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Cannabinoid Receptor 2 Participates in Amyloid-β Processing in a Mouse Model of Alzheimer's Disease but Plays a Minor Role in the Therapeutic Properties of a Cannabis-Based Medicine

Ester Aso[a,b,*], Pol Andrés-Benito[a,b], Margarita Carmona[a,b], Rafael Maldonado[c] and Isidre Ferrer[a,b]
[a]*Institut de Neuropatologia, Servei d'Anatomia Patològica, IDIBELL-Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Spain*
[b]*CIBERNED, Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas, Instituto Carlos III, Spain*
[c]*Laboratori de Neurofarmacologia, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain*

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Abstract. The endogenous cannabinoid system represents a promising therapeutic target to modify neurodegenerative pathways linked to Alzheimer's disease (AD). The aim of the present study was to evaluate the specific contribution of CB_2 receptor to the progression of AD-like pathology and its role in the positive effect of a cannabis-based medicine (1:1 combination of Δ^9-tetrahidrocannabinol and cannabidiol) previously demonstrated to be beneficial in the AβPP/PS1 transgenic model of the disease. A new mouse strain was generated by crossing AβPP/PS1 transgenic mice with CB_2 knockout mice. Results show that lack of CB_2 exacerbates cortical Aβ deposition and increases the levels of soluble $A\beta_{40}$. However, CB_2 receptor deficiency does not affect the viability of AβPP/PS1 mice, does not accelerate their memory impairment, does not modify tau hyperphosphorylation in dystrophic neurites associated to Aβ plaques, and does not attenuate the positive cognitive effect induced by the cannabis-based medicine in these animals. These findings suggest a minor role for the CB_2 receptor in the therapeutic effect of the cannabis-based medicine in AβPP/PS1 mice, but also constitute evidence of a link between CB_2 receptor and Aβ processing.

Keywords: AβPP/PS1 mice, Alzheimer's disease, amyloid, cannabinoid receptor 2, cognitive impairment, Δ^9-tetrahidrocannabinol and cannabidiol, tau, therapy

INTRODUCTION

CB_2 receptors are one of the main components of the endogenous cannabinoid system, a lipid signaling network involved in the maintenance of cellular homeostasis in the central nervous system and other peripheral tissues. These $G_{i/o}$-coupled receptors are mainly located in immune system cells including

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*Correspondence to: Ester Aso, Institut de Neuropatologia, Servei d'Anatomia Patològica, IDIBELL-Hospital Universitari de Bellvitge, C/Feixa Llarga s/n, 08907 L'Hospitalet de Llobregat, Spain. Tel.: +34 93 2607452; Fax: +34 93 2607503; E-mail: aso@bellvitgehospital.cat.

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microglia. The stimulation of CB_2 receptors by endogenous or exogenous cannabinoid compounds modulates an array of signaling pathways that in the end lead to immune cell migration and control of cytokine release [1]. Thus, CB_2 receptors are implicated in the reduction of pro-inflammatory molecules in response to harmful stimuli [2]. However, relatively low CB_2 receptor expression is also observed in brain neurons under certain conditions [3–6]. Moreover, recent findings reveal that CB_2 receptors are involved in several physiological processes related to neuronal activity [4, 5, 7, 8].

Alzheimer's disease (AD), the most frequent neurodegenerative disease, is characterized by the presence of amyloid-β (Aβ) deposition and neuronal tau hyperphosphorylation in brain, accompanied by energetic failure, oxidative stress, and neuroinflammation [9, 10]. Previous studies have shown the participation of CB_2 receptors in AD. CB_2 receptor levels are increased in postmortem brains, specifically in astrocytes and predominantly in microglia surrounding Aβ plaques [11–14]. Pharmacological stimulation of these receptors facilitates Aβ removal [15–17], reduces neuroinflammation [12, 16–21], and facilitates cognitive improvement [16, 17, 21] in different animal models of the disease. Moreover, CB_2 receptors modulate oxidative stress damage and tau hyperphosphorylation in AD models [21, 22]. However, the pathophysiological role of CB_2 receptors in the progression of the disease remains to be elucidated.

A recent report has shown that administration of a combination of two botanical extracts derived from the plant *Cannabis sativa*, one of them enriched in Δ^9-tetrahydrocannabinol (Δ^9-THC) and the other in cannabidiol (CBD), results in cognitive improvement together with reduction of several pathologic parameters in AβPP/PS1 transgenic mice, a well-established murine model of AD-related pathology [23]. In the same line, positive effects of the combination of Δ^9-THC and CBD botanical drug substances (BDS) have been reported on the altered behavior in a murine model of tauopathy [24]. Moreover, some previous findings demonstrated a beneficial effect of Δ^9-THC [25] and CBD [26–28] separately in other AD models. The mechanism of action of such compounds is still not fully understood. Δ^9-THC and CBD act on different signaling pathways [29, 30], and the fact that their combination produces better therapeutic effects than that resulting from treatment with only one of the components suggests a cumulative effect or a positive interaction between the two compounds. Moreover, it is important to notice that other phytocannabinoids and terpens, including tetrahydrocannabivarin, cannabigerol, cannabichromene among others, which were initially considered inactive compounds but that have recently demonstrated to exert *per se* additional therapeutic effects, are also present in minor proportions in these botanical extracts. A synergy between the main components (Δ^9-THC and CBD) and such other phytocannabinoids might also occur when using cannabis botanical extracts, resulting in a potentiation or inhibition of their activity, in a summation of effects and/or in pharmacokinetic and metabolic interactions [31].

The aim of the present study was to evaluate the specific contribution of CB_2 receptor to the progression of the AD-like pathology and its role in the positive effect of the 1:1 combination of Δ^9-THC BDS and CBD BDS in AβPP/PS1 transgenic mice.

MATERIALS AND METHODS

Animals

A new mouse strain was generated by crossing male AβPP/PS1 mice (AβPPswe and PS1dE9) [32] purchased from Jackson Laboratories (Bar Harbor, Maine, USA) with female CB_2 knockout (KO) mice [33] obtained from the European Mutant Mouse Archive (Helmholtz Zentrum, München, Germany), both strains in a C57BL/6 background. The resulting AβPP/PS1/CB_2 heterozygous (HET) males were then crossed with wild-type (WT)/CB_2 HET females to obtain the animals for the present study. The genotype of the pups was evaluated with the polymerase chain reaction (PCR) technique using genomic DNA isolated from tail clips. CB_2 HET mice were ruled out for the subsequent experiments. Memory performance was evaluated in WT/CB_2 WT, WT/CB_2 KO, AβPP/PS1/CB_2 WT, and AβPP/PS1/CB_2 KO male littermates aged 3 and 6 months. The pharmacological study was carried out in males aged 6 months (early symptomatic phase in AβPP/PS1 mice). Animals were maintained under standard animal housing conditions in a 12-h dark-light cycle with free access to food and water. Mice were randomly assigned to treatment groups and the experiments were conducted under blind experimental conditions. All animal procedures were carried out following the guidelines of the European Communities Council Directive 2010/63/EU and with the approval of the local ethical committees of the University of Barcelona and University Pompeu Fabra.

Pharmacological treatment

Δ^9-THC BDS (containing 67.1% Δ^9-THC, 0.3% CBD, 0.9% cannabigerol, 0.9% cannabichromene, and 1.9% other phytocannabinoids) and CBD BDS (containing 64.8% CBD, 2.3% Δ^9-THC, 1.1% cannabigerol, 3.0% cannabichromene, and 1.5% other phytocannabinoids) were supplied by GW Pharmaceuticals Ltd (Cambridge, UK). The 1:1 mixture of both extracts (Δ^9-THC BDS 0.75 mg/kg + CBD BDS 0.75 mg/kg) was dissolved in 5% ethanol, 5% Tween, and 90% saline, and injected intra-peritoneally (i.p.) in a volume of 10 mL/kg body weight. The selection of the dose was based on previous studies revealing a therapeutic effect of the Δ^9-THC +CBD BDS combination in AβPP/PS1 mice [34]. Animals were treated once a day for 5 weeks with the extracts (WT/CB_2WT, $n = 7$; AβPP/PS1/CB_2WT, $n = 6$; WT/CB_2 KO, $n = 8$; AβPP/PS1/CB_2 KO, $n = 8$) or the corresponding vehicle (WT/CB_2WT, $n = 8$; AβPP/PS1/CB_2WT, $n = 5$; WT/CB_2 KO, $n = 7$; AβPP/PS1/CB_2 KO, $n = 8$). After a 4-day washing period, animals were subjected to behavioral evaluation.

Behavioral evaluation of cognitive performance

Two-object recognition test

On day 1, mice were habituated for 9 min to a V-maze 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; the time that mice spent exploring each object was recorded. Twenty-four hours 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. Object recognition index (RI) was calculated as the difference between the time spent exploring the novel (T_N) and the familiar object (T_F) divided by the total time spent exploring the two objects [$RI = (T_N - T_F)/(T_N + T_F)$]. Animals exhibiting memory impairments showed a lower RI.

Active avoidance test

After the two-object recognition test, the animals were allowed to rest for 7 days before starting the active avoidance test. 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, mice were placed in the shuttle box for 10 min before the start of each session to allow them to explore the box.

At the end of the behavioral testing, the animals were sacrificed by cervical dislocation, and their brains were rapidly removed from the skulls and processed for study. One hemisphere was dissected on ice, immediately frozen, and stored at –80ºC until use. The other hemisphere was fixed in 4% paraformaldehyde and processed for immunohistochemistry.

Aβ immunohistochemistry

Fixed tissue samples were embedded in paraffin, and coronal sections, 4 μm thick, were cut with a microtome. Consecutive de-waxed sections were incubated with 98% formic acid (3 min) and then treated with citrate buffer (20 min) to enhance antigenicity. Then endogenous peroxidases were blocked by incubation in 10% methanol-1% H_2O_2 solution (15 min). Sections were blocked with 3% normal horse serum solution and then incubated at 4°C overnight with the primary antibody against total Aβ (clone 6F/3D 1:50, Dako, Glostrup, Denmark), $A\beta_{40}$ (1:100, Merck Millipore, Billerica, MA, USA), or $A\beta_{42}$ (1:50, Merck Millipore). Sections were subsequently rinsed and incubated with biotinylated secondary antibody (Dako). Peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Sections were lightly counterstained with hematoxylin. After staining, the sections were dehydrated and cover-slipped for observation under a Nikon Eclipse E800 microscope (Nikon Imaging Inc., Tokyo, Japan). The cortical total Aβ burden was calculated as the percentage of the area of amyloid deposition in plaques with respect to the total cortical area (0.6 mm^2) in 9 pictures taken from 3 different sections

(–0.1 mm, –1.5 mm, and –2.0 mm from bregma) of the each animal brain (3 pictures per section corresponding to cingular/retrospenial and motor cortex, somatosensory cortex, and piriform/entorrhinal cortex), corresponding to the main regions where Aβ deposition is observed in AβPP/PS1 mice [23, 24]. Two sections of the hippocampus of each animal (–1.5 mm and –2.0 mm from bregma) were used for quantification of the hippocampal Aβ burden, calculated as the percentage of the amyloid deposition in plaques with respect to the total hippocampal area in each section. The ratio between $A\beta_{42}$ and $A\beta_{40}$ deposition in each plaque was calculated by comparing the specific staining with each antibody in at least 15 cortical plaques per animal in consecutive sections. Quantifications were performed by a researcher blind for the corresponding genotype or treatment of each section. Aβ quantification was calculated using the Analysis tool of the Adobe® Photoshop® CS4 software (Adobe Systems Inc., San Jose, CA, USA). All the AβPP/PS1-treated animals were analyzed.

Aβ soluble quantification: Enzyme-linked immunosorbent assay (ELISA)

Fresh-frozen mouse brain cortex was 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 Molecular Systems, Pleasanton, CA, USA). Homogenate was spun 100,000 g × 1 h, and the supernatant was saved as the soluble fraction for Aβ quantification. $A\beta_{40}$ and $A\beta_{42}$ Human ELISA kits (Invitrogen™ Corporation, Camarillo, CA, USA) were used to quantify the levels of $A\beta_{40}$ and $A\beta_{42}$ peptides in the brain soluble fractions. Quantitative determination was carried out according to the manufacturer's instructions. $A\beta_{40}$ and $A\beta_{42}$ levels were normalized to the total amount of protein from each individual sample (BCA method, Thermo Fisher Scientific, Wilmington, DE, USA). All the AβPP/PS1-treated animals were analyzed.

Double-labeling immunofluorescence

De-waxed sections were incubated with 98% formic acid (3 min) for Aβ immunofluorescence and then treated with citrate buffer (20 min) to enhance antigenicity. Sections were stained with a saturated solution of Sudan black B for 10 min (Merck Millipore) to block lipofuscin autofluorescence, and then rinsed in 70% ethanol and washed in distilled water. After a blockade with 10% fetal bovine serum (90 min), the sections were incubated at 4ºC overnight with combinations of primary antibodies against Aβ (clone 6F/3D 1:50, Dako), glial fibrillary acidic protein (GFAP; 1:250, Dako), IBA1 (1:250, Wako, Richmond, VA, USA) or phospho-Tau (Thr181) (1:250, Merck Millipore). After washing, the sections were incubated with Alexa488 or Alexa546 fluorescence secondary antibodies against the corresponding host species (1:400, Molecular Probes, Eugene, OR, USA). Then they were washed and mounted in Immuno-Fluore Mounting medium (ICN Biomedicals, Solon, OH, USA), sealed, dried overnight, and examined with a Nikon Eclipse E800 microscope coupled to a camera (Jenoptik I Optical Systems, Germany) controlled from the ProgRes® CapturePro 2.7 software (Jenoptik). For each animal, the specific GFAP, IBA1 and phospho-Tau (Thr181) immunostaining density was calculated in reference to the Aβ plaque area in 5 pictures taken from the cingular/retrospenial and motor cortices, somatosensory cortex or piriform/entorrhinal cortex using the Adobe® Photoshop® CS4 software. No differences were observed in the glial or tau phosphorylation response depending on cortical region. The criteria for choosing such plaques were (1) medium in size, (2) with a condensed center and (3) not associated to vascular processes or to other plaques.

Statistical analysis

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. Statistical analysis was performed with the SPSS® Statistics v21.0 software (IBM, New York, NY, USA). The normality of the data was assessed with the Shapiro-Wilk test and as a consequence parametric statistical tests were used for the analysis of all the data in the study. The frequency of observed genotypes was analyzed with Pearson's chi-squared test (χ^2) test. Memory performance at different ages was analyzed with two-way ANOVA with AβPP/PS1 transgene and CB_2 mutation as between factors, followed by Tukey's *post hoc* when required. Cognitive evaluation after chronic treatment was analyzed with three-way ANOVA with AβPP/PS1 transgene, CB_2 mutation and treatment as between factors, followed by Tukey's *post hoc* when required. Aβ and glia or phosphorylated tau quantifications were analyzed with two-way ANOVA with CB_2 mutation and treatment as between factors, followed by Tukey's *post hoc* when required. In all the experiments, the significance level was set at $p < 0.05$.

RESULTS

Viability and memory performance of AβPP/PS1 deficient for CB_2 receptors

As shown in Fig. 1A, crossing AβPP/PS1/CB_2 HET males with WT/CB_2 HET females resulted in the generation of pups bearing six different genotypes. The Pearson's chi-squared test (χ^2) test revealed that observed frequencies of each genotype were not significantly different from the expected frequencies according to Mendelian laws: 50% WT and 50% AβPP/PS1, and for each one of these genotypes 25% CB_2 WT, 50% CB_2 HET and 25% CB_2 KO. Thus, CB_2 receptor deficiency does not compromise the viability of AβPP/PS1 and WT mice. Moreover, the mortality of CB_2 mutant mice was not increased in AβPP/PS1 and WT mice, at least up to six months of age, when the chronic treatment started. None of the genotypes exhibited physical abnormalities.

A

	WT			AβPP/PS1		
N = 214	CB_2 WT	CB_2 HET	CB_2 KO	CB_2 WT	CB_2 HET	CB_2 KO
Observed	28	54	28	28	51	25
Expected	26.8	53.5	26.8	26.8	53.5	26.8

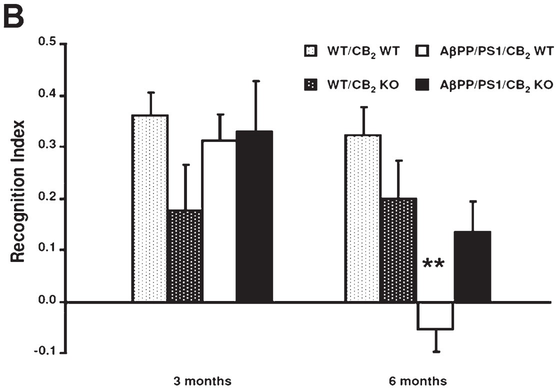

Fig. 1. A) Distribution of the genotypes in the offspring from mating of AβPP/PS1/CB_2 heterozygous males (HET) and WT/CB_2 HET females. The observed genotype frequencies were not significantly different from the expected frequencies according to the Mendelian laws. N, total number of mice studied. B) Memory performance of animals aged 3 months (left; pre-symptomatic phase in AβPP/PS1 mice) and 6 months (right; early symptomatic phase) in the two-object recognition test. CB_2 deletion does not accelerate memory impairment in AβPP/PS1 mice. In contrast, WT/CB_2 KO exhibit a tendency toward decrease in the recognition index at 3 and 6 months with respect to WT/CB_2 WT littermates. Data are expressed as the mean values ± SEM. $^{**}p < 0.01$ compared to WT/CB_2 WT mice.

In order to evaluate a potential acceleration of the AD-related cognitive impairment in the AβPP/PS1/CB_2 KO mice, memory performance of mutant mice was assessed at 3 (pre-symptomatic phase in AβPP/PS1 mice) and 6 (early symptomatic phase) months of age. No significant difference between genotypes was observed at 3 months of age. In contrast, two-way ANOVA revealed a significant effect of the AβPP/PS1 transgene ($F_{(1,34)} = 8.644$, $p < 0.01$), no effect of CB_2 deletion, but interaction between the two genotypes ($F_{(1,34)} = 4.492$, $p < 0.05$) at 6 months of age. Subsequent *post hoc* test revealed memory impairment in AβPP/PS1/CB_2 WT mice when compared to WT/CB_2 WT littermates ($p < 0.01$). WT/CB_2 KO mice exhibited a tendency to reduce memory impairment at 3 and 6 months although without statistical significance. This fact contributed to the absence of significant memory impairment in AβPP/PS1/CB_2 KO mice when compared to WT/CB_2 KO littermates at 6 months, in spite of the lower recognition index exhibited by AβPP/PS1/CB_2 KO mice (Fig. 1B). Therefore, it can be concluded that CB_2 deficiency does not accelerate memory impairment in AβPP/PS1 mice.

CB_2 receptor deficiency does not reduce the cognitive improvement induced by natural cannabinoids in AβPP/PS1 mice

Daily administration of Δ^9-THC BDS and CBD BDS (0.75 mg/kg each botanical extract i.p.) for 5 weeks at the early stages of the symptomatic phase (6 months) blunted the memory impairment observed in vehicle-treated AβPP/PS1 mice when compared to WT animals as revealed by the two-object recognition test both in CB_2 WT and CB_2 KO mice (Fig. 2A). Details of the three-way ANOVA are shown in Supplementary Table 1. Subsequent Tukey's *post hoc* tests revealed memory impairment in vehicle-treated AβPP/PS1/CB_2 WT mice with respect to WT/CB_2 WT mice ($p < 0.001$). In spite of the lower recognition index exhibited by AβPP/PS1/CB_2 KO treated with vehicle, no significant difference was observed when compared to WT/CB_2 KO mice treated with vehicle, likely due to the WT/CB_2 KO tendency to show reduced memory performance with respect WT/CB_2 WT littermates. Δ^9-THC + CBD BDS significantly increased the recognition index of AβPP/PS1/CB_2 WT ($p < 0.01$) and AβPP/PS1/CB_2 KO ($p < 0.01$) mice when compared to vehicle-treated littermates bearing the same genotype.

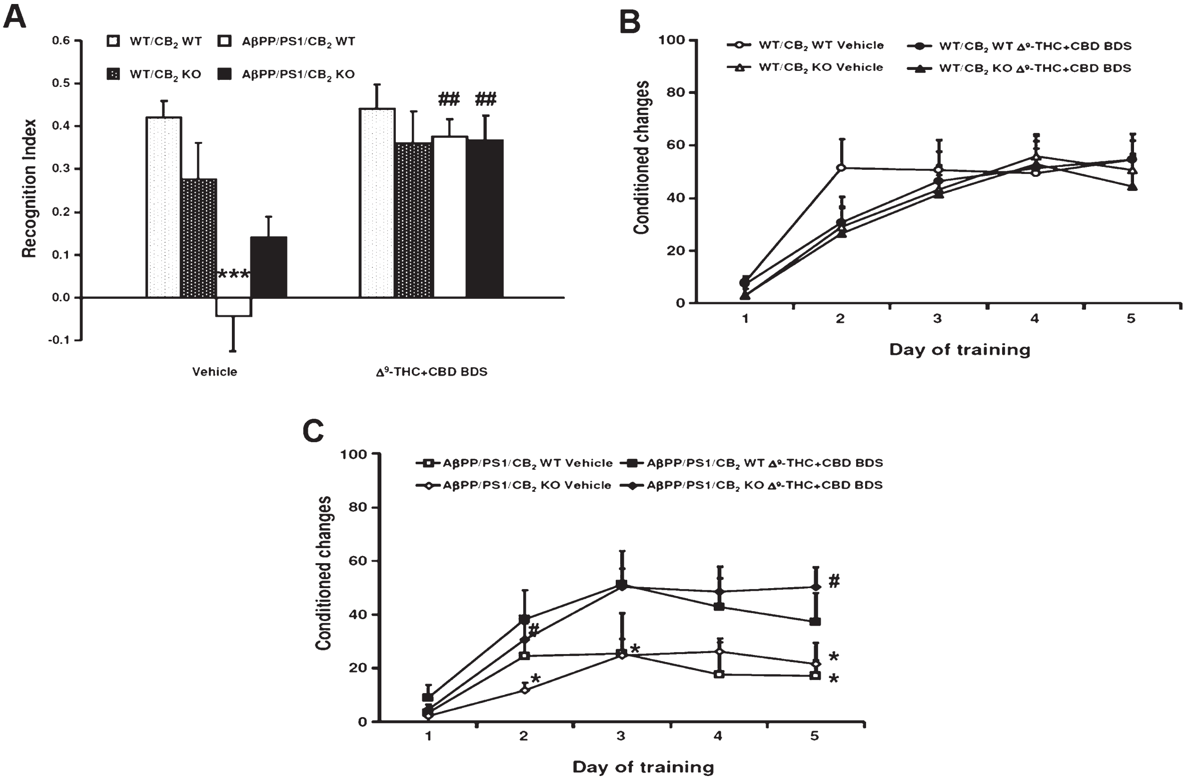

Fig. 2. A) Memory performance of animals treated during the early symptomatic stage (6 months): AβPP/PS1/CB$_2$ WT mice chronically treated with vehicle exhibit significant reduction in the recognition index when compared to corresponding wild-type littermates. Similar memory impairment is observed in vehicle-treated AβPP/PS1/CB$_2$ KO mice although the reduction in the recognition index with respect to WT/CB$_2$ KO does not reach statistical significance. Chronic Δ^9-THC + CBD BDS (0.75 mg/kg each, i.p.) administration induces memory improvement in AβPP/PS1 independently of the presence or absence of CB$_2$ receptor when compared to corresponding control group. B, C) The number of conditioned changes in the active avoidance test is recorded during 5 consecutive days to evaluate learning performance. B) No significant differences in the number of conditioned changes during the 5 days are observed in any of the groups of mice not bearing the AβPP/PS1 transgene. C) Vehicle-treated AβPP/PS1/CB$_2$ WT exhibit a significant reduction in learning performance compared to WT/CB$_2$ WT on day 5. The number of conditioned changes achieved by vehicle-treated AβPP/PS1/CB$_2$ KO is reduced on days 2, 3, and 5 with respect to WT/CB$_2$ KO. In contrast, AβPP/PS1 mice chronically treated with the combination of Δ^9-THC + CBD BDS did not evidence such learning impairment at any time point independently of the presence or absence of CB$_2$ receptor. A significant treatment effect is observed in AβPP/PS1/CB$_2$ KO mice on days 2 and 5. Data are expressed as the mean values ± SEM. $^*p < 0.05$, $^{***}p < 0.001$ compared to animals not bearing the AβPP/PS1 transgene; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to vehicle-treated littermates.

Learning performance was evaluated with the active avoidance test in order to assess the effects of the natural cannabinoids on a more complex cognitive task. Details of the three-way ANOVA are shown in Supplementary Table 1; comparisons between groups were assessed with Tukey's *post hoc* test when interaction between factors was significant. In the active avoidance test, WT/CB$_2$ KO mice did not exhibit any tendency to reduce their cognitive performance with respect WT/CB$_2$ WT littermates as occurred in the two object recognition task. The number of achieved conditioned changes was reduced in vehicle-treated AβPP/PS1/CB$_2$ WT mice on day 5 ($p < 0.05$), and in vehicle-treated AβPP/PS1/CB$_2$ KO mice on day 2 ($p < 0.05$), day 3 ($p < 0.05$), and day 5 ($p < 0.05$) when compared with vehicle-treated WT/CB$_2$ WT and WT/CB$_2$ KO animals, respectively (Fig. 2B, C). In contrast, AβPP/PS1/CB$_2$ WT and AβPP/PS1/CB$_2$ KO mice chronically treated with the combination of Δ^9-THC + CBD BDS did not show learning impairment at any time when compared to the corresponding treated WT controls (Fig. 2B, C). Moreover, a significant improvement was observed in AβPP/PS1/CB$_2$ KO mice chronically treated with Δ^9-THC + CBD BDS on day 2 ($p < 0.05$) and day 5 ($p < 0.05$) when compared to vehicle-treated AβPP/PS1/CB$_2$ KO littermates. These results demonstrate that Δ^9-THC + CBD BDS rescued AβPP/PS1 learning impairment in the active avoidance paradigm when administered at the beginning of the symptomatic stage in CB$_2$

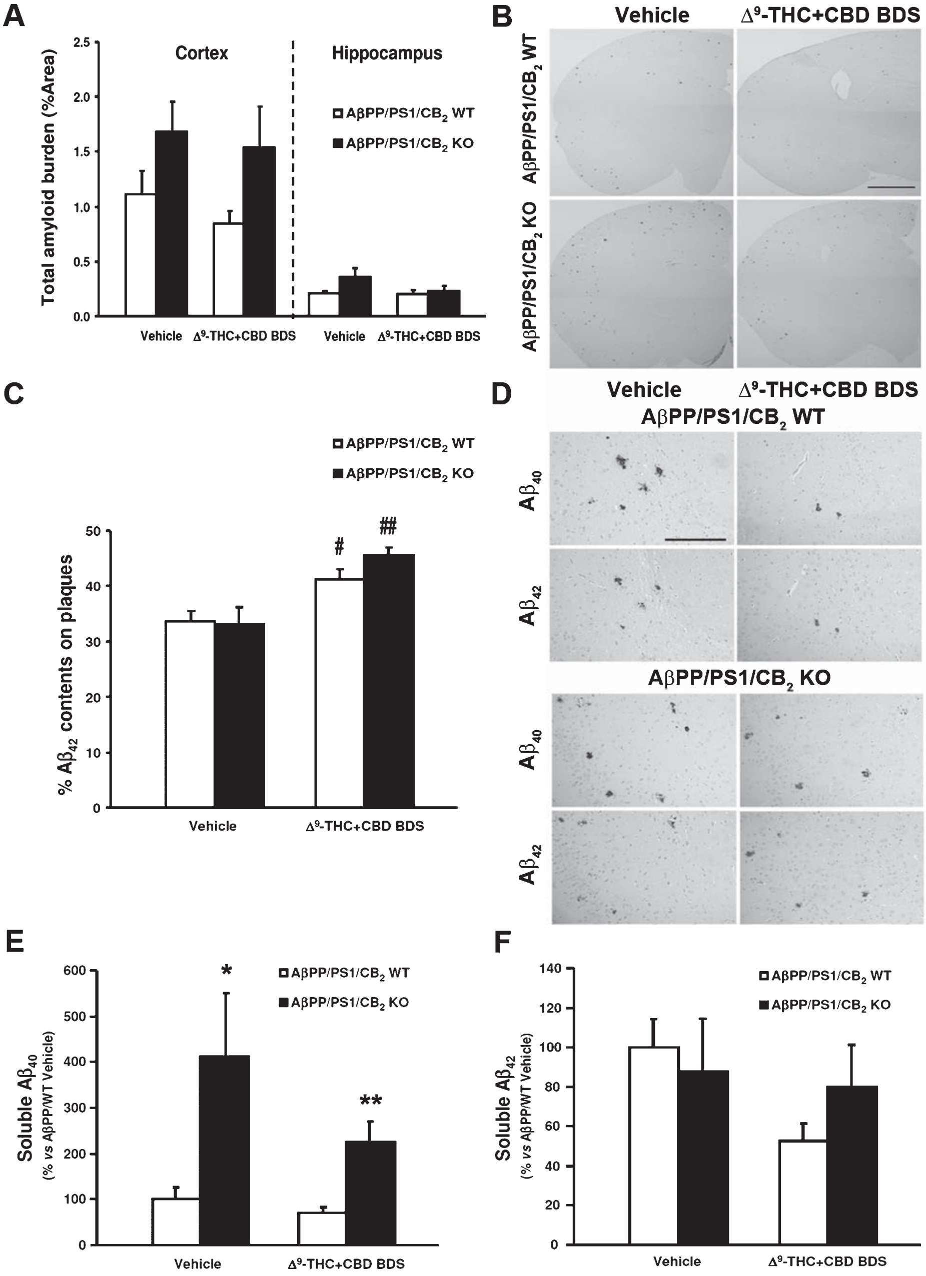

Fig. 3. A) Total Aβ burden quantification in cortical sections reveals increased Aβ deposition in AβPP/PS1/CB_2 KO mice (black bars) compared to AβPP/PS1/CB_2 WT littermates (open bars) at the end of the treatment period, but no significant effect induced by the Δ^9-THC + CBD BDS in any of the AβPP/PS1 groups. B) Low magnification images showing representative brain sections stained with a specific antibody against total Aβ. Scale bar represents 1000 μm. C) Δ^9-THC + CBD BDS significantly increases the percentage of $A\beta_{42}$ in plaques, calculated as the contents of $A\beta_{42}$ with respect to the total $A\beta_{40}$ + $A\beta_{42}$ deposition, in both AβPP/PS1/CB_2 WT and AβPP/PS1/CB_2 KO mice. D) Representative images of $A\beta_{40}$ and $A\beta_{42}$ specific immunoreactivity in consecutive cortical sections of AβPP/PS1/CB_2 WT (upper) and AβPP/PS1/CB_2 KO (bottom) mice chronically treated with vehicle (left) or with Δ^9-THC + CBD BDS (right). Scale bar represents 200 μm. E) Increased soluble $A\beta_{40}$ levels in cortical homogenates from AβPP/PS1 mice deficient for CB_2 receptor; Δ^9-THC + CBD BDS does not modify $A\beta_{40}$ levels in AβPP/PS1/CB_2 WT and AβPP/PS1/CB_2 KO mice evaluated with ELISA. F) No significant effect of genotype or treatment is observed in cortical soluble $A\beta_{42}$ levels in spite of a tendency toward $A\beta_{42}$ protein level reduction in AβPP/PS1/CB_2 WT when compared to vehicle-treated controls. Counts expressed as mean values ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ compared to AβPP/PS1/CB_2 WT.

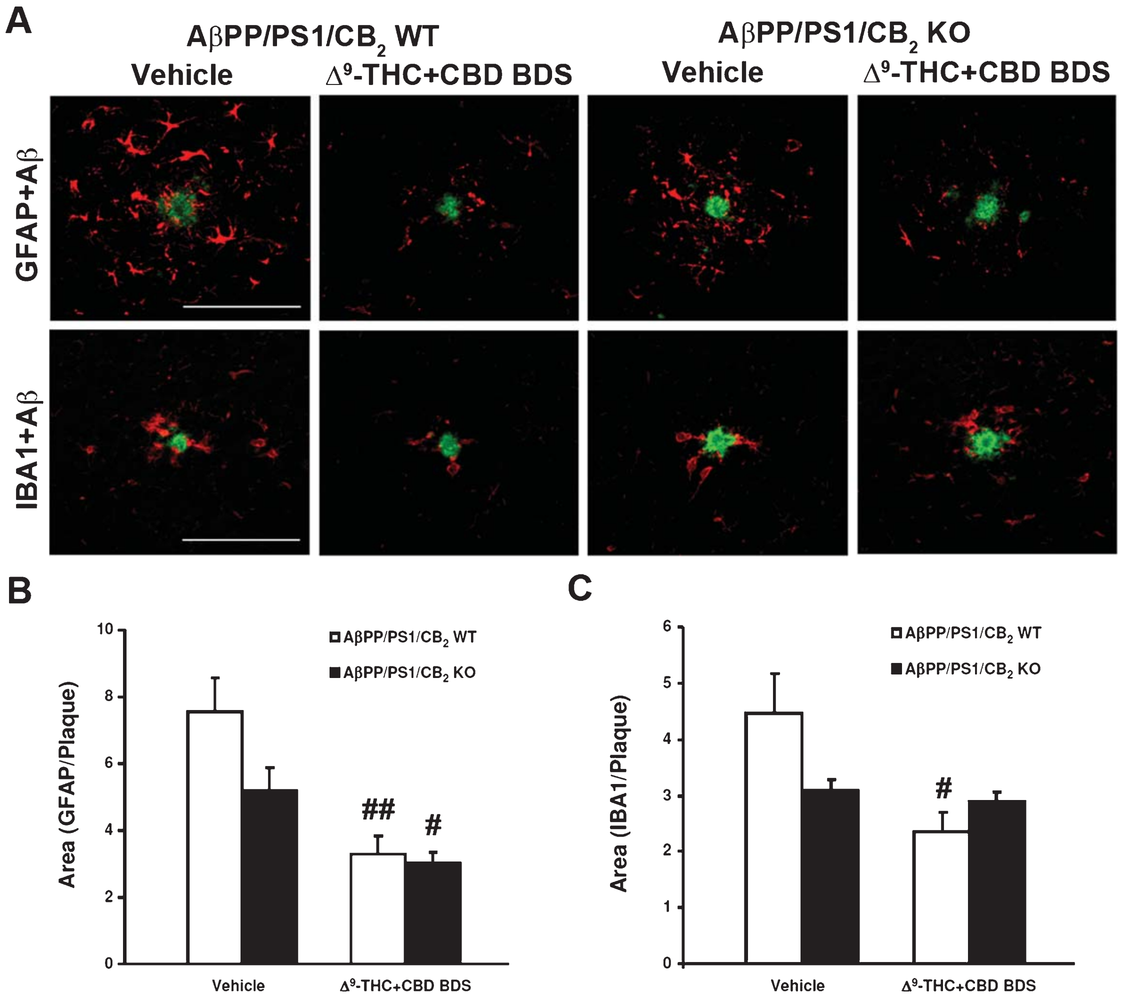

Fig. 4. A) Representative images of double GFAP (red, upper panels) and IBA1 (red, lower panels) and Aβ (green) immunoreactivity in cortical sections of AβPP/PS1 mice chronically treated during the early symptomatic phase with natural cannabinoids. Scale bar represents 100 μm. B) Quantification of GFAP staining around Aβ plaques reveals a significant reduction in the astroglial response in both AβPP/PS1/CB_2 WT and AβPP/PS1/CB_2 KO mice chronically treated with Δ^9-THC + CBD BDS. C) Quantification of IBA1 staining around Aβ plaques reveals a tendency toward microglial cell reduction in AβPP/PS1/CB_2 KO and a significant reduction in microglial response in Δ^9-THC + CBD BDS-treated AβPP/PS1/CB_2 WT, but not in AβPP/PS1/CB_2 KO mice, when compared to corresponding vehicle control groups. Data are expressed as the mean values ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to vehicle-treated littermates.

functional and CB_2 KO backgrounds. Together, these results suggest that CB_2 receptors do not play a relevant role in the cognitive improvement induced by natural cannabinoids in AβPP/PS1 mice.

Deficiency in CB_2 receptors alters Aβ processing but not the Δ^9-THC + CBD BDS effect on Aβ plaque composition in AβPP/PS1 mice

At 3 months of age, no significant difference was observed between the cortical Aβ burden in AβPP/PS1/CB_2 WT (%Area with Aβ plaques: 0.06 ± 0.01) and AβPP/PS1/CB_2 KO mice (%Area with Aβ plaques: 0.05 ± 0.01). No Aβ plaques were observed in hippocampus of AβPP/PS1 mice at 3 months of age. However, AβPP/PS1/CB_2 KO mice exhibited an increase in the total Aβ deposition with respect to AβPP/PS1/CB_2 WT littermates at the end of the chronic treatment period (≈ 8 months of age), independently of the treatment received (Fig. 3A, B). Two-way ANOVA revealed a significant genotype effect (Cortex: $F_{(1,32)} = 6.418$, $p < 0.05$; Hippocampus: $F_{(1,29)} = 5.030$, $p < 0.05$), but no treatment effect and no interaction between the two

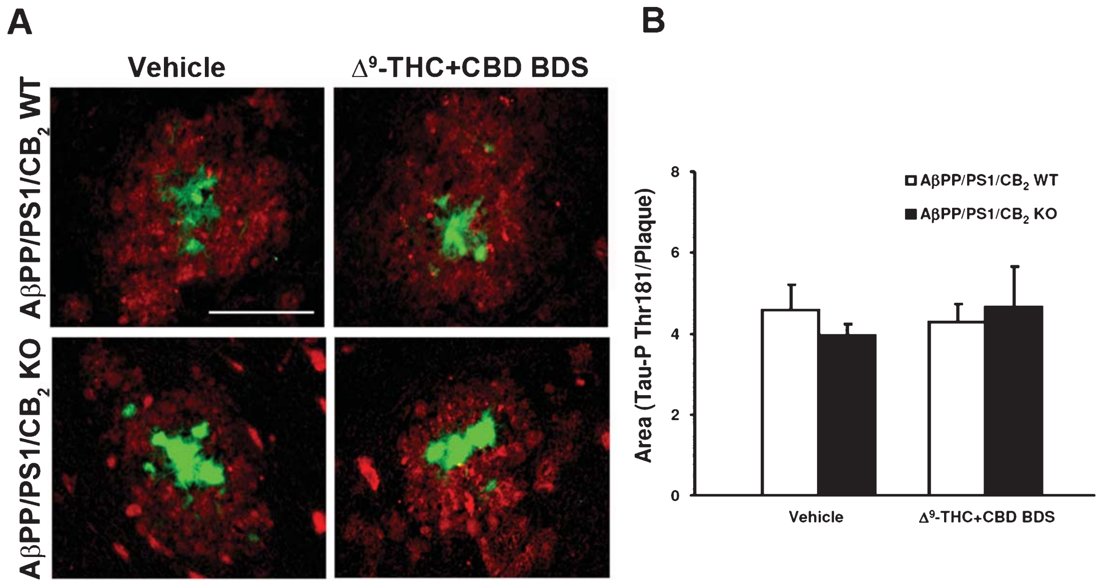

Fig. 5. A) Representative images of double phospho-tau (Thr181) (red) and Aβ (green) immunoreactivity in cortical sections of AβPP/PS1/CB$_2$ WT (upper panels) and AβPP/PS1/CB$_2$ KO (lower panels) mice chronically treated with vehicle (left) or Δ^9-THC + CBD BDS (right). Scale bar represents 50 μm. B) Quantification of phospho-tau (Thr181) staining in the vicinity of Aβ plaques reveals no significant effect of CB$_2$ receptor genotype or treatment on tau phosphorylation in AβPP/PS1 mice. Data are expressed as the mean values ± SEM.

factors. As expected, Aβ burden was more relevant in cortex than hippocampus in AβPP/PS1 mice. The deficiency in CB$_2$ receptors did not alter the composition of plaques and did not reduce the increase in the Aβ$_{42}$/Aβ$_{40}$ ratio in plaques induced by Δ^9-THC + CBD BDS both in AβPP/PS1/CB$_2$ WT ($p < 0.05$) and AβPP/PS1/CB$_2$ KO ($p < 0.01$) mice (Fig. 3C, D), as revealed with the two-way ANOVA (Treatment effect: $F_{(1,15)} = 18.130$, $p < 0.001$; Genotype effect and interaction: not significant) and subsequent *post hoc* tests. An increase in the soluble levels of Aβ$_{40}$, but not Aβ$_{42}$, was observed in the cortex of AβPP/PS1/CB$_2$ KO mice treated with vehicle ($p < 0.05$) and with Δ^9-THC + CBD BDS ($p < 0.01$) as revealed with two-way ANOVA (Genotype effect: $F_{(1,25)} = 14.390$, $p < 0.001$; Treatment effect and interaction: not significant) and subsequent *post hoc* tests (Fig. 3E, F).

CB$_2$ receptor plays a role in the microglial response to Aβ deposition induced by natural cannabinoids in AβPP/PS1 mice

Astrocytic and microglial responses to Aβ plaques were evaluated with double-labeling immunofluorescence and densitometric quantification. Two-way ANOVA revealed a treatment effect ($F_{(1,15)} = 3.548$, $p < 0.001$) but no genotype effect or interaction between the two factors in the astroglial response. Subsequent *post hoc* tests indicated that the combination of Δ^9-THC + CBD BDS reduced the astrocytic reactivity in both AβPP/PS1/CB$_2$ WT ($p < 0.01$) and AβPP/PS1/CB$_2$ KO mice ($p < 0.05$) when compared to corresponding vehicle-treated controls (Fig. 4A, B). Regarding microglial reactivity, two-way ANOVA revealed a significant effect of treatment ($F_{(1,16)} = 8.104$, $p < 0.05$), but no genotype effect and no interaction between the two factors ($F_{(1,16)} = 5.550$, $p < 0.05$). Subsequent *post hoc* tests indicated that the number of microglial cells associated with Aβ plaques was significantly reduced by Δ^9-THC + CBD BDS in AβPP/PS1/CB$_2$ WT ($p < 0.05$), but not in AβPP/PS1/CB$_2$ KO mice when compared to the corresponding vehicle-treated group (Fig. 4A, C). A tendency without statistical significance to reduce numbers of microglial cells around Aβ plaques was observed in vehicle-treated AβPP/PS1/CB$_2$ KO mice when compared to AβPP/PS1/CB$_2$ WT littermates.

Tau phosphorylation in the vicinity of Aβ plaques is not modified by CB$_2$ receptor deficiency or natural cannabinoids treatment in AβPP/PS1 mice

AβPP/PS1 mice do not produce neurofibrillary tangles at any age but they present small amounts of hyperphosphorylated tau in dystrophic neurites surrounding Aβ plaques, which are suggested to be a consequence of the detrimental effect of soluble Aβ in neurons and to contribute to the neurodegenerative process [35]. For these reasons, we decided to evaluate the levels of tau phosphorylated at Thr181, the most abundantly phosphorylated site in our animal model of AD, by double-immunofluorescence and quantitative densitometry. No significant differences related to CB_2 receptor deficiency and/or treatment were observed in the levels of tau phosphorylated at the Thr181 site in the vicinity of Aβ plaques in AβPP/PS1 mice (Fig. 5A, B).

DISCUSSION

AβPP/PS1 transgenic mice lacking the CB_2 receptor generated in the present study revealed that this cannabinoid receptor plays a minor role in the therapeutic effects of the combination of Δ^9-THC + CBD BDS natural cannabinoids but demonstrated a link between CB_2 receptor and AD progression.

CB_2 receptor deficiency does not compromise the viability of AβPP/PS1 and WT mice at least up to 6 months of age. Lack of CB_2 receptor induces a tendency to memory impairment in WT mice in the two object recognition test, according to previous reports [36], although does not accelerate the memory impairment in AβPP/PS1 mice. However, AβPP/PS1/CB_2 KO mice exhibit increased Aβ deposits (cerebral cortex and hippocampus) and cortical $A\beta_{40}$ soluble levels at 6 (early symptomatic phase), but not at 3 (pre-symptomatic phase) months of age. Similar observations have been described in another AD model bearing CB_2 receptor deletion [22]. These findings suggest a role for CB_2 receptor in Aβ clearance rather than in the production of this peptide. This hypothesis is also supported by previous studies showing that CB_2 receptor pharmacological activation facilitates Aβ transport through the choroid plexus [16] and Aβ removal by immune cells [15, 17]. The present results together with recent reports [37] also indicate that CB_2 receptor deletion in AβPP/PS1 mice results in a reduction of the microglial, but not astroglial, response to Aβ deposition. Thus, altered microglial responses in AD models lacking CB_2 receptor are accompanied by modification of the levels of molecules involved in neuroinflammation [22, 37]. Moreover, we have also evaluated the levels of hyperphosphorylated tau protein in the Aβ plaques surrounding area because, although AβPP/PS1 mice present only small amounts of hyperphosphorylated tau that are never on a par with those seen in AD brains, they are supposed to reflect a detrimental effect of soluble Aβ and to contribute to the neurodegenerative process [35]. Additional interest on the study of tau phosphorylation in AβPP/PS1 mice lacking CB_2 receptors derived from previous evidence showing a specific role for these cannabinoid receptors in tau phosphorylation by using pharmacological and genetic models [21, 22]. Nevertheless, AβPP/PS1/CB_2 KO mice exhibit similar levels of hyperphosphorylated tau in the dystrophic neurites associated to Aβ plaques than CB_2 receptors sufficient AβPP/PS1 mice, suggesting that these cannabinoid receptors do not play a crucial role in tau phosphorylation in our animal model of AD. These findings collectively demonstrate a role for CB_2 receptors in the pathological progression of AD primarily related to Aβ processing.

The administration of Δ^9-THC + CBD BDS, containing mainly Δ^9-THC with a mixed action on CB_1 and CB_2 receptors and CBD with activity on other receptors [30], at a therapeutic dose in AβPP/PS1 mice [23] is as effective in AβPP/PS1/CB_2 KO mice as in AβPP/PS1 mice not lacking CB_2 receptors. Thus, the combination of the two natural cannabis extracts reduces memory and learning impairment, increases $A\beta_{42}$ contents in plaques, and decreases the astroglial reactivity to Aβ deposition in AβPP/PS1 mice as previously described [23] independently of the presence or absence of CB_2 receptors. In contrast, Δ^9-THC + CBD BDS administration has no effect on the microglial reactivity or tau phosphorylation around Aβ plaques in AβPP/PS1/CB_2 KO mice. These results suggest that other mechanisms apart from CB_2 receptor signaling are involved in the positive effects of these natural cannabinoids in AβPP/PS1 mice. Some potential targets involved in such effects might be CB_1, GPR55 or other G-coupled receptors for which Δ^9-THC or CBD compounds exhibited certain activity. However, further research is needed to unravel the specific contribution of such receptors on the beneficial effect produced by the natural cannabinoids on the AD model.

In conclusion, the contribution of CB_2 receptors to the therapeutic properties of the cannabis-based medicine composed mainly of Δ^9-THC and CBD is minor although CB_2 receptors participate in the progression of the AD pathology in our animal model.

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SUPPLEMENTARY MATERIAL

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