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Cannabinoid receptor stimulation is anti-inflammatory and improves memory in old rats

Yannick Marchalant, Francesca Cerbai, Holly M. Brothers, Gary L. Wenk *

Department of Psychology, Psychology Building, Ohio State University, Columbus, OH 43210, USA

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Abstract

The number of activated microglia increase during normal aging. Stimulation of endocannabinoid receptors can reduce the number of activated microglia, particularly in the hippocampus, of young rats infused chronically with lipopolysaccharide (LPS). In the current study we demonstrate that endocannabinoid receptor stimulation by administration of WIN-55212-2 (2 mg/kg day) can reduce the number of activated microglia in hippocampus of aged rats and attenuate the spatial memory impairment in the water pool task. Our results suggest that the action of WIN-55212-2 does not depend upon a direct effect upon microglia or astrocytes but is dependent upon stimulation of neuronal cannabinoid receptors. Aging significantly reduced cannabinoid type 1 receptor binding but had no effect on cannabinoid receptor protein levels. Stimulation of cannabinoid receptors may provide clinical benefits in age-related diseases that are associated with brain inflammation, such as Alzheimer's disease.

Keywords: Cannabinoid receptors; Inflammation; Activated microglia; Aging; Spatial memory

1. Introduction

Microglial cells play a pivotal role as immune effectors in the central nervous system 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 cytotoxic proteins, reactive oxygen species or complement (Akiyama et al., 2000; Kim and de Vellis, 2005). Recent evidence suggests that the aging process induces morphological changes in microglia (Streit, 2006), and treatment with NSAIDs does not reduce microglia activation in old rats, in contrast to their effectiveness in young rats (Hauss-Wegrzyniak et al., 1999), thus raising the possibility of microglial senescence (Perry et al., 1993; Streit, 2006).

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 (Klein, 2005; Marchalant et al., 2007). The endocannabinoid system is comprised of two G-protein-coupled receptors designated as CB1 and CB2 (Pertwee, 2005); 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 exogenous cannabinoids (Pertwee, 2005; Tsou et al., 1998). CB2 receptors are expressed by immune and hematopoietic cells peripherally (Begg et al., 2005), and may be expressed on neurons in the brainstem and the brain (Benito et al., 2003; Gong et al., 2006; Onaivi et al., 2006; Van Sickle et al., 2005) although their presence in the brain is controversial (Munro

Abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; aCSF, artificial cerebral spinal fluid; AD, Alzheimer's disease; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; CBr, cannabinoid receptors; DG, dentate gyrus; EC, entorhinal cortex; LPS, lipopolysaccharide; NMDA, *N*-methyl-D-aspartate; PBS, phosphate buffer saline; TBS, Tris buffer saline; 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

* Corresponding author. Tel.: +1 614 688 3404; fax: +1 614 688 4733.
E-mail addresses: marchalant.1@osu.edu (Y. Marchalant), cerbai.1@osu.edu (F. Cerbai), brothers.23@osu.edu (H.M. Brothers), wenk.6@osu.edu (G.L. Wenk).

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et al., 1993). Two endogenous ligands for these receptors, arachydonylethanolamine and 2-arachidonoylglycerol (Stella, 2004), influence immune responses by inhibiting cytokine release and other anti-inflammatory actions (Klein et al., 2003; Klein, 2005). In vitro, microglia expresses CB receptors and release cytokines in response to exposure to LPS or beta-amyloid protein (Facchinetti et al., 2003; Ramirez et al., 2005; Sheng et al., 2005). Astrocytes may also synthesize and release endocannabinoids (Walter et al., 2002). Although stimulation of CB1 receptors, e.g. by administration of Δ^9-tetrahydrocannabinol, can impair performance in rats, mice or monkeys (Castellano et al., 2003), we previously demonstrated that stimulation of the CB1/2 receptors using a low dose of (*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) significantly reversed the LPS-induced microglia activation in young rats without attenuating the neuroinflammation-induced performance impairment observed in the water pool task (Marchalant et al., 2007). Because normal aging is associated with increased levels of microglial activation, in the current study, we investigated for anti-inflammatory and memory enhancing effects of CB1/2 receptor stimulation in normal aged rats.

2. Methods

2.1. Subjects and surgical procedures

Eighteen young (3 months old) and 24 old (23 months old) male F-344 rats (Harlan Sprague–Dawley, Indianapolis, IN) were singly housed in Plexiglas cages with free access to food and water. The rats were maintained on a 12/12-h light–dark cycle in a temperature-controlled room (22 °C) with lights off at 08:00. All rats were given health checks, handled upon arrival and allowed at least 1 week to adapt to their new environment prior to surgery.

WIN-55212-2 (Sigma, St. Louis, MO, 0.5 or 2 mg/kg day, $n = 28$) or the vehicle (dimethylsulfoxide (100%) DMSO Sigma, St. Louis, MO, $n = 14$) were chronically infused for 21 days subcutaneously using an osmotic minipump (Alzet, Cupertino, CA, model 2ML4, to deliver 2.5 μl/h). Rats were assigned to one of the following six groups: young + vehicle ($n = 6$), old + vehicle ($n = 8$), young + WIN 0.5 mg/kg day ($n = 6$), old + WIN 0.5 mg/kg day ($n = 8$), young + WIN 2 mg/kg day ($n = 6$), old + WIN 2 mg/kg day ($n = 8$). Behavioral testing began on day 14 post surgery.

2.2. Water pool testing

Spatial learning ability was assessed using a 170 cm diameter water maze with grey walls. The water was maintained at 26–28 °C. The pool was in the center of a 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 10 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 using Noldus Ethovision 3.1 tracking and analysis system (Noldus, Leesburg, VA).

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. The results were analyzed by ANOVA followed by post hoc comparisons according to the method of Fisher.

2.3. 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. 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 μm) 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, Pharmigen, San Diego, CA) was used to visualize activated microglia cells. This antibody is directed against Class II major histocompatibility complex (MHC II) antigen. After quenching endogenous peroxydase/activity and blocking nonspecific binding, the sections were incubated (4 °C) overnight with the primary antibody (OX-6). 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). Sections were then incubated for 1 h (22 °C) with avidin-biotinylated horseradish peroxydase (Vectastain, Elite ABC kit, Vector, Burlingame, CA). After washing again in PBS, the sections were incubated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Vector, Burlingame, CA) as chromogen. The reaction was stopped by washing the section with PBS. No staining was detected in the absence of the primary or secondary antibodies. Sections were mounted on slides, air-dried and coverslipped with cytoseal (Allan Scientific, Kalamazoo, MI) 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). Briefly, areas of interest were determined as previously reported in detail (Rosi et al., 2005), their surface measured, and the immunoreactive cells numerated, allowing us to determine a number of immunoreactive cells by millimeter square in the areas of interest.

2.4. Double immunofluorescence staining

Free floating sections were mounted on slides and air-dried. The tissues were then processed as described previously (Rosi et al., 2005). Briefly, after washing in TBS solution the polyclonal rabbit anti-CB1 (Sigma, St. Louis, MO, dilution 1:500) was applied. After 24 h of incubation at 4 °C, the sections were incubated for 2 h at room temperature with the secondary anti-rabbit biotinylated antibody (Vector, Burlingame, CA), followed by incubation with Avidin + Biotin amplification system (Vector, Burlingame, CA) for 45 min. The staining was visualized using the TSA fluorescence system CY3 (Perkin-Elmer Life Sciences, Emeryville, CA). After washing in TBS solution, the tissues were quenched and blocked again and incubated with either of the following: a monoclonal antibody to MHC-II (OX-6, Pharmigen, San Diego, CA, final dilution 1:400), the monoclonal antibody anti-GFAP (Chemicon, Temecula, CA, final dilution 1:500), the monoclonal antibody anti-neuronal nuclei (Chemicon, Temecula, CA, final dilution 1:500), the monoclonal antibody OX-42 (BD Pharmingen, San Jose, CA final dilution 1:400) or the monoclonal antibody NMDA-R1 (Chemicon, Temecula, CA, final dilution 1:250) for 24 h at 4 °C. Before applying the biotinylated monoclonal secondary rat absorbed antibody (Vector, Burlingame, CA) for 2 h, the tissue was incubated with Avidin Biotin Blocking Kit (Vector, Burlingame, CA) for 30 min to block cross-reaction with the primary staining. Following treatment with an Avidin + Biotin amplification system (Vector, Burlingame, CA), the staining was visualized with a TSA fluorescence system CY5 (Perkin-Elmer Life Sciences, Emeryville, CA) and the nuclei were counterstained with Sytox-Green (Molecular Probes, Eugene, OR). No staining was detected in the absence of the primary or secondary antibodies.

2.5. Western blots

Western immunoblotting was carried out as described in Giovannini et al. (2001). Briefly, rats were sacrificed, areas of interest microdissected and transferred to ice-cold microcentrifuge tubes and kept at −70 °C until use. On experiment day, tissues were homogenized on ice directly into the Eppendorf tube (20 strokes, 1 stroke/s) using the lysis buffer (composition (mM): 50 Tris–HCl, pH 7.5, 50 NaCl, 10 EGTA, 5 EDTA, 2 sodium pyrophosphate, 4 para-nitrophenylphosphate, 1 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin and 30 μg/ml aprotinin, 0.1% SDS). Immediately after homogenization the quantity of protein was determined using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). An appropriate volume of 2 × loading buffer was added to the homogenates, and samples were boiled for 5 min. Samples (40 μg of proteins per well) were loaded onto a 10% SDS-PAGE gel and resolved by standard electrophoresis. The proteins were then transferred electrophoretically onto nitrocellulose membrane (Hybond-C extra; Amersham, Arlington Heights, IL, USA) using a transfer tank kept at 4 °C, with a constant current of 12 mA. Membranes were blocked for 1 h at room temperature with a blocking buffer (BB, 5% non-fat dry milk in TBS containing 0.05% Tween 20, TBS-T), then probed for 2 h at room temperature using primary antibody for CB1 (rabbit polyclonal, 1:500; Sigma, Saint-louis, MO, USA). After washing in TBS-T (three washes, 15 min each), the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:7500; Pierce, Rockeford, Illinois, USA), and proteins were visualized using chemiluminescence (Super Signal West Pico Chemioluminescent Substrate, Pierce, Rockeford, Illinois, USA). In order to normalize the values of CB1, we used actin. Membranes were stripped by Restore Western Blot Stripping Buffer (Pierce, Rockeford, Illinois, USA) (15 min, room temperature), blocked in BB for 1 h at room temperature and probed for 2 h at room temperature using antibodies for actin (1:10,000; NEB), incubated in the secondary antibody and developed. All primary antibodies were dissolved in BB, while secondary antibodies were dissolved in TBS-T. After development of the film, the bands were acquired as TIFF files, and the density of the bands was quantified by a densitometric analysis performed using Quantity One for Windows (Biorad, Beverly, MA, USA) software. CB1 values were expressed as percentage of actin run in the same Western blot analysis.

2.6. [^{3}H] SR141716A in vitro binding

Each rat was briefly anesthetized using isoflurane gas and then quickly sacrificed by decapitation. The brains were quickly removed and bilateral samples of hippocampus were dissected as described previously (Wenk and Barnes, 2000) and quickly frozen on dry ice and then stored (@ −70 °C) until assayed for [^{3}H] SR141716A binding to CB1 receptors according to the method of Steffens et al. (2005). Membrane fractions were prepared from the tissues by Homogenization in 10 volumes of the following buffer (50 mM Tris–HCl, 1.0 mM EGTA, 3 mM $MgCl_2$, 0.1% bovine serum albumin, pH 7.4), repeated centrifugation (1000 × *g* for 10 min @ 4 °C followed by 10,000 × *g* for 10 min @ 4 °C). The supernatant was discarded and the remaining pellet was resuspended (10 vols) in the same buffer. The assays were conducted in an incubation volume of 500 μl containing [^{3}H] SR141716A (4.0 ηM, 55 Ci/mmol, NEN) and 80–100 μg of membrane protein. Incubation was carried out at 30 °C for 60 min and terminated by dilution with 4 ml of ice-cold homogenizing buffer followed immediately by filtration using a

Brandell 24-well harvester through GF/C filters that had been presoaked in 0.03% polyethylenimine, pH 7.0. Bound radioactivity was determined with a Tri-Carb 1800TR liquid scintillation analyser (Packard). Specific binding was defined as total binding minus binding in the presence of the CB_1 receptor antagonist *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251, 1 μM). Differences in the number of receptor binding sites were analyzed by ANOVA.

3. Results

Chronic infusion of DMSO and WIN-55212-2 were well tolerated by all rats.

3.1. Behavior

An ANOVA performed on the latency results obtained in the water maze task revealed a significant effect of testing day ($F_{5,134} = 20.709$, $p < 0.0001$, see Fig. 1), age ($F_{1,196} = 13.4$, $p < 0.0001$ across all days of testing), and drug treatment ($F_{5,192} = 16.9$, $p < 0.0001$ for days 2–4). For young rats, there was a significant interaction between treatment and testing day ($F_{2,381} = 6.691$, $p = 0.0014$). On days 2 and 3 the performance of young rats given the low dose of WIN was significantly ($p < 0.05$) worse as compared to young rats given the vehicle. In contrast, on day 4, the performance of young rats given the high dose of WIN was significantly ($p < 0.05$) better as compared to the young rats given the vehicle. For old rats, there was a significant interaction between treatment and testing day ($F_{2,405} = 13.999$, $p < 0.0001$). The performance of old rats given the high dose of WIN was significantly better ($p < 0.05$) as compared to the old rats given the vehicle. The performance of old rats given the low dose of WIN did not differ from the old rats given the vehicle. For young rats, WIN treatment did not impair performance during the probe trial (platform removed, percentage of time spent in platform quadrant: vehicle, 35% ± 3.2; WIN 0.5, 48.6% ± 4; WIN 2, 38.2% ± 8.5). For old rats, the high WIN dose (2 mg/kg day) significantly improved performance during the probe trial (44.5% ± 9.2 versus 26.4% ± 1.8 for vehicle group).

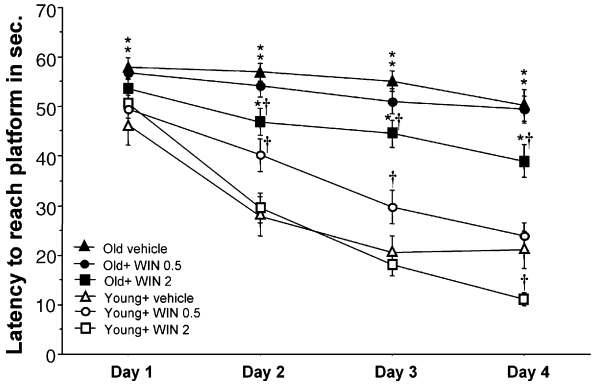

Fig. 1. Water maze performance. During days 2 and 3, young rats receiving the 0.5 mg/kg WIN dose (open circle) performed significantly worse than rats in the other two young groups (open triangle and open square) ($^{\dagger}p < 0.05$). On day 4, the young rats receiving the 2 mg/kg WIN dose (open square) performed significantly better than the other two groups of young rats (open triangle and circle, $^{\dagger}p < 0.05$). Old rats were significantly impaired as compared to young rats ($^{*}p < 0.05$). WIN-55212-2 2 mg/kg (closed square) improved performance of age rats (closed triangle, $^{\dagger}p < 0.05$).

3.2. Histology

Immunostaining (Fig. 2A) revealed activated microglia (OX-6-immunopositive) cells distributed throughout the hippocampus of old 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). Young rats (with vehicle, WIN 0.5 or WIN 2, Fig. 2A, a–c) had very few, mildly activated microglia evenly scattered throughout the brain, consistent with results from previous studies (Hauss-Wegrzyniak et al., 1998; Marchalant et al., 2007). The highest dose of WIN reduced the number of immunostained microglia within the hippocampus of old rats (Fig. 2A, d–f).

3.3. Regional microglia cell counts

The number of activated microglia per millimeter square was determined in four different area of interest: dentate gyrus (DG), CA3 and CA1 regions of the hippocampus and the entorhinal cortex (EC) (Fig. 2B). These brain regions were examined because of their importance for spatial learning (Nadel and de Vellis, 2000). An ANOVA revealed an overall main effect of age on the number of immunostained microglia ($F_{1,61} = 7.626$, $p < 0.0076$) with a significant increase due to age in the dentate gyrus ($F_{1,13} = 9.766$, $p = 0.008$) and in CA3 ($F_{1,13} = 24.931$, $p = 0.0002$). The highest dose of WIN reduced the number of immunostained microglia in the CA3 ($F_{5,9} = 15.303$, $p = 0.0004$).

3.4. CB1 receptors

CB1 immunoreactivity was found in the hippocampus, striatum, amygdala as well as in the somatosensory, cingulate and entorhinal cortices; the distribution of these immunoreactive cells did not vary across age or treatment groups and was consistent with previous reports (Tsou et al., 1998; Katona et al., 2006; Marchalant et al., 2007). The quantity of CB1 protein present in the hippocampus determined by western blotting technique (Fig. 3) also did not vary significantly across age or treatment groups ($F_{5,79} = 0.516$, $p = 0.7631$). In contrast, an ANOVA revealed a significant main effect of age ($F_{1,20} = 6.13$, $p < 0.05$) upon the number of [^{3}H] SR141716A binding sites in the hippocampus (Table 1), but no main effect of drug treatment ($F_{5,16} = 1.161$, $p = 0.37$).

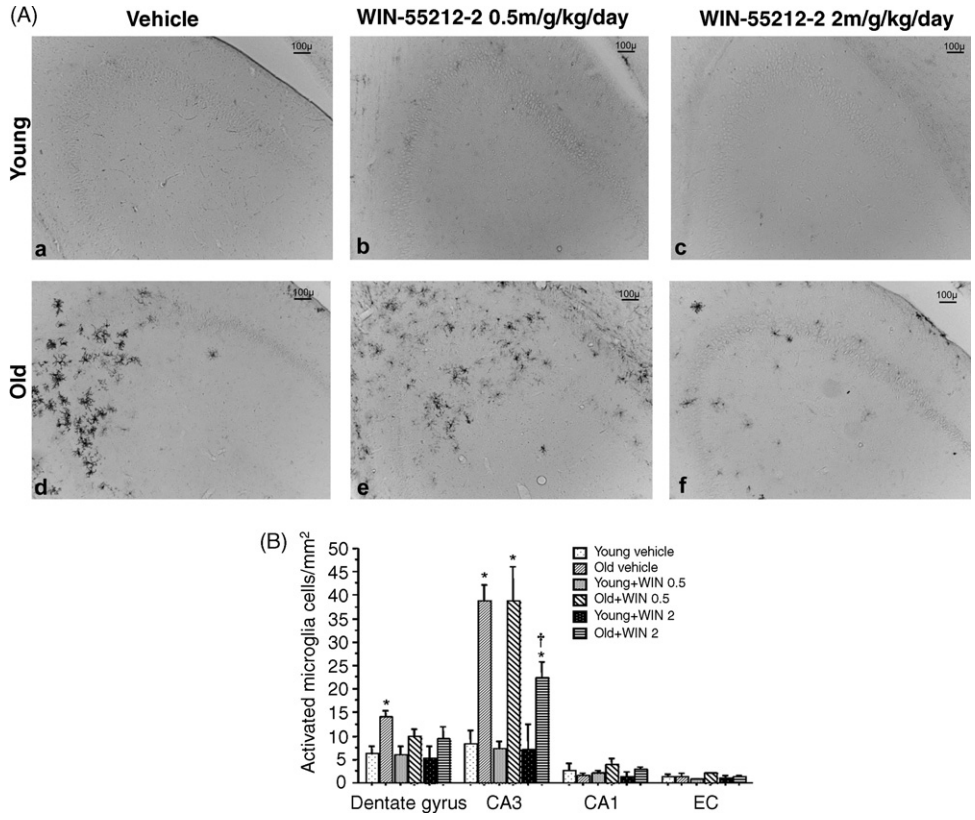

Fig. 2. (A) Activated microglia in the CA3 region. Note the diminution of activated microglia cells in this region of rats treated with 2 mg/kg day of WIN-55212-2 (f), as compared to the normal aged rats (d). Scale bar: 100 μm. (B) Density of activated microglial cells in different areas of interest. Microglia density in the dentate gyrus and CA3 of aged animal were significantly different from their young control group ($^{*}p < 0.05$). The infusion of the highest dose (2 mg/kg day) of WIN-55212-2 reversed partially the aged induction of activated microglia ($^{\dagger}p < 0.05$). No significant effect of aging or treatment was found for either CA1 or the entorhinal cortex.

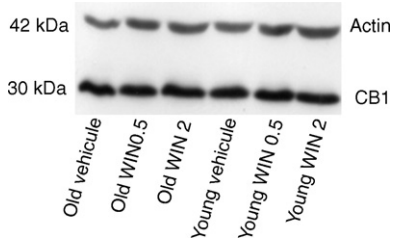

Fig. 3. Protein expression of CB1 receptors in the hippocampus. No changes in protein expression were found between groups for CB1 receptors in the hippocampus of rats.

3.5. No co-localization between CB1 receptors and resting or activated microglial cells

Double-immunofluorescence staining for CB1 receptors and resting (OX-42, Fig. 4E, e) or activated microglial (OX-6, Fig. 4A, a) performed on the brains of all groups did not show any co-localization. These results suggest that CB1 receptors are not present on microglial cells in aged rats or following a treatment with a CBr agonist.

3.6. No co-localization between CB1 and astrocytes

Double-immunofluorescence staining for CB1 receptors and GFAP-positive astrocytes performed on the brains of all groups did not show any co-localization (Fig. 4C, c). These results suggest that CB1 are not present on astrocytes in aged rats or following treatment with a CBr agonist.

3.7. Co-localization between CB1 and neurons

Double-immunofluorescence staining for CB1 receptors and neurons performed on the brains of all groups demon-

Table 1
Hippocampal CB1 receptor binding assay

fmol/mg of protein ± S.D.	Vehicle	WIN 0.5 mg/kg day	WIN 2 mg/kg day
Young (3 months)	10.089 ± 2.715	8.548 ± 4.931	9.322 ± 3.343
Old (23 months)	5.995 ± 2.259	5.038 ± 3.817	6.631 ± 3.644

An overall aging effect (young vs. aged rats) was observed ($p < 0.05$). No other significant differences between groups were observed.

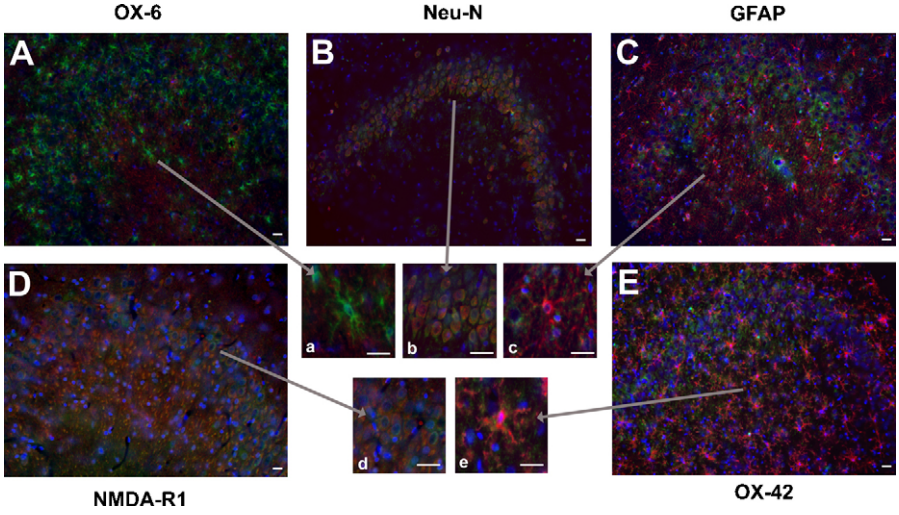

Fig. 4. Immunoreactivity (IR) of CB1 in the CA3 region of the hippocampus. All scale bars: 50 μm. (A) CB1-IR (red) do not co-localized with OX-6-IR (green), as magnified in (a). (B) CB1-IR (green) and NeuN-IR (red) do co-localize, as magnified in (b). (C) CB1-IR (green) and GFAP-IR (red) do not co-localize as magnified in (c). (D) CB1-IR (green) and NMDA-R1-IR (red) do co-localize as magnified in (d). (E) CB1-IR (green) and cd11b-IR (red) do not co-localize as magnified in (e). CB1-IR thus seems to be located only within neurons in the hippocampus, particularly with neurons expressing NMDA receptors. These photomicrographs are representative of the staining observed in all groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

strated a strong spatial co-localization of CB1 receptors and the soma of neurons (Fig. 4B, b). These results are consistent with the hypothesis that CB1 receptors are closely associated with neurons in the regions of interest and particularly within the CA3 region of the hippocampus. Moreover double-immunofluorescence staining for CB1 and NMDA-R1 performed on the brains of all groups demonstrated a strong spatial co-localization of CB1 receptors and NMDA-R1 receptors on neuronal cell bodies and dendritic processes (Fig. 4D, d). These results suggest that CB1 receptors are closely associated with glutamatergic receptors in the regions of interest and particularly in the CA3 region of the hippocampus.

4. Discussion

The results demonstrate that a CB1/CB2 receptor agonist, WIN-55212-2, can effectively reduce microglial cell activation associated with normal aging. The effects of this drug were not dependent upon direct CB1 receptor stimulation on microglia or astrocytes, were region dependant and significantly attenuated the aged-associated impairment in a spatial memory task. No significant changes were observed in the protein expression of the CB1 receptor in the hippocampus, with the exception of a decrease in CB1 receptor density in aged rats as compared to young rats that is consistent with a previous report (Berrendero et al., 1998). CB receptor expression is altered (diminution of CB1-immunoreactive neurons) in AD (Ramirez et al., 2005). Endocannabinoids may be neuroprotective via their ability to modulate inflammatory response of neurons and glia to β-amyloid, control the release of TNF-α, nitric oxide and glutamate and reduce calcium influx via NMDA channels (Klein et al., 2003; Klein, 2005; Marchalant et al., 2007; Piomelli, 2003; Ramirez et al., 2005; Takahashi and Castillo, 2006; Waksman et al., 1999). Cannabinoids can attenuate oxidative stress and its subsequent toxicity (Hampson and Grimaldi, 2001) or induce the expression of brain-derived neurotrophic factor following the infusion of kainic acid (Marsicano et al., 2003). Interestingly, WIN-55212-2 can inhibit the IL-1 signaling pathway in human astrocytes through a pathway that may not involve CB1 or CB2 cannabinoid receptors (Curran et al., 2005). We previously demonstrated that WIN-55212-2 administration can reduce LPS-induced chronic neuroinflammation in young rats (Marchalant et al., 2007).

The mechanism underlying the effect of WIN-55212-2 treatment upon microglial activation is unknown but is likely indirect. Our results suggest that CB1 receptors are not co-localized with microglia (in their resting or activated state) or GFAP-positive astrocytes within the hippocampus of normal aged rats. CB1 receptors were co-localized with NMDA receptors on neurons (Fig. 3) in the CA3 region of the hippocampus in young and aged rats. We have recently shown that selective antagonism of NMDA receptors can also reduce microglia activation (Rosi et al., 2006) similar to that reported in the current study suggesting an indirect influence of CB receptors upon microglia activation that might be linked to the modulation of glutamate synaptic transmission or neuronal activity. We speculate that the activation of endocannabinoid receptors in the presence of brain inflammation may restore a proper calcium influx via NMDA channels in a manner similar to that described for the NMDA channel antagonist memantine (Rosi et al., 2006; Wenk et al., 2006). Activated microglial cells also increase their expression of GABA-b receptors; stimulation of GABA-b receptors can

reduce microglia activation (Kuhn et al., 2004) and reduce the release of glutamate and GABA in the entorhinal cortex (Thompson et al., 2007). Therefore, our results are consistent with the hypothesis that CB receptors on hippocampal neurons modulate glutamatergic and GABAergic function (Katona et al., 1999; Katona et al., 2006; Takahashi and Castillo, 2006) and this leads to reduced microglia activation. This mechanism may underlie the neuroprotective effects of cannabinoids (Nagayama et al., 1999; Parmentier-Batteur et al., 2002; Pryce et al., 2003).

Importantly, the benefits of cannabinoid receptor stimulation occurred at a dose that did not impair performance in a spatial memory task, indeed the performance of aged rats was significantly improved. This finding is particularly relevant for elderly for patients suffering with diseases associated with brain inflammation, e.g. AD, Parkinson's disease or multiple sclerosis. The current report is the first to our knowledge to demonstrate the anti-inflammatory actions of cannabinoid therapy in aged animals and strongly advocate an cannabinoid-based therapy for neuroinflammation-related diseases (Klein, 2005), as well as a potential tool to reduce the impairment in memory processes occurring during normal aging.

Conflict of interest

None of the authors have conflicts of interest.

Disclosure statement

We certify that the experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23) revised 1996. We also certify that the formal approval to conduct the experiments has been obtained from the animal subjects review board from Ohio State University.

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