1–17.
[24] O. Busquets, M. Ettcheto, M. Pallàs, et al., Long-term exposition to a high fat diet favors the appearance of β-amyloid depositions in the brain of C57BL/6J mice. A potential model of sporadic Alzheimer's disease, Mech. Ageing Dev. (2016) in press.
[25] I. Pedrós, D. Petrov, M. Allgaier, et al., Early alterations in energy metabolism in the hippocampus of APPswe/PS1dE9 mouse model of Alzheimer's disease, Biochim. Biophys. Acta – Mol. Basis Dis. 1842 (2014) 1556–1566.
[26] M. Ettcheto, D. Petrov, I. Pedrós, et al., Hypercholesterolemia and neurodegeneration. comparison of hippocampal phenotypes in LDLr knockout and APPswe/PS1dE9 mice, Exp. Gerontol. 65 (2015) 69–78.
[27] Y.H. Li, J. Li, Y. Huang, et al., Gastroprotective effect and mechanism of amtolmetin guacyl in mice, World J. Gastroenterol. 10 (2004) 3616–3620.
[28] P. Eikelenboom, E. van Exel, J.J. Hoozemans, et al., Neuroinflammation – an early event in both the history and pathogenesis of Alzheimer's disease, Neurodegener. Dis. 7 (2010) 38–41.
[29] M. Garcia-Alloza, E.M. Robbins, S.X. Zhang-Nunes, et al., Characterization of amyloid deposition in the APPswe/PS1dE9 mouse model of Alzheimer disease, Neurobiol Dis. 24 (2006) 516–524.
[30] L.E. Rojo, J.A. Fernández, A.A. Maccioni, et al., Neuroinflammation: implications for the pathogenesis and molecular diagnosis of Alzheimer's Disease, Arch. Med. Res. 39 (2008) 1–16.
[31] E. Sánchez-López, M. Ettcheto, M.A. Egea, M. Espina, A.C. Calpena, J. Folch, A. Camins, M.L. García, New potential strategies for Alzheimer's disease prevention: pegylated biodegradable dexibuprofen nanospheres administration to APPswe/PS1dE9, Nanomedicine 13 (3) (2017) 1171–1182.
[32] L. Ruan, Z. Kang, G. Pei, et al., Amyloid deposition and inflammation in APPswe/PS1dE9 mouse model of Alzheimer's disease, Curr. Alzheimer Res. 6 (2009) 534–540.
[33] S. Habbas, M. Santello, D. Brecker, et al., Neuroinflammatory TNFα impairs memory via astrocyte signaling, Cell 163 (2015) 1730–1741.
[34] F.M. Laird, BACE1, a major determinant of selective vulnerability of the brain to amyloid- amyloidogenesis, is essential for cognitive, emotional, and synaptic functions, J. Neurosci. 25 (2005) 11693–11709.
[35] J. Baranello, L. Robert, K. Bharani, et al., Amyloid-beta protein clearance and degradation (ABCD) pathways and their role in Alzheimer's disease, Curr. Alzheimer Res. 12 (2015) 32–46.
[36] J. Wang, L. Tan, H.F. Wang, et al., Anti-Inflammatory drugs and risk of Alzheimer's Disease: an updated systematic review and meta-analysis, J. Alzheimers Dis. 44 (2015) 385–396.
[37] K. Iqbal, I. Grundke-Iqbal, Alzheimer neurofibrillary degeneration: significance, etiopathogenesis, therapeutics and prevention: Alzheimer review series, J. Cell Mol. Med. 12 (2008) 38–55.
[38] M. Kitazawa, Lipopolysaccharide-induced inflammation exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease, J. Neurosci. 25 (2005) 8843–8853.
[39] I. Carreras, A.C. McKee, J.K. Choi, et al., R-flurbiprofen improves tau, but not Aß pathology in a triple transgenic model of Alzheimer's disease, Brain Res. 1541 (2013) 115–127.
[40] H. Patzke, U. Maddineni, R. Ayala, et al., Partial rescue of the p35-/- brain phenotype by low expression of a neuronal-specific enolase p25 transgene, J. Neurosci. 23 (2003) 2769–2778.
[41] O. Hantschel, G. Superti-Furga, Regulation of the c-Abl and Bcr-Abl tyrosine kinases, Nat. Rev. Mol. Cell Biol. 5 (2004) 33–44.
[42] F. Trinchese, S. Liu, F. Battaglia, et al., Progressive age-related development of Alzheimer-like pathology in APP/PS1 mice, Ann. Neurol. 55 (2004) 801–814.
[43] W. Zhang, M. Bai, Y. Xi, et al., Early memory deficits precede plaque deposition in APPswe/PS1dE9 mice: involvement of oxidative stress and cholinergic dysfunction, Free Radic. Biol. Med. 52 (2012) 1443–1452.
[44] A.F. Teich, R.E. Nicholls, D. Puzzo, et al., Synaptic therapy in Alzheimer's Disease: a CREB-centric approach, Neurotherapeutics 12 (2015) 29–41.
[45] Y. Chen, X. Huang, Y. Zhang, et al., Alzheimer's β-secretase (BACE1) regulates the cAMP/PKA/CREB pathway independently of β-amyloid, J. Neurosci. 32 (2012) 11390–11395.

PUBLICACIÓN III

Peripheral and central effects of memantine in a mixed preclinical mice model of obesity and familial Alzheimer's disease

Miren Ettcheto, Elena Sánchez-López, Yaiza Gómez-Mínguez, Henrry Cabrera, Oriol Busquets, Carlos Beas-Zarate, Maria Luisa García, Eva Carro, Gemma Casadesus, Carme Auladell, Manuel Vaquez-Carrera, Jaume Folch and Antoni Camins (2018) *Molecular neurobiology*

Resumen

Hay evidencias crecientes de que la obesidad asociada a la DMT2 y la edad son factores de riesgo para el desarrollo de la enfermedad de Alzheimer EA. Sin embargo, los mecanismos moleculares por los cuales la obesidad interacciona con el βA para promover el declive cognitivo mantiene sin esclarecerse. La memantina (MEM), antagonista del receptor de NMDA, actualemente es utilizado como fármaco para el tratamiento de la EA. No obstante, pocos estudios han analizado su efecto en un modelo genético preclínico de esta enfermedad neurodegenerativa exacerbado por la obesidad inducida por la ingesta de una dieta rica en grasa.

Por ello, el objetivo del presente estudio es esclarecer los efectos de la MEM en el modelo familiar de la EA, el cual presenta alteraciones en memoria y aprendizaje y resistencia a la insulina inducida por la ingesta de dieta grasa. Además de intentar determinar los posibles mecanismos moleculares que conectan ambas, la EA y la DMT2 patologías.

El presente estudio se realizó con ratones salvajes C57BL/6 (Wild type; WT) y APP/PS1. Estos animales fueron alimentados tanto con dieta normal como con dieta rica grasa hasta los 6 meses de edad, además de ser tratados con agua suplementada con MEM, a una dosis de 30 mg/kg, durante los últimos 12 meses.

Nuestro estudio demostró que la MEM mejora las alteraciones metabólicas producidas por la dieta rica en grasa en este modelo de EA familiar. La evaluación del comportamiento confirmó que el tratamiento también incrementa las habilidades de aprendizaje y disminuye la pérdida de memoria en los ratones APP/PS1. Además, nuestros resultados demostraron que la MEM mejora la vía de señalización de la insulina mediante la regulación al alza de los niveles proteicos de la proteína cinasa B (PKB; AKT), así como, del elemento de unión a la respuesta de cAMP (en ingles cAMP response element binding; CREB), modulando la vía amiloidogénica, lo que se traduce en una disminución de β amiloide (βA). Asimismo, en este estudio se ha observado que la MEM activa moléculas involucradas en la vía de la insulina hepática, tales como el sustrato del receptor de la insulina 2 (IRS2), el cual juega un papel esencial en la regulación de la resistencia a la insulina en el hígado.

Los presentes resultados aportan nuevos conocimientos con relación al papel de la MEM no solo en lo que respecta al tratamiento de la EA, sino que también como posible aplicación para desórdenes metabólicos periféricos donde el βA juega un papel clave, como es el caso de la DMT2.

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Peripheral and Central Effects of Memantine in a Mixed Preclinical Mice Model of Obesity and Familial Alzheimer's Disease

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Abstract

There is growing evidence that obesity associated with type 2 diabetes mellitus (T2DM) and aging are risk factors for the development of Alzheimer's disease (AD). However, the molecular mechanisms through which obesity interacts with β-amyloid (Aβ) to promote cognitive decline remains poorly understood. Memantine (MEM), a *N*-methyl-D-aspartate receptor antagonist, is currently used for the treatment of AD. Nonetheless, few studies have reported its effects on genetic preclinical models of this neurodegenerative disease exacerbated with high-fat diet (HFD)-induced obesity. Therefore, the present research aims to elucidate the effects of MEM on familial AD HFD-induced insulin resistance and learning and memory impairment. Furthermore, it aspires to determine the possible underlying mechanisms that connect AD to T2DM. Wild type and APPswe/PS1dE9 mice were used in this study. The animals were fed with either chow or HFD until 6 months of age, and they were treated with MEM-supplemented water (30 mg/kg) during the last 12 weeks. Our study demonstrates that MEM improves the metabolic consequences produced by HFD in this model of familial AD. Behavioural assessments confirmed that the treatment also improves animals learning abilities and decreases memory loss. Moreover, MEM treatment improves brain insulin signalling upregulating AKT, as well as cyclic adenosine monophosphate response element binding (CREB) expression, and modulates the amyloidogenic pathway, which, in turn, reduced the accumulation of Aβ. Moreover, this drug increases the activation of molecules involved with insulin signalling in the liver, such as insulin receptor substrate 2 (IRS2), which is a key protein regulating hepatic

Jaume Folch and Antoni Camins are senior co-authors.

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resistance to insulin. These results provide new insight into the role of MEM not only in the occurrence of AD treatment, but also in its potential application on peripheral metabolic disorders where Aβ plays a key role, as is the case of T2DM.

Keywords Memantine · Obesity · Diabetes · APPswe/PS1dE9 · High-fat diet · Alzheimer's disease

Introduction

Currently, there is no effective treatment for Alzheimer's disease (AD), a degenerative condition affecting more than 40 million people worldwide [1–7]. The main pathological hallmarks of this neurodegenerative disease are the accumulation of β-amyloid (Aβ) plaques in extracellular spaces, as well as in the blood vessels, and the aggregation of the microtubule protein TAU in neurofibrillary tangles (NFTs) in neurons [3–8].

Memantine (MEM) is a drug approved for the treatment of moderate to severe AD. It is a low-affinity uncompetitive voltage-dependent *N*-methyl-D-aspartate receptor (NMDAR) antagonist, which in pathological conditions, blocks the excessive activation of the NMDAR [5, 9, 10]. In a recent study, Alley and colleagues reported that MEM decreases the production of Aβ peptide in a preclinical model of AD [11]. This effect is probably mediated through drug secretase enzyme inhibition and also its modulation of amyloid precursor protein (APP) endocytosis [11, 12].

Moreover, recent studies have suggested that AD is a brain-specific diabetes (cerebral diabetes or diabetes type 3) [2, 13–21]. Preclinical and clinical epidemiological studies have confirmed this association and have demonstrated that impaired metabolic parameters such as hyperglycaemia and hyperinsulinemia are positively correlated with AD development [14, 15, 19, 20]. In addition, it has been reported that AD brains exhibit defective insulin signalling and decreased responsiveness to insulin [17]. Thus, all this data correlates positively with the type 3 diabetes theory [19]. Furthermore, current research approaches proposed intranasal insulin administration as a treatment for AD, improving memory impairment in healthy adults without affecting circulating levels of insulin or glucose [2]. Consequently, insulin may have important therapeutic implications at early and/or intermediate phases of AD because improves AD-related cognitive deficits [2, 14, 20–22]. Recently, Wu and colleagues observed peripheral metabolic changes in plasma and liver extracts of an AD mice model, suggesting that brain Aβ is not only involved in the formation of amyloidal plaques in the brain but also causes metabolic changes in peripheral tissues such as the liver [23]. These data are in agreement with previous studies of Zhang and colleagues reporting that Aβ exerts liver damage effects causing peripheral insulin resistance [24, 25]. Furthermore, Clarke and colleagues reported that intracerebral injected Aβ cause peripheral glucose intolerance and insulin resistance through and inflammatory processes in the hypothalamus [15]. In this line, β-25-35 was injected into the hypothalamus and an increase in plasma glucose levels was demonstrated due to enhanced hepatic glucose production [26, 27]. Taken together, these results suggest that AD should be evaluated as a body-wide human disease and reveals an important interaction between brain and peripheral tissues on its development.

In this sense, it has been demonstrated that MEM preserves cerebral energy status during experimentally induced hypoglycaemia. Moreover, in a recent study, Bahramian and colleagues reported that the combination of insulin and MEM was effective in improving memory loss [22]. Therefore, the drug MEM due to the mechanism trough Aβ decreasing could be useful in order to treat also type 2 diabetes mellitus.

Due to the emerging hypothesis relating AD with the metabolic insulin pathway, the aim of present research is to clarify if MEM improves central and peripheral pathologies in a preclinical mixed model of type 2 diabetes mellitus (T2DM) and familial AD. For this purpose, APPswe/PS1dE9 (APP/PS1) mice were fed with a high-fat diet (HFD), which has been demonstrated to induce glucose intolerance and insulin resistance in previous research from our group [28]. Our working hypothesis is that HFD favours brain AD neuropathology and, at the same time, Aβ is involved in peripheral metabolic alterations thus forming a positive feedback cycle that will be alleviated with MEM through Aβ inhibition. Therefore, MEM could be a suitable treatment in order to modify and/or forestall this damaging process.

Material and Methods

Animals and Treatment

In this study, 6-month-old male C57BL/6J wild type (WT) and APP/PS1 mice were used. These transgenic mice express a Swedish (K594M/N595L) mutation of a chimeric mouse/human APP (mo/huAPP695swe), together with the human exon-9-deleted variant of presenilin 1 (PS1) (PS1-dE9). Animals were divided into four groups (WT, APP/PS1) fed with high fat diet (45% fat mainly from hydrogenated coconut oil, Research Diets Inc., New Brunswick, USA) and two of them additionally treated with supplemented water containing MEM at a dose of 30 mg/kg/day since the 4 months of age until their sacrifice (see Fig. 1 of supplementary material). Treatments were weekly prepared and the drinking water was replaced by a new bottle once per week. The dose was calculated and prepared by weighting the mice once per week, and the results of MEM dose were obtained based on the fact

that the mice estimate to drink around 3.2 ml per day on the first place. Moreover, the water intake was measured by weighting the bottles with the treatment at the beginning and at the end of the week. To assure that the treatment was correct, plasma MEM levels were recorded. At least, 10 animals per group were used. All animals were given access to food or water ad libitum and kept under controlled temperature, humidity and light conditions. Every effort was made to reduce the number of animals used and minimize their suffering. Mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

MEM was quantified on plasma. Blood samples were extracted from the facial vein and samples were centrifuged during 20 min at 2000 r.p.m. adding EDTA (10 μl K_2EDTA 18 mg/ml) to avoid blood coagulation. Mice were sacrificed by cervical dislocation and immediately brains were removed and preserved at − 80 °C. Amantadine was added as internal standard and MEM extraction was carried out using organic solvents diethyl ether–chloroform for blood samples). Solvents were evaporated under nitrogen flow, and samples were reconstituted with methanol [29].

Glucose and Insulin Tolerance Tests

Intraperitoneal glucose tolerance tests (IP-GTT) and insulin tolerance tests (ITT) were performed in accordance with the previously published guidelines [28].

For IP-GTT, mice were fasted, at least, for 6 h. Blood glucose levels in the tail vein were measured at 0, 5, 15, 30, 60, and 120 min after intraperitoneal glucose injection at a dose of 1 g/kg (diluted in water) with Accu-Chek Aviva blood glucose meter (Roche; Mannheim, Germany).

ITT was performed through human insulin intraperitoneal injection at a dose of the 0.25 IU/kg diluted in saline (Humulina Regular, 100 IU/ml/Lilly, S.A.; Madrid, Spain). Blood glucose levels were measured at 0, 15, 30, 45, 60, and 90 min after the insulin administration. If during this time blood glucose levels dropped to below 20 mg/dl, 1 g/kg glucose was administered to counteract the effects of insulin, in order to reduce animal suffering.

Cognitive Function Tests

Morris Water Maze

Spatial memory and learning were assessed by the Morris water maze (MWM). Briefly, a white platform was submerged on a white-water tank (temperature 21 ± 2 °C) in the middle of the northeast quadrant. Behavioural data were acquired and analysed using a computerized video tracking system. A 6-day navigation test was performed with five trials per day and a probe trail. Animals were allowed to swim on the water tank for 60 s and allowed to remain on the platform for 10 s. If after 60 s a mouse was not able to find the platform, it was guided to it and left there for 30 s. The probe trail was performed the day after the last training. This day, the hidden platform was removed, and the mice were released from the southwest quadrant and allowed to swim for 60 s [30]. Results were calculated individually for each animal. At least, 10 animals per group were used.

Novel Object Recognition Test

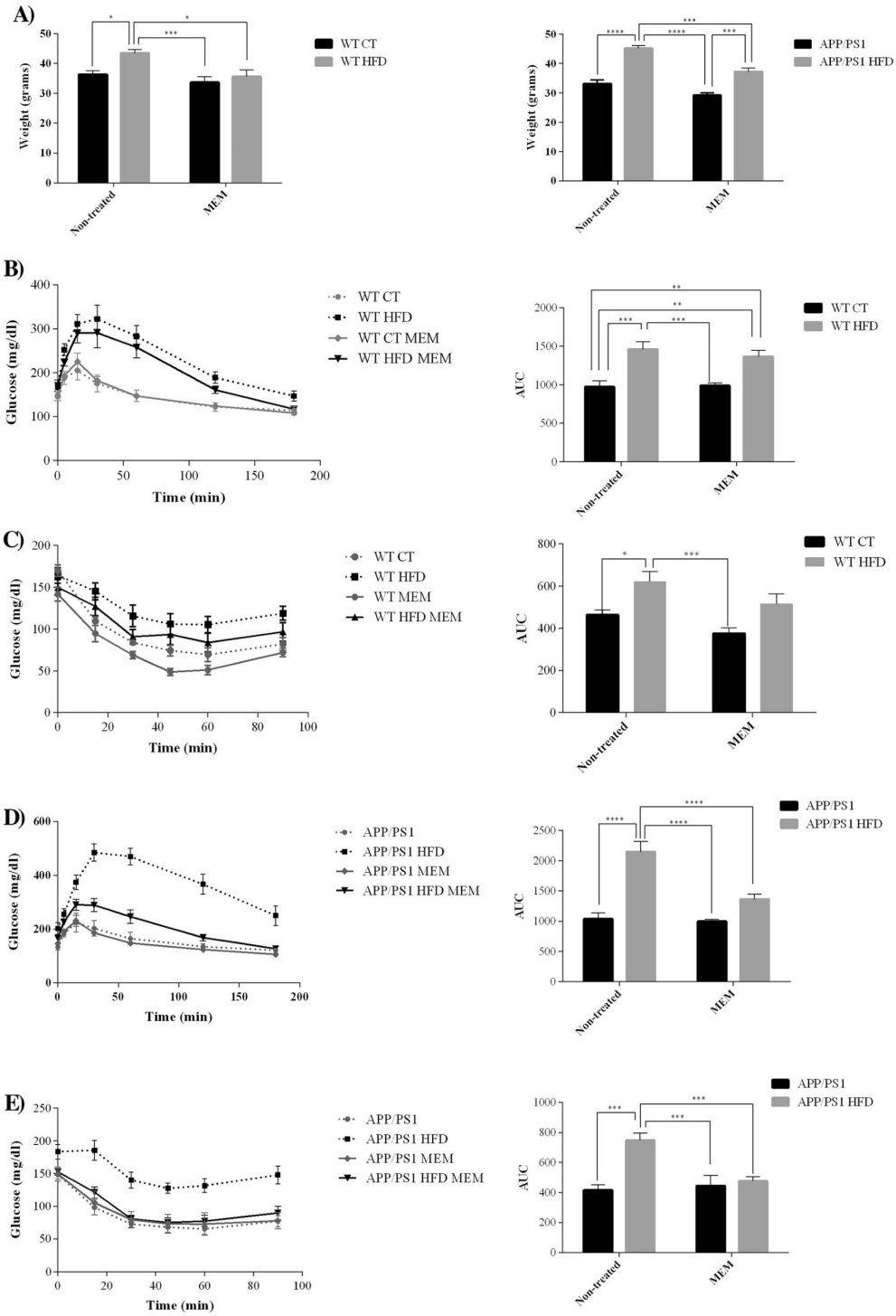
The hippocampal-dependent recognition memory of mice was assessed through novel object recognition test (NORT) [28]. Briefly, the first 3 days of the test each animal was left to get used to the circular open field box of 100 cm of diameter without any objects (10 min/session). On the fourth day, mice were left for 10 min to explore two identical objects (A + A). On the fifth day, each mouse was exposed for 10 min to a familiar object A and a novel object, namely B. Between each trial, the objects and the open field box were cleaned with soap and water in order to avoid the presence of olfactory signs. Recorded videos were analysed and data was measured by discrimination index (DI), which was defined as (exploration time of new object − old object)/total exploration time) for each mouse, measured in seconds. Exploration was defined as sniffing or touching an object. Mice with a total exploration time of < 5 s for an object were removed from analyses.

Western Blot Method

At 6 months, four to five animals per group were sacrificed by cervical dislocation. To perform brain and liver protein extraction, tissues were homogenized in lysis buffer (50 mM Tris HCl pH: 7.4, 150 mM NaCl, 5 mM EDTA, 1%Triton X-100) and protease (Complete, Roche Diagnostics, Barcelona, Spain) and phosphatase inhibitors were added. Protein concentration was determined using Pierce BCA Protein Assay kit (Pierce Company, Rockford, MI, USA), and samples were stored at − 80 °C until their use. Aliquot of samples containing 10 μg of protein was analysed by Western blot, and the experimental procedure was carried out as previously described [28]. The measurements were expressed and normalized with GAPDH, used as a control loading protein. The antibodies used are detailed in Table 1 of the Supplementary material.

Real-Time PCR Method

The RNA of hippocampus and liver was isolated through Trizol-based extraction method (Life Technologies Corporation; Carlsbad, CA, USA). Briefly, the tissue was homogenized with Trizol, chloroform was added immediately, and RNA was precipitated from the aqueous phase using


A)
Weight (grams)
WT CT
WT HFD
Non-treated
MEM
APP/PS1
APP/PS1 HFD
B)
Glucose (mg/dl)
Time (min)
WT CT MEM
WT HFD MEM
AUC
C)
WT MEM
D)
APP/PS1 MEM
APP/PS1 HFD MEM
E)

◀ **Fig. 1** **a** Body weight comparison of the different groups and genotypes at the end of the study. **b** Glucose tolerance test (GTT) results of WT mice (left side corresponding to all the points assessed, right side corresponding to the AUC calculation). **c** Insulin tolerance test (ITT) results of WT mice (left side corresponding to all the points assessed, right side corresponding to the AUC calculation). **d** Glucose tolerance test (GTT) results of APP/PS1 mice (left side corresponding to all the points assessed, right side corresponding to the AUC calculation). **e** Insulin tolerance test (ITT) results of APP/PS1mice (left side corresponding to all the points assessed, right side corresponding to the AUC calculation). Values are expressed as mean ± S.E.M.; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. $n = 10$ animals/group

isopropanol at −20 °C. The pellet was reconstituted in RNAse-free water, and RNA concentration was measured using a NanoDropTM 1000 Spectrophotometer (Thermo Scientific, MA, USA). Two microgram of total RNA were reversely transcribed with the High Capacity cDNA Reverse Transcription Kit, according to manufacturer's protocol (Applied Biosystems). Equivalent amounts of cDNA of each animal were used to perform quantitative real-time PCR using SYBRGreen® qPCR Master Mix (K0253, Pierce, Thermo Fisher Scientific) through StepOnePlusTM Real-Time PCR System (Applied Biosystems). Samples were run by triplicate and the values were normalized to *Gapdh*. Primers of analysed genes are detailed in Table 2 of Supplementary Material [28].

Immunofluorescence and Thioflavin Staining

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were stored in the same solution overnight O/N at 4 °C and cryoprotected in 30% sucrose-PFA-PB solution. Samples were frozen at −80 °C, and coronal sections of 20 μm of thickness were obtained using a cryostat (Leica Microsystems, Wetziar, Germany).

Briefly, to detect plaque deposition, brain slices were incubated with 0.002% thioflavin S (ThS 0.002%, Sigma-Aldrich) washed twice with ethanol 50% and rinsed with PBS 0.1 M. Afterwards, the sections were incubated with 0.1 μg/ml Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) during 15 min in the dark at room temperature and washed with PBS-T followed by PBS. To quantify them, similar and comparable histological areas were selected, particularly the hippocampus and the cortex, and they were manually counted by three blinded investigators. Moreover, the area was measured using ImageJ software [30].

For the immunofluorescence, free-floating coronal sections were rinsed in 0.1 mol/L PBS pH 7.35 and after they in PBST (PBS 0.1 M, 0.2% Triton X-100). Then, they were preincubated in a blocking solution (10% foetal bovine serum (FBS), 1% Triton X-100, PBS 0.1M+ 0.2% gelatin) for 2 h at room temperature. Later, sections were washed with PBST (PBS 0.1 M, 0.5% Triton X-100) five times for 5 min each and incubated O/N at 4 °C with different primary antibodies (Table 4, Supplementary material). On the second day, brain slices were washed again with PBST (PBS 0.1 M, 0.5% Triton X-100) five times for 5 min and incubated with the corresponding secondary antibody for 2 h at room temperature. After that, sections were counterstained with 0.1 μg/ml Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA).

All the samples were mounted onto gelatinised slides with Fluoromount medium (Sigma-Aldrich, St. Louis, MO, USA). And image acquisition was performed with and epifluorescence microscope filter (BX41, Laboratory Microscope, Melville, NY-Olympus America Inc.)

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6. Data were expressed as mean ± SEM. Significant differences were determined by two-way ANOVA followed by Tukey's post-hoc test. The level of significance for the acceptance was $p < 0.05$.

Results

MEM Treatment Improves HFD-Related Pathology Only in APP/PS1 Mice

Before sacrifice, drug levels were recorded confirming that the treated animals showed a steady-state plasma concentration of 0.90 μM ± 0.18 in accordance with the reported therapeutic drug levels [31]. As expected, HFD intake leads to a progressive diet-induced obesity, showing a significant increase of body weight in both, WT ($p < 0.05$) and APP/PS1 ($p < 0.0001$) mice compared to animals fed with chow diet (Fig. 1a). This weight increase was accompanied by impaired glucose and insulin tolerance in WT ($p < 0.001$; $p < 0.0001$ respectively), and APP/PS1 ($p < 0.05$; $p < 0.001$) mice as described previously [28]. Moreover, our results demonstrated that MEM impedes body weight increasing in HFD-fed mice in both genotypes, WT ($p < 0.05$) and APP/PS1 ($p < 0.001$). Interestingly, IP-GTT and ITT showed that there were no differences in glucose blood concentrations between APP/PS1 HFD mice treated with MEM and animals fed with normal diet, but the levels were significantly higher in APP/PS1 HFD mice without MEM treatment ($p < 0.0001$). By contrast, in the case of WT mice, there were no differences between treated and no treated WT HFD mice (Fig. 1b–e).

Taken these data into account, we focused our research on the effects of MEM in APP/PS1 mice fed with both HFD and chow (control, CT) diet.

MEM Treatment Improves Cognitive Alterations in APP/PS1 Mice Fed with HFD

In this study, we first evaluated whether MEM treatment would provide functional rescue of cognitive deterioration in

transgenic mice by using both, the NORT and MWM cognitive tests. The training results shown in Fig. 2a confirmed the impaired learning ability of APP/PS1 and APP/PS1 HFD mice compared with WT during the 6 days of training. In addition, it can be observed that APP/PS1 mice fed with HFD and treated with MEM showed a significant improvement on the test performance compared to non-treated groups (APP/PS1 $p < 0.01$; APP/PS1 HFD $p < 0.05$). Interestingly, both groups of treated APP/PS1 mice demonstrated a significant increase in DI of NORT compared to non-treated mice ($p < 0.05$), thus indicating that the drug enhanced memory effects (Fig. 2b).

At the molecular level, we evaluated protein kinase A (PKA), p-CREB (Ser133) and CREB due to their direct involvement in the memory consolidation process. As shown in Fig. 2c, PKA protein levels were significantly increased in APP/PS1 MEM mice compared to non-treated animals ($p < 0.01$). In the case of p-CREB, MEM-treated mice showed a significant increase compared to non-treated animals ($p < 0.001$ for APP/PS1 and $p < 0.05$ for mice fed with HFD). Thus, these data indicate that MEM treatment could improve the memory process through the p-CREB pathway. Afterwards, mRNA expression of early genes implicated in memory consolidation like *Bdnf* and *cFos* were tested. As observed in Fig. 2d, *Bdnf* mRNA expression did not show any changes, by contrast, *cFos* was dreadfully lower in APP/PS1 mice fed with HFD but significantly increased in the same mice with MEM treatment ($p < 0.05$). Furthermore, as shown in Fig. 2e, MEM induced a consistent increase in synaptophysin (SYP) labelling in the hippocampus of MEM treated APP/PS1 mice compared to vehicle-treated APP/PS1 mice.

Finally, Western blot detection of NMDAR1, NMDAR2β and pNMDAR2β (Tyr1472) did not show significant differences among groups. However, as can be observed in Fig. 3b, *Nmdar2b* mRNA expression of MEM treated groups was significantly decreased compared with APP/PS1 HFD (MEM APP/PS1, $p < 0.05$; MEM APP/PS1 HFD, $p < 0.01$).

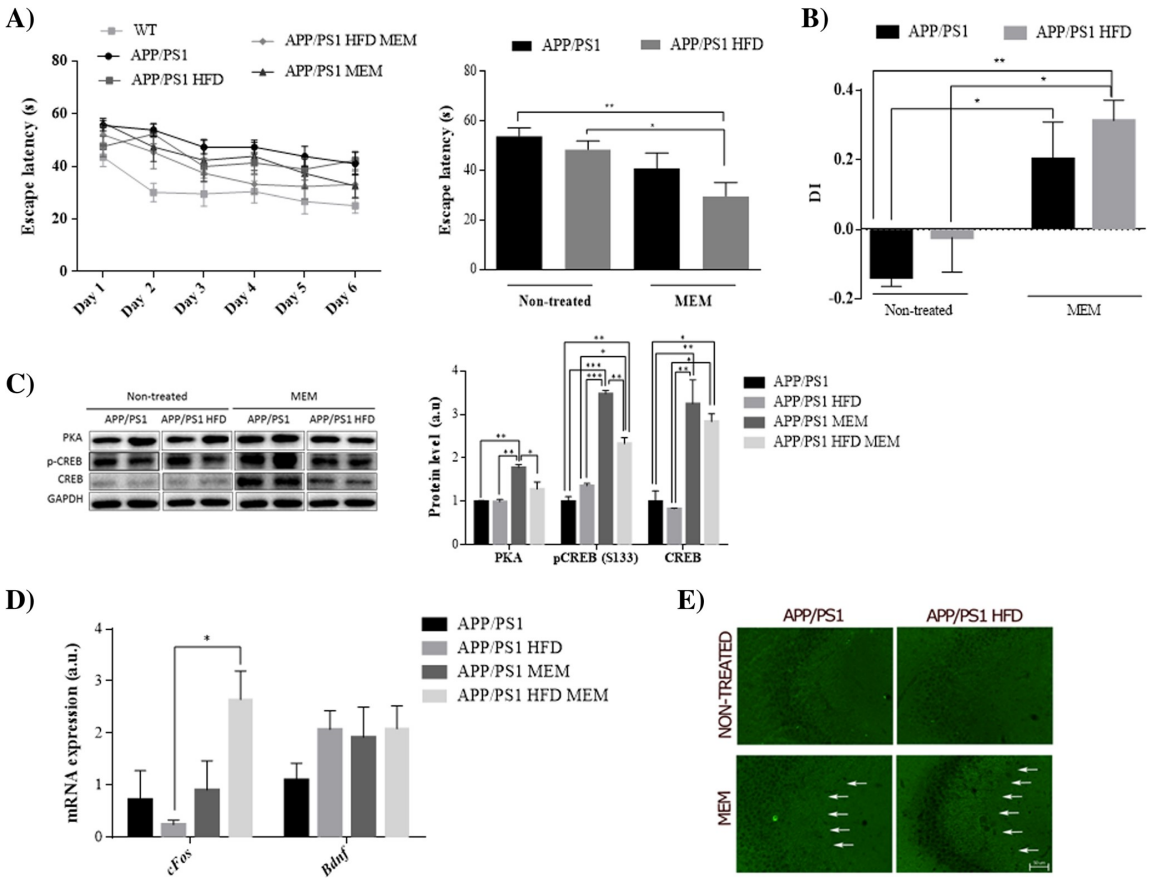


Fig. 2 Cognition analysis. **a** Escape latency evolution during the training days and on the probe trial. **b** DI corresponding to the NORT. **c** Protein levels of PKA, p-CREB and CREB measured by Western blotting. **d** mRNA levels of *Bdnf* and *cFos*. **e** Synaptophysin levels in hippocampus. Values are expressed as mean ± S.E.M.; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. $n = 10$ animals/group

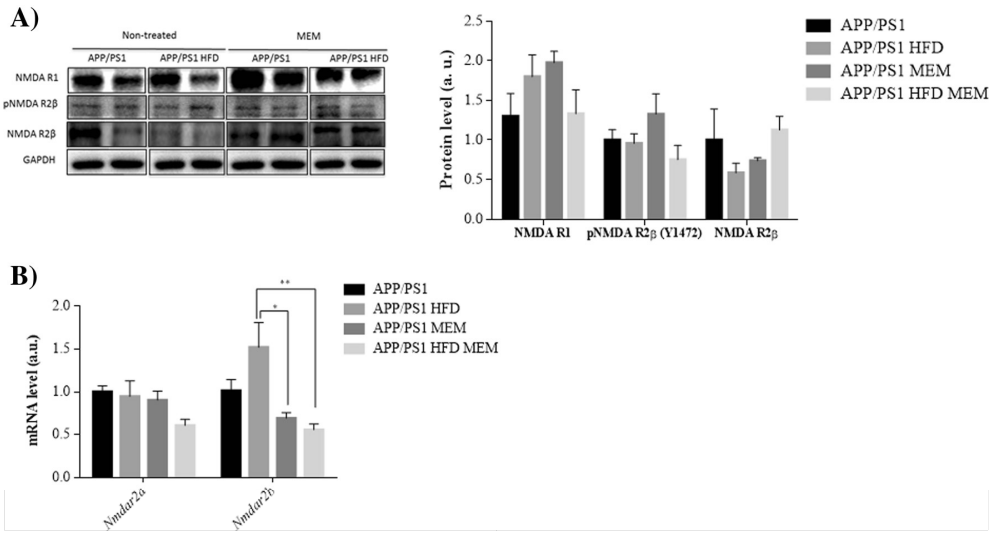


Fig. 3 NMDA receptor studies. **a** Western blot of NMDA R1, NMDA R2β and pNMDA R2β and **b** mRNA of *Nmdar2a* and *Nmdar2b*. Values are expressed as mean ± S.E.M.; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. $n = 5$ animals/group

MEM Reduces Aβ Plaques and APP Processing in APP/PS1 Mice Fed with HFD

The number of Aβ plaques detected in different areas of the brain (hippocampi and cortex) was significantly higher in mice APP/PS1 HFD compared to mice APP/PS1 fed with control diet ($p < 0.001$). Furthermore, our data demonstrated that MEM treatment lowered Aβ plaque burden in the brain of APP/PS1 mice ($p < 0.05$) and APP/PS1 HFD ($p < 0.0001$). Besides, the area of the plaques was smaller in MEM groups (Fig. 4a). Thus, the effects of MEM on APP processing and its underlying mechanisms were investigated by Western blot. As shown in Fig. 4b, quantitative analysis demonstrated a significant decrease in full-length APP protein levels in the hippocampus of MEM-treated APP/PS1 fed with HFD mice against non-treated mice ($p < 0.05$). However, no significant differences were observed among the groups fed with CT diet. Interestingly, BACE protein levels were significantly decreased in mice treated with MEM in both APP/PS1 ($p < 0.001$) and APP/PS1 HFD ($p < 0.01$) groups compared to non-treated APP/PS1. By contrast, ADAM10, a protein involved in non-amyloidogenic pathway, showed a significant increase in APP/PS1 HFD MEM treated animals compared to APP/PS1 MEM mice ($p < 0.01$). As observed in Fig. 4c, mRNA results did not show significant differences among groups.

MEM Reduces Neuroinflammation in APP/PS1 Fed with HFD

GFAP-immunoreactive astrocytes and IBA1-immunoreactive microglia were abundant in the cerebral cortex of non-treated APP/PS1 mice compared with MEM-treated APP/PS1 mice (Fig. 4d). Furthermore, mRNA expression of *Il6* was significantly increased in APP/PS1 mice fed with HFD ($p < 0.05$) and MEM treatment reduced its expression ($p < 0.05$). In the case of *Tnfα*, two-way ANOVA analyses demonstrated a significant effect of MEM ($p < 0.01$) (Fig. 4e).

MEM Improves Insulin Pathway in the Hippocampus of APP/PS1 Mice Fed with a HFD

Our results showed a significant increase in PTP1B protein levels in APP/PS1 HFD mice compared to APP/PS1 ($p < 0.01$), which were significantly reduced in animals treated with MEM ($p < 0.001$). Moreover, p-AKT showed a significant increase in MEM treated animals ($p < 005$). In the case of pGSK3β, two-way ANOVA analyses demonstrated a significant effect in the treatment ($p < 0.05$). No differences were observed in AKT and GSK3β protein levels (Fig. 4f).

MEM Improves Insulin Pathway in the Liver of APP/PS1 Mice Fed with a HFD

As shown in Fig. 5a, hepatic IR protein levels showed a significant increase in APP/PS1 MEM mice compared to non-treated group ($p < 0.05$). However, although p-IR showed a slight increase with MEM treated groups compared to non-treated group, there were not significant differences. p-IRS2 and IRS2 were significantly increased in APP/PS1 MEM in comparison with non-treated ($p < 0.01$) and APP/PS1 HFD mice ($p < 0.05$). In addition, our data demonstrated a significant reduction of p-AKT ($p < 0.05$) and p-GSK3β ($p < 0.05$) in APP/PS1 mice fed

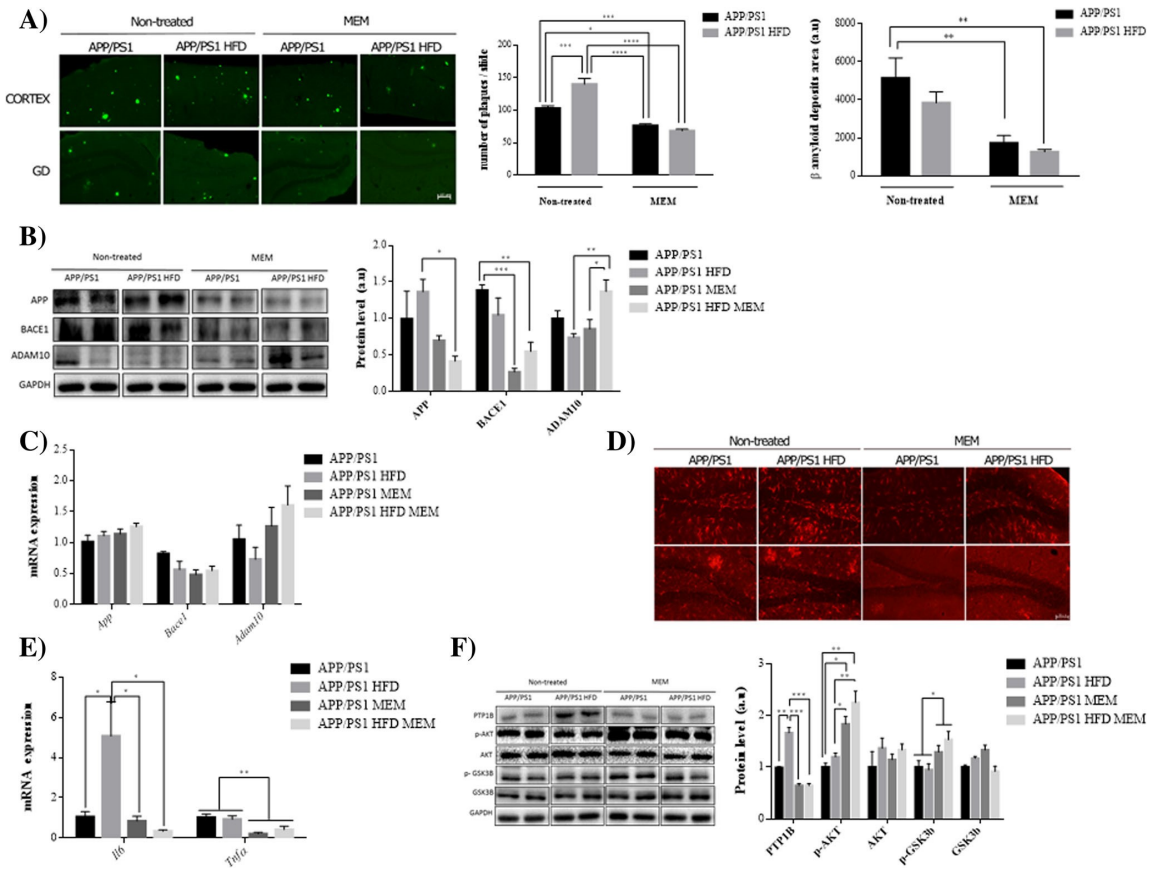


Fig. 4 **a** Aβ plaques immunostaining, counting and plaques area. DG refers to dentate gyrus. **b** Representative GAPDH-normalized immunoblot images and quantification ($n = 4$–6) of molecules related to amyloid signalling (APP, BACE1 and ADAM10) of the hippocampal extracts of HFD-fed 6-month-old APP/PS1 and MEM treated mice. **c** mRNA expression levels of *App*, *Bace1* and *Adam10*. **d** GFAP and Iba-1 immunostaining against astrocytes and microglia, respectively. **e** mRNA expression levels of *il6* and *tnfα*. **f** Representative GAPDH-normalized immunoblot images and quantification ($n = 4$–6) of molecules related to insulin signalling (PTP1B, p-AKT, AKT, p-GSK-3β and GSK3β) in the hippocampal extracts of HFD-fed 6-month-old APP/PS1 and MEM treated mice. Values are expressed as mean ± S.E.M.; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. $n = 5$ animals/group

with HFD; by contrast, p-GSK3B was significantly increased in both APP/PS1- and APP/PS1 HFD-treated animals ($p < 0.01$ and $p < 0.05$ respectively). Lastly, no differences were observed among experimental groups regarding AKT; however, GKS3β protein levels were significantly increased in APP/PS1 MEM compared to APP/PS1 ($p < 0.001$), APP/PS1 HFD ($p < 0.01$) and APP/PS1 HFD MEM ($p < 0.01$).

Evaluation of the Inflammatory Process on APP/PS1 Mice Liver

The expression of inflammatory markers on the liver was also evaluated. No differences were observed relative to TNFα and NFκβ protein levels among groups. Finally, to determine if MEM effects in the peripheral parameters were mediated by NMDA hepatic or cerebral receptor blockade, the levels of NMDAR1 protein and mRNA expression in the liver were determined. Our data demonstrated the absence of this protein in the liver compared to hippocampus tissue (Fig. 5c). Furthermore, mRNA expression of *Nmdar2a* and *Nmdar2b* was analysed by RT-PCR and no amplification was observed (data not shown).

Discussion

Numerous epidemiological studies have demonstrated an increased incidence of AD in patients with type T2DM and vice versa; however, the mechanistic interactions between them remain unknown [32–37].

The present study demonstrates that MEM improves significantly the neuropathological hallmarks of AD such as the

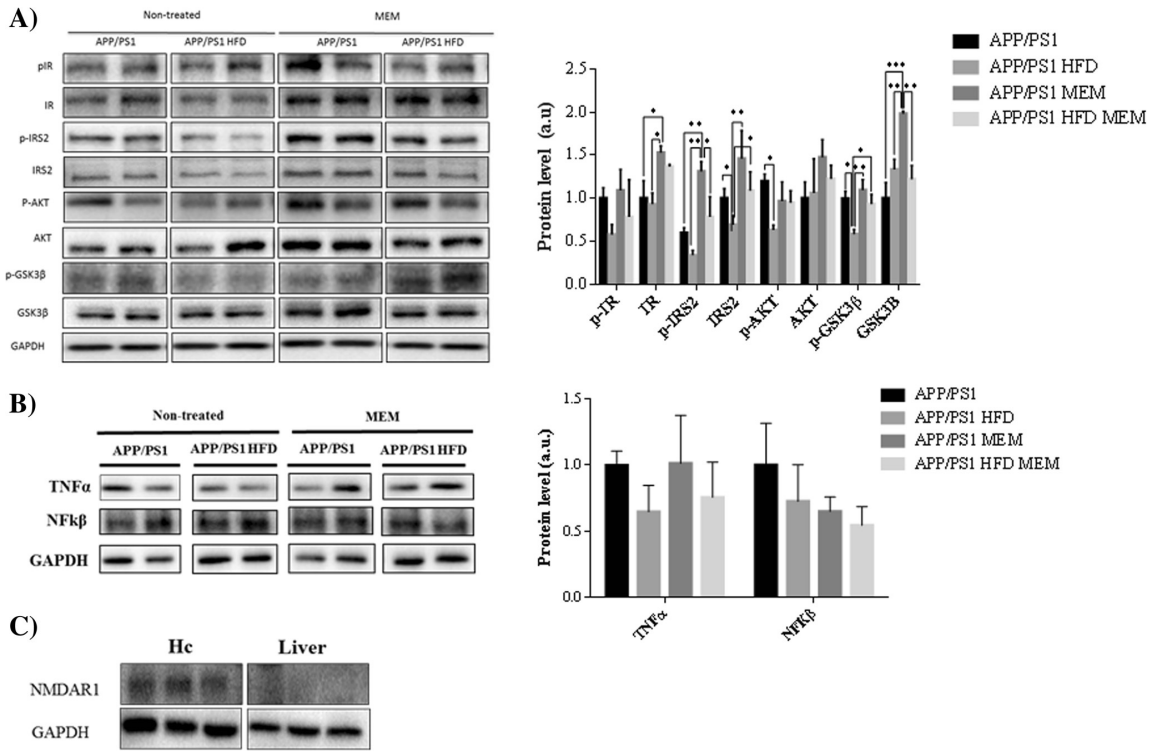


Fig. 5 Liver analyses. **a** Representative GAPDH-normalized immunoblot images and quantification ($n = 4–6$) of molecules implicated in insulin signalling pathway in the liver extracts of HFD-fed 6-month old APP/PS1 and MEM treated. **b** TNFα and NFκβ protein levels. **c** NMDAR1β protein levels. Values are expressed as mean ± S.E.M. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. $n = 5$ animals/group

reduction of plaque formation in APP/PS1 mice fed with HFD promoting the non-amyloidogenic pathway (α-secretase modulation), the inhibition of β-secretase and the decrease in APP protein levels. Likewise, MEM treatment markedly ameliorated learning and memory deficits in mice, enhancing insulin signalling pathway activation, blocking PTP1B protein levels, inhibiting glial activation and enhancing synaptic protein levels in obese APP/PS1 mice. Likewise, in the current

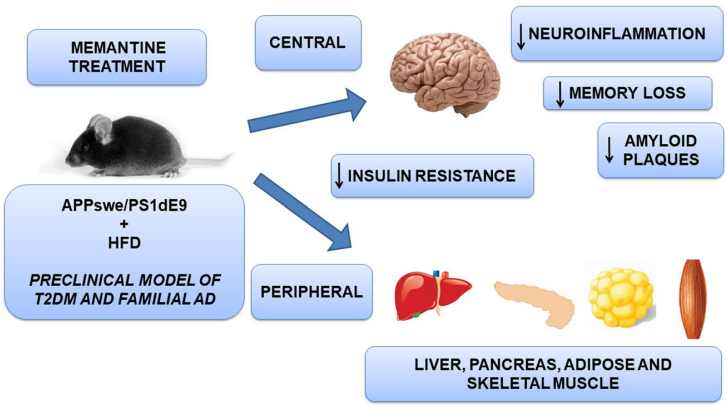


Fig. 6 MEM improves preclinical AD neuropathology improving cognition and decreasing neuroinflammation and brain amyloid levels. Moreover, MEM blocks NMDA receptors and prevents insulin resistance. At the peripheral level, the potential beneficial metabolic effects of MEM are also mediated by the inhibition of peripheral (pancreatic) NMDA receptors and the improvement in insulin resistance mediated by the decrease of Aβ1-42 levels. Therefore, Aβ1-42 could be a link between central AD and peripheral metabolic alterations

study, MEM also reversed learning and memory loss induced by HFD intake.

Moreover, our data demonstrated that MEM improves peripheral parameters such as HFD-feeding-induced hyperglycaemia, insulin resistance and body weight gain in APP/PS1 mice. Nevertheless, we did not observe this effect in WT mice fed with HFD. These results indicate that MEM should have effect on a component at the brain level, probably Aβ peptide, which must contribute synergistically with HFD causing the insulin resistance process in APP/PS1 mice. Furthermore, our results would reinforce the previous data reported by Clarke and colleagues who demonstrated that Aβ affected the hypothalamus and caused peripheral metabolic alterations in glucose and insulin. On this metabolic process, neuroinflammation seems to play a prominent role in peripheral alterations in glucose homeostasis in APP/PS1 mouse model of AD [15].

Thus, we demonstrate that (i) a diabetic condition enhances cognitive dysfunction associated with an impairment in brain and liver insulin signalling, (ii) Aβ pathology may adversely affect and favour liver diabetic insulin resistance and (iii) finally, we report that MEM improves central and peripheral Aβ pathology, being, in our best knowledge, the first report which describes peripheral effects by MEM.

Likewise, although recent data suggest that pancreatic peripheral NMDA receptors are involved in blood insulin and glucose regulation, we suggest that these peripheral effects of MEM are not mediated by interaction of this drug with hepatic NMDA receptors, since our data indicates that there is no expression of these receptors in the liver [38–40]. Accordingly, we confirm the importance of NMDAR antagonists in modulating and improving peripheral glucose and insulin in a preclinical diabetic model [38–42].

Recent research is increasingly highlighting AD may be considered as a systemic disorder, where pathological lesions are not only localized in the brain [43–47]. In addition, it has been reported that deposits of Aβ peptides can be found in different non-neuronal tissues such as the heart, liver and pancreas [44]. Thus, we propose that brain-synthesized Aβ could mediate peripheral insulin resistance through several possible mechanisms: (i) by binding directly to insulin receptor in the liver and inhibiting the downstream signalling pathway, (ii) linking peripheral receptors such as NMDA pancreatic receptors and (iii) through a hypothalamic Aβ brain effects regulating the liver. Arrieta-Cruz and colleagues demonstrated that direct in vivo hypothalamic β25–35 administration alters the periphery regulation of glucose, showing that Aβ plays an important role in regulating glucose homeostasis [26, 27]. Although in the present study we do not evaluate the effects of MEM in the hypothalamus, we hypothesize that, probably, MEM could block hypothalamic NMDA receptors improving liver pathology mediated by Aβ.

Moreover, Zhang and colleagues demonstrated that Aβ induces insulin resistance in hepatocytes by activating the JAK2/STAT3/SOCS-1 signalling pathway and suggest an important role of peripheral Aβ in modulating systemic insulin sensitivity and glucose metabolism [24, 25]. They demonstrated that APP/PS1 mice treated with anti-Aβ neutralizing antibodies improved fasting blood glucose and insulin sensitivity, proposing that Aβ is the responsible of development of insulin resistance in vivo. These results suggest that Aβ is required for the development of hyperglycaemia and insulin resistance in APP/PS1 mice model of familial AD, pointing out the protective role of lowering peripheral Aβ in insulin sensitivity. Working with the same preclinical model, Macklin and colleagues confirmed previous data reported by our group and others that the dysregulation of glucose homeostasis seen in APP/PS1 mice occurred prior to the appearance of amyloid plaques or cognitive decline and in this research peripheral glucose alterations were detected as early as 2 months of age [48].

Here, we report that MEM improves insulin signalling in liver. This is evidenced by an increase in IRS2 in APP/PS1 mice fed with HFD. In addition, it has been published that an alteration in the IRS2 in hepatocytes is a very early event in the onset of hepatic insulin resistance and that it precedes β cell failure [49, 50]. IRS2 and its downstream targets PI 3-kinase, AKT and GSK3β are components of insulin action in the liver, suggesting that impaired IRS2-mediated signalling may represent a major event in the development of hepatic insulin resistance in APP/PS1 mice fed with a HFD [49]. Thus, an increase in the levels of this protein in the liver of APP/PS1 HFD MEM results in a positive balance to decrease insulin resistance and, therefore, it could constitute a potential benefit of MEM in the peripheral system. Moreover, in addition to its role in hepatic carbohydrate metabolism, IRS-2 is involved in the regulation of apoptotic cell death in hepatocytes and an increase in its expression has demonstrated to exert a protective role in cell death [49]. Therefore, the ability of MEM treatment to increase hepatic IRS2 and/or its downstream signalling needs to be extensively investigated for a new potential treatment in T2DM and additional liver pathologies.

In previous studies, several authors have reported that the beneficial effects of the usage of NMDAR antagonists for T2DM came through the blockade of NMDAR pancreatic receptors [38–42]. Although we do not discard this possibility, we also suggest that MEM modulation of cerebral and plasmatic Aβ levels could be an additional mechanism involved in the improvement of insulin sensitivity [11, 12, 51]. Moreover, since

the liver plays a fundamental role in the clearance of plasma Aβ, the observed beneficial effects of MEM in the liver may contribute to AD improvement and other peripheral diseases associated with hyperglycaemia and diabetes such as diabetic nephropathy [22, 44, 51–54].

In summary, the results reported in the present study show that MEM improves insulin resistance in vivo, in part, through modulation of IRS2 hepatic signalling and improving brain insulin resistance in APP/PS1 mice fed with HFD. Current results with MEM treatment confirm the implication of Aβ as a contributor in the development of both AD and metabolic disorders, including T2DM (Fig. 6). Accordingly, the present results and that of other groups raise an interesting question: Can Aβ peptide favour or contribute to the appearance of a new type of peripheral diabetes? The answer to this question would be based on the preclinical results in APP/PS1 mice and other preclinical AD models that demonstrate a peripheral glucose intolerance and insulin resistance in these mice. In addition, if Aβ has a pathological role in T2DMs, drugs that lower brain Aβ levels would be of clinical efficacy for T2DM and could open a new avenue for the treatment of this metabolic disease.

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Authors' Contributions All the authors had directly participated on the execution and planning of the manuscript

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Compliance with Ethical Standards Mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

Conflict of Interest The authors declare that there is no conflict of interest.

References

1. Masters CL, Bateman R, Blennow K, Rowe CC, Spearling RA, Cummings JL (2015) Alzheimer's disease. Nat Rev Dis Primers 1:15056
2. Craft S (2012) Alzheimer disease: insulin resistance and AD: extending the translational path. Nat Rev Neurol 8(7):360–362. https://doi.org/10.1038/nrneurol.2012.112
3. Ritchie CW, Molinuevo JL, Truyen L, Satlin A, Van der Geyten S, Lovestone S (2016) Development of interventions for the secondary prevention of Alzheimer's dementia: the European Prevention of Alzheimer's Dementia (EPAD) project. Lancet Psychiatry 3(2): 179–186. https://doi.org/10.1016/S2215-0366(15)00454-X
4. Querfurth HW, LaFerla FM (2010) Alzheimer's disease. N Engl J Med 362(4):329–344. https://doi.org/10.1056/NEJMra0909142
5. Mangialasche F, Solomon A, Winblad B, Mecocci P, Kivipelto M (2010) Alzheimer's disease: clinical trials and drug development. Lancet Neurol 9:702e716
6. Selkoe DJ (2011) Resolving controversies on the path to Alzheimer's therapeutics. Nat Med 17(9):1060–1065. https://doi.org/10.1038/nm.2460
7. Selkoe DJ, Hardy J (2016) The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med 8(6):595–608. https://doi.org/10.15252/emmm.201606210
8. Viola KL, Klein WL (2015) Amyloid β oligomers in Alzheimer's disease pathogenesis, treatment, and diagnosis. Acta Neuropathol 129(2):183–206. https://doi.org/10.1007/s00401-015-1386-3
9. Lipton SA (2004) Paradigm shift in NMDA receptor antagonist drug development: molecular mechanism of uncompetitive inhibition by memantine in the treatment of Alzheimer's disease and other neurologic disorders. J Alzheimers Dis 6:S61eS74
10. Danysz W, Parsons CG, Mobius HJ, Stoffler A, Quack G (2000) Neuroprotective and symptomatological action of memantine relevant for Alzheimer's disease—a unified glutamatergic hypothesis on the mechanism of action. Neurotox Res 2(2–3):85–97. https://doi.org/10.1007/BF03033787
11. Alley GM, Bailey JA, Chen D, Ray B, Puli LK, Tanila H, Banerjee PK, Lahiri DK (2010) Memantine lowers amyloid-beta peptide levels in neuronal cultures and in APP/PS1 transgenic mice. J Neurosci Res 88(1):143–154. https://doi.org/10.1002/jnr.22172
12. Ito K, Tatebe T, Suzuki K, Hirayama T, Hayakawa M, Kubo H, Tomita T, Makino M (2017) Memantine reduces the production of amyloid-β peptides through modulation of amyloid precursor protein trafficking. Eur J Pharmacol 798:16–25. https://doi.org/10.1016/j.ejphar.2017.02.001
13. Accardi G, Caruso C, Colonna-Romano G, Camarda C, Monastero R, Candore G (2012) Can Alzheimer disease be a form of type 3 diabetes? Rejuvenation Res 15(2):217–221. https://doi.org/10.1089/rej.2011.1289
14. Bedse G, Di Domenico F, Serviddio G, Cassano T (2015) Aberrant insulin signaling in Alzheimer's disease: current knowledge. Front Neurosci 9:204. https://doi.org/10.3389/fnins.2015.00204
15. Clarke JR, Lyra E Silva NM, Figueiredo CP, Frozza RL, Ledo JH, Beckman D, Katashima CK, Razolli D et al (2015) Alzheimer-associated Aβ oligomers impact the central nervous system to induce peripheral metabolic deregulation. EMBO Mol Med 7(2): 190–210. https://doi.org/10.15252/emmm.201404183
16. De Felice FG, Benedict CA (2015) Key role of insulin receptors in memory. Diabetes 64(11):3653–3655. https://doi.org/10.2337/dbi15-0011
17. De Felice FG, Lourenco MV, Ferreira ST (2014) How does brain insulin resistance develop in Alzheimer's disease? Alzheimers Dement 10(1 Suppl):S26–S32. https://doi.org/10.1016/j.jalz.2013.12.004
18. De Felice FG, Ferreira ST (2014) Inflammation, defective insulin signaling, and mitochondrial dysfunction as common molecular denominators connecting type 2 diabetes to Alzheimer Disease. Diabetes 63(7):2262–2272. https://doi.org/10.2337/db13-1954
19. de la Monte SM, Wands JR (2005) Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. J Alzheimer Dis 7(1):45–61. https://doi.org/10.3233/JAD-2005-7106
20. de la Monte SM (2017) Insulin resistance and neurodegeneration: progress towards the development of new therapeutics for Alzheimer's disease. Drugs 77(1):47–65. https://doi.org/10.1007/s40265-016-0674-0

21. de la Monte SM (2012) Brain insulin resistance and deficiency as therapeutic targets in Alzheimer's disease. Curr Alzheimer Res 9(1):35–66. https://doi.org/10.2174/156720512799015037
22. Bahramian A, Rastegar K, Namavar MR, Moosavi M (2016) Insulin potentiates the therapeutic effect of memantine against central STZ-induced spatial learning and memory deficit. Behav Brain Res 311:247–254. https://doi.org/10.1016/j.bbr.2016.05.046
23. Wu J, Fu B, Lei H, Tang H, Wang Y (2016) Gender differences of peripheral plasma and liver metabolic profiling in APP/PS1 transgenic AD mice. Neuroscience 332:160–169. https://doi.org/10.1016/j.neuroscience.2016.06.049
24. Zhang Y, Zhou B, Deng B, Zhang F, Wu J, Wang Y, Le Y, Zhai Q (2013) Amyloid-β induces hepatic insulin resistance in vivo via JAK2. Diabetes 62(4):1159–1166. https://doi.org/10.2337/db12-0670
25. Zhang Y, Zhou B, Zhang F, Wu J, Hu Y, Liu Y, Zhai Q (2012) Amyloid-β induces hepatic insulin resistance by activating JAK2/STAT3/SOCS-1 signaling pathway. Diabetes 61(6):1434–1443. https://doi.org/10.2337/db11-0499
26. Arrieta-Cruz I, Knight CM, Gutiérrez-Juárez R (2015) Acute exposure of the mediobasal hypothalamus to amyloid-β25-35 perturbs hepatic glucose metabolism. J Alzheimers Dis 46(4):843–848. https://doi.org/10.3233/JAD-131865
27. Arrieta-Cruz I, Gutiérrez-Juárez R (2016) The role of insulin resistance and glucose metabolism dysregulation in the development of Alzheimer's disease. Rev Investig Clin 68(2):53–58
28. Petrov D, Pedrós I, Artiach G, Sureda FX, Barroso E, Pallàs M, Casadesús G, Beas-Zarate C et al High-fat diet-induced deregulation of hippocampal insulin signaling and mitochondrial homeostasis deficiencies contribute to Alzheimer disease pathology in rodents. Biochim Biophys Acta 1852:1687–1699
29. Almeida AA, Campos DR, Bernasconia G, Calafatti S, Barros FAP, Eberlin MN, Meurer EC, Paris EG et al (2007) Determination of memantine in human plasma by liquid chromatography–electrospray tandem mass spectrometry: application to a bioequivalence study. J Chrom B 848:31–316
30. Sánchez-López E, Ettcheto M, Egea MA, Espina M, Calpena AC, Folch J, Camins A, García ML (2017) New potential strategies for Alzheimer's disease prevention: pegylated biodegradable dexibuprofen nanospheres administration to APPswe/PS1dE9. Nanomedicine 13(3):1171–1182. https://doi.org/10.1016/j.nano.2016.12.003
31. MG H, Ikeda R, Wada M, Kuroda N, Abdel-Wadood HM, Mohamed HA, Nakashima K (2013) Interaction study of acetylcholinesterase inhibitors on pharmacokinetics of memantine in rat plasma by HPLC-fluorescence method. Biomed Chromatogr 27:1685–1689
32. Talbot K, Wang H, Kazi H, Han L, Bakshi KP, Stucky A et al (2012) Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF1 resistance, IRS1 dysregulation, and cognitive decline. J Clin Invest 122(4):1316–1338. https://doi.org/10.1172/JCI59903
33. Biessels GJ, Reagan LP (2015) Hippocampal insulin resistance and cognitive dysfunction. Nat Rev Neurosci 16(11):660–671. https://doi.org/10.1038/nrn4019
34. Schwartz MW, Porte D Jr (2005) Diabetes, obesity, and the brain. Science 307(5708):375–379. https://doi.org/10.1126/science.1104344
35. McNay EC, Recknagel AK (2011) Brain insulin signaling a key component of cognitive processes and a potential basis for cognitive impairment in type 2 diabetes. Neurobiol Learn Mem 96(3):432–442. https://doi.org/10.1016/j.nlm.2011.08.005
36. Plucińska K, Dekeryte R, Koss D, Shearer K, Mody N, Whitfield PD, Doherty MK, Mingarelli M et al (2016) Neuronal human BACE1 knockin induces systemic diabetes in mice. Diabetologia 59(7):1513–1523. https://doi.org/10.1007/s00125-016-3960-1
37. Schiöth HB, Craft S, Brooks SK, Frey WH, Benedict C (2011) Brain insulin signaling and Alzheimer's disease: current evidence and future direction. Mol Neurobiol 46(1):4–10. https://doi.org/10.1007/s12035-011-8229-6
38. Lechin F, van der Dijs B, Pardey-Maldonado B, Rivera JE, Lechin ME, Baez S (2009) Amantadine reduces glucagon and enhances insulin secretion throughout the oral glucosetolerance test: central plus peripheral nervous system mechanisms. Diab Metab Syndr Obes 2:203–213
39. Marquard J, Otter S, Welters A, Stirban A, Fischer A, Eglinger J, Herebian D, Kletke O et al (2015) Characterization of pancreatic NMDA receptors as possible drug targets for diabetes treatment. Nat Med 21:363–372
40. Marquard J, Stirban A, Schliess F, Sievers F, Welters A, Otter S, Fischer A, Wnendt S et al (2016) Effects of dextromethorphan as add-on to sitagliptin on blood glucose and serum insulin concentrations in individuals with type 2 diabetes mellitus: a randomized, placebo-controlled, double-blinded, multiple crossover, single-dose clinical trial. Diabetes Obes Metab 18:100–103
41. Welters A, Lammert E, Mayatepek E, Meissner T (2017) Need for better diabetes treatment: the therapeutic potential of NMDA receptor antagonists. Klin Padiatr 229(1):14–20. https://doi.org/10.1055/s-0042-117831
42. Wollheim CB, Maechler P (2015) Beta cell glutamate receptor antagonists: novel oral antidiabetic drugs? Nat Med 21(4):310–311. https://doi.org/10.1038/nm.3835
43. Morris JK, Honea RA, Vidoni ED, Swerdlow RH, Burns JM (2014) Is Alzheimer's disease a systemic disease? Biochim Biophys Acta 1842(9):1340–1349. https://doi.org/10.1016/j.bbadis.2014.04.012
44. Wang J, Gu BJ, Masters CL, Wang YJ (2017) A systemic view of Alzheimer disease—insights from amyloid-β metabolism beyond the brain. Nat Rev Neurol 13(10):612–623. https://doi.org/10.1038/nrneurol.2017.111
45. Troncone L, Luciani M, Coggins M, Wilker EH, Ho CY, Codispoti KE, Frosch MP, Kayed R et al (2016) Aβ amyloid pathology affects the hearts of patients with Alzheimer's disease: Mind the Heart. J Am Coll Cardiol 68(22):2395–2407. https://doi.org/10.1016/j.jacc.2016.08.073
46. Liu Y-H, Wang Y-R, Xiang Y, Zhou H-D, Brian Giunta NB, Mañucat-Tan, Tan J, Zhou X-F et al (2015) Clearance of amyloid-beta in Alzheimer's disease: shifting the action site from center to periphery. Mol Neurobiol 51:1–7
47. Desai GS, Zheng C, Geetha T, Mathews ST, White BD, Huggins KW, Zizza CA, Broderick TL et al (2014) The pancreas-brain axis: insight into disrupted mechanisms associating type 2 diabetes and Alzheimer's disease. J Alzheimers Dis 42(2):347–356. https://doi.org/10.3233/JAD-140018
48. Macklin L, Griffith CM, Cai Y, Rose GM, Yan XX, Patrylo PR (2017) Glucose tolerance and insulin sensitivity are impaired in APP/PS1 transgenic mice prior to amyloid plaque pathogenesis and cognitive decline. Exp Gerontol 88:9–18. https://doi.org/10.1016/j.exger.2016.12.019
49. Valverde AM, González-Rodríguez A (2011) IRS2 and PTP1B: two opposite modulators of hepatic insulin signalling. Arch Physiol Biochem 117(3):105–115. https://doi.org/10.3109/13813455.2011.557386
50. Lavin DP, White MF, Brazil DP (2016) IRS proteins and diabetic complications. Diabetologia 59(11):2280–2291. https://doi.org/10.1007/s00125-016-4072-7
51. Rajasekar N, Nath C, Hanif K, Shukla R (2016) Inhibitory effect of memantine on streptozotocin-induced insulin receptor dysfunction, neuroinflammation, amyloidogenesis, and neurotrophic factor decline in astrocytes. Mol Neurobiol 53(10):6730–6744. https://doi.org/10.1007/s12035-015-9576-5

52. Roshanravan H, Kim EY, Dryer SE (2016) NMDA receptors as potential therapeutic targets in diabetic nephropathy: increased renal NMDA receptor subunit expression in Akita mice and reduced nephropathy following sustained treatment with memantine or MK-801. Diabetes 65(10):3139–3150. https://doi.org/10.2337/db16-0209
53. White MF (2014) IRS2 integrates insulin/IGF1 signalling with metabolism, neurodegeneration and longevity. Diabetes Obes Metab 16(Suppl 1):4–15. https://doi.org/10.1111/dom.12347
54. Baquedano E, Burgos-Ramos E, Canelles S, González-Rodríguez A, Chowen JA, Argente J, Barrios V, Valverde AM et al (2016) Increased oxidative stress and apoptosis in the hypothalamus of diabetic male mice in the insulin receptor substrate-2 knockout model. Dis Model Mech 9(5):573–583. https://doi.org/10.1242/dmm.023515