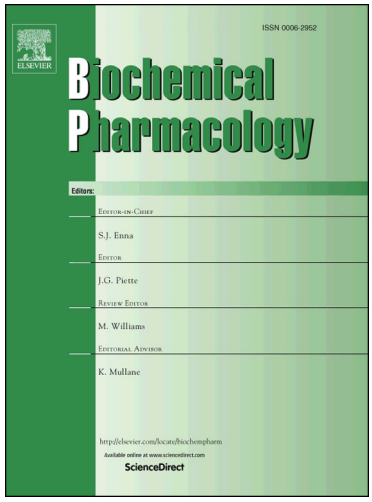

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PII: S0006-2952(18)30327-7
DOI: https://doi.org/10.1016/j.bcp.2018.08.007
Reference: BCP 13240

To appear in: *Biochemical Pharmacology*

Received Date: 30 May 2018
Accepted Date: 6 August 2018

Please cite this article as: E. Aso, P. Andrés-Benito, I. Ferrer, Genetic deletion of CB_1 cannabinoid receptors exacerbates the Alzheimer-like symptoms in a transgenic animal model, *Biochemical Pharmacology* (2018), doi: https://doi.org/10.1016/j.bcp.2018.08.007

Genetic deletion of CB_1 cannabinoid receptors exacerbates the Alzheimer-like symptoms in a transgenic animal model

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Abstract

Activating CB_1 cannabinoid receptor has been demonstrated to produce certain therapeutic effects in animal models of Alzheimer's disease (AD). In this study, we evaluated the specific contribution of CB_1 receptor to the progression of AD-like pathology in double transgenic APP/PS1 mice. A new mouse strain was generated by crossing APP/PS1 transgenic mice with CB_1 knockout mice. Genetic deletion of CB_1 drastically reduced the survival of APP/PS1 mice. In spite that CB_1 mutant mice bearing the APP/PS1 transgene developed normally, they suddenly died within the first two months of life likely due to spontaneous seizures. This increased mortality could be related to an imbalance in the excitatory/inhibitory transmission in the hippocampus as suggested by the reduced density of inhibitory parvalbumin positive neurons observed in APP/PS1 mice lacking CB_1 receptor at 7 weeks of age. We also evaluated the AD-like phenotype of APP/PS1 mice heterozygous for the CB_1 deletion at 3 and 6 months of age. The memory impairment associated to APP/PS1 transgene was accelerated in these mice. Neither the soluble levels of Aβ or the density of Aβ plaques were modified in APP/PS1 mice heterozygous for CB_1 deletion at any age. However, the reduction in CB_1 receptor expression decreased the levels of PSD-95 protein in APP/PS1 mice, suggesting a synaptic dysfunction in these animals that could account for the acceleration of the memory impairment observed. In summary, our results suggest a crucial role for CB_1 receptor in the progression of AD-related pathological events.

Keywords: Alzheimer's disease, cannabinoid receptor 1, APP/PS1 mice, cognitive impairment, amyloid

1. Introduction

The $G_{i/o}$-coupled CB_1 cannabinoid receptors are widely expressed within the central nervous system, in both neurons and glial cells. They are mostly located at the terminals of neurons where they act as modulators of excitatory and inhibitory neurotransmission, regulating important brain functions including cognition (Howlett, 2005). Moreover, CB_1 activity mediates protection against excitotoxicity and the induction of repair mechanisms in response to neuronal damage (Marsicano et al, 2003; Fagan and Campbell, 2014). Based on the neuroprotective properties of CB_1 receptor activation, a number of studies have explored the role of this receptor in several neurodegenerative diseases, including Alzheimer's disease (AD). AD is the most common form of dementia, histologically characterized by the dysfunctional accumulation of amyloid-β peptide (Aβ) and tau hyper-phosphorylation in major brain regions contributing to memory and cognition (Ferrer, 2012; Selkoe, 2012). Alterations in the expression or functionality of CB_1 receptors have been described both in AD patients' brains (Ramírez et al, 2005; Solas et al, 2013; Manuel et al, 2014) and AD animal models (Kalifa et al, 2011; Aso et al, 2012a). Interestingly, a similar reduction in CB_1 levels was observed in both human and animal models brains at advanced stages of the AD, in contrast with the increase in CB_1 expression during the asymptomatic stages (Aso et al, 2012a; Manuel et al, 2014). These findings suggest an attempt of CB_1 receptor to compensate for the initial synaptic impairment occurring at the early asymptomatic stages of AD and reinforce the hypothesis that targeting the CB_1 receptor could offer a strategy against AD. In fact, pharmacological stimulation of CB_1 receptors with natural or synthetic agonists resulted in a reduction of the neurotoxic effect of amyloid-ß peptide (Aß) in some *in vitro* models (Noonan et al, 2010; Chen et al, 2011a; Aso et al, 2012a; Janefjord et al, 2014) and in a reversion of the Aß-induced memory impairment in different *in vivo* models (Aso et al,

2012a; Haghani et al, 2012; Aso et al, 2015). The alleviation of the AD-like symptoms in these models seems not to be directly related to a significant contribution of CB_1 receptors in Aβ production, aggregation or clearance since no modifications in such parameters were reported in two different transgenic AD models after chronic treatment with specific CB_1 agonists (Aso et al, 2012a; Chen et al, 2010). However, a role for CB_1 receptor on APP processing cannot be completely ruled out because APP23 transgenic mice deficient for CB_1 receptor exhibited reduced APP protein levels and Aβ plaque deposition, likely due to changes in intracellular APP transport, although those animals presented enhanced cognitive deficits (Stumm et al, 2013).

Considering these evidence, the aim of the present study was to further explore the specific contribution of CB_1 receptor to the progression of AD in APP/PS1 transgenic mice deficient for CB_1 receptor. We have chosen this animal model of AD because it reproduces some of the most relevant features of the disease, including cognitive impairment and Aβ plaques from the age of six months onwards (Borchelt et al, 1997; Aso et al, 2012b). Aβ deposition is accompanied in APP/PS1 mice by altered mitochondria and increased oxidative damage, post-translational modifications and accumulation of altered proteins at the dystrophic neurites surrounding plaques, likely due to aberrant degradation pathways (Aso et al, 2012b). However, cognitive decline in this AD model is assumed to correlate with the levels of soluble oligomeric species, which are known to play a critical role in the AD pathogenicity (Walsh and Selkoe, 2004). Thus, we have performed a time-course evaluation of the aberrant Aβ production and memory impairment, in CB_1 receptor deficient APP/PS1 transgenic mice.

2. Materials and methods

2.1. Animals

Double transgenic APP(swe)/PS1(1dE9) (APP/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 (Borchelt et al, 1997; Aso et al, 2012b). A new mouse strain was generated by crossing male APP/PS1 mice purchased from Jackson Laboratories (Bar Harbor, Maine, USA) with female CB_1 knockout (-/-) mice (Zimmer et al, 1999) obtained from the European Mutant Mouse Archive (Helmholtz Zentrum, München, Germany), both strains in a C57BL/6 background. The resulting APP/PS1/CB_1 heterozygous (+/-) males were then crossed with wild-type (WT)/CB_1 +/- females to obtain the animals for the present study. The genotype of the pups was analyzed with the polymerase chain reaction (PCR) technique using genomic DNA isolated from tail clips obtained at postnatal day 21 or at embryonic day 13.5. Animals were maintained under standard animal housing conditions in a 12-h dark-light cycle with free access to food and water. The experiments were conducted by experienced researchers blind for the genotype of the animals. 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 committee of the University of Barcelona.

2.2. Behavioral evaluation of cognitive performance: Two-object recognition test

Memory performance was evaluated in WT/CB_1 +/+, WT/CB_1 +/-, WT/CB_1 -/-, APP/PS1/CB_1 +/+ and APP/PS1/CB_1 +/- male littermates aged 3 and 6 months. On day 1, mice were placed for 9 min in a V-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.

2.3. Brain samples collection

At the end of the behavioral testing, animals ageing 3 and 6 months were killed and their brains rapidly removed from the skull and processed for study. A group of mice, including APP/PS1/CB_1 -/-, were sacrificed at 7 weeks of age for brain samples collection. 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.

2.4. 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 (Aβ, 3 min) and then treated with citrate buffer (Aβ, parvalbumin, NeuN, 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 4ºC overnight with the primary antibodies against Aβ (clone 6F/3D 1:50, Dako, Glostrup, Denmark), parvalbumin (PV, 1:1,000, Sigma-Aldrich, St Louis, MO, USA) or the neuronal marker NeuN (1:100, Millipore, Burlington, MA, USA). Sections were subsequently rinsed and incubated with biotinylated secondary antibodies (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, approximately) 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 APP/PS1 mice. Density of PV^+ and $NeuN^+$ cells in hippocampus of each animal brain was calculated by counting the number of immunostained cells from 3 different sections (-1.5 mm, -1.7 mm and -1.9 mm from bregma, approximately) with respect the total hippocampal area in the section (mm^2). Quantifications were performed by a researcher blind for the corresponding genotype of each section. Aβ quantification and hippocampal area were calculated using the Analysis tool of the Adobe® Photoshop® CS4 software (Adobe Systems Inc., San Jose, CA, USA). The number of APP/PS1 mice analyzed for each genotype (CB_1 +/+ and CB_1 +/- or CB_1 -/-) and age (<2, 3 or 6 months) was n = 5-10.

2.5. 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. $Aβ_{40}$ 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. Quantitative determination was carried out according to the manufacturer's instructions. $Aβ_{40}$ and $Aβ_{42}$ levels were normalized to the

total amount of protein from each individual sample (BCA method, Thermo Fisher Scientific, Wilmington, DE, USA). The number of APP/PS1 mice analyzed for each genotype (CB_1 +/+, CB_1 +/-, CB_1 -/-) and age (<2, 3 and 6 months) was n = 5-6.

2.6. Gel electrophoresis and western blotting

Brain samples of mice aging 3 months were homogenized in RIPA lysis buffer (50mM Tris/HCl buffer, pH 7.4 containing 2mM EDTA, 0.2% Nonidet P-40, 1mM PMSF, protease, and phosphatase inhibitor cocktails, Roche Molecular Systems, USA). The homogenates were centrifuged for 15 min at 13,000 rpm. Protein concentration was determined with the BCA method (Thermo Scientific). Equal amounts of protein (20 μg) for each sample were loaded and separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) gels and transferred onto nitrocellulose membranes (Amersham, Freiburg, Germany). Non-specific bindings were blocked by incubation in 3% albumin in PBS containing 0.2% Tween for 1 h at room temperature. After washing, membranes were incubated overnight at 4°C with the antibodies against postsynaptic density protein-95 (PSD-95) (1:500, Invitrogen) and synaptophysin (1:4,000, Novocastra). Protein loading was monitored using an antibody against β-actin (1:10,000, Abcam). Membranes were then incubated for 1 h in the appropriate HRP- conjugated secondary antibody (1:2,000, Dako), and immunocomplexes were revealed by chemiluminescence reagent (ECL, Amersham). Densitometric quantification was carried out with TotalLab v2.01 software (Pharmacia, Sweden). Bands were normalized to β-actin. Six animals per genotype were analyzed.

2.7. Statistical analysis

Statistical analysis was performed with the SPSS® Statistics v21.0 software (IBM, New York, NY, USA). The frequency of observed genotypes was analyzed with Pearson's chi-squared test (χ^2) test. Survival curve was analyzed with Kaplan-Meier test. Memory

performance, PV^+ cells quantification and PSD-95 levels were analyzed with two-way ANOVA with APP/PS1 transgene and CB_1 mutation as between factors, followed by Tukey's *post hoc* when required. Aß quantifications were analyzed with a Student *t*-test. In all the experiments, the significance level was set at $p < 0.05$.

3. Results

3.1 Reduced survival of APP/PS1 deficient for CB_1 receptors

As shown in Fig. 1A, crossing APP/PS1/CB_1 +/- males with WT/CB_1 +/- females resulted in the generation of pups bearing six different genotypes. The Pearson's chi-squared test revealed that observed frequencies of each genotype were significantly different ($\chi^2_{(2)}$ = 26.71, $p < 0.001$) from the expected frequencies according to Mendelian laws (50% WT and 50% APP/PS1, and for each one of these genotypes 25% CB_1 +/+, 50% CB_1 +/- and 25% CB_1 -/-). Specifically, the number of APP/PS1 knockout for CB_1 receptor was drastically reduced at postnatal day 21, when the tail clips were collected for DNA isolation and genotyping. In contrast, the observed frequencies of each genotype were not significantly modified from the expected ones when analyzed in pups at embryonic day 13.5 (78 pups analyzed, data not shown), suggesting that CB_1 deficiency does not affect embryonic implantation or early development (before embryonic day 13.5) in APP/PS1 mice. Most importantly, none of the APP/PS1/CB_1 -/- mice survived more than two months, in spite they exhibited a healthy appearance and no external physical abnormalities. In at least three cases, during the daily handling of the animals, the researchers witnessed the sudden death of the APP/PS1/CB_1 -/- mice at their home cage following spontaneous seizures. Kaplan-Meier test applied to the survival curve calculated up to six months of age (without considering those animals sacrificed at 7 weeks or 3 months of age for brain samples collection) revealed a significant increase in the mortality of CB_1 mutant mice bearing the APP/PS1 transgene compared to WT mice ($\chi^2_{(5)}$ = 257.21, $p < 0.001$; Fig. 1B). It is noticeable that the survival of APP/PS1/CB_1 +/- mice and WT/CB_1 -/- mice was also significantly reduced when compared to control animals at six months of age.

3.2. Characterization of APP/PS1/CB_1 -/- brain samples

Because of the serendipitous observation of the sudden death of a few APP/PS1/CB_1 -/- mice after spontaneous seizures, we hypothesized that the increased mortality of these mutant mice could be related to an imbalance in the excitatory/inhibitory neurotransmission in brain areas such as hippocampus, which is closely related to the occurrence of seizures. We observed that the genetic deletion of CB_1 receptors reduced the density of PV^+ inhibitory neurons in the hippocampus of APP/PS1 mice (Fig. 2A-C), as revealed two-way ANOVA (interaction between APP/PS1 and CB_1 genotypes, $F_{(1, 13)} = 5.014$, $p < 0.05$; no significant effect of APP/PS1 or CB_1 genotypes) and subsequent *post hoc* test ($p < 0.05$ respect to WT/CB_1 -/-). The reduction in the density of neurons was specific for inhibitory PV^+ cells since no differences were observed in the total number of neurons in the hippocampus, as revealed the NeuN immunostaining quantification (data not shown). No significant modifications in PV^+ cells density were observed in APP/PS1/CB_1 +/- mice at 3 or 6 months, in spite of a tendency to decrease at 6 months when compared to APP/PS1/CB_1 +/+ animals (data not shown). Importantly, the decreased density of inhibitory neurons was not related with an increment of Aβ peptide production in APP/PS1/CB_1 -/-. Thus, the levels of soluble Aβ peptide were not modified in mutant mice (Fig. 2D) and no Aβ plaques were observed in APP/PS1/CB_1 -/- mice at 7 weeks of age (data not shown).

3.3. CB_1 reduced expression accelerates the memory impairment associated to APP/PS1 transgene

In order to evaluate a potential contribution of CB_1 receptor deficiency to the progression of the AD-related cognitive impairment in APP/PS1 mice, and considering the impossibility to test CB_1 -/- mice due to their premature mortality, memory performance was assessed at 3 (pre-symptomatic phase in APP/PS1 mice) and 6 (early symptomatic phase) months of age in APP/PS1/CB_1 +/- mice and corresponding controls. As expected,

at 6 months of age APP/PS1 mice exhibited an evident memory impairment (Fig. 3B). Thus, two-way ANOVA revealed a significant effect of the APP/PS1 transgene ($F_{(1, 37)}$ = 35.772, $p < 0.001$), CB_1 deficiency ($F_{(2, 37)}$ = 4.571, $p < 0.05$) but no interaction between the two genotypes. Subsequent Tukey's *post hoc* test revealed memory impairment in APP/PS1/CB_1 +/+ ($p < 0.001$ respect to WT/CB_1 +/+), APP/PS1/CB_1 +/- ($p < 0.001$ respect to WT/CB_1 +/-) and WT/CB_1 -/- ($p < 0.05$ respect to WT/CB_1 +/+ and $p < 0.01$ respect to WT/CB_1 +/-). However, in spite that APP/PS1 mice at 3 months do not exhibit memory impairment normally, a significant memory deficit was observed in APP/PS1/CB_1 +/-, indicating an acceleration of the cognitive impairment when CB_1 activity is reduced (Fig. 3A). Specifically, two-way ANOVA revealed a significant effect of the APP/PS1 transgene ($F_{(1, 46)}$ = 5.672, $p < 0.05$), but no CB_1 genotype effect or interaction between the two factors at 3 months of age. Subsequent Tukey's *post hoc* test revealed a reduction in the recognition index in APP/PS1/CB_1 +/- when compared to APP/PS1/CB_1 +/+ ($p < 0.05$) and to WT/CB_1 +/- ($p < 0.05$).

3.4. Reduction of CB_1 expression does not alter Aβ processing but modifies synaptic composition in APP/PS1 mice

We evaluated next whether the acceleration in memory impairment could be related to a modification in the Aβ processing, since Aβ peptide is known to induce cognitive disturbances in APP/PS1 mice. However, no significant difference was observed between the cortical Aβ burden (Fig. 4A-B) or soluble Aβ contents neither at 3 (data not shown) or 6 months of age in APP/PS1/CB_1 +/- mice (Fig. 4C). These findings suggest that memory impairment in APP/PS1/CB_1 +/- could be related to enhanced negative consequences of Aβ production rather than to an increase in Aβ levels. We next evaluated the levels of postsynaptic density protein-95 (PSD-95) and the presynaptic protein synaptophysin, as molecular markers of synaptic density and integrity. As shown in Fig.

4D, APP/PS1/CB_1 +/- exhibited at 3 months of age a significant reduction in the PSD-95 contents when compared to WT/CB_1 +/- ($p < 0.05$). No modifications in the levels of synaptophysin were observed (Fig. 4E).

4. Discussion

A number of previous reports suggested that targeting the CB_1 receptor during the early stages could offer a strategy against AD, based on the neuroprotective effects against Aβ toxicity observed in certain experimental models after treatment with CB_1 receptor agonists (Noonan et al, 2010; Chen et al, 2011; Aso et al, 2012; Haghani et al, 2012; Janefjord et al, 2014; Aso et al, 2015). The present study aimed to further explore the specific contribution of CB_1 receptors to the progression of the cognitive impairment and the aberrant Aβ production typically occurring in AD by using APP/PS1 transgenic mice as an animal model of the disease.

We generated a new mouse strain by crossing APP/PS1 transgenic mice with CB_1 knockout mice. Intriguingly, the observed frequency of APP/PS1 mice lacking the CB_1 receptor at postnatal day 21 was significantly reduced respect the expected frequency according to Mendelian laws, in spite that CB_1 deficiency does not affect embryonic implantation or early development of transgenic animals, as revealed the genotyping of mice at embryonic day 13.5. These results indicate that a significant number of APP/PS1/CB_1 -/- mice died during late intrauterine life or shortly after birth. Most importantly, none of the APP/PS1 mice mutant for CB_1 receptor survived more than two months, revealing a major role for CB_1 receptor in the viability of these transgenic mice. The mechanisms underlying the increased mortality of APP/PS1 mice lacking CB_1 receptor remain to be fully elucidated. However, based on the serendipitous observation of the premature death of a few APP/PS1/CB_1 -/- mice after spontaneous seizures, we hypothesized that deficiency in CB_1 receptor could lead to an imbalance in the excitatory/inhibitory neurotransmission leading to enhanced excitability in response to Aβ peptide production that might ultimately cause lethal seizures. Our hypothesis is based on the facts that (i) patients and AD models exhibit increased seizure susceptibility

associated to Aβ peptide overproduction (Palop et al, 2007; Irizarry et al, 2012), which is increased with the progression of the disease (Amatniek et al, 2006; Cloyd et al, 2006) and (ii) the key role played by CB_1 receptor in the control of neuronal excitability and epileptiform seizures (Lutz, 2004; Monory et al, 2006). In agreement with this hypothesis, we observed in APP/PS1/CB_1 -/- mice at 7 weeks of age a decrease in the density of GABAergic inhibitory PV^+ cells in the hippocampus. GABAergic function is enhanced in this brain region in certain AD models and likely serves to counteract Aβ-induced neuronal overexcitation (Palop et al, 2007). Thus, the deficiency in the inhibitory mechanisms suggested by the decrease in PV^+ cells density in APP/PS1/CB_1 -/- mice, together with the already known role of CB_1 receptors on hippocampal glutamatergic neurons on providing on-demand endocannabinoid-dependent protection against excitotoxic seizures (Monory et al, 2006), could account for an aberrant hyperexcitability and the consequent incidence of life-threatening seizures in these APP/PS1 mice lacking CB_1 receptors. The specific loss of PV^+ cells in APP/PS1/CB_1 -/- mice could be related with the increased susceptibility to Aβ toxicity of GABAergic interneurons already reported both in patients and APP/PS1 mice (Davies et al 1980, Ramos et al 2006, Takahashi et al 2010, Verret et al 2012). Neither the soluble levels of $A\beta_{40}$ or $A\beta_{42}$, the most toxic form of this peptide, were altered in APP/PS1/CB_1 -/- mice, suggesting that CB_1 deficiency impaired the mechanisms of defense against Aβ detrimental effects rather than the production of this peptide.

Considering the impossibility to characterize the progressive cognitive impairment of APP/PS1/CB_1 -/- mice due to their premature death, we evaluated memory performance of APP/PS1 mice expressing reduced levels of CB_1 receptor (APP/PS1/CB_1 +/-) at 3 and 6 months of age, corresponding respectively to the pre-symptomatic and to the early symptomatic phase in APP/PS1 mice. Noticeably, the reduced CB_1 receptor expression

resulted in the acceleration of the memory impairment usually evidenced by APP/PS1 from 6 months of age, since APP/PS1/CB_1 +/- exhibited a significant reduction in the recognition index in the two-object recognition test at 3 months of age. This finding agrees with previous reports demonstrating that APP23 transgenic mice deficient for CB_1 receptor presented enhanced cognitive deficits (Stumm et al, 2013). WT/CB_1 -/- also exhibited a memory deficit at 6 months of age, in accordance with previous reports demonstrating an accelerated age-related decline in cognitive functions in the absence of CB_1 receptors (Bilkei-Gorzo et al, 2005). Neither the levels of soluble Aβ peptide or Aβ deposition were affected by the reduced CB_1 receptor expression in APP/PS1/CB_1 +/- mice, supporting our assumption about a major role for CB_1 receptor in the defense against Aβ detrimental effects rather than in the production of this peptide. However, Stumm et al (2013) revealed that APP23 transgenic mice knockout for CB_1 receptor exhibited reduced APP protein levels and Aβ plaque deposition and a recent report demonstrated that APP is able to alter membrane localization and inhibitory signalling activity of CB_1 receptor in the hippocampus of Tg2576 mice (Maccarrone et al, 2018). Thus, a direct or indirect participation of CB_1 receptor in Aβ processing cannot be completely ruled out.

Aβ peptide is known to induce synaptic dysfunction during early stages of AD by deregulating molecular organization of the postsynaptic density (Liu et al, 2010; Oyelami et al, 2016). Because of this, we evaluated the levels of PSD-95, one of the major scaffolding protein in the excitatory postsynaptic density and a potent regulator of synaptic strength (Chen et al, 2011b), and the levels of the presynaptic component synaptophysin, which are known to be reduced in APP/PS1 mice at advanced stages of the neurodegenerative process (Woo et al, 2015; Mitew et al, 2013). Interestingly, APP/PS1/CB_1 +/- exhibited at 3 months of age, when APP/PS1 mice still present normal

levels, a significant reduction in the PSD-95 contents but no modifications of the synaptophysin levels. PSD-95 protein has been previously demonstrated to play a key role in the retrograde synaptic signaling elicited by CB_1 receptor to regulate the activity-dependent inhibition of synaptic strength in the brain (Chen et al, 2011c). Thus, PSD-95 reduction could lead to an alteration of the synaptic dynamics that could ultimately account for the accelerated cognitive impairment observed in APP/PS1/CB_1 +/- animals. In summary, the present study reveals that CB_1 receptor blockade reduces the life expectancy of APP/PS1 mice and accelerates their cognitive impairment, suggesting a relevant role of this receptor in the progression of AD.

Acknowledgements

We thank Vanessa Reig-Sorribes for teaching in embryonic dissection and evaluation. The authors' work is supported by CIBERNED (Institute of Health Carlos III, Spanish Ministry of Economy and Competitiveness).

Statement of Interest

None of the authors declare any conflict of interest.

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Figure 1: A. Distribution of the six possible genotypes in the offspring from mating of APP/PS1/CB_1 heterozygous males (+/-) and WT/CB_1 +/- females at postnatal day 21. The observed genotype frequencies were significantly different from the expected frequencies according to the Mendelian laws, mainly due to the drastic reduction in the number of APP/PS1/CB_1 -/- mice. N: total number of mice studied. **B.** Survival curve calculated up to six months of age revealed none of the APP/PS1/CB_1 -/- mice survived more than two months from birth. The viability of APP/PS1/CB_1 +/- mice and WT/CB_1 -/- mice was also significantly reduced when compared to control animals at six months of age.

Figure 2: Reduction of the density of PV^+ inhibitory neurons in the hippocampus of APP/PS1/CB_1 -/- at 7 weeks of age. **A.** Low magnification images showing representative hippocampal sections stained with a specific antibody against PV. Scale bar represents 500 μm. **B.** Higher magnification of brain areas indicated by black squares. Scale bar represents 100 μm. **C.** Quantification of PV^+ density in the hippocampus based on 3 pictures from each animal and 5-6 animals per group. **D.** No significant effect of genotype was observed in the levels of cortical soluble $A\beta_{40}$ or $A\beta_{42}$ peptide at 7 weeks of age (n = 5-6 per genotype). Data are expressed as the mean values ± SEM. * $p < 0.05$ compared to WT/CB_1 -/-.

Figure 3: CB_1 reduced expression accelerates memory impairment in APP/PS1 mice. **A.** Memory performance of animals aged 3 months (pre-symptomatic phase in APP/PS1 mice) in the two-object recognition test was significantly reduced in APP/PS1/CB_1 +/- when compared to APP/PS1/CB_1 +/+ or WT/CB_1 +/- mice. **B.** At 6 months of age (early symptomatic phase), all the APP/PS1 animals, independently of CB_1 genotype, exhibited memory impairment in the two-object recognition test. Moreover, WT/CB_1 -/- also showed a decrease in the recognition index. Data are expressed as the mean values ± SEM (n = 9-17 animals per genotype). * $p < 0.05$, *** $p < 0.001$ compared to WT mice bearing

the same genotype for CB_1 receptor. # $p < 0.05$ compared to APP/PS1/CB_1 +/+. $ $p < 0.05$ compared to WT/CB_1 +/+. $ p 0.01 compared to WT/CB_1 +/-. N.A.: Not Applicable.

Figure 4: A. Representative images of Aβ specific immunostaining in cortical sections of APP/PS1/CB_1 +/+ (left) and APP/PS1/CB_1 +/- (right) mice at 3 (upper) and 6 (bottom) months of age. Arrow heads indicate Aβ plaques. Scale bar represents 200 μm. **B.** Aβ burden quantification in cortical sections reveals no alteration in the Aβ deposition in APP/PS1/CB_1 +/- mice (black bars) compared to APP/PS1/CB_1 +/+ littermates (open bars) at 3 or 6 months of age (n = 5-10 animals per genotype). **C.** Neither the soluble $Aβ_{40}$ or $Aβ_{42}$ levels of APP/PS1/CB_1 +/- mice (black bars) were significantly modified respect APP/PS1/CB_1 +/+ littermates (open bars) in cortical homogenates from animals ageing 6 months (n = 5-6 animals per genotype). **D.** *Upper*: Representative immunoblots for PSD-95 and corresponding β-actin loading control. *Bottom*: Quantification of the PSD-95 levels in brain homogenates of animals ageing 3 months revealed a significant reduction in APP/PS1/CB_1 +/- when compared to WT/CB_1 +/- mice (n = 6 animals per genotype). **E.** *Upper*: Representative immunoblots for synaptophysin and corresponding β-actin loading control. *Bottom*: Quantification of the synaptophysin levels in brain homogenates of animals ageing 3 months revealed no significant alterations due to genotype (n = 6 animals per group). Data are expressed as mean values ± SEM. * $p < 0.05$ compared to WT/CB_1 +/-.

Figure 1

A

N = 507	WT			APP/PS1		
	+/+	+/-	-/-	+/+	+/-	-/-
Observed	63	146	72	62	132	29
Expected	70,3	140,5	70,3	70,3	140,5	70,3

B

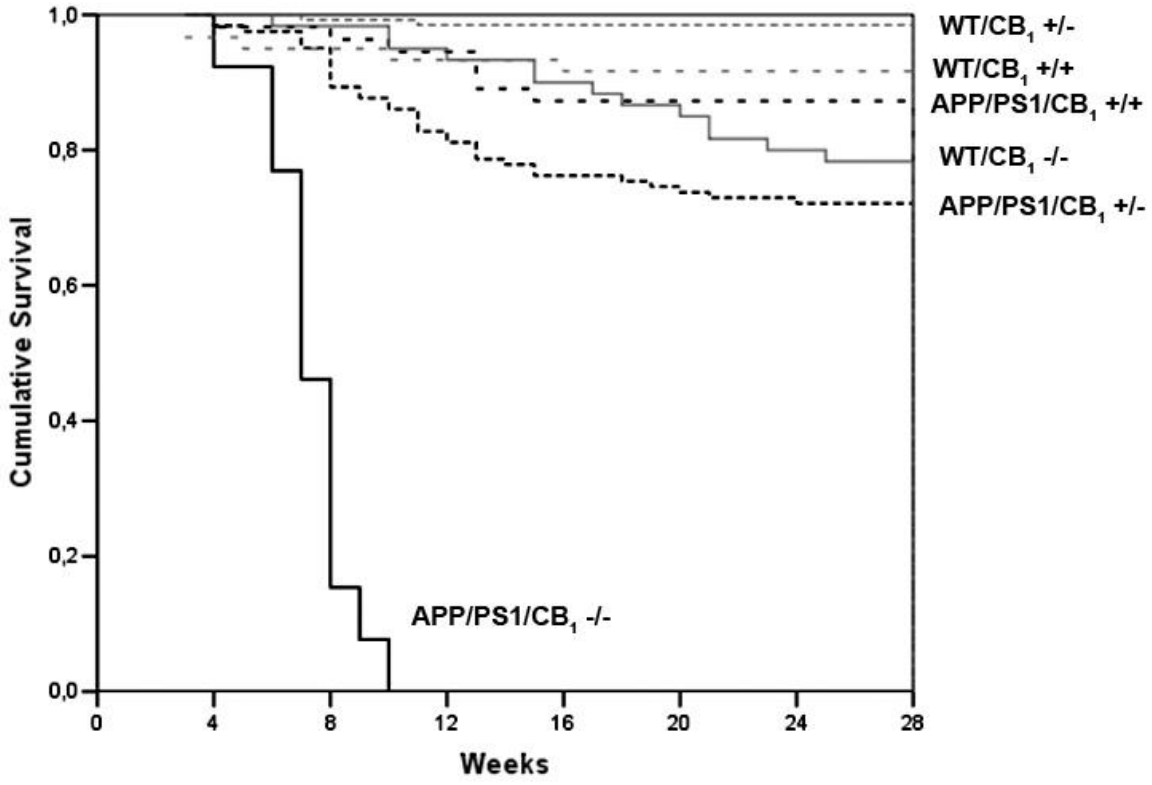

Figure 2

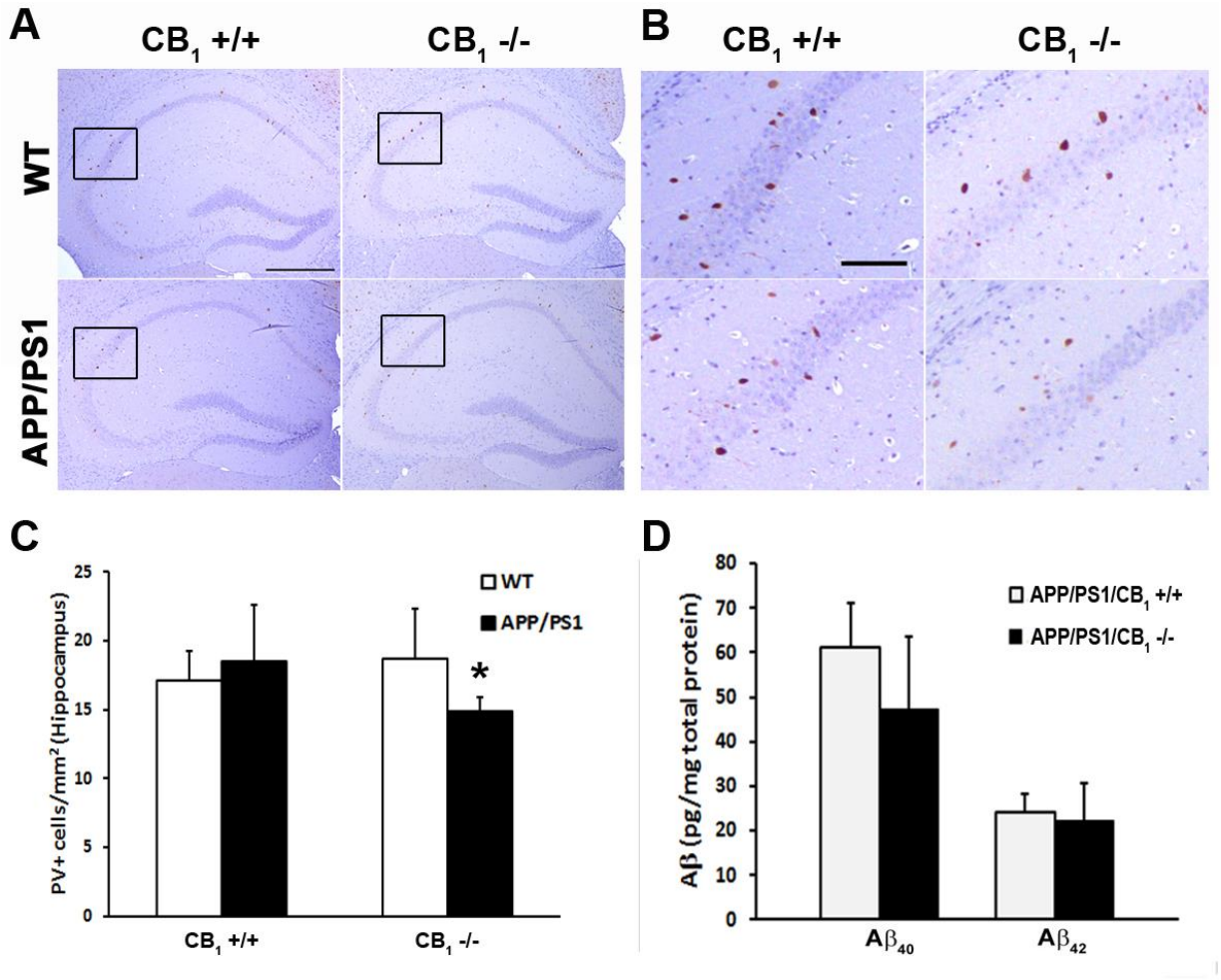

Figure 3

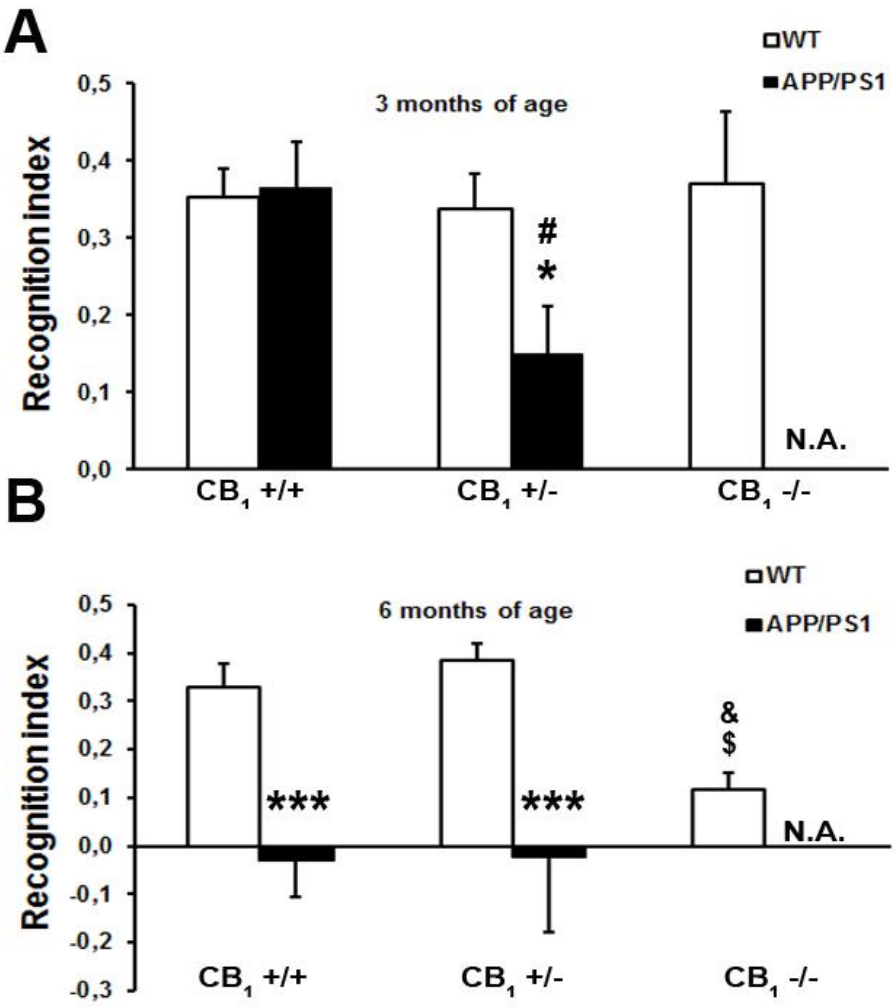

Figure 4