

ANTI-INFLAMMATORY PROPERTY OF THE CANNABINOID AGONIST WIN-55212-2 IN A RODENT MODEL OF CHRONIC BRAIN INFLAMMATION

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Abstract—Cannabinoid receptors (CB_r) stimulation induces numerous central and peripheral effects. A growing interest in the beneficial properties of manipulating the endocannabinoid system has led to the possible involvement of CB_r in the control of brain inflammation. In the present study we examined the effect of the CB_r agonist, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate (WIN-55212-2), on microglial activation and spatial memory performance, using a well-characterized animal model of chronic brain inflammation produced by the infusion of lipopolysaccharide (LPS, 250 ng/h for 3 weeks) into the fourth ventricle of young rats. WIN-55212-2 (0.5 or 1.0 mg/kg/day, i.p.) was administered for 3 weeks. During the third week of treatment, spatial memory ability was examined using the Morris water-maze task. We found that 0.5 and 1 mg/kg WIN-55212-2 reduced the number of LPS-activated microglia, while 1 mg/kg WIN-55212-2 potentiated the LPS-induced impairment of performance in the water maze task. Cannabinoid receptors 1 were not expressed by microglia and astrocytes, suggesting an indirect effect of WIN-55212-2 on microglia activation and memory impairment. Our results emphasize the potential use of CB_r agonists in the regulation of inflammatory processes within the brain; this knowledge may lead to the use of CB_r agonists in the treatment of neurodegenerative diseases associated with chronic neuroinflammation, such as Alzheimer disease. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cannabinoid receptors, inflammation, activated microglia, Alzheimer's disease, LPS, spatial memory.

Microglial cells play a pivotal role as immune effectors in the CNS and may participate in the initiation and progression of neurological disorders, such as Alzheimer's disease (AD), Parkinson's disease and multiple sclerosis by

releasing harmful molecules such as pro-inflammatory cytokines, reactive oxygen species or complement proteins (Akiyama et al., 2000; Kim and de Vellis, 2005). Many of the pathological, immunological, biochemical and behavioral changes seen in these and other neurodegenerative diseases can be reproduced in young rats by chronic infusion of lipopolysaccharide (LPS) into the fourth ventricle (Hauss-Wegrzyniak et al., 1998, 1999). Chronic infusion of LPS results in the activation of microglia within hippocampus and entorhinal cortex (EC), brain regions involved in learning and memory formation (Hauss-Wegrzyniak et al., 1998; Rosi et al., 2004). Chronic brain inflammation is associated with impaired spatial memory, impaired induction of long-term potentiation, a loss of *N*-methyl-D-aspartate (NMDA) receptors, astrogliosis, elevated cytokines and related pro-inflammatory proteins and transcription factors (Hauss-Wegrzyniak et al., 1998, 1999; Rosi et al., 2004).

The endocannabinoid system may regulate many aspects of the brain's inflammatory response, including the release of pro-inflammatory cytokines and modulation of microglial activation (Neumann, 2001; Klein, 2005). The endocannabinoid system is composed of two G-protein-coupled receptors designated as cannabinoid receptor 1 (CB₁) and cannabinoid receptor 2 (CB₂), although not all endocannabinoid effects can be explained only by these two receptors (Begg et al., 2005). CB₁ receptors are expressed in the brain and are responsible for most of the behavioral effects of the cannabinoids. CB₂ receptors are expressed by immune and hematopoietic cells peripherally (Begg et al., 2005), and seem to be expressed on neurons in the brainstem and the brain (Van Sickle et al., 2005; Gong et al., 2006; Onaivi et al., 2006) although their presence in the brain is controversial (Munro et al., 1993). Two endogenous ligands for these receptors, arachidonyl ethanolamine and 2-arachidonoylglycerol (Stella, 2004), influence immune responses by inhibiting cytokine release and other anti-inflammatory actions (Klein et al., 2003; Klein, 2005). Microglia also express cannabinoid receptors (CB_r) and release cytokines in response to exposure to LPS or beta-amyloid protein; this effect can be inhibited by prior cannabinoid treatment (Facchinetti et al., 2003; Ramirez et al., 2005; Sheng et al., 2005). Astrocytes may also synthesize and release endocannabinoids (Walter et al., 2002). CB₁ receptors have been widely studied because of their role in the psychoactive effects of the *Cannabis sativa* plant (Δ^9 -tetrahydrocannabinol or Δ^9 -THC). Δ^9 -THC can impair performance in rats, mice or monkeys under multiple experimental conditions (Castellano et al., 2003).

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Abbreviations: aCSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; CB₁, cannabinoid receptor 1; CB₂, cannabinoid receptor 2; CB_r, cannabinoid receptors; DG, dentate gyrus; EC, entorhinal cortex; LPS, lipopolysaccharide; MHC II, class II major histocompatibility complex; NMDA, *N*-methyl-D-aspartate; PBS, phosphate buffer saline; TBS, Tris-buffered saline; WIN, WIN-55212-2; (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate Δ^9 -THC, Δ^9 -tetrahydrocannabinol.

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Therefore, in the current study, we investigated the effect of a CBR agonist on microglial activation and spatial memory in a rodent model of chronic brain inflammation induced by LPS infusion into the fourth ventricle. We used the number of immunoreactive microglia (activated) as a biomarker of brain inflammation (Hauss-Wegrzyniak et al., 1998, 1999; Rosi et al., 2004, 2005) to evaluate the potential anti-inflammatory properties of the WIN-55212-2 compound.

EXPERIMENTAL PROCEDURES

Subjects and surgical procedures

Fifty-four young (3 months old) male F-344 rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) were singly housed in Plexiglas cages with free access to food and water. The rats were maintained on a 12-h light/dark cycle in a temperature-controlled room (22 °C) with lights off at 08:00 h. All rats were given health checks, handled upon arrival and allowed at least 1 week to adapt to their new environment prior to surgery.

Artificial cerebrospinal fluid (aCSF $n=26$) or LPS (Sigma, St. Louis, MO, USA, *E. coli* serotype 055:B5, TCA extraction, 1.0 mg/ml dissolved in aCSF, $n=28$) were chronically infused for 21 days through a cannula implanted into the fourth ventricle that was attached (via Tygon tubing, 0.06 O.D., and an osmotic pump connect, model 3280P, Plastics One, Roanoke, VA, USA) to an osmotic minipump (Alzet, Cupertino, CA, USA, model #2004, to deliver 0.25 μ l/h, Hauss-Wegrzyniak et al., 1998). The aCSF vehicle contained (in mM) 140 NaCl; 3.0 KCl; 2.5 CaCl₂; 1.0 MgCl₂; 1.2 Na₂HPO₄, adjusted to pH 7.4. Rats infused with either aCSF or LPS were also administered daily the synthetic cannabinoid CB1/CB2 receptor agonist (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate (WIN-55212-2, Sigma, 0.5 or 1.0 mg/kg, i.p.) or vehicle (dimethylsulfoxide (100%), DMSO, Sigma). Rats were assigned to one of the following six groups: aCSF+vehicle, LPS+vehicle, aCSF+WIN (0.5 mg/kg), LPS+WIN (0.5 mg/kg), aCSF+WIN (1 mg/kg), LPS+WIN (1 mg/kg). Drug or vehicle administration began the first day after surgery and continued throughout the behavioral testing, which began on day 14 of the LPS or aCSF infusion.

Behavioral testing

Spatial learning ability was assessed using a 185 cm diameter water maze with white walls. The water was maintained at 26–28 °C and made opaque by adding white, non-toxic, paint. The pool was in the center of a 2.3×2.73×2.5m room with multiple visual stimuli on the wall as distal cues, and a chair and a metal board against the wall of the pool as proximal cues. The circular escape platform was 11.5 cm in diameter. For the spatial (hidden-platform) version of the water task, a circular escape platform was present in a constant location, submerged about 2.5 cm below the water surface. The rats were tracked by an overhead video camera connected to a VP114 tracking unit (HVS Image, Hampton, UK). Custom software was used to store and analyze each rat's latency to find the submerged platform during each trial.

Each rat performed three training blocks per day (two training trials per block) for 4 days (24 trials total), with a 60-min inter-block interval. On each trial, the rat was released into the water from one of seven locations spaced evenly at the side of the pool, which varied randomly from trial to trial. After the rat found the escape platform or swam for a maximum of 60 s, it was allowed to remain on the platform for 30 s. To control for possible drug-induced deficits in visual acuity and swimming ability, the same rats were also tested on a second version of this task. In this version, a

visible platform raised 2 cm above the surface of the water was moved randomly to one of four locations in the tank after each trial. A total of four visible-platform trials were performed. Drug administration was performed 20 min prior to the behavioral testing. The results, i.e. latency (s) to find the hidden platform, were analyzed by ANOVA followed by post hoc comparisons according to the method of Bonferroni/Dunn.

Histological procedures

After behavioral testing was completed, each rat was deeply anesthetized with isoflurane and prepared for a transcardiac perfusion of the brain with cold saline containing 1 U/ml heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were then removed and the placement of the cannula in the fourth ventricle was confirmed. The brains were then post-fixed 1 h in the same fixative and then stored (4 °C) in phosphate buffer saline (PBS), pH 7.4. Free-floating coronal sections (40 μ m) were obtained using a vibratome from perfused tissues for staining with standard avidin/biotin peroxidase or fluorescence labeling methods. The monoclonal antibody OX-6 (final dilution 1:400, Pharmingen, San Diego, CA, USA) was used to visualize activated microglia cells. This antibody is directed against class II major histocompatibility complex (MHC II) antigen. After quenching endogenous peroxidase/activity and blocking nonspecific binding, the sections were incubated (4 °C) overnight with primary antibodies directed against the specific epitope (MCH II). Thereafter, the sections were incubated for 2 h (22 °C) with the secondary monoclonal antibody, rat adsorbed biotinylated horse anti-mouse immunoglobulin G (final dilution 1:200, Vector, Burlingame, CA, USA). Sections were then incubated for 1 h (22 °C) with avidin-biotinylated horseradish peroxidase (Vectastain, Elite ABC kit, Vector). After washing again in PBS, the sections were incubated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Vector) as chromogen. The reaction was stopped by washing the section with buffer. No staining was detected in the absence of the primary or secondary antibodies. Sections were mounted on slides, air-dried and coverslipped with cytooseal (Allan Scientific, Kalamazoo, MI, USA) mounting medium. The location of immunohistochemically-defined cells was examined by light microscopy. Quantification of cell density in the reconstructed hippocampal coronal sections was assessed with MetaMorph imaging software (Universal Image Corporation, West Chester, PA, USA). Briefly, areas of interest were determined as previously reported in detail (Rosi et al., 2005), their surface measured, and the immunoreactive cells enumerated, allowing us to determine a number of immunoreactive cells by millimeter square in the areas of interest.

A polyclonal antibody directed against the first 99 amino-acid residue from the human CB1 (final dilution 1:500, Affinity Bioreagents, Golden, CO, USA) was used to visualize CB1 receptors. After quenching endogenous peroxidase/activity and blocking nonspecific binding, the sections were rinsed in 0.1 M TRIS buffer (TB), pH 7.4, for 15min, in 0.1 M Tris-buffered saline (TBS), pH 7.4, for 15 min, blocked using 2% avidin in TBS for 30 min, rinsed in TBS for 30 min, blocked using 2% biotin for 30 min, and finally rinsed in TBS for 30 min. The tissue sections were then incubated in the anti-CB1 (diluted 1:500) overnight at 4 °C. The antibody was diluted in a solution containing 0.1% Triton X-100 and 1% NGS in 0.1 M TBS. These sections were then rinsed in TBS for 60 min and incubated in biotinylated goat anti-rabbit IgG (diluted 1:200) for 90 min at room temperature. The sections were rinsed with TBS for 60 min and incubated for 1 h (22 °C) with avidin-biotinylated horseradish peroxidase (Vectastain, Elite ABC kit, Vector). The sections were then rinsed with TBS for 30 min and then incubated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride as chromogen. The reaction was stopped by washing the section with buffer. No staining was detected in the absence of the primary or secondary antibodies. Sections were mounted on slides, air-dried for 24 h. Counterstaining (Cresyl Violet) was performed before slides

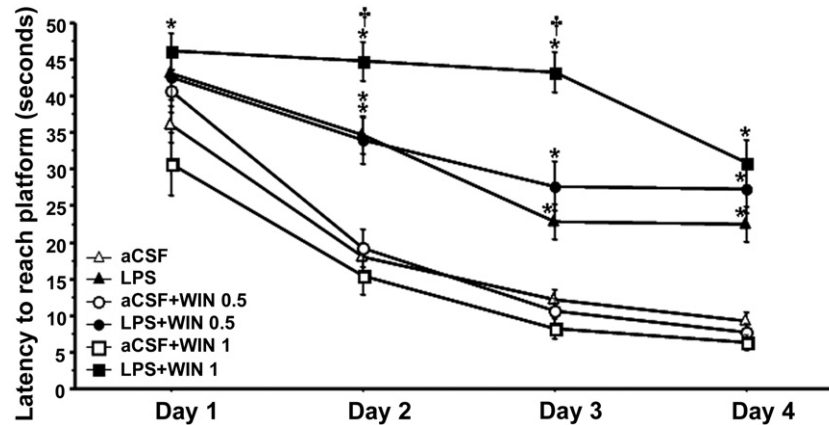


Fig. 1. Water maze performance. On days 2 and 3, all LPS-infused animals (closed triangles, squares and circles) were significantly impaired ($*P<0.0033$, Bonferroni/Dunn post hoc test) compared with their control groups. On days 2 and 3, LPS+WIN 1 rats were significantly impaired ($†P<0.0033$, Bonferroni/Dunn post hoc test) compared with the LPS+vehicle rats. WIN-55212-2 did not impair performance (open squares and circles) of aCSF-infused rats and did not attenuate the impairment due to the LPS infusion (closed squares and circles).

were coverslipped with cytoaseal (Allan Scientific) mounting medium.

Double immunofluorescence staining

Free floating sections were mounted on slides and air-dried under a hood. The tissues were then processed as described in detail in Rosi et al., 2005. After washing in TBS solution the polyclonal rabbit anti-CB1 (Affinity Bioreagents, dilution 1:500) was applied. After 48 h of incubation at 4 °C, the sections were incubated for 2 h at room temperature with the secondary anti-rabbit biotinylated antibody (Vector), followed by incubation with avidin+biotin amplification system (Vector) for 45 min. The staining was visualized using the TSA fluorescence system CY3 (PerkinElmer Life Sciences, Emeryville, CA, USA). After washing in TBS solution, the tissue was quenched and blocked again and incubated with the monoclonal antibody OX-6 (Pharmigen, final dilution 1:400) for 24 h or with the monoclonal antibody anti-GFAP (Chemicon, Temecula, CA, USA, final dilution 1:500) for 24 h. Before applying the biotinylated monoclonal secondary rat adsorbed antibody (Vector) for 2 h, the tissue was incubated with Avidin Biotin Blocking Kit (Vector) for 30 min to block cross reaction with the primary staining. Following treatment with an avidin+biotin amplification system (Vector), the staining was visualized with TSA fluorescence system CY5 (PerkinElmer Life Sciences) and the nuclei were counterstained with Sytox-Green (Molecular Probes, Eugene, OR, USA). No staining was detected in the absence of the primary or secondary antibodies.

RESULTS

Chronic infusion of LPS and WIN-55212-2 injections was well tolerated by all rats: they gained weight normally for the duration of the study.

Behavior

No significant difference in locomotor activity (swim speed) was found across groups, $P>0.1$. An ANOVA performed on the latency results obtained in the water maze task revealed an overall main effect of testing day ($F_{5,218}=16.057$, $P<0.0001$) for all groups (see Fig. 1) and an overall group effect (LPS versus aCSF) upon latency for each day of testing ($F_{1,328}=11.367$, $P=0.0008$ for day1; $F_{1,328}=85.681$,

$P<0.0001$ for day2; $F_{1,328}=109.756$, $P<0.0001$ for day3; $F_{1,328}=96.25$, $P<0.0001$ for day 4). There was no significant effect of treatment except for day 3 ($F_{2,328}=5.788$, $P=0.0034$). There was a significant interaction between group and drug on days 2 and 3 ($F_{2,328}=3.982$, $P=0.0196$ for day 2; $F_{2,328}=12.641$, $P<0.0001$ for day 3). Post hoc analyses of each testing day revealed a significant treatment effect on day 1, where LPS+WIN 1 rats were significantly impaired compared with the aCSF+WIN 1 rats ($P=0.0015$). On days 2 and 3, all LPS-infused rats were significantly ($P<0.0033$) impaired, as compared with their respective control groups. On day 4, all LPS-infused rats were significantly ($P<0.0033$) impaired as compared with their respective aCSF controls. There was a significant interaction between treatment and LPS-infused animals ($F_{2,113}=5.026$, $P=0.081$). Post hoc analyses of each testing day revealed a significant treatment effect on days 2 and 3; performance of LPS+WIN one rats was significantly ($P<0.0033$) worse as compared with the LPS+vehicle rats on those both days. Overall, the drug treatment (0.5 or 1 mg/kg) did not significantly impair performance of aCSF-infused rats and did not attenuate the impairment induced by the LPS infusion. The 1 mg/kg treatment, that did not cause any impairment in aCSF-infused rats, but did worsen the impairment observed in LPS-infused rats, demonstrating an interaction between inflammation and the highest dose of the drug used.

Histology

Immunostaining for OX-6 (Fig. 2A) revealed numerous highly activated microglia cells distributed throughout the hippocampus of LPS-infused rats (Fig. 2A d). The activated microglia had a characteristic bushy morphology with increased cell body size and contracted and ramified processes (Fig. 2A d). Rats infused with aCSF had very few mildly activated microglia evenly scattered throughout the brain, consistent with results from previous studies (Hauss-Wegrzyniak et al., 1998; Rosi et al., 2005). No

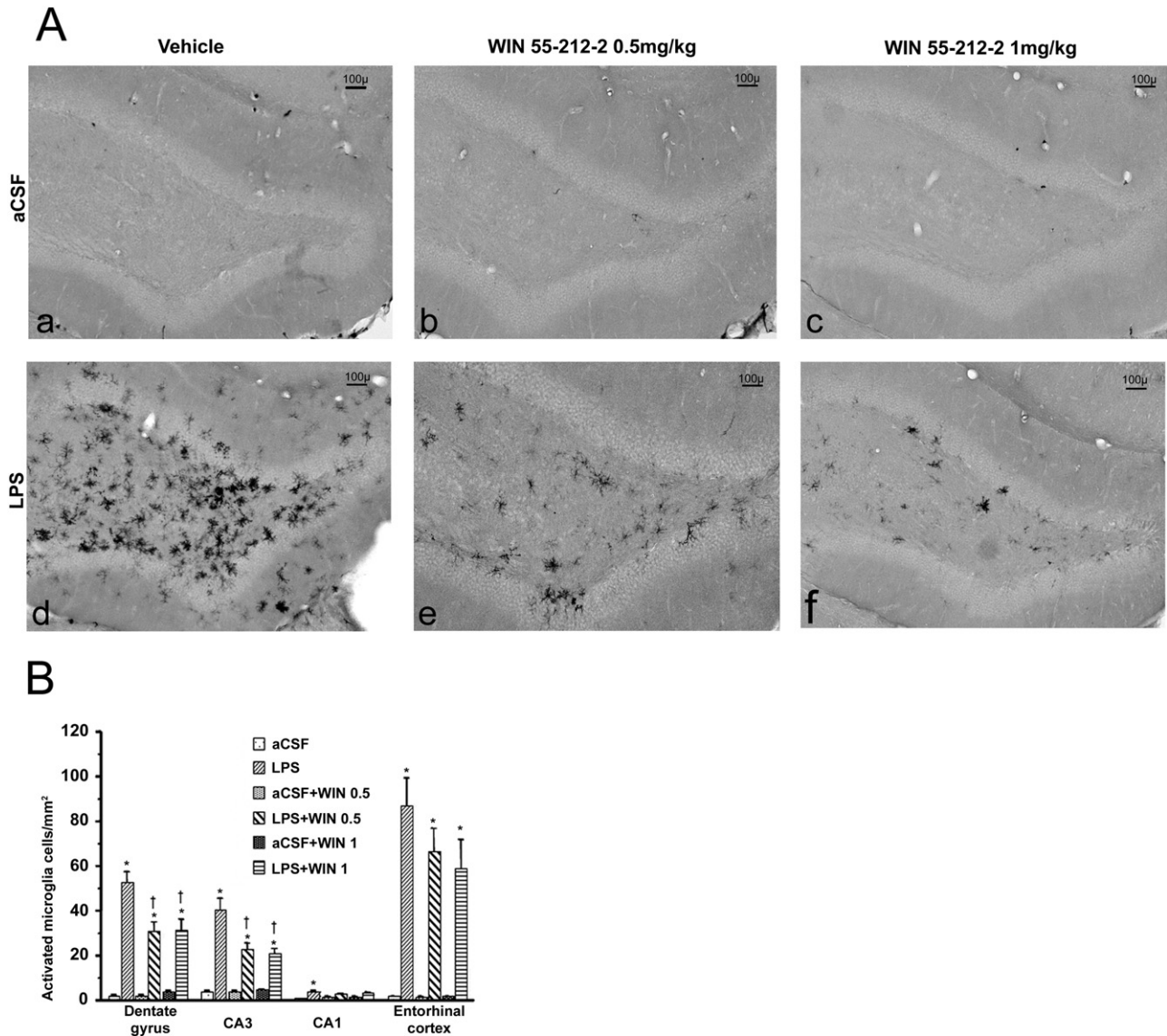


Fig. 2. (A) Activated microglia in the DG. Note the diminution of activated microglia cells in the DG of animals treated with either doses of WIN 55212-2 (e, f) compare with the LPS+vehicle group (d) (B) Density of activated microglial cells in different areas of interest. Similar results were obtained for the two doses of WIN-55212-2 used in the DG and CA3 areas. For both of them, LPS infused animal were significantly different from their control group (* $P < 0.0033$, Bonferroni/Dunn post hoc test). The injection of both doses (0.5 and 1 mg/kg) reversed partially the LPS induction of activated microglia († $P < 0.0033$, Bonferroni/Dunn post hoc test). No significant effect of the treatment was found for either CA1 or the EC.

difference in staining was evident between the aCSF group and the aCSF rats injected with either dose of WIN (Fig. 2A a–c). In LPS-infused rats that were also treated with WIN (Fig. 2A e–f) the OX-6 antibody stained fewer activated microglial cells.

Activated microglia cell counts

The number of activated microglia per millimeter square was determined in four different brain regions: dentate gyrus (DG), CA3 and CA1 regions of the hippocampus and the EC (Fig. 2B). These brain regions were examined for their known implication in spatial learning (Nadel and Land, 2000). An ANOVA of the data revealed an overall main region effect ($F_{5,210} = 16.057$, $P < 0.0001$) for all

groups and an overall main effect of the infusion of LPS in each region examined ($F_{5,48} = 32.557$, $P < 0.0001$ for DG; $F_{5,48} = 23.552$, $P < 0.0001$ for CA3; $F_{5,48} = 3.828$, $P = 0.0053$ for CA1; $F_{5,48} = 19.308$, $P < 0.0001$ for EC).

In the DG, CA3 and EC, all LPS-infused rats had a significantly ($P < 0.0033$) higher density of activated microglia compared with their respective control groups, consistent with our previous results (Rosi et al., 2005). In the DG and CA3 the number of activated microglia was significantly ($P < 0.0033$) reduced in LPS-infused rats given either dose of WIN, as compared with rats in the LPS+vehicle group (DG, 41.4% reduction for 0.5 mg/kg and 40.6% reduction for 1 mg/kg; CA3, 43.7% reduction for WIN 0.5 mg/kg and 49.4% reduction for WIN 1 mg/kg). In

the EC, despite a 24.2% reduction for WIN 0.5 mg/kg and 32.4% reduction for WIN 1 mg/kg, no significant difference was found between the LPS-infused groups. In the CA1 region of the hippocampus, there was a significant ($P < 0.0033$) difference only between the aCSF and LPS+vehicle groups. Overall, the effects of WIN were not dose-dependent in the DG and CA3 and not significant within the CA1 or EC brain regions.

CB1 receptors

The distribution of the CB1 receptors observed following our staining is in accordance with previous studies (Tsou et al., 1998) (Fig. 3a). Neuronal CB1 immunoreactivity was found in the hippocampus, striatum, amygdala as well as in the somatosensory, cingulate cortex and EC. The apparent density of immunoreactive cells in the areas of interest (DG, CA3, CA1, or EC) did not vary across groups.

No co-localization between CB1 receptors and activated microglial cells

Double-immunofluorescence staining for CB1 and activated microglial cells performed on the brains of all LPS-infused groups did not show any co-localization (Fig. 3b). These results indicate that CB1 receptors are not present on activated microglial cells in response to LPS infusion or treatment with a CBr agonist.

No co-localization between CB1 and astrocytes

Double-immunofluorescence staining for CB1 and astrocytes performed on the brains of all LPS-infused groups did not show any co-localization (Fig. 3c). These results indicate that CB1 are not present on astrocytes in response to LPS infusion or treatment with a CBr agonist.

DISCUSSION

The results demonstrate that a CB1/CB2 agonist, WIN-55212-2, prevents microglial cell activation during LPS-induced chronic neuroinflammation in young rats. The effects of this agonist were not dependent on direct CB1 receptors stimulation of microglia or astrocytes, were region dependent and did not reverse the LPS-induced impairment in a spatial memory task. The neurodegeneration associated with AD may result from prolonged activation of microglia and a chronic elevation of cytokines and nitric oxide (Akiyama et al., 2000; Streit, 2004) leading to a cascade of self-propagating cellular events that alters the expression of CBr (Minghetti and Levi, 1998; Bernardino et al., 2005; Klein, 2005). Endocannabinoids are implicated in the modulation of the central inflammatory response by neurons and glia (Klein et al., 2003; Klein, 2005) and may have a neuroprotective role in several neuroinflammation related diseases (Klein, 2005). Cannabinoid agonists can prevent the activation of microglia by β -amyloid, reduce the subsequent release of TNF- α (Ramirez et al., 2005), and attenuate the induction and release of nitric oxide by cultured microglia (Waksman et al., 1999). An inhibition of glutamate release by cannabinoids and the subsequent

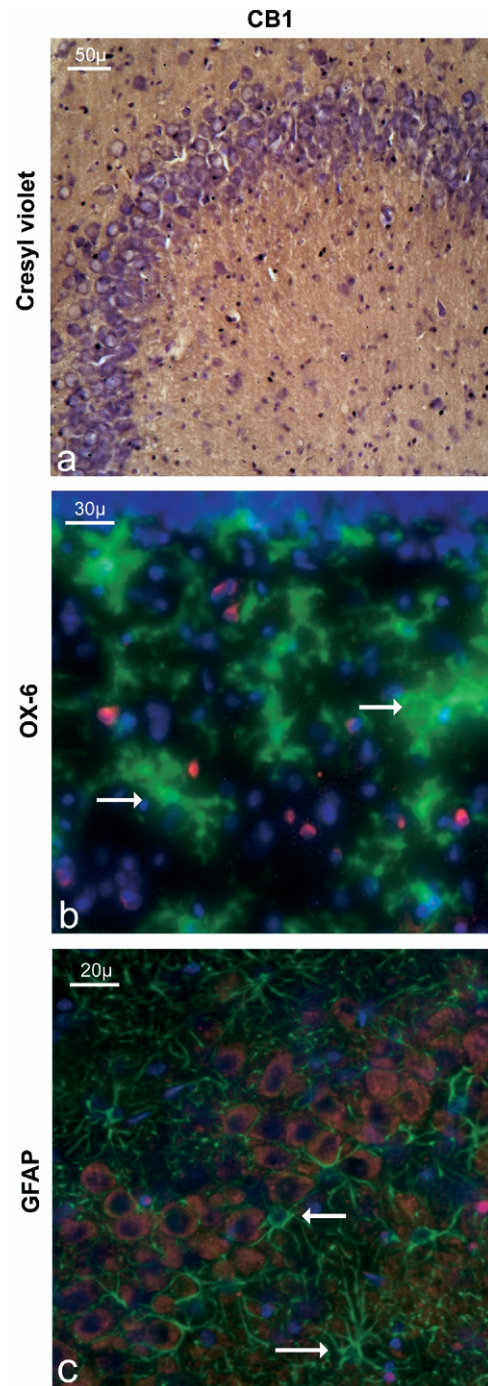


Fig. 3. Immunoreactivity (IR) of CBr in the CA3 region of the hippocampus. (a) CB1-IR (DAB stain) is seen in the cytoplasm of all CA3 neuronal cell bodies (counterstaining with Cresyl Violet). (b) CB1-IR (red) and MCH-II-IR (green) do not co-localize, as indicated by white arrows. (c) CB1-IR (red) and GFAP-IR (green) do not co-localize as indicated by white arrows. CB1-IR thus seems to be located only within neurons in the hippocampus. These photomicrographs were obtained from only LPS-infused rats that are representative of the staining observed in all groups.

reduction of the calcium influx via NMDA channels (Piomelli, 2003; Takahashi and Castillo, 2006) have also been demonstrated. Additionally, cannabinoids can attenuate

oxidative stress and subsequent toxicity (Hampson and Grimaldi, 2001) and induce the expression of brain-derived neurotrophic factor following the infusion of kainic acid (Marsicano et al., 2003).

CB₁ are expressed in senile plaques (Benito et al., 2003; Ramirez et al., 2005) and the number of CB₁ receptor-immunoreactive neurons is greatly reduced in areas of microglial activation in the AD brain (Ramirez et al., 2005). In contrast, we did not find evidence for changes in CB₁ receptor expression on hippocampal neurons in response to brain inflammation or CBr stimulation. This absence of changes in CB₁ receptors on neurons in our model may be related to the fact that the chronic infusion of LPS into young rats does not induce senile plaque formation (Hauss-Wegrzyniak et al., 1998) or neuronal loss in the hippocampus (Rosi et al., 2005), important factors that likely influence the expression of CBr-positive neuron in postmortem studies on AD brains from aged humans. The LPS infusion into the fourth ventricle produces widespread activation of glia and impaired performance in the water maze task (Hauss-Wegrzyniak et al., 1998). In contrast, an infusion of amyloid into a lateral ventricle (Ramirez et al., 2005) produced a more localized and limited glial activation (frontal cortex) and performance impairment that responded positively to treatment with WIN-55212-2 (Ramirez et al., 2005). This difference may explain why WIN-55212-2 in our model did not reverse the impairment induced by LPS in the fourth ventricle.

In the current study, CB₁ receptors were not co-localized with MCH II-positive microglia or GFAP-positive astrocytes within the hippocampus; the presence of these receptors appeared to be solely neuronal (Fig. 3). This suggests an indirect role of CBr upon microglia that is linked to the modulation of neuronal activity by stimulation of the endocannabinoid system. Our results are consistent with previous *in vivo* findings (Tsou et al., 1998) that describe CBr receptors located on hippocampal neurons, and their modulator role upon glutamatergic and GABAergic function (Takahashi and Castillo, 2006; Katona et al., 1999). CBr are present on astrocytes and microglial cells taken from humans, monkeys, rats and mice when studied *in vitro* (Stella 2004). The contradictory findings we report may be related to many factors, such as the different species that have been studied, the microenvironment of the cells (*in vivo* vs. *in vitro*), antibody sensitivity and selectivity, and the age or pathological condition under examination. Further experiments must be performed to determine clearly the characteristic and changes of the endocannabinoid system in our model and thus explain our present findings.

We have previously speculated that LPS induces a cascade of inflammatory processes that leads to an elevation in extracellular glutamate and activation of NMDA receptors (Wenk et al., 2006). The selective antagonism of NMDA receptors reduced microglia activation (Rosi et al., submitted for publication) similar to that reported in the current study using WIN-55212-2. Taken together these findings are consistent with the hypothesis that the ability of endocannabinoids to reduce the release of glutamate

within the hippocampus underlies the reduction of microglia activation by WIN-55212-2.

Interestingly, our results demonstrated an interaction between the presence of brain inflammation and the actions of the cannabinoid agonist at a dose (1 mg/kg/day i.p. of WIN-55212-2) that did not impair performance in the control rats. Surprisingly, those doses of agonist (0.5 and 1 mg/kg/day i.p. of WIN-55212-2) also did not improve performance, as might be expected given the results of our previous investigations using more typical anti-inflammatory drugs (Hauss-Wegrzyniak et al., 1999). We speculate that this was because the WIN-55212-2 treatment was not able to reverse totally the activation of microglia that had been induced by the chronic LPS infusion; in the current study, the reduction in the number of activated microglia was only about 40–50%, in contrast to the ability of a non-steroidal anti-inflammatory drug's ability to reduce the number of activated microglia almost completely. This partial reduction in microglia activation might thus not be sufficient to reverse the spatial memory impairment produced by the LPS infusion. This result is of particular importance with regard to patients suffering with a disease associated with brain inflammation, e.g. AD, Parkinson's disease or multiple sclerosis, who are also using marijuana.

The current report is the first to our knowledge to demonstrate the modulatory role of cannabinoids in an animal model of chronic neuroinflammation, pointing out the effectiveness of a CBr agonist on the consequences of LPS-mediated neuroinflammation at a dose (0.5 mg/kg/day i.p. of WIN-55212-2) that does not impair performance in a spatial memory task. These results further advocate for the manipulation of the endocannabinoid system to diminish the consequences of neuroinflammation in progression of AD and others inflammation-related diseases (Klein, 2005).

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