

CB₁ Agonist ACEA Protects Neurons and Reduces the Cognitive Impairment of A β PP/PS1 Mice

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Abstract. The present study shows that chronic administration of the Cannabinoid receptor type 1 (CB₁) receptor agonist arachidonyl-2-chloroethylamide (ACEA) at pre-symptomatic or at early symptomatic stages, at a non-amnesic dose, reduces the cognitive impairment observed in double A β PP(swe)/PS1(1dE9) transgenic mice from 6 months of age onwards. ACEA has no effect on amyloid- β (A β) production, aggregation, or clearance. However, ACEA reduces the cytotoxic effect of A β ₄₂ oligomers in primary cultures of cortical neurons, and reverses A β -induced dephosphorylation of glycogen synthase kinase-3 β (GSK3 β) *in vitro* and *in vivo*. Reduced activity of GSK3 β in ACEA-treated mice is further supported by the reduced amount of phospho-tau (Thr181) in neuritic processes around A β plaques. In addition, ACEA-treated mice show decreased astroglial response in the vicinity of A β plaques and decreased expression of the pro-inflammatory cytokine interferon- γ in astrocytes when compared with age-matched vehicle-treated transgenic mice. Our present results show a beneficial effect of ACEA at both the neuronal, mediated at least in part by GSK3 β inhibition, and glial levels, resulting in a reduction of reactive astrocytes and lower expression of interferon- γ . As a consequence, targeting the CB₁ receptor could offer a versatile approach for the treatment of Alzheimer's disease.

Keywords: Alzheimer's disease, astrogliosis, cannabinoid receptor, CB₁ cognition, GSK3 β , neuroprotection, transgenic mice

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disorder affecting one in eight people aged 65 and older in Western countries [1]. The limited effectiveness of current therapies against AD

highlights the need for intensified research efforts devoted to developing new agents for preventing or retarding the disease process. In recent years, interest has increasingly focused on the potential neuroprotective properties of cannabinoids in AD [2]. The endocannabinoid system is composed of at least two well-characterized cannabinoid G_{i/o}-coupled receptors, CB₁ and CB₂, their endogenous ligands, and the enzymes related to their synthesis and degradation [3]. The CB₁ receptor is widely expressed within the central nervous system [4], in both neurons and glial cells

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[3, 5], where it regulates important brain functions [6, 7]. Moreover, CB₁ receptor plays a role in protection against neurotoxicity [8] and in the induction of repair mechanisms in response to neuronal damage [9]. In contrast, CB₂ receptor is mainly expressed in the immune system, including microglia [10]. The activation of CB₂ receptor reduces the microglial production of pro-inflammatory molecules [11], which is also implicated in the control of neural survival [12]. Thus, the attention paid to cannabinoids in AD is mainly due to their ability to reduce neuroinflammation through the activation of CB₁ and CB₂ receptors [13–16], but also through reducing the harmful action of amyloid- β peptide (A β) and promoting the brain's intrinsic repair mechanisms [17]. Among the neuroinflammation-independent mechanisms associated with cannabinoid-induced neuroprotection against A β , the CB₁ receptor plays a remarkable role. In this line, recent studies have reported that the activation of CB₁ receptor preserves neuron viability by reducing A β -induced lysosomal membrane permeabilization [18] and by suppressing pro-apoptotic signaling pathways [19]. The diversity of mechanisms involved in the neuroprotective role of CB₁ receptor in AD suggests that targeting this receptor could represent a versatile approach toward the treatment of AD. Based on this premise, the present study is specifically focused on the potential properties of a CB₁ receptor agonist in an animal model of AD. We selected the synthetic agonist arachidonyl-2-chloroethylamide (ACEA) because of its high affinity and specificity to the CB₁ receptor [20].

Double A β PP(swe)/PS1(1dE9) (A β PP/PS1) mice are used in the present study as a model of familial AD because they reproduce some of the most relevant features of the disease, including cognitive impairment and several pathological alterations such as A β plaques, dystrophic neurites around A β deposition, and synaptic abnormalities from the age of six months onwards [21, 22]. A β PP/PS1 mice do not replicate neurofibrillary tangles observed in AD brains, but do exhibit hyperphosphorylated tau protein in the vicinity of A β plaques, as also observed in A β PP Tg2576 mice [23]. Therefore, we consider that A β PP/PS1 mice represent valuable tools for the evaluation of novel therapeutic strategies against AD.

In the present study, we provide data revealing a reduction in the cognitive impairment of A β PP/PS1 mice treated during pre-symptomatic and early symptomatic stages with a non-amnesic dose of a CB₁ receptor agonist, supporting the hypothesis that

cannabinoid compounds may have potential use in the treatment of AD.

MATERIALS AND METHODS

Primary cultures of cortical neurons

Cortical cells were isolated from 18-day-old OF1 mouse embryos. The procedure was approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. Cortex were aseptically dissected and trypsinized. Cells were seeded in phenol-red-free Dulbecco's Modified Eagle Medium (DMEM) plus 10% horse serum on to 1% poly-L-Lysine coated plates. After 120 min, medium was removed and neurobasal medium (high-glucose phenol-red-free DMEM; Gibco BRL) was added containing 1% B27 supplement (Gibco BRL), plus antibiotics. On day 3 of culture, cells were treated with 2 μ M 1- β -D-arabinofuranosylcytosine (Sigma) for 24 h to eliminate proliferating non-neuronal cells. Cultured cortical cells were used for the experiments on day 10.

Cannabinoid protection assays in cortical neurons

Primary cultures of mouse cortical neurons (7.5×10^4 cells/300 μ L/well) were assayed in neurobasal supplemented with B27 without antioxidants in 24 well culture-plates. Cells were treated with 0.1 or 1 μ M ACEA, and then PBS (control) or 1 μ M A β oligomers were added to wells. Cells were incubated for 24 h. Cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Briefly, 33 μ L of MTT stock solution (5 mg/mL) was added and after 2 h the reaction was stopped with 300 μ L of DMSO. MTT reduction was determined in a plate reader spectrophotometer at 540 and 650 nm. Control cells were taken as 100%.

Animals

The experiments were carried out on male A β PP/PS1 mice and wild-type littermates aged 3, 6, or 12 months at the beginning of the study. The generation of mice expressing the human mutated forms A β PPswe and PS1dE9 (A β PP/PS1) has been already described [21]. In the present work, identification of transgenic mice was carried out as follows: genomic DNA was isolated from 1-cm tail clips and genotyped by polymerase chain reaction (PCR) technique using

the PCR conditions proposed by Jackson Laboratory. Animals were maintained under standard animal housing conditions in a normal 12-h dark-light cycle with free access to food and water. The sample size for experimentation was computed using the Power and Precision software (Biostat, Englewood, NJ, USA), assuming a power of 95% and no missing data. Animal procedures were conducted according to ethical guidelines (European Community Council Directive 86/609/EEC) and approved by the local ethical committee (UB-IDIBELL).

Drugs and pharmacological treatment

The selective CB₁ receptor agonist ACEA was supplied by Tocris Bioscience[®] (Bristol, UK). ACEA (1.5 mg/kg) was dissolved in 5% ethanol, 5% Tween, and 90% saline, and this mixture was injected intraperitoneally (i.p.) in a volume of 10 mL/kg body weight. Animals treated during the pre-symptomatic phase received one daily administration for 5 weeks with ACEA (wild-type, $n=10$; A β PP/PS1, $n=8$) or the corresponding vehicle (wild-type, $n=9$; A β PP/PS1, $n=7$) starting at 3 months of age. The behavioral testing was performed when animals were six months of age. A second group of animals were treated during the early symptomatic phase. These mice aged 6 months were treated once daily for 5 weeks with ACEA (wild-type, $n=10$; A β PP/PS1, $n=9$) or the corresponding vehicle (wild-type, $n=6$; A β PP/PS1, $n=7$). After 10 days of washing period, animals were subjected to behavioral evaluation. A third group of 6-month-old mice ($n=5$) was used for the acute experiment to evaluate the GSK3 β levels. Animals were administered ACEA (1.5 mg/kg) and sacrificed 30 min later. Their brains were dissected on ice, immediately frozen, and stored at -80°C until processing.

Behavioral evaluation of cognitive performance

Two-object recognition test

This paradigm was performed in a V-maze (Panlab, Barcelona, Spain) because it improves the exploration time of the animals with respect to a classical open field. On day 1, mice were habituated for 9 min, allowing them to freely explore the apparatus. On the second day, mice were placed for 9 min in the maze, where two identical objects were situated at the end of the arms, and the time that the mice spent exploring each object was recorded. Then, 24 h after the training session, animals were placed again in the

V-maze where one of the two familiar objects was replaced by a novel object. The time that the animals spent exploring the two objects was recorded and an object recognition index was calculated as the difference between the time spent exploring the novel and the familiar object, divided by the total time spent exploring the two objects. Animals exhibiting memory impairments revealed a lower object recognition index [24].

Active avoidance test

After the two-object recognition test, the animals were allowed to rest for 4 days before starting the active avoidance test. Then, the mice were trained to avoid an aversive stimulus associated with the presentation of a conditioned stimulus (CS) in a two-way shuttle box apparatus (Panlab, Barcelona, Spain). The CS was a light (10 W) switched on in the compartment in which the mouse was placed. The CS was received 5 s before the onset of the unconditioned stimulus (US) and overlapped it for 25 s. At the end of the 30-s period, both CS and US were automatically turned off. The US was an electric shock (0.2 mA) continuously applied to the grid of the floor. A conditioned response was recorded when the animal avoided the US by changing from the compartment where it received the CS to the opposite compartment within the 5 s period after the onset of the CS. If animals failed to avoid the shock, they could escape it by crossing during the US (25 s) and this was recorded as unconditioned response. Between each trial session, there was an inter-trial interval of 30 s. Animals were subjected to five daily 100-trial active avoidance sessions. Each day, the mice were placed in the shuttle box for 10 min before the start of each session to allow them to explore the box. Data are expressed as the total number of conditioned changes, converted to the area under the curve (AUC) using a standard trapezoid method.

Tissue collection

At the end of the behavioral testing, the animals were sacrificed and their brains were removed. One brain hemisphere was dissected on ice, immediately frozen and stored at -80°C until processing for the A β soluble quantification. The other brain hemisphere was fixed in 4% paraformaldehyde and processed for immunohistochemistry.

A β immunohistochemistry

Tissue samples were embedded in paraffin and coronal sections (4 μ m) were cut with a microtome. De-waxed sections were incubated with 98% formic acid (3 min) and then treated with citrate buffer (20 min) to enhance antigenicity. Then, the endogenous peroxidases were blocked by incubation in 10% methanol-1% H₂O₂ solution (15 min). Sections were blocked with 3% normal horse serum solution and then incubated at 4°C overnight with the primary antibodies against A β (1 : 50, Dako, Clone 6F/3D). Sections were subsequently rinsed and incubated with biotinylated secondary antibody (Dako), followed by EnVision+ system peroxidase (Dako) and finally with the chromogen diaminobenzidine and H₂O₂. Some sections were incubated without the primary antibody. No immunostaining was detected in these sections. Sections were lightly counterstained with haematoxylin. After staining, the sections were dehydrated and cover-slipped for microscopic observation. The A β burden in neocortex was calculated as the percentage of the A β deposition area with respect to the total area in 9 representative pictures, corresponding to the main regions where A β deposition is observed in A β PP/PS1 mice (Fig. 4A). One section of the hippocampus was used for similar quantification of the A β burden. Sections from all the A β PP/PS1 animals were evaluated by using the Analysis tool of the software Adobe® Photoshop® CS4. This Analysis tool allows selection by color range and quantification of the specific immunostaining density of each picture.

A β soluble quantification: Enzyme-linked immunosorbent assay (ELISA)

Fresh-frozen mouse brain cortexes were homogenized in 4 volumes (wt:vol) of TBS extraction buffer (140 mM NaCl, 3 mM KCl, 25 mM Tris (pH 7.4), 5 mM EDTA and protease inhibitor cocktail (Roche)). Homogenate was spun 100,000 g \times 1 h, and the supernatant saved as the soluble fraction for A β quantifications. The A β ₄₀ and A β ₄₂ Human ELISA kits (Invitrogen™ Corporation, Camarillo, CA, USA) were used to quantify the levels of A β ₄₀ and A β ₄₂ proteins in the brain soluble fractions, respectively. The quantitative determination was carried out according to the manufacturer's instructions. A β ₄₀ and A β ₄₂ levels were normalized to the total amount of protein from each individual sample. A ratio was calculated of the A β ₄₂ levels with respect to those of A β ₄₀.

A β aggregation

The A β ₄₂ (Sigma) stock solution was prepared by dissolving the peptide in DMSO to a final concentration of 15 μ g/ μ L. The turbidometric assay was carried out at room temperature, within a dark chamber in a 96-well plate under continuous shaking (300 rpm). Each well contained 100 ng/ μ L A β ₄₂ dissolved in 100 μ L PBS pH 5.5 and 0.1 or 1 μ M ACEA. Absorbance at 405 nm was followed over time.

Double-labeling immunofluorescence

CB₁ and A β or GFAP double-immunofluorescence

For CB₁ and A β or GFAP double-immunostaining, free-floating sections were incubated with 98% formic acid (3 min) to enhance antigenicity and then thoroughly washed in PBS. Tissue permeabilization was facilitated by incubation with 0.25% TX-100 together with 10% normal goat serum for non-specific binding blocking for 90 min at room temperature. Sections were then incubated with the combination of primary antibodies against CB₁ (1 : 500, Frontier Science Co. Ltd, Japan) and A β (1 : 50, Boehringer-Mannheim) or GFAP (1 : 250, Dako) overnight at 4°C. After washing, the sections were incubated for 60 min with Alexa488 or Alexa546 (1 : 400, Molecular Probes) fluorescence secondary antibodies against the corresponding host species and subsequently washed in PBS. Then, nuclei were stained with DRAQ5™ (1 : 2000, Biostatus Ltd, Leicestershire, UK), thoroughly washed, mounted onto polylysine-coated slides in Immuno-Fluore Mounting medium (ICN Biomedicals), sealed, and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope.

Densitometric quantification of CB₁

The presence of dystrophic neurites in the vicinity of the A β plaques is supposed to indicate that the integrity of the neurons is compromised in the areas directly influenced by A β deposition in A β PP/PS1 mice [22]. In contrast, the functionality of the neurons far from A β plaques should be more preserved since there is no evidence of dystrophic neurites in such areas. Thus, in order to estimate the influence of A β deposition in the CB₁ receptor cortical density, the CB₁ protein expression levels were evaluated in an area free from the A β plaque as well as in the vicinity of A β deposition. The percentage of the CB₁ staining area was calculated (a) with respect to the total cortex (wild-type mice) or to a 225 μ m \times 225 μ m area in the case

of an area free from A β deposition (A β PP/PS1) and (b) with respect to an area equivalent to 4 times the A β plaque (arbitrary reference based on the dystrophic neurites and presence of reactive gliosis and taken in order to normalize the A β extension), both in 5 representative pictures taken from the neocortex of each animal ($n=5$ per group) using the software Adobe[®] Photoshop[®] CS4.

Glial, tau phosphorylation and A β or Interferon- γ (IFN- γ) double-immunofluorescence

In the case of the glial, tau phosphorylation and A β or IFN- γ double immunostaining, de-waxed sections were stained with a saturated solution of Sudan black B (Merck) for 30 min to block the autofluorescence of lipofuscin granules present in cell bodies, then rinsed in 70% ethanol and washed in distilled water. The sections were treated with 98% formic acid (3 min, in the case of A β immunostaining) and with citrate buffer to enhance antigenicity, and then incubated at 4°C overnight with combinations of primary antibodies against A β (1 : 50, Dako), IFN- γ (1 : 50, Millipore) and tau-P(Thr181) (1 : 250, Calbiochem), GFAP (1 : 250, Dako) or Iba1 (1 : 250, Wako). After washing, the sections were incubated with Alexa488 or Alexa546 (1 : 400, Molecular Probes) fluorescence secondary antibodies against the corresponding host species. After washing, the sections were mounted in Immuno-Fluore Mounting medium (ICN Biomedicals, Solon, OH, USA), sealed, and dried overnight. Sections were examined with an Olympus BX51 microscope.

Densitometric quantification of glia and tau phosphorylation around A β plaques

Astrocytic and microglial responses to A β deposition, as well as tau phosphorylation, were evaluated by densitometric quantification of GFAP and Iba1 or tau-P(Thr181) protein expression levels around A β plaques, respectively. The GFAP, Iba1, and tau phosphorylation immunostaining was in reference to the A β plaque area in 5 representative pictures taken from the neocortex of each animal ($n=5$ per group) using the software Adobe[®] Photoshop[®] CS4.

Densitometric quantification of IFN- γ expression in astrocytes

IFN- γ immunostaining colocalized with GFAP, but not Iba1, immunostaining. Thus, the expression levels of IFN-g were evaluated by densitometric quantification and in reference to the GFAP immunostained area in 5 representative pictures taken from the neocortex

of each animal ($n=5$ per group) using the software Adobe[®] Photoshop[®] CS4.

P-Ser9-glycogen synthase kinase-3 β (GSK3 β) immunolabeling assay

Primary cultures of mouse cortical neurons (7.5×10^4 cells/300 μ L/well) were seeded on to poly-L-coated coverslips and assayed in Neurobasal supplemented with B27 without antioxidants in 24-well culture plates. Cells were incubated for 30 min with 1 μ M ACEA and PBS (control) or 1 μ M A β oligomers. Cells were incubated for 24 h. Then, neurons were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Immunostaining was done with polyclonal rabbit anti-p-Ser9-GSK3 β antibody (1 : 200; Calbiochem) or monoclonal rabbit anti-tubulin (1 : 1000; Sigma). Secondary antibodies were Alexa Fluor 488 goat anti-rabbit (1 : 700; Molecular Probes) and Alexa Fluor 555 goat anti-rabbit (1 : 1000; Molecular Probes). Coverslips were mounted and analysed under a confocal microscope.

P-Ser9-GSK3 β quantification

Cortical neurons

Primary cultures of mouse cortical neurons (10^6 cells/2 mL/well) were assayed in Neurobasal supplemented with B27 without antioxidants in 6-well culture plates. Cells were pre-incubated for 15 min with 0.5 μ M Rimonabant (a specific CB₁ inhibitor). Then, ACEA 1 μ M and PBS (control) or 1 μ M A β oligomers were added to wells. Cells were incubated for 30 min and lysed (137 mM NaCl; 20 mM Tris-HCl, pH 8.0; 1% NP-40; 10% glycerol; 1 mM sodium vanadate; 5 mM sodium pyrophosphate; 100 mM NaF; 40 mM glycerol phosphate; 1 mM PMSF; 0.15 μ M aprotinin; 11 μ M leupeptine; 1.5 μ M pepstatine).

Wild-type and A β PP/PS1 mice

Wild-type and A β PP/PS1 mice were acutely treated with vehicle or ACEA as indicated in section 2.4. Frozen brain areas were dounce-homogenized in the same lysis buffer described above. Samples were homogenized in 30 μ l lysis buffer/mg wet weight.

Immunoblot analysis

After 20 min of incubation with lysis buffer in agitation at 4°C, both cell and brain samples were centrifuged for 30 min at 16,000 g, and the supernatant was recovered and stored at -80°C. Protein content

was determined using the DC Protein Assay (Bio-Rad) following the manufacturer's instructions.

GSK3 β and phospho-Ser9 GSK3 β levels were quantified by western blotting. Equal amounts of lysates (20 μ g per lane) were separated by SDS-PAGE (8%) before electrophoretic transfer on to nitrocellulose membrane (Bio-Rad, Spain). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (100 mM NaCl, 10 mM Tris, pH7.4) with 0.1% Tween 20 (TTBS) and 5% nonfat milk. Afterwards, membranes were incubated overnight with the monoclonal rabbit anti-phospho-Ser9 anti-GSK3 β (1 : 1000; Cell Signaling Technology) in TTBS with 5% nonfat milk, and polyclonal rabbit anti-GSK3 β (1 : 1000, Chemicon) in TTBS with 5% bovine serum albumin. The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit (1 : 10000; Sigma). Samples were incubated 1 h at room temperature. Bands were visualized by enhanced chemiluminescence detection (ECL AdvanceTM, Amersham Biosciences). The value of active phospho-Ser9 GSK3 β was normalized to the amount of total GSK3 β in the same sample and expressed as a percentage of control treatment.

Statistical analyses

Data were analyzed by two-way ANOVA with genotype and treatment or age as between factors, followed by Tukey's *post hoc* test when required. Learning data (conditioned changes) were analyzed by three-way ANOVA with day (repeated measures), genotype and treatment as between factors. A β , glia, tau, and IFN- γ quantifications were analyzed by Student's *t*-test. The *in vitro* experiment data were evaluated statistically using one-way ANOVA followed by Tukey's *post hoc* test. In all the experiments, the significance level was set at $p < 0.05$.

RESULTS

ACEA protection against A β -induced neurotoxicity

As a preliminary approach to the study of the potential cannabinoid protection in A β PP/PS1 mice, we tested whether the CB₁ receptor agonist ACEA could induce a protective effect against A β -induced neurotoxicity *in vitro*. In the present study, we observed that ACEA protected cortical neurons against A β oligomer insult (Fig. 1). One-way ANOVA revealed significant effect of A β oligomer in cell viability ($F_{(3,14)} = 12.97$, $p < 0.001$). Tukey's *post hoc* test indicated that the exposure to A β oligomer produced a reduction in the

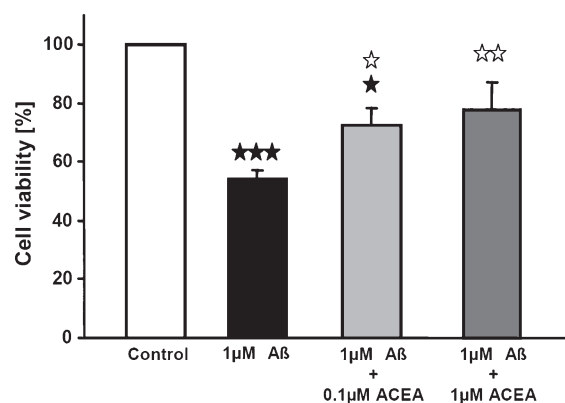


Fig. 1. Neuroprotection by ACEA against A β oligomer neurotoxicity in primary cultures of cortical neurons. Cell viability of cortical neurons exposed to A β_{42} oligomers in the absence or presence of ACEA. Data are the mean \pm SEM of 3–6 independent experiments. * $p < 0.05$, *** $p < 0.001$ compared to control. * $p < 0.05$, ** $p < 0.01$ compared to A β (one-way ANOVA followed by Tukey's *post hoc* analysis).

cell viability ($p < 0.001$), which was also significant in the presence of 0.1 μ M ACEA ($p < 0.05$), compared to control cells. Interestingly, a significant increase in viability was observed in cells exposed to A β oligomer in the presence of the 0.1 ($p < 0.05$) or 1 μ M ACEA ($p < 0.01$) when compared to A β -treated cells.

Progressive age-dependent loss of CB₁ receptor in the neocortex of A β PP/PS1 mice

In an attempt to assess the relevance of CB₁ receptor in AD, we evaluated the expression levels of this receptor in the neocortex of A β PP/PS1 mice, an animal model of familial AD [21], at different stages of the neurodegenerative progression by immunofluorescence techniques and quantitative densitometry. Our results revealed a progressive age-dependent reduction in the levels of CB₁ receptor in A β PP/PS1 mice from 6 months when compared to age-matched wild-type littermates (Fig. 2). Two-way ANOVA indicated a significant effect of age ($F_{(2,64)} = 16.84$, $p < 0.001$) and interaction between age and genotype ($F_{(2,64)} = 6.61$, $p < 0.001$) in the cortical areas free from A β deposition (Fig. 2Y). Subsequent one-way ANOVA revealed an age effect in both A β PP/PS1 ($F_{(2,29)} = 15.82$, $p < 0.001$) and wild-type animals ($F_{(1,32)} = 9.41$, $p < 0.01$). Tukey's *post hoc* test showed a significant reduction in CB₁ levels in A β PP/PS1 aged 12 months when compared to 3- and 6-month-old mice ($p < 0.001$ and $p < 0.01$, respectively), in contrast to the increase in CB₁ immunostaining in wild-type littermates at 6

compared to 3 months of age ($p < 0.05$). However, the levels of CB₁ were also reduced in wild-type mice at 12 months when compared to 6 months of age ($p < 0.001$). Comparing genotypes, we observed an increase in the CB₁ levels in A β PP/PS1 aged 3 months ($p < 0.05$) but reduced CB₁ immunostaining at 6 months ($p < 0.05$) and 12 months ($p < 0.01$) when compared to age-matched wild-type littermates.

In the area surrounding the A β plaques, a reduction in the CB₁ immunostaining in A β PP/PS1 animals aged 12 months was also observed when compared to 6-month-old mice ($t_{(23)} = 5.39$, $p < 0.001$) (Fig. 2Z).

The effect of ACEA on the cognitive performance of A β PP/PS1 mice treated during the pre-symptomatic phase

We evaluated the effect of a CB₁ receptor agonist *in vivo* at the cognitive level in our animal model of AD. A β PP/PS1 mice chronically treated for 5 weeks with ACEA during the pre-symptomatic phase did not show the cognitive impairment exhibited in the two-object recognition test by vehicle control A β PP/PS1 mice at the age of 6 months (Fig. 3B left).

Similarly, A β PP/PS1 mice chronically treated with ACEA during the pre-symptomatic phase did not exhibit the learning impairment exhibited by A β PP/PS1 mice chronically treated with vehicle in the active avoidance test at the age of 6 months (Fig. 3C and 3D left). See Table 1 for statistical details.

ACEA treatment during early stages of the symptomatic phase partially reversed the cognitive deficits in A β PP/PS1 mice

The daily stimulation of CB₁ receptors for 5 weeks at the early stages of the symptomatic phase (6 months) reversed the cognitive impairment exhibited by A β PP/PS1 mice on the two-object recognition test (Fig. 3B, right).

In contrast, the CB₁ agonist was not able to significantly reduce the impairment shown by A β PP/PS1 mice aged 6 months at the beginning of the treatment in the active avoidance paradigm (Fig. 3C right). See Table 2 for statistical details.

Cortical and hippocampal A β quantification

One possible mechanism used by ACEA to protect A β PP/PS1 mice would be a direct effect reducing A β production. To address this point, we quantified the

A β burden in neocortex and hippocampus (Fig. 4) as well as the soluble A β production and aggregation (Fig. 5) after ACEA treatment. We found that chronic treatment with ACEA did not significantly modify the A β burden in the cortex of A β PP/PS1 mice either when they were chronically treated during the pre-symptomatic phase or during the early stages of the symptomatic phase (Fig. 4B and C). Similarly, the A β burden was not modified in the hippocampus, a region where A β deposition starts later than in the neocortex (Fig. 4D). ACEA did not significantly modify the A β ₄₀ or A β ₄₂ protein levels (Fig. 5A) or the ratio between them (Fig. 5B) in the soluble cortical fraction of A β PP/PS1, either when they were chronically treated during the pre-symptomatic phase or during the early stages of the symptomatic phase. A possible direct effect of ACEA on A β aggregation due to its hydrophobic nature was ruled out since A β fibrillation was not affected by the presence of 0.1 or 1 μ M ACEA (Fig. 5C).

Chronic treatment with ACEA did not modify the expression of CB₁ receptor levels in the neocortex of A β PP/PS1 mice

In order to evaluate a possible down-regulation of CB₁ receptor after chronic exposure to the specific agonist ACEA, we analyzed the levels of this receptor in the neocortex of treated animals by immunofluorescence. Our results indicated that the ACEA dose used in our study did not significantly modify the levels of CB₁ receptor in the neocortex of A β PP/PS1 mice (Fig. 6). Two-way ANOVA indicated a significant effect of genotype ($F_{(1,51)} = 8.26$, $p < 0.01$) but no interaction between genotype and treatment in the CB₁ immunostaining in the cortical areas free from A β deposition (Fig. 6M). Tukey's *post hoc* test revealed a reduction in the CB₁ levels in vehicle-treated A β PP/PS1 mice ($p < 0.05$), but not in ACEA-treated animals, with respect to wild-type littermates.

Reduction of the astrocytic responses associated with A β deposition after ACEA treatment

Hypertrophic astrocytes and reactive microglia were observed in the vicinity of A β plaques in A β PP/PS1 mice. Double immunofluorescence techniques revealed that chronic stimulation with the CB₁ agonist ACEA produced a reduction in the area of astrocytes surrounding the A β plaques when compared to vehicle-treated animals during the

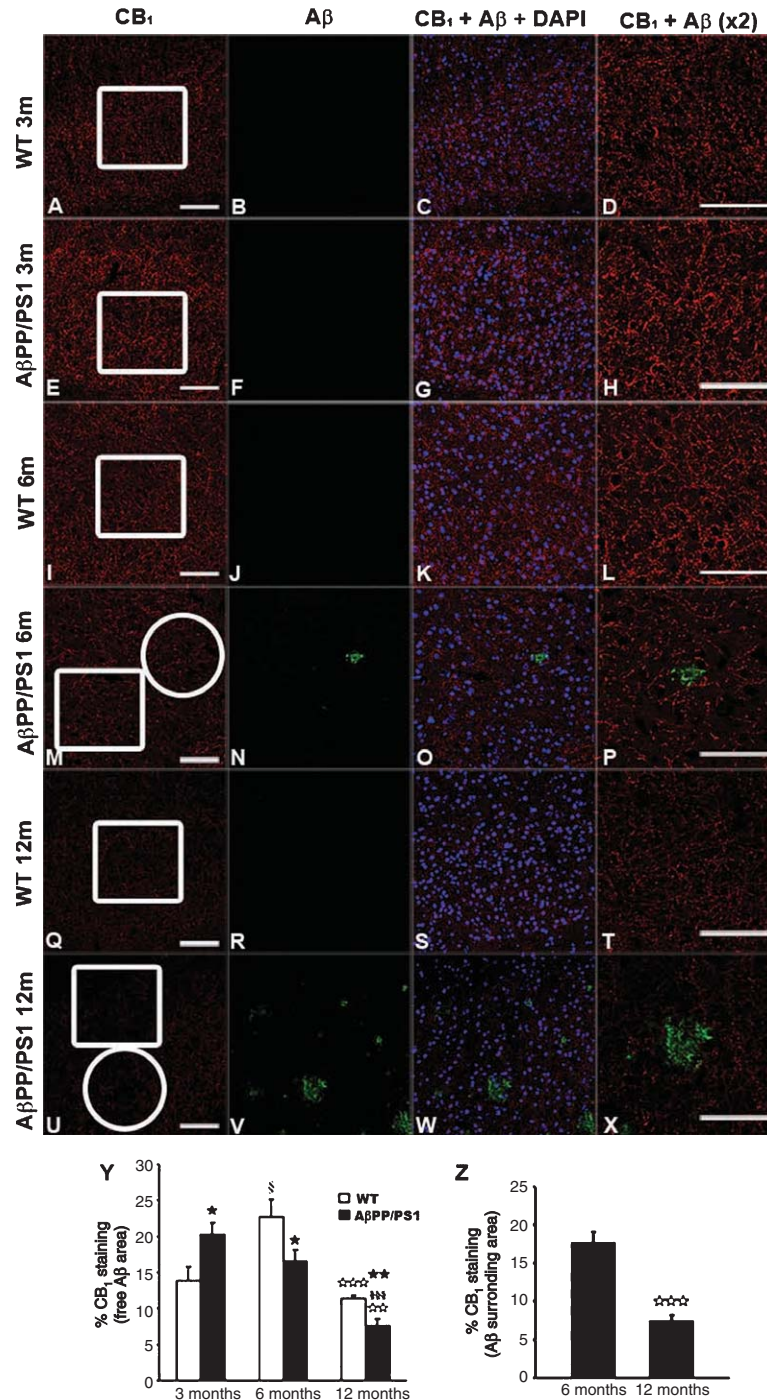


Fig. 2. Representative images of the double-immunofluorescence for CB₁ receptor (red, A, E, I, M, Q, and U) and A β (green, B, F, J, N, R, and V) in coronal sections of wild-type and A β PP/PS1 mice aged 3 months (A to H), 6 months (I to P), or 12 months (Q to X), indicating an age-dependent CB₁ downregulation in the cortical areas free from amyloid deposition (white-line squares, A, E, I, M, Q, and U. A, E, I, and Q magnified in D, H, L, and T), as well as in the areas surrounding amyloid plaques (white-line circles, A, E, I, M, Q, and U. M and U magnified in P and X). C, G, K, O, S, and W: merge. Nuclei are stained in blue. Scale bars represent 75 μ m. Quantification of CB₁ labeling in wild-type and A β PP/PS1 mice aged 3, 6, or 12 months revealed an age-dependent CB₁ downregulation in (Y) the cortical area free from A β deposition as well as in (Z) the area surrounding A β plaques. Data are expressed as the mean values \pm SEM ($n=5$ pictures from each animal, 3 animals per group). $\star p < 0.05$, $\star\star p < 0.01$ compared to wild-type. $\star\star\star p < 0.001$, $\star\star\star\star p < 0.0001$ compared to 6 months. $\S p < 0.05$, $\S\S\S p < 0.001$ compared to 3 months (two-way ANOVA followed by Tukey's *post hoc* analysis).

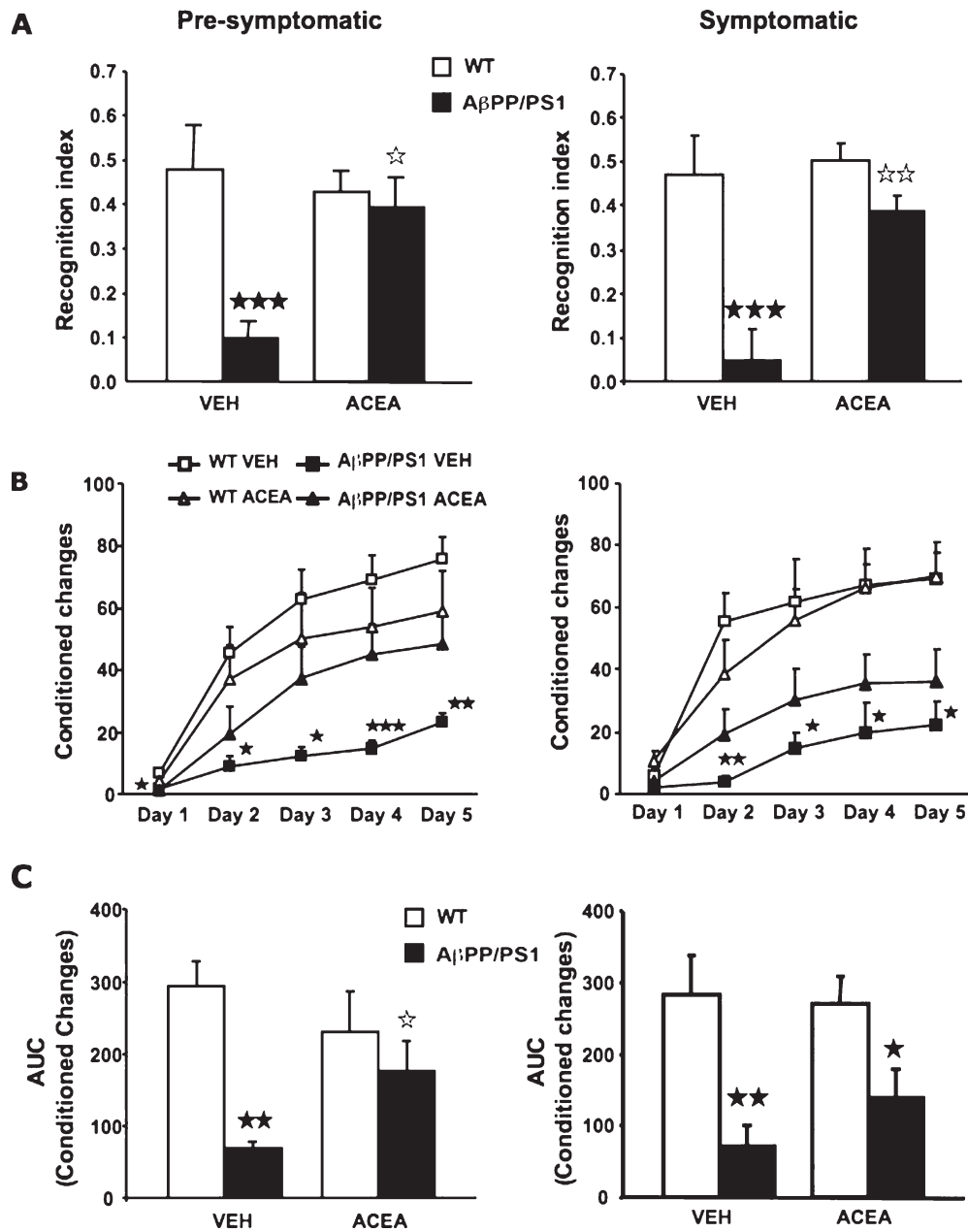


Fig. 3. Cognitive improvement of A β PP/PS1 mice chronically treated with ACEA during the pre-symptomatic phase (left: treatment beginning at 3 months) or during the beginning of the symptomatic phase (right: treatment beginning at 6 months). A) Acute administration of ACEA at the dose utilized in the present study (1.5 mg/kg, i.p.) does not produce amnesia-like effects in wild-type mice when evaluated in the two-object recognition test. B) Memory performance in the V-maze at 6 months of age (left) or at 8 months of age (right). A β PP/PS1 mice chronically treated with vehicle exhibited a significant reduction in the recognition index when compared to corresponding wild-type littermates. Chronic ACEA administration completely reversed the A β PP/PS1 memory deficiency when compared to vehicle-treated animals in both pre-symptomatic (left) and symptomatic (right) groups of animals. C) Active avoidance test shows a decrease in the learning performance in vehicle-treated A β PP/PS1 mice when compared with age-matched wild littermates. This learning impairment was not evidenced after ACEA chronic administration in animals treated during the pre-symptomatic phase (left), in contrast to the symptomatic group (right). D) Statistical analysis from the Area Under the Curve (AUC) representing the data from the active avoidance test revealed a significant reduction in the learning performance of A β PP/PS1 mice treated with vehicle during the pre-symptomatic phase, but an improvement in ACEA-treated animals (left). However, this improvement was not observed in animals treated with ACEA at the early symptomatic phase. Data are expressed as the mean values \pm SEM ($n=6-10$ per group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to wild-type mice. * $p<0.05$, ** $p<0.01$ compared to vehicle-treated animals (two-way ANOVA and Tukey's *post hoc* test).

Table 1
Statistical analysis of the ACEA effects in the pre-symptomatic phase at the cognitive level in AβPP/PS1 mice

	Pre-symptomatic phase							
	Memory	Learning (Active avoidance)						
	Recognition index	Conditioned changes	Day1	Day2	Day3	Day4	Day5	AUC
Three-way ANOVA								
Factors								
Day (repeated)	N.A.	$F_{(4,115)} = 20.68,$ $p < 0.001$						N.A.
Genotype	N.A.	$F_{(1,115)} = 44.70,$ $p < 0.001$						N.A.
Treatment	N.A.	N.S.						N.A.
Interaction								
Day × genotype	N.A.	N.S.						N.A.
Day × treatment	N.A.	N.S.						N.A.
Genotype × treatment	N.A.	$F_{(1,115)} = 15.51,$ $p < 0.001$						N.A.
Day × genotype × treatment	N.A.	N.S.						N.A.
Two-way ANOVA								
Factors								
Genotype	$F_{(1,29)} = 16.06,$ $p < 0.001$		$F_{(1,23)} = 8.44,$ $p < 0.01$	$F_{(1,23)} = 9.82,$ $p < 0.01$	$F_{(1,23)} = 8.99,$ $p < 0.01$	$F_{(1,23)} = 12.23,$ $p < 0.01$	$F_{(1,23)} = 11.76,$ $p < 0.01$	$F_{(1,23)} = 12.28,$ $p < 0.01$
Treatment	N.S.		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Interaction	$F_{(1,29)} = 11.80,$ $p < 0.01$		N.S.	N.S.	N.S.	$F_{(1,23)} = 6.51,$ $p < 0.05$	$F_{(1,23)} = 5.32,$ $p < 0.05$	$F_{(1,23)} = 4.56,$ $p < 0.05$
Tukey's <i>post hoc</i> test								
WT Veh vs AβPP/PS1 Veh	$p < 0.001$		$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.001$	$p < 0.01$	$p < 0.01$
WT Veh vs WT ACEA	N.S.		N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
AβPP/PS1 Veh vs AβPP/PS1 ACEA	$p < 0.05$		N.A.	N.A.	N.A.	N.S.	N.S.	$p < 0.05$
WT ACEA vs AβPP/PS1 ACEA	N.S.		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Three-way ANOVA with day (repeated measures), genotype and treatment as between-subjects factors was applied for learning analysis. A sinteraction between genotype and treatment was significant, subsequent two-way ANOVA with genotype and treatment as between-subjects factors was performed. For memory studies, two-way ANOVA with genotype and treatment as between-subjects factors was applied. When one factor or interaction between factors were significant, comparisons between groups were performed by Tukey's *post hoc* test. N.A., not applicable. N.S., not significant difference. See Materials and methods for details.

Table 2
Statistical analysis of the ACEA effects in the early symptomatic phase at the cognitive level in AβPP/PS1 mice

	Symptomatic phase							AUC
	Memory	Learning (Active avoidance)					AUC	
	Recognition index	Conditioned changes						
		Day1	Day2	Day3	Day4	Day5		
Three-way ANOVA								
Factors								
Day (repeated)	N.A.	$F_{(4,127)} = 16.40$, $p < 0.001$					N.A.	
Genotype	N.A.	$F_{(1,127)} = 60.88$, $p < 0.001$					N.A.	
Treatment	N.A.	N.S.					N.A.	
Interaction								
Day × genotype	N.A.	N.S.					N.A.	
Day × treatment	N.A.	N.S.					N.A.	
Genotype × treatment	N.A.	$F_{(1,127)} = 4.59$, $p < 0.05$					N.A.	
Day × genotype × treatment	N.A.	N.S.					N.A.	
Two-way ANOVA								
Factors								
Genotype	$F_{(1,27)} = 20.13$, $p < 0.001$	N.S.	$F_{(1,23)} = 13.04$, $p < 0.001$	$F_{(1,25)} = 18.19$, $p < 0.001$	$F_{(1,25)} = 25.90$, $p < 0.001$	$F_{(1,25)} = 27.24$, $p < 0.001$	$F_{(1,25)} = 25.53$, $p < 0.001$	
Treatment	$F_{(1,27)} = 10.33$, $p < 0.01$	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	
Interaction	$F_{(1,27)} = 7.05$, $p < 0.05$	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	
Tukey's <i>post hoc</i> test								
WT Veh vs AβPP/PS1 Veh	$p < 0.001$	N.A.	$p < 0.01$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.01$	
WT Veh vs WT ACEA	N.S.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	
AβPP/PS1 Veh vs AβPP/PS1 ACEA	$p < 0.01$	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	
WT ACEA vs AβPP/PS1 ACEA	N.S.	N.A.	N.S.	N.S.	N.S.	N.S.	$p < 0.05$	

Three-way ANOVA with day (repeated measures), genotype and treatment as between-subjects factors was applied for learning analysis. As interaction between genotype and treatment was significant, subsequent two-way ANOVA with genotype and treatment as between-subjects factors was performed. For memory studies, two-way ANOVA with genotype and treatment as between-subjects factors was applied. When one factor or interaction between factors were significant, comparisons between groups were performed by Tukey's *post hoc* test. N.A., not applicable. N.S., not significant difference. See Materials and methods for details.

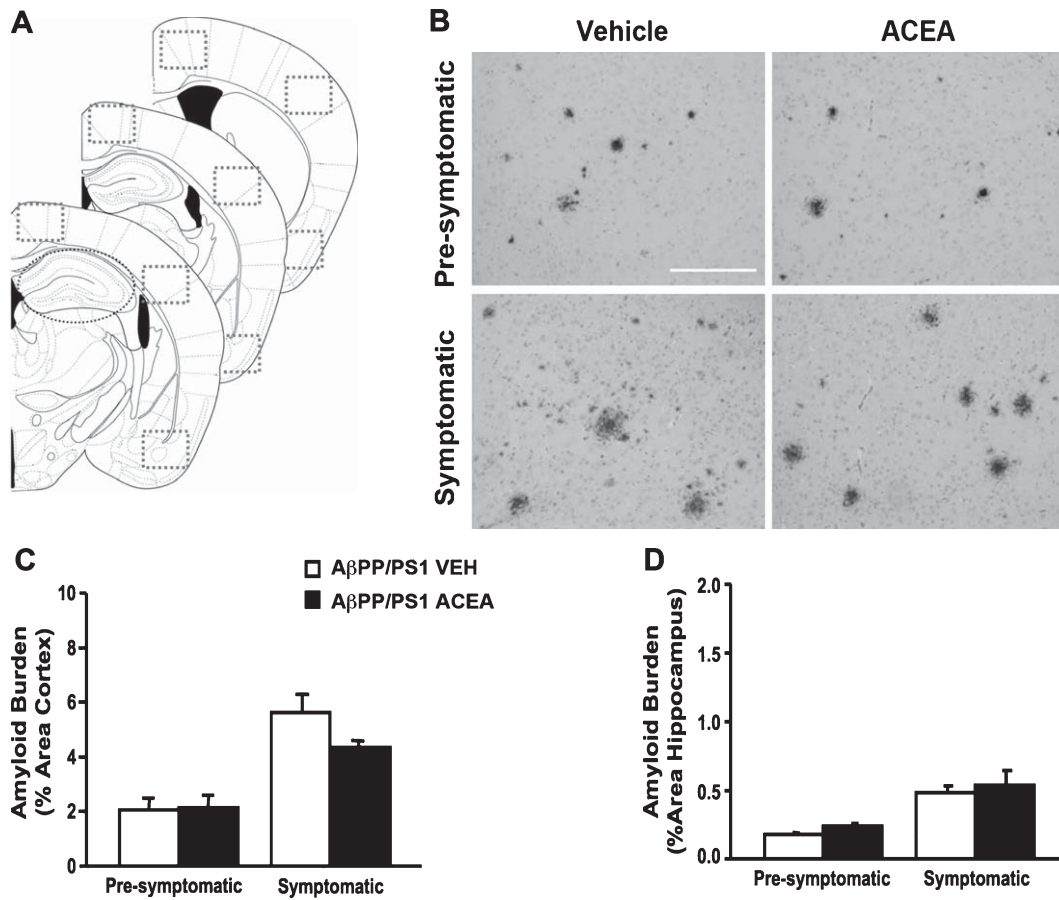


Fig. 4. A) Schematic representation of the 9 cortical areas (dashed squares) and the hippocampal section (dotted ellipse) evaluated for A β burden in each animal (B). Representative images of the A β immunoreactivity in cortical sections of A β PP/PS1 mice treated during the pre-symptomatic phase (upper panels) or during the early symptomatic phase (lower panels). Scale bar represents 100 μ m. C) Cortical A β burden in A β PP/PS1 mice was not modified by the chronic ACEA treatment during the pre-symptomatic phase or during the early symptomatic phase. D) Compared to cortex, the A β burden in hippocampus of A β PP/PS1 mice was relatively low. ACEA did not modify the hippocampal A β burden during the pre-symptomatic phase or during the early symptomatic phase. Counts are expressed as the mean values \pm SEM ($n = 7-9$ per group).

pre-symptomatic ($t_{(8)} = 3.43, p < 0.01$) or symptomatic phase ($t_{(8)} = 3.24, p < 0.05$) (Fig. 7A, B, and E). In contrast, ACEA did not significantly modify the microglial activation in the area surrounding the A β plaques at any phase (Fig. 7C, D, and F).

The effect of ACEA was not dependent upon direct CB $_1$ stimulation on astrocytes since CB $_1$ was not expressed in astrocytes in the neocortex of A β PP/PS1 mice, as evidenced by the lack of colocalization between CB $_1$ and GFAP immunostaining (Fig. 8A). However, a reduction in the expression of the pro-inflammatory IFN- γ protein was observed in the ACEA-treated A β PP/PS1 astrocytes ($t_{(8)} = 2.12, p < 0.05$), suggesting a possible mechanism explaining the effect of ACEA in those animals (Fig. 8B-F). IFN- γ expression was absent in microglia (data not shown).

ACEA reduced the GSK3 β phosphorylation at Ser9 induced by A β in vitro and in vivo

A β has been reported to induce GSK3 β phosphorylation at Tyr216 as a harmful mechanism which involved the downstream phosphorylation of β -catenin [25]. Interestingly, antioxidants protect against A β neurotoxicity, increasing GSK3 β phosphorylation at Ser9 [25] and avoiding β -catenin inactivation. Since cannabinoids have been previously reported to induce GSK3 β phosphorylation at Ser9 [26], we addressed the relationship of GSK3 β with neuroprotection against A β challenge. ACEA treatment prevented the decrease in phospho-Ser9-GSK3 β levels induced by A β in cortical neurons, as revealed by double-immunofluorescence and western blotting techniques

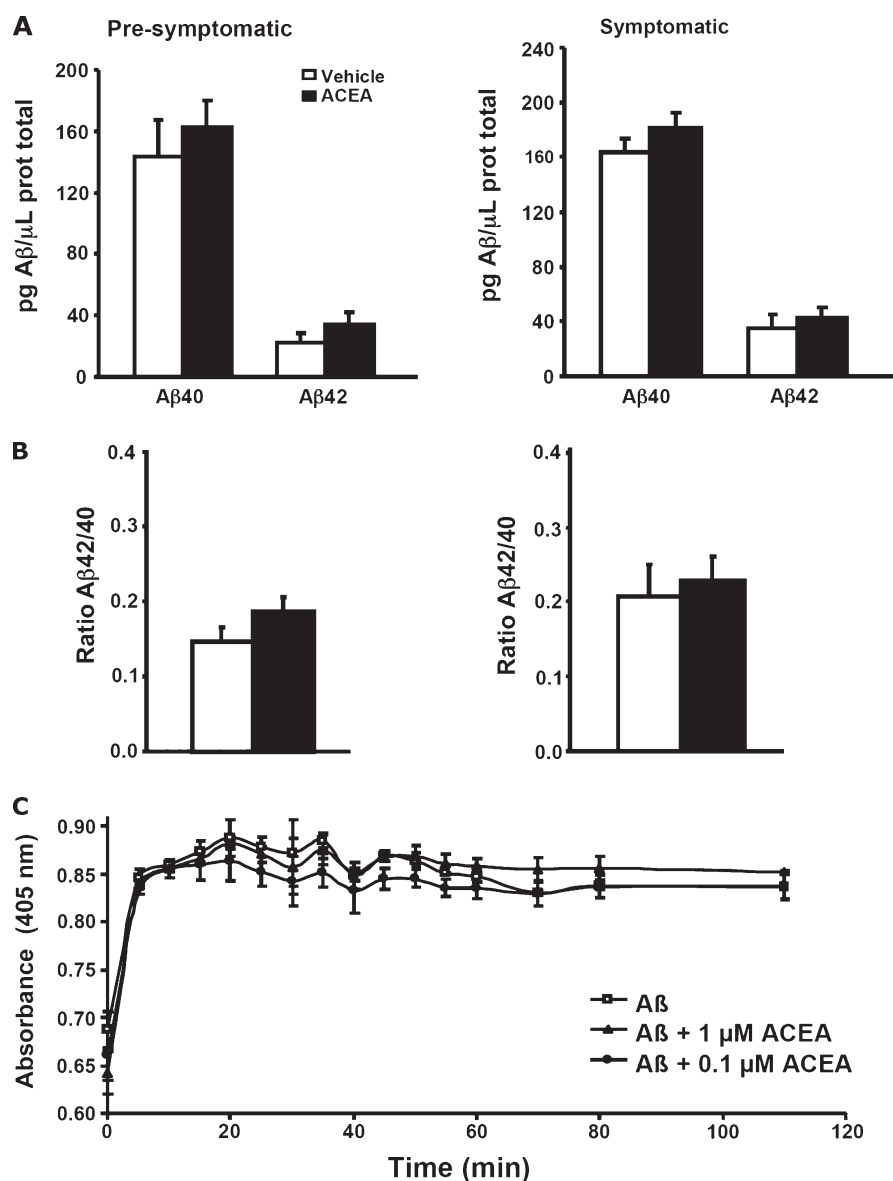


Fig. 5. A) Soluble A β ₄₀ and A β ₄₂ concentrations or (B) the ratio between the two soluble A β forms were not modified in cortical homogenates from A β PP/PS1 mice chronically treated with ACEA during the pre-symptomatic phase (left) or during the early symptomatic phase (right) when compared to corresponding vehicle-treated controls. Data are expressed as the mean values \pm SEM ($n = 3-6$ per group). C) Turbidometric analysis of A β ₄₂ aggregation in the presence of 0.1 and 1 μ M ACEA. This CB₁ agonist was not able to modify the A β aggregation kinetics *in vitro*. Data are the mean \pm SEM of 3 independent experiments.

(Fig. 9), correlating with the observed neuroprotection (Fig. 1). This ACEA effect was significantly avoided when a specific antagonist of CB₁ receptors was used (rimonabant; Fig. 9B and C). For western blotting analysis, one way-ANOVA indicated a treatment effect ($F_{(3,12)} = 11.90$, $p < 0.001$). Subsequent Tukey's *post hoc* test revealed a significant

reduction of phospho-Ser9-GSK3 β levels in neurons challenged with A β ($p < 0.05$), which was reduced by ACEA ($p < 0.01$). Rimonabant blocked the ACEA-induced effect ($p < 0.01$). Moreover, A β PP/PS1 mice acutely treated with ACEA exhibited higher p-Ser9-GSK3 β levels in cortical homogenates when compared to vehicle-treated animals, as revealed by

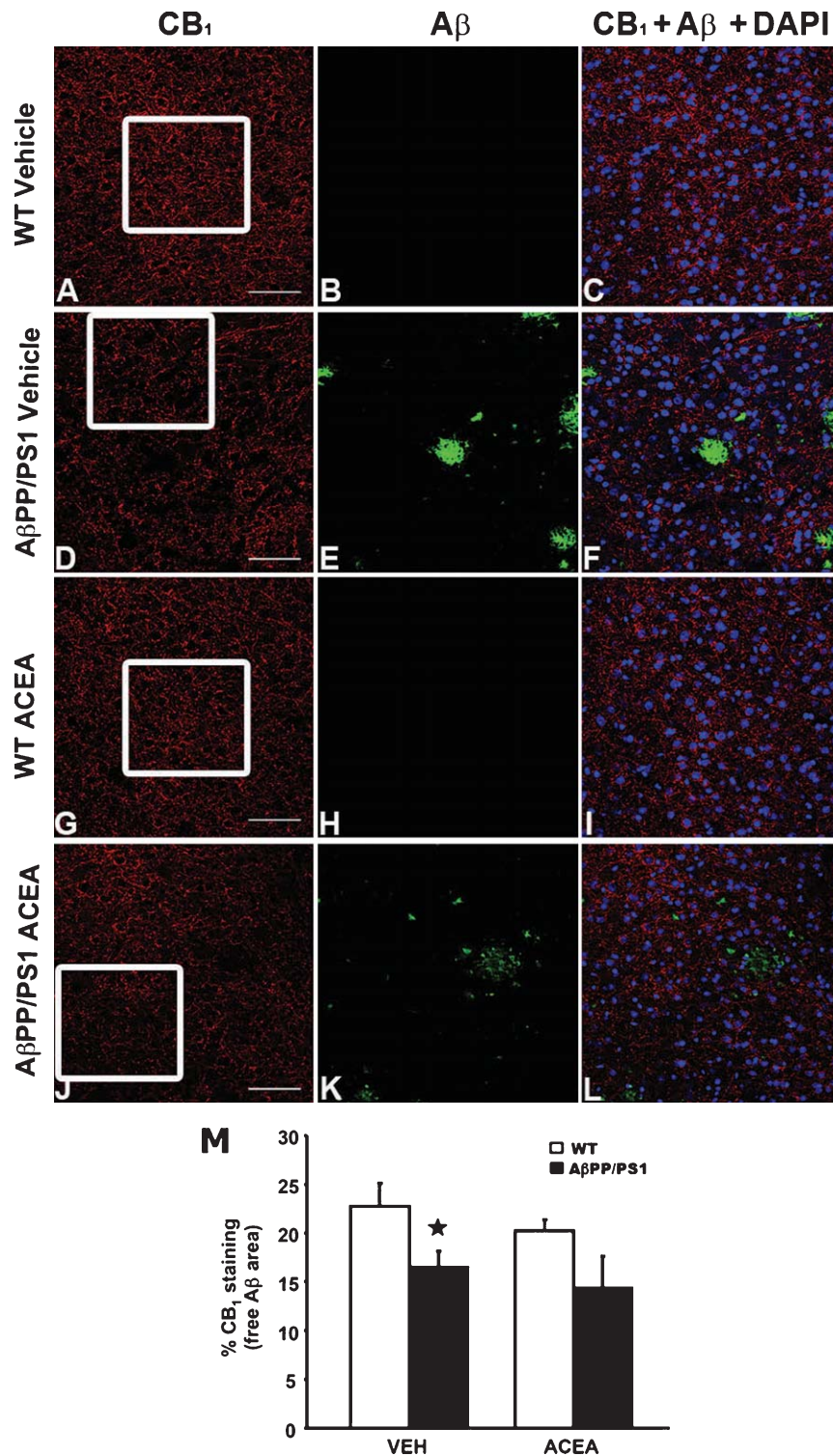


Fig. 6. Representative images of the CB₁ (red, A, D, G, and J) and A β (green, B, E, H, and K) double-immunofluorescence in coronal sections of wild-type (Vehicle: A to C; ACEA: G to I) and A β PP/PS1 mice (Vehicle: D to F; ACEA: J to L). C, F, I, and L) merge. Nuclei are stained in blue. Scale bar represents 75 μ m. M) Densitometric quantification of CB₁ labeling revealed no CB₁ downregulation in the free A β cortical areas (white-line squares, A, D, G, and J) in chronically ACEA-treated animals. Data are expressed as the mean values \pm SEM ($n=5$ pictures from each animal, 3 animals per group) * $p < 0.05$ compared to wild-type mice (two-way ANOVA and Tukey's *post hoc* test).

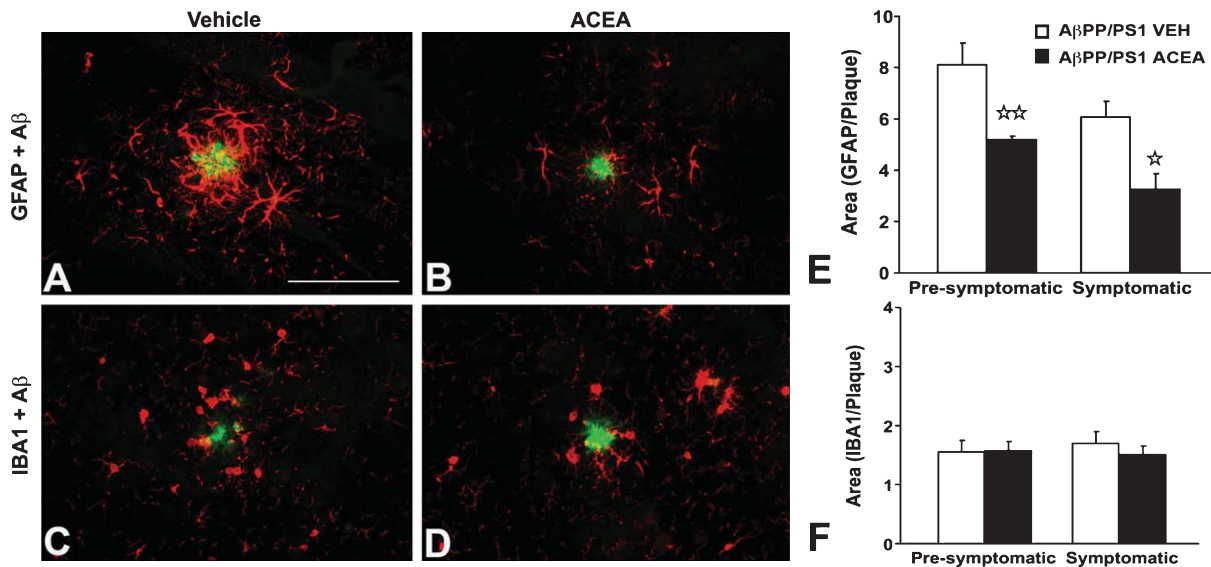


Fig. 7. Double immunofluorescent staining of glial cells (red) and A β plaques (green). A to B) Astroglial response in the surrounding A β plaque area. Antibody against GFAP was used to specifically stain astrocytes. ACEA induced a reduction in the astroglial reactivity (B). C to D) Microglial response in the surrounding A β plaque area. Antibody against Iba1 was used to specifically stain microglia. Scale bar represent 100 μ m. E) Quantification of the GFAP density with respect to A β plaque areas indicated a significant reduction in the astroglial response after chronic treatment with ACEA at both pre-symptomatic and symptomatic stages. However, no difference was observed in the microglial response in ACEA-treated animals (F). Data are expressed as the mean values \pm SEM ($n = 5$ pictures from each animal, 5 animals per group). * $p < 0.05$, ** $p < 0.01$ compared to vehicle-treated mice (Student's t test analysis).

two-way ANOVA (Genotype, $F_{(1,16)} = 5.79$, $p < 0.05$; Treatment, ($F_{(1,16)} = 10.61$, $p < 0.01$) and Tukey's *post hoc* test ($p < 0.05$) (Fig. 9D and E).

Reduced tau phosphorylation at Thr181 in the area surrounding A β deposition in ACEA-treated A β PP/PS1 mice

Considering the evidence demonstrating that the neuronal microtubule-associated protein tau is highly phosphorylated by GSK3 β , including the Thr181 site [27, 28], and the relevance of tau in AD, we aimed to examine whether ACEA could diminish tau phosphorylation. Double immunofluorescence techniques revealed that only small amounts of phospho-tau could be seen in the area surrounding mature plaques in A β PP/PS1 mice during the early symptomatic phase. However, ACEA was able to reduce the expression of tau phosphorylated at Thr181 in the vicinity of A β plaques in A β PP/PS1 mice ($t_{(8)} = 2.57$, $p < 0.05$) (Fig. 10).

DISCUSSION

Here we provide behavioral and molecular findings supporting the preventive and therapeutic properties

of the CB $_1$ cannabinoid receptor agonist ACEA in a familial AD transgenic mouse model when administered at pre-symptomatic or early symptomatic stages of the disease.

In a preliminary study, we observed that the CB $_1$ receptor agonist ACEA conferred neuroprotection against the cytotoxic effect of A β_{42} oligomers to cortical neurons in culture. This result was in agreement with a previous study revealing that the elevation of the endogenous cannabinoid 2-AG, a full agonist for cannabinoid receptors, was also capable of preventing and suppressing A β -induced neurodegeneration and apoptosis of hippocampal neurons in culture [19]. Considering these observations, we aimed to test whether ACEA could also present beneficial properties in an *in vivo* model of AD.

As a first step, we evaluated the availability and distribution of the CB $_1$ receptor in double transgenic A β PP/PS1 mice at different stages of the neurodegenerative process, since alterations in the levels of the ACEA target could compromise the effectiveness of the cannabinoid compound. Previous studies based on human postmortem brain samples suggested that the CB $_1$ receptor could be involved in the pathophysiology of the disease. The analysis of AD brains revealed reduced CB $_1$ expression in neurons farther from the

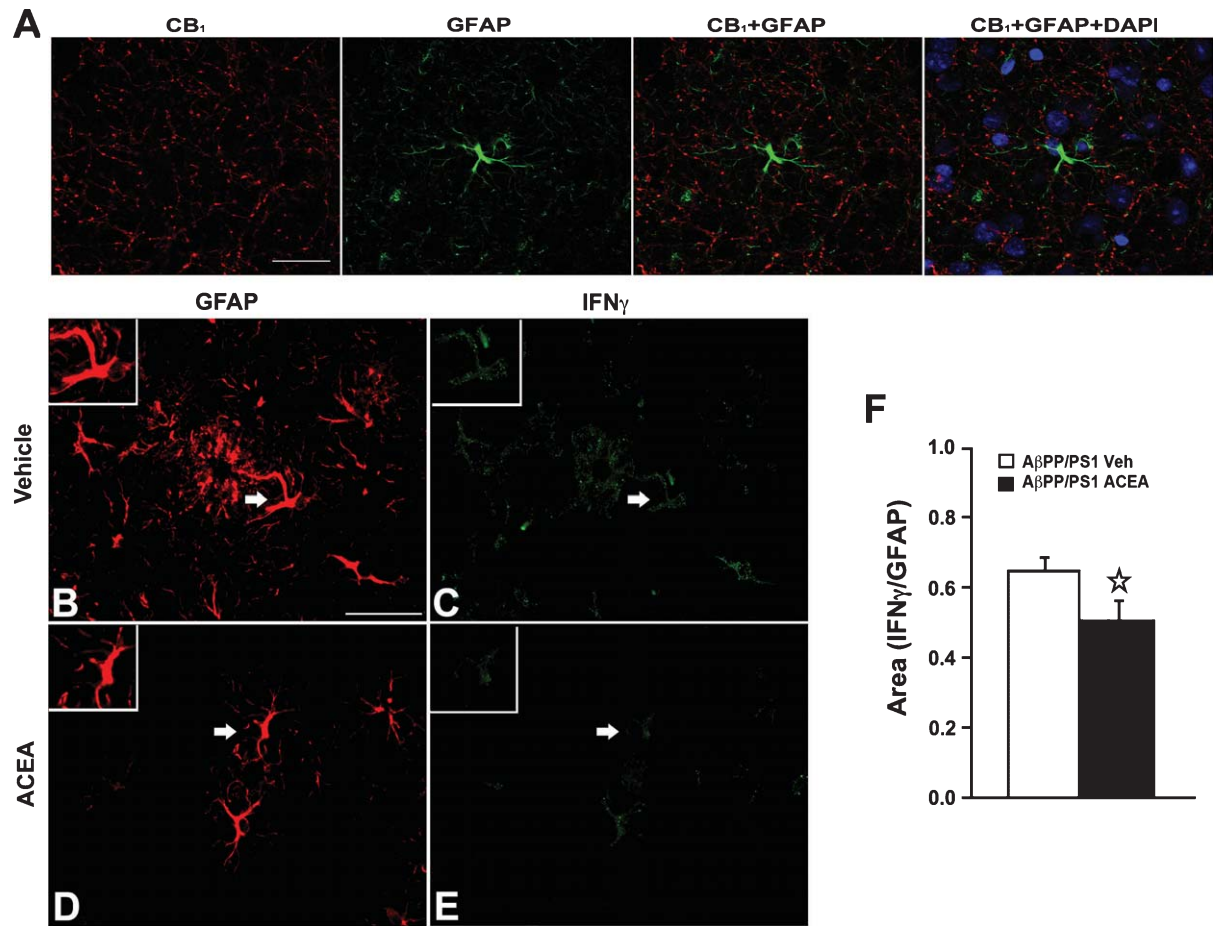


Fig. 8. A) Double immunofluorescent staining of CB₁ (red) and GFAP (green) showed no CB₁ receptor expression in astrocytes in the neocortex of A β PP/PS1 mice. Nuclei are stained in blue. Scale bar represents 75 μ m. B to E) The pro-inflammatory cytokine IFN- γ (green) is specifically expressed in the astrocytes (GFAP, red). *Inset*, higher magnification of the astrocytes indicated by white arrows. F) Quantification of the IFN- γ density with respect to GFAP area revealed a reduction in the expression of this pro-inflammatory cytokine in A β PP/PS1 mice chronically treated with ACEA when compared to vehicle-treated mutants. Scale bar represents 50 μ m. Data are expressed as the mean values \pm SEM ($n = 5$ pictures from each animal, 5 animals per group). $\star p < 0.05$ compared to vehicle-treated mice (Student's t test analysis).

plaque [13, 29]. However, some others reported no changes in CB₁ receptor levels in AD brains [30, 31]. In agreement with the first studies, A β PP/PS1 mice presented a substantial reduction of CB₁ receptor levels from 6 months of age in the cortical areas not associated with A β deposition as well as in the area surrounding A β plaques, which was age-dependently aggravated. Similar CB₁ reductions were recently reported in the hippocampus of the same animal model of AD [32]. Moreover, A β PP/PS1 mice presented higher levels of CB₁ receptor in the cortex than wild-type mice at 3 months of age, suggesting a possible mechanism attempting to reduce the latent neurodegenerative process in mutant mice. These results point out on one hand that CB₁ receptor signaling efficacy could be compromised in advanced pathological stages,

exacerbating the ongoing neurodegeneration, as has been also suggested for the normal age-related decline of cognitive functions [33]. On the other hand, these findings suggest the importance of evaluating the effect of the CB₁ receptor agonist at early stages of the neurodegenerative process, when the levels of CB₁ receptor are still preserved.

Some earlier reports indicated that the administration of natural and synthetic cannabinoids or endocannabinoid reuptake blockers in rodents reduced the pro-inflammatory responses and memory impairment associated with the intracerebral inoculation of A β peptide [13, 15, 34]. In contrast, some others did not succeed in revealing beneficial effects of a potent synthetic cannabinoid in an AD model [35]. Interestingly, our study reveals for the first time

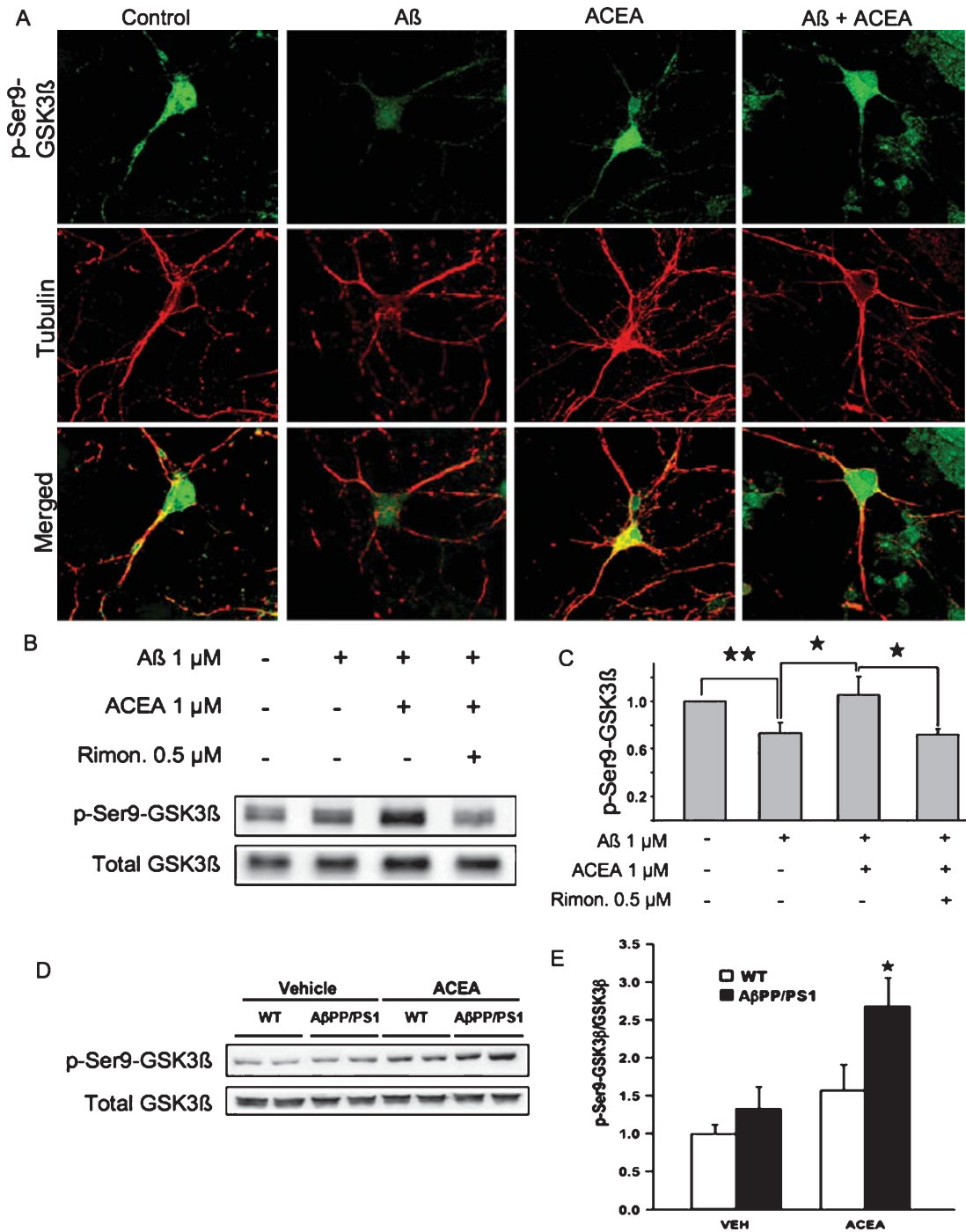


Fig. 9. ACEA increased the GSK3 β phosphorylation (Ser9) in cortical primary cultures when challenged with A β ₄₂ oligomers and in the cerebral cortex of A β PP/PS1 mice. A) Double immunofluorescent staining of phospho-GSK3 β (green) and tubulin (red) in cortical primary culture challenged with 1 μ M A β ₄₂ oligomers and treated with 1 μ M ACEA. B) Representative western blot revealing the CB₁-dependent ACEA reversion of the A β -induced dephosphorylation of GSK3 β in neuronal cultures and the inhibition of ACEA effect when the CB₁ blocker rimonabant is present. C) Data are the mean \pm SEM of 6 independent experiments performed by western blot. * p < 0.05; ** p < 0.005 (one-way ANOVA followed by Tukey's *post hoc* analysis). D) Representative western blot showing the increase in the GSK3 β phosphorylation (Ser9) in cortical homogenates after acute administration of ACEA (1.5 mg/kg) in A β PP/PS1 mice. E) Data are the mean \pm SEM of 5 mice per group. * p < 0.05 (two-way ANOVA and Tukey's *post hoc* test).

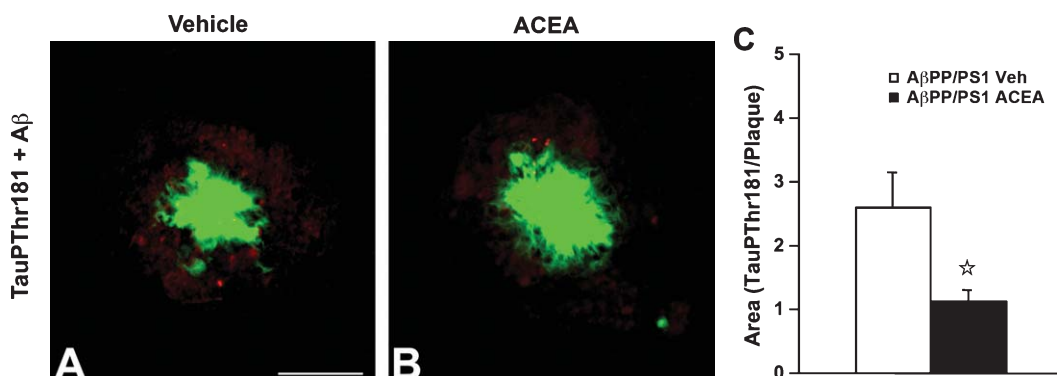


Fig. 10. Only small amounts of phospho-tau were seen in the area surrounding mature plaques in A β PP/PS1 mice at 8 months of age (A). However, ACEA was able to reduce the expression of tau phosphorylated at Thr181 (red) in the area surrounding A β plaques (green) in A β PP/PS1 mice (B). C) Densitometric quantification of the phospho-tau Thr181 respect to A β plaque areas. Data are expressed as the mean values \pm SEM ($n=5$ pictures from each animal, 5 animals per group) $^{*}p<0.05$ compared to vehicle-treated A β PP/PS1 animals (Student's t test analysis).

positive behavioral effects of a selective CB₁ receptor agonist in a transgenic animal model of the disease that mimics the progressive cognitive deficiency and the A β deposition occurring in familial AD brains [21]. Thus, chronic treatment with a non-amnesic dose of the CB₁ receptor agonist ACEA during the pre-symptomatic phase of the pathology prevented the cognitive impairment exhibited by A β PP/PS1 mice at 6 months of age. Furthermore, chronic administration of ACEA to A β PP/PS1 animals aged 6 months at the beginning of the experiment partially reversed their cognitive deficits, improving memory but not the performance of a more complex learning task, such as the active avoidance paradigm, at the end of the treatment. These data suggest that the efficacy of the cannabinoid compounds could be inversely proportional to the disease progression stage at the beginning of the treatment. Importantly, chronic treatment with ACEA did not induce a CB₁ down-regulation in the neocortex of A β PP/PS1 mice, as was previously observed after prolonged exposure to different cannabinoid compounds [36, 37], suggesting that the low ACEA dose employed in the present study did not induce a tolerance to the CB₁ stimulation effects.

The activation of CB₁ receptor has been widely reported to impair learning and memory. High doses of CB₁ agonists impair memory formation and produce deficits in working and short-term memory by regulating neurotransmission and selectively affecting encoding processes [24, 38]. However, it is important to highlight that our data were obtained in a different scenario, for different reasons. First, we administered a non-amnesic dose of CB₁ agonist,

which did not produce memory impairment after acute administration (data not shown). Second, we administered the cannabinoid compounds to animals continuously exposed to A β insult. The endogenous cannabinoid system is known to trigger different mechanisms devoted to maintaining cellular homeostasis and protecting neurons against the deleterious consequences of toxic molecules. Thus, CB₁ receptor promotes protection against excitotoxicity [8, 9] and against other insults related to neurodegenerative processes [39–41]. Considering these previous reports and our present observations about the reduction of the A β -induced neurotoxicity in the ACEA-treated cortical neuron culture, the beneficial cognitive effects observed after the chronic ACEA treatment could be directly related with the neuroprotection against the A β insult conferred by the stimulation of CB₁ receptors. This neuroprotective effect is supported by the demonstrated capacity of ACEA to reverse the A β -induced dephosphorylation of GSK3 β in neuronal cultures. Similarly, the acute administration of ACEA increased the GSK3 β Ser9 phosphorylation in mice. Our results are in line with previous studies demonstrating that the stimulation of CB₁ receptor activates the pro-survival PI3K/Akt pathway, leading to the inactivation of GSK3 β by phosphorylation at Ser9 [26]. GSK3 β is known to play an important role in mediating neuronal fate and synaptic plasticity [42]. In AD, GSK3 β is considered as a possible link between A β peptide and the neuronal microtubule-associated tau protein [27], since A β promotes GSK3 β over-activation, which in turn accounts for tau hyperphosphorylation and which subsequently reduces the ability of tau to promote microtubule assembly

[27, 28]. Moreover, GSK3 β over-activity has also been related to other hallmarks of AD such as memory impairment and the inflammatory responses mediated by microglia [43–45]. Taking into account this evidence, we evaluated whether the ACEA-induced reduction in GSK3 β activity correlated with alterations in tau phosphorylation in mice. Effectively, the levels of tau phosphorylated at the Thr181 site, which is a target of the GSK3 β kinase activity [28], were decreased in the area surrounding A β plaques in ACEA-treated A β PP/PS1 mice. This result is in agreement with a previous study demonstrating that CB₁ receptor selective activation reduced tau protein hyperphosphorylation in co-cultured neurons [46]. Thus, our results suggest that the ability of ACEA to diminish the deleterious impact of GSK3 β could be a possible mechanism explaining the positive effect of this CB₁ receptor agonist in A β PP/PS1 mice. However, the reduction in tau phosphorylation by itself deserves to be considered with caution, since our animal model of AD presents only small amounts of phospho-tau in dystrophic neurites, which are never on a par with those seen in AD brains, and which do not produce neurofibrillary tangles at any age in A β PP/PS1 mice [22]. Thus, the contribution of the abnormal tau phosphorylation to the neurodegenerative process occurring in A β PP/PS1 mice is assumed to be minor.

In addition, the cognitive improvement was associated with the reduction of the astroglial reactivity in the vicinity of A β plaques after chronic ACEA treatment. This finding is in agreement with a previous report revealing that ACEA was able to blunt A β -induced reactive astrogliosis *in vitro* and in A β -inoculated rats [15]. However, this observation cannot be explained by a direct effect of ACEA through astrocytic CB₁ stimulation since we were not able to reveal CB₁ expression in reactive astrocytes in the neocortex of A β PP/PS1. The presence and functional significance of CB₁ receptors in astrocytes is controversial [5]. While several studies have shown their presence in cultured astrocytes and associated their activity to the reduction of inflammatory mediators [47–51], few studies reported astrocytic CB₁ expression in specific brain areas and suggested a role of CB₁ receptor in neuron-astrocyte communication [52–55]. In line with the studies relating CB₁ receptors to the regulation of inflammatory mediators, our results revealed an ACEA-induced reduction in the expression of the pro-inflammatory cytokine IFN- γ in astrocytes. Interferons represent crucial modulators of the central and peripheral immune responses

and previous studies demonstrated the capability of the endocannabinoid system to modulate interferon levels [56], supporting the idea that the reduction in the inflammatory processes mediated by interferons could be a mechanism accounting for the CB₁ agonist's positive effect in A β PP/PS1 mice. However, further studies are needed in order to address the implication of such observations in the cognitive improvement reported in ACEA-treated A β PP/PS1 mice, as well as to increase knowledge of the mechanisms underlying the reduced astroglial reactivity observed in those animals.

Regarding the microglial response to A β deposition, ACEA was not able to modify the microglia activation. The apparent controversy with respect to previous studies indicating cannabinoid-induced reductions in microglial responses to A β [13, 16] could be explained by the fact that such reports were based on mixed CB₁/CB₂ agonists or on microglial cell lines. CB₁ receptor is known to be expressed in microglial cells barely under culture conditions [5], so the lack of effect of a specific CB₁ agonist observed in the microglia of our brain samples is not unexpected. On the other hand, these observations suggest that the use of a non-selective agonist for CB₁/CB₂ receptors could probably provide a combined effect of the CB₁-mediated reduction in neurotoxicity and astroglial response to A β , with CB₂-mediated reduction of microglial toxicity, resulting in a higher benefit.

Our present findings indicate that CB₁ receptors do not participate significantly in the production, aggregation, or clearance of the A β in A β PP/PS1 mice. Thus, ACEA did not change A β production attending to both A β ₄₀ and the more fibrillogenic A β ₄₂ soluble forms in mouse brain. Moreover, ACEA did not produce any effect on A β aggregation *in vitro*, which correlated with a lack of difference in the A β burden on treated animals. Similar results were previously reported for another synthetic cannabinoid in another animal model of AD [35]. Hence, considering that ACEA did not alter A β processing, we may conclude that the protection conferred on neurons challenged with A β by decreasing GSK3 β activity, the reduction of astroglial reactivity and the decreased production of pro-inflammatory proteins such as IFN- γ could be the major effects mediating ACEA-induced cognitive improvement in A β PP/PS1 mice.

In summary, our present results reinforce the hypothesis that targeting the endocannabinoid system could offer a versatile approach for the development of novel therapeutic strategies against AD.

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