Delineating the Efficacy of a Cannabis-Based Medicine at Advanced Stages of Dementia in a Murine Model

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Abstract. Previous reports have demonstrated that the combination of Δ\textsuperscript{9}-tetrahydrocannabinol (Δ\textsuperscript{9}-THC) and cannabidiol (CBD) botanical extracts, which are the components of an already approved cannabis-based medicine, reduce the Alzheimer-like phenotype of A\textsuperscript{β}PP/PS1 transgenic mice when chronically administered during the early symptomatic stage. Here, we provide evidence that such natural cannabinoids are still effective in reducing memory impairment in A\textsuperscript{β}PP/PS1 mice at advanced stages of the disease but are not effective in modifying the A\textsuperscript{β} processing or in reducing the glial reactivity associated with aberrant A\textsuperscript{β} deposition as occurs when administered at early stages of the disease. The present study also demonstrates that natural cannabinoids do not affect cognitive impairment associated with healthy aging in wild-type mice. The positive effects induced by Δ\textsuperscript{9}-THC and CBD in aged A\textsuperscript{β}PP/PS1 mice are associated with reduced GluR2/3 and increased levels of GABA-A R\textsubscript{α}1 in cannabinoid-treated animals when compared with animals treated with vehicle alone.

Keywords: Advanced stages, Alzheimer’s disease, cannabidiol, Δ\textsuperscript{9}-tetrahydrocannabinol, dementia

INTRODUCTION

Dementia is a general term referring to a progressive decline in cognitive abilities occurring usually in the elderly due to a variety of causes. Among the symptoms, memory loss, language impairment, attention deficiencies, and difficulty in reasoning and judgment are the most prominent. Etiologies of dementia may be divided into two broad categories: (i) degenerative, reflecting pathological processes which are intrinsic to the central nervous system and usually involve aberrant protein processing, and (ii) non-degenerative, which include acquired or secondary dementias linked to vascular, endocrine, traumatic, and other primary central and systemic diseases. Alzheimer’s disease (AD) is the most common type of dementia, accounting for 60 to 80\% of cases, and is characterized by the presence in the brain of extracellular deposits of amyloid-β (A\textsuperscript{β}), a peptide derived from the aberrant processing of the transmembrane amyloid-β protein precursor (A\textsuperscript{β}PP),
and intracellular neurofibrillary tangles composed of hyperphosphorylated microtubule-associated protein tau. AD is also associated with neuroinflammation and oxidative stress, two pathological processes that exacerbate neurodegeneration during AD progression [1, 2].

Recent studies have demonstrated the therapeutic effects of several compounds acting on the endocannabinoid system in neurodegenerative diseases such as AD [3–6]. The endocannabinoid system is a complex network of cellular receptors and signaling molecules [7] highly expressed in brain and targeted by cannabis derivatives which, when activated, provides neuroprotection by reducing neuronal damage, neuroinflammation, and oxidative stress, as well as promoting intrinsic repair mechanisms [3]. Thus, chronic stimulation with selective synthetic agonists of CB1 and CB2 receptors, the most well-known cannabinoid receptors, reduces cognitive impairment and brain alterations associated with Aβ production, in at least three different animal models of AD [8–11]. Moreover, the combination of Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD), two phytocannabinoids produced by the plant Cannabis sativa, reduces the pathological phenotype in mouse models of AD and tauopathy when administered at early stages of the disease [12, 13]. These natural compounds are the two main components of Sativex®, which is a well-tolerated medicine prescribed for the treatment of spasticity associated with multiple sclerosis.

The aim of the present study was to broaden our knowledge about the potential beneficial effect of the Δ9-THC and CBD combination in reducing dementia symptoms at advanced stages of the disease. The present study was designed to evaluate the effect of this cannabis-based medicine administered in old AβPP/PS1 mice and in aged wild-type littersmates. Our results may contribute to increased understanding of the possible use of the Δ9-THC and CBD combination in demented patients.

MATERIALS AND METHODS

Animals

The experiments were carried out in male AβPP/PS1 mice and wild-type-like (WT) littermates aged 12 months at the onset of the study and in male WT mice aged 3 months (non-aged controls) with a C57Bl6J genetic background. The generation of mice expressing the human mutated AβPPswe and PS1dE9 has been described elsewhere [14]. All the animals used in this study derived from 7 breeder pairs. Animals were maintained under standard animal housing conditions (static isolation caging, 3-4 animals per cage) 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 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 committee of the University of Barcelona.

Pharmacological treatment

Δ9-THC-enriched botanical extract (containing 67.0% Δ9-THC, 0.8% CBD, 1.2% cannabigerol, 0.9% cannabichromene and 3.2% other phytocannabinoids) and CBD-enriched botanical extract (containing 62.7% CBD, 3.6% Δ9-THC, 1.4% cannabigerol, 5.7% cannabichromene and 1.8% other phytocannabinoids) were supplied by GW Pharmaceuticals Ltd (Cambridge, UK). The extracts (Δ9-THC 0.75 mg/kg + CBD 0.75 mg/kg) were dissolved in 5% ethanol, 5% Tween, and 90% saline, and this combination was administered intraperitoneally (i.p.) in a single injection in a volume of 10 mL/kg body weight. The human equivalent dose (HED) calculated with the formula for dose translation based on body surface area [15] corresponds to 0.04 mg/kg for each cannabinoid, which is equivalent to the administration of a single Sativex® oromucosal spray (2.8 mg Δ9-THC + 2.8 mg CBD) in a human being weighing 70 kg. At this dose, the compound lacks psychoactivity. Groups of animals were treated once a day for 5 weeks with the extracts or with vehicle alone. The number of animals included in each group was as follows: WT 12 months treated with cannabinoids, n = 9; vehicle alone, n = 8; AβPP/PS1 12 months with cannabinoids, n = 11; AβPP/PS1 12 months treated with vehicle alone, n = 10; WT aged 3 months treated with cannabinoids, n = 8; treated with vehicle alone, n = 7. After a 10-day washing period, animals were subjected to behavioral evaluation.

Cognitive evaluation and sample collection

Memory performance was evaluated with the two-object recognition test. On day 1, mice were placed for 9 min in a V-maze, in which two identical objects were situated at the ends of the arms; the time that the mice spent exploring each object was recorded.
Then, 24 h after the training session, animals were placed again for 9 min in the V-maze, with one of the two familiar objects replaced by a novel object. The time that the animals spent exploring the two objects was recorded and an object recognition index (RI) was calculated, as the difference between the time spent exploring the novel object (TN) and the familiar object (TF) divided by the total time spent exploring the two objects [RI = (TN- TF)/(TN+ TF)].

At the end of the behavioral testing, the animals were killed and their brains rapidly removed from the skull and processed for study. One hemisphere was dissected on ice, immediately frozen, and stored at −80°C 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 mm 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% H2O2 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), Aβ40 (1:100, Merck Millipore, Billerica, MA, USA) or Aβ42 (1:50, Merck Millipore). Sections were subsequently rinsed and incubated with biotinylated secondary antibodies (Dako). Peroxidase reaction was visualized with diaminobenzidine and H2O2. Sections were slightly 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 mm²) 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/retrosplenial and motor cortex, somatosensory cortex, and piriform/entorhinal cortex). The areas selected were the main regions of the cerebral cortex in which Aβ is deposited in AβPP/PS1 mice. In addition, two sections of the hippocampus of each animal (−1.5 mm and −2.0 mm from bregma) were used for quantification of Aβ burden in the hippocampus, calculated as the percentage of the amyloid deposition in plaques with respect to the total hippocampal area in each section. The percentage of Aβ42 contents in each plaque was calculated by comparing the specific Aβ42 staining with respect to total Aβ42 + Aβ40 staining in at least 25 cortical plaques per animal in consecutive sections. A researcher who did not know the treatment received in each group performed the quantifications. 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 at 100,000 g for 1 h, and the supernatant was saved as the soluble fraction for Aβ quantification. Aβ40 and Aβ42 Human ELISA kits (Invitrogen™ Corporation, Camarillo, CA, USA) were used to quantify the levels of Aβ40 and Aβ42 peptides in the brain soluble fractions. Quantitative determination was carried out according to the manufacturer’s instructions. Aβ40 and Aβ42 levels were normalized to the total amount of protein from each individual sample (BCA method, Thermo Fisher Scientific, Wilmington, DE, USA). All treated animals were analyzed but only AβPP/PS1 mice were used for quantification because, as expected, no specific immunolabeling was observed in any WT mice.

**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 30 min (Merck Millipore) to block lipofuscin autofluorescence, 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 fibrillar acidic protein (GFAP; 1:250, Dako) and IBA1 (1:250, Wako,
Richmond, VA, USA). After washing, the sections were incubated with Alexa488 and Alexa546 fluorescence secondary antibodies against the corresponding host species (1:400, Molecular Probes, Eugene, OR, USA). The sections 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.

The specific GFAP and IBA1 immunostaining density was calculated in reference to the Aβ plaque area in 5 representative pictures taken from the cortex of each animal using the Adobe® Photoshop® CS4 software. All the AβPP/PS1-treated animals were used for quantifications.

**Gel electrophoresis and western blotting**

Frozen samples of the somatosensory cortex were homogenized in RIPA lysis buffer (50 mM Tris/HCl buffer, pH 7.4 containing 2 mM EDTA, 0.2% Nonidet P-40, 1 mM 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 thioredoxin 2 (Txn2, 13 kDa, rabbit, 1:1,000, Proteintech, Chicago, IL, USA), wingless-related integration site 16 (Wnt16, 37 kDa, rabbit, 1:1,000, Proteintech, Chicago, IL, USA), synaptotagmin (47 kDa, mouse, 1:10,000, Abcam), glutamate receptor 2/3 (GluR2/3, 110 kDa, rabbit, Merck Millipore), and gamma-aminobutyric acid receptor A subunit α1 (GABA-A Rα1, 51 kDa, rabbit, Santa Cruz Biotechnology, Dallas, TX, USA). Protein loading was monitored using an antibody against β-tubulin (50 kDa, 1:10,000, Abcam). Membranes were then incubated for 1 h in the appropriate HRP-conjugated secondary antibodies (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-tubulin. Six animals per group were analyzed.

**Statistical analysis**

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 parametric statistical tests were used for the analysis of all the data in the study. Memory data were analyzed with two-way ANOVA by group (depending on age and genotype) and treatment, followed by Tukey’s post hoc test. Protein quantifications with western blotting were analyzed using two-way ANOVA with genotype and treatment as between factors. Aβ and glia quantifications were analyzed with Student’s t-test. In all the experiments, the significance level was set at $p < 0.05$.

**RESULTS**

*Natural cannabinoids reduce memory deficits in AβPP/PS1 mice at advanced stages of AD-like pathology but have no beneficial effects in aged WT mice*

Daily administration of $\Delta^9$-THC and CBD (0.75 mg/kg each botanical extract i.p.) for 5 weeks at advanced stages of the symptomatic phase (12 months) blunted the memory impairment observed in vehicle-treated AβPP/PS1 mice, as revealed by the two-object recognition test (Fig. 1). Two-way ANOVA revealed a significant group effect [$F(1,39) = 7.579, p < 0.01$] and treatment effect [$F(1,39) = 4.840, p < 0.05$] and interaction between the two factors [$F(2,39) = 6.810, p < 0.01$). Subsequent Tukey’s post hoc tests revealed that vehicle-treated WT mice aged 12 months ($p < 0.05$) and vehicle-treated AβPP/PS1 mice aged 12 months ($p < 0.001$) exhibited memory impairment when compared to corresponding non-aged control mice. Interestingly, $\Delta^9$-THC + CBD significantly increased the recognition index of 12-month-old AβPP/PS1 mice when compared to vehicle-treated littermates ($p < 0.001$). However, the treatment was not effective in reducing the memory impairment exhibited by 12-month-old WT mice. Treated mice were also tested in the active avoidance test, which is a more complex task and involves both memory and learning processes. However, aged WT mice evidenced difficulties to perform...
Daily administration of Δ⁹-THC + CBD (0.75 mg/kg each botanical extract i.p.) for 5 weeks blunts memory impairment of AβPP/PS1 mice at advanced stages (12 months). However, it is not effective in reducing memory impairment in 12-month-old WT littermates. Data are expressed as the mean values ± SEM; ***p<0.001 treatment effect; #p<0.05, ###p<0.001 compared to vehicle-treated non-aged control mice.

The combination of Δ⁹-THC and CBD does not alter Aβ processing in AβPP/PS1 mice at advanced stages of the disease

Chronic treatment with the combination of Δ⁹-THC + CBD did not significantly modify total Aβ burden in the neocortex and hippocampus of AβPP/PS1 mice (Fig. 2A, B). In contrast to what was previously observed at early stages of AD-like pathology in AβPP/PS1 mice [11], Δ⁹-THC + CBD was not able to reduce the level of soluble Aβ₄₂ and Aβ₄₀ when administered at advanced stages of the disease (Fig. 2E). In the same line, natural cannabinoids failed to induce a change in the composition of Aβ plaques similar to that observed when treatment was applied at early stages [13]. Despite an upward trend (p = 0.119), the percentage of Aβ₄₂ in each plaque was not significantly increased in treated mice (Fig. 2C, D). None of the assessed Aβ forms was detectable in WT littermates (data not shown).

Gliosis related to Aβ deposition was not modified in AβPP/PS1 mice treated at advanced stages

In contrast to our previous observations showing reduced astroglial and microglial reactivity in the vicinity of Aβ deposits in AβPP/PS1 mice treated with Δ⁹-THC + CBD during the early symptomatic stages [13], the present results show that the same combination of natural cannabinoids was not able to reduce glial reactivity in AβPP/PS1 mice when administered at advanced stages of the disease (Fig. 3).

Δ⁹-THC and CBD combination modifies the levels of several synaptic and neurotransmission markers

In order to evaluate the possible molecular mechanisms underlying the beneficial effects of Δ⁹-THC + CBD on AβPP/PS1 mice at advanced stages, we analyzed, using western blotting, selected proteins linked to redox metabolism, and synapses and neurotransmission in cortical homogenates (Fig. 4). First, we evaluated the levels of the redox protein Txn2 and the signaling protein Wnt16. However, two-way ANOVA revealed no significant effect of treatment independently of genotype, and no interaction between the two factors on the levels of Txn2 or Wnt16 in cortical homogenates of AβPP/PS1-mice treated at advanced stages. Next, we evaluated the levels of certain proteins involved in the regulation of synaptic plasticity and function, including SNAP25, PSD-95 and synaptotagmin. Δ⁹-THC + CBD induced an effect on the levels of SNAP25 (Genotype effect: F(1,18) = 0.61, N.S.; Treatment effect: F(1,18) = 7.07, p < 0.05; Interaction: F(1,18) = 3.08, N.S.). Thus, chronic treatment reduced the amounts of SNAP25 protein in AβPP/PS1 mice, which were elevated in comparison to WT littermates, when compared to AβPP/PS1 mice treated with vehicle alone (p < 0.05). In contrast, cannabinoids did not reduce the elevated levels of PSD-95 observed in AβPP/PS1 mice (Genotype effect: F(1,19) = 6.03, p < 0.05; Treatment effect: F(1,19) = 0.01, N.S.; Interaction: F(1,19) = 0.29, N.S.). When compared to WT mice, the increase in PSD-95 only reached statistical significance in Δ⁹-THC + CBD-treated AβPP/PS1 mice (p < 0.05), although a clear tendency was also observed in vehicle-treated AβPP/PS1 mice. Two-way ANOVA revealed no significant effect of treatment or genotype, nor interaction between the two factors, at the level of synaptotagmin in treated mice. Finally, we evaluated the protein levels of the metabotropic glutamate receptor 2/3 (GluR2/3) and the ionotropic GABA-A receptor α₁. Two-way ANOVA revealed that chronic treatment significantly modulated both GluR2/3 (Genotype effect: F(1,19) = 16.492,
Fig. 2. A) Representative images of the Aβ immunoreactivity in somatosensory cortex (top) and hippocampus (bottom) in vehicle- (left) and Δ⁹-THC + CBD-treated (right) AβPP/PS1 mice. Scale bars represent 200 μm (cortex) or 500 μm (hippocampus). B) Total Aβ burden quantification reveals no treatment effect in cortex and hippocampus in aged AβPP/PS1 mice. C) Representative images of the Aβ40 (top) and Aβ42 (bottom) immunoreactivity in consecutive cortical sections of vehicle- (left) and Δ⁹-THC + CBD-treated (right) AβPP/PS1 mice aged 12 months at the beginning of the study. Scale bar represents 50 μm. D) Quantification of the percentage of Aβ42 contents with respect to total Aβ42 + Aβ40 levels in each plaque reveals no significant effect in spite of a tendency towards increase in Δ⁹-THC + CBD-treated mice. E) The combination of Δ⁹-THC + CBD does not modify soluble Aβ40 and Aβ42 levels in cortical homogenates from AβPP/PS1 mice treated at advanced stages of the disease. Counts are expressed as the mean values ± SEM.

\( p < 0.001 \); Treatment effect: \( F_{(1,19)} = 0.09, \) N.S.; Interaction: \( F_{(1,19)} = 7.09, p < 0.05 \) and GABA-A Rα1 receptors (Genotype effect: \( F_{(1,19)} = 0.23, \) N.S.; Treatment effect: \( F_{(1,19)} = 1.93, \) N.S.; Interaction: \( F_{(1,19)} = 8.93, p < 0.01 \). Thus, subsequent post hoc tests showed that Δ⁹-THC + CBD significantly
reduced the levels of GluR2/3 in AβPP/PS1 mice compared to vehicle-treated (p < 0.05) and WT littermates (p < 0.01), and increased the GABA-A Rα1 levels with respect to vehicle-treated AβPP/PS1 mice (p < 0.05), which exhibited reduced levels compared to WT animals (p < 0.05).

DISCUSSION

During the last decade, targeting of the endogenous cannabinoid system has emerged as a promising therapy against Alzheimer-type dementia because endocannabinoid signaling modulates some pathological processes linked to neurodegenerative processes, including protein misfolding, neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress [3–6]. However, most evidence is based on observations in experimental models at early stages of the disease. The present study analyzes the efficacy of a cannabis-based medicine, composed essentially of Δ9-THC and CBD, at advanced stages of Alzheimer-type dementia in AβPP/PS1 transgenic mice. Collaterally, we evaluated the therapeutic profile of the Δ9-THC and CBD combination in WT littermates used as a model of aging. We chose the combination of these two natural cannabinoids because, although previous findings revealed that both Δ9-THC and CBD given alone resulted in beneficial cognitive effects in AβPP/PS1 mice [13, 16, 17], the combination of Δ9-THC and CBD was more effective than either cannabinoid alone in these transgenic animals when administered at early stages [13].

The present results show that treatment with Δ9-THC and CBD at non-psychoactive doses reduces memory impairment occurring in AβPP/PS1 mice at 12 months of age when the progression of the disease is at an advanced stage. These findings might appear to contradict the evidence about the cognitive impairment induced by Δ9-THC exposure in healthy mice [18]. However, the doses employed revealing memory impairments in mice [18] are much higher than the dose used here showing a neuroprotective effect. Second, the brain context of young healthy mice exposed to high doses of Δ9-THC is different from that of aged AD mice, since molecular reorganization of the endogenous cannabinoid system and altered neuronal signaling are found in AD [19]. These differences may explain different Δ9-THC-linked effects in these two groups of mice. Finally, the combination of natural cannabinoids administered in the present study is composed of Δ9-THC in a similar proportion to CBD, which is known to antagonize Δ9-THC-induced deficits in memory [20].

The Δ9-THC- and CBD-enriched extract combination administered at advanced stages of the disease does not modify Aβ burden or the glial reactivity associated with aberrant Aβ deposition in AβPP/PS1 mice, in contrast to the effect when administered at early stages of the disease [13]. Thus, these findings suggest that natural cannabinoids modulate mechanisms other than Aβ processing and glial reactions.
which contribute to cognitive improvement revealed in AβPP/PS1 mice treated at advanced stages of the disease. We may speculate that the mechanisms involved in this cannabinoid-induced beneficial effect on memory performance are specific for neurodegenerative dementia since the Δ⁹-THC and CBD combination is ineffective at reducing mild cognitive impairment in old WT littermates used as a model of behavior deterioration linked to old age.

Among the potential molecular mechanisms involved in the beneficial effects induced by the Δ⁹-THC and CBD combination in AβPP/PS1 mice treated at advanced stages of the disease, we focused our attention on evaluating three groups of proteins: (i) Txn2 and Wnt16, two proteins that play a role in the Δ⁹-THC + CBD-induced effects on AβPP/PS1 mice when treated at early stages of the disease [13], (ii) SNAP25, which is associated with synaptosome and contributes to the formation of the exocytotic fusion complexes in neurons, PSD-95, located in the post-synaptic density of neurons and involved in receptors clustering, modulation of ion channels and associated signaling proteins, and synaptotagmin, a presynaptic Ca²⁺ sensor involved in the regulation of the traffic of synaptic vesicles and neurotransmitter release, as markers of synaptic plasticity and function, and (iii) GluR2/3 and GABA-A Rα₁, both involved in the regulation of neurotransmission in cortical brain areas. The selection of these markers was based on previous evidence about the role of cannabinoids in modulating synaptic signaling and function [21, 22]. In contrast to what happened when Δ⁹-THC and CBD were administered at early stages [13], the combination of these natural cannabinoids fails to modify the levels of the redox protein Txn2 and the signaling molecule Wnt16 in the somatosensory cortex homogenates in WT and AβPP/PS1-treated mice with cannabinoids or with vehicle alone: thioredoxin 2 (Txn2), wingless-related integration site (Wnt16), synaptosome associated protein-25 (SNAP25), post-synaptic density protein-95 (PSD-95), synaptotagmin, glutamate receptor 2/3 (GluR2/3) and gamma-aminobutyric acid receptor A α₁ (GABA-A Rα₁). A) Representative immunoblots for Txn2, Wnt16, SNAP25, PSD-95, synaptotagmin, GluR2/3, GABA-A Rα₁, and corresponding β-tubulin loading control indicating the molecular weight of proteins. B) Optical density quantification of each immunoblot with respect to WT-vehicle group which is considered as control. No significant change in the total amount of Txn2 or Wnt16 is observed in any group. However, the Δ⁹-THC + CBD combination normalizes SNAP25 levels but fails to reduce levels of PSD-95 or to modify synaptotagmin protein levels. Δ⁹-THC + CBD modifies the expression of certain neurotransmission-related molecules. Thus, AβPP/PS1-treated mice exhibit significantly lower levels of GluR2/3 and increased levels of GABA-A Rα₁ protein when compared with vehicle-treated transgenic mice. Densitometric quantifications are expressed as mean values ± SEM. *p < 0.05 treatment effect. #p < 0.05, ##p < 0.01, genotype effect.
cortex of aged AβPP/PS1 mice. However, the combination of Δ⁹-THC and CBD induces some changes in markers of synaptic function, which might be related to the cognitive improvement observed in AβPP/PS1 mice treated during advanced stages of the disease. Thus, chronic treatment normalized the levels of the pre-synaptic SNAP25, but not the post-synaptic PSD-95 protein, in AβPP/PS1 mice. Increased levels of synaptic proteins such as SNAP25 were previously associated with aberrant neuritic sprouting in brain areas affected by Aβ synthesis and deposition in AD [23], suggesting that Δ⁹-THC and CBD might contribute to reducing this pathological process. More importantly, AβPP/PS1 mice treated with Δ⁹-THC and CBD at advanced stages exhibit a reduction in GluR2/3 expression levels accompanied by an increase in the expression of GABA-A Receptors. These results suggest that natural cannabinoids alter the imbalance of excitatory versus inhibitory neural activity in the somatosensory cortex of aged AβPP/PS1 mice [24, 25]. In fact, Aβ has been demonstrated to inhibit long-term plasticity processes associated with learning and memory through suppression of CB₁-dependent GABAergic synaptic disinhibition [26]. Moreover, CB₁ receptor activity in GABAergic neurons protects against age-dependent cognitive decline [27]. Therefore, it may be speculated that the combination of Δ⁹-THC and CBD facilitates inhibitory GABAergic activity in the somatosensory cortex by mitigating the deleterious effect of Aβ on GABAergic function, and subsequently on cognitive performance, via activation of CB₁ receptors. Δ⁹-THC is also known to depress glutamate synaptic transmission via CB₁ receptor activation, affecting glutamate release, inhibiting receptors and transporters function, reducing enzyme activity, and disrupting glutamate synaptic plasticity after prolonged exposure [28]. Thus, the reduction of GluR2/3 expression observed on treated mice might be the result of such depressed glutamatergic activity induced by chronic stimulation of CB₁ receptor. These modifications could contribute to counteract the alterations in neural excitability observed in AβPP/PS1 mice [24, 25] and in turn to improve cognitive performance. However, further experimental evidence will be needed to confirm the specific role of CB₁ receptors in such effects.

In light of these observations, and of the fact that Sativex® is devoid of psychoactivity and is well-tolerated at the dose proposed in our studies [29], potential translation to human beings might offer a safe and effective therapeutic agent in advanced Alzheimer-type dementia.

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