Alzheimer's
Research & Therapy

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Cannabidiol as a multifaceted therapeutic agent: mitigating Alzheimer's disease pathology and enhancing cognitive function

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Abstract

Background Cannabidiol (CBD), the second most abundant phytocannabinoid in Cannabis sativa, has garnered significant interest due to its non-psychoactive nature and diverse receptor interactions.

Methods This study employs in vitro and in vivo methodologies to validate CBD's potential as a treatment for Alzheimer's disease (AD) by addressing key hallmarks of the condition and promoting neuroprotective effects on spatial memory.

Results Our findings demonstrate CBD's ability to decrease pTau and Aβ aggregation and to mitigate their axonal transport between cortical and hippocampal neurons. Moreover, CBD treatment was shown to reduce neuroinflammation, as CBD was able to skew microglia towards a neuroprotective M2 phenotype while attenuating proinflammatory cytokine release in the 5xFAD AD mouse model. Notably, daily CBD injections (10 mg/Kg) for 28 days in 5xFAD mice resulted in significant improvements in both short- and long-term spatial memory. The study also reveals CBD's capacity to partially revert neurite formation loss induced by Aβ, Tau, and pTau proteins, suggesting a potential role in promoting neuronal plasticity. Additionally, CBD treatment led to a reduction in reactive oxygen species (ROS) formation and increased neuronal viability in the presence of AD-associated protein aggregates.

Conclusions These multifaceted effects of CBD, ranging from molecular-level modulation to behavioral improvements, underscore its potential as a comprehensive therapeutic approach for AD. The findings not only support CBD's neuroprotective properties but also highlight its ability to target multiple pathological processes simultaneously, offering a promising avenue for future AD treatment strategies.

Keywords CBD, Alzheimer's disease, CB_1R, CB_2R, Axonal transport, β-amyloid, Tau, PTau

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Introduction

Alzheimer's disease (AD) is nowadays the most common form of dementia worldwide, with two-thirds of cases in individuals over 65 years old. It is estimated that more than fifty million people are affected by this condition [1]. AD is caused by a combination of genetic, lifestyle, and environmental factors, being age the main risk. Thus, these numbers will increase in the coming years due to the global aging of the population, doubling the number of cases in the following decades.

Two different AD subtypes exist: familial and sporadic. At the molecular level, both express plaques formed by the parenchymal amyloid-β (Aβ) deposition and intraneuronal accumulation of hyperphosphorylated Tau protein [2–4]. These aberrant protein aggregates lead to inflammation and oxidative stress, but also to microglial activation, astrogliosis, characteristic losses of neurons, neuropil, and synaptic elements, provoking a progressive impairment of cognitive and behavioral functions including comprehension, language, memory, attention, reasoning, and judgment. Nowadays, this pathology has no cure, only treatments to counteract the symptomatology. In this sense, seven available drugs have been approved: an antagonist of the N-methyl-D-aspartate (NMDA) receptor (memantine), three acetylcholinesterase inhibitors (rivastigmine, donepezil, and galantamine) [5], and three monoclonal antibodies against ß-amyloid protein (aducanumab, donanemab and lecanemab) [6, 7].

The Endocannabinoid System (ECS) plays an essential role in cognitive function and brain memory in different aspects [8]. The Cannabinoid System is made up of three main components: the CB_1 and CB_2 cannabinoid receptors, that are known to interact forming CB_1R-CB_2R heteromers [9], being CB_1R the most expressed membrane receptor at the CNS level, and the CB_2 receptor having a strong involvement in the immune system [10], the endocannabinoid compounds, and the enzymes of synthesis and degradation of these. Cannabinoids are divided into endocannabinoids, anandamide and 2-AG (2-arachidonoylglycerol) are the most studied with greater involvement at a physiological level, synthetic cannabinoids, and phytocannabinoids, which are those compounds directly extracted from the *Cannabis sativa* plant.

Published data point to CB_2R as a key player to combat neuroinflammation, as CB_2R expression is increased in activated microglia [9]. On the other hand, CB_1R seems to have a more important role in neuroprotection [11]. CB_1R expression shows complex changes in AD, with some studies indicating initial hyperactivity in certain brain areas that later decrease with disease progression, while other areas show unchanged or even decreased CB_1R levels compared to controls [12]. However, increased expression levels of 2-AG and its degrading enzyme monoacylglycerol lipase (MAGL) have been found in AD patients [13].

Cannabis sativa contains more than 500 distinct compounds, and 120 are classified as phytocannabinoids, showing different structures and pharmacological properties [14]. Cannabinoids are involved in synaptic responsiveness and plasticity [15]. Specifically, low doses of Δ^9-THC (Δ^9-tetrahydrocannabinol) have been demonstrated to induce neurogenesis and reduce Aβ toxicity and plaque deposition in rodents' hippocampus and other dementia symptoms in both preclinical and clinical studies [16]. Cannabidiol (CBD) is the second most abundant phytocannabinoid; it comprises up to 40% of the total extracted compounds. It can bind to both cannabinoid receptors, acting as an allosteric modulator at nanomolar concentrations on both receptors [17, 18] and binding to the orthosteric site of CB_1R at micromolar concentrations. It shows a biased functionality [19] with anti-oxidant [20], anti-neuroinflammatory [21], anxiolytic [22], antipsychotic, and neuroprotective properties. Moreover, multiple basic and clinical assays have demonstrated its potential to combat different pathological conditions such as schizophrenia, social phobia, post-traumatic stress, depression, bipolar disorder, sleep disorders, epilepsy, substance abuse and dependence, and neurodegenerative diseases such as Parkinson's [23]. Specifically, different studies demonstrate the capacity of CBD to decrease neuroinflammation and reactive gliosis as well as to induce neurogenesis, preventing the development of cognitive deficits in AD rodent models [24]. Meanwhile, it is important to point out that apart from cannabinoid receptors, CBD induces some of its effects by activating a broad spectrum of other receptors, such as some orphan receptors plus serotonin ($5\text{-}HT_{1\ A}$), peroxisome proliferator-activated receptors (PPARs), vanilloid receptors, adenosine receptors, N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors opioid receptors, and dopamine receptors [25, 26].

Hence, the main objective of the study consists of investigating the potential beneficial effects of cannabidiol in the neurodegenerative disease of Alzheimer's. We have focused our attention on studying the effect of CBD on memory impairment in 5xFAD and CL2006 AD models, protein aggregation and axonal transport, glia activation, neuronal plasticity, ROS formation and neuronal death. The interest in CBD relies on the lack of psychoactive effects and its beneficial effects in different pathophysiology conditions, converting it into a powerful compound with multiple benefits for AD treatment.

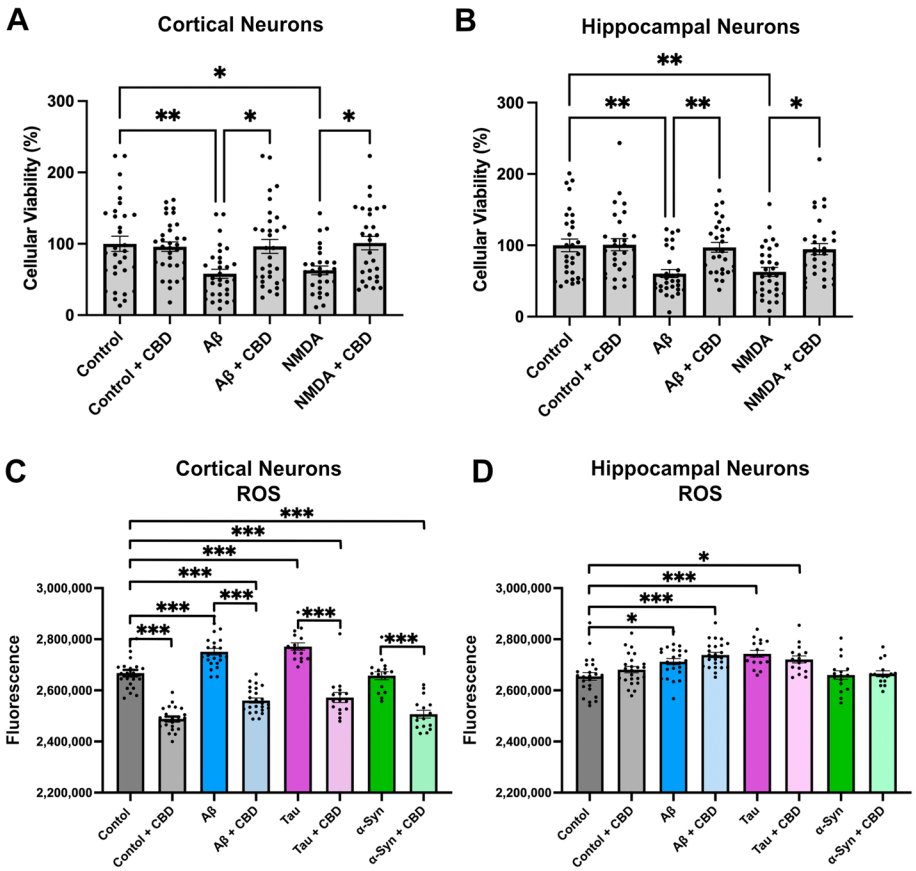

Fig. 1 Determination of cell viability and ROS species in primary neurons treated with Aß$_{1-42}$ or Tau aggregates. **A**, **B**. Cellular viability determination was performed in primary cultures of cortical (**A**) or hippocampal (**B**) neurons from C57BL/6 J mice treated with NMDA (30 μM), Aβ_{1-42} (500 nM) or vehicle, and subsequently stimulated with CBD (200 nM) or vehicle for 48 h. Cells were stained with Trypan Blue and sampled in a Neubauer chamber. The unstained (viable) and stained (nonviable) cells were counted separately, and the percentage of viability was calculated as the total number of viable cells/total number of cells × 100. Experiments were performed in samples from 6 different pregnant females. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test was used for statistical analysis. *$p < 0.05$, *$p < 0.005$ ***$p < 0.001$. **C**, **D** Detection of ROS species levels was determined using Dihydroethidium as an indicator. Cells were treated with Aβ_{1-42} (500 nM), Tau (1 μM), α-synuclein (4 μM) or vehicle, and subsequently stimulated with CBD (200 nM) or vehicle for 48 h. Values are the mean ± S.E.M. of 5 independent experiments. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$

Results

CBD stimulation increases neuronal viability while decreasing ROS species after Aβ treatment

In AD, neuronal death is correlated with memory impairment, one of the main symptoms of the pathology [27]. To better understand the effect of CBD on neuronal death, primary cultures of cortical and hippocampal neurons were seeded in 6 well plates at 50% confluency for 12 days. After, neurons were treated with Aβ_{1-42} (500 nM) and then stimulated with CBD or vehicle for 48 h. Results showed that Aβ_{1-42} treatment induced around 45% of neuronal death that was practically reverted in cells treated with CBD (Fig. 1A, B). A similar experiment was performed in primary cultures of cortical and hippocampal neurons treated with NMDA (30 μM) or vehicle and then stimulated with CBD for 48 h. NMDA toxicity induced around 40% of neuronal death. CBD stimulation counteracted this effect, restoring neuronal survival to similar values as those of cells treated with a vehicle (Fig. 1A, B). Then, CBD stimulation recovers neuronal survival from Aβ_{1-42} and NMDA toxicity to levels similar to those of vehicle-treated cells.

AD is also associated with an increase in oxidative stress. To depict CBD's potential role in reactive oxygen species (ROS) formation, primary cultures of cortical neurons were treated with Aβ_{1-42} (500 nM), Tau (1 μM), or α-synuclein (4 μM) as control and then stimulated with CBD or vehicle for 48 h. After analyzing the fluorescence emitted by the indicator Dihydroethidium, it was observed that all protein aggregates induced a significant

increase in the formation of ROS species in cortical neurons that was counteracted by CBD treatment (Fig. 1C). However, in hippocampal neurons, only Aß$_{1-42}$ and Tau protein aggregates induced a significant effect, that was not altered by CBD treatment (Fig. 1D).

CBD treatment improves neurite patterning while decreasing protein aggregates formation in neuronal primary cultures treated with Aβ, Tau, and pTau proteins

Another characteristic neuropathological change of AD is the loss and alteration of synaptic elements that evolve in parallel with amyloid plaques and neurofibrillary tangles (NFTs) accumulation [28]. In this sense, we first evaluated the Aβ$_{1-42}$ effect on neurite pattering. To detect neurite pattering, an immunocytochemistry assay was conducted in cortical neuronal primary cultures treated with Aβ$_{1-42}$ (500 nM), Tau (1 μM), or pTau (1 μM) protein aggregates for 48 h. Synapses were detected with an anti-Nectin antibody over neurons stained with an anti-F-actin antibody conjugated to an Alexa 488 fluorophore. Data demonstrates an essential loss in neurite formation after 48 h of Aβ$_{1-42}$, Tau, and pTau treatments (around 55%, 60%, and 65%, respectively). Then, to evaluate the potential neurogenic effect of CBD, the experiment was repeated, treating cortical neuronal primary cultures with CBD (200 nM) for 48 h. It was observed that cannabidiol partially reverted the neurite pattern loss induced by Aβ$_{1-42}$, Tau, and pTau proteins (Fig. 2A,C). The same experiment was repeated with hippocampal neurons, obtaining similar results (Fig. 2D). As a control, we tested the effect of α-synuclein, which is main component of Lewy bodies, and a hallmark of several neurodegenerative diseases such as Parkinson's. Contrary to Aβ$_{1-42}$, Tau and pTau proteins, the neurite loss induced by α-synuclein was not counteracted by CBD treatment (Fig. 2A,C,D).

To analyze the capacity of CBD to reduce protein aggregate formation, neuronal primary cultures were treated with Aβ$_{1-42}$ (500 nM), Tau (1 μM), or pTau (1 μM) protein aggregates and subsequently stimulated with CBD (200 nM) for 48 h. Results in Fig. 2E, F indicate that CBD treatment induced an important reduction in Aβ$_{1-42}$ (61%), Tau (82%), and also pTau (69%) aggregation. These results agree with the involvement of cannabinoids in altering Tau aggregation [29]. As a control, CBD's capacity to decrease α-synuclein (4 μM) aggregation was tested (Fig. 2E, F). This reduction in protein aggregation could underlie the mechanism explaining the beneficial effects induced by CBD in AD.

CBD treatment decreases Aβ$_{1-42}$, Tau and pTau axonal transport in neuronal primary cultures

One of the mechanisms that could slow down the progression of Alzheimer's disease is the decrease in the transport of abnormal aggregates of pTau and beta-amyloid proteins. In this sense, microfluidic devices with two different chambers were prepared to demonstrate CBD's potential to regulate protein axonal transport. As Tauopathies extend further to the entorhinal-hippocampal connection, the first chamber was cultured with cortical neurons and treated with Aβ$_{1-42}$ (500 nM), Tau (1 μM), pTau (1 μM) or α-synuclein (4 μM) as control, while the second chamber was cultured with non-treated hippocampal neurons. Then, cortical neurons were treated with CBD or vehicle for 48 h. Immunocytochemistry assays demonstrated that Aβ$_{1-42}$ aggregates were transported from cortical to hippocampal neurons (Fig. 3A). Interestingly, neurons treated with CBD showed a strong decrease in Aβ$_{1-42}$ axonal transport. Although the deposits of Aβ forming plaques are one of the most important features of Alzheimer's disease, this pathology also exhibit tangles of twisted fibers of Tau protein. Then, the axonal transport of Tau protein was analyzed. First, Tau aggregates were shown to be transported from cortical neurons in chamber 1 to hippocampal neurons in chamber 2 in the microfluidic device. Furthermore,

(See figure on next page.)

Fig. 2 Effect of CBD over neurite patterning and Aβ, Tau, and pTau protein aggregation. **A-D** On DIV 10, cortical and hippocampal neurons were treated with Aβ$_{1-42}$ (500 nM), Tau (1 μM), pTau (1 μM) or α-synuclein (4 μM) and subsequently stimulated with CBD (200 nM) or vehicle for 48 h. Neurite patterning was detected by immunocytochemistry using an anti-Nectin 3 antibody (Abcam, 1/1000). Neurons were detected with the anti-F-actin antibody fused to an Alexa 488 fluorophore (ThermoFisher, 1/400). Cell nuclei were stained with Hoechst (blue). **C**, **D** Neurite quantification was performed over segments of 15 μm. Each red dot represents a neurite formation. Values are the mean ± S.E.M. of 9 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ versus control condition. **B** A schematic representation of the section length used to quantify the neurite formation. **E**, **F** Cortical neurons were treated with Aβ$_{1-42}$ (500 nM), Tau (1 μM), pTau (1 μM) or α-synuclein (4 μM) and subsequently treated with CBD (200 nM) or vehicle for 48 h. Protein aggregates were detected by immunocytochemistry using rabbit anti-Aβ (1/100, ab201060), rabbit anti-Tau (1/100, Abcam ab32057), rabbit anti-pTau (S396) (1/100, Abcam ab109390) or mouse anti-α-synuclein (1/100, ab1903) antibodies and subsequently marked with a Cy3 anti-rabbit (1/200, Jackson InmunoResearch) secondary antibody (red). Cell nuclei were stained with Hoechst (blue). Values are the mean ± S.E.M. of 5 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used for statistical analysis *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. Scale bar: 10 μm

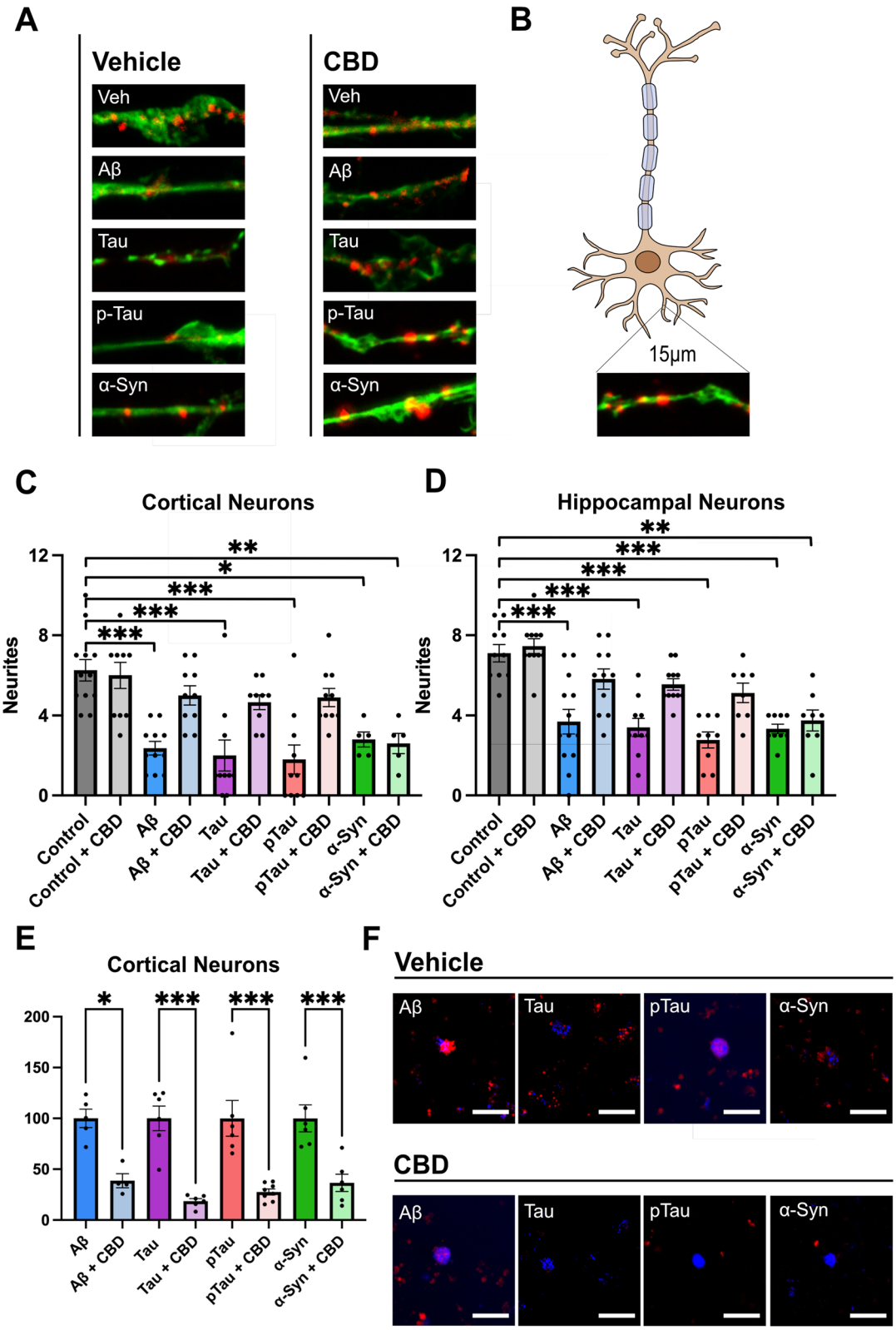

Fig. 2 (See legend on previous page.)

CBD treatment significantly reduced the spread of Tau from cortical to hippocampal cells (Fig. 3B). Tau protein is highly phosphorylated in AD at several residues, specifically Ser202 and Thr205 being the most common ones. Then, it was also investigated pTau axonal transport. Results indicated that pTau protein is less transported than Tau protein, while CBD treatment induced an important decrease in pTau spread from cortical to hippocampal neurons (Fig. 3C). Axonal transport of α-synuclein, a protein that forms aggregates in Parkinson's disease, was investigated as a control. Confocal images showed low levels of α-synuclein transport from cortical to hippocampal neurons, even lower in those neurons treated with CBD (Fig. 3D).

CBD injection in 5xFAD mice polarizes microglia to a neuroprotective M2 phenotype

Microglia, the brain's resident immune cells, can polarize into different phenotypes in response to several signals. To test CBD involvement in microglia polarization, the 5xFAD mice model of AD was injected with one daily dose of CBD (10 mg/Kg) for 4 weeks. AD is a multifactorial pathology that mainly affects cortical and hippocampal areas [30]). In this sense, cortical and hippocampal brain sections of the AD mice model 5xFAD treated or not with CBD, as well as control mice, were analyzed by immunohistochemistry. First, activated microglia was detected by Iba1 primary antibody and a secondary Cy3 conjugated anti-rabbit antibody in cortical sections. As shown in Fig. 4A, 5xFAD mice exhibited a significant increase in activated microglia compared to control mice, which was not affected by CBD treatment. However, when analyzing Arginase I staining, a marker of the M2 neuroprotective phenotype, a significant increase was observed in 5xFAD animals compared to controls, that was even more pronounced in those animals injected with CBD (Fig. 4B). The inducible nitric oxide synthase (iNOS), a marker of the microglia M1 proinflammatory phenotype, was also analyzed. The signal in the AD mice model triplicated the fluorescence of control mice (Fig. 4C), while CBD injections reduced this effect.

These results show that CBD injections polarize microglia to an M2 neuroprotective phenotype in the AD mice model 5xFAD, indicating a neuroprotective role of CBD. The expression of oligodendrocyte precursor cells that differentiate in mature oligodendrocytes providing myelin to neurons was assessed by detecting the oligodendrocyte transcription factor 2 (Olig2). Similar Olig2 immunoreactivity was detected when comparing 5xFAD animals and controls. However, CBD-treated animals showed a significant increase in the number of oligodendrocyte precursor cells (Fig. 4D). Finally, the amount of reactive astroglia was identified by the antibody against the glial fibrillary acidic protein (GFAP). After quantification with Andy's algorithm Fiji's plug-in, a significant increase in GFAP immunoreactivity was observed in 5xFAD animals compared to control mice. However, this effect was completely reverted in animals treated with CBD (Fig. 4E). These results demonstrate that CBD treatment promotes oligodendrocyte precursor cell expression while decreases activated astroglia cells in the AD mice model 5xFAD. When the same experiments were performed in hippocampal neurons, similar results were obtained (Fig. 5). The only difference was observed in Olig2 immunoreactivity, where CBD treatment showed no increase in comparison to 5xFAD or control mice.

CBD reduces IL-1ß while increases IL-10 expression in microglial primary cultures of 5xFAD mice

AD is also characterized by differences in cytokines release compared to physiological conditions. On the one hand, IL-1ß is related to a proinflammatory condition. On the other hand, IL-10 is related to an anti-inflammatory state, which can protect neurons and promote a healthier brain environment [31]. Therefore, microglial primary cultures of WT or 5xFAD mice were treated with CBD (200 nM) for 48 h. Interestingly, after collecting the supernatant it was observed that 5xFAD mice showed a significant increase in IL-1ß levels that was counteracted

(See figure on next page.)

Fig. 3 Detection of $A\beta_{1-42}$, Tau and pTau axonal transport in mice cortical and hippocampal neurons. Mice cortical and hippocampal neurons were grown in the microfluidic devices (**A-D**). On DIV 10 and once the axons fully crossed the microgrooves (150 µm distance), $A\beta_{1-42}$ (500 nM) (**A**), Tau (1 µM) (**B**), pTau (1 µM) (**C**) or α-synuclein (4 µM) (**D**) proteins were added into the top left well of each device, together with CBD (200 nM) or vehicle for 48 h. Neurons were labeled with a rabbit anti-Aβ antibody (1/100, ab201060), rabbit anti-Tau antibody (1/100, Abcam ab32057), rabbit anti-pTau (S396) antibody (1/100, Abcam ab109390) or mouse anti-α-synuclein antibody (1/100, ab1903) and subsequently marked with a Cy3 anti-rabbit (1/200, Jackson InmunoResearch) secondary antibody (red). Following 2 h of incubation, cells were washed and subsequently imaged using a confocal microscope with 25X (yellow squares) and 40X (green squares) objectives (Zeiss LSM 880). The bar graph shows the quantification of the amount of fluorescence in the microfluidic channel opposite to the treated channel in comparison with total fluorescence. Values are the mean ± S.E.M. of 3 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used for statistical analysis *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. **E** A schematic representation of the microfluidic device and the location of the treatment, allowing migration through the axons in the microchannels. Scale bar: 20 µm

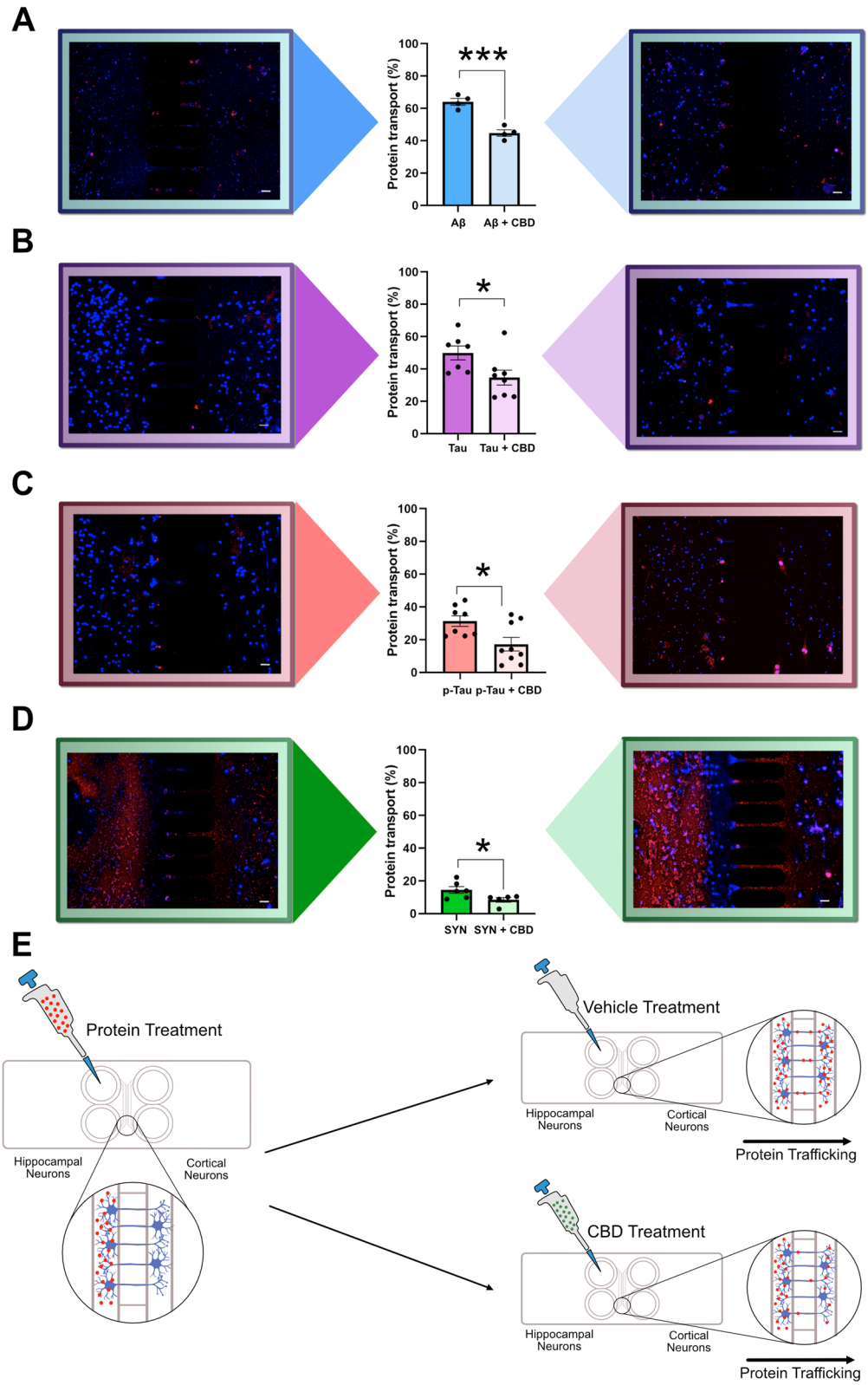

Fig. 3 (See legend on previous page.)

in cells treated with CBD (Fig. 6A). However, both WT and 5xFAD mice showed similar levels of IL-10 that were significantly increased in the presence of CBD (Fig. 6B).

After that, it was analyzed whether CBD was also capable of decreasing $A\beta_{1-42}$ aggregation. Thus, sections of 5xFAD mice treated or not with daily injections of CBD at 10 mg/Kg were analyzed by immunohistochemistry. Results indicate the capacity of CBD treatment to reduce $A\beta_{1-42}$ expression in cortex but also in hippocampus (Fig. 6C, B).

Finally, the capacity of CBD to reduce AD progression was also analyzed by detecting Tau axonal transport in the 5xFAD mouse model. Then, microfluidic devices were cultured with 5xFAD neuronal primary cultures and treated with pTau (1 µM) protein. By immunocytochemistry, it was observed that CBD induces a decrease in pTau transport also in the 5xFAD AD mouse model (Fig. 6E), and thus, it could reduce the progression of the pathology in AD mouse models.

5xFAD CBD injected mice show a reduction in cannabinoid CB_1 and CB_2 receptors expression

An important increase in receptor expression has been observed in neuroinflammation processes [32]. To evaluate the expression levels of cannabinoid receptors in 5xFAD mice, a quantitative PCR was performed with the mRNA obtained from dissected cortex and hippocampus. In Fig. 6F-I, two-fold expression of the cannabinoid CB_1 receptor can be observed in 5xFAD animals compared to control mice (C57BL/6) both in the cortex and hippocampus. As expected, the analysis of CB_2R expression showed a stronger increase with eight- and five-fold higher expression in 5xFAD cortex and hippocampus compared to control mice, respectively (F ig. 6G, I). When the quantitative PCR was performed in animals that had received a daily injection with CBD (10 mg/Kg) for 4 weeks, it was observed that cannabinoid CB_1 receptor expression returned to levels similar to those of control mice in both tissues, cortex, and hippocampus (Fig. 6F, H). Cannabinoid CB_2 receptor expression showed similar levels to those of control mice in the hippocampus, while a two-fold increase of expression was found in the cortex (Fig. 6G, I).

Cognitive improvement after CBD treatment in the in vivo animal models *C. elegans* and 5XFAD mice

Cognitive deficits, particularly impairments in spatial memory, are hallmark symptoms of AD, significantly impacting the quality of life of the affected individuals. Studying potential interventions that could mitigate these cognitive deficits is crucial for developing effective treatments. Cannabidiol, known for its neuroprotective properties, could be an interesting strategy to improve cognitive impairment in AD.

To evaluate the impact of CBD on the progression of AD, the CL2006 strain, one of the best characterized transgenic AD strains, was employed. This strain contains the Punc-54::$A\beta_{1-42}$ transgene, which results in progressive adult-onset paralysis, that has been correlated with increased levels of Aβ aggregates. As shown in Fig. 6H-J, treatment with CBD at 10 µM and 100 µM resulted in improved motility of CL2006 worms compared to the CL2006 control group. Accordingly, CBD treatment significantly increased travel distance and speed (Fig. 6J-L), confirming the delay of the progressive adult-onset paralysis in CL2006. We also measured the Aß deposits in the head of the *C. elegans* strain CL2006, demonstrating that CBD treatment at both 10 µM and 100 µM concentrations shows efficacy to reduce the degree of Aβ oligomerization. In addition, Fig. 6M shows Aβ deposits detected in CL2006. These findings strongly suggest that the administration of CBD holds promise in mitigating the progression of Aβ-induced AD in *C. elegans*, offering a potential avenue for future research and treatment.

To investigate any potential role of cannabidiol in cognitive improvement, spatial memory was evaluated in the AD mice model 5xFAD treated or not with daily injections of CBD at 10 mg/Kg. The exploration time during the familiarization phase of the novel object recognition test (NORT) task was unchanged by CBD treatment. The short-term memory was evaluated with the NORT 2 h after the first trial-familiarization. Results revealed that 5xFAD animals exhibited a significant cognitive deficit (Fig. 6N) compared to control mice (C57BL/6). However, CBD treatment strongly reduced short-term memory loss (Fig. 6N). Then, the long-term memory was analyzed with the novel object

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Fig. 4 Iba1, Arginase I, iNOS, Olig2, and GFAP immunohistochemistry in cortical sections from 5xFAD mice injected with CBD. **A-E**. Cortical sections from 5xFAD mice (5xFAD), 5xFAD mice treated with i.p. injection of pure CBD (5xFAD + CBD) and control Wild Type mice (WT), were analyzed by immunohistochemistry using specific anti-Iba1, anti-Arginase I, anti-iNOS, anti-Olig2 and anti-GFAP antibodies. Confocal microscopy images (stacks of 3 consecutive panels) show protein immunoreactivity in red over Hoechst-stained nuclei (blue). Protein immunoreactivity was quantified using the Andy's algorithm Fiji's plug-in. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test was used for statistical analysis. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. Scale bar: 20 µm

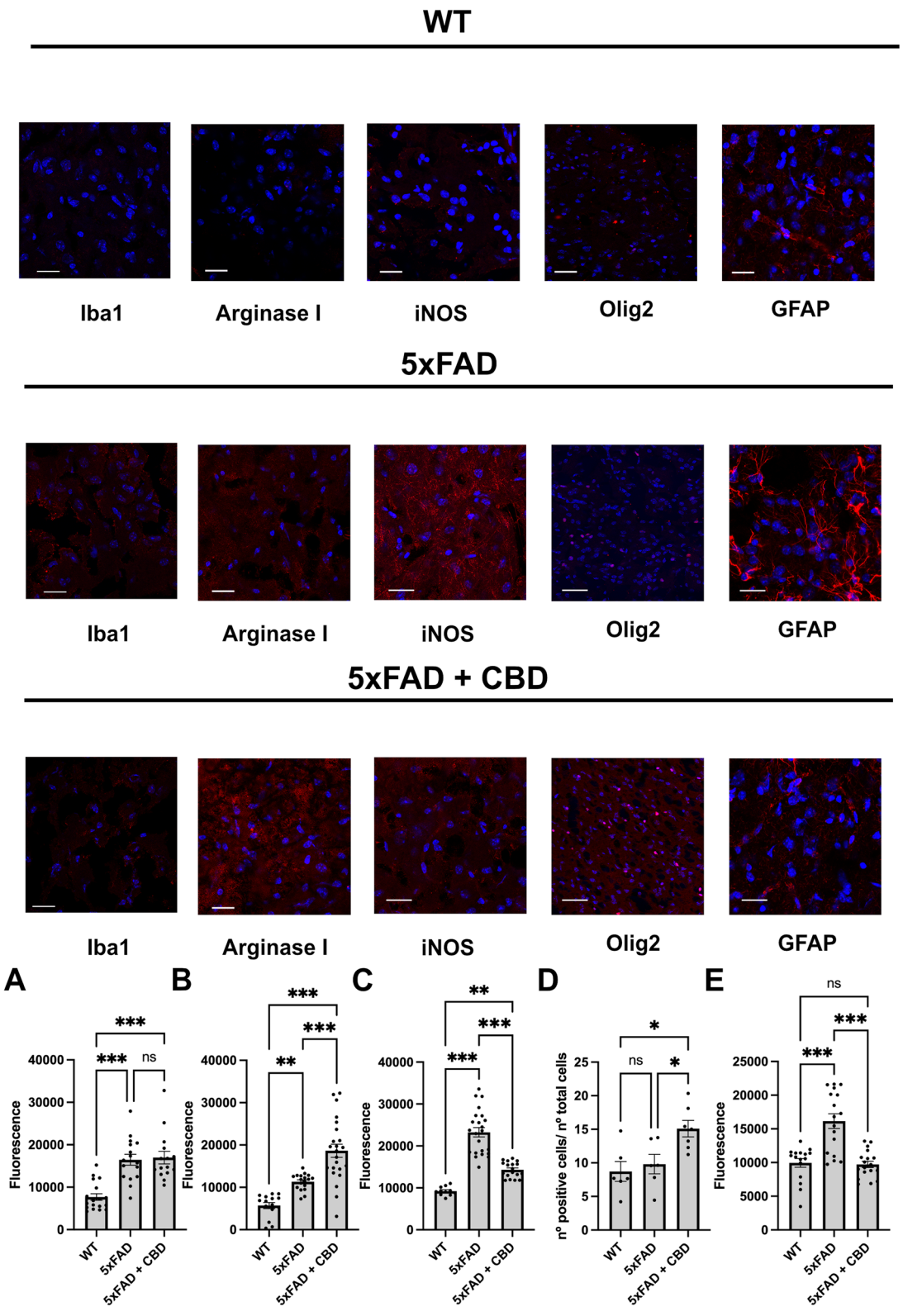

Fig. 4 (See legend on previous page.)

recognition test (NORT) 24 h after the first trial familiarization. Again, an important deficit in cognition was observed that was practically reverted in those animals after 4 weeks with daily injections of CBD at 10 mg/Kg (Fig. 6O).

These data present CBD as a potential target to combat not only molecular deficits in AD but also cognitive impairment, offering a promising therapeutic approach that could potentially slow disease progression and improve quality of life for AD patients.

CBD beneficial effects in AD models are mediated by CBD binding to cannabinoid receptors

CBD can bind different receptors such as serotoninergic, adenosine, opioid or cannabinoid receptors between others. We questioned if CBD beneficial effects on AD models were due to CBD binding to cannabinoid receptors. Then, neuronal primary cultures were pretreated with CB_1R selective antagonist, rimonabant, the CB_2R selective antagonist, SR144528 or vehicle for 30 min and subsequently treated with $Aß_{1-42}$ and stimulated with CBD or vehicle during 48 h. After, neuronal plasticity was determined by detecting neurite pattering. As observed in Fig. 7B, CBD recovered nerite loss due to $Aß_{1-42}$ treatment and this effect was counteracted by rimonabant but not by SR144528 (Fig. 7A, B). Furthermore, CBD improvement of cell viability after $Aß_{1-42}$ treatment was also blocked by rimonabant, but not by SR144528 (Fig. 7C). Finally, microglia primary cultures were pretreated with rimonabant, SR144528 or vehicle for 30 min and then treated with $Aß_{1-42}$ and CBD for 48 h more. Results indicated that both rimonabant and SR144528 blocked CBD induced increase in the neuroprotective M2 microglia phenotype, detected by Arginase I immunocytochemistry (Fig. 7D, E).

These results demonstrate that CBD beneficial effects on neuronal primary cultures treated with $Aß_{1-42}$ depend on CBD binding to CB_1R while CBD beneficial effects on $Aß_{1-42}$ induced activated microglia depend on CBD binding to both CB_1R and CB_2R.

Discussion

The findings of this study provide compelling evidence for the potential of cannabidiol as a therapeutic agent in Alzheimer's disease, demonstrating its ability to address multiple pathological aspects of the disease through both in vitro and in vivo methodologies. The results highlight CBD's capacity to reduce neuroinflammation, decrease pTau and Aβ aggregation, and promote neuroprotective effects, ultimately leading to improvements in spatial memory.

One of the key findings of this study is CBD's positive impact on neuronal plasticity. Neuronal plasticity, the brain's ability to form and reorganize synaptic connections, is crucial for learning, memory, and cognitive function. In AD, this plasticity is severely compromised, contributing to cognitive decline. The current study demonstrates that CBD treatment partially recovers neurite formation loss induced by $Aβ_{1-42}$, Tau, and pTau proteins. This effect on neuronal plasticity is particularly significant as it suggests CBD's potential to not only halt disease progression but also to promote neuronal repair and regeneration.

On the one hand, it has been proposed that CBD shows neuroprotection and antioxidant properties on β-amyloid peptide-induced toxicity in cultured rat PC12 cells [20] and also modulates microglial cell function in vitro [33, 34]. More specifically, it has been described that cannabinoids prevent Aβ-induced microglial activation. In this sense, we analyzed microglial phenotype in the AD mice model 5xFAD, that expresses human PSEN1 and APP transgenes with a total of five mutations linked to Alzheimer's disease: the M146L and L286 V mutations in PSEN and the Swedish (K670 N/M671L), Florida (I716 V), and London (V717I) mutations in APP. By immunohistochemistry assays we demonstrated that CBD treatment shows a neuroprotective role in the AD mouse model 5xFAD in cortical and hippocampal neurons by i) polarizing microglia to a neuroprotective M2 phenotype ii) decreasing the activated astroglia cells and iii) increasing the expression of oligodendrocyte precursor cells expression in cortex. These results agree with previous data showing that the endocannabinoid AEA shows anti-inflammatory and neuroprotective effects [35].

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Fig. 5 Iba1, Arginase I, iNOS, Olig2 and GFAP immunohistochemistry in hippocampal sections from 5xFAD mice injected with CBD. **A-E**. Hippocampal sections from 5xFAD mice (5xFAD), 5xFAD mice treated with i.p. injection of pure CBD (5xFAD + CBD) and control Wild Type mice (WT) were analyzed by immunohistochemistry using specific anti-Iba1, anti-Arginase I, anti-iNOS, anti-Olig2 and anti-GFAP antibodies. Confocal microscopy images (stacks of 3 consecutive panels) show protein immunoreactivity in red over Hoechst-stained nuclei (blue). Protein immunoreactivity was quantified using the Andy's algorithm Fiji's plug-in. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test was used for statistical analysis. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$. Scale bar: 20 µm

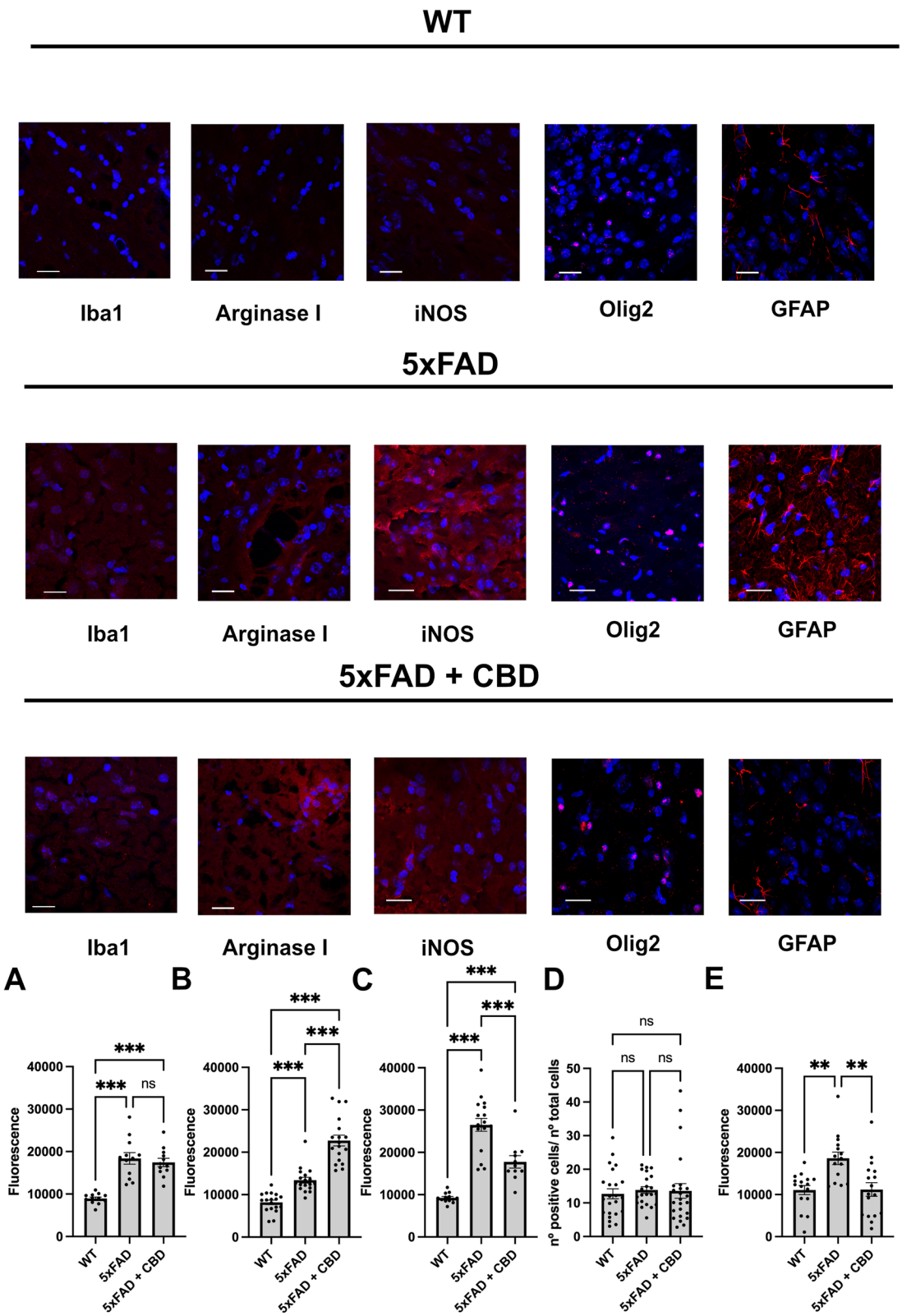


Fig. 5 (See legend on previous page.)

CBD can bind to both CB_1 and CB_2 cannabinoid receptors; however, different data point to CB_2R as responsible for microglia skew to a neuroprotective phenotype. For instance JWH-133, a selective CB_2R agonist, attenuates microglial activation and downregulates the concentrations of pro-inflammatory mediators in pneumococcal infection in vitro and in vivo [36]. Moreover, in the $APP_{Sw/Ind}$ AD mice model, CB_2R activation favors the M2 microglia phenotype [37, 38]. Consequently, the induced effect of CBD over microglia polarization seems to be mediated through CB_2R activation.

The role of plaques and tangles in AD is still unknown. Different studies propose a close relationship between plaque formation and Tau phosphorylation. For example, it has been demonstrated that Aβ-pE [3] and pTau Ser202/Thr205 levels strongly correlate in murine and human tissues, suggesting that Aβ-pE [3] could amplify Tau phosphorylation [39]. Somehow, experts believe that Aβ and pTau aggregates could contribute to blocking neuronal cell communication, inducing cell death, causing memory failure, behavioral changes, and other AD symptoms. On the other hand, Sativex administration, a cannabis extract used to combat neuropathic pain, spasticity, and other symptoms of multiple sclerosis, has shown an important reduction in phosphorylated Tau, GSK3 expression, and levels of Aβ oligomers in transgenic mice model and human [33, 40]. In this regard, CBD has shown an important inhibition of β-amyloid-induced Tau protein hyperphosphorylation and nitric oxide production [41, 42].

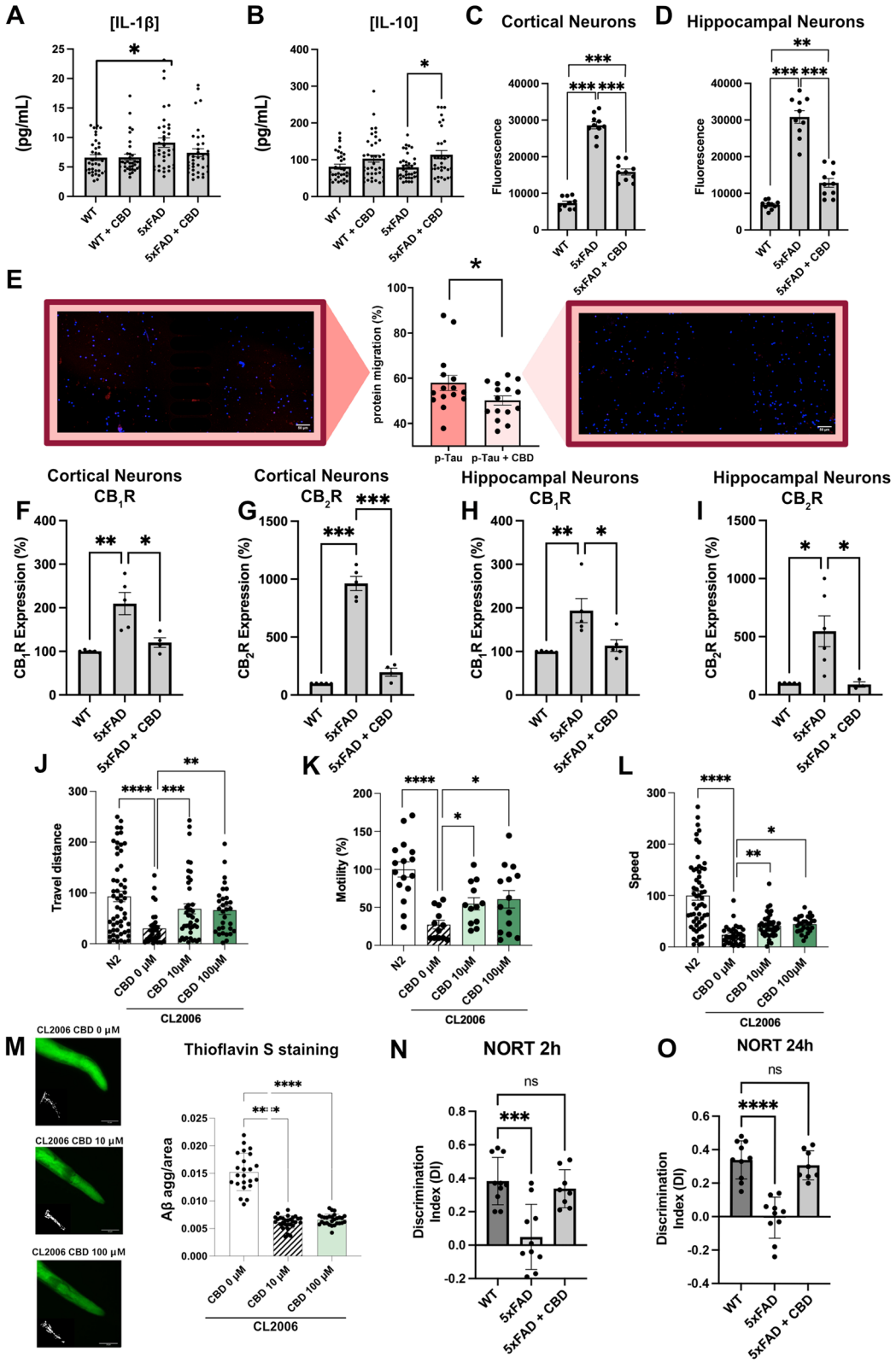
One possibility to stop AD progression would consist on reducing the anterograde Aβ and pTau axonal transport by reducing the spread of aberrant proteins between different neuronal regions and thus decreasing the neuroinflammation and neurodegeneration patterns. After analyzing $Aβ_{1–42}$, Tau, and pTau axonal transport from cortical to hippocampal neurons in microfluidic devices of two chambers, we observed that all $Aβ_{1–42}$, Tau, and pTau proteins had spread from one neuron to another. Moreover, treatment with CBD for 48 h significantly decreased $Aβ_{1–42}$, Tau and pTau axonal transport, being the effect over $Aβ_{1–42}$ the strongest decrease.

Our results show that CBD decreases the formation of ROS species induced by $Aß_{1–42}$, Tau, and pTau and recovers neuronal survival after both $Aβ_{1–42}$ peptide and NMDA-induced toxicity in primary cortical neurons. These data agree with those published in primary neuronal cultures that show a strong antioxidant effect of CBD against glutamate toxicity [43] and protection from amyloid β-$peptide_{25–35}$-induced neurotoxicity [44]. In this context, transmission electron microscopy assays have also shown that CBD treatment up-regulated the autophagy pathway in hippocampal neurons of APP/PS1 mice model of AD [45].

It is well accepted that cannabinoid CB_2 receptor is upregulated in proinflammatory processes both in the

(See figure on next page.)

Fig. 6 Effect of CBD over IL-10 and IL-1ß cytokine levels and short- and long-term memory on 5xFAD mice. **A**, **B** Microglia cultured from 5xFAD (5xFAD) and control mice (WT) were treated with CBD (200 nM) or vehicle. After 2 days, cell supernatant was collected, and proinflammatory (IL-1ß) (A) and anti-inflammatory (IL-10) (B) cytokines levels were quantified using an ELISA detection kit as described in methods. Values are the mean ± S.E.M. of 3 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used for statistical analysis (*$p < 0.05$, versus 5xFAD condition). **C**, **D** Quantification of $Aß_{1–42}$ aggregates was performed in sections of cortical (**C**) and hippocampal (**D**) 5xFAD mice treated with i.p. injection of pure CBD (5xFAD + CBD) or vehicle (5xFAD) and control Wild Type mice (WT) by immunohistochemistry. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used for statistical analysis (**$p < 0.01$, ***$p < 0.001$ versus WT animals). **E** Cortical neurons of 5xFAD mice were grown in microfluidic devices. On DIV 10, pTau (1 µM) protein was added into the top left well of each device. On DIV 11, neurons were treated with CBD (200 nM) or vehicle. Neurons were labeled with a rabbit anti-pTau (S396) antibody (1/100, Abcam ab109390) and subsequently marked with a Cy3 anti-rabbit (1/200, Jackson InmunoResearch) secondary antibody (red). Cells were imaged using a confocal microscope with 25X (yellow squares) and 40X (green squares) objectives (Zeiss LSM 880). The bar graph shows the quantification of the amount of fluorescence in the microfluidic channel opposite to the treated channel in comparison with total fluorescence. Values are the mean ± S.E.M. of 5 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used for statistical analysis *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. Scale bar: 20 µm. **F**, **G**, **H**, **I** Quantification of CB_2R and CB_1R mRNA extracted from the cortex (**F**, **G**) or hippocampus (**H**, **I**) of 5xFAD mice treated with i.p. injection of pure CBD (5xFAD + CBD) or vehicle (5xFAD) and control Wild Type mice (WT) was performed by qPCR analysis and normalized with the b-actin housekeeping gene. **J**, **K**, **L**, **M** The transgenic CL2006 strain (dvIs2 [pCL12(unc-54/human Aβ peptide 1–42 minigene) + rol-6(su1006)]) were treated with CBD 10 µM, 100 µM or vehicle and analyzed by motility assays (**J**, **K**, **L)** or ThS-positive particles detection (**M**). Representative images of the head region of the CL2006 strain from each group were tested. Results were compared to WT N2 animals treated with vehicle. Values are the mean ± S.E.M. of 4 independent experiments with 50–60 worms in each group. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used for statistical analysis. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ ****$p < 0.0001$. **N**,**O** A novel object recognition test (NORT) was performed 2 h (Short-term memory) or 24 h (Long term memory) after the first trial familiarization. Experiments were performed in samples from 28 different animals (10 WT animals, 10 5xFAD animals, and 8 5xFAD animals treated with CBD (10 mg/Kg)). One-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used for statistical analysis. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$



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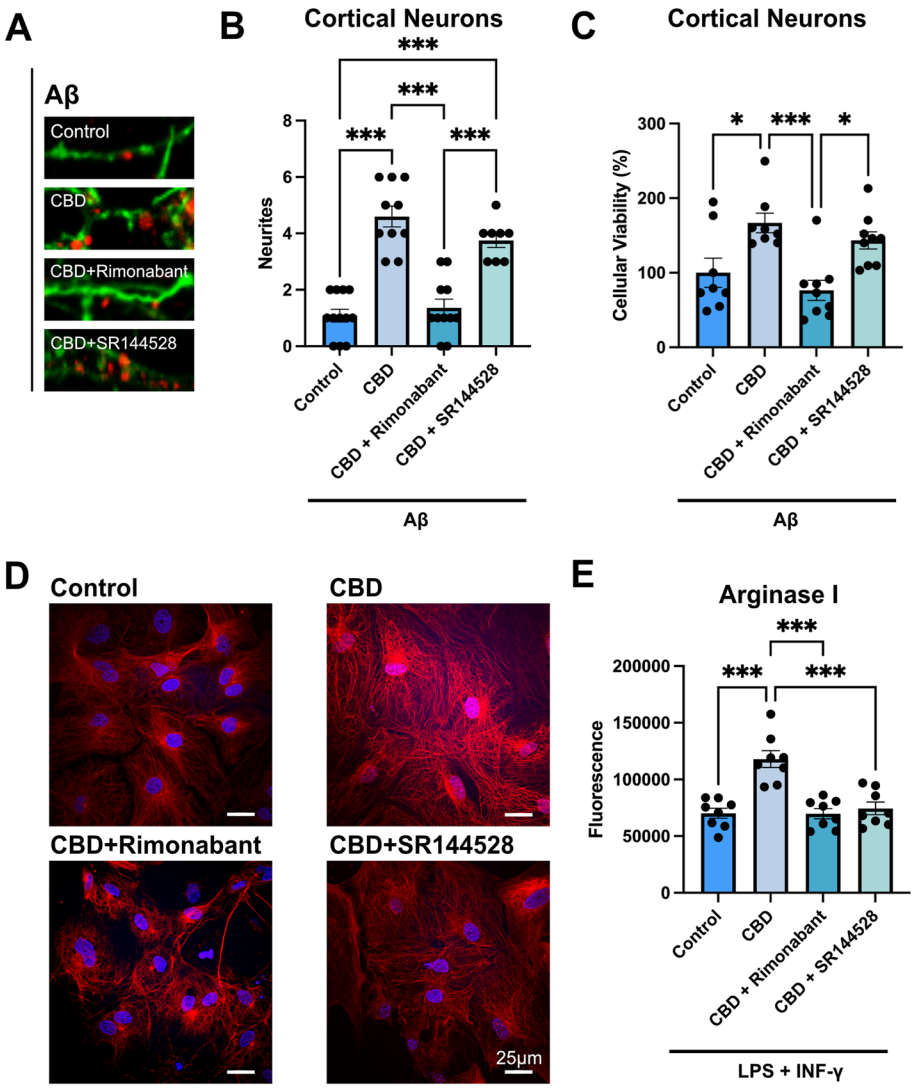

Fig. 7 Analysis of CB_1R and CB_2R antagonists eficacy to block CBD induced activity. **A-C** On DIV 10, Cortical neurons were incubated with CB_1R (Rimonabant) or CB_2R (SR144528) selective antagonists or vehicle for 30 min and subsequently treated with $A\beta_{1\text{-}42}$ (500 nM) and CBD (200 nM) or vehicle, for 48 h. Neurite patterning was detected by immunocytochemistry (**A**, **B**) and the Trypan Blue unstained (viable) and stained (nonviable) cells were counted separately, and the percentage of viability was calculated as the total number of viable cells/total number of cells × 100 (**C**). Neurite quantification was performed over segments of 15 µm. Values are the mean ± S.E.M. of 5 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ versus control condition. **D**, **E** Microglia primary cultures were activated with 1 µM lipopolysaccharide (LPS) and 200 U/mL interferon- (IFN-γ) and incubated for 30 min with rimonabant or SR144528 selective antagonists or vehicle. After, cells were treated with Aβ (500 nM) and CBD (200 nM) or vehicle for 48 h. Arginase I was detected by immunocytochemistry and quantified using the Andy's algorithm Fiji's plug-in. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Bonferroni's multiple comparison post-hoc test was used for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar: 25 µm

periphery, such as in the inflammatory bowel disease [46], and in the central nervous system, being upregulated in the hippocampus and entorhinal cortex of AD patients in neuritic plaque-associated microglia [47, 37]. In the same line, we have detected an upregulation of not only CB_2R, but also CB_1R, although at lower levels, in 5xFAD mice. Interestingly, the expression of both cannabinoid receptors returned to levels like those of control mice after daily CBD injections for one month.

Other well-established effect of CBD is its capacity to decrease AD-associated gene expression, including genes coding for Aβ production, such as the beta- and gamma-secretase or proteins responsible for Tau phosphorylation [48]. Likewise, in the hippocampus of Aβ-induced

neuroinflammation mice, CBD avoids the expression of proinflammatory glial peptides [48]. In this sense, here we described that CBD treatment decreases $A\beta_{1-42}$, Tau and pTau protein aggregation. Due to protein aggregate-induced cytotoxicity, this significant result could underlie other beneficial effects induced by CBD. In parallel, the involvement of cannabidiol in promoting neurogenesis has been shown [24]. To deepen in CBD-induced effects, we further analyzed neurite formation in neuronal primary cultures treated with $A\beta_{1-42}$, Tau, and pTau proteins in the presence or in the absence of CBD. The capacity of CBD to recover neuronal plasticity has been explained in the analyzed data. In this sense, we have observed that CBD treatment can partially decrease the neurite formation loss induced by $A\beta_{1-42}$ and also Tau and pTau proteins treatment.

It has been demonstrated that hemiparkinsonian females in the estrus phase and males show different answers in the nociceptive tests after different doses of CBD therapy. Thus, CBD therapy seems effective for parkinsonism-induced nociception [49]. In this line, as control, it was tested if α-synuclein, the characteristic protein aggregates of Parkinson's disease, could alter neuronal plasticity. An important decrease in neurite formation was observed, however, CBD treatment was not able to counteract this effect, contrary to what happens upon $A\beta_{1-42}$, Tau and pTau proteins treatment. This result was intriguing, due to the fact that CBD was able to reduce α-synuclein aggregation.

Finally, the capacity of CBD to combat cognitive impairment in AD was evaluated. Most studies point to the involvement of CBD in preventing cognition deficits in neurodegenerative diseases. Some examples are the improvement of social recognition memory in the double transgenic APP ×PS1 mouse model of AD [50], the beneficial effects observed in neuropsychiatric symptoms in AD patients after CBD consumption [51, 52], the preserved memory in APP/PS1 transgenic mice when Δ^9-THC, CBD or both are chronically administrated in botanical extracts, the attenuation of contextual conditioned fear in rats induced by cannabidiol [53] or the prevention of the development of a social recognition deficit in AD transgenic mice [50]. Despite the large amount of data, there continues to be some controversy, as the improvement in spatial memory detected in 14-month-old female TAU58/2 transgenic mice [54] was not observed in 4-month-old male TAU58/2 transgenic mice [55]. In our hands, the NORT test presents CBD as a potential tool to combat cognitive impairment as it demonstrates that daily CBD injections can revert short- and long-term memory deficits in AD mice model 5xFAD. We have also used the CL2006 strain, which contains the Punc-54::$A\beta_{1-42}$ transgene, as a model for ß-amyloid toxicity, although it does not show any tauopathy. The CL2006 strain treated with CBD showed improved motility and a decrease in Aß aggregation. These results highlight the capacity of CBD to delay the progressive onset paralysis in CL2006 strain, and thus suggest CBD as a promising compound to mitigate AD progression.

Therefore, in this work we have demonstrated a wide spectrum of beneficial effects induced by CBD, ranging from the molecular to the behavioral level, and which may be based on the reduction of the formation of beta-amyloid plaques and the prevention of phosphorylated Tau tangles [48]. More studies are required to underscore the mechanisms of action of CBD and to translate the preclinical studies into clinical assays.

The multifaceted effects of CBD observed in this study – from reducing pathological protein aggregation and spread, to modulating neuroinflammation and enhancing neuronal plasticity – highlight its potential as a comprehensive treatment approach for AD. Unlike current AD treatments that typically target single aspects of the disease, or the new anti-amyloid monoclonal antibodies, that can produce little benefits while showing harmful effects [6], CBD's broad spectrum of effects addresses multiple pathological processes simultaneously. Moreover, despite the capacity of CBD to bind different receptors, these effects are due to CBD binding to canabinoid CB_1R in neurons and to CB_1R and CB_2R in microglia.

In conclusion, this study provides robust evidence for CBD's therapeutic potential in AD, demonstrating its ability to modulate key pathological processes and improve cognitive outcomes. The findings on neuronal plasticity are particularly promising, suggesting that CBD may not only slow disease progression but also promote neural repair. While further research is needed to fully elucidate the mechanisms of CBD's effects and to translate these findings to human patients, this study lays a strong foundation for the continued exploration of CBD as a novel treatment strategy for Alzheimer's disease.

Material and methods

Reagents

CBD was purchased from Cerilliant (Texas, US). The antibodies used were the following: Monoclonal mouse anti-Arginase I (ref. 610,708, BD Bioscience), monoclonal mouse anti-iNOS (MA5-17,139, Invitrogen), polyclonal goat anti-GFAP (PA5-18,598, Invitrogen), polyclonal goat anti-Iba1 (ab107159, Abcam), monoclonal mouse anti-Olig2 (ab216020, Abcam), polyclonal rabbit anti-Nectin 3 (ab63931, Abcam) and polyclonal rabbit anti-F-actin antibody fused to an Alexa 488 fluorophore (A12379, ThermoFisher). N-methyl-D-aspartate (NMDA) was purchased from Tocris Bioscience (Bristol, United Kingdom). α-synuclein was prepared as described [56] and

Tau and pTau proteins were kindly provided by Prof. J. Avila (CBM, UAM-CSIC, Madrid, Spain). Detailed descriptions of the elaboration and processing of proteins can be found elsewhere [57].

Neuronal primary cultures

To prepare primary cultures of cortical and hippocampal neurons, brains from fetuses of pregnant C57BL/6 J mice were removed (gestational age: 19 days). Neurons were isolated as described in Hradsky et al. [58]. Briefly, the samples were dissected and, after a careful removal of the meninges, digested for 20 min at 37ºC with 0.25% trypsin. Trypsinization was stopped by adding an equal volume of culture medium (supplemented DMEM). Cells were brought to a single-cell suspension by repeated pipetting followed by passage through a 100 µm-pore mesh. Pelleted (5 min, 200 ×g) cells were resuspended in supplemented DMEM and seeded at a density of 3.5×10^5cells/mL in 6-well plates. The day after, medium was replaced by neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 2% (v/v) B27 medium (GIBCO). Neuronal cultures were assayed 12 days after. Using NeuN as a marker, it was detected a percentage of neurons in the culture > 90%.

Microglial primary cultures

Primary cultures of microglia were obtained from 2–3-day-old pups. Cells were isolated as described in [59] and plated at a confluence of 40,000 cells/0.32 cm^2. Briefly, the samples were dissected, carefully stripped of their meninges and digested with 0.25% trypsin for 20 min at 37 ºC. Trypsinization was stopped by repeated washes with Hanks0 balanced salt solution (HBSS composition: 1.26 mM $CaCl_2$, 5 mM KCl, 0.44 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 137 mM NaCl, 0.34 mM Na_2HPO_4 and 10 mM Hepes, pH: 7.4). Cells were brought to a single cell suspension by repeated pipetting followed by passage through a 100 µM-pore mesh. Cells were then resuspended in supplemented DMEM and seeded at a density of 3.5×10^5 cells/mL in 96 well plates for detection of inflammatory and anti-inflamatory cytokines. Cultures were maintained at 37 ºC in a humidified 5% CO_2 atmosphere. Immunodetection of specific markers (CD-11b) showed that microglia preparations contained at least 98% microglial cells.

Preparation of human α-synuclein fibrils

α-synuclein fibrils were prepared by shaking purified recombinant α-synuclein as described [57, 60]. Briefly, purified recombinant α-synuclein (5 mg/ml) containing 30 mM Tris–HCl (pH 7.5), 10 mM DTT, and 0.1% sodium azide were incubated for 7 days at 37ºC in a horizontal shaker at 200 rpm, then ultracentrifuged at 113,000 ×g for 20 min at 25ºC. The pellets were washed with saline and ultracentrifuged as before. The resulting pellets were collected as α-synuclein fibrils and resuspended in 30 mM Tris–HCl (pH 7.5). The fibrils were fragmented using a cup horn sonicator (Sonifier® SFX, Branson) at 35% power for 180 s (total 240 s, 30 s on, 10 s off) [57]. Before use, aliquots were left at room temperature and placed in PBS 1× (pH 7.2) to a final concentration of 0.1 µg/µL. These preparations were subjected to 60 pulses of sonication (runtime 30 s: 0.5 s on, 0.5 s off in a BBR03031311 digital SONIFIER sonicator). Sonicated fibril preparations were diluted in pre-warmed medium and immediately added to cells.

Viability assay

Neurons were resuspended with neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 2% (v/v) B27 (GIBCO). Trypan blue staining was performed mixing 1 part of 0.4% trypan blue and 1 part of cell suspension in a plastic tube. After ~3 min of incubation at room temperature, 10 µl of the mixture was sampled in a Neubauer chamber. The unstained (viable) and stained (nonviable) cells were counted separately, and the percentage of viability was calculated as: total number of viable cells/total number of cells ×100.

Detection of ROS levels

Cortical neuronal primary cultures distributed in 96 well microplates were treated with $A\beta_{1-42}$ (500 nM), Tau (1 µM), α-synuclein (4 µM) or vehicle and subsequently stimulated with 200 nM CBD or vehicle for 48 h. The day of the experiment, cells were washed twice with PBS and treated with a solution of Dihydroethidium (ThermoFisher) 5 µM diluted with PBS for 10 min and the fluorescence signal was determined in fluorimeter VICTOR Nivo Multimode Microplate Reader (Perkinelmer, Waltham, Massachusetts) using the excitation/emission filters of 518/605 nm at 20 min.

Neurite patterning determination

Cortical neuronal primary cultures were treated with rimonabant (1 µM) or SR144528 (1 µM) for 30 min and subsequently treated with $A\beta_{1-42}$ (500 nM), Tau (1 µM), pTau (1 µM) or α-synuclein (4 µM) and with CBD (200 nM) or vehicle for 48 h on DIV 10. Then, cells were fixed in 4% paraformaldehyde for 15 min and then washed twice with PBS containing 20 mM glycine followed by permeabilization with the same buffer containing 0.2% Triton X-100 (15 min incubation). Samples were treated for 1 h with blocking solution (PBS containing 1% bovine serum albumin) and labeled with polyclonal rabbit anti-Nectin 3 antibody (Abcam, 1/1000). Neurons were

detected with anti-F-actin antibody fused to an Alexa 488 fluorophore (ThermoFisher, 1/400). Then, sections were incubated at RT for 2 h with a Cy3-conjugated anti-rabbit secondary antibody (1/200, 711–166-152, Jackson ImmunoResearch). Samples were washed several times with PBS and mounted with 30% Mowiol (Calbiochem, San Diego, CA, USA). Nuclei were stained with Hoechst (1/100). Samples were observed under a Zeiss 880 confocal microscope (Leica Microsystems, Wetzlar, Germany). Quantification of neurite formation was performed over segments of 15 µm. Each red dot represents a neurite formation.

Protein aggregation detection

Cortical neuronal primary cultures were treated with $A\beta_{1-42}$ (500 nM), Tau (1 µM), α-synuclein (4 µM) or vehicle and subsequently stimulated with 200 nM CBD or vehicle for 48 h. Then, cells were fixed in 4% paraformaldehyde for 15 min and then washed twice with PBS containing 20 mM glycine before permeabilization with the same buffer containing 0.2% Triton X-100 (15 min incubation). The samples were treated for 1 h with blocking solution (PBS containing 1% bovine serum albumin) and labeled with a rabbit anti-Aβ antibody (1/100, ab201060), a rabbit anti-Tau antibody (1/100, Abcam ab32057), a rabbit anti-phospo-Tau (S396) antibody (1/100, Abcam ab109390) or a mouse anti-α-synuclein antibody (1/100, ab1903) and subsequently marked with a Cy3 anti-rabbit (1/200, Jackson InmunoResearch) secondary antibody (red). Following 2 h of incubation, cells were washed and subsequently imaged using confocal microscope with 25X (yellow squares) and 40X (green squares) objectives (Zeiss LSM 880).

Age-synchronized CL2006 worms were fixed in 4% paraformaldehyde/PBS, pH 7.5, for 24 h at 4 °C, and permeabilized in 5% fresh β-mercaptoethanol, 1% Triton X-100, 125 mm Tris, pH 7.5, at 37 °C for another 24 h. The nematodes were stained with 0.125% Thioflavin S (ThS) (CAS# 1326–12-1, Sigma Aldrich, St Louis, MO, USA) in 50% ethanol for 2 min, de-stained in 50% EtOH for 2 min, washed three times with PBS, and transferred in approximately 10 µL volume on a droplet of Fluoromount G (CAT#17,984–25, Electron Microscopy Sciences, Hatfield, PA, USA) on a glass slide for microscopy. Fluorescence images were acquired using a 20 Å ~ objective of a fluorescence microscope. Amyloid deposits in the head regions of the worms were quantified by counting the number of ThS-positive spots using ImageJ, and were expressed as Aß deposits/anterior area.

Protein trafficking

Microfluidic standard neuronal devices (with 150 µm microgroove barriers located between the channels; AXISTM AXon Investigation System, EMD Millipore) were handled according to the manufacturer's protocol.

The pure cortical neurons from mouse embryos (E19) were isolated from neuronal primary cultures. Before neuronal seeding, each assembled device was coated with poly-D-lysine (0.1 mg/mL, Gibco A3890401) for 1 h. After, ten microliters of cell suspension (5–6 million neurons/mL) were introduced to both chambers of each device by passive pumping. After 30 min incubation at 37°C to allow cell attachment, 200 µL of neurobasal medium (supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 2% (v/v) B27 supplement (Gibco)) was added. Neurons were maintained at 37 °C in humidified 5% CO_2 atmosphere. Medium was replaced every three days in each device (a 50 µL difference in media volume was maintained to prevent spontaneous diffusion).

On DIV 10, once the axons fully crossed the microgrooves (150 µm distance) into the axonal compartment of a device, $A\beta_{1-42,}$Tau, pTau, or α-synuclein were added into the compartment A of each device and maintained for 48 h. On DIV 12, neurons were treated with CBD (200 nM) or vehicle for 48 h more. Neurons were labeled with a rabbit anti-Aβ antibody (1/100, ab201060), a rabbit anti-Tau antibody (1/100, Abcam ab32057), a rabbit anti-phospo-Tau (S396) antibody (1/100, Abcam ab109390) or a mouse anti-α-synuclein antibody (1/100, ab1903) and subsequently marked with a Cy3 anti-rabbit (1/200, Jackson InmunoResearch) secondary antibody (red). Following 2 h of incubation, cells were washed and subsequently imaged using a confocal microscope Zeiss LSM 880 with 25X and 40X objectives.

CL2006 animal model

The study employed two strains of C. elegans: the WT N2 and the transgenic CL2006 strain (dvIs2 [pCL12(unc-54/human Aβ peptide 1–42 minigene) + rol-6(su1006)]). Both strains were obtained from the C. elegans Genetic Center (CGC). The N2 strain was maintained at 20 °C, while the CL2006 strain was kept at 16 °C in a temperature-controlled incubator on a solid nematode growth medium (NGM) seeded with Escherichia coli (E. coli) OP50 strain as a food source.

C. elegans motility assay

For the CBD effects, we followed a detailed procedure as described in [61]. In essence, a suspension of the L1 larval stage, containing 25–35 larvae, was incubated with 20 µL of inactivated E. coli and 25 µL of different CBD concentrations (10 µM and 100 µM) in a 96-well plate. The movements of the worms were then meticulously recorded for 20 min using the WMicroTracker mini (Phylumtech S.A., Argentina). The travel distance

and movement speed were further evaluated using the WMicrotracker SMART equipment (Phylumtech S.A., Argentina).

5xFAD mouse model

Wild-type (Wt) and 5xFAD (n= 28) male mice of 4-month-old were used to perform cognitive and molecular studies. We divided these mice randomly into three groups: Wt Control (n= 10), 5xFAD Control (n= 10), and 5xFAD treated with the phytocannabinoid cannabidiol (CBD) (5xFAD CBD (10 mg/Kg); n= 8). The sample size for the intervention was chosen following previous studies in our laboratory and using one of the available interactive tools (http://www.biomath.info/ power/index.html). Experimental groups received either one dose of vehicle (2% w/v, Tween 80 (Fischer, USA) or one daily dose of 10 mg/Kg/day of CBD dissolved in 2% Tween 80 via oral gavage for 4 weeks. Animals had free access to food and water and were kept under standard temperature conditions (22 ± 2 °C) and 12 h:12 h light–dark cycles (300 lx/0 lx). After the treatment period, cognitive tests were performed in the animals.

Studies and procedures involving mouse behavior test, brain dissection and extractions followed the ARRIVE and standard ethical guidelines (European Communities Council Directive 2010/63/EU and Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research, National Research Council 2003) and were approved by Bioethical Committees from the University of Barcelona and the Government of Catalonia. All efforts were made to minimize the number of animals used and their suffering.

Brain sampling

Mice were sacrificed under deep anesthesia (i.e., injection of diazepam/ketamine) and transcardially perfused with saline and 4% paraformaldehyde. Brains were harvested and embedded in paraffin to obtain coronal Sects. (30 µm thick) using a cryostat LEICA CM3050 S (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry

Brain slices were fixed in 4% paraformaldehyde for 15 min and then washed twice with PBS containing 20 mM glycine before permeabilization with the same buffer containing 0.2% Triton X-100 (15 min incubation). The samples were treated for 1 h with blocking solution (PBS containing 1% bovine serum albumin) and labeled with monoclonal mouse anti-Arginase I (1/100, ref. 610,708, BD Bioscience), monoclonal mouse anti-iNOS (1/200, MA5-17,139, Invitrogen), polyclonal goat anti-GFAP (1/100, PA5-18,598, Invitrogen), polyclonal goat anti-Iba1 (1/100, ab107159, Abcam) or monoclonal mouse anti-Olig2 (1/200, ab216020, Abcam) or rabbit anti-Aβ antibody (1/100, ab201060) as primary antibodies and a Cy3-conjugated anti-mouse IgG (1/200, 715–166-150, Jackson ImmunoResearch), a Cy3-conjugated anti-goat IgG (1/200, 705–167-003, Jackson ImmunoResearch) or a Cy3 anti-rabbit (1/200, Jackson InmunoResearch) as secondary antibodies. The samples were washed several times and mounted with 30% Mowiol (Calbiochem, San Diego, CA, USA). Nuclei were stained with Hoechst (1/100). Samples were observed under a Zeiss 880 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Detection of inflammatory and anti-inflamatory cytokines

Microglia derived from 5xFAD mice (5xFAD) and control Wild Type mice (WT) were cultured in 96-well plates. After a 12-day incubation period, cells were exposed to CBD (200 nM) or vehicle. Following a 5-day treatment, the cell supernatant was harvested and centrifugated for 5 min at 500xG. Levels of proinflammatory (IL-1ß) and anti-inflammatory (IL-10) cytokines were quantified using an ELISA detection kit (KE10003, Proteintech) according to supplier instructions.

Polymerase chain reaction (PCR)

The hippocampus and cortex were isolated from C57BL/6 J mice at age 4-month-old. After decapitation, the hippocampus and cortex were dissected and immediately transferred to PBS. mRNAs were isolated with TRIzol™ Reagent protocol and treated with RNase-Free DNase (Qiagen). The purity was verified with a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Single strand cDNA was synthesized from the extracted RNA (2 µg) using a MLV-reverse transcriptase (Fisher Scientific), Random Hexamers and oligo-dT. The qPCR was conducted with the cDNA and PowerUp SYBR Green Master Mix (AppliedBiosystems). Determinations were conducted using real-time PCR technique on an Applied Biosystems QuantStudio3 device in 96-well plates.

The primer pair for the gene that codifies for CB_1R was CCAAGAAAAGATGACGGCAG (forward) and AGG ATGACACATAGCACCAG (reverse). The primer pair for the gene that codifies for CB_2R was GGGTCGACT CCAACGCTATC (forward) and AGGTAGGCGGGT AACACAGA (reverse). Primers for beta-actin were used as an internal control (forward, TTGACATCCGTAAAG ACCTC; backward, AGGAGCCAGAGCAGTAAT). Each experiment included a template-free control. The PCR products were analyzed by the DNA melting curve. The relative quantities of Cnr1 and Cnr2 PCR products were estimated with respect to the amount of the house keeping gene beta-actin product using the ΔCt method: %beta-actin = (2 Ct of Gapdh – Ct of CB_2R) × 100.

Novel object recognition test

The protocol employed was a modification of Ennanceur and Delacour (1988). The experimental apparatus used for this test was a 90-degree, two-arm, 25-cm-long, 20-cm-high maze of black polyvinyl chloride. Light intensity in the middle of the field was 30 lx. Briefly, the first mouse was individually habituated to the apparatus for 10 min per day for 3 days. On day 4, the mice were allowed to freely explore two identical objects (A and A or B and B) placed at the end of each arm for 10-min acquisition trial (First trial-familiarization). The mouse was then removed from the apparatus and returned to its home cage. Then, a 10-min retention trial (second trial) was carried out for 2 (Short-term memory) or 24 h (Long term memory) later. During the Short-term memory retention, objects A and B were placed in the maze replacing one of them (A and B or B and A), and the times that the animal took to explore the new object (TN) and the old object (TO) were recorded. The mice were tested again 24 h after the acquisition trial, with a new object and an object identical to the new one in the previous trial (A and C or B and C). The time that mice explored the Novel object (TN) and Time that mice explored the Old object (TO) were measured from the video recordings from each trial session. A Discrimination index (DI) was defined as (TN-TO)/(TN + TO). Exploration of an object by a mouse was defined as pointing the nose towards the object at a distance ≤ 2 cm and/or touching it with the nose. Turning or sitting around the object was not considered exploration. In order to avoid object preference biases, objects A and B were counterbalanced so that one-half of the animals in each experimental group were first exposed to object A and then to object B, whereas the other half first saw object B and then object A. The maze, the surface, and the objects were cleaned with 70% ethanol between the animals' trials to eliminate olfactory cues.

Data handling and statistical analysis

Data were analyzed blindly. Data are presented as the mean ± SEM. Statistical analysis was performed with SPSS 18.0 software. The test of Kolmogorov–Smirnov with the correction of Lilliefors was used to evaluate normal distribution and the test of Levene to evaluate the homogeneity of variance. Significance was analyzed by one-way ANOVA and Bonferroni's multiple comparison post hoc test. Significance was considered when $p < 0.05$.

Authors' contributions

IR, JL, JBR, AB-S and CG-F: Data Curation, Formal Analysis, Investigation, Visualization, IR-R, RF and MP: Writing - original draft; and Writing - review & editing. GN: Conceptualization, Funding Acquisition, Investigation, Project Administration, Supervision, Validation, Writing - original draft; and Writing - review & editing.

Funding

This work was partially supported by the Spanish Ministry of Economy and Innovation with FEDER funds (PID2020-113430RB-I00 and 2021-126600OB-I00). The research group of the University of Barcelona is considered of excellence (grup consolidat #2021 SGR 554 00304) by the Regional Catalonian Government. Generalitat de Catalunya (2021 SGR 00304).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Animal handling, sacrifice, and further experiments were conducted according to the guidelines set in Directive 2010/63/EU of the European Parliament and the Council of the European Union that is enforced in Spain by National and Regional organisms; the 3R rule (replace, refine, reduce) for animal experimentation was also taken into account. By the current legislation, protocol approval is not needed if animals are sacrificed to obtain a specific tissue. All animal experiments have been conducted according to ARRIVE guidelines.

Competing interests

The authors declare no competing interests.

Received: 17 February 2025 Accepted: 7 May 2025
Published online: 20 May 2025

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