CB₁ agonist ACEA protects neurons and reduces the cognitive impairment of AβPP/PS1 mice

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Abstract

The present study shows that chronic administration of the CB₁ receptor agonist ACEA at pre-symptomatic or at early symptomatic stages, at a non-amnesic dose, reduces the cognitive impairment observed in double AβPP(swe)/PS1(1dE9) transgenic mice from 6 months of age onwards. ACEA has no effect on Aβ production, aggregation and clearance. However, ACEA reduces the cytotoxic effect of Aβ₄₂ oligomers in primary cultures of cortical neurons, and reverses Aβ-induced dephosphorylation of glycogen synthase kinase-3β (GSK3β) in vitro and in vivo. Reduced activity of GSK3β in ACEA-treated mice is further supported by the reduced amount of phospho-tau (Thr181) in neuritic processes around Aβ plaques. In addition, ACEA-treated mice show decreased astroglial response in the vicinity of Aβ 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 GSK3β 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, transgenic mice, CB₁ cannabinoid receptor, cognition, GSK3β, neuroprotection, astrogliosis

Abbreviations: amyloid-β peptide (Aβ); amyloid-β precursor protein (AβPP); presenilin 1 (PS1); Alzheimer’s disease (AD), Arachidonyl-2-chloroethylamide (ACEA)
1. 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 [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, 14, 15, 16], but also through reducing the harmful action of amyloid-β peptide (Aβ) and promoting the brain’s intrinsic repair mechanisms [17]. Among the neuroinflammation-independent mechanisms associated with cannabinoid-induced neuroprotection against Aβ, 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 Aβ-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 ACEA because of its high affinity and specificity to the CB₁ receptor [20].

Double transgenic AβPP(swe)/PS1(1dE9) (AβPP/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 Aβ plaques, dystrophic neurites around Aβ deposition, and synaptic abnormalities from the age of six months onwards [21, 22]. AβPP/PS1 mice do not replicate neurofibrillary tangles observed in AD brains, but do exhibit hyperphosphorylated tau protein in the vicinity of Aβ plaques, as also observed in AβPP Tg2576 mice [23]. Therefore, we consider that AβPP/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 AβPP/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.
2. Materials and methods

2.1 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 µM 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.

2.2 Cannabinoid protection assays in cortical neurons

Primary cultures of mouse cortical neurons (7.5x10⁴ cells/300 µL/well) were assayed in neurobasal supplemented with B27 without antioxidants in 24 well culture-plates. Cells were treated with 0.1 or 1 µM ACEA, and then PBS (control) or 1 µM Aβ 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 µL of MTT stock solution (5 mg/mL) was added and after 2 h the reaction was stopped with 300 µL of DMSO. MTT reduction was determined in a plate reader spectrophotometer at 540 and 650 nm. Control cells were taken as 100%.

2.3 Animals

The experiments were carried out on male AβPP/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 AβPPswe and PS1dE9 (AβPP/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).

2.4 Drugs and pharmacological treatment

The selective CB$_1$ receptor agonist arachidonyl-2-chloroethylamide (ACEA) was supplied by Tocris Bioscience\textsuperscript{®} (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; AβPP/PS1, n = 8) or the corresponding vehicle (wild-type, n = 9; AβPP/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; AβPP/PS1, n = 9) or the corresponding vehicle (wild-type, n = 6; AβPP/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 GSK3β 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.

2.5 Behavioral evaluation of cognitive performance

2.5.1 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].

2.5.2 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 sec before the onset of the unconditioned stimulus (US) and overlapped it for 25 sec. At the end of the 30-sec 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 sec period after the onset of the CS. If animals failed to avoid the shock, they could escape it by crossing during the US (25 sec) and this was recorded as unconditioned response. Between each trial session, there was an inter-trial interval of 30 sec. 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.

**2.6 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 Aβ soluble quantification. The other brain hemisphere was fixed in 4% paraformaldehyde and processed for immunohistochemistry.

**2.7 Aβ immunohistochemistry**

Tissue samples were embedded in paraffin and coronal sections (4 µm) 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 Aβ (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 Aβ burden in neocortex was calculated as the percentage of the Aβ deposition area with respect to the total area in 9 representative pictures, corresponding to the main regions where Aβ deposition is observed in AβPP/PS1 mice (Figure 4A). One section of the hippocampus was used for similar quantification of the Aβ burden. Sections from all the AβPP/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.

2.8 Aβ 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 × 1 h, and the supernatant saved as the soluble fraction for Aβ quantifications. The Aβ40 and Aβ42 Human ELISA kits (Invitrogen™ Corporation, Camarillo, CA, USA) were used to quantify the levels of Aβ40 and Aβ42 proteins in the brain soluble fractions, respectively. The quantitative determination was carried out according to the manufacturer’s instructions. Aβ40 and Aβ42 levels were normalized to the total amount of protein from each individual sample. A ratio was calculated of the Aβ42 levels with respect to those of Aβ40.

2.9 Aβ aggregation

The Aβ42 (Sigma) stock solution was prepared by dissolving the peptide in DMSO to a final concentration of 15 μg/μL. 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/μL Aβ42 dissolved in 100 μL PBS pH 5.5 and 0.1 or 1 μM ACEA. Absorbance at 405 nm was followed over time.

2.10 Double-labeling immunofluorescence

2.10.1 CB1 and Aβ or GFAP double-immunofluorescence

For CB1 and Aβ 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 Aβ (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.

2.10.2 Densitometric quantification of CB₁

The presence of dystrophic neurites in the vicinity of the Aβ plaques is supposed to indicate that the integrity of the neurons is compromised in the areas directly influenced by Aβ deposition in AβPP/PS1 mice [22]. In contrast, the functionality of the neurons far from Aβ plaques should be more preserved since there is no evidence of dystrophic neurites in such areas. Thus, in order to estimate the influence of Aβ deposition in the CB₁ receptor cortical density, the CB₁ protein expression levels were evaluated in an area free from the Aβ plaque as well as in the vicinity of Aβ deposition. The percentage of the CB₁ staining area was calculated (a) with respect to the total cortex (wild-type mice) or to a 225 μm x 225 μm area in the case of an area free from Aβ deposition (AβPP/PS1) and (b) with respect to an area equivalent to 4 times the Aβ plaque (arbitrary reference based on the dystrophic neurites and presence of reactive gliosis and taken in order to normalize the Aβ extension), both in 5 representative pictures taken from the neocortex of each animal (n=5 per group) using the software Adobe® Photoshop® CS4.

2.10.3 Glial, tau phosphorylation and Aβ or Interferon-γ (IFN-γ) double-immunofluorescence

In the case of the glial, tau phosphorylation and Aβ 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 Aβ immunostaining) and with citrate buffer to enhance antigenicity, and then incubated at 4°C overnight with combinations of primary antibodies against Aβ (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.

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

2.10.3 Densitometric quantification of IFN-γ expression in astrocytes
IFN-γ immunostaining colocalized with GFAP, but not Iba1, immunostaining. Thus, the expression levels of IFN-γ 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.

2.11 P-Ser9-glycogen synthase kinase-3β (GSK3β) immunolabelling assay
Primary cultures of mouse cortical neurons (7.5x10^4 cells/300 μL/ 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 μM ACEA and
PBS (control) or 1 µM Aβ oligomers. Cells were incubated for 24 hrs. Then, neurons were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Immunostaining was done with polyclonal rabbit anti-p-Ser9-GSK3β 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.

2.12 P-Ser9-GSK3β quantification

Cortical neurons

Primary cultures of mouse cortical neurons (10⁶ cells/2mL/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 µM Rimonabant (a specific CB₁ inhibitor). Then, ACEA 1 µM and PBS (control) or 1 µM Aβ 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; 1mM PMSF; 0.15 µM aprotinin; 11 µM leupeptine; 1.5 µM pepstatine).

Wild-type and AβPP/PS1 mice

Wild-type and AβPP/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 µl lysis buffer/mg wet weight.

Immunoblot analysis

After 20 min of incubation with lysis buffer in agitation at 4ºC, both cell and brain samples were centrifuged for 30 min at 16,000 g, and the supernatant was recovered and stored at -80ºC. Protein content was determined using the DC Protein Assay (Bio-Rad) following the manufacturer's instructions.
GSK3β and phospho-Ser9 GSK3β levels were quantified by western blotting. Equal amounts of lysates (20 µg per lane) were separated by SDS-PAGE (8%) before electrophoretic transfer on to nitrocellulose membrane (Bio-Rad, Spain). Membranes were blocked for 1 hour at room temperature in Tris-buffered saline (100mM NaCl, 10mM 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-GSK3β (1:1000; Cell Signaling Technology) in TTBS with 5% nonfat milk, and polyclonal rabbit anti-GSK3β (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 hour at room temperature. Bands were visualized by enhanced chemiluminescence detection (ECL AdvanceTM, Amersham Biosciences). The value of active phospho-Ser9 GSK3β was normalized to the amount of total GSK3β in the same sample and expressed as a percentage of control treatment.

2.13 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. Aβ, 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.
3. Results

3.1 ACEA protection against Aß-induced neurotoxicity

As a preliminary approach to the study of the potential cannabinoid protection in AßPP/PS1 mice, we tested whether the CB$_1$ receptor agonist ACEA could induce a protective effect against Aß-induced neurotoxicity \textit{in vitro}. In the present study, we observed that ACEA protected cortical neurons against Aß oligomer insult (Figure 1). One-way ANOVA revealed significant effect of Aß oligomer in cell viability ($F_{(3, 14)} = 12.97, p < 0.001$). Tukey’s \textit{post hoc} test indicated that the exposure to Aß oligomer produced a reduction in the cell viability ($p < 0.001$), which was also significant in the presence of 0.1 µM ACEA ($p < 0.05$), compared to control cells. Interestingly, a significant increase in viability was observed in cells exposed to Aß oligomer in the presence of the 0.1 ($p < 0.05$) or 1 µM ACEA ($p < 0.01$) when compared to Aß-treated cells.

3.2 Progressive age-dependent loss of CB$_1$ receptor in the neocortex of AßPP/PS1 mice

In an attempt to assess the relevance of CB$_1$ receptor in AD, we evaluated the expression levels of this receptor in the neocortex of AßPP/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 CB$_1$ receptor in AßPP/PS1 mice from 6 months when compared to age-matched wild-type littermates (Figure 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 Aß deposition (Figure 2Y). Subsequent one-way ANOVA revealed an age effect in both AßPP/PS1 ($F_{(2, 29)} = 15.82, p < 0.001$) and wild-type animals ($F_{(1, 32)} = 9.41, p < 0.01$). Tukey’s \textit{post hoc} test showed a significant reduction in CB$_1$ levels in AßPP/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 CB$_1$ 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 AβPP/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 Aβ plaques, a reduction in the CB1 immunostaining in AβPP/PS1 animals aged 12 months was also observed when compared to 6-month-old mice (t(23) = 5.39, p < 0.001) (Figure 2Z).

3.3 The effect of ACEA on the cognitive performance of AβPP/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. AβPP/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 AβPP/PS1 mice at the age of 6 months (Figure 3B left). Similarly, AβPP/PS1 mice chronically treated with ACEA during the pre-symptomatic phase did not exhibit the learning impairment exhibited by AβPP/PS1 mice chronically treated with vehicle in the active avoidance test at the age of 6 months (Figure 3C and 3D left). See Table 1 for statistical details.

3.4 ACEA treatment during early stages of the symptomatic phase partially reversed the cognitive deficits in AβPP/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 AβPP/PS1 mice on the two-object recognition test (Figure 3B right).
In contrast, the CB₁ agonist was not able to significantly reduce the impairment shown by AβPP/PS1 mice aged 6 months at the beginning of the treatment in the active avoidance paradigm (Figure 3C right). See Table 2 for statistical details.

### 3.5 Cortical and hippocampal Aβ quantification

One possible mechanism used by ACEA to protect AβPP/PS1 mice would be a direct effect reducing Aβ production. To address this point, we quantified the Aβ burden in neocortex and hippocampus (Figure 4) as well as the soluble Aβ production and aggregation (Figure 5) after ACEA treatment. We found that chronic treatment with ACEA did not significantly modify the Aβ burden in the cortex of AβPP/PS1 mice either when they were chronically treated during the pre-symptomatic phase or during the early stages of the symptomatic phase (Figure 4B and 4C). Similarly, the Aβ burden was not modified in the hippocampus, a region where Aβ deposition starts later than in the neocortex (Figure 4D). ACEA did not significantly modify the Aβ₄₀ or Aβ₄₂ protein levels (Figure 5A) or the ratio between them (Figure 5B) in the soluble cortical fraction of AβPP/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 Aβ aggregation due to its hydrophobic nature was ruled out since Aβ fibrillation was not affected by the presence of 0.1 or 1 µM ACEA (Figure 5C).

### 3.6 Chronic treatment with ACEA did not modify the expression of CB₁ receptor levels in the neocortex of AβPP/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 AβPP/PS1 mice (Figure 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 Aß deposition (Figure 6M). Tukey’s *post hoc* test revealed a reduction in the CB₁ levels in vehicle-treated AßPP/PS1 mice (p < 0.05), but not in ACEA-treated animals, with respect to wild-type littermates.

**3.7 Reduction of the astrocytic responses associated with Aß deposition after ACEA treatment**

Hypertrophic astrocytes and reactive microglia were observed in the vicinity of Aß plaques in AßPP/PS1 mice. Double immunofluorescence techniques revealed that chronic stimulation with the CB₁ agonist ACEA produced a reduction in the area of astrocytes surrounding the Aß plaques when compared to vehicle-treated animals during the pre-symptomatic (*t*(8) = 3.43, p < 0.01) or symptomatic phase (*t*(8) = 3.24, p < 0.05) (Figure 7A, B and E). In contrast, ACEA did not significantly modify the microglial activation in the area surrounding the Aß plaques at any phase (Figure 7C, D and F).

The effect of ACEA was not dependent upon direct CB₁ stimulation on astrocytes since CB₁ was not expressed in astrocytes in the neocortex of AßPP/PS1 mice, as evidenced by the lack of colocalization between CB₁ and GFAP immunostaining (Figure 8A). However, a reduction in the expression of the pro-inflammatory IFN-γ protein was observed in the ACEA-treated AßPP/PS1 astrocytes (*t*(8) = 2.12, p < 0.05), suggesting a possible mechanism explaining the effect of ACEA in those animals (Figure 8B-F). IFN-γ expression was absent in microglia (data not shown).

**3.8 ACEA reduced the GSK3ß phosphorylation at Ser9 induced by Aß in vitro and in vivo**

Aß has been reported to induce GSK3ß phosphorylation at Tyr216 as a harmful mechanism which involved the down-stream phosphorylation of β-catenin [25]. Interestingly, antioxidants protect against Aß neurotoxicity, increasing GSK3ß phosphorylation at Ser9 [25] and avoiding β-catenin inactivation. Since cannabinoids have been previously reported to induce
GSK3β phosphorylation at Ser9 [26] we addressed the relationship of GSK3β with neuroprotection against Aβ challenge. ACEA treatment prevented the decrease in phospho-Ser9-GSK3β levels induced by Aβ in cortical neurons, as revealed by double-immunofluorescence and western blotting techniques (Figure 9), correlating with the observed neuroprotection (Figure 1). This ACEA effect was significantly avoided when a specific antagonist of CB₁ receptors was used (rimonabant; Figure 9B and 9C). 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-GSK3β levels in neurons challenged with Aβ \( (p < 0.05) \), which was reduced by ACEA \( (p < 0.01) \). Rimonabant blocked the ACEA-induced effect \( (p < 0.01) \). Moreover, AβPP/PS1 mice acutely treated with ACEA exhibited higher p-Ser9-GSK3β levels in cortical homogenates when compared to vehicle-treated animals, as revealed by 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 \( (p < 0.05) \) (Figure 9D and 9E).

3.9 Reduced tau phosphorylation at Thr181 in the area surrounding Aβ deposition in ACEA-treated AβPP/PS1 mice

Considering the evidence demonstrating that the neuronal microtubule-associated protein tau is highly phosphorylated by GSK3β, 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 AβPP/PS1 mice during the early symptomatic phase. However, ACEA was able to reduce the expression of tau phosphorylated at Thr181 in the vicinity of Aβ plaques (green) in AβPP/PS1 mice \( t(8) = 2.57, p < 0.05 \).
4. Discussion

Here we provide behavioral and molecular findings supporting the preventive and therapeutic properties of the CB₁ 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 CB₁ receptor agonist ACEA conferred neuroprotection against the cytotoxic effect of Aβ₄₂ 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 Aβ-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 CB₁ receptor in double transgenic AβPP/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 post-mortem brain samples suggested that the CB₁ receptor could be involved in the pathophysiology of the disease. The analysis of AD brains revealed reduced CB₁ expression in neurons farther from the plaque [13, 29]. However, some others reported no changes in CB₁ receptor levels in AD brains [30, 31]. In agreement with the first studies, AβPP/PS1 mice presented a substantial reduction of CB₁ receptor levels from 6 months of age in the cortical areas not associated with Aβ deposition as well as in the area surrounding Aβ plaques, which was age-dependently aggravated. Similar CB₁ reductions were recently reported in the hippocampus of the same animal model of AD [32]. Moreover, AβPP/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 Aβ 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 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 Aβ 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 AβPP/PS1 mice at 6 months of age. Furthermore, chronic administration of ACEA to AβPP/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 AβPP/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 Aβ 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, 40, 41]. Considering these previous reports and our present observations about the reduction of the Aβ-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 Aβ insult conferred by the stimulation of CB1 receptors. This neuroprotective effect is supported by the demonstrated capacity of ACEA to reverse the Aβ-induced dephosphorylation of GSK3β in neuronal cultures. Similarly, the acute administration of ACEA increased the GSK3β 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 GSK3β by phosphorylation at Ser9 [26]. GSK3β is known to play an important role in mediating neuronal fate and synaptic plasticity [42]. In AD, GSK3β is considered as a possible link between Aβ peptide and the neuronal microtubule-associated tau protein [27], since Aβ promotes GSK3β over-activation, which in turn accounts for tau hyper-phosphorylation and which subsequently reduces the ability of tau to promote microtubule assembly [27, 28]. Moreover, GSK3β over-activity has also been
related to other hallmarks of AD such as memory impairment and the inflammatory responses mediated by microglia [43, 44, 45]. Taking into account this evidence, we evaluated whether the ACEA-induced reduction in GSK3β 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 GSK3β kinase activity [28], were decreased in the area surrounding Aβ plaques in ACEA-treated AβPP/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 GSK3β could be a possible mechanism explaining the positive effect of this CB1 receptor agonist in AβPP/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 AβPP/PS1 mice [22]. Thus, the contribution of the abnormal tau phosphorylation to the neurodegenerative process occurring in AβPP/PS1 mice is assumed to be minor.

In addition, the cognitive improvement was associated with the reduction of the astrogial reactivity in the vicinity of Aβ plaques after chronic ACEA treatment. This finding is in agreement with a previous report revealing that ACEA was able to blunt Aβ-induced reactive astrogliosis in vitro and in Aβ-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 AβPP/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, 48, 49, 50, 51], few studies reported
astrocytic CB₁ expression in specific brain areas and suggested a role of CB₁ receptor in neuron-astrocyte communication [52, 53, 54, 55]. In line with the studies suggesting a role of CB₁ receptors in 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 AβPP/PS1 mice. However, further studies are needed in order to address the implication of such observations in the cognitive improvement reported in ACEA-treated AβPP/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 Aβ 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 Aβ [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 Aβ, 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 Aβ in AβPP/PS1 mice. Thus, ACEA did not change Aβ production attending to both Aβ₄₀ and the more fibrillogenic Aβ₄₂ soluble forms in
mouse brain. Moreover, ACEA did not produce any effect on Aβ aggregation in vitro, which correlated with a lack of difference in the Aβ 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 Aβ processing, we may conclude that the protection conferred on neurons challenged with Aβ by decreasing GSK3β 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 AβPP/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.
Acknowledgements:

We thank Arnau Busquets and Andrés Ozaita for kind advice about behavioral evaluation. We are indebted to Margarita Carmona and Alfredo López-Salcedo for their excellent assistance in immunohistochemical experiments and imaging analysis, respectively. This study was supported by grants from the Spanish Ministry of Health (FIS PI08/0582 to IF; PI07/0593 to FJM), ERA-NET-NEURON (IF), Agrupació Mútua Foundation (XVII Award in the Elderly Field, to IF) and Mutua Madrileña Foundation (IF).
References


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Figure 1: Neuroprotection by ACEA against Aβ oligomer neurotoxicity in primary cultures of cortical neurons. Cell viability of cortical neurons exposed to Aβ_{42} oligomers in the absence or presence of ACEA. Data are the mean ± SEM of 3-6 independent experiments. ★ p < 0.05, ★★★ p < 0.001 compared to control. ☆ p < 0.05, ☆☆ p < 0.01 compared to Aβ (one-way ANOVA followed by Tukey’s post hoc analysis).

Figure 2: Representative images of the double-immunofluorescence for CB1 receptor (red, A, E, I, M, Q and U) and Aβ (green, B, F, J, N, R and V) in coronal sections of wild-type and AβPP/PS1 mice aged 3 months (A to H), 6 months (I to P) or 12 months (Q to X), indicating an age-dependent CB1 down-regulation 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 μm. Quantification of CB1 labeling in wild-type and AβPP/PS1 mice aged 3, 6 or 12 months revealed an age-dependent CB1 down-regulation in (Y) the cortical area free from Aβ deposition as well as in (Z) the area surrounding Aβ plaques. Data are expressed as the mean values ± SEM (n = 5 pictures from each animal, 3 animals per group). ★ p < 0.05, ★★ p < 0.01 compared to wild-type. ☆☆ p < 0.01, ☆☆☆ p < 0.001 compared to 6 months. § p < 0.05, §§§ p < 0.001 compared to 3 months (two-way ANOVA followed by Tukey’s post hoc analysis).

Figure 3: Cognitive improvement of AβPP/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). AβPP/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 AβPP/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 AβPP/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 AβPP/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 ± 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).

Figure 4: (A) Schematic representation of the 9 cortical areas (dashed squares) and the hippocampal section (dotted ellipse) evaluated for Aβ burden in each animal (B). Representative images of the Aβ immunoreactivity in cortical sections of AβPP/PS1 mice treated during the pre-symptomatic phase (upper panels) or during the early symptomatic phase (lower panels). Scale bar represents 100 μm. (C) Cortical Aβ burden in AβPP/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 Aβ burden in hippocampus of AβPP/PS1 mice was relatively low. ACEA did not modify the hippocampal Aβ burden during the pre-symptomatic phase or during the early symptomatic phase. Counts are expressed as the mean values ± SEM (n = 7-9 per group).
Figure 5: (A) Soluble Aβ40 and Aβ42 concentrations or (B) the ratio between the two soluble Aβ forms were not modified in cortical homogenates from AβPP/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 ± SEM (n = 3-6 per group). (C) Turbidometric analysis of Aβ42 aggregation in the presence of 0.1 and 1 µM ACEA. This CB1 agonist was not able to modify the Aβ aggregation kinetics in vitro. Data are the mean ± SEM of 3 independent experiments.

Figure 6: Representative images of the CB1 (red, A, D, G and J) and Aβ (green, B, E, H and K) double-immunofluorescence in coronal sections of wild-type (Vehicle: A to C; ACEA: G to I) and AβPP/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 µm. (M) Densitometric quantification of CB1 labeling revealed no CB1 down-regulation in the free Aβ cortical areas (white-line squares, A, D, G and J) in chronically ACEA-treated animals. Data are expressed as the mean values ± 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).

Figure 7: Double immunofluorescent staining of glial cells (red) and Aβ plaques (green). A to B: Astroglial response in the surrounding Aβ 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 Aβ plaque area. Antibody against Iba1 was used to specifically stain microglia. Scale bar represent 100 µm (E): Quantification of the GFAP density with respect to Aβ 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 ± 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).

**Figure 8:** (A) Double immunofluorescent staining of CB1 (red) and GFAP (green) showed no CB1 receptor expression in astrocytes in the neocortex of AβPP/PS1 mice. Nuclei are stained in blue. Scale bar represents 75 μm. 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 AβPP/PS1 mice chronically treated with ACEA when compared to vehicle-treated mutants. Scale bar represents 50 μm. Data are expressed as the mean values ± SEM (n = 5 pictures from each animal, 5 animals per group). ☆ p < 0.05 compared to vehicle-treated mice (Student’s t test analysis).

**Figure 9:** ACEA increased the GSK3β phosphorylation (Ser9) in cortical primary cultures when challenged with Aβ42 oligomers and in the cerebral cortex of AβPP/PS1 mice. (A) Double immunofluorescent staining of phospho-GSK3β (green) and tubulin (red) in cortical primary culture challenged with 1 μM Aβ42 oligomers and treated with 1 μM ACEA. (B) Representative western blot revealing the CB1-dependent ACEA reversion of the Aβ-induced dephosphorylation of GSK3β in neuronal cultures and the inhibition of ACEA effect when the CB1 blocker rimonabant is present. (C) Data are the mean ± 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 GSK3β phosphorylation (Ser9) in cortical homogenates after acute administration of ACEA (1.5 mg/kg) in AβPP/PS1 mice. (E) Data are the mean ± SEM of 5 mice per group. ★ p<0.05 (two-way ANOVA and Tukey’s post hoc test).
**Figure 10:** Only small amounts of phospho-tau were seen in the area surrounding mature plaques in AβPP/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 Aβ plaques (green) in AβPP/PS1 mice (B). C: Densitometric quantification of the phospho-tau Thr181 density with respect to Aβ plaque areas. Data are expressed as the mean values ± SEM (n = 5 pictures from each animal, 5 animals per group) ★ p < 0.05 compared to vehicle-treated AβPP/PS1 animals (Student’s t test analysis).
Table 1: Statistical analysis of the ACEA effects in the pre-symptomatic phase at the cognitive level in AβPP/PS1 mice.

<table>
<thead>
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Three-way ANOVA Factors

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Tukey’s post hoc test

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Three-way ANOVA with day (repeated measures), genotype and treatment as between-subjects factors was applied for learning analyses. 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.
Table 2: Statistical analysis of the ACEA effects in the early symptomatic phase at the cognitive level in AβPP/PS1 mice.

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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 was 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.
Figure 1

![Bar chart showing cell viability percentages for different conditions: Control, 1µM Aβ, 1µM Aβ + 0.1µM ACEA, and 1µM Aβ + 1µM ACEA. The chart includes error bars and statistical symbols for significance.](image-url)
Figure 2

<table>
<thead>
<tr>
<th></th>
<th>CB&lt;sub&gt;t&lt;/sub&gt;</th>
<th>Aβ</th>
<th>CB&lt;sub&gt;t&lt;/sub&gt; + Aβ + DAPI</th>
<th>CB&lt;sub&gt;t&lt;/sub&gt; + Aβ (x2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/T 3m</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>APP/PS1 3m</td>
<td>E</td>
<td>F</td>
<td>G</td>
<td>H</td>
</tr>
<tr>
<td>WT 6m</td>
<td>I</td>
<td>J</td>
<td>K</td>
<td>L</td>
</tr>
<tr>
<td>APP/PS1 6m</td>
<td>M</td>
<td>N</td>
<td>O</td>
<td>P</td>
</tr>
<tr>
<td>WT 12m</td>
<td>Q</td>
<td>R</td>
<td>S</td>
<td>T</td>
</tr>
<tr>
<td>APP/PS1 12m</td>
<td>U</td>
<td>V</td>
<td>W</td>
<td>X</td>
</tr>
</tbody>
</table>

![Graph showing % CB<sub>t</sub> staining and % Aβ surrounding area](image)

* 0.05 < p < 0.1
** p < 0.01
*** p < 0.001
Figure 6

<table>
<thead>
<tr>
<th></th>
<th>CB₁</th>
<th>Aβ</th>
<th>CB₁ + Aβ + DAPI</th>
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</thead>
<tbody>
<tr>
<td>WT Vehicle</td>
<td><img src="A" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="C" alt="Image" /></td>
</tr>
<tr>
<td>APP/PS1 Vehicle</td>
<td><img src="D" alt="Image" /></td>
<td><img src="E" alt="Image" /></td>
<td><img src="F" alt="Image" /></td>
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<tr>
<td>WT ACEA</td>
<td><img src="G" alt="Image" /></td>
<td><img src="H" alt="Image" /></td>
<td><img src="I" alt="Image" /></td>
</tr>
<tr>
<td>APP/PS1 ACEA</td>
<td><img src="J" alt="Image" /></td>
<td><img src="K" alt="Image" /></td>
<td><img src="L" alt="Image" /></td>
</tr>
</tbody>
</table>

**M**

<table>
<thead>
<tr>
<th></th>
<th>% CB₁ staining (free Aβ area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH</td>
<td>![Image](bar chart)</td>
</tr>
<tr>
<td>ACEA</td>
<td>![Image](bar chart)</td>
</tr>
</tbody>
</table>

* indicates significant difference.
Figure 8

(A) CB₁, GFAP, CB₁+GFAP, CB₁+GFAP+DAPI

(B) Vehicle

(C) ACEA

(F) Area (IFNγ/GFAP)

- APP/PS1 VEH
- APP/PS1 ACEA

※
Figure 10

[Images of fluorescence microscopy showing Vehicle (A) and ACEA (B) treated samples with quantification of TauPThr181 in panel C with statistical significance indicated by asterisk.]