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Cannabidiol and Other Cannabinoids Reduce Microglial Activation In Vitro and In Vivo: Relevance to Alzheimer's Disease

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ABSTRACT

Microglial activation is an invariant feature of Alzheimer's disease (AD). It is noteworthy that cannabinoids are neuroprotective by preventing β-amyloid (Aβ)-induced microglial activation both in vitro and in vivo. On the other hand, the phytocannabinoid cannabidiol (CBD) has shown anti-inflammatory properties in different paradigms. In the present study, we compared the effects of CBD with those of other cannabinoids on microglial cell functions in vitro and on learning behavior and cytokine expression after Aβ intraventricular administration to mice. CBD, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-*d*,*e*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone [WIN 55,212-2 (WIN)], a mixed CB_1/CB_2 agonist, and 1,1-dimethylbutyl-1-deoxy-Δ^9-tetrahydrocannabinol [JWH-133 (JWH)], a CB_2-selective agonist, concentration-dependently decreased ATP-induced (400 μM) increase in intracellular calcium ($[Ca^{2+}]_i$) in cultured N13 microglial cells and in rat primary microglia. In contrast, 4-[4-(1,1-dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethyl-bicyclo[3.1.1]hept-2-ene-2-methanol [HU-308 (HU)], another CB_2 agonist, was without effect. Cannabinoid and adenosine A_{2A} receptors may be involved in the CBD action. CBD- and WIN-promoted primary microglia migration was blocked by CB_1 and/or CB_2 antagonists. JWH and HU-induced migration was blocked by a CB_2 antagonist only. All of the cannabinoids decreased lipopolysaccharide-induced nitrite generation, which was insensitive to cannabinoid antagonism. Finally, both CBD and WIN, after subchronic administration for 3 weeks, were able to prevent learning of a spatial navigation task and cytokine gene expression in β-amyloid-injected mice. In summary, CBD is able to modulate microglial cell function in vitro and induce beneficial effects in an in vivo model of AD. Given that CBD lacks psychoactivity, it may represent a novel therapeutic approach for this neurological disease.

Introduction

Alzheimer's disease (AD) is characterized by β-amyloid (Aβ) deposition in senile plaques, neurofibrillary tangles, selective neuronal loss, and progressive cognitive deficits. Another invariant feature of the neurological disease is glial activation and is considered to be responsible of the ongoing inflammatory condition occurring in AD brain (Akiyama et al., 2000). Microglial activation is also present in AD experimental models in vivo, such as rats injected with Aβ either focally or intraventricularly, and in transgenic models of the

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ABBREVIATIONS: AD, Alzheimer's disease; Aβ, β-amyloid peptide; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; WIN, WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-*d*,*e*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone; JWH, JWH-133; JWH-133, 1,1-dimethylbutyl-1-deoxy-Δ^9-tetrahydrocannabinol; HU, HU-308, 4-[4-(1,1-dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethyl-bicyclo[3.1.1]hept-2-ene-2-methanol; LPS, lipopolysaccharide; iNOS, inducible nitric-oxide synthase; SCR, scrambled peptide; FCS, fetal calf serum; PCR, polymerase chain reaction; ANOVA, analysis of variance; SR1, SR141716, *N*-piperidino-5-(4-chlorophenyl)-l-(2, 4-dichlorophenyl)-4-methylpyrazole-3-carboxamide; SR2, SR144528, *N*-[(1S)-*endo*-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; CGS, CGS21680, 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine; ZM, ZM241385, 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl}phenol; VDM11, *N*-(4-hydroxy-2-methylphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide.

disease (Masliah et al., 1996; Jantzen et al., 2002; Ramírez et al., 2005). Furthermore, Aβ addition to cultures induces microglial activation, reflected in increased secretion of toxic species such as NO and cytokines (Combs et al., 2001; Ramírez et al., 2005). Given that microglial activation may result in neurodegeneration, currently, it is considered that pharmacological targeting of microglial activity may be a feasible therapeutic strategy for neurodegenerative diseases in general and for AD in particular.

Cannabinoids, whether plant-derived, synthetic, or endocannabinoids, exert their functions through activation of cannabinoid receptors, two of which have been well characterized to date: CB_1 and CB_2 (Howlett et al., 2002; Piomelli, 2003). Cannabinoids are neuroprotective against excitotoxicity and acute brain damage, both in vitro and in vivo (van der Stelt et al., 2002; Mechoulam and Shohami, 2007). Several mechanisms account for the neuroprotection afforded by this type of drug such as blockade of excitotoxicity, reduction of calcium influx, antioxidant properties of the compounds, or enhanced trophic factor support. A decrease in proinflammatory mediators brought about by cannabinoids (Walter and Stella, 2004) may be also involved in their neuroprotection. Indeed, several reports have shown that cannabinoids reduce NO and cytokine generation and/or their mRNA expression in microglia cultures (Waksman et al., 1999; Puffenbarger et al., 2000; Facchinetti et al., 2003). Cannabidiol (CBD), the major plant-derived nonpsychotropic constituent of marijuana, is of potential therapeutic interest in different disease conditions (e.g., inflammation) (Mechoulam et al., 2007). Oral treatment with CBD decreases edema and hyperalgesia in a rat paw model of carrageenan-induced inflammation (Costa et al., 2004). A single dose of the phytocannabinoid reduces tumor necrosis factor-α (TNF-α) levels in lipopolysaccharide (LPS)-injected mice and improves collagen-induced arthritis (Malfait et al., 2000). CBD binds to CB receptors with low affinity (Showalter et al., 1996) and may exert cannabinoid receptor-independent effects as well. For instance, CBD inhibition of the equilibrative nucleoside transporter, which results in enhancement of adenosine signaling through A2A receptors, is involved in its immunosuppressive effects (Carrier et al., 2006).

In the context of AD, CBD has shown to be neuroprotective against the Aβ addition to cultured cells. Several mechanisms seem to be involved, including CBD reduction of oxidative stress and blockade of apoptosis (Iuvone et al., 2004), τ-phosphorylation inhibition through the Wnt/β-catenin pathway (Esposito et al., 2006a), and the decrease in iNOS expression and nitrite generation (Esposito et al., 2006b). We have shown that cannabinoids prevent Aβ-induced neurodegeneration by reducing microglial activation (Ramírez et al., 2005), and both CB_1 and CB_2 receptors in microglia participate in such an action. More importantly, cannabinoids prevented microglial activation, loss of neuronal markers, and cognitive deficits in Aβ-treated rats (Ramírez et al., 2005). In vivo, CBD also suppressed neuroinflammation in mice injected with Aβ into the hippocampus by inhibiting the increased glial fibrillary acidic protein and iNOS expression, along with nitrite and interleukin-1β generation (Esposito et al., 2007). However, previous studies have not investigated the effects of CBD on microglial cell function.

Taken together, these results prompted us to study the effects CBD in comparison with other cannabinoids on functions involved in microglial activation, namely intracellular calcium levels, migration, and NO generation in cultured microglial cells. To that end, we have used [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-*d*,*e*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone [WIN 55,212-2 (WIN)], a mixed CB_1/CB_2 agonist (Howlett et al., 2002) and 1,1-dimethylbutyl-1-deoxy-Δ^9-tetrahydrocannabinol [JWH-133 (JWH)] and 44-[4-(1,1-dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-methanol [HU-308 (HU)]s as selective CB_2 agonists (Hanus et al., 1999; Huffman et al., 1999). Furthermore, we assessed whether these cannabinoids administered to Aβ-injected mice were able to counteract inflammation and the cognitive deficits.

Materials and Methods

Materials

Aβ1–40 (NeoMPS, Strasbourg, France) was dissolved in phosphate buffer (1.72 mg/ml) and aged at 37°C for 24 h ("fibrillar" peptide), being vortexed several times during that period, and aliquots were stored at −80°C until use. The control peptide was not subjected to ageing ("soluble" peptide). Aggregation of all peptides was confirmed by electron microscopy after staining with 2% uranyl acetate or 1% tungstic acid. A peptide containing the same 11 amino acids of Aβ25–35 fragment but with a scrambled sequence (SCR; Neosystem France, Strasbourg, France) was used as an additional control. The scrambled peptide was dissolved in oxygen-free distilled water at a concentration of 2.5 mg/ml and stored at −80°C until used. WIN and JWH were from Tocris Bioscience (Bristol, UK); CBD and HU were provided by one of us (R. Mechoulam); and *N*-piperidino-5-(4-chlorophenyl)-l-(2, 4-dichlorophenyl)-4-methylpyrazole-3-carboxamide [SR141716 (SR1)] (Rinaldi-Carmona et al., 1994) and *N*-[(1S)-*endo*-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide [SR144528 (SR2)] (Rinaldi-Carmona et al., 1998) were kindly donated by Sanofi-Synthelabo (Montpellier, France). Each of these compounds was dissolved in dimethyl sulfoxide at 10 mM concentration, and aliquots were stored at −80°C. Before their use, drugs were diluted in appropriate solvent (e.g., phosphate-buffered saline or cell culture medium) and dimethyl sulfoxide never exceeded 0.1% in cell culture experiments. Cell culture reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Salts and other reagents were of analytical grade from Merck (Darmstadt, Germany).

Cell Cultures and Treatments

Primary Rat Microglial Cultures. Primary mixed glial cultures were prepared from neonatal rat cortex as described previously (Ramírez et al., 2005). In brief, mechanically dissociated cortices were seeded onto 75-cm^2 flasks in Dulbecco's modified Eagle's medium (Lonza France Sàrl, Paris, France), supplemented with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA) and 40 μg/ml gentamicin. Cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C, and the medium was changed the day after seeding and once every week afterward. When confluence was reached, after being cultured for 2 to 3 weeks, flasks were shaken for 2 to 3 h at 230 rpm at 37°C, and floating cells were pelleted and seeded onto poly-(lysine)-coated 96-well plates in medium with 0.1% FCS. The cultures were at least 99% pure, as judged by immunocytochemical criteria. Drugs were added in one tenth of the final volume to maintain the aggregation of peptides.

Because of the poor yield of the microglial cultures, we took advantage of microglial cell lines N13 and BV-2 to construct concentration-response curves with the cannabinoids under study. Thereafter, concentrations that approximated the EC_{50} were assayed in primary microglial cultures. Furthermore, the involvement of either CB_1 or CB_2 receptors in the microglial cell functions, difficult to study in vivo, was investigated.

BV-2 Microglial Cells. The transformed microglial cell line (v-raf/v-mic) was obtained from the Interlab Cell Line Collection (National Institute for Cancer Research and Advanced Biotechnology Center, Geneva, Italy).The cells were grown in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal calf serum and 2 mM glutamine, detached from the flasks by manual shaking, and seeded onto poly(L-lysine) (10 μg/ml)-coated plates.

N13 Microglial Cells. In other experiments, the microglial cell line N13 was used (Righi et al., 1989). Cells were cultured in RPMI 1640 containing 10% FCS in F75 flasks, and the medium was changed every 2 or 3 days. At confluence, the cells were trypsinized (trypsin-EDTA; Sigma-Aldrich) and seeded onto poly(L-lysine)-coated plates in medium containing 0.1% FCS.

Measurement of $[Ca^{2+}]_i$. Microglial cells, either N13 cells or primary rat microglia, were plated in 96-well plates (90.000 cells/well in 100 μl of medium) in RPMI culture medium with 0.1% FCS for 1 day. After washing twice with Krebs' solution (105 mM NaCl; 5 mM KCl; 10 mM HEPES-sodium; 5 mM $NaHCO_3$; 60 mM mannitol; 5 mM sucrose; 0.5 mM $MgCl_2$; and 1.3 mM $CaCl_2$, pH 7.4), the cells were loaded with 50 μl/well of 10 μM Fura-2 containing 0.2% Pluronic acid in Krebs' solution for 30 min and 37°C. Antagonists were added to the dye solution at final concentration in the corresponding wells. Then, the wells were washed twice with Krebs' solution, and 40 μl were added to each well, adding the antagonist to final concentration where appropriate. The intracellular calcium concentration was estimated by alternatively exciting with 340 and 380 nm and measuring the fluorescence emission at 510 nm in a fluorescence plate reader (Fluostar Optima; BMG Labtech GmbH, Offenburg, Germany) at 37°C. After 15-s reading, 10 μl of control (Krebs' buffer) or a 5-fold concentrated agonist solution was added to each well by means of the injector system, and the fluorescence was measured for an additional 1 to 2 min. The ratio of emission levels at 340 and 380 nm was calculated at each time point. The conversion to intracellular calcium concentration was performed using calibrating solutions consisting in 5 μM ionomycin in Krebs' buffer (maximal response) or 5 μM ionomycin plus 20 mM EGTA in Krebs' buffer (minimal response). In addition, any background signal was subtracted as measured in nonloaded wells. The increase in $[Ca^{2+}]_i$ was expressed as a percentage of the peak level in comparison with the preinjection baseline according to the following formula: $\Delta[Ca^{2+}]_i = [(\text{peak} - \text{baseline})/\text{baseline}] \times 100$. In other experiments, data were normalized versus the ATP response. In preliminary experiments, it was shown that the reduction of extracellular calcium, without added calcium in the buffer and addition of EGTA 10 mM, decreased the intracellular calcium levels by 50%.

Migration Studies in Chemotaxis Chambers. Chemotaxis through porous membranes was assessed according to Boyden with some modifications. N13 cells or primary microglial cells (90,000 cells) were seeded onto the upper compartment of inserts (6.5 mm diameter) with 8-μm porous polycarbonate membranes in 24-well plates (Transwell Costar 3422; Corning Life Sciences, Lowell, MA).

In preliminary experiments, it was confirmed that to obtain a migratory response, it was necessary to activate the cells by exposure to LPS for 24 h, in agreement with previous studies (Cui et al., 2002). Under these conditions, LPS dose-dependently (1500–6000 ng/ml) or the chemotactic peptide fMLP (25–200 nM) induced migration of N13 cells after 3 h of its addition to the cultures (data not shown). Cells were treated by the addition of LPS (3 μg/ml final concentration; from *Escherichia coli* 0127:B8, Difco Laboratories, Detroit, MI) in culture medium to both compartments. After 24 h, the medium was changed, and the treatments were added to the lower compartment. Three hours later, cells were fixed with 4% paraformaldehyde for 30 min and stained with Coomassie Brilliant Blue (0.2% in 10% acetic acid/40% methanol). The insert membrane was cut and mounted onto a microscope slide, and cells on the lower face of the filter were counted by phase-contrast microscopy by an observer unaware of the treatments (four fields per condition in triplicate) in an Axiovert Zeiss microscope at 400× magnification.

Nitrite Assay. These experiments were performed with BV-2 cells. Cells were plated (50,000 cells/well in 100 μl of medium) onto 96-well precoated plates in RPMI culture medium containing 0.1% FCS. After 24 h, the culture cells were treated with LPS (300 ng/ml) alone or with the cannabinoids, and they were cultured for an additional 24 h. In preliminary studies, we determined the appropriate LPS concentration and incubation time. Nitrite oxide production was assessed by the colorimetric Griess reaction (Sigma-Aldrich), which detects nitrite (NO_2^-), a stable reaction product of NO and molecular oxygen, in cell cultures supernatants. Eighty microliters of each sample was incubated with 80 μl of Griess reagent for 15 min, and absorbance was measured at 540 nm in a microplate reader. The nitrite concentration was determined from a sodium nitrite standard curve.

Immunocytochemistry

Immunostaining of microglial cell cultures was performed after fixation with paraformaldehyde (4% paraformaldehyde in 0.1 M phosphate buffer) for 30 min, followed by rinses with phosphate-buffered saline as described previously (Ramírez et al., 2005). The cells were incubated with the different antibodies overnight at 4°C. Dilutions of antibodies were as follows: polyclonal anti-CB_1 (Dr. K. Mackie, University of Washington, Seattle, WA), 1:900; polyclonal anti-CB_2 (Thermo Fisher Scientific, Waltham, MA), 1:900, and biotinylated tomato lectin (Sigma-Aldrich), 1:150. Development was conducted by the ABC method (Thermo Fisher Scientific), and immunoreactivity was visualized by 3,3′-diaminobenzidine oxidation as chromogen, with nickel enhancement. Omission of primary or secondary antibodies resulted in no immunostaining. Specificity of anti-CB_1 and anti-CB_2 staining was assessed by preabsorption of the antibodies with the antigenic peptides (kindly given by Dr. K. Mackie), which completely abolished labeling.

Aβ-Injected Mice. All experiments were performed according to ethical regulations on the use and welfare of experimental animals of the European Union and the Spanish Ministry of Agriculture, and the procedures were approved by the bioethical committee of the Consejo Superior de Investigaciones Cientificas.

These animals were used as a partial AD model, which develops glial activation and cognitive deficit in learning a spatial task (Ramírez et al., 2005). C57/Bl6 mice of 3 months of age were intraventricularly injected with 2.5 μg of fibrillar Aβ or saline (5 μl). The Hamilton syringe used for intraventricular injections was repeatedly washed with distilled water followed by flushing with 1 mg/ml bovine serum albumin solution, which reduces drastically binding to glass. This procedure was performed before every injection. The next day, the intraperitoneal treatment with the cannabinoids (20 mg/kg CBD; 0.5 mg/kg HU-308, JWH, and WIN) was initiated. During the first week, the mice were treated daily, then for 2 weeks, they were treated 3 days/week. Performance in the Morris water maze was conducted at the same time of the day (9:00 AM to 2:00 PM). To determine spatial learning, rats were trained to find a hidden platform in a water tank 100 cm in diameter. Four trials per day with different start positions, each 30 min apart, were conducted for 5 days (Ramírez et al., 2005), and latency to reach the platform was recorded. Cutoff time to find the platform was 60 s, and mice failing to find the platform were placed on it and left there for 15 s. Data acquisition was performed with a video camera (Noldus Information Technology, Wageningen, the Netherlands). The animals were sacrificed 18 days after the Aβ injection, and their brains were dissected, frozen, and stored at −80°C until assayed.

Analysis of mRNA Levels by Quantitative Real-Time PCR

Total RNA from cortex was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). To avoid interference with potential genomic DNA amplification, we treated 1 μg of total RNA with 1 μl of DNase I (Invitrogen) plus 1 μl of 10× buffer (Invitrogen). The samples were incubated at 37°C for 15 min. EDTA (25

mM) was added to the mixture and the samples were incubated at 65°C for 15 min to heat-inactivate the DNase I. Then, the samples were incubated at 40°C for 1 min. The reaction was collected after centrifugation at 10,000 rpm (pulse), and 1 μg of DNA-free RNA was used for reverse transcription. For cDNA synthesis, a total of 1 μg of RNA from the different samples was reverse-transcribed for 75 min at 42°C using 5 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) in the presence of 20 U of RNasin (Promega). The real-time PCR was performed in 25 μl using the fluorescent dye SYBR Green Master mix (Applied Biosystems, Foster City, CA) and a mixture of 5 pmol of reverse and forward primers. The primers used were for TNF-α, forward primer 5′ CATCTTCTCAAAATTCGAGTGACAA 3′, and reverse primer 5′ TGGGAGTAGACAAGGTACAACCC 3′ (fragment size 175, 30 cycles for linear range), and for IL-6, forward primer 5′ GAGGATACCACTCCCAACAGACC 3′, and reverse primer 5′ AAGTGCATCATCGTTGTTCATACA 3′ (fragment size 141, 30 cycles for linear range). Quantification was performed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). PCR cycles proceeded as follows: initial denaturation for 10 min at 95°C, then 40 cycles of denaturation (15 s, 95°C), annealing (30 s, 60°C), and extension (30 s, 60°C). The melting-curve analysis showed the specificity of the amplifications. Threshold cycle, which inversely correlates with the target mRNA level, was measured as the cycle number at which the reporter fluorescent emission appears above the background threshold (data not shown). Data analysis is based on the ΔCT method with normalization of raw data to a housekeeping gene (β-actin). All of the PCRs were performed in triplicate.

Statistical Analysis

Statistical significance analysis was assessed by using one-way or two-way analysis of variance (ANOVA) followed by unpaired Student's t test (Prism software version 5.0; GraphPad Software Inc., San Diego, CA). A value of $p < 0.05$ was considered significant.

Results

Expression of CB_1 and CB_2 in Microglial Cell Line N13. N13 cells have been immortalized from primary mice microglial cell cultures and shown to express microglial markers and release several cytokines upon LPS stimulation while being capable of FcR-mediated phagocytosis (Righi et al., 1989). Previous evidence has shown that primary microglia and BV-2 microglial cells express both CB_1 and CB_2 (Walter et al., 2003; Ramírez et al., 2005). Accordingly, the expression of cannabinoid receptor subtypes by N13 microglial cells was assessed by immunocytochemistry and compared with that in cultured primary microglia. As expected, the N13 cells expressed both receptors (Fig. 1, top). Immunoreaction was present in the cell membrane and excluded the nuclei. Similar results were obtained in rat microglial cells (Fig. 1, bottom). Therefore, microglial cells should be susceptible of activation by the different cannabinoid agonists selected for the present study.

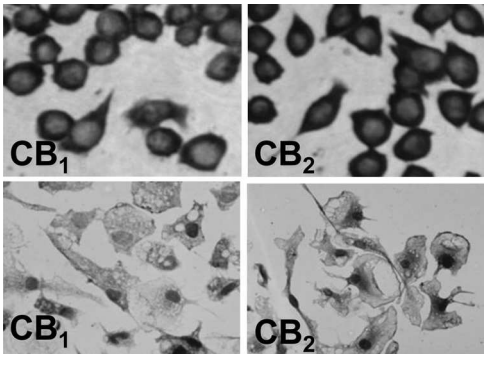

Fig. 1. N13 microglial cells and rat primary microglia express CB_1 and CB_2 receptors. Cells were immunostained with anti-CB_1 (1:900 dilution) and anti-CB_2 antibodies (1:900 dilution). Top, N13 cells; bottom, primary microglia. Initial magnification, 200×.

Cannabinoid Agonists Inhibit ATP-Induced Intracellular Calcium Increase in Cultured Microglia. Variations in intracellular calcium concentration ($[Ca^{2+}]_i$) underlie several important cell signaling functions, and they are involved in microglia activation. Indeed, an increase in extracellular ATP, released by dying neurons and glia, can interact with purinergic receptors, increase $[Ca^{2+}]_i$, and activate microglia (Färber and Kettenmann, 2006). On the other hand, cannabinoid agonists have been shown to inhibit calcium responses in a variety of cells, including glial cells (Mato et al., 2009).

Indeed, we found that ATP increased $[Ca^{2+}]_i$ in a concentration-dependent manner (10–400 μM; data not shown) in N13 cells, reaching concentrations as high as 700 nM (nearly a 3-fold increase over basal levels) with the highest concentration tested. Intracellular calcium increased almost immediately after adding ATP to the N13 cells (see Fig. 2 C), and after reaching its maximal, even in the presence of ATP, it returned to baseline calcium levels.

For the subsequent experiments, we selected the ATP concentration of 400 μM that may mimic the high concentrations released by dying neurons and glia (e.g., pathological concentrations). Cannabinoids per se did not affect basal $[Ca^{2+}]_i$ levels when added to the cultures in a wide concentration range (10–1000 nM). However, CBD did reduce ATP-induced $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 2A), and the maximal effect attained was a 25% reduction. The effect of CBD in N13 microglia was not changed in the presence of either the CB_1- or the CB_2-selective antagonists (100 nM; Fig. 2E), which per se showed no effect (data not shown). The $[Ca^{2+}]_i$ response of primary microglia to ATP was different from that observed in N13 cells, because after reaching its peak effect, approximately 20 s after its addition to the culture, it was maintained at least for 50 s (Fig. 2, B and C). It is noteworthy that the CB_2 antagonist fully reversed the CBD effect (Fig. 2, B and F). WIN reduced ATP-induced intracellular calcium (Fig. 2), and although both antagonists blocked its effect in N13 cells (Fig. 2E), the CB_2-selective agonist fully reversed its effect in microglia (Fig. 2F). Finally, JWH also decreased the calcium intracellular levels after ATP addition. This effect was counteracted by the CB_2-selective antagonist in primary microglia (Fig. 2F), although it was not the case in N13 cells (Fig. 2E). HU-308 did not change ATP-induced increase in $[Ca^{2+}]_i$ at any concentration tested (10–300 nM; data not shown).

Previous work has shown that the immunosuppressive effects of CBD involved the activation of adenosine receptors, given its blockade of the equilibrative nucleoside transporter (Carrier et al., 2006). To ascertain the possible involvement of a similar mechanism responsible for the CBD reduction of ATP-induced calcium responses in microglial cells, we examined whether an A_{2A} agonist could mimic the CBD action (Fig. 3). In fact, 2-[*p*-(2-carboxyethyl) phenethylamino]-5′-*N*-ethylcarboxamidoadenosine [CGS21680 (CGS)], an A_{2A} agonist (Klotz et al., 1998), decreased at the same extent the ATP-induce increase in $[Ca^{2+}]_i$ in N13 and primary microglial cells (Fig. 3, A and B), which was blocked by 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-

a][1,3,5]triazin-5-ylamino]ethyl]phenol [ZM241385 (ZM)], an A_{2A} antagonist (Palmer et al., 1995), that per se had no effect. Of note is that ZM was able to inhibit the CBD effect both in N13 and primary microglial cells (Fig. 3, C and D). Therefore, we have shown that CBD and other cannabinoids counteract the effects of ATP on microglial cells by a mechanism involving both cannabinoid receptor-dependent and independent mechanisms.

CBD and Other Cannabinoids Promote Microglial Cell Migration. The Aβ peptide (Tiffany et al., 2002) and different cannabinoids trigger migration (Walter et al., 2003) that may subserve a beneficial function as a requisite for phagocytosing aggregated Aβ. Therefore, migration of microglial cells through porous membranes toward the lower chamber containing the compounds was investigated.

The mixed CB_1/CB_2 agonist WIN and the CB_2-selective agonist JWH promoted N13 cell migration (Fig. 4, A and B). Note that in these experiments, the cells changed their oval morphology (Fig. 1) to a fully round morphology with no lamellipodia (Fig. 4 B). Migration was similarly increased (approximately 20% compared with controls) by the two agonists (200 nM). The CB_1-selective agonist SR1 did not affect migration, whereas the CB_2-selective antagonist SR2 induced a partial although statistically significant effect on migration (approximately 10% increase; Fig. 2A). The effect of WIN on N13 cells was not altered by either of the cannabinoid antagonists, but full prevention of its migratory effect was obtained when both antagonists were combined. In contrast, the CB_2 antagonist completely blocked the response induced by JWH, whereas the CB_1 antagonist had no effect (Fig. 4A). Fibrillar Aβ (2 μM) induced microglial migration (20%) in comparison with vehicle-treated control cultures (Fig. 4C) and to those treated with a scrambled peptide, which had no effect. Aβ combined with the cannabinoids exerted a similar effect to that obtained with the agonists alone (data not shown). Primary microglial cells showed a more robust response (2.5-fold compared with controls) upon the addition of Aβ, LPS, or the cannabinoid agonists (Fig. 4C). The migratory effect induced by CBD and WIN was fully reversed by either of the selective antagonists (Fig. 4C). In contrast, the migration promoted by JWH and HU were only inhibited by the CB_2 antagonist (Fig. 4C). Taken together, these results indicate that CBD and the other cannabinoids promote microglial cell migration in a cannabinoid receptor-dependent manner.

NO Generation Is Inhibited by Cannabinoids. Cannabinoids inhibit LPS NO synthase stimulation and NO generation as reflected in nitrate accumulation in the culture media (Waksman et al., 1999). Given that LPS challenge did not generate nitrites in N13 cells, BV-2 microglial cells were used in these experiments.

Nitrite generation, measured in the culture media after LPS stimulation, was concentration-dependently reduced by all the cannabinoids tested (Fig. 5). According to their IC_{50}, their relative potencies were CBD = HU-308>JWH-133≫WIN (Fig.

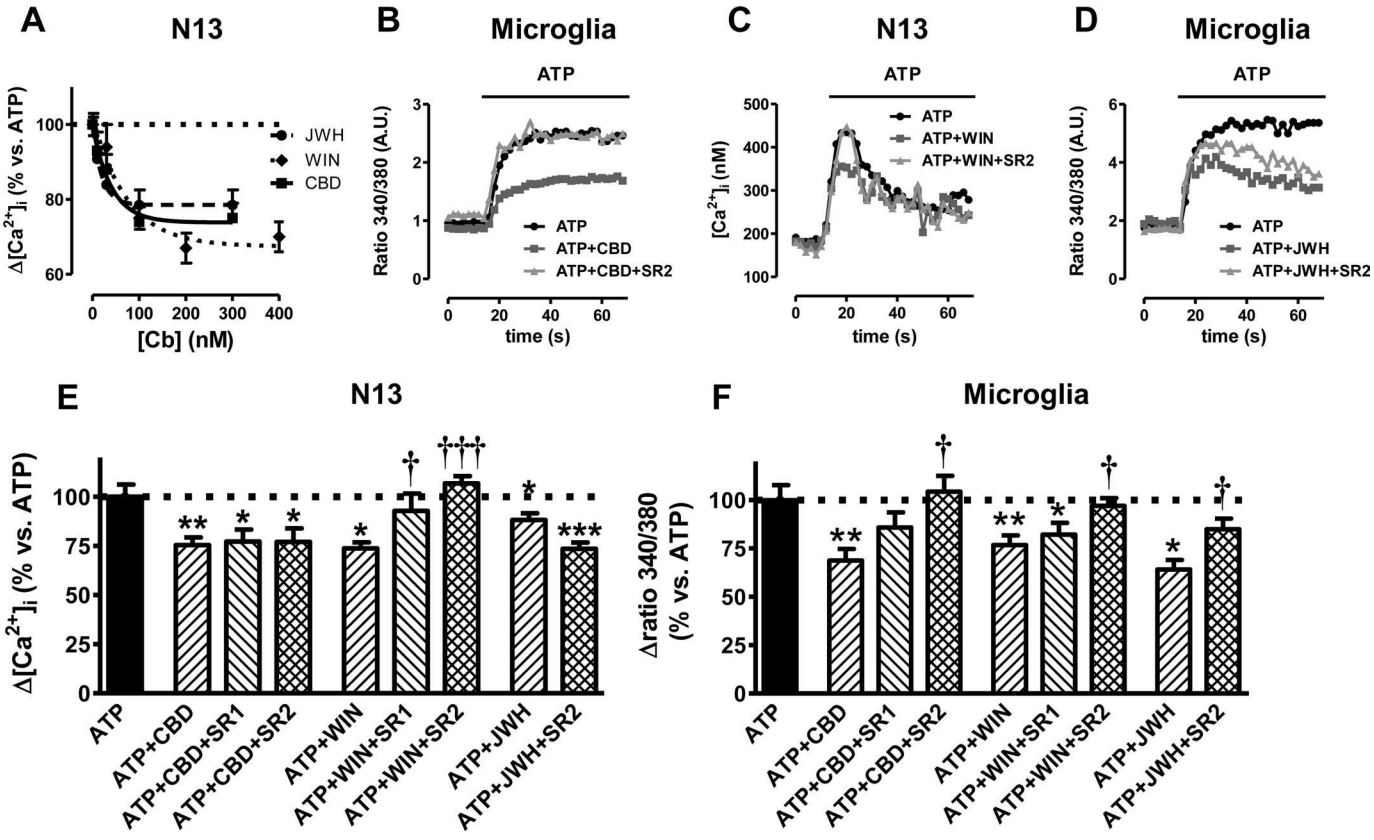

Fig. 2. CBD, WIN, and JWH inhibited the ATP-induced increase in intracellular calcium in cultured N13 and primary microglial cells. A, concentration-dependent inhibition of ATP-induced increase in $[Ca^{2+}]_i$ by cannabinoids in N13 cells. The effect of CBD (B; 100 nM), of WIN (C; 400 nM), and of JWH (D; 100 nM) was reverted by SR2 antagonist (100 nM). Effect of cannabinoids on ATP-induced increase in $[Ca^{2+}]_i$ in N13 cells (E) and an primary microglial cells (F). The antagonist SR2 blocked the effect of WIN in N13 cells and the effect of all cannabinoids in primary microglia. Results are mean ± S.E.M. of three independent experiments with multiple well replicates. Statistical analysis was done by one-way ANOVA followed by Student's *t* test; *, $P < 0.05$ and **, $P < 0.01$ versus ATP, and †, $P < 0.05$ and †††, $P < 0.001$ versus ATP + cannabinoid.

5A). At a concentration near their IC_{50}, the inhibition of nitrite generation was greater in primary microglia (Fig. 5C) in comparison with BV-2 cells (Fig. 8B). Nitrite inhibition by cannabinoids was resistant to the antagonists tested (data not shown), and these results suggest that this microglial response is independent of cannabinoid receptor activation.

Cannabinoids Counteract Aβ-Induced Cognitive Impairment and Increased Cytokine Gene Expression. Given that several parameters of microglial activation in vitro were affected by CBD and other cannabinoids, we sought to determine whether these compounds were able to prevent some features of a pharmacological AD model. First, we examined whether cognitive impairment of Aβ-injected mice was affected by subchronic treatment. Mice injected with Aβ showed increased latencies to find the hidden platform in comparison with control animals (SCR + vehicle). Both CBD at a dose of 20 mg/kg and WIN 0.5 mg/kg were able to prevent the cognitive impairment shown by the animals in the Morris water maze (Fig. 6A). In fact, from the third training day, the mice showed a significant reduction in the latency to reach the hidden platform in comparison with Aβ-injected mice, and they behaved similar to the controls (SCR + vehicle). In contrast, neither JWH-133 nor HU-308 ameliorated the cognitive impairment of the animals (Fig. 6B).

Gene expression of two different proinflammatory cytokines, TNF-α and IL-6, were measured in cerebral cortex of the animals treated with CBD and WIN. CBD did not alter the increased TNF-α gene expression observed in the AD mice model (Fig. 6C), and WIN partially reduced it. However, the levels of IL-6, which were dramatically increased (6-fold) in the Aβ-injected mice, were markedly decreased by both cannabinoids (Fig. 6D).

Discussion

Cannabinoids have been shown to be promising agents for the treatment of different neurodegenerative conditions. In particular, agents that are devoid of psychoactive effects, solely mediated by CB_1 receptor interaction, would be interesting for its translation into the Clinic. This is the case of CBD, which has very low affinity for cannabinoid receptors, or CB_2-selective agonists.

Mobilization of intracellular calcium constitutes an important second messenger in cells and in microglia can be considered central for many inflammatory-mediated responses, affecting enzymes, ion channels, and gene transcription. In this work, we have studied the modulation by cannabinoids of ATP-induced responses in microglial cells in culture. Microglial cells are endowed of different purinergic receptors (Light et al., 2006), which may account for the concentration-dependent increase in $[Ca^{2+}]_i$ (Kettenmann et al., 1993; Möller et al., 2000). The identity of the P2 receptors responsible for that increase has not been established in the present study, but P2Y leads to calcium release from intracellular stores, whereas P2X activation results in influx through the cationic ion channel. It is noteworthy that the calcium response was different in the N13 cell line compared with primary rat microglia. In the first case, it caused a desensitizing response that, after reaching its maximum, subsided in approximately 40 s in the presence of ATP, but in primary

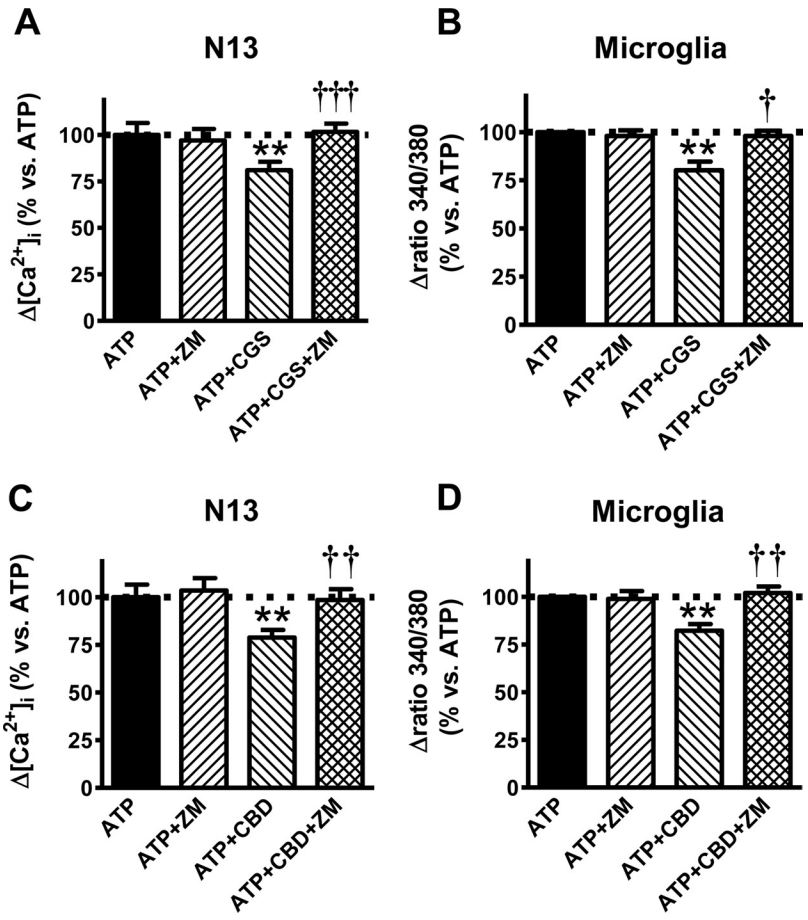

Fig. 3. Implication of A_{2A} adenosine receptor in the effect of CBD on ATP-induced increase in intracellular calcium. Activation of A_{2A} receptor by CGS (100 nM) inhibited the ATP-induced increase in intracellular calcium and was reverted by the A_{2A} antagonist ZM (1000 nM) in N13 cells (A) and primary microglia (B). The implication of A_{2A} receptors was confirmed by the reversion of CBD effect on intracellular calcium by the ZM antagonist in N13 (C) and primary microglia (D). Results are mean ± S.E.M. of three independent experiments with multiple well replicates. Statistical analysis was done by one-way ANOVA followed by Student's t test; **, $P < 0.01$ versus ATP, and †, $P < 0.05$, ††, $P < 0.01$, †††, $P < 0.001$ versus ATP + CGS or ATP + CBD.

microglial cells, the response was sustained. Dying neurons release high amounts of ATP; therefore, we decided to use those pathological concentrations for subsequent experiments. CBD and the other cannabinoid agonists, with the exception of HU, decreased ATP-induced $[Ca^{2+}]_i$, both in N13 microglial cells and in primary rat microglia in culture. The effect of the compounds was either independent of cannabinoid receptor activation (e.g., CBD and JWH in N13 cells), given that it was resistant to cannabinoid receptor antagonism, or CB_2 receptor-mediated (WIN in N13 cells and all compounds in primary microglia). The cannabinoid receptor independence of the effect of CBD in N13 cells prompted us to investigate a possible A_{2A} receptor mediation. We found that the A_{2A}-selective agonist CGS (Klotz et al., 1998) also reduced the ATP-induced $[Ca^{2+}]_i$ to a similar extent and the inhibition elicited by CBD and CGS was blocked by the

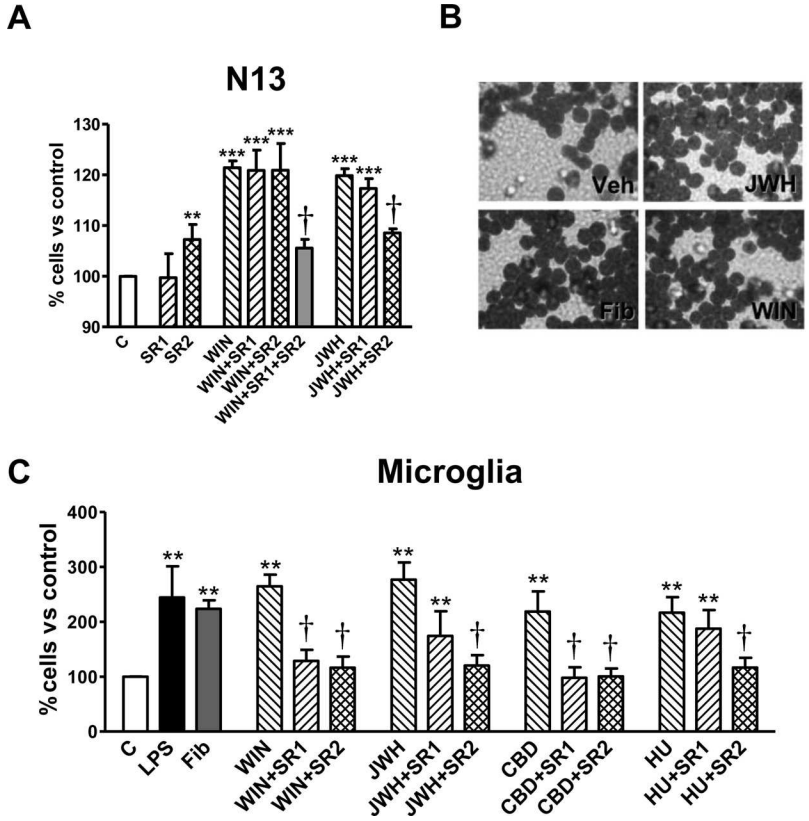

Fig. 4. Cannabinoid agonists promote microglial cell migration. WIN and JWH (200 nM) promoted N13 cell migration (A and B), which was inhibited by the combination of CB_1 and CB_2 antagonist (100 nM each), or the CB_2 antagonist (SR2), respectively. Cannabinoid agonists promoted primary microglia migration, which was blocked by CB_1 and/or CB_2 antagonism (C). Fib, fibrillar $A\beta_{1-40}$ (2 μM). Results are the mean ± S.E.M. of four independent experiments in duplicate. Statistical analysis was done by one-way ANOVA followed by Student's t test; **, $P < 0.01$, ***, $P < 0.001$ versus control (no treatment); and †, $P < 0.05$ versus the cannabinoid agonist alone.

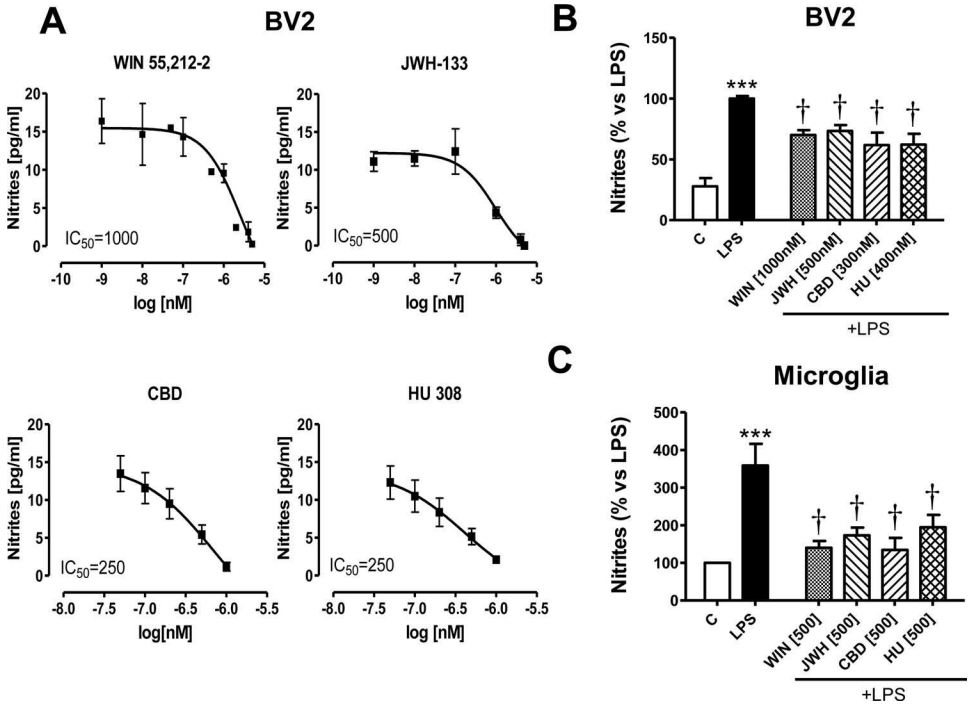

Fig. 5. Cannabinoid agonists decreased LPS-induced nitrite generation in microglial cells. A, cannabinoid agonists concentration-dependently inhibited LPS-induced nitrite generation in BV-2 microglial cells. Cannabinoid agonist inhibition of LPS-induced nitrite generation in BV-2 (B) and primary microglial cells (C). Agent concentrations are depicted. In B and C, results are expressed as a percentage of control. Nitrite levels were the following: BV-2 cells: control, 2.47 ± 0.21, and LPS-induced, 12.54 ± 0.98 pg/ml; primary microglia: control, 3.65 ± 0.40, and LPS-induced, 18.5 ± 1.2. Results are mean ± S.E.M. of four independent experiments in duplicate. Statistical analysis was done by one-way ANOVA followed by Student's t test; ***, $P < 0.001$ versus control (vehicle); and †, $P < 0.05$ versus LPS.

A_{2A}-selective antagonist ZM (Palmer et al., 1995). Those results indicate that cannabinoid inhibition of the intracellular calcium increase brought about by high concentrations of ATP in microglial cells may be mediated by cannabinoid or A_{2A} receptors.

In some works in which micromolar concentrations of cannabinoids enhance $[Ca^{2+}]_i$, ryanodine receptors seem to be involved. Indeed, the increase in $[Ca^{2+}]_i$ induced by ACEA and JWH-133 was partially blocked by a ryanodine antagonist in RIN insulinoma cells (De Petrocellis et al., 2007). The involvement of intracellular calcium stores in cannabidiol elevation of $[Ca^{2+}]_i$ has also been described in hippocampal cells (neurons and glia) in culture (Drysdale et al., 2006). Given that we found a decrease by cannabinoids of ATP-induced increase in $[Ca^{2+}]_i$ and that in microglial cells, CB_1 (e.g., in the case of WIN) and/or CB_2 antagonists were effective at blocking their effect, we have not addressed the involvement of ryanodine receptors.

Activation of microglia by Aβ is associated with chemotactic responses toward it, consistent with the extensive clustering of activated microglia at sites of Aβ deposition in AD brain. Furthermore, Aβ induces migration across porous membranes through the interaction with chemotactic receptors such as the formyl peptide receptors FPR2 and FPR-like 1 receptor, its human counterpart (Cui et al., 2002). Indeed, we observed that the Aβ peptide induced chemotactic responses in cultured microglia cells. Cannabinoids, whether plant-derived or endocannabinoids, have been shown to induce migration of BV-2 microglial cells (Walter et al., 2003). In the present work, the synthetic cannabinoids WIN and JWH promoted migration at similar concentrations in the N13 cell line, and their effect was greater in primary microglia. According to the work of Walter et al. (2003), the cannabinoid effects were mediated by CB_2 and abnormal cannabidiol receptors. The results of the present work also show the involvement of CB_2 receptors in migration of N13 and primary microglial cells. Indeed, the two CB_2-selective agonists JWH and HU promoted it, and their effect was completely blocked by the CB_2-selective antagonist but unchanged by the CB_1-selective antagonist, as expected. However, in our hands, CB_1 receptor activation was effective as well. In fact, the migration induced by WIN, a mixed CB_1/CB_2 agonist not tested previously, and by CBD was prevented by both selective antagonists.

In agreement with previous work that has shown decreased NO generation by microglia in the presence of cannabinoids (Waksman et al., 1999), we observed a concentration-dependent decrease in nitrites in the culture media of LPS-stimulated microglia. Although in BV-2 microglial cells CBD and HU seemed to be more potent at decreasing nitrites, in primary microglia, all of the cannabinoid agonists were equipotent. The effect of cannabinoids were independent of cannabinoid receptor activation, given that it was unaltered by the selective antagonists used in the present study.

We and other authors have shown that treatment with cannabinoid agonists and agents that increase endocannabinoid availability, such as the inhibitor of endocannabinoid reuptake *N*-(4-hydroxy-2-methylphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide (VDM11), are able to prevent Aβ-induced cognitive deficits (Ramírez et al., 2005; van der Stelt et al., 2006). Furthermore, in the work by van der Stelt et al. (2006), it was found that VDM11 treatment inhibited different glial parameters (cyclooxygenase-2, iNOS, S100β) in hippocampus, which were increased by Aβ intracortical injection. In the present work, Aβ-injected mice subjected to subchronic systemic administration of WIN or CBD showed better performance in the Morris water maze compared with vehicle-treated animals. In this paradigm, the two selective CB_2 agonists were ineffective in that respect, although JWH-prolonged oral administration showed beneficial effects in the transgenic model of AD Tg APP (A. M. Martín-Moreno, B. Brera, E. Carro, M. Delgado, M. A. Pozo, N. Innamorato, A. Cuadrado, and M. L. de Ceballos, in preparation). The possible involvement of glial activation modulation was assessed by measuring cytokine gene expression. In mice injected with Aβ, both TNF-α and IL-6 expression was markedly increased. WIN and CBD treatment abolished IL-6 expression increase, and WIN partially reduced that of TNF-α. Therefore, in an in vivo short-term pharmacological model of AD, cannabinoids showed beneficial behavioral effects

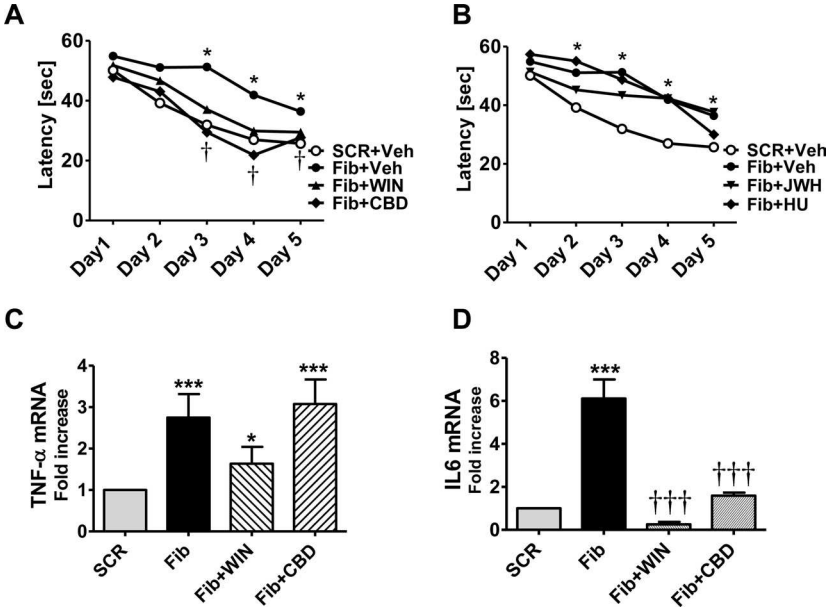

Fig. 6. CBD and WIN 55,212-2 prevented Aβ-induced learning deficit and cytokine expression. Mice received a single Aβ intraventricular injection (2.5 μg/5 μl) and were treated with the cannabinoid agonists daily (see *Materials and Methods* for details). Training in the water maze was conducted during the second week of treatment. CBD and WIN prevented Aβ-induced cognitive impairment (A), whereas HU and JWH were without effect (B). WIN and CBD prevented IL-6 increased gene expression induced by Aβ (D), but did not affect TNFα increase (C). Results are mean ± S.E.M. of eight mice per group. In the water maze, results S.E.M., which were less than 15%, were omitted for the sake of clarity. SCR, scrambled peptide; Fib, fibrillar $A\beta_{1-40}$. Statistical analysis of water maze performance was done by two-way ANOVA followed by Student's *t* test: *, $P < 0.05$ versus SCR-treated mice; †, $P < 0.05$ versus Aβ-injected mice and of cytokine expression by one-way ANOVA: *, $P < 0.05$ and ***, $P < 0.001$ versus SCR, and †††, $P < 0.001$ versus Fib.

that seem to be mediated through glial activation modulation.

The pharmacology of endocannabinoids and phytocannabinoids seems to be increasingly complicated. According to binding studies performed in cells transfected with the CB_1 and CB_2 human receptors (Showalter et al., 1996), CBD has shown very low affinity (approximately 2–4 μM). However, one interesting finding of this work is that CBD exerts several effects on microglial function in the high nanomolar range and at similar concentrations of other cannabinoids tested. Moreover, many of CBD effects seem to be CB_2 receptor-mediated. Expression of CB_2 receptors in normal brain is negligible and only measurable by quantitative PCR. This fact can explain that the interaction of CBD with CB_2 receptors has been unnoticed and unraveled in studies involving microglial cells.

There is no doubt that in AD, a pronounced inflammation occurs in which astrocytes and microglial cells are involved and Aβ, which is central to AD pathology and is at least in part responsible of it. However, the inflammatory response accounts for both detrimental and beneficial effects in the pathology. Indeed, activated microglia release toxic molecules, such as NO and proinflammatory cytokines, as initial players that may induce neurodegeneration. At the same time, these cells release trophic factors and thereafter migrate to affected brain areas and phagocytose dead neurons and Aβ deposits, therefore contributing to neuroprotection. The resulting outcome of the inflammatory process would be the combination of both effects. It is noteworthy that cannabinoids seem to differentially regulate those separate cellular events of activated microglial in a positive direction. On the one hand, these compounds effectively counteract Aβ-mediated increase in the proinflammatory cytokine TNF-α and the ensuing neurodegeneration after its administration in vitro and in vivo (Ramírez et al., 2005). In contrast, as shown here, cannabinoids promote migration, a cellular mechanism that ultimately will allow the removal of the deposited Aβ peptide. Therefore, this kind of drug with neuroprotective and anti-inflammatory effects (Walter and Stella, 2004) may be of interest in the prevention of AD inflammation, in particular CB_2-selective agonists, which are devoid of psychoactive effects (Hanus et al., 1999).

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Authorship Contributions

Participated in research design: Mechoulam, Cuadrado, and de Ceballos.

Conducted experiments: Martín-Moreno, Reigada, Ramírez, and Innamorato.

Contributed new reagents or analytic tools: Mechoulam.

Performed data analysis: Martín-Moreno, Reigada, Ramírez, Innamorato, and de Ceballos.

Wrote or contributed to the writing of the manuscript: Mechoulam, Cuadrado, and de Ceballos.

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