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Antidiabetic polypill improves central pathology and cognitive impairment in a mixed model of Alzheimer's disease and type 2 diabetes

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Running title: Antidiabetic pill reduces Alzheimer´s pathology

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Abstract

Type 2 diabetes (T2D) is an important risk factor to suffer dementia, being Alzheimer's disease (AD) the most common form. Both, AD and T2D, are closely related to ageing and with a growing elderly population it might be of relevance to explore new therapeutic approaches that may slow or prevent central complications associated with metabolic disorders. Therefore, we propose the use of the antidiabetic polypill (PP), a pharmacological cocktail, commonly used by T2D patients that includes metformin, aspirin, simvastatin and an angiotensin-converting enzyme inhibitor. In order to test the effects of PP at the central level, we have long-term treated a new mixed model of AD-T2D, the APP/PS1xdb/db mouse. We have analyzed AD pathological features and the underlying specific characteristics that relate AD and T2D. As expected, metabolic alterations were ameliorated after PP-treatment in diabetic mice, supporting a role for PP in maintaining pancreatic activity. At central level, PP reduced T2D-associated brain atrophy, showing both neuronal and synaptic preservation. Tau and amyloid pathology were also reduced after PP treatment. Furthermore, we observed a reduction of spontaneous central bleeding and inflammation after PP treatment in diabetic mice. As consequence, learning and memory processes were improved after PP treatment in AD, T2D and AD-T2D mice. Our data provide the basis to further analyze the role of PP, as an alternative or adjuvant, to slow down or delay the central complications associated with T2D and AD.

Key words: Alzheimer's disease, type 2 diabetes, polypill, amyloid-beta, tau, hemorrhage, inflammation.

Introduction

The increase in life expectancy is contributing to a significant increase in the pathologies associated with aging. Among these, Alzheimer's disease (AD) and vascular dementia (VaD) are the most prevalent forms of dementia [1]. The main neuropathological features of AD are senile plaques (SP), neurofibrillary tangles, and neuronal and synaptic loss, with the latter correlating most strongly with the severity of the disease [2]. On the other hand, VaD is a heterogeneous disease that includes multiple microinfarcts, ischemic disease and microvascular damage [3]. AD pathology regularly coexists with vascular injury, making the borderlines between AD and VaD blurred in many cases [3]. The underlying causes of dementia are not completely understood and while aging remains the main risk factor to suffer AD, metabolic related alterations also increase the risk to develop AD. Following this idea, hyperinsulinemia and type 2 diabetes (T2D) are the metabolic factors that show a stronger association with dementia [4-5]. In recent years, multiple studies have reported the impact of insulin dysfunction and diabetes on tau pathology [6-7]. In addition, central nervous system insulin receptors are highly expressed in regions relevant for cognition, such as the cortex and hippocampus. This is consistent with evidence showing that insulin influences memory [3]. It has also been suggested that Aβ clearance, along interstitial fluid drainage pathways, can be reduced when there is central vascular damage [8] and T2D is also closely related with vascular dysfunction [9]. Insulin may regulate Aβ levels by modulation of β and γ secretases [10] and Aβ oligomers may also interfere with insulin signalling in hippocampal neurons [11]. It is therefore feasible that metabolic alterations may also underlie the crosstalk between T2D and AD [12].

At present, there are no effective treatments for AD and current pharmacological approaches are limited to anti-cholinesterase drugs or memantin. For this reason, new treatments that may ameliorate the symptoms of AD are urgently needed. The anti-T2D polypill (PP) might be a useful tool to reduce the number of deaths and serious complications caused by diabetes [13]. PP contains several drugs commonly used to treat T2D, including metformin, aspirin, a generic statin, and a angiotensin-converting enzyme inhibitor (ACEi) [13]. Moreover, a commercially available version of a PP, that combines aspirin, a statin and an ACEi, is already available in Europe (Trinomia®). The pathological basis for the relationship between T2D and AD-VaD may also support PP, as a therapy that may treat, prevent or delay central pathology in dementias and T2D. Previous studies have focused on the isolated components of the PP to treat dementia,

however to our knowledge the so-called PP has not been assessed at central level. In our hands, long-term PP treatment improved metabolic alterations observed in diabetic mice (both db/db and APP/PS1xdb/db). At central level, we detected a significant improvement in amyloid pathology and reduced tau hyperphosphorylation, as well as brain integrity preservation. Furthermore, spontaneous central bleeding and neuroinflammation were reduced after PP-treatment. These effects were accompanied by a significant improvement of learning and memory deficits after long-term treatment, supporting a role for PP in the management of central pathology associated with AD and T2D.

Material and Methods

1. Animals and treatments

AD-T2D mice were produced by cross-breeding APPswe/PS1dE9 with db/db mice [14-15]. Both females and males were included and a similar number of males and females were used in each experiment. The animals were randomly divided into groups and received PP included in the diet from 4 to 26 weeks of age. Dosage of individual PP components were selected from previous studies using individual drugs: metformin (200 mg/Kg/day)[16], simvastatin (40 mg/kg/day) [17], aspirin (5 mg/kg/day) [18], perindopril (0.5 mg/kg/day) [19] and included in food pellets (SAFE A04, France). Dosing was adjusted to daily food intake (~6 g for diabetic mice and ~3,6 for non diabetic mice), in line with previous studies [20]. Briefly, powder diet was thoroughly mixed with the powered drugs and distilled water (50ml of distilled water per 100g of diet), compacted in cubes and dried at 37ºC in a stove (Raypa Incuterm, Spain) overnight. New food batches were prepared every 48h. All experimental procedures were approved by the Animal Care and Use Committee of the University of Cadiz, in accordance with the Guidelines for Care and Use of experimental animals (European Commission Directive 2010/63/UE and Spanish Royal Decree 53/2013).

2. Plasma determinations

Body weight, postprandial blood glucose and plasma insulin levels were determined before treatment and every 4 weeks until sacrifice at 26 weeks. Total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglycerides were measured in plasma by enzymatic colorimetric method with autoanalyzer Cobas c 8000 at 26 weeks of age (Roche Diagnostic®) following manufacturer's indications. ACE

activity was measured by Infinity ACE assay (Thermo Fisher, USA) following manufacturer´s indications. All determinations were perfomed in 5-15 mice (Control n=11, Control-PP N=15, APP/PS n=12, APP/PS1-PP n=12, db/db n=10, db/db-PP=10, APP/PSxdb/db n=5, APP/PS1xdb/db-PP n=5).

3. Morris water maze (MWM)

Spatial cognition was analyzed as previously described [15]. The maze consisted of a round tank of water (0.95 m in diameter) with four equal virtual quadrants indicated with geometric cues mounted on the walls. The escape platform was 2-3 cm below the surface and camouflaged with calcium carbonate. A camera was mounted above the maze and attached to a computer and software. Water temperature was 21±1ºC. Testing was conducted in two phases: acquisition and retention. The acquisition consisted of 4 trials/day for 4 consecutive days with the platform submerged. During this phase, the platform was located in the quadrant 2. The time limit was 60 s/trial with an intertrial interval of 10 min. If the animal did not find the platform, it was placed on it for 10 s. The retention phase started a day after acquisition phase was completed, and consisted in a single trial with the platform removed. Time required to locate the platform in the acquisition phase, percentage of time spent in quadrant 2 during the retention phase and swim speed were analyzed using Smart software (Panlab, Spain). MWM studies include 7-16 mice pre group (Control n=12, Control-PP N=16, APP/PS n=15, APP/PS1-PP n=12, db/db n=10, db/db-PP=8, APP/PSxdb/db n=8, APP/PS1xdb/db-PP n=7).

4. Actimetry and novel object discrimination (NOD) task

Episodic memory was assessed with the NOD test as previously described [21] with minor modifications. We assessed spontaneous locomotor activity prior the commencement of the NOD test. Briefly, on day 1, mice were allowed to explore a transparent rectangular box (22 cm long x 44 cm width x 40 cm high) for 30 minutes. Animals were recorded, and distance travelled and speed were measured by Smart software (Panlab, Spain). NOD test commenced on day 2 and animals were exposed to two objects, for habituation purposes, not used again during the object exploration task. On day 3, each mouse received two sample trials and a test trial. On the first sample trial, mice were placed into the centre of the box containing 4 copies of a novel object (blue balls) arranged in a triangle-shaped spatial configuration and allowed to explore them for 5 min. After a delay of 30 min, the mice received a second sample trial with 4 novel objects (red cones), arranged in a quadratic-shaped spatial configuration, for 5 min. After a delay of 30 min, the mice received a test trial with 2 copies of the object from sample trial 2

("recent" objects) placed at the same position, and two copies of the object from sample trial 1 ("familiar" objects) placed one of them at the same position ("old non displaced" object) and the another in a new position ("familiar displaced" object). Integrated episodic memory for "what", "where" and "when" was analyzed as previously described [21]: "what" was defined as the difference in time exploring familiar and recent objects, "where" was defined as the difference in time exploring displaced and non displaced objects and "when" was defined as the difference between time exploring familiar non displaced and recent non displaced objects. Actimetry and NOD test studies included 7-16 mice per group (Control n=12, Control-PP N=16, APP/PS n=15, APP/PS1-PP n=12, db/db n=10, db/db-PP=8, APP/PSxdb/db n=8, APP/PS1xdb/db-PP n=7).

5. Cresyl violet staining

Brain sections were selected 1 mm apart (from 1.5 to -3.5 mm from Bregma). Sections were mounted and dehydrated in 70% ethanol for 15 minutes before incubation in cresyl violet as previously described [22]. Images were acquired using an optical Olympus Bx60 microscope with an attached Olympus DP71 camera and Cell F software (Olympus, Hamburg, Germany). Cortex and hippocampus cross-sectional areas were measured using Image J software in 5-7 mice per group (Control n=6, Control-PP N=7, APP/PS n=7, APP/PS1-PP n=5, db/db n=7, db/db-PP=7, APP/PSxdb/db n=6, APP/PS1xdb/db-PP n=7).

6. Prussian blue staining

Presence of spontaneous haemorrhages was assessed postmortem by Prussian blue iron staining and neutral red counterstaining. Consecutive sections to those used for cresyl violet staining were processed as previously described [22], and images were analyzed using Image J software to quantify haemorrhage burden in the cortex and hippocampus of all groups studied (Control n=7, Control-PP N=5, APP/PS n=5, APP/PS1-PP n=4, db/db n=6, db/db-PP=4, APP/PSxdb/db n=5, APP/PS1xdb/db-PP n=5).

7. Total-tau and phospho-tau levels

Total tau and tau phosphorylation levels were measured in cortical and hippocampal samples. Tissue was homogenized in lysis buffer (Cell Signaling, USA) and supplemented with a protease and phosphatase inhibitor cocktail (Sigma, USA). The homogenates were sonicated and centrifuged at 4 ºC for 5 min at 15.000 g. Supernatants

were collected and protein concentration determined by Bradford protein assay (Biorad, Germany). Proteins were separated on 10% acrylamide-bisacrylamide gels, followed by electrophoretic transfer to PVDF membranes (Schleicher & Schuell, Keene, NH). Membranes were then immersed in blocking buffer (Invitrogen, USA) for 1 h and incubated overnight at 4ºC with primary antibodies for anti-phospho-tau antibody (1:1000) (clon AT8, Fisher Scientific, Waltham, Ma). Membranes were washed and then incubated with chemiluminescent inmunodetection system for mouse and rabbit primary antibodies respectively (Invitrogen, Carlsbad, USA). Membranes were washed and signal was detected using Novex AP Chemiluminescent Substrate (Invitrogen, USA) and Kodak Biomax Light Film (Sigma, USA). After stripping, membranes were incubated with anti-total tau (1:1000) (DAKO, Glostrup, Denmark). Immunoblots were semi-quantified by measuring the optical density (OD) of each protein band on scanned film using the ImageJ software. Each band was normalized to β-actin (1:2.500.000; Sigma, USA) optical density, and phospho tau/ total tau ratio was represented from 6-9 mice per group (Control n=8, Control-PP N=6, APP/PS n=7, APP/PS1-PP n=7, db/db n=9, db/db-PP=7, APP/PSxdb/db n=6, APP/PS1xdb/db-PP n=6).

8. Aβ40 and Aβ42 levels

Soluble and insoluble Aβ40 and Aβ42 were quantified in frozen cortex and hippocampus using colorimetric ELISA kits (Wako, Japan) as previously described with minor modifications [23]. Tissue (5-10 mg) was homogenized in 50µl of lysis buffer with inhibitor cocktail (Thermo Scientific Pierce, Spain) and centrifuged 14.500 rpm for 12 min at 4°C. Supernatants were diluted 1:300 for soluble Aβ40 and 42 levels. The resultant pellet was then extracted with 50µl of 70% formic acid and then centrifuged. Insoluble fraction was neutralized with 1M Tris (pH 11) and diluted 1:10. Standard curves were made using human Aβ40 and Aβ42 standards provided in the kit. Absorbance was measured spectrophotometrically at 450 nm (MQX200R2, Biotek instruments, Burlington VT, USA) and data were expressed as pmol/g tissue (Control n=6, Control-PP N=6, APP/PS n=10, APP/PS1-PP n=10, db/db n=6, db/db-PP=6, APP/PSxdb/db n=14, APP/PS1xdb/db-PP n=14).

10. Senile plaques and microglia immunostaining

After pretreatment in 70% formic acid, sections were incubated in anti-Iba1 (Wako, Osaka, Japan) (1:1.000) and anti Aβ (4G8, Covance, Greenfield, IN, USA) (1:2000) antibodies overnight at 4º C in 0.5% NGS. Secondary antibodies Alexa Fluor 594 and Alexa Fluor 488 (Molecular Probes, OR, USA) (1:1.000) were used. Sections were mounted and photographed using a fluorescent microscope (Laser Microdissection System CellCut Version 4.3, Molecular Machines & Industries AG, Switzerland). SP burden was analyzed in the cortex and hippocampus. Microglia burden was also quantified using Image J software. Regions of interest were marked around SP (up to 50µm from the plaque border) to quantify microglia burden in the proximity of amyloid deposits. Microglia burden was also measured in random SP-free areas from APP/PS1 and APP/PS1xdb/db mice, and in animals without plaques (Control and db/db mice), as previously described [14, 24] (Control n=5, Control-PP n=5, APP/PS n=4, APP/PS1-PP n=4, db/db n=5, db/db-PP=4, APP/PSxdb/db n=4, APP/PS1xdb/db-PP n=5).

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11. Neuron immunostaining senile plaques staining

Sections were blocked in 3% BSA with 0.1% Tritón-X in PBS for 1h, followed by anti-NeuN antibody (Chemicon) incubation (1:200) overnight at 4º C, in 0.5% BSA. Conjugated goat anti-mouse Alexa 594 antibody was used as secondary antibody. Sections were washed and stained with DAPI 1mg/ml (Sigma, St. Louis, MO, USA) (1:3000) and thioflavin S staining was used to label SP. Sections were mounted and photographed using a Laser Olympus U-RFL-T fluorescent microscope (Olympus, Japan) and MMIcellTools software. Aβ burden was analyzed in the cortex and hippocampus. The percentage of NeuN-positive cells (normalized by total cells stained with DAPI) was quantified in the cortex and hippocampus from 3 mice per group, close (up to 50µm from the plaque border) and far from plaques (>50µm from SP).

12. Array tomography

Synaptic density was assessed by array tomography as previously described [25] with minor modifications. Cortical samples were fixed in 4 % PFA for 4 h, dehydrated and immersed into LR White resin (Electron Microscopy Sciences) and polymerized overnight at 53 °C. Sections were rehydrated for 5 min with 50 mM glycine in TBS and blocked for 5 min in 0.05 % Tween and 0.1 % BSA. Ribbons were incubated on “day1” with antibodies against synaptophysin (Abcam, USA) and PSD95 (Cell signaling, USA)

(1:100) overnight at 4ºC. Secondary antibodies Alexa Fluor 594 and 488 (Invitrogen, USA) were incubated (1:50) in blocking buffer for 30 min. Sections were counterstained with 0.01 mg/mL DAPI for 5 min. Images were obtained along the ribbon using a Zeiss Axio Imager Z2 epifluorescent microscope with a CoolSnap digital camera and AxioImager software with array tomography macros (Carl Zeiss, Ltd, Cambridge UK). High-resolution images were obtained with a 63×1.4 NA Plan Apochromat objective. Coverslips were washed twice in TBS and stripped with stripping buffer (0.2 M NaOH and 0.02 % SDS in TBS). Coverslips were washed with TBS and quickly washed 3 times in H_2O_{dd}. The staining protocol was then repeated ("day2") with antibodies against synapthophysin (1:100) and AW7 (1:1.000) for plaques (Dr. Dominic Walsh, USA). Images from "day1" and "day2" were aligned, and regions of interest (crops) chosen in the neuropil. The combined role of the SP halo effect and metabolic alterations on the synaptic density was assessed in both SP free areas (distance from SP >50 µm) and close to plaque borders (distance from SP <50 µm). Synaptic puncta and volumes were generated using MATLAB scripts from Dr. Spires-Jones laboratory in 4-5 mice per group (Control n=5, Control-PP N=5, APP/PS n=5, APP/PS1-PP n=5, db/db n=4, db/db-PP=5, APP/PSxdb/db n=5, APP/PS1xdb/db-PP n=5).

13. Statistical analysis

Student t test for independent samples, one-way ANOVA for independent samples followed by Tukey b or Tamhane tests, or 2 way ANOVA were used to compare the groups under study. SPSS v.20 software was used for all statistical analysis.

Results

1. PP treatment improves metabolic parameters in db/db and APP/PS1xdb/db mice

As previously described [14], body weight was significantly increased in db/db and APP/PS1xdb/db mice up to 26 weeks of age (Figure 1A). We detected a significant weekXgroup effect when body weight was compared between groups by two-way ANOVA [$F_{(35,373)}$=10.98, **p<0.01]. Further differences on weekly intervals were detected by one way ANOVA for independent samples followed by Tukey b or Tamhane tests as required (week 4 [$F_{(7,66)}$=9.20, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 8 [$F_{(7,72)}$=46.93, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 12 [$F_{(7,79)}$=32.09, ‡‡p<0.01 vs. Control, Control-PP,

APP/PS1 and APP/PS1-PP], week 16 [$F_{(7,79)}$=35.21, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db and APP/PS1xdb/db; ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 20 [$F_{(7,77)}$=33.17 ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP; ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db and APP/PS1xdb/db], week 24 [$F_{(7,78)}$=25.33 ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP; ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db and APP/PS1xdb/db]) (Figure 1A).

We detected a significant weekXgroup effect when we analyzed postprandial glucose levels [$F_{(35,374)}$=7.97, **p<0.01]. Further analysis was performed by one-way ANOVA followed by Tukey b or Tamnhane tests as required. PP treatment reduced glucose levels in diabetic mice after 4 weeks of treatment (8 weeks of age), and this effect was more pronounced in APP/PS1xdb/db after 12 weeks under PP-treatment (week 4 [$F_{(7,72)}$=3.085, p=0.07], week 8 [$F_{(7,71)}$=52.67, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP; ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 12 [$F_{(7,79)}$=34.49, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP; ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 16 [$F_{(7,78)}$=60.43, **p<0.01 vs. rest of the groups; ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP; ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 20 [$F_{(7,76)}$=39.54, **p<0.01 vs. rest of the groups; ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP; ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 24 [$F_{(7,77)}$=49.04, **p<0.01 vs. rest of the groups; ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP; ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP] (Figure 1B).

We also observed a significant weekXgroup effect when insulin levels were compared [$F_{(35,370)}$=1,65, **p<0.01]. Insulin levels were significantly increased in all diabetic mice before the commencement of treatment at 4 weeks of age (week 4 [$F_{(7,72)}$=7.74, ‡‡ p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP]). Reduction of insulin levels were more pronounced in APP/PS1xdb/db mice, and PP treatment rescued insulin levels in both db/db-PP and APP/PS1xdb/db-PP mice after 4 weeks of treatment (Figure 1C) (week 8 [$F_{(7,69)}$=19.27, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP; ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db and APP/PS1xdb/db], week 12 [$F_{(7,78)}$=23.66, ††p<0.01 vs. Control, Control-PP, APP/PS1,

APP/PS1-PP, db/db and APP/PS1xdb/db; ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 16 [$F_{(7,73)}$=22.16, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db and APP/PS1xdb/db; ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], week 20 [$F_{(7,77)}$=14.12, ††p<0.01 vs. Control, Control-PP, APP/PS1, db/db and APP/PS1-PP], week 24 [$F_{(7,79)}$=8.9, ††p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP]) (Figure 1C). Altogether, metabolic assessment revealed an early exhaustion of beta-pancreatic cells in db/db and APP/PS1xdb/db mice, leading to reduced insulin production and higher glucose levels, with this effect partially restored in treated mice.

Lipid profile was significantly affected in diabetic mice and total cholesterol levels were increased in db/db mice [$F_{(7,70)}$=3.64, ††p=0.002 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP], while HDL levels were significantly reduced in db/db and APP/PS1xdb/db mice. PP treatment reduced these effects, although differences did not reach statistical significance [$F_{(7,72)}$=1.88, p=0.084]. PP treatment also reduced LDL levels in db/db and APP/PS1xdb/db mice [$F_{(7,71)}$=3.76, ††p=0.002 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP and ##p=0.002 vs. APP/PS1-PP]. PP treatment partially controlled triglyceride levels [$F_{(7,67)}$=4.13, ††p=0.001 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP]. Plasmatic ACE activity was reduced after PP treatment reduced in db/db mice, reaching statistical significance in APP/PS1xd/db animals [$F_{(7,71)}$=2.72, †p=0.015 vs. Control, Control, APP/PS1, db/db, APP/PS1xdb/db] (Table 1).

2. PP treatment improves cognitive impairment in APP/PS1, db/db and APP/PS1xdb/db mice

Episodic memory in the NOD was significantly impaired in APP/PS1 and db/db mice and a worsening effect was observed in APP/PS1xdb/db, as previously described [14-15]. However, an overall improvement was observed after PP treatment (Figure 1D). A significant recovery was observed in APP/PS1xdbdb-PP treated mice when "what" paradigm was analyzed [$F_{(7,226)}$=7,72, ††p<0.01 vs. Control, Control-PP, db/db-PP and APP/PS1xdb/db-PP, ##p<0.01 vs. Control and Control-PP, ‡‡p<0.01 vs. Control] (Figure 1D). For the "where" paradigm, we observed cognitive alterations in APP/PS1, db/db and APP/PS1xdb/db and PP treatment significantly improved diabetic mice performance [$F_{(7,232)}$=13.05, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP, ##p<0.01 vs. Control and Control-PP]. Alterations observed in the "when" paradigm did not reach statistical significance [$F_{(7,237)}$=1.73, p=0.101] (Figure 1D).

In the MWM, learning and memory abilities were impaired in APP/PS1 and db/db mice, showing a synergistic effect when both pathologies coexist in APP/PS1xdb/db mice, as previously described [14-15]. PP treatment partially rescued these alterations and this effect was more robust in APP/PS1xdbdb-PP mice (Figure 1E). We detected a significant groupXday effect by 2-way ANOVA [$F_{(21,1196)}$=59.36, **p=0.01] during the acquisition phase in the MWM test and further differences were analyzed by one-way ANOVA for independent samples followed by Tukey b test. Reduced acquisition times were observed in APP/PS1 mice, db/db and APP/PS1xdb/db mice on PP-treatment and the effect of PP treatment was more pronounced in the APP/PS1xdb/db-PP group (Figure 1E). Day 1: [$F_{(3,341)}$=5.50, ##p<0.01 vs. Control and Control-PP], day 2 [$F_{(7,304)}$=17.33, ##p<0.01 vs. Control and Control-PP, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP and db/db-PP], day 3 [$F_{(7,315)}$=26.05, ##p<0.01 vs. Control and Control-PP, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP], day 4 [$F_{(7,312)}$=267.60, ♯♯p<0.01 vs. Control, Control-PP and APP/PS1-PP, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP, **p<0.01 vs. rest of the groups] (Figure 1E). Spatial memory was assessed in the MWM test 24 h after completing the acquisition phase. We observed that db/db and APP/PS1 animals spent less time in quadrant 2, where the platform used to be located, this effect was more severe and statistically significant in APP/PS1xdb/db mice. However, PP-treatment partially rescued spatial memory, reaching statistical significance in APP/PS1xdb/db-PP mice [$F_{(7,77)}$=3.42, ††p=0.003 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdbdb-PP] (Figure 1F).

Since db/db and APP/PS1xdb/db mice are overweight, motor activity was also evaluated and no differences were detected among groups (Control: 21481.05±2413.03cm; Control-PP: 19973.33±1002.48 cm; APP/PS1: 25125.66±1507.01 cm; APP/PS1-PP: 23087.96±2865.09 cm; db/db: 197.92,30±2383.34 cm; db/db-PP: 18129.18±2086.45 cm/s; APP/PS1xdb/db: 22445.00±3175.14 cm; APP/PS1xdb/db-PP: 19320.71±3259.73 cm; [$F_{(7,49)}$=1.06, p=0.401]). Swimming speed in the MWM test was significantly reduced in diabetic mice (both db/db and APP/PS1xdb/db) and this effect was maintained after PP treatment (Control: 23.71±0.41cm/s, Control-PP: 23.14±0.35 cm/s, APP/PS1: 23.44±0.55 cm/s, APP/PS1-PP: 23.44±0.51 cm/s, db/db: 12.99±0.91 cm/s, db/db-PP: 10.83±1.11 cm/s, APP/PS1xdb/db: 10.56±1.32 cm/s, APP/PS1xdb/db-PP:

10.02±1.22 cm/s), suggesting that observed cognitive improvement in treated mice was not due to motor activity improvement.

3. PP treatment ameliorates brain atrophy and neuronal loss in diabetic mice

Brain atrophy was observed in db/db mice and this effect was more severe in APP/PS1xb/db mice as previously described [15, 25]. Long-term PP treatment partially recovered this situation. These differences were readily detectable when we compared brain weight by one-way ANOVA followed by Tukey b test [$F_{(7,79)}$=72.39, **p<0.01 vs. rest of the groups, ‡‡p<0.01 vs. Control, control-PP, APP/PS1, APP/PS1-PP and db/db-PP, ††p<0.01. vs. Control, control-PP, APP/PS1 and APP/PS1-PP] (Figure 2A). Further cresyl violet staining revealed a significant reduction of cortical thickness, which was partially restored after PP treatment in db/db mice, this effect was statistically significant in APP/PS1xdb/db mice (Figure 2B and 2D). Differences were detected by one-way ANOVA followed by Tukey b test [$F_{(7,297)}$=22.17, **p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP]. While a similar profile was observed in the hippocampus, differences did not reach statistical significance [$F_{(7,152)}$=0.113, p=0.997] (Figure 2C).

When we analyzed NeuN-positive cells, we detected significant reductions in db/db and APP/PS1xdb/db mice, whilst PP treatment significantly improved the number of NeuN-positive cells [$F_{(7,2022)}$=5.65, ‡‡p<0.01 vs. Control, Control-PP, db/db-PP and APP/PS1xdb/db-PP, ##p<0.01 vs. Control]. Similarly, reduced NeuN+/DAPI ratio in APP/PS1 and APP/PS1xdb/db mice was improved after PP treatment in the proximity of SP [$F_{(7,2722)}$=4.20, ‡‡p=0.08 vs. APP/PS1xdb/db-PP] (Figure 2E and 2F).

4. PP treatment rescues synaptic loss in db/db and APP/PS1xdb/db mice

Array tomography on synaptophysin and PSD95 was performed to evaluate pre and postsynaptic densities respectively. Synaptic density was reduced in the proximity of SP in APP/PS1 and APP/PS1xdb/db mice as previously described [25], and no differences were observed after PP treatment among groups in areas close to SP for PSD95 [$F_{(3,396)}$=1.91, p=0.127] and synaptophysin [$F_{(3,395)}$=0.838, p=0.474]) (Figure 3A, 3B and 3C). When we analyzed SP free areas, we observed that even in the absence of amyloid pathology, db/db mice showed reduced pre- and post-synaptic densities, indicating that T2D may impair synapses. This synaptic loss was exacerbated in APP/PS1xdb/db mice,

probably due to a synergistic effect between amyloid pathology and T2D central pathology. PP-treatment completely reversed PSD95 loss in db/db and APP/PS1xdb/db mice [$F_{(7,1065)}$=14.45, **p<0.01 vs. rest of the groups, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP and APP/PS1xdb/db-PP] and a significant improvement was also observed in synaptophysin [$F_{(7,1064)}$=8.23, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, ‡‡p<0.01 vs. Control and Control-PP, ##p<0.01 vs. Control-PP] (Figure 3A, 3B and 3C).

5. PP treatment modifies amyloid pathology in APP/PS1 and APP/PS1xdb/db mice

Diffuse and dense SP burdens were measured by 4G8 immunostaining and thioflavin S staining respectively. Cortical SP burden in APP/PS1xdb/db was reduced when compared with APP/PS1 mice, as previously described in this animal model [14-15]. PP treatment did not affect APP/PS1xdb/db SP burden, however it ameliorated SP deposition in APP/PS1 mice [$F_{(3,106)}$=28.098, **p<0.01 vs. rest of the groups, ††p<0.01 vs. APP/PS1xdb/db and APP/PS1xdb/db-PP] and a similar profile was observed when thioflavin S burden was analyzed [$F_{(3,105)}$=8.98, ††p<0.01 vs. APP/PS1xdb/db and APP/PS1xdb/db-PP] (Figure 4A and 4B). The same trend was observed when the hippocampus was analyzed after 4G8 immunostaining [$F_{(3,52)}$=9.47, ††p<0.01 vs. APP/PS1xdb/db and APP/PS1xdb/db-PP] and thioflavin S staining [$F_{(3,45)}$=3.37, ‡‡p=0.026 vs. APP/PS1xdb/db] (Figure 4A). We also analyzed cortical insoluble Aβ levels by ELISA: Aβ40 levels were significantly higher in APP/PS1 mice compared to APP/PS1xdb/db mice, and a significant reduction was observed after PP treatment [$F_{(3,16)}$=4.4, *p=0.019 vs. rest of the groups], in accordance with our previous results on SP burden. A similar profile was observed when insoluble Aβ42 levels were measured, although differences did not reach statistical significance [$F_{(3,16)}$=2.27, p<0.075] (Figure 4C). We observed a similar pattern in the hippocampus for insoluble Aβ40 levels [$F_{(3,18)}$=6.13, **p=0.005 vs. rest of the groups] and Aβ42 [$F_{(3,16)}$=2.67, p=0.083] (Figure 4C).

Interestingly, when we analyzed cortical soluble Aβ40 levels, we detected a shift towards more toxic soluble species in APP/PS1xdb/db mice compared with APP/PS1 animals, as previously described [14]; and PP-treatment reduced this effect [$F_{(3,20)}$=3.41, ‡p<0.035 vs. APP/PS1xdb/db-PP] (Figure 4C). A similar trend was observed when soluble Aβ42 levels were analyzed, although differences did not reach statistical significance [$F_{(3,19)}$=2.61, p=0.081] (Figure 4C). Soluble Aβ levels showed a similar

trend in the hippocampus, although differences did not reach statistical significance (soluble Aβ40 [$F_{(3,20)}$=2.01, p=0.144], soluble Aβ42 [$F_{(3,19)}$=2.13, p=0.130]) (Figure 4C).

6. PP treatment reduces tau phosphorylation

Tau phosphorylation was increased in db/db mice and further increased in APP/PS1xdb/db mice, as previously described [14-15]. PP-treatment limited tau hyperphosphorylation both in the cortex ([$F_{(7,49)}$=2.67, †p=0.02 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP]) (Figure 4D and 4E) and the hippocampus ([$F_{(7,52)}$=5.84, ##p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdbd/db-PP, ††p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP]) (Figure 4D).

7. PP reduces central inflammation in diabetic mice

In the cortex, when analyzed in the proximity of SP, microglia burden was higher in APP/PS1 mice compared with APP/PS1xdb/db mice, as previously described [15]. PP treatment significantly reduced microglia burden both in APP/PS1 and APP/PS1xdb/db mice [$F_{(3,1064)}$=34.46, **p<0.05 vs. rest of the groups, ††p<0.01 vs. APP/PS1 and APP/PS1-PP, ‡‡p<0.01 vs. APP/PS1] (Figure 5A and 5B). In free SP areas we observed an increase in microglia burden in db/db and APP/PS1xdb/db mice as previously described [14], and this effect was reduced by PP treatment [$F_{(7,3078)}$=27.34, ##p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP, ‡‡p<0.01 vs. Control, Control-PP and PP/PS1-PP] (Figure 5A and 5B). When we analyzed the hippocampus, we did not detect significant differences in the proximity [$F_{(3,161)}$=3.90, p=0.1, no further differences detected by Tukey b test] or in SP free areas [$F_{(7,585)}$=1.65, p=0.118] (Figure 5A).

8. Spontaneous central bleeding is reduced in db/db and APP/PS1xdb/db mice after long-term PP treatment

Cortical spontaneous bleeding was increased in the cortex from db/db mice, and this effect was more pronounced in APP/PS1xdb/db mice, while PP treatment reversed this effect [$F_{(7,218)}$=2.7, †p=0.011 vs. Control, Control-PP, APP/PS1-PP and db/db-PP] (Figure 5C and 5D). This increase in hemorrhage burden was due to an increase in

hemorrhage size and number of hemorrhages/mm^2 (data not shown). A similar profile was observed in the hippocampus although differences did not reach statistical significance when hemorrhage burden was analyzed ([$F_{(7,114)}$=0.742, p=0.637]) (Figure 5C).

Discussion

AD patients are in a tremendous need of new alternatives to treat or slow down the dementia process. The close relationship with T2D [26-27] opens the door to explore therapeutic options, including strict metabolic control, that could ultimately prevent or delay central nervous system complications. Since T2D is a multifactor disease its treatment is usually approached in different ways and PP (metformin, aspirin, a generic statin and an ACEi in its composition) has been proposed as a relevant opportunity to reduce T2D and its associated complications [13]. Previous epidemiological studies have reported the beneficial effects, at central level, of isolated PP components [28-31]. However, since we have not performed pharmacological studies we can hardly point towards an additive or synergistic effect. To our knowledge, no previous studies have addressed the potential beneficial effects of the antidiabetic PP at central level. We have tested this approach after long-term treatment of our mixed T2D-AD model, the APP/PS1xdb/db mouse. This animal model, resulting from crossing classical models of AD (APPswe/PS1dE9 mouse) and T2D (db/db mouse) has been previously characterized in our laboratory [14-15]. Since leptin has been widely implicated in cognition [32] and long-term potentiation [33], as well as in AD [34-35] we cannot exclude that our observations might be, at least partially, due to the lack of the leptin receptor in our animals. However, db/db mice have been largely used as a reference model to study type 2 diabetes for the last 4 decades. It resembles not only hyperglycaemia, but also, associated comorbidities observed in the disease such, as obesity or dyslipidemia, providing a more complete version of the illness. While plasma levels of individual components of PP were not determined, and this aspect should be carefully taken into consideration, previous studies in mice have reported aspirin [36] and simvastatin [37] blood levels after oral administration. Also, both central and peripheral effects of individual components of PP have been shown in different mouse models after similar interventions [16, 38-40]

Metabolic assessment revealed that glucose levels were reduced after PP-treatment, as expected. These results are consistent with the maintenance of insulin levels in diabetic

mice after the treatment, and support the role of PP in β-pancreatic preservation [41]. Lipid profile was improved and ACE activity was slightly reduced after PP treatment. Previous studies have also reported the beneficial effects of statin treatments [42-43] or ACE inhibition [44] on AD and T2D related complications. Therefore, we can not point towards a single mechanism that justifies improvement observed after PP treatment. On the other hand, this is precisely the objective of this study, since the combination of the PP components are regularly used by T2D patients.

We observed that brain atrophy in db/db and APP/PS1xdb/db mice was not detected in PP treated groups. Previous studies from our lab have shown that proliferation and neurogenesis are not affected in db/db mice at 4 weeks of age [45]. Moreover, recent studies on APP/PS1xdb/db mice, from 4 to 36 weeks of age [14-15], show that brain atrophy and cortical thinning seem to be preserved at early stages of the disease. Therefore, even though we cannot exclude that deeper assessment could reveal early maturation abnormalities, it seems that detected brain atrophy in APP/PS1xd/db mice are not due to abnormal development, but to a later degeneration.

Severe cognitive deterioration has been reported when T2D coexists with dementia [14-15, 46]. Since leptin has been widely implicated in cognition [32] and long-term potentiation [33], as well as in AD [34-35] we cannot exclude that our, at least partially, could be due to altered leptin signaling. We observed cognitive impairment in APP/PS1xdb/db mice, which was ameliorated by PP treatment. Our data are in accordance with previous studies on isolated PP components. While the role of metformin remains controversial [28, 47], it has been reported that brain penetrating ACEis improve cognitive impairment in AD transgenic mice [19] and in patients with cerebrovascular disease [40] or T2D [31]. Moreover, ACE serum activity has been correlated with AD severity [48], supporting ACEi as an interesting therapeutic alternative at this level. Aspirin effects on cognition are arguable [49], however low-dose aspirin is readily available and effective in reduction of vascular and platelet aggregation [50]. Therefore, even a slight beneficial effect on risk or progression of AD might justify this treatment [51].

As previously described, APP/PS1xdb/db mice present fewer SP and increased soluble, more toxic, Aβ species [14-15] while PP treatment significantly reduced this effect. Interestingly, PP treatment also reduced SP burden in APP/PS1 mice, supporting its role in amyloid pathology even in the absence of metabolic disorders. Our data suggest that PP may interfere with Aβ regular deposition at different levels, depending on the

metabolic status, and these observations are in concordance with previous studies with isolated PP components. NSAIDs may reduce soluble Aβ42 levels and plaque burden in AD mice [52-53]. It has also been shown that statins can reduce Aβ levels and amyloid precursor protein C-terminal fragment [54]. Also, simvastatin can induce Aβ degradation, by increasing neprilisin secretion [55]. While the role of metformin in amyloid pathology remains doubtful [56-57], it may reduce BACE1 expression and activity in cell and animal models [58]. Furthermore, metformin can reduce Aβ42 levels in diabetic mice [16] and AD related pathology [58], supporting the combined effects of all PP components.

Increased tau phosphorylation has been observed in T2D and mixed AD-T2D animal models [15, 22] and PP treatment significantly reduced tau hyperphosphorylation. Our results are in agreement with previous studies on tau phosphorylation after individually administering PP components. Metformin may induce tau pro-aggregation [59], however it also shows a positive effect on tau pathology inducing tau dephosphorylation via AMPK/mTOR and PP2A [60]. Likewise, simvastatin may improve tau pathology through decreasing neurofibrillary tangles, probably by reducing microglia burden [61]. Central inflammation is also a common feature in AD and T2D. In this sense, the anti-inflammatory effects of metformin [62], simvastatin [63] and perindopril [64] at central level have been recently described. In our hands, PP treatment ameliorated microglia burden. While microglia activation does not seem to be affected to the same extent in the hippocampus, previous studies have also reported that the hippocampus is not affected to the same extend in APP/PS1, db/db and APP/PS1xdb/db mice [14-15], making differences among groups harder to detect. It has been reported that reduced microglia activation might be derived from its effects on tau and amyloid pathology. However, it is also feasible that PP treatment may specifically reduce central inflammation. On the other hand we can not exclude that the reduction in the NeuN/DAPI ratio, observed in db/db and APP/PS1xdb/db mice, might also reflect an increased inflammatory process, that is partially restored after PP treatment, ultimately accounting for the limited effect of PP treatment of synaptic densities in the areas close to SP.

Both T2D and AD have been closely related to vascular dysfunction, and preceding studies have reported increased spontaneous bleeding in db/db mice [22]. Also, a synergistic vascular damage is observed when AD and T2D are set together [3, 14-15]. While we have not analyzed other brain regions or addressed white matter disease in our

mice, previous studies have pointed out that diabetic mice have significantly increased white matter damage after induced stroke [65]. We observed a reduction of cortical microhemorrhages in our mixed model after long-term PP treatment, in line with previous studies on individual PP components: aspirin [50], simvastatin [63], perindopril [66] and metformin [38], supporting a role for PP cocktail to ameliorate vascular pathology.

Altogether, PP might be an appealing approach that combines inexpensive, safe and approved components (metformin+aspirin+statin+perindopril). PP interferes with tau and amyloid pathology, inflammation, and spontaneous bleeding, and improves cognitive impairment. Our data provide the basis to further analyze the role of PP, as an alternative or adjuvant, to slow down or delay the central pathology associated with T2D and AD.

Author's contribution: CI-G performed the experiments, analyzed the data and drafted the manuscript, JJR-R, CH-B, RJ and FH-P performed the experiments and analyzed the data, TSJ analyzed the data, drafted and reviewed the manuscript, MGA conceived the study, analyzed the experiments and wrote the manuscript approved by all authors.

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Figure legends

Figure 1. **PP-treatment improves plasma parameters in db/db and APP/PS1xdb/db mice and rescues cognitive impairment**. **A)** Body weight was significantly higher in diabetic mice (db/db and APP/PS1xdb/db) when compared with Control and APP/PS1 groups, and PP treatment also contributed to body weight gain (weeks 4, 8, 12: ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP; weeks 16, 20 and 24: ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db and APP/PS1xdb/db, ‡‡ p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP). **B)** Blood glucose levels were reduced after PP treatment in diabetic mice and this effect was significantly increased in APP/PS1xdb/db mice after 12 weeks of treatment (week 4: p=0.07; weeks 8 and 12: ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP; weeks 16, 20 and 24: **p<0.01 vs. rest of the groups, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP). **C)** Insulin levels were significantly increased in diabetic mice (db/db and APP/PS1xdb/db) before the commencement of the treatment, at 4 weeks of age, when compared with Control and APP/PS1 mice. Insulin exhaustion was more pronounced in APP/PS1xdb/db mice than in db/db mice and PP treatment rescued insulin levels in diabetic mice (week 4: ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP; weeks 8, 12 and 16: ‡‡p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db and APP/PS1xdb/db, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP; weeks 20 and 24: ‡‡p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db and APP/PS1xdb/db. **D)** Episodic memory was significantly improved after PP treatment, as observed in the NOD test. A significant improvement was observed in "what" and "where" paradigms for APP/PS1, db/db and APP/PS1xdb/db mice after PP, when compared with untreated mice: "what" (††p<0.01 vs. Control, Control-PP, db/db-PP and APP/PS1xdb/db-PP, ##p<0.01 vs. Control and Control-PP, ‡‡p<0.01 vs. Control), "where" (‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP, ##p<0.01 vs. Control and Control-PP). While a similar profile was observed for "when" paradigm, differences did not reach statistical significance (p=0.101). **E)** Spatial learning impairment in the MWM test was partially reverted after PP treatment in APP/PS1 mice. db/db and APP/PS1xdb/db mice on PP also presented significantly shorter times to find the hidden platform along the acquisition phase, when compared with untreated littermates. (day 1: ##p<0.01 vs. Control and Control-PP; day 2: ##p<0.01 vs. Control

and Control-PP, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP and db/db-PP; day 3: ##p<0.01 vs. Control and Control-PP, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP; day 4: ♯♯p<0.01 vs. Control, Control-PP and APP/PS1, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP, **p<0.01 vs. rest of the groups). **F)** In the retention phase of the MWM, when the platform was removed from the pool, we also observed that memory impairment in db/db and APP/PS1xdb/db mice was completely recovered after PP administration (††p=0.003 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdbdb-PP).

Figure 2. PP treatment improves central atrophy in db/db mice. A) Brain weight was significantly reduced in db/db and APP/PS1xdb/db mice when compared to Control and APP/PS1 mice. Long-term PP treatment significantly reduced brain weight loss, although PP-treated groups did not reach Control values (**p<0.01 vs. rest of the groups, ‡‡p<0.01 vs. Control, control-PP, APP/PS1, APP/PS1-PP and db/db-PP, ††p<0.01. vs. Control, control-PP, APP/PS1 and APP/PS1-PP). **B)** Similarly, cortical size compromise in db/db mice was improved after PP treatment and this effect reached statistical significance in APP/PS1xdb/db mice (**p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP, ††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP). **C)** When we measured hippocampus size, differences did not reach statistical significance among groups (p=0.997). **D)** Illustrative example of cresyl violet staining where cortical thinning, observed in db/db and APP/PS1xdb/db mice, improves after PP treatment. Scale bar=250 µm. **E)** Immunostaining for NeuN-possitive cells was reduced in SP-free areas, both in db/db and APP/PS1xdb/db mice. PP treatment significantly improved this situation and the number of NeuN-possitive cells was increased both in db/db-PP and APP/PS1xdb/db mice (‡‡p<0.01 vs.Control, Control-PP, db/db-PP and APP/PS1xdb/db-PP, ##p<0.01 vs. Control). Also, in the proximity of SP, reduced NeuN+/DAPI ratio in APP/PS1 and APP/PS1xdb/db mice improved after PP when compared to untreated animals (‡‡p=0.08 vs. APP/PS1xdb/db-PP). **F)** Illustrative example of NeuN (red) immunostaining and DAPI (blue) counterstain. PP treatment restores NeuN+/DAPI ratios in APP/PS1, db/db and APP/PS1xdb/db mice. Scale bar: 50 µm.

Figure 3. PP-treatment preserves synaptic density in db/db and APP/PS1xdb/db treated mice. A) No differences were detected among groups when PSD95 densities were analyzed by array tomography in the proximity of the SP (p=0.127). In SP free areas, PSD95 densities were lower in db/db mice and this effects worsened in APP/PS1xdb/db mice. Long-term PP treatment significantly increased PS95 densities in db/db-PP and APP/PS1xdb/db-PP groups, that reached control values (**p<0.01 vs. rest of the groups, ‡‡p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP and APP/PS1xdb/db-PP). **B)** Similarly, synaptophysin densities, analyzed by array tomography, were not significantly different among groups in the proximity of the SP, (p=0.474). However, in SP free areas, synaptophysin densities were significantly lower in db/db and APP/PS1xdb/db mice. PP treatment significantly improved synaptophysin densities in db/db and APP/PS1xdb/db treated mice (††p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, ‡‡p<0.01 vs. Control and Control-PP, ##p<0.01 vs. Control-PP). **C)** Illustrative example of SP (anti-AW7 in grey) and synaptic proteins PSD95 (green), synapthophysin (red) and nuclear stain with DAPI (blue). Scale bar=10 µm.

Figure 4. PP treatment ameliorates amyloid pathology and reduces tau hyperphosphorylation. A) SP burden was significantly lower in APP/PS1xdb/db mice when compared with APP/PS1 animals. PP treatment reduced amyloid SP burden in APP/PS1 mice after 4G8 immunostainning and thioflavin S staining (TS) (**p<0.01 vs. rest of the groups, ††p<0.01 vs. APP/PS1xdb/db and APP/PS1xdb/db-PP). A similar profile was observed in the hippocampus after 4G8 immunostaining (††p<0.01 vs. APP/PS1xdb/db and APP/PS1xdb/db-PP]) and TS staining (‡‡p=0.026 vs. APP/PS1xdb/db). **B)** Illustrative example of SP deposition after 4G8 immunostaining (red). Scale bar=250 µm. **C)** Cortical soluble Aβ40 levels were slightly increased in APP/PS1xdb/db mice when compared with APP/PS1 animals and PP reduced this effect (‡p<0.035 vs. APP/PS1xdb/db-PP). Soluble Aβ42 levels showed similar profile although differences did not reach statistical significance (p=0.075). When we analyzed insoluble Aβ levels, we observed a similar profile to that detected for SP burden: insoluble Aβ40 levels were significantly higher in APP/PS1 mice when compared with APP/PS1xdb/db mice, however PP treatment reduced insoluble Aβ40 in the APP/PS1 group (*p=0.019 vs. rest of the groups). A similar profile was observed for Aβ42 levels although differences did not reach statistical significance (p=0.081). Hippocampus presented a similar profile with higher soluble Aβ levels in APP/PS1xdb/db mice that

were reduced after PP treatment, however differences did not reach statistical significance (Aβ40, p=0.144; Aβ42, p=0.130). Insoluble Aβ40 levels were higher in APP/PS1 mice and this effect was counterbalanced after PP treatment (**p=0.005 vs. rest of the groups). Insoluble Aβ42 were also slightly increased in APP/PS1 mice and reduced after PP treatment, although differences did not reach statistical significance (p=0.083). **D)** Tau phosphorylation, analyzed by western-blot, showed that increased hyperphosphorylation in the cortex from db/db and APP/PS1xdb/db mice was significantly reduced after PP treatment in both groups (†p=0.02 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP). A similar profile was observed in the hippocampus where PP significantly reduced tau phosphorylation in db/db and APP/PS1xdb/db mice (##p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdbd/db-PP, ††p<0.01 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP). **G)** Illustrative example of cortical tau phosphorylation in all groups under study.

Figure 5. PP treatment reduces central inflammation and spontaneous bleeding. A) Microglia burden, measured after IBA-1 immunohistochemistry, was increased in the proximity of cortical SP in APP/PS1 mice, when compared with APP/PS1xdb/db mice. Long-term PP treatment significantly reduced microglia burden, both in APP/PS1 and APP/PS1xdb/db mice (**p<0.05 vs. rest of the groups, ††p<0.01 vs. APP/PS1 and APP/PS1-PP, ‡‡p<0.01 vs. APP/PS1). In areas far from SP, increased microglia burden in db/db and APP/PS1xdb/db mice was also counterbalanced by PP treatment (##p<0.01 vs. Control, Control-PP, APP/PS1, APP/PS1-PP, db/db-PP and APP/PS1xdb/db-PP, ‡‡p<0.01 vs. Control, Control-PP and APP/PS1-PP). Hippocampal microglia burden showed a similar trend, however differences among groups were not statistically significant close (p=0.1) or far from SP (p=0.118). **B)** Representative image of SP (anti-4G8 immunostaining, red) and microglia inmunostaining (anti-IBA1 immunostaining, green) in the cortex. Scale bar=25 μm. **C)** Hemorrhage burden, analyzed by Prussian blue staining, was also significantly increased in the cortex from APP/PS1xdb/db mice, while PP treatment reduced spontaneous bleeding in APP/PS1xdb/db-PP mice, that reached control values (*p=0.011 vs. Control, Control-PP, APP/PS1-PP and db/db-PP). A similar profile was detected in the hippocampus although differences did not reach statistical significance (p=0.637). **D)** Illustrative example of cortical hemorrhages stained with Prussian blue. Green arrows point at individual hemorrhages. Scale bar=200 μm.

Table 1. Lipid profile in APP/PS1xdb/db mice after PP treatment

Group	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	Triglycerides (mg/dl)	ACE activity (U/L)
Control	78.91±4.72	40.55±8.58	23.64±2.00	52.36± 2.53	125.19±11.01
Control-PP	84.56±4.72	36.31±6.76	24.13±2.05	52.07± 2.33	117.31±15.68
APP/PS1	76.55±4.79	32.45±6.50	25.00±2.50	56.18± 3.43	133.61±10.62
APP/PS1-PP	82.75±3.68	33.67±6.67	20.83±1.13	57.00± 3.59	114.74±16.39
db/db	133.13±16.87††	16.22±6.08	40.78±6.91††	85.00±17.50	93.37±17.50
db/db-PP	116.43±14.33	21.14±8.50	28.29±3.41	70.29± 7.32	70.29±17.94
APP/PS1xdb/db	111.50±14.33	9.71±5.40	38.50±9.03	101.00±19.78††	149.32±14.42
APP/PS1xdb/db-PP	89.43±16.83	25.86±8.71	31.57±3.21##	72.00± 6.24	56.65±16.55†

Total cholesterol levels were increased in db/db mice and PP treatment counterbalanced this effect (††p=0.002 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP). HDL levels were reduced in db/db and APP/PS1xdb/db mice and PP treatment improved this effect, although differences did not reach statistical significance (p=0.084). Increased LDL levels in db/db and APP/PS1xdb/db mice were reduced after PP treatment (††p=0.002 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP and ##p=0.002 vs. APP/PS1-PP). Increased triglycerides levels in APP/PS1xdb/db mice were reduced after PP treatment (††p=0.001 vs. Control, Control-PP, APP/PS1 and APP/PS1-PP). ACE activity was reduced in db/db mice after PP treatment and this effect reached statistical significance in APP/PS1xdb/db (†p=0.015 vs. Control, APP/PS1, db/db and APP/PS1xdb/db).

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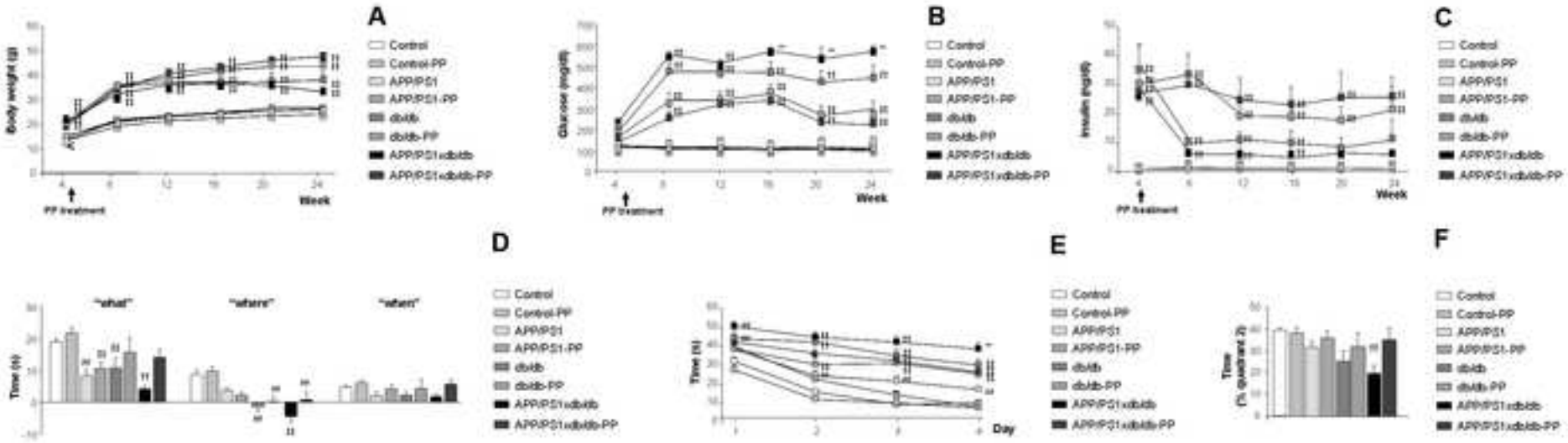

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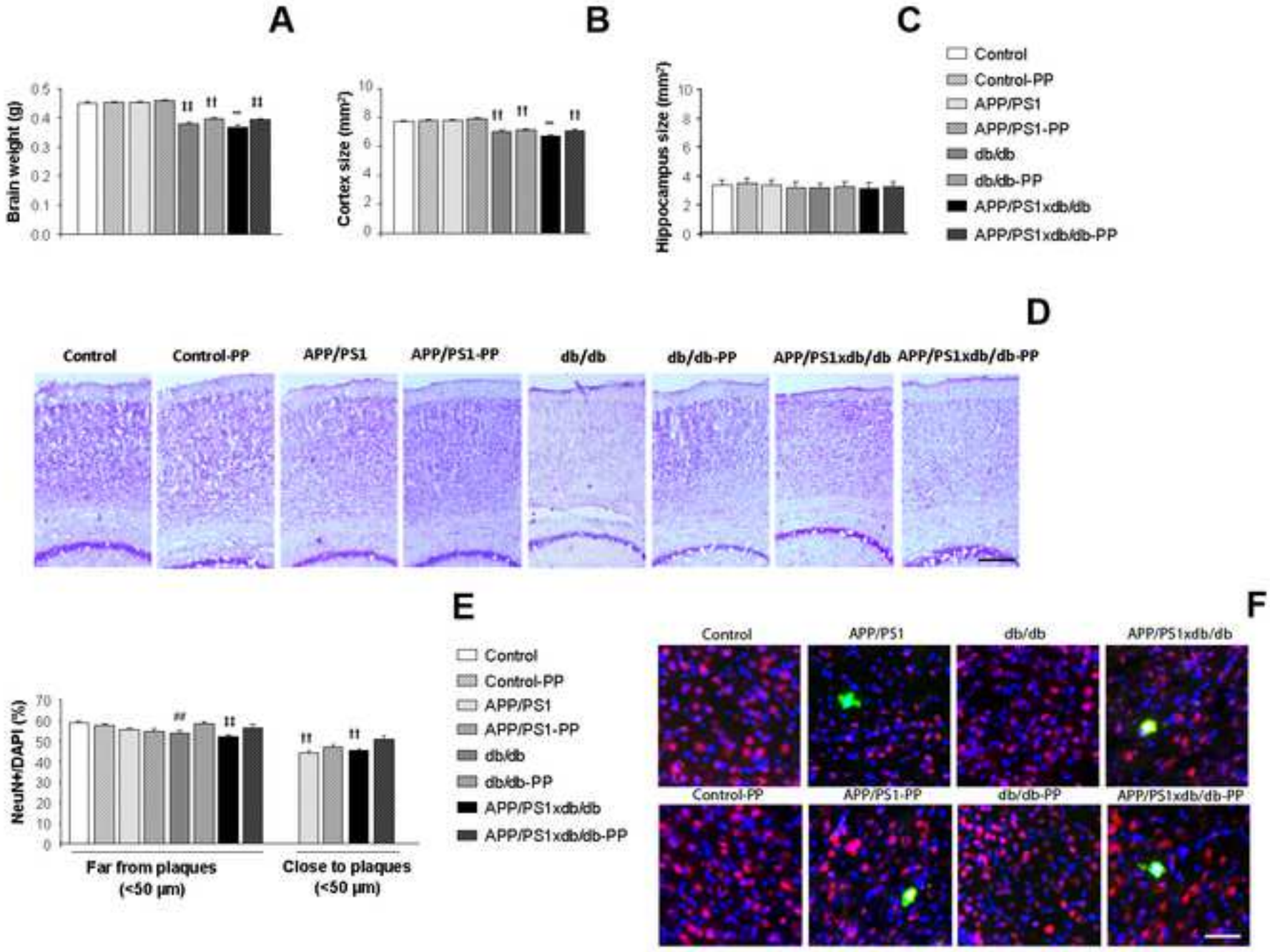

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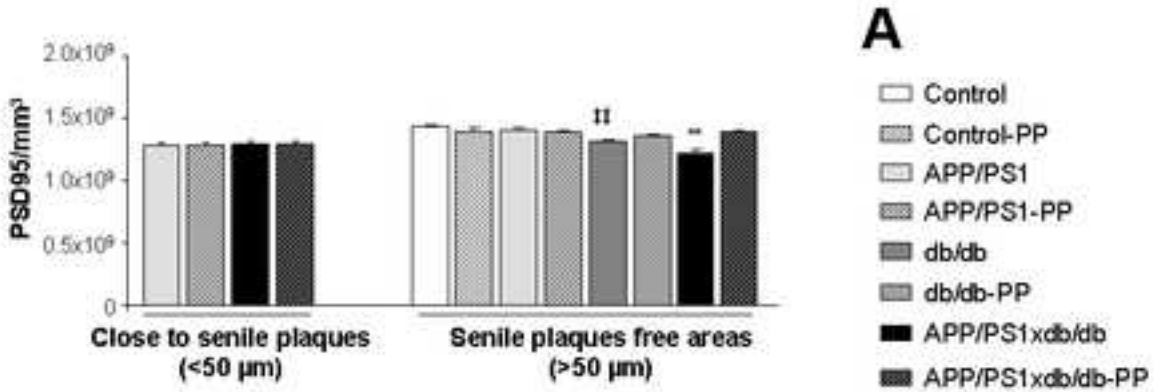

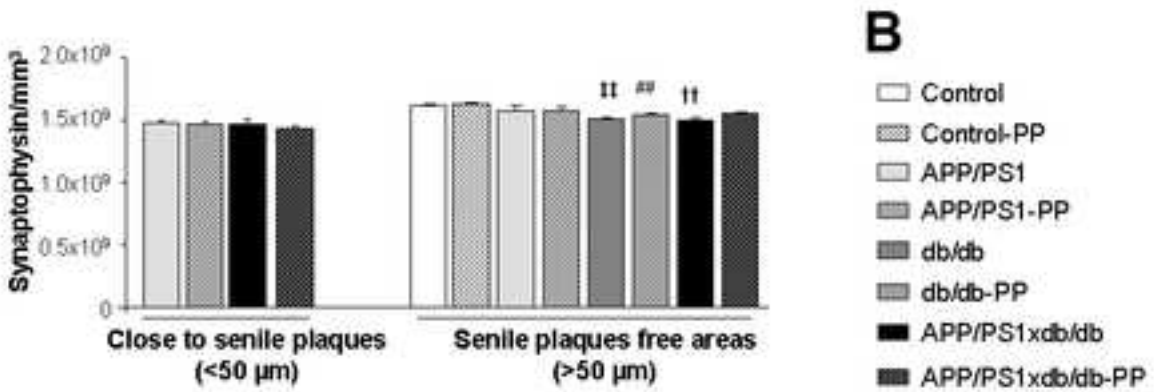

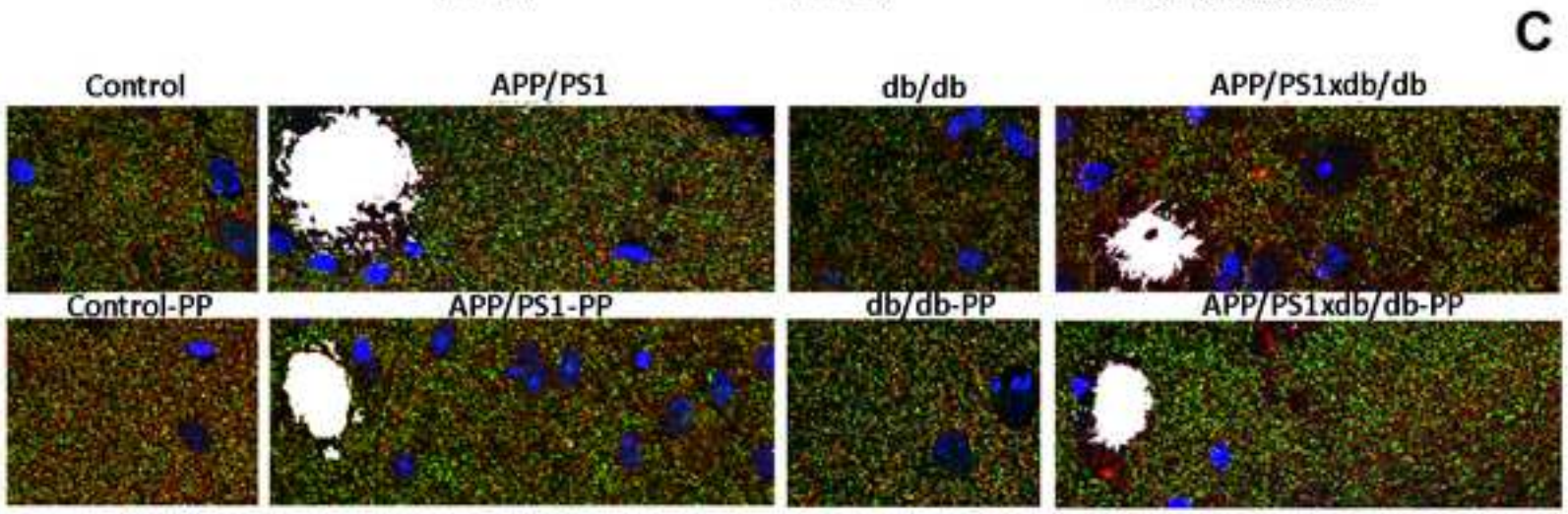

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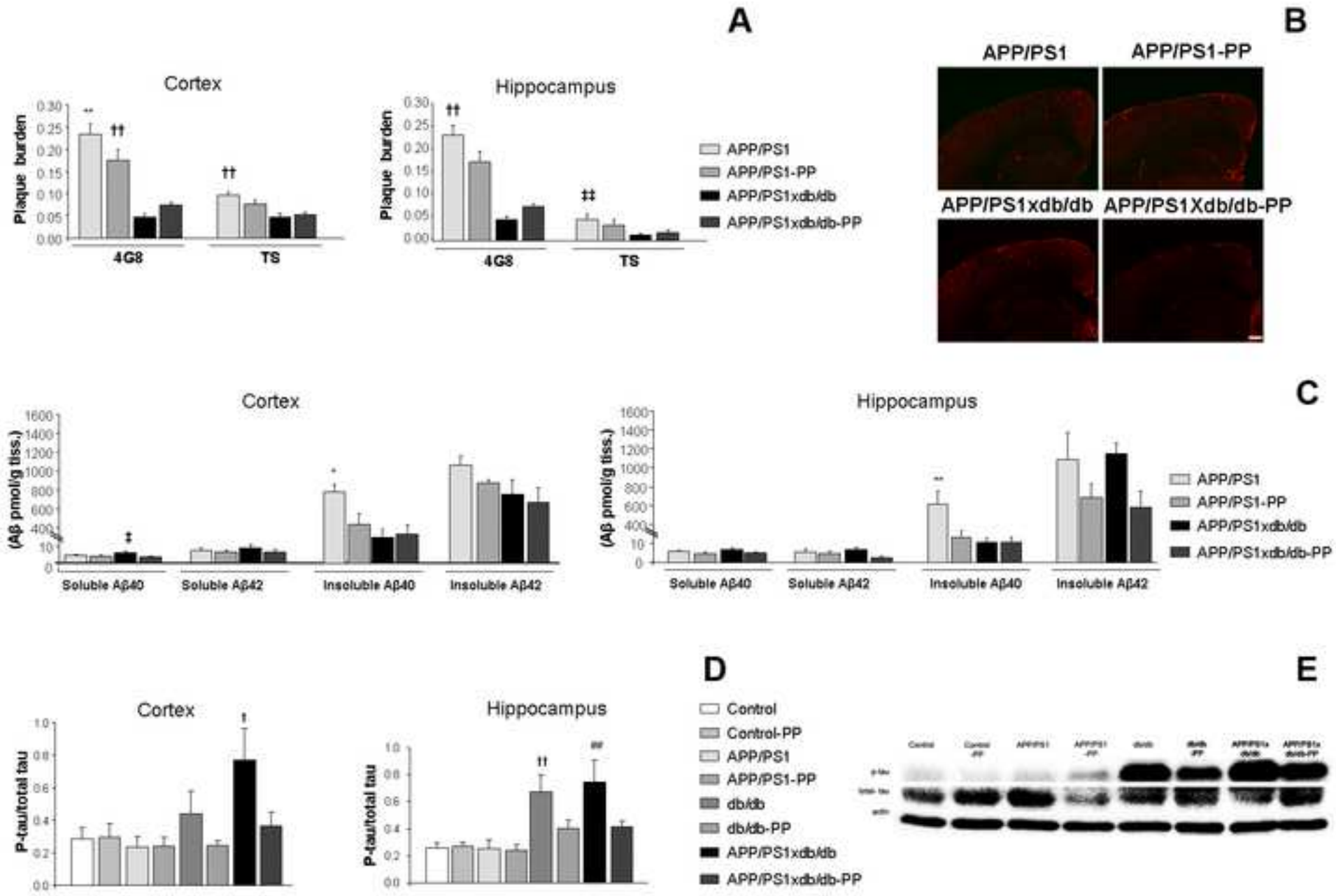

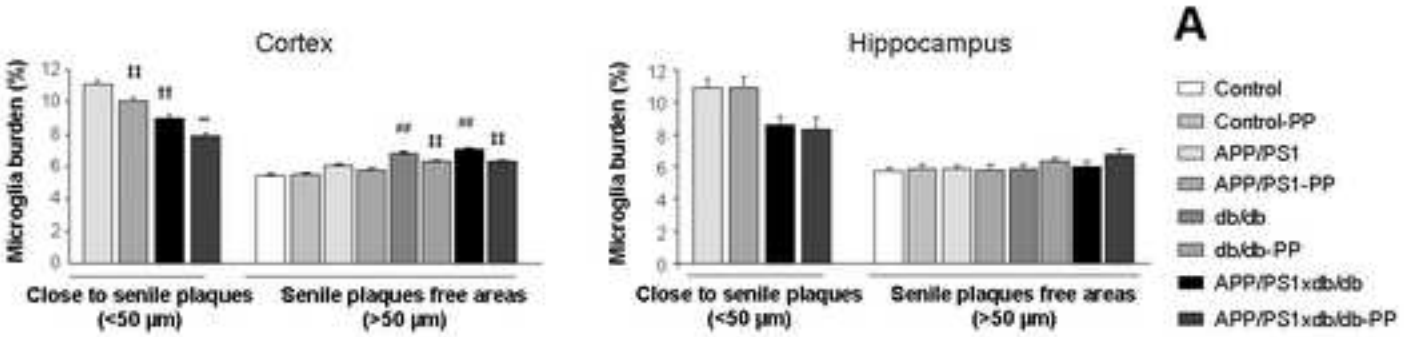
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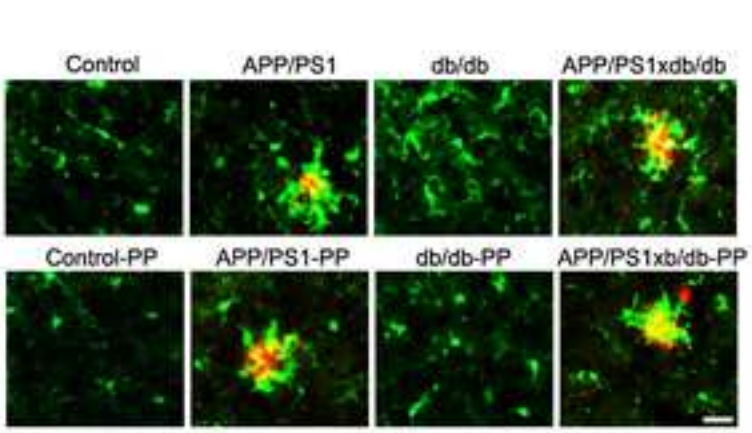
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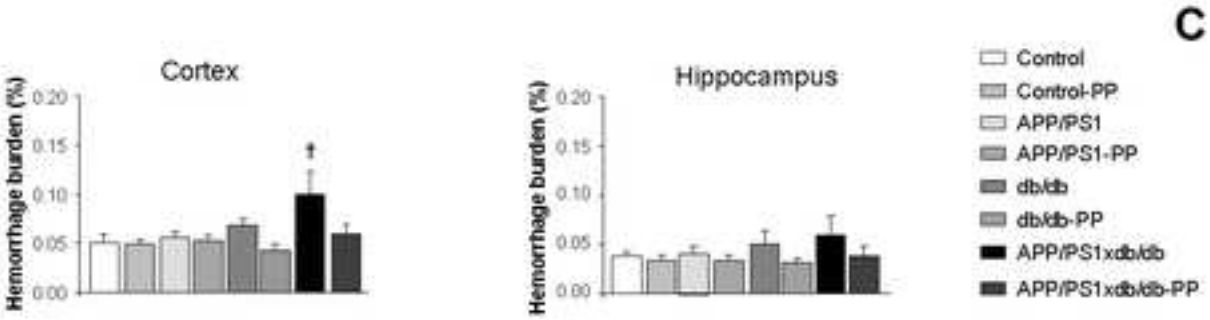
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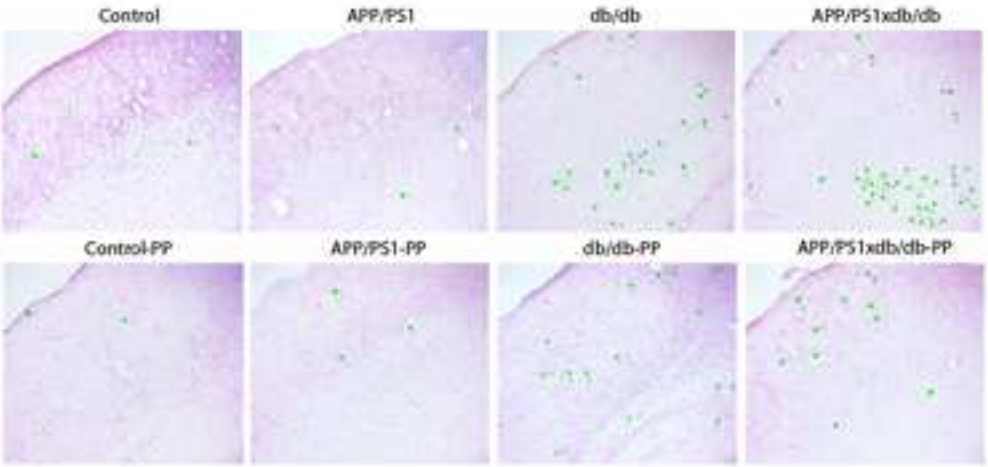
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CI-G performed the experiments, analyzed the data and drafted the manuscript, JJR-R, CH-B, RJ and FH-P performed the experiments and analyzed the data, TSJ analyzed the data, drafted and reviewed the manuscript, MGA conceived the study, analyzed the experiments and wrote the manuscript approved by all authors.
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