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Cannabinoids attenuate the effects of aging upon neuroinflammation and neurogenesis☆

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ABSTRACT

WIN-55,212-2 (WIN-2) can elicit anti-inflammatory and cognitive-enhancing effect in aged rats. The current study was designed to determine the differential role of the endocannabinoid receptor sub-types 1 (CB1) and 2 (CB2) and transient receptor potential vanilloid 1 receptor (TRPV1) in the reduction of age-associated brain inflammation and their effects on neurogenesis in the dentate gyrus of aged rats. Our results demonstrate that 1) the antagonist actions of WIN-2 at the TRPV1 receptor are responsible for the reduction in microglial activation and 2) the agonist actions of WIN-2 at CB1/2 receptors can trigger neurogenesis in the hippocampus of aged rats. Chronic treatment with WIN-2 established an anti-inflammatory cytokine profile within the hippocampus. Our results provide insight into the role of the endocannabinoid and vanilloid systems upon two different and detrimental aspects of normal and pathological aging, chronic neuroinflammation and decline in neurogenesis.

Introduction

Inflammatory changes are closely related to the clinical manifestations of many age-related diseases of the brain (Akiyama et al., 2000). A central element of the brain's inflammatory response during neurodegenerative diseases is activated microglial cells (Akiyama et al., 2000; Hauss-Wegrzyniak et al., 1998; Streit, 2004). Clinical benefits might be achieved by adjusting the microglial response to one which will benefit recovery and protect neurons from further damage rather than contributing to the degeneration (Prince et al., 1998; Blasko et al., 2004, 2007). Recent studies suggest that drugs targeting the endocannabinoid system may prove useful in the treatment of diseases associated with neuroinflammation, including multiple sclerosis, Parkinson's disease (PD) and Alzheimer's disease (AD, Maresz et al., 2005; Ramirez et al., 2005; Eljaschewitsch et al., 2006; Marchalant et al., 2007, 2008a, 2008b). The current challenge is to avoid targeting the components of this neurotransmitter system that are responsible for its psychoactive proclivities. The endocannabinoid system in the brain is composed of two major molecules derived from arachidonic acid, arachidonoylethanolamide or anandamide and 2-arachidonoyl glycerol or 2-AG, that bind primarily to endocannabinoid receptors type 1 (CB1) and type 2 (CB2) along with the transient receptor potential vanilloid type 1 receptor (TRPV1). We have previously demonstrated the anti-inflammatory effect of a low dose of WIN-55,212-2 (WIN-2) in young rats chronically infused with lipopolysaccharide into the 4th ventricle (I.C.V) and normal aged rats (Marchalant et al., 2007, 2008a). However, it is not known whether the anti-inflammatory effect of WIN-2 was achieved through the CB1, CB2 or TRPV1 receptors because WIN-2 is an agonist at the CB1 and CB2 and an antagonist at the TRPV1. Moreover, the relation between neuroinflammation and neurogenesis during normal and pathological aging also remains unclear (Ekdahl et al., 2008, for review). Neurogenesis occurs in the dentate gyrus (DG) via the mitosis of neural progenitor cells located in the subgranular zone (SGZ) (Gage et al., 1998; Kokaia and Lindvall, 2003; Lledo et al., 2006) and may play an important role in the maintenance of hippocampal functions such as learning and memory (Van Praag et al., 2000; Shors et al., 2001; Aimone et al., 2006). Neurogenesis in the DG declines dramatically during normal aging and this decline may underlie many aspects of cognitive deficits of dementia associated with Alzheimer's disease (Galvan and Bredesen, 2007 for review; Chen et al., 2008). Recent studies suggest that the endocannabinoid system may control the proliferation of newly generated neurons via either CB1 receptors (Aguado et al., 2005, 2007) or CB2 receptors (Goncalves et al., 2008). The current study

Abbreviations: 5′-I-RTX, 5′-Iodo-resiniferatoxin; AD, Alzheimer's disease; BrdU, Bromodeoxyuridine; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; CBr, cannabinoid receptors; DG, Dentate gyrus; DCX, Double cortin; EC, Entorhinal cortex; LPS, Lipopolysaccharide; NMDA, N-methyl-D-aspartate; PBS, phosphate buffer saline; SGZ, Subgranular zone; SR 141716A, 5-(p-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-piperidinopyrazole-3-carboxamide hydrochloride; SR 144528, 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; TBS, Tris buffer saline; TRPV1, Transient receptor potential vanilloid 1; 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.

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determined the differential role of the CB1, CB2 and TRPV1 receptors in the reduction of age-associated brain inflammation and in the control of neurogenesis in the DG of the aging hippocampus. This information may lead to more effective and selective manipulation of the endocannabinoid system in the aging brain.

Experimental procedures

Subjects and surgical procedures

Fifty four aged (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 were maintained on a 12/12 h light–dark cycle in a temperature-controlled room (22 °C) with lights off at 0900. All rats were given health checks, handled upon arrival and allowed at least one week to adapt to their new environment prior to surgery.

WIN-2 (2 mg/kg/day, Sigma, St-Louis, MO), Rimonabant, a selective CB1 antagonist (SR141716 or SR1, Sanofi-Aventis, Montpellier, France, 3 mg/kg/day), SR144528, a selective CB2 antagonist (SR2, Sanofi-Aventis, Montpellier, France, 1 mg/kg/day) or the vehicle (dimethylsulfoxide/Polyethylene glycol (50/50v) DMSO/PEG Sigma, St-Louis, MO) were chronically infused for 21 days subcutaneously (s.c.) using an osmotic minipump (Alzet, Cupertino, CA, model 2ML4, to deliver 2.5 µl/h) implanted into the dorsal abdomen area. The selective TRPV1 antagonist, 5′-I-RTX (5′-Iodoresiniferatoxin, Tocris, MO, 1 µg/kg/day) was chronically infused via an indwelling cannula into the 4th ventricle for 21 days using an osmotic minipump (Alzet, Cupertino, CA, model 2004, to deliver 0.25 µl/h) according to a previously described surgical implantation approach (Hauss-Wegrzyniak et al., 1998). The doses of SR1, SR2 and 5′-I-RTX were previously demonstrated to effectively antagonize CB1, CB2 and TRPV1 receptors, respectively (Rinaldi-Carmona et al., 1995, 1998; Jhaveri et al., 2005).

Rats were assigned to one of the six following groups: vehicle (n=11), WIN (n=9), WIN+SR1 (n=9), WIN+SR2 (n=10), WIN+SR1/SR2 (n=8), and 5′-I-RTX (n=7).

Histological procedures

After three weeks of drug treatment each rat was deeply anesthetized with isoflurane and prepared for a transcardial 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 post-fixed overnight in the same fixative and then stored (4 °C) in phosphate buffered saline (PBS), pH 7.4. Free-floating coronal sections (40 µm) were obtained using a vibratome for staining with standard avidin/biotin peroxidase or fluorescence labeling methods.

Single immunocytochemistry

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. The polyclonal antibody anti-capsaicin receptor (TRPV1, final dilution 1:250, Chemicon, Temecula, CA) was used to visualize TRPV1 receptors. The antibody doublecortin (DCX, final dilution 1:250, Santa Cruz, CA) was used to visualize progenitors and immature granule cells. All procedures were conducted as previously described (Rosi et al., 2005). Briefly, after quenching endogenous peroxidase activity and blocking nonspecific binding, the sections were incubated (4 °C) overnight (or 48 h for DCX) with the primary antibody. 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) for OX-6, rat adsorbed biotinylated horse anti-rabbit Immunoglobulin G (final dilution 1:150, Vector, Burlingame, CA) for TRPV1 and rat adsorbed biotinylated horse anti-goat immunoglobulin G (final dilution 1:200, Vector, Burlingame, CA) for DCX. 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 sections 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). To quantify the activated microglia, the hippocampal coronal sections were analyzed with Meta-Morph imaging software (Universal Imaging Corporation, West Chester, PA). The hippocampus was divided into three areas of interest (CA1, CA3, and the DG). After drawing these regions in each reconstructed image, a threshold tool was used to detect all of the OX-6 staining, and the area of each object was measured. All images were analyzed using the same threshold settings. The detected objects ranged from 5 to 2000 mm^2. After creating a distribution curve, only those objects of sizes >65 mm^2 were included in the analysis (the average size was 100 mm^2). This object size was chosen to match more accurately the size of activated microglial cells and therefore significantly reduce sampling errors. The resulting number of objects was then corrected using the total area of each region of interest, and the number of objects per region (in millimeter square) was reported.

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 antibody anti-capsaicin receptor (TRPV1, final dilution 1:250, Chemicon, Temecula, CA) 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 (PerkinElmer 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), or the monoclonal antibody OX-42 (BD Pharmingen, San Jose, CA final dilution 1:400) for 24 h (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 (PerkinElmer 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.

RT-PCR

Samples of hippocampus were microdissected and total RNA was extracted by homogenization (Ultra-Turrax T8, IKA Works, Wilmington, NC) using an RNeasy Mini Kit (Qiagen, Valencia CA) according to manufacturer's protocol. Extracted RNA was suspended in 30 µL of RNase-free water and RNA concentration was determined by a spectrophotometer (NanoDrop ND-1000, Wilmington, DE). The

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following inventoried primers and probes (Applied Biosystems, Foster City, CA) were used: *IL-1 beta* (Assay ID: Rn00580432_m1), *TNF-alpha* (Assay ID: Rn99999017_m1), *IL-1RA* (Rn00573488_m1), *IL-6* (Assay ID: Rn01410330_m1). A TaqMan 18S rRNA primer and probe set (labeled with VIC dye: Applied Biosystems, Foster City, CA) were used as a control gene for relative quantification. Amplification was performed on an ABI 7000 Sequencing Detection System by using Taqman Universal PCR master mix. The universal two-step RT-PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

ELISA

Samples of hippocampus were microdissected, weighed and homogenized in buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, and 0.5% sodium deoxycholate) containing protease inhibitors (Bennett et al., 2003). Homogenates were centrifuged (12,000 rpm) for 20 min at 4°C; the resuspended pellets were centrifuged (12,000 rpm) for 20 min at 4 °C to obtain total cell lysates. Protein concentrations were determined using bovine serum albumin as a standard (Bradford, 1976). Tissue homogenates were stored (−80 °C) until assayed in duplicate using sandwich enzyme-linked immunosorbent assay kits for IL-RA(R&D Systems, Minneapolis, MN), TNF-α, IL-6 and IL-1β (Biosource, Carlsbad, California) according to the manufacturer's guidelines. Optical density of each plate was read (450 nm filter on a Bio-Rad Benchmark microplate reader, Richmond, CA) and the mean optical density of each sample was calculated.

Statistical analysis

All statistical analyses were performed using Statview software (SAS Institute Inc., NC). Results are expressed as means ± SD. Statistical

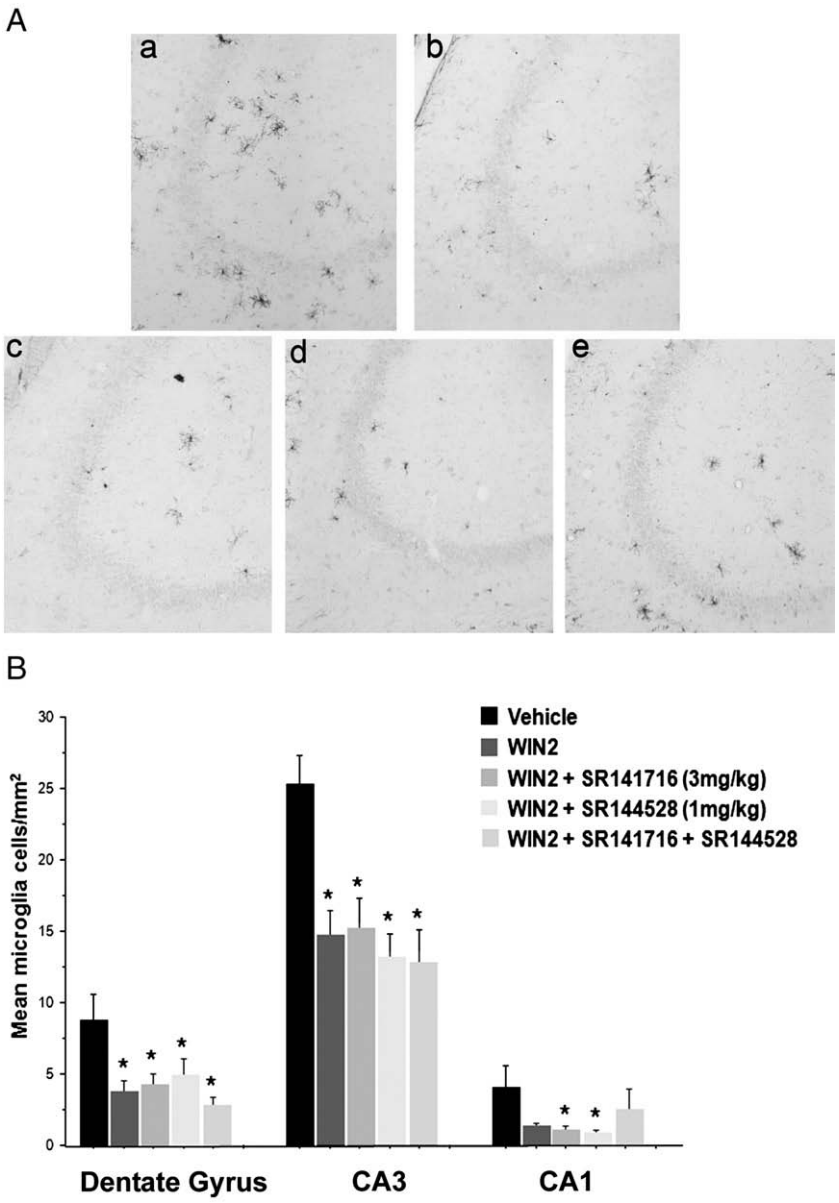

Fig. 1. (A) Activated microglia in the CA3 region. Note the diminution of activated microglia cells in this region of rats treated with 2 mg/kg/day of WIN-55,212-2 (b), as compared to the normal aged rats (a). None of the CB1/2 receptor antagonists, alone or in combination, could reverse the anti-inflammatory effect induce by WIN-2 (c–e). Magnification 160×. (B) Density of activated microglial cells in different areas of interest. The infusion of 2 mg/kg/day of WIN-55,212-2 partially reversed the aged induction of activated microglia (*$p<0.05$) in the dentate gyrus and CA3 region. None of the drugs' combination of CB1 and/or CB2 antagonist used to counteract the anti-inflammatory of WIN-55,212-2 had any significant effect.

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analysis were performed using ANOVA and when a significant effect was found ($p<0.05$) a post hoc analysis was made using the Fisher's PLSD test. A p-value lower than 5% was considered significant for each analysis.

Results

Chronic infusion of all drugs and vehicle were well tolerated by all rats and all rats gained weight normally during the treatment regimen.

Histology

Immunostaining (Fig. 1A) revealed activated microglia (OX-6-immunopositive) cells distributed throughout the hippocampus of old rats (Fig. 1A-a). The activated microglia had a characteristic bushy morphology with increased cell body size and contracted and ramified processes consistent with our previous study (Marchalant et al., 2008a). WIN-2 reduced the number of immunostained microglia within the hippocampus of old rats when administered alone (Fig. 1A-b) or in combination with SR1, SR2, SR1+SR2 (Fig. 1A-c,d,e).

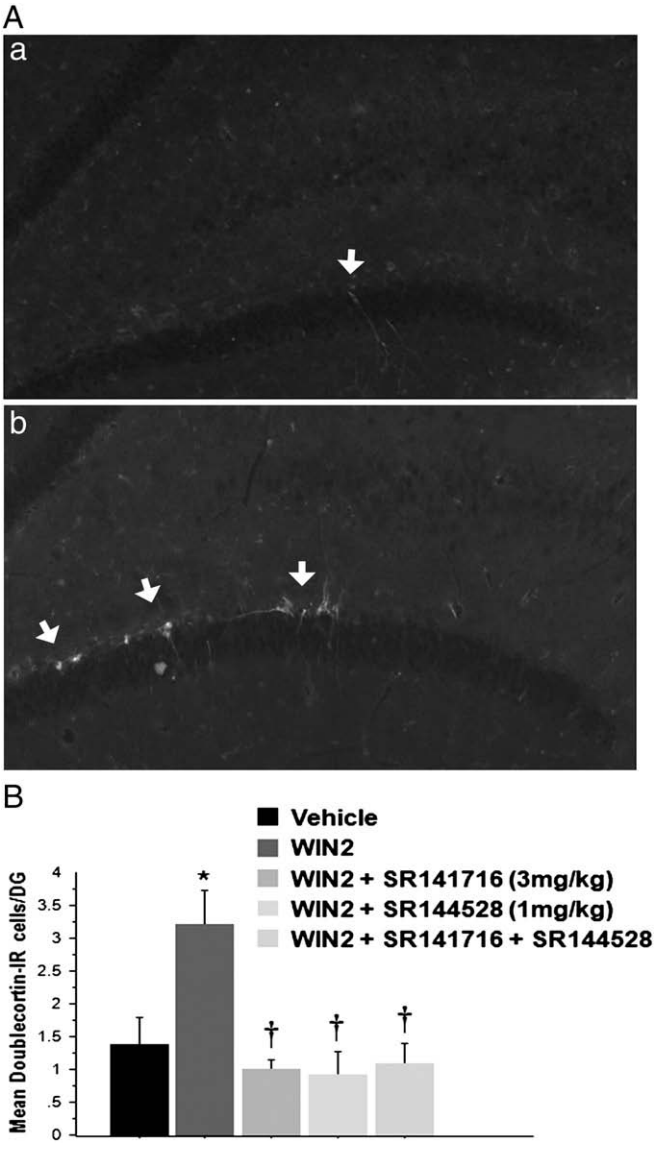

Fig. 2. (A) Double cortin immunoreactive (DCX-IR) cells in the dentate gyrus region. Note the increase in DCX-IR cells in this region of rats treated with 2 mg/kg/day of WIN-55,212-2 (b), as compared to the normal aged rats (a). Magnification 160×. (B) Average number of DCX-IR cells in the dentate gyrus. The infusion of 2 mg/kg/day of WIN-55,212-2 increased significantly the number of DCX-IR cells (*$p<0.05$) in the dentate gyrus. All drugs' combination of CB1 and/or CB2 antagonist did significantly blocked the increase in DCX-IR induced by WIN-55,212-2 (†$p<0.05$).

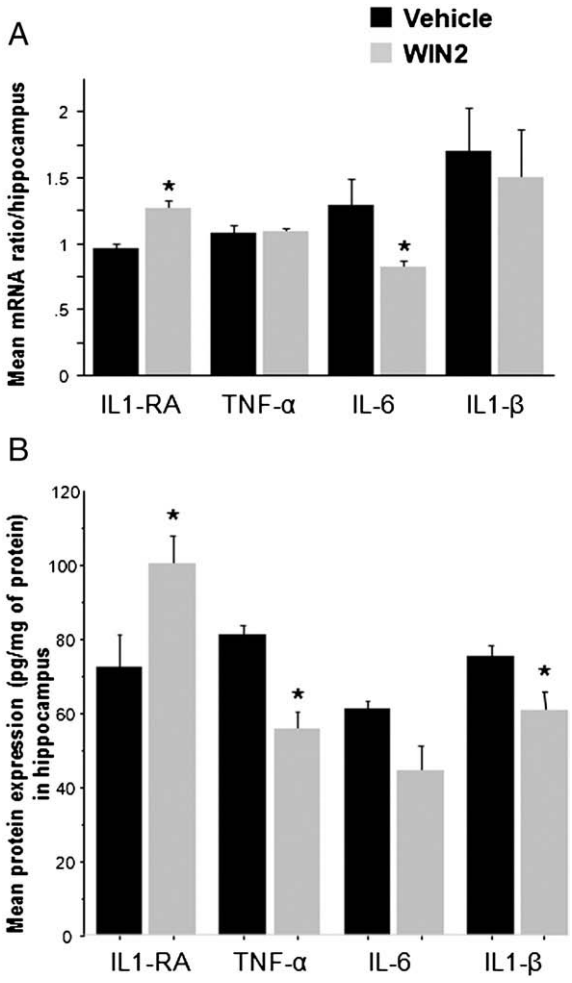

Fig. 3. Cytokine expression profile following 4 weeks of treatment with WIN-55,212-2. (A) Changes in mRNA cytokine expression in the hippocampus of old rats treated with or without WIN-55,212-2. Hippocampal level of *IL1-RA* and *IL-6* mRNA expression were significantly different after the 4 week treatment (*$p<0.05$) compared to old control rats. (B) Changes in cytokine protein level in the hippocampus of old rats treated with or without WIN-55,212-2. Hippocampal protein level of IL1-RA, TNF-α and IL1-β were significantly different after the 4 week treatment (*$p<0.05$) compared to old control rats.

Regional microglia cell counts

The number of OX-6-immunoreactive (OX6-IR) microglia per millimeter square was determined in 3 different areas of interest: DG, CA3 and CA1 regions of the hippocampus (Fig. 1B). These brain regions were examined because of their importance for spatial learning (Nadel and Land, 2000). An ANOVA revealed an overall effect of the treatments on the number of OX-6-immunoreactive microglia in the DG ($F_{8,\ 36}=5.053$, $p=0.0003$), in the CA3 ($F_{8,\ 36}=5.424$, $p=0.0002$) but not in the CA1 region ($F_{8,\ 36}=1.375$, $p=0.2403$). In the DG, and in the CA3 region, all treatments reduced significantly the number of OX-6-IR cells compare to vehicle treated rats (*$p<0.05$, Fisher's PLSD post-hoc test).

Effect of WIN-2 on neurogenesis

Although very few doublecortin-IR (DCX-IR) cells were detected in the DG of aged animals infused with the vehicle, WIN-2 treatment

significantly increased the number of DCX-IR cells (Fig. 2A). The number of DCX-IR cells per DG was determined (Fig. 2B). An ANOVA revealed an overall effect of treatment on the number of DCX-IR cells in the dentate gyrus ($F_{4,\ 256}=5.545$, $p=0.0008$). WIN-2 significantly increased DCX-IR compare to vehicle treated rats ($^{*}p<0.05$, Fisher's PLSD post-hoc test). All combinations of WIN-2 with CB1 and/or CB2 antagonist returned DCX-IR cells to vehicle infused level ($^{\dagger}p<0.05$, Fisher's PLSD post-hoc test).

Effect of 4 weeks infusion of WIN-2 upon cytokine expression profile

After 4 weeks of subcutaneous (s.c.) administration of WIN-2, the mRNA level of 4 cytokines, one anti-inflammatory (*IL1-RA*) and 3 pro-inflammatory (*TNFα*, *IL-1β*, *IL-6*), were measured within the hippocampus (Fig. 3A; expressed as a ratio between mRNA and control gene's mRNA). No significant difference were found between vehicle treated and WIN-2 infused animals for *TNF-α* ($F_{1,8}=0.016$, $p=0.903$) or *IL-1β* ($F_{1,8}=0.146$, $p=0.712$). However, a significant decrease was found after WIN-2 treatment for *IL-6* ($F_{1,8}=7.475$, $p=0.026$) while a significant increase was observed for *IL1-RA* ($F_{1,8}=15.056$, $p=0.0047$).

The protein level of those cytokines was also determined by ELISA (Fig. 3B). No significant difference was found between vehicle treated and WIN-2 animals for IL-6 ($F_{1,6}=5.603$, $p=0.056$). However, WIN-2 treatment produced a significant decrease in TNF-α ($F_{1,6}=24.67$, $p=0.025$) and IL-1β ($F_{1,6}=6.372$, $p=0.045$) while a significant increase was observed for IL1-RA ($F_{1,6}=6.171$, $p=0.0475$) in the hippocampus.

Effects of the TRPV1 antagonist 5′I-RTX on neuroinflammation and neurogenesis

The selective TRPV1 antagonist 5′I-RTX reduced the number of OX-6-immunoreactive microglia cells within the CA3 region of the hippocampus as compared to vehicle-treated rats (Fig. 4A, $F_{2,12}=8.547$, $p=0.0049$). Treatment with 5′I-RTX did not influence the number of DCX-IR cells (Fig. 4B). Indeed, no significant difference was found between vehicle and 5′I-RTX treated rats (ANOVA, $^{\dagger}p<0.05$, Fisher's PLSD post-hoc test) while a significant difference was observed between WIN-2 and vehicle treated rats and between WIN-2 and 5′I-RTX treated rats (ANOVA, $^{*}p<0.05$, Fisher's PLSD post-hoc test).

Co-localization between TRPV1 and other cell types in the hippocampus

As previously described by others using the same antibody (Tóth et al., 2005), a strong TRPV1 immunoreactivity was observed in the hippocampus (Fig. 5A) and the cortex, mostly within the soma of neurons. The staining was punctate, non-homogenous and not restricted to the plasma membrane.

Double-immunofluorescence staining for TRPV1 receptors and neurons (NeuN) demonstrated a strong spatial co-localization between TRPV1-IR and the soma of neurons in the hippocampus across all groups (Fig. 5Ba–c). These results are consistent with studies showing that TRPV1 receptors are closely associated with neurons in the regions of interest; DG, CA1 and the CA3 region of the hippocampus (Tóth et al., 2005).

Double-immunofluorescence staining for TRPV1 receptors and astrocytes (GFAP-IR, Fig. 5Bd–f), activated microglia (OX-6-IR, Fig. 5Bg–i) and total microglia (OX-42, Fig. 5B j–l) did not demonstrate any co-localization between these biomarkers. These results suggest that TRPV1 are not present on astrocytes or on microglial cells in aged rats or in response to WIN-2 treatment.

Discussion

Our results demonstrate for the first time that i) antagonism of TRPV1 in the brain reduces chronic microglia activation in an aged brain; ii) CB1 and CB2 agonism can reverse the effect of aging on the production of new neurons in the hippocampus and that iii) *both* CB1 and CB2 are required to produce this neurogenic effect.

The current study is the first to demonstrate that the specific components of the endocannabinoid system can be differentially targeted to counteract two detrimental aspects of normal aging, chronic neuroinflammation and the decline in neurogenesis, that have been implicated in age- or disease-related decline in cognitive function (Akiyama et al., 2000; Greenberg and Jin, 2006).

TRPV1 receptors and neuroinflammation

Impaired spatial memory in rats parallels the age-associated appearance of inflammation within the hippocampus (Hauss-Wegrzyniak et al., 1999; Gemma and Bickford, 2007). The ability of WIN-2

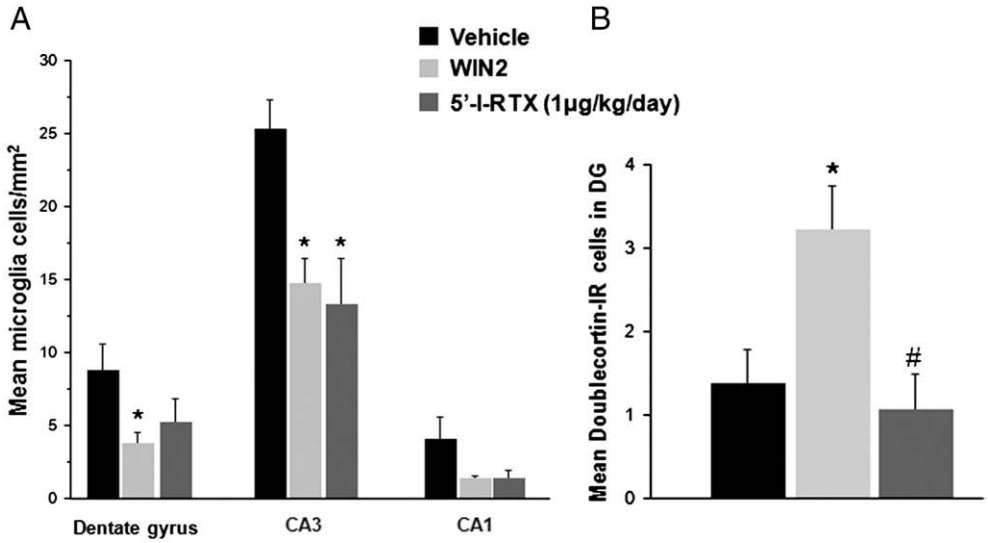

Fig. 4. Effect of 5′-Iodo-resiniferatoxin on inflammation and neurogenesis in the hippocampus of old rats. (A). Density of activated microglial cells in different areas of interest. The infusion of 1 µg/kg/day I.C.V of 5′-I-RTX for 4 weeks reproduced the reduction of the number of activated microglia observed following 2 mg/kg/day of WIN-55,212-2 in the CA3 region of the hippocampus ($^{*}p<0.05$). (B) Average number of DCX-IR cells in the dentate gyrus. The infusion of 0.4 µg/day I.C.V of 5′-I-RTX for 4 weeks did not reproduce the increased number of DCX-IR cells observed following 2 mg/kg/day of WIN-55,212-2 in the dentate gyrus region of the hippocampus ($^{\#}p<0.05$).

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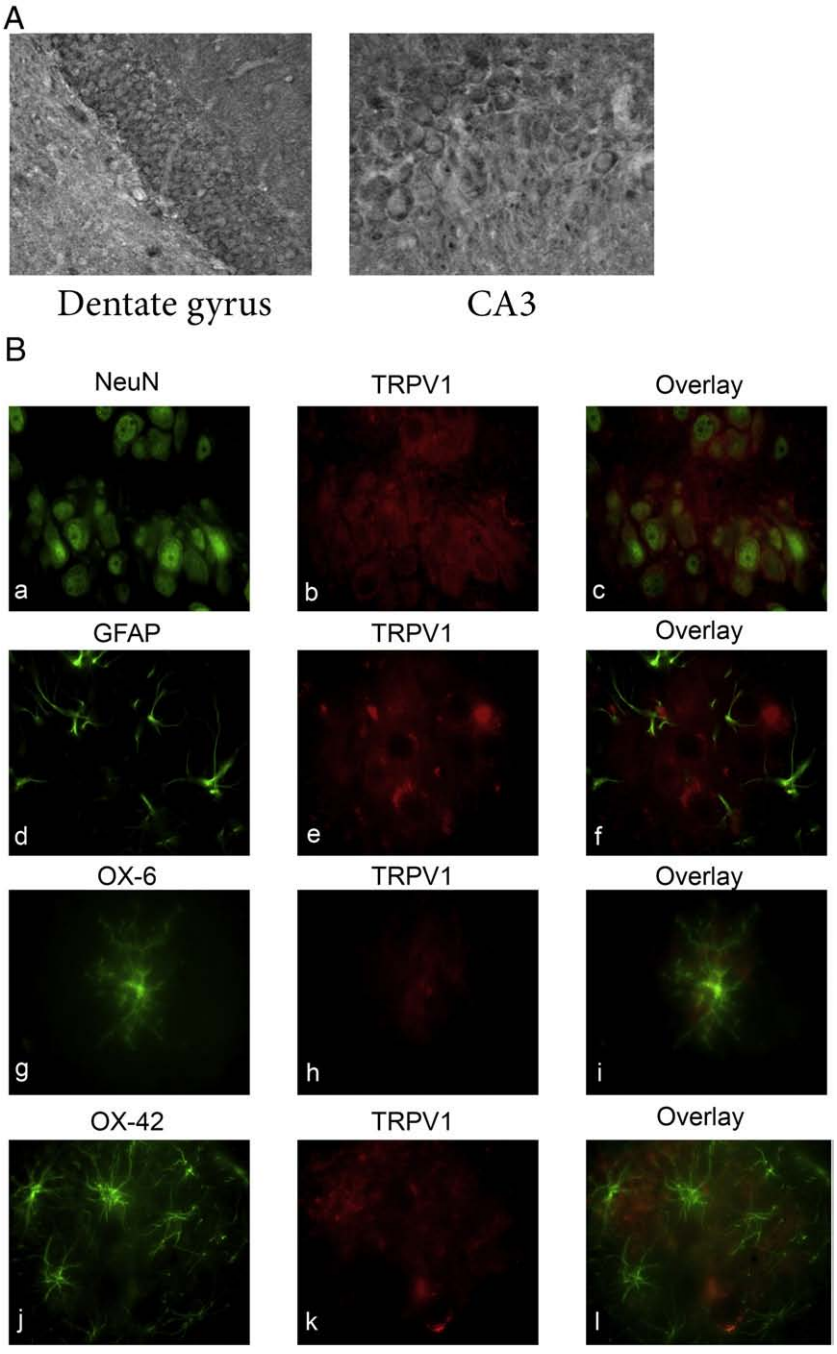

Fig. 5. Immunoreactivity (IR) of TRPV1 in the CA3 region of the hippocampus. All magnification 800×. (a–c) NeuN-IR (green) and TRPV1-IR (red) do co-localize in the CA3 region of the hippocampus. (d–f), GFAP-IR (green) and TRPV1-IR (red) do not co-localize in the CA3 region of the hippocampus. (g–i) OX-6-IR (green) and TRPV1-IR (red) do not co-localize in the CA3 region of the hippocampus. (j–l) OX-42-IR (green) and TRPV1-IR (red) do not co-localize in the CA3 region of the hippocampus. TRPV1-IR thus seems to be located primarily within neurons in the hippocampus. These photomicrographs are representative of the staining observed in all groups.

to reduce microglial activation in the aged rat brain was therefore both unique and encouraging as microglia in those animals are unresponsive to long term therapy with non-steroidal anti-inflammatory drugs (Rozovsky et al., 1998; Hauss-Wegrzyniak et al., 1999). In contrast, treatment with WIN-2 significantly decreased microglial activation in the CA3 and DG. Our results indicate that antagonism of the TRPV1 receptor is sufficient to reduce microglial activation in the CA3 region of the hippocampus in aged rats and highlights another important role for the TRPV1 receptor in addition to its known involvement in pain modulation (Cui et al., 2006) and hippocampal LTD (Gibson et al., 2008). One potential mechanistic explanation for why the decrease in microglial activation within the CA1 and DG following treatment with 5′I-RTX did not reach statistical significance is that these two regions exhibit fewer microglia than the CA3, and therefore might be less susceptible to the actions of WIN-2, i.e. there was a floor-effect.

Chronic neuroinflammation in the hippocampus is associated with increased levels of endogenous glutamate and increased entry of calcium ions via NMDA glutamate receptors and the inappropriate activation of calcium-dependent enzymes, proteins and genes (Bezzi et al., 1998; Rosi et al., 2005, 2006; Wenk et al., 2006). Treatment with the NMDA channel antagonist memantine restores normal calcium homeostasis, reduces over-expression of specific genes and proteins and restores normal performance in the water maze task in young inflamed rats (Rosi et al., 2005, 2006). In the current study,

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WIN-2 suppressed neuroinflammation via antagonism of the TRPV1 receptor, however, TRPV1 receptors in the hippocampus were primarily localized on neurons, thereby suggesting an indirect modulation of glial cells via post-synaptic TRPV1 receptor modulation. Blockade of TRPV1 receptors by 5′I-RTX can reduce the entry of calcium ions into neurons (Van der Stelt et al., 2005). The reduced activation of microglia in the current study may be due to the ability of WIN-2 to antagonize TRPV1 receptors and thereby reduce the influx of calcium ions into neurons and restore the subsequent neuron-glia communication to an anti-inflammatory state in a manner similar to that previously described (Rosi et al., 2006). We hypothesize that a major underlying problem in the development of age-associated neurodegenerative disorders is a failure of the glia to respond appropriately to neuronal signaling; therefore, the glia fail to convert from a pro-inflammatory activation state to an anti-inflammatory activation state, leading to excessive calcium entry and neuronal injury.

Chronic WIN-2 activity on cytokine mRNA and protein expression

A pro-inflammatory environment characterized by elevated levels of cytokines and activated microglia is associated with many age-related diseases of the brain, including AD, multiple sclerosis and PD (Akiyama et al, 2000; Wenk and Hauss-Wegrzyniak, 2003). Therapeutic approaches using standard anti-inflammatory drugs have not produced clinical benefits in patients with AD (ADAPT, 2007; Kang et al., 2007) in contrast to the findings of numerous epidemiological studies of people using non-steroidal anti-inflammatory drugs that suggest that long term use of these drugs can slow the onset of AD symptoms (Rozzini et al., 1996; Prince et al., 1998). We have previously shown that microglia in the brains of aged rats are unresponsive to long term therapy with non-steroidal anti-inflammatory drugs (Rozovsky et al., 1998; Hauss-Wegrzyniak et al., 1999). Therefore, the timing of the anti-inflammatory drug therapy is critical because microglia may become progressively unresponsive to standard therapeutic interventions, such as cyclooxygenase inhibitors or steroids, with normal aging. In the current study, the anti-inflammatory profile, i.e. a down-regulation of pro-inflammatory cytokines (TNFα, IL-1β, IL-6) and up-regulation of an anti-inflammatory cytokine (IL1-RA), observed following 4 weeks infusion of WIN-2 is of great interest because it demonstrates that long term manipulation of the endogenous cannabinoid and vanilloid systems can alter the profile of pro- and anti-inflammatory cytokines leading to a reduced level of neuroinflammation. Further experiments are needed to determine whether selective TRPV1 antagonists alone are capable of sustained anti-inflammatory effects in the aged brain.

CB receptors and neurogenesis

Neurogenesis is thought to be involved in normal hippocampal function as well as in brain repair following injury (Verret et al., 2007 for review). Newly generated cells are produced in the subgranular zone of the DG and the subventricular zone along the lateral ventricle in the forebrain. A growing interest in the fate of these cells during normal aging and neurodegenerative disease has generated in finding novel ways to stimulate neurogenesis in order to treat various neurological disorders (Greenberg and Jin, 2006). Results from our lab (unpublished) and others (Hwang et al., 2007, 2008) using DCX as a marker of neuronal progenitors indicates that aging is associated with a decrease in newly generated neurons in various species of aged animals. Other methods of detection of newly generated cells exist, notably retroviral incorporation and bromo-deoxyuridine (BrdU), but they may present a drawback when studying aging and brain inflammation: the intracranial injection necessary to perform retroviral incorporation can trigger lesions as well as inflammation (Yamada et al., 2004). Although BrdU is commonly used in young animals this approach may exhibit altered entry properties in the brain during aging (Cameron and Mckay, 2001).

Our current study indicates that the stimulation of both subtypes of CB receptors are necessary in order for WIN-2 to induce neurogenesis in the DG of the hippocampus of aged rats, as determined by examination of DCX-immunoreactive cells in this region. Blocking either CB1 or CB2 receptors was sufficient to prevent the increase in neurogenesis due to the WIN-2 treatment. Our results using the TRPV1 receptor antagonist 5′-I-RTX are consistent with the hypothesis that the CB1/2 agonist properties of WIN-2, and not its antagonist actions at the TRPV1 receptor, were responsible for the increase in neurogenesis. Therefore, the pharmacological action of WIN-2 that underlies the increase in neurogenesis appears to be unrelated to its ability to reduce microglia activation; blocking either or both of the CB receptors was insufficient to prevent the reduction in microglial activation by WIN-2 treatment but negatively affected neurogenesis. Therefore, our results confirm previous reports that during developmental or pathological conditions (Aguado et al, 2005, 2007; Goncalves et al., 2008; Mulder et al., 2008) the endocannabinoid system regulates the proliferation and migration of newly generated neurons. Moreover, our current results demonstrate that both CB1 and CB2 receptors are required in an aged brain to restore the proliferation of new neurons in the hippocampus.

Overall, the results of the current study are encouraging because they identify a pharmacological approach targeting the CB and TRPV1 receptors that can produce short-term therapeutic benefits on cognitive function (Marchalant et al., 2008a), partially restore an anti-inflammatory environment and stimulate neurogenesis in an aged brain. This multi-target approach may prove to be neuroprotective against neurodegenerative processes associated with inflammation-related pathological aging as well as during normal aging.

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