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CB_2 Cannabinoid Receptor Agonist Ameliorates Alzheimer-Like Phenotype in AβPP/PS1 Mice

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Abstract. The specific CB_2 cannabinoid receptor agonist JWH-133 induced cognitive improvement in double AβPP/PS1 transgenic mice, a genetic model of Alzheimer's disease. This effect was more pronounced when administered at the pre-symptomatic rather than the early symptomatic stage. The cognitive improvement was associated with decreased microglial reactivity and reduced expression of pro-inflammatory cytokines IL-1β, IL-6, TNFα, and IFNγ. In addition, JWH-133 reduced the expression of active p38 and SAPK/JNK, increased the expression of inactive GSK3β, and lowered tau hyperphosphorylation at Thr181 in the vicinity of amyloid-β plaques. Moreover, JWH-133 produced a decrease in the expression of hydroxynonenal adducts, and enhanced the expression of SOD1 and SOD2 around plaques. In contrast, the chronic treatment with JWH-133 failed to modify the amyloid-β production or deposition in cortex and hippocampus. In conclusion, the present study lends support to the idea that stimulation of CB_2 receptors ameliorates several altered parameters in Alzheimer's disease such as impaired memory and learning, neuroinflammation, oxidative stress damage and oxidative stress responses, selected tau kinases, and tau hyperphosphorylation around plaques.

Keywords: Alzheimer's disease, CB_2 cannabinoid receptor, cognition, neuroinflammation, oxidative stress, tau kinases

INTRODUCTION

Alzheimer's disease (AD) is considered to be the result of a multifactorial neurodegenerative process in which, in addition to amyloid-β (Aβ) deposition and neuronal tau hyperphosphorylation, numerous pathways are altered, leading to impaired energy processing, oxidative stress damage, and inflammation [1, 2].

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Cannabinoid compounds have emerged as potential therapeutic agents against AD because of their known multifaceted neuroprotective properties [3, 4]. The endocannabinoid system is composed of at least two well-characterized cannabinoid $G_{i/o}$-coupled receptors, CB_1 and CB_2, their endogenous ligands, and the enzymes related to their synthesis and degradation [5]. The CB_1 receptor is widely expressed within the central nervous system, in both neurons and glial cells, where it regulates important brain functions [6, 7]. Recent studies have shown that low non-psychotropic doses of specific CB_1 agonists exhibited potential therapeutic properties in AD [8]. However, among the

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extensive range of natural and synthetic cannabinoids, major attention in AD has been paid to CB_2 receptor specific agonists because of their lack of psychoactive properties. CB_2 receptors are mainly expressed in the immune system, including microglial cells [9, 10], but relatively lower expression of CB_2 receptors in neurons has been proposed as well [11–14]. In AD postmortem brains, CB_2 receptors have been reported to be overexpressed in Aβ plaques-associated microglia, suggesting these receptors as potential therapeutic targets [15]. The activity of CB_2 receptors is implicated in the reduction of pro-inflammatory molecules in response to harmful stimuli [16] and in the control of neural survival [17]. In line with these observations, activation of CB_2 receptors had the beneficial effects of inducing cognitive improvement and reducing neuroinflammatory response in two different *in vivo* models of AD [18–21]. Moreover, stimulation of CB_2 receptors produced Aβ removal by human macrophages, at least in the context of very specific settings [22], and lowered the Aβ levels in transgenic AβPP2576 mice after a prolonged oral administration [20]. Certain cannabinoids were also capable of reducing tau phosphorylation via CB_2 receptors in PC12 cells and in cultured neurons [23, 24]. However, other effects of CB_2 receptor agonists as therapeutic agents in AD need further study. Thus, the present study was aimed to evaluate the effects of the CB_2 receptor specific agonist JWH-133 in AβPP/PS1 mice, a transgenic model of AD that mimics the progressive cognitive deficiency and neurodegenerative process occurring in this disease [25, 26], not only at the inflammatory and amyloid level but also at the oxidative stress and tau kinases levels because of their relevance in AD progression [1, 2].

MATERIALS AND METHODS

Animals

The experiments were carried out on male AβPP/PS1 mice and wild-type littermates aged 3 months (pre-symptomatic) or 6 months (early symptomatic) at the beginning of the study [26]. The generation of mice expressing the human mutated forms AβPPswe and PS1dE9 has been already described [25]. 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 conducted according to ethical guidelines (European Communities Council Directive 86/609/EEC) and approved by the local ethical committee (UB-IDIBELL).

RNA extraction and Cnr2 gene expression quantification

Frozen cortical samples obtained from male AβPP/PS1 mice and wild-type littermates aged 6 months were used to evaluate the basal expression of the gene coding for CB_2 receptor (Cnr2). Isolation of total RNA was carried out according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen® GmbH, Hilden, Germany). cDNA was prepared from 1 μg total RNA using random hexamers and SuperScript II RNase H- reverse transcriptase (Invitrogen, Eugene, OR, USA). For each sample, SYBR Green real-time quantitative PCR was run on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA) with a melting curve dissociation protocol. The primer sequences were designed using Primer Express software and resulted as follows: forward 5'-GGTCGACTCCAACGCTATCTTC-3' and reverse 5'-GTAGCGGTCAACAGCGGTTAG-3'. Xpnpep1 was used as housekeeping gene for normalization. Samples were analyzed by the double delta CT (ΔΔCT) method.

Pharmacological treatment

The selective CB_2 receptor agonist JWH-133 ($K_i = 677$ nM for CB_1 and 3.4 nM for CB_2) [27] was supplied by Tocris Bioscience® (Bristol, UK). JWH-133 (0.2 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 JWH-133 or the corresponding vehicle starting at 3 months of age ($n = 6$–10 per group). Then, the behavioral testing was performed when animals were six months because they are known to exhibit the first cognitive deficiencies at this age [26]. A second group of animals were treated during the early symptomatic phase. These 6-month-old mice were treated once a day for 5 weeks with JWH-133 or the corresponding vehicle ($n = 6$–10 per group). After 10 days of washing period, animals were subjected to behavioral evaluation. This period was considered enough to ensure that the animals were void of the direct effect of the compound during the behavioral performance, considering

the long half-life of cannabinoid compounds in plasma [28].

Behavioral evaluation of cognitive performance and sample collection

Two-object recognition test

This paradigm was performed in a V-maze (Panlab, Barcelona, Spain), as previously described [8]. Animals exhibiting memory impairments revealed decreased object recognition index calculated as the difference between the time the animals spent exploring either the novel or the familiar object, divided by the total time spent exploring the two objects.

Active avoidance test

The learning performance of the animals was evaluated in a two-way shuttle box apparatus (Panlab), where mice were trained to avoid an aversive stimulus associated with the presentation of a conditioned stimulus during 5 consecutive days, as previously described [8]. The total number of conditioned responses was recorded.

At the end of the behavioral testing, the animals were killed and their brains rapidly removed from the skull and processed for study. One brain hemisphere was dissected on ice, immediately frozen and stored at −80°C until used for the quantification of soluble Aβ and for cytokine analysis. The other brain hemisphere was fixed in 4% paraformaldehyde and processed for immunohistochemistry.

Aβ and synaptophysin 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 (Aβ, 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% 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 Aβ (clone 6F/3D, 1:50, Dako, Glostrup, Denmark) or synaptophysin (1:100, Dako). Sections were subsequently rinsed and incubated with biotinylated secondary antibody (Dako), followed by EnVision+ system peroxidase (Dako) and finally with the chromogen diaminobenzidine and H_2O_2. Sections were lightly counterstained with haematoxylin. After staining, the sections were dehydrated and cover-slipped for observation under a Nikon Eclipse E800 microscope (Nikon Imaging Inc., Tokyo, Japan; Objective: 20×). The cortical Aβ burden was calculated as the percentage of the area of amyloid deposition in plaques with respect to the total area in 9 representative pictures taken from the cerebral cortex of each animal, corresponding to the main regions where Aβ deposition is observed in AβPP/PS1 mice. Three sections of the hippocampus of each animal were used for quantification of the hippocampal Aβ burden. The cortical and hippocampal Aβ burden of all the AβPP/PS1 animals were calculated using the Adobe® Photoshop® CS4 software (Adobe Systems Inc., San Jose, CA, USA), as previously described [8].

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 quantifications. The $Aβ_{40}$ and $Aβ_{42}$ Human ELISA kits (Invitrogen™ Corporation, Camarillo, CA, USA) were used to quantify the levels of $Aβ_{40}$ and $Aβ_{42}$ peptides in the brain soluble fractions. The quantitative determination was carried out according to the manufacturer's instructions, as previously described [8]. $Aβ_{40}$ and $Aβ_{42}$ levels were normalized to the total amount of protein from each individual sample.

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, or with a 0.04% borohydride solution (20 min) for hydroxynonenal (HNE) immunohistochemistry. All sections were stained with a saturated solution of Sudan black B for 30 min (Merck Millipore, Billerica, MA, USA) to block lipofuscin autofluorescence, 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 4°C 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), interferon-γ (IFNγ, 1:50, Merck Millipore), phospho-tau (Thr181) (1:250, Merck

Millipore), phospho-p38 (Thr180/Tr182) (1:100, Merck Millipore), phospho-stress-activated protein kinase/Jun-amino-terminal kinase (Thr183/Tyr185) (phosphor-SAPK/JNK 1:100, Cell Signalling, Beverly, MA, USA), phospho-glycogen synthase kinase-3β (Ser9) (phospho-GSK3β, 1:200, Merck Millipore), HNE (1:1000, Merck Millipore), superoxide dismutase 1 (SOD1, 1:1,000, Novocastra-Leica Biosystems, Nussloch, Germany), SOD2 (1:500, Stressgen-Gentaur, Brussels, Belgium), proliferating cell nuclear antigen (PCNA, 1:500, Oncogene Science, Cambridge, MA, USA), and voltage-dependent anion channel (VDAC, 1:100, Calbiochem, Denmark). 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 an Nikon Eclipse E800 microscope (Objective: 40×; Red filter: Excitation 540-25 nm- Emission 605-55 nm; Green filter: Excitation 480-30 nm- Emission 605-55 nm). The specific GFAP, Iba1, phospho-tau, phospho-p38, phospho-SAPK/JNK, phospho-GSK3β, HNE, SOD1 and SOD 2 immunostaining density was calculated in reference to the Aβ plaque area in 5 representative pictures taken from the cortex of each animal ($n = 5$ per group) using the Adobe® Photoshop® CS4 software. In the case of IFNγ, specific density was in reference to the GFAP immunostained area.

Cytokine quantification

The murine IL1β, IL-6, IL10, and TNFα Mini ELISA Development kits (Peprotech™, Rocky Hill, NJ, USA) were used to quantify the levels of these cytokines in cortical brain homogenates (RIPA buffer). The quantitative determination was carried out according to the manufacturer's instructions. Cytokine levels were normalized to the total amount of protein from each individual sample and expressed with respect to the control group.

Statistical analyses

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. Data were analyzed with two-way ANOVA with genotype and treatment as between factors, followed by Tukey's *post hoc* when required. Aβ burden and immunofluorescence quantifications were analyzed with Student's *t*-test. In all the experiments, the significance level was set at $p < 0.05$.

RESULTS

CB_2 gene expression appears to be upregulated in AβPP/PS1 mice

In cortical homogenates of non-treated AβPP/PS1 mice aging 6 months, we observed a clear tendency to upregulate the CB_2 gene expression levels when compared to wild-type littermates (WT: 1.00 ± 0.09; AβPP/PS1: 1.39 ± 0.19; $n = 14$–15; $p = 0.071$). This result is in line with the reported CB_2 receptor upregulation in AD [15] and supports the hypothesis of this receptor as a potential target against AD.

Cognitive improvement of AβPP/PS1 mice treated with JWH-133 during the pre-symptomatic stage

AβPP/PS1 mice treated for 5 weeks with JWH-133 (0.2 mg/kg) during the pre-symptomatic stage (mice aged 3 months) did not exhibit the memory impairment revealed by the two-object recognition test in vehicle-treated AβPP/PS1 mice at the age of 6 months when compared to wild-type animals ($p < 0.001$) (Fig. 1A). Thus, JWH-133 significantly increased the recognition index of AβPP/PS1 mice when compared to vehicle-treated animals ($p < 0.01$). Similarly, AβPP/PS1 mice treated with JWH-133 during the pre-symptomatic stage significantly improved their learning performance when compared to vehicle-treated AβPP/PS1 mice in the active avoidance test at the age of 6 months (Fig. 1C). For statistic details see Supplementary Table 1 (available here: http://dx.doi.org/10.3233/JAD-130137).

JWH-133 treatment during early stages of the symptomatic phase partially reversed the cognitive deficits in AβPP/PS1 mice

Daily stimulation of CB_2 receptors for 5 weeks at the early stages of the symptomatic stage (6 months) reversed the memory impairment exhibited by vehicle-treated AβPP/PS1 mice on the two-object recognition test when compared to wild-type animals ($p < 0.01$) (Fig. 1B). Accordingly, JWH-133 significantly increased the recognition index of AβPP/PS1 mice when compared to vehicle-treated littermates ($p < 0.001$).

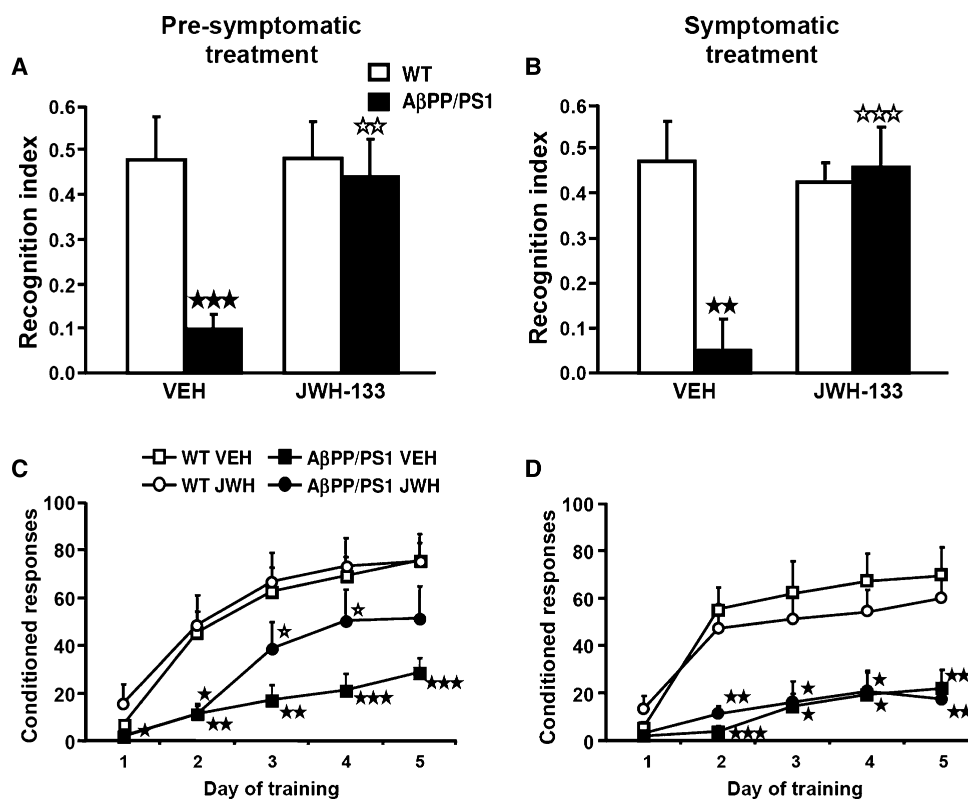

Fig. 1. Cognitive improvement of AβPP/PS1 mice chronically treated with JWH-133 during the pre-symptomatic stage (3 months, left panels) and during the beginning of the symptomatic stage (6 months, right panels). A, B) Memory performance in the V-maze. JWH-133 chronic administration during the pre-symptomatic (A) or symptomatic (B) stages reverses AβPP/PS1 memory deficiency when compared to vehicle-treated animals. C, D) Learning impairment exhibited by AβPP/PS1 mice in the active avoidance test during the five consecutive days of training is not evidenced after chronic JWH-133 administration during the pre-symptomatic stage (C). In contrast, JWH-133 treatment during the symptomatic stage is not able to modify learning deficiencies in AβPP/PS1 mice (D). Data are expressed as the mean values ± SEM of conditioned responses (n = 6–10 per group). ★$p < 0.05$, ★★$p < 0.01$, ★★★$p < 0.001$, genotype effect; ☆$p < 0.05$, ☆☆$p < 0.01$, ☆☆☆$p < 0.001$, treatment effect.

However, JWH-133 did not significantly rescue AβPP/PS1 learning impairment in the active avoidance paradigm when administered at the beginning of the symptomatic stage (Fig. 1D). These results reveal that the efficacy of CB_2 receptor agonists is greater when the treatment is started at an early pre-symptomatic stage of the disease. For statistical details, see Supplementary Table 1.

JWH-133 treatment did not modify the cortical and hippocampal Aβ burden, the cortical soluble fraction, or synaptic markers in AβPP/PS1 mice

Chronic treatment with JWH-133 in AβPP/PS1 mice beginning at the age of either 3 or 6 months did not significantly modify the Aβ burden in the cortex or the hippocampus, a region where Aβ deposition in our murine AD model starts later and is less intense than in the cortex [26] (Fig. 2A, C). Similarly, no difference was observed in the $Aβ_{40}$ or $Aβ_{42}$ protein levels of AβPP/PS1 cortical soluble fraction in either of the two settings (Fig. 2B). Moreover, no significant JWH-133 effect was observed in the cortical or hippocampal expression of synaptophysin, considered as a marker of synapses, and VDAC around the Aβ deposition, which indicates the extension of polymorphous inclusions in dystrophic neurites (Supplementary Figure 1).

Modulation of the gliosis associated with Aβ deposition and cytokine expression following JWH-133 treatment at the early symptomatic stage in AβPP/PS1 mice

Because of the potential relevance for therapeutic intervention, the next studies were carried out with the cortical samples obtained from the AβPP/PS1 mice chronically treated during the early symptomatic stage (6 months of age).

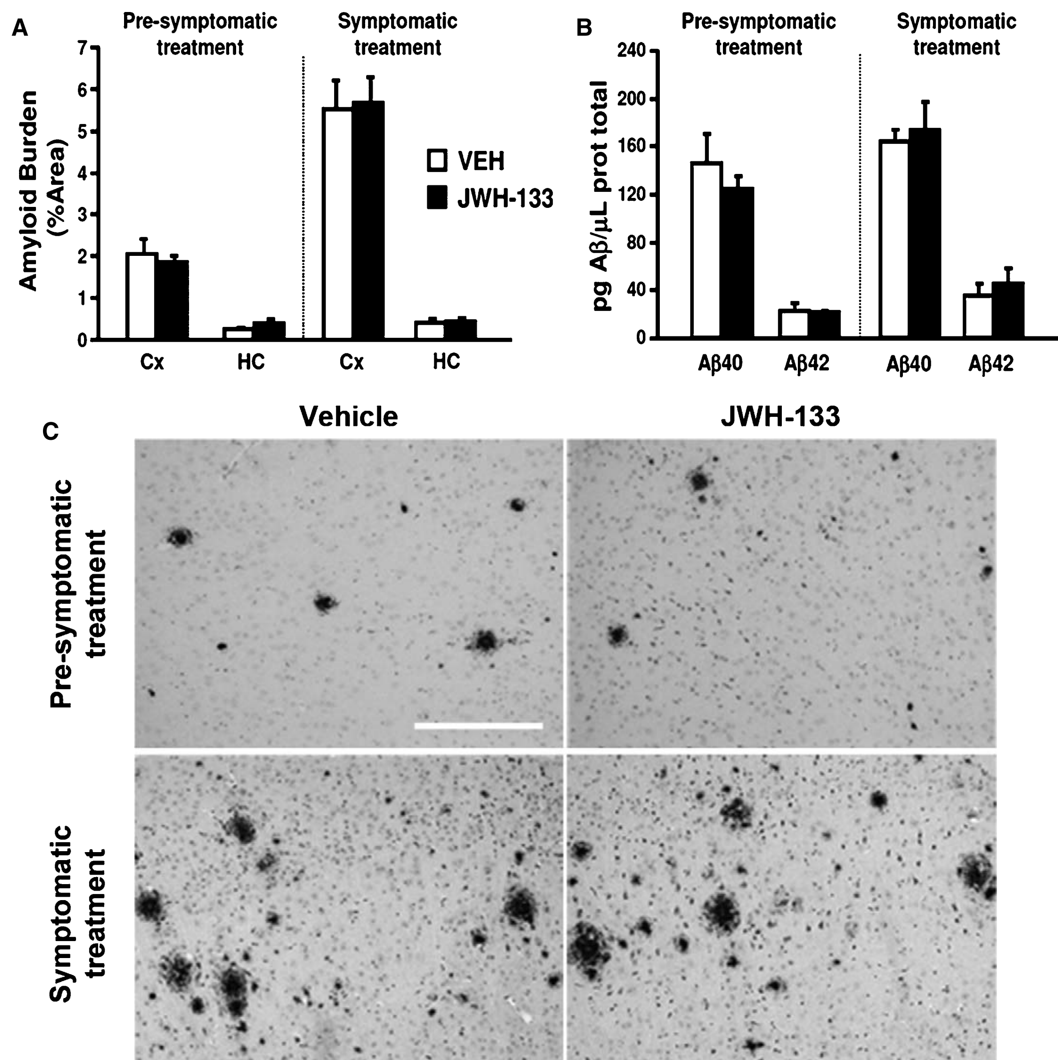

Fig. 2. A) Cortical (Cx) or hippocampal (HC) Aβ burden in AβPP/PS1 mice is not modified by chronic JWH-133 treatment during the pre-symptomatic stage or during the early symptomatic stage. Compared to cortex, the Aβ burden in hippocampus of AβPP/PS1 mice is relatively low. B) Neither $A\beta_{40}$ nor $A\beta_{42}$ cortical soluble fractions are modified in AβPP/PS1 mice chronically treated with JWH-133 during the pre-symptomatic stage or during the early symptomatic stage. Data are expressed as the mean values ± SEM ($n = 6$ per group). C) Representative images of the Aβ immunoreactivity in cortical coronal sections of AβPP/PS1 mice treated during the pre-symptomatic stage (upper panels) and during the early symptomatic stage (lower panel). Scale bar represents 100 μm.

Double-immunofluorescence revealed that CB_2 receptor stimulation did not produce any significant effect on the number of astrocytes around Aβ plaques (Fig. 3A to C). However, the number of microglial cells associated with Aβ plaques was significantly reduced by JWH-133 ($p < 0.001$) in comparison to vehicle-treated AβPP/PS1 animals (Fig. 3D to F). Reduction in microglial response was associated with a decrease in the expression levels of several pro-inflammatory cytokines. Thus, a reduction in the pro-inflammatory cytokine IFNγ was revealed by densitometric quantification in astrocytes surrounding Aβ plaques in JWH-133-treated AβPP/PS1 animals (Fig. 3G to K, $p < 0.001$). Moreover, the ELISA results demonstrated that JWH-133 was also able to mitigate ($p < 0.05$ when compared to vehicle in all the cases) the increase in the pro-inflammatory cytokine IL-1β ($p < 0.001$), IL-6 ($p < 0.001$), and TNFα ($p < 0.05$) levels exhibited by AβPP/PS1 with respect to control wild-type littermates (Fig. 3L to N). For statistical details, see Supplementary Table 1. Similar JWH-133-induced reduction in the levels of the anti-inflammatory cytokine IL-10 ($p < 0.05$) was also observed with respect to vehicle-treated AβPP/PS1

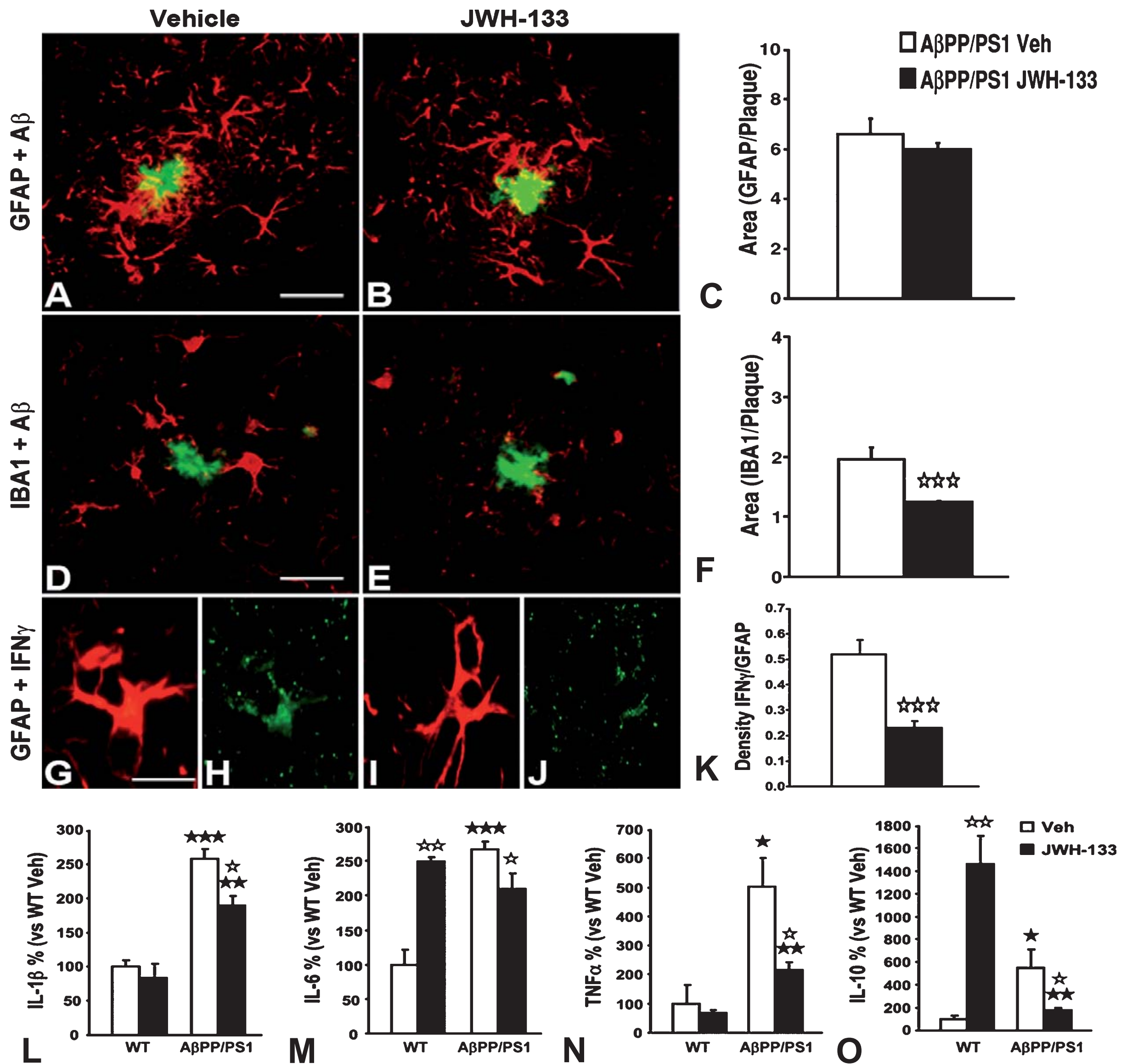

Fig. 3. JWH-133 reduces microglial and pro-inflammatory responses in AβPP/PS1 mice during the early symptomatic stage. A–C) Double-immunofluorescence staining of GFAP (red) and Aβ (green) reveals no effect of JWH-133 on astroglial reactivity around plaques. D–F) In contrast, Iba1 staining (red) reveals a reduction of microglia surrounding Aβ plaques (green) after treatment. Scale bar represent 100 μm. The evaluation of several cytokines confirms the anti-inflammatory effect of the CB_2 agonist in AβPP/PS1. Densitometric quantification of the levels of IFNγ (green) expressed by astrocytes (red) revealed a reduction in JWH-133-treated AβPP/PS1 mice (G–K) ($n = 5$ per group). Scale bar represents 50 μm. Similarly, the CB_2 agonist was able to mitigate the increase of pro-inflammatory cytokines IL-1β (L), IL-6 (M), and TNFα (N), and to decrease the levels of the anti-inflammatory cytokine IL-10 (O) in the cortex of AβPP/PS1, as measured by ELISA ($n = 6$ per group). However, JWH-133 increases the levels of IL-6 and IL-10 in wild mice. Data are expressed as the mean values ± SEM. ★$p < 0.05$, ★★$p < 0.01$ ★★★$p < 0.001$ genotype effect; ☆$p < 0.05$, ☆☆$p < 0.01$, ☆☆☆$p < 0.001$ treatment effect.

animals (Fig. 3O), which exhibited increased IL-10 levels when compared to vehicle wild-type animals ($p < 0.05$).

Interestingly, chronic administration of JWH-133 induced an increase in the levels of IL-6 ($p < 0.01$) and IL-10 ($p < 0.001$) (Fig. 3), and increased the number of microglial cells observed in the cortex of wild-type animals (See Supplementary Figure 2). Increased numbers of microglial cells were not associated with higher levels of proliferating (PCNA positive) Iba1-immunoreactive cells (Supplementary Figure 3).

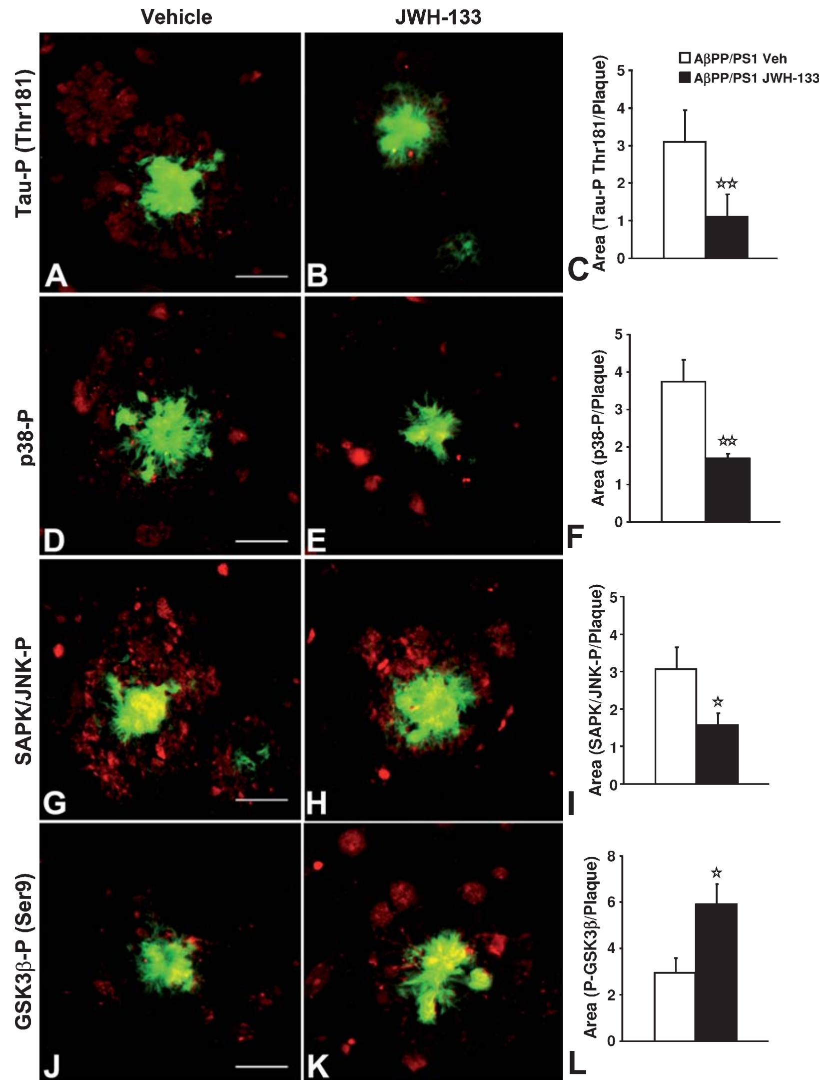

Fig. 4. Double-immunofluorescence staining of Aβ (green) and phospho-tau (Thr181) and related kinases (red). A–C) JWH-133 reduces the amount of tau phosphorylated at the Thr181 in the area surrounding Aβ plaques in AβPP/PS1 mice treated during the early symptomatic stage. D–F) A reduction in the activity of p38 kinase (p38-P) is observed after chronic treatment with the CB_2 agonist during the early symptomatic stage. G–I) A similar decrease in the activity of the SAPK/JNK (SAPK/JNK-P) is observed in the vicinity of Aβ plaques in JWH-133-treated animals. J–L) The activity of GSK3β is also reduced around Aβ plaques in treated animals, as indicated by the increased immunoreactivity of the inactivated form of GSK3β phosphorylated at Ser9. Scale bars represent 75 µm. Data are expressed as the mean values ± SEM ($n = 5$ per group). ☆$p < 0.05$, ☆☆$p < 0.01$ compared to vehicle-treated animals.

Decreased tau phosphorylation and selected tau kinase activity in the vicinity of Aβ plaques by JWH-133 in AβPP/PS1 mice

Even although our animal model of AD presents with only small amounts of phospho-tau in dystrophic neurites, which do not produce neurofibrillary tangles at any age in AβPP/PS1 mice and are never on a par with those seen in AD brains [26], we decided to evaluate the levels of phospho-tau because they are known to have a detrimental effect on soluble Aβ and to contribute to the neurodegenerative process [29].

Double-immunofluorescence and quantitative densitometry showed significant reduction ($p < 0.01$) of tau phosphorylated at the Thr181 site in neuritic processes surrounding Aβ plaques in JWH-133-treated AβPP/PS1 mice (Fig. 4A to C). This was associated with the decreased activity of three well-known tau kinases, as revealed by the local reduced expression of active stress-induced kinases p38 (phosphorylated

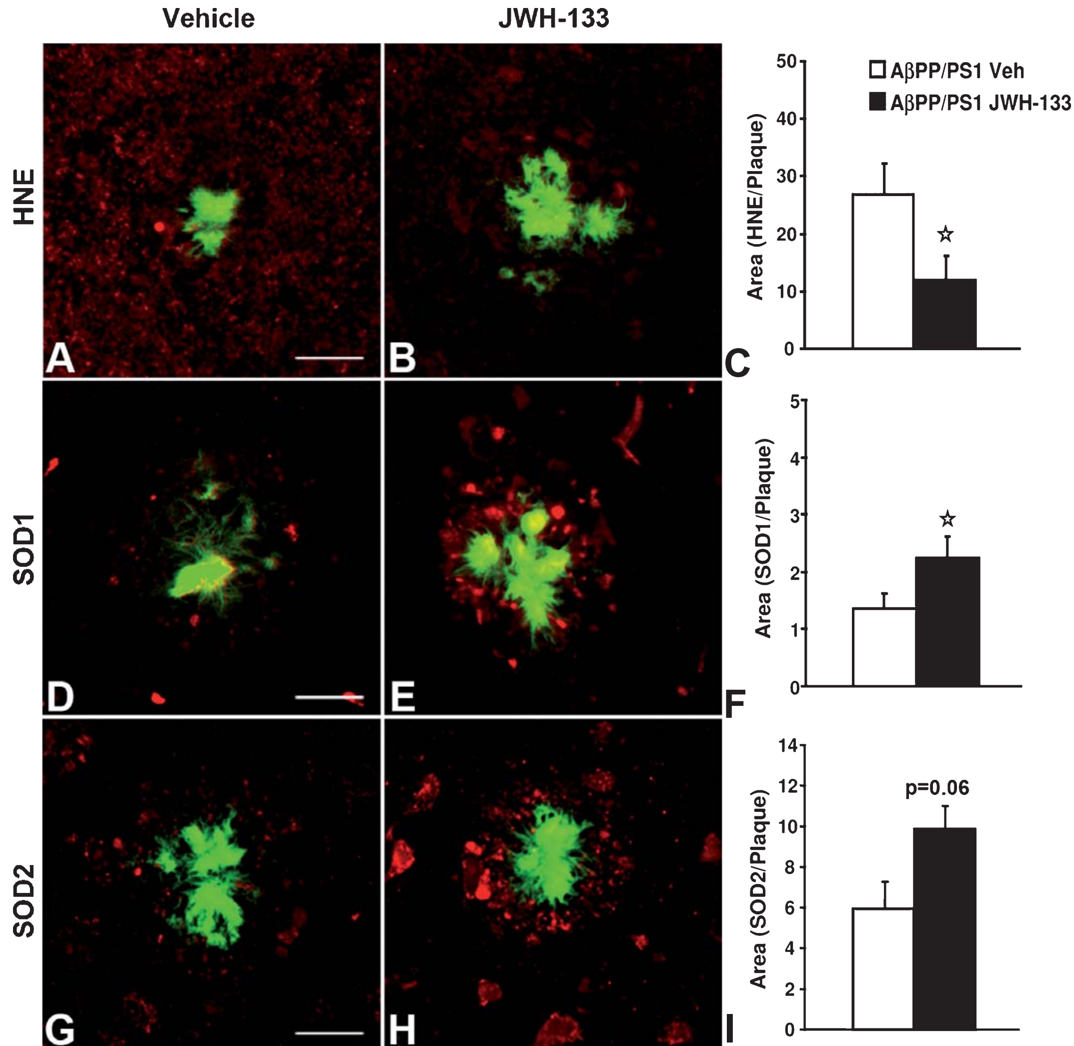

Fig. 5. A–C) Decreased staining of HNE (red) in the area surrounding Aβ plaques (green) in AβPP/PS1 mice treated with JWH-133 during the early symptomatic stage. This is accompanied by an increase in the expression of SOD1 (D–F) and SOD2 (G–I) (red) around Aβ plaques (green) (D–I). Scale bars represent 75 μm. Data are expressed as the mean values ± SEM ($n = 5$ per group). ☆$p < 0.05$ compared to vehicle-treated animals.

at Thr180/Tr182) (Fig. 4D to F, $p < 0.01$) and SAPK/JNK (phosphorylated at Thr183/Tyr185) (Fig. 4G to I, $p < 0.05$), and the increased expression of the inactive form of GSK3β (GSK3β phosphorylated at Ser9) (Fig. 4J to L, $p < 0.05$) in JWH-133-treated AβPP/PS1 mice when compared to transgenic animals treated with vehicle.

Reduction of oxidative stress damage by JWH-133 in AβPP/PS1 mice

Considering the reduction induced by JWH-133 in the activity of the SAPK/JNK and p38 kinases and the well-documented induction of both enzymes by the oxidative stress [30, 31], we evaluated if the CB_2 agonist could also reduce the consequences of oxidative stress occurring in AβPP/PS1 mice. With this purpose, we quantified by densitometry the HNE adducts immunostaining in cortical areas surrounding the Aβ deposition in animals. HNE immunostaining was significantly reduced ($p < 0.05$) in the vicinity of the Aβ plaque area in AβPP/PS1 animals chronically treated with JWH-133 (Fig. 5A to C). This was accompanied by increased SOD1 (Fig. 5D to F, $p < 0.05$) and SOD2 (Fig. 5G to I, $p = 0.06$) immunoreactivity around Aβ plaques.

DISCUSSION

The present observations show that the administration of the specific CB_2 agonist JWH-133 prevented cognitive impairment in AβPP/PS1 transgenic mice when the administration was started at pre-symptomatic stages (3 months), and reversed the long-term recognition memory decline but did not improve the aversive avoidance learning capacity when the treatment was started at symptomatic stages (6 months). This positive cognitive effect was associated with reduced inflammation manifested as decreased microglial reaction and reduced expression of cytokines IL-1β, IL-6, TNFα, and IFNγ. These observations are in agreement with those already reported in Tg2576 mice bearing the AβPP Swedish mutation and in animals inoculated with Aβ [18–21, 32, 33], and can be related with the direct role of CB_2 receptors in inhibiting the microglia-mediated neurotoxicity [18]. The described upregulation of CB_2 receptors in AD brains [15], which is also apparent in AβPP/PS1 mice, is suggested to account for an attempt to reduce the ongoing neurodegenerative process.

However, JWH-133 was not able to reduce Aβ plaques and soluble Aβ in the cortex of AβPP/PS1 mice. We also confirmed that the CB_2 agonist failed to modify the Aβ burden in the hippocampus. In spite of the low affectation exhibited by AβPP/PS1 mice in the hippocampus compared to cortex [26], this brain area was also evaluated for the Aβ burden because of its relevant role in learning and memory. The lack of JWH-133 effect on Aβ processing in our model of AD is in contrast with two previous studies showing that activation of CB_2 receptors stimulated Aβ removal *in vitro* by human macrophages [22] and in Tg2576 mice by favoring Aβ transport through the choroid plexus [20]. However, we should take into consideration that the experimental conditions were different, as the demonstration of Aβ removal *in vitro* was carried out using frozen human tissue and cultured macrophages exposed to a determinate amount of Aβ fibrils [22] and the *in vivo* experiments were performed in a different animal model exposed to a more prolonged treatment than the described in the present study.

The JWH-133 treatment did not affect the synaptophysin density in cortex or hippocampus and the content of VDAC-positive polymorphous inclusions in dystrophic neurites of Aβ plaques. Thus, we have no morphological evidence about a neuroprotective effect of JWH-133 in AβPP/PS1 mice. However, we consider that the cognitive improvement is evidence by itself of the preserved neuronal functionality in JWH-133-treated animals.

In addition to the effects on inflammatory responses, the present results show for the first time other beneficial effects induced by the chronic stimulation of CB_2 receptors. Thus, AβPP/PS1 mice treated with JWH-133 exhibited reduced levels of tau hyperphosphorylation at Thr181 in dystrophic neurites surrounding Aβ plaques. Although the levels of phospho-tau are scarce when compared to AD brains and do not produce neurofibrillary tangles at any age in AβPP/PS1 mice, they are a characteristic feature of our animal model of AD and are supposed to be a detrimental effect of soluble Aβ forms [26, 29]. Then, the JWH-133-induced reduction of tau hyperphosphorylation at Thr181 reveals a protection against deleterious effects of Aβ and a preservation of tau functionality by CB_2 stimulation. Previous studies reported the ability of non-selective cannabinoids to modulate tau phosphorylation but failed to demonstrate a specific role for CB_2 receptors [23, 24]. Reduced tau hyperphosphorylation may be explained by decreased activity of GSK3β, p38,and SAPK/JNK kinases in the vicinity of Aβ plaques [34], as observed following chronic JWH-133 administration in AβPP/PS1 mice. To our knowledge, this is the first report indicating a specific role of CB_2 receptors in the modulation of GSK3β. A previous study revealed the normalization of the phospho-GSK3β levels in other AD model by mixed CB_1/CB_2 agonist prolonged administration, but failed to demonstrate a positive effect by the specific CB_2 agonist JWH-133 [20]. This apparent discrepancy with our results could be due to the different experimental approach, since the previous study assessed the levels of the inactive form of GSK3β in cortical homogenates, rather than in the specific brain areas directly affected by Aβ deposition. Increased GSK3β activity has been related to the memory impairment and the inflammatory responses mediated by microglia in AD [35]. Thus, the JWH-133-induced reduction in the GSK3β activity could account for the reduction of the AD-like phenotype in AβPP/PS1 mice beyond the effect on tau phosphorylation. Regarding the other two tau kinases evaluated, a previous study performed on human coronary smooth muscle cell culture revealed that CB_2 agonists were able to attenuate the activation of p38 and SAPK/JNK induced by the pro-inflammatory cytokine TNFα [36]. Therefore, it seems reasonable to assume that the reduction in the levels of TNFα in our JWH-133-treated AβPP/PS1 mice may provide an explanation, at least in part, for the decreased activity of p38 and SAPK/JNK in our paradigm. However, other

mechanisms may participate in the activation of these two kinases, such as Aβ-mediated oxidative stress [30, 31, 37]. In this line, reduced HNE immunoreactivity and enhanced SOD1 and SOD2 immunoreactivity in the vicinity of plaques in JWH-133-treated compared with vehicle-treated AβPP/PS1 mice, indicates that CB_2 receptors have the capacity to modulate oxidative stress responses and oxidative stress damage. The present observations, although novel in AD models, are in accordance with previous studies suggesting a role for CB_2 receptors as antioxidant mediators in other neurodegenerative settings [38–40].

Collateral observations also deserve a comment. Surprisingly, we observed that administration of JWH-133 to wild mice induced an increase in the number of microglial cells and enhanced expression of IL-6 and IL-10. The reasons for this increase are not clear, as no increase in the proliferation of microglia can be proven. However, we cannot rule the possibility that CB_2 receptor activation enhances microglial migration as shown in other paradigms [41]. Nevertheless, the clinical implications appear to be limited, as the behavior of these animals did not manifest any apparent deleterious effect.

In summary, our present results reveal that the CB_2 cannabinoid agonist JWH-133 is able to ameliorate cognitive symptoms in an experimental model of AD by reducing inflammation, stress oxidative responses to Aβ, GSK3β activity, and tau hyperphosphorylation around Aβ plaques. These findings reinforce the hypothesis that the activation of CB_2 cannabinoid receptor, which is devoid of psychoactive side effects, could be considered a potential target for the development of novel therapeutic strategies against AD.

SUPPLEMENTARY MATERIAL

Supplementary material is available here: http://dx.doi.org/10.3233/JAD-130137

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