Cannabiods and Autoimmune diseases

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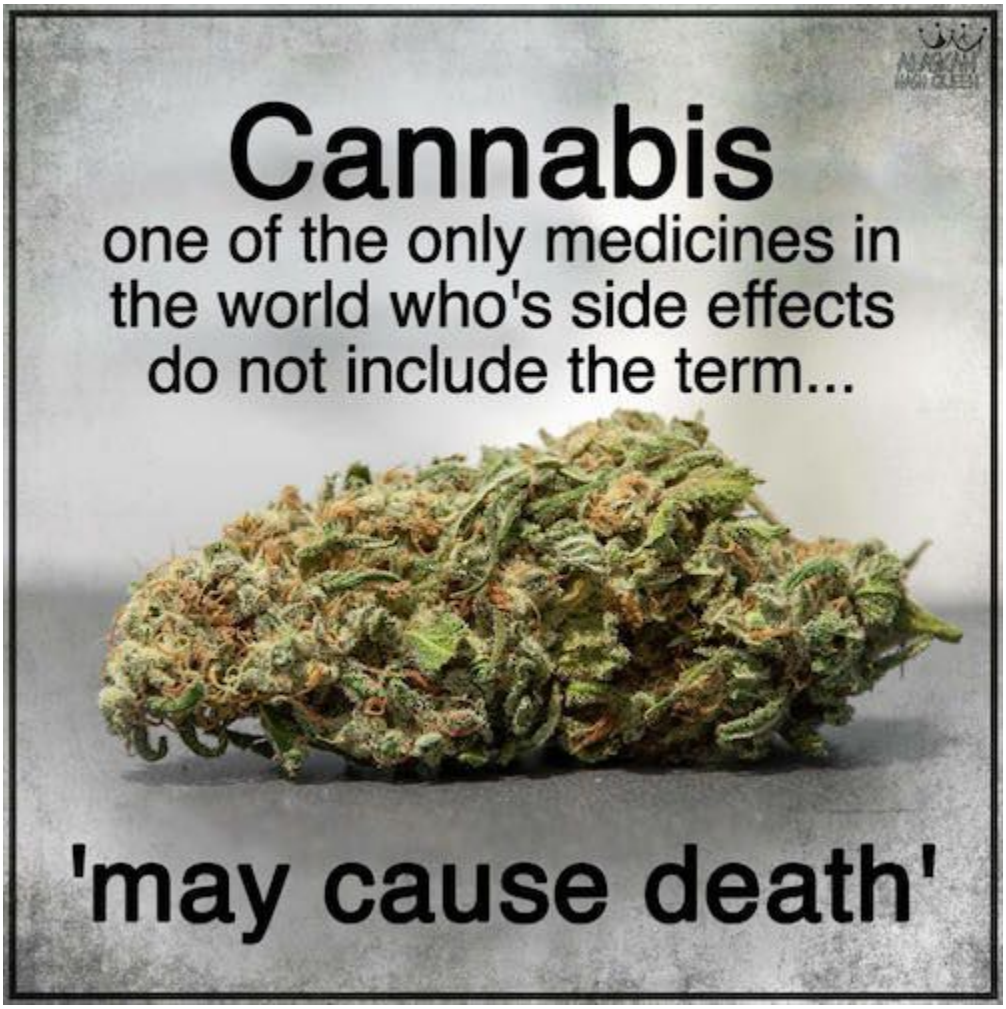

http://www.nature.com/nm/journal/v13/n4/abs/nm1561.html

Nature Medicine 13, 492 - 497 (2007)
Published online: 1 April 2007 | :10.1038/nm1561

Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB_1 on neurons and CB_2 on autoreactive T cells

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The cannabinoid system is immunomodulatory and has been targeted as a treatment for the central nervous system (CNS) autoimmune disease multiple sclerosis. Using an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), we investigated the role of the CB_1 and CB_2 cannabinoid receptors in regulating CNS autoimmunity. We found that CB_1 receptor expression by neurons, but not T cells, was required for cannabinoid-mediated EAE suppression. In contrast, CB_2 receptor expression by encephalitogenic T cells was critical for controlling inflammation associated with EAE. CB_2-deficient T cells in the CNS during EAE exhibited reduced levels of apoptosis, a higher rate of proliferation and increased production of inflammatory cytokines, resulting in severe clinical disease. Together, our results demonstrate that the cannabinoid system within the CNS plays a critical role in regulating autoimmune inflammation, with the CNS directly suppressing T-cell effector function via the CB_2 receptor.

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http://www.jleukbio.org/content/78/1/231.short

Reduced endocannabinoid immune modulation by a common cannabinoid 2 (CB2) receptor gene polymorphism: possible risk for autoimmune disorders

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Abstract

Immune system responsiveness results from numerous factors, including endogenous cannabinoid signaling in immunocytes termed the “immunocannabinoid” system. This system can be an important signaling pathway for immune modulation. To assess the immunomodulating role of the cannabinoid 2 (CB2) receptor, we sought polymorphisms in the human gene, identified a common dinucleotide polymorphism, and investigated its effect on endocannabinoid-induced inhibition of T lymphocyte proliferation. The CB2 cDNA 188–189 GG/GG polymorphism predicts the substitution of glutamine at amino acid position 63 by arginine. T lymphocytes from CB2 188–189 GG/GG homozygotes had approximately twofold reduction of endocannabinoid-induced inhibition of proliferation compared with cells from CB2 188–189 AA/AA homozygotes. In GG/GG subjects, the reduced endocannabinoid inhibitory response was highly significant for N-arachidonylglycine and nearly significant for 2-arachidonylglycerol, and a specific CB2 receptor antagonist partially blocked these effects. Also, patients with autoimmune diseases had an increased prevalence of the homozygous GG/GG genotype. Collectively, these results demonstrate reduced endogenous fatty acid amide immunomodulatory responses in individuals with the CB2 188–189 GG/GG genotype and suggest that this CB2 gene variation may be a risk factor for autoimmunity. The results also support the proposition that the CB2 receptor may represent a novel pharmacological target for selective agonists designed to suppress autoreactive immune responses while avoiding CB1 receptor-mediated cannabinoid adverse effects.

http://onlinelibrary.wiley.com/doi/10.1111/j.1471-4159.2005.03380.x/full

Modulation of the cannabinoid CB_2 receptor in microglial cells in response to inflammatory stimuli

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- First published: Full publication history
- DOI: 10.1111/j.1471-4159.2005.03380.x View/save citation
- Cited by (CrossRef): 187 articles Check for updates

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Abstract

The cannabinoid system is known to be important in neuronal regulation, but is also capable of modulating immune function. Although the CNS resident microglial cells have been shown to express the CB_2 subtype of cannabinoid receptor during non-immune-mediated pathological conditions, little is known about the expression of the cannabinoid system during immune-mediated CNS pathology. To examine this question, we measured CB_2 receptor mRNA expression in the CNS of mice with experimental autoimmune encephalomyelitis (EAE) and, by real-time PCR, found a 100-fold increase

in CB_2 receptor mRNA expression during EAE onset. We next determined whether microglial cells specifically express the CB_2 receptor during EAE, and found that activated microglial cells expressed 10-fold more CB_2 receptor than microglia in the resting state. To determine the signals required for the up-regulation of the CB_2 receptor, we cultured microglial cells with combinations of γ-interferon (IFN-γ) and granulocyte) macrophage-colony stimulating factor (GM-CSF), which both promote microglial cell activation and are expressed in the CNS during EAE, and found that they synergized, resulting in an eight to 10-fold increase in the CB_2 receptor. We found no difference in the amount of the CB_2 receptor ligand, 2-arachidonylglycerol (2-AG), in the spinal cord during EAE. These data demonstrate that microglial cell activation is accompanied by CB_2 receptor up-regulation, suggesting that this receptor plays an important role in microglial cell function in the CNS during autoimmune-induced inflammation.

Abbreviations used

AA

arachidonic acid

AEA

N-arachidonoylethanolamine

2-AG

2-arachidonoylglycerol

BM

bone marrow

CB

cannabinoid

EAE

experimental autoimmune encephalomyelitis

eCB

endocannabinoid

GM-CSF

granulocyte macrophage-colony stimulating factor

IFN-γ

: γ-interferon

LC-MS

: liquid chromatography-mass spectrometry

MS

: multiple sclerosis

PBS

: phosphate-buffered saline

The endogenous cannabinoid (CB) system includes the endocannabinoids (eCBs) and their G protein-coupled receptors, CB_1 and CB_2 (Porter and Felder 2001; Klein *et al*. 2003). The CB_1 receptor is expressed primarily by neurons (Matsuda *et al*. 1990) and can also be found at low levels in cells of the immune system (Kaminski *et al*. 1992). In contrast, the CB_2 receptor is primarily expressed by immune cells (Munro *et al*. 1993; Galiègue *et al*. 1995). Ligands for the CB_1 and CB_2 receptors include *N*-arachidonoylethanolamine (anandamide; AEA) (Devane *et al*. 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al*. 1995; Sugiura *et al*. 1995), with 2-AG produced at nanomolar amounts in the brain and spinal cord (Baker *et al*. 2001). eCB contents are regulated by synthesis and degradation; AEA is metabolized intracellularly by fatty acid amide hydrolase (Schmid *et al*. 1985) and 2-AG by monoglyceride lipase (Saario *et al*. 2004). Both enzymes result in the production of arachidonic acid (AA), the precursor to the immune-regulatory prostaglandins and other eicosanoids.

Activation of neuronal CB_1 receptors by eCBs results in inhibition of adenylyl cyclase (Kim and Thayer 2001) and decreased neurotransmitter release through blockade of voltage-operated calcium channels (Wilson and Nicoll 2001; Guo and Ikeda 2004). Because of the high CB_1 receptor density in the hippocampus, cerebellum, amygdala, cerebral cortex and striatum, there are implications for eCB involvement in the modulation of memory, emotion, pain and movement (De Petrocellis *et al*. 2003; Freund *et al*. 2003). In addition, the eCB system has been reported to modulate immune functions through the CB_2 receptor, which is expressed by T cells, macrophages, B cells and NK cells (Klein *et al*. 2003). Of the two major eCBs, evidence points to 2-AG as the primary eCB agonist at the CB_2 receptor (Sugiura *et al*. 2000). 2-AG has been shown to serve as a chemoattractant, inducing migration of microglia (Walter *et al*. 2003), dendritic cells (Maestroni 2004), macrophage-like cells and peripheral monocytes (Kishimoto *et al*. 2003) through a CB_2 receptor-dependent mechanism.

The monocyte lineage microglial cell is the only resident hematopoietic cell in the CNS. In the healthy brain, microglial cells are quiescent and exhibit a ramified morphology (Ling and Wong 1993).

However, during early development and after brain injury microglia become activated, resulting in a change in morphology and the acquisition of phagocytic function and the production of pro-inflammatory cytokines (Woodroofe *et al*. 1991). Using the experimental autoimmune encephalomyelitis (EAE) animal model of the human CNS autoimmune demyelinating disease, multiple sclerosis (MS), we have recently shown that microglial cells become activated and proliferate in the CNS prior to the onset of clinical disease and the infiltration of peripheral macrophages into the site of inflammation (Ponomarev *et al*. 2005a). Additional evidence supporting a role for microglial cell activation in the progression of EAE is a study by Heppner *et al.*, where mice expressing a transgene in microglial cells containing the herpes simplex thymidine kinase gene exhibited attenuated EAE when administered ganciclovir (Heppner *et al*. 2005).

The precise role of the eCB system in microglial cell activation and inflammation progression in the CNS has not yet been elucidated. Evidence supporting such a role are our studies showing that a rat microglial cell line synthesizes 2-AG, which increases cell proliferation via a CB_2 receptor-dependent mechanism (Carrier *et al*. 2004). In addition, it has been shown that CB_2 receptor expression, which is normally undetectable in the healthy CNS (Munro *et al*. 1993; Galiègue *et al*. 1995; Buckley *et al*. 2000), undergoes a major modulatory change in relation to cell activation (Carlisle *et al*. 2002). For example, in chronic pain models associated with peripheral nerve injury, CB_2 receptor expression is induced in the spinal cord, coinciding with the appearance of activated microglial cells (Zhang *et al*. 2003). Also, Alzheimer's disease is associated with plaques that contain activated microglial cells and increased CB_2 receptor expression (Benito *et al*. 2003). Although CB_2 receptor expression by activated microglial cells is suggested by these studies, activated microglial cells are not distinguishable from macrophages infiltrating the CNS from the periphery immunohistochemically. One of the goals of the present study was to determine the contribution of microglia and infiltrated peripheral cells to the up-regulation of CB_2 receptor expression during CNS inflammation.

Microglial cells and the CB system have also been implicated in inflammation in the CNS associated with MS. MS is characterized by microglial activation and neurological deficits including sensory deficits, motor weakness, tremor and ataxia (Compston and Coles 2002). Clinical studies have suggested beneficial effects of cannabinoids in MS (Pertwee 2002). In EAE, Δ^9-tetrahydrocannabinol (THC), a plant-derived cannabinoid that binds to both CB_1 and CB_2 receptors, delayed the onset of disease when administered prior to disease induction (Lyman *et al*. 1989). Administration of synthetic cannabinoids to mice with chronic, relapsing EAE resulted in a reduction in spasticity and tremors (Baker *et al*. 2001). Mice with Theiler's murine encephalomyelitis administered cannabinoids exhibited a significant improvement in neurological deficits, decreased microglial activation and decreased numbers of infiltrating T cells in the CNS (Arévalo-Martín *et al*. 2003). Taken together, these studies suggest that CB receptor activation could have a beneficial role in the progression of EAE.

To better understand the role of the CB receptors and the eCBs in the CNS during autoimmune inflammation, we have examined tissue content of the eCBs and expression of their receptors in the

CNS during EAE. We report here that spinal cord contents of eCBs and AA remained constant during the EAE time course. However, CNS CB_2 receptor mRNA was up-regulated approximately 100-fold at the onset of EAE disease, while CB_1 receptor mRNA was not altered. At the onset of EAE, both activated microglial cells and peripheral macrophages expressed identical levels of CB_2 receptor mRNA, which was 10-fold higher than resting microglial cells and the encephalitogenic T cells. Treatment of cultured microglial cells from adult mice (Ponomarev *et al.* 2005b) with the cytokines γ-interferon (IFN-γ) and granulocyte macrophage-colony stimulating factor (GM-CSF), both highly expressed in the CNS at EAE onset, produced a 10-fold increase in CB_2 receptor mRNA expression. These data demonstrate that EAE is accompanied by a dramatic up-regulation of CB_2 receptor expression in the CNS, both in resident microglia and infiltrating macrophages. These studies extend the findings of other laboratories, suggesting that the beneficial effects of cannabinoid ligand treatment for EAE and MS could result, in part, from CB_2 receptor interactions.

Material and methods

Mice

B10.PL (H-2^{μ}) and C57BL/6 (H-2^{b}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The MBP-TCR transgenic mice expressing a T cell receptor transgene specific for the acetylated NH_2-terminal peptide of myelin basic protein (Ac1–11) bound to I-A^{u} have been previously described (Dittel *et al.* 1999). (B10.PL/C57BL/6)F1 mice (H-$2^{\mu/b}$) were generated in our animal colony. Animals were housed at the Biomedical Research Center of the Medical College of Wisconsin. All animal protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

EAE induction

Sublethaly irradiated (360 rad) 7–8-week-old B10.PL mice were injected i.v. with 1×10^6 T cells from MBP-TCR CD4 T cell lines generated from MBP-TCR-transgenic mice as previously described (Dittel *et al.* 1999). Individual animals were assessed daily for clinical signs of EAE and scored using a scale from 1 to 5 as follows: (0) no disease; (1) limp tail and/or hind limb ataxia; (2) hind limb paresis; (3) hind limb paralysis; (4) hind and fore limb paralysis; (5) death. The disease time course was subdivided into several points for subsequent analysis: pre-clinical (day 0); onset (day 7); peak of disease (day 10); recovering (day 21).

mRNA purification and cDNA isolation

Total RNA was extracted from whole spinal cords of phosphate-buffered saline (PBS)-perfused B10.PL mice, on the indicated days after EAE induction, using TRIzol (Invitrogen, Carlsbad, CA, USA) after mechanical homogenization. Total mRNA from CNS mononuclear cells and from cultured microglial

cells was isolated using the Dynabeads mRNA DIRECT Micro Kit (Dynal Biotech ASA, Oslo, Norway), according to the manufacturer's instructions. cDNA was synthesized as previously described using Superscript II reverse transcriptase (Invitrogen) (Dittel *et al.* 1997).

CB_2 receptor mRNA quantitation by real-time RT-PCR

CB_2 receptor mRNA was quantified by real-time RT-PCR using SYBR-Green as the detection agent as previously described (Ponomarev *et al.* 2004). The real-time PCR amplifications were performed with the iCycler iQ (Bio-Rad, Hercules, CA, USA) in 25 µL reaction volumes containing cDNA, primers and IQ Supermix (Bio-Rad), according to the manufacturer's instructions. Thermal cycling proceeded with one amplification cycle of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 60°C for 1 min and 75°C for 1 min. Threshold values of CB_2 receptor expression were normalized to GAPDH expression using standard curves generated for each sample by a series of four consecutive 10-fold dilutions (1–1 × 10^3) of the cDNA template. All reactions were performed in triplicate and the data were analyzed using iQ Cycler analyzing software. The following sequence-specific primers were used in RT-PCR. GAPDH: forward, 5′-TTCACCACCATGGAGAAGGC-3′, reverse 5′-GGCATGGACTGTGGTCATGA-3′; and CB_2 receptor: forward 5′-TCTGTGTTACCCGCCTACCT-3′, reverse 5′-GTGGGGAAAGCTCAGAGCAG-3′. Specificity of the RT-PCR reaction was controlled by the generation of melting curves; PCR efficiencies were 100% ± 15% and correlation coefficients were 0.97–0.99.

Bone marrow (BM) chimeras

Six-week-old B10.PL mice were lethally irradiated (950 rad) and transplanted with 7 × 10^6 BM-derived cells from (B10.PL/C57BL/6)F1 mice; they were allowed to reconstitute for at least 8 weeks as previously described (Ponomarev *et al.* 2005a).

Mononuclear cell isolation and cell sorting

BM chimera mice 10 days after EAE induction were anesthetized and perfused with 25 mL ice-cold PBS. The brains and spinal cords were then harvested, homogenized and strained through a 75 µm nylon filter. Mononuclear cells were purified using a 40%/70% discontinous Percoll gradient (Sigma, St Louis, MO, USA) and after blocking of FcR (mAb 2.4G2), were stained as follows: anti-CD45-PE, anti-CD11b-CyChrome (eBioscience, San Diego, CA, USA), anti-H-$2K^b$-FITC, anti-CD4-Biotin (BD Bioscience), combined with APC-Cy7-Strepavidin (Biolegend, San Diego, CA, USA). Staining was performed on ice for 20 min, the cells were washed and, as previously described (Ponomarev *et al.* 2005a), sorted into four populations: (i) peripheral macrophages ($CD11b^+CD45^{hi}$H-$2K^{b+}$), (ii) resting microglial cells ($CD11b^+CD45^{low}$H-$2K^{b-}$), (iii) activated microglial cells ($CD11b^+CD45^{hi}$H-$2K^{b-}$) and (iv) the encephalitogenic T cells ($CD4^+$H-$2K^{b-}$) using a FACS Aria Cell Sorting System (BD Bioscience).

Tissue preparation for eCB and arachidonic acid quantification

B10.PL mice with EAE were killed by swift decapitation on the indicated days. Spinal cords were removed and immediately frozen on dry ice. Samples were weighed and placed in borosilicate glass tubes containing 2 mL acetonitrile (Sigma) with 84 pm[^{2}H8] AEA, 186 pm[^{2}H8]2-AG and 33 pm[^{2}H8]AA (Cayman Chemical, Ann Arbor, MI, USA). Tissue samples were homogenized briefly with a glass rod until flaky in appearance and sonicated for 1 h. Samples were incubated overnight at −20°C to precipitate proteins, then centrifuged at 1500 *g*. Supernatant fluids were removed to a fresh glass tube and evaporated to dryness under N_2. The samples were resuspended in 500 µL acetonitrile to recapture any lipids adhering to the glass, and dried again. Remaining lipids were resuspended in 25 µL acetonitrile, 20 µL of which were transferred to a glass vial for mass spectrometry.

Mass spectrometry

Identity and relative quantities of eCBs were determined using electrospray ionization liquid chromatography-mass spectrometry (LC-MS) following the addition of deuterated AEA, 2-AG and AA. Samples (5 µL) were analyzed using electrospray ionization LC-MS (Agilent 1100 LC/MSD, SL model) and separated on a reverse-phase C18 column (Kromasil, 250 × 2 mm; Phenomenex, Torrance, CA, USA) using mobile phase A (deionized water, 0.005% glacial acetic acid) and mobile phase B (acetonitrile, 0.005% glacial acetic acid). Samples were eluted at a flow rate of 200 µL/min for 25 min by a linear gradient of 25% solvent A to 75% solvent B, then held at 100% solvent B for 10 min. Drying gas flow was 12 L/min, drying gas temperature was 350°C, nebulizer pressure was 241 kPA and capillary voltage was 3000 V. Selective ion monitoring in positive mode allowed for the detection of AEA (*m/z* = 348; retention time = 10.00 min), 2-AG and 1(3)-arachidonylglycerol (*m/z* = 379; retention time = 17.50 min), and AA (*m/z* = 303; retention time = 21.52 min). Limits of detection for eCBs were 2.88 fm/L for AEA, 132 fm/L for 2-AG and 1.64 fm/L for AA. Because 2-AG is often seen as a doublet due to isomerization to 1(3)-arachidonylglycerol, total yield was calculated by adding the areas of both peaks.

Microglial cell culture

Mononuclear cells from the CNS of 4-week-old B10.PL mice were isolated and cultured as previously described (Ponomarev *et al*. 2005b). Briefly, the isolated microglial cells were resuspended in Dulbecco's modification of Eagle's medium (Cellgro, Herndon, VA, USA) supplemented with 50 µm 2-mercaptoethanol, 2 mm l-glutamine, 50 µg/mL gentamicin and 10% fetal calf serum (FCS). Cells were plated at 5×10^6/mL in 12-well flat-bottom tissue culture plates in the presence of M-CSF (10 ng/mL) (R & D Systems, Minneapolis, MN, USA). The purity of the cultures was checked using immunohistochemical staining for astrocytes with anti-glial fibrillary acidic protein antibody (Zymed Laboratories Inc., San Francisco, CA, USA) with FITC conjugated rat IgG (eBioscience), and microglial cells with anti-CD11b-PE (eBioscience). Cultures were glial fibrillary acidic protein negative and CD11b positive. After 4 weeks, the microglial cell cultures were stimulated with IFN-γ (150 U/mL) and GM-CSF (50 ng/mL) (eBioscience) alone or both together, or with lipopolysaccharide

(LPS) (100 ng/mL) (Sigma) for 24 h. These optimal culture conditions were experimentally determined. The cells were removed from the culture plates using cell dissociation solution (Sigma).

Results

The level of CB_2 receptor mRNA level is up-regulated in the CNS during EAE

As shown in Fig. 1(a), EAE clinical disease in B10.PL mice has an onset between days 7–10, peaks at day 14 and exhibits spontaneous remission by day 21. Real-time RT-PCR was used to quantify CB_2 receptor mRNA in the spinal cord extracts from B10.PL mice on days 0 (control), 3, 7, 10 and 14 following EAE induction. We found that low levels of the CB_2 receptor mRNA were detectable in the spinal cord of control unmanipulated B10.PL mice, a level that was not altered 3 days after EAE induction. As EAE disease progressed, we found that message for the CB_2 receptor was increased dramatically starting at disease onset on day 7 and was increased 100-fold on day 10, the day all the mice showed signs of EAE clinical disease (Fig. 1b). No significant changes in CB_2 receptor mRNA expression were observed in spinal cord extracts from mice subjected to sublethal irradiation only (data not shown). In addition, we observed no change in the expression of CB_1 receptor mRNA during EAE progression (data not shown).

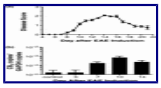

Figure 1.

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CB_2 receptor mRNA is up-regulated in the CNS during EAE. EAE was induced in B10.PL mice and individual mice were scored daily starting on day 4 after disease induction. (a) Data are shown as the average daily EAE score ± SEM of 16 mice starting on day 4. (b) On days 0, 3, 7, 10 and 14 after EAE induction, mice were randomly chosen and total RNA was isolated from the spinal cord; 10 µg of RNA from each mouse was combined prior to cDNA generation. CB_2 receptor mRNA was quantified by real-time RT-PCR. Quantitative PCR results are presented as a ratio of the number of specific copies to the number of GAPDH copies with the SEM shown. The data shown are the average of nine mice from three separate groups of three. The asterisk above the bar indicates a statistically significant ($p < 0.05$) increase in CB_2 receptor mRNA compared with the control as determined by the unpaired *t*-test.

CB_2 receptor mRNA level is up-regulated in activated microglial cells

Because the CB_2 receptor is primarily expressed by hematopoietic cells, we determined which immune cells found in the CNS were the source of the CB_2 receptor expression in the EAE mice spinal cords.

We examined CB_2 receptor expression by macrophages, microglial cells and the encephalitogenic T cells because they are the primary immune cells present in the CNS inflammatory lesions associated with EAE. Since microglial cells and infiltrating macrophages cannot be distinguished phenotypically, we generated MHC mismatched BM chimeras by transplanting B10.PL mice (H-$2^{\mu/u}$) with BM from (B10.PL/C57BL/6)F1 mice (H-$2^{\mu/b}$) (Ponomarev *et al.* 2005a). In these chimeras, the peripheral macrophages are distinguished from microglial cells by the expression of H-$2K^b$. Ten days after EAE induction, at the peak of CB_2 receptor mRNA content in the spinal cord extracts (Fig. 1b), we examined CB_2 receptor mRNA content in the following four cell populations: (i) peripheral macrophages, (ii) resting microglial cells, (iii) activated microglial cells and (iv) the encephalitogenic T cells used for EAE induction. This was accomplished by sorting total CNS mononuclear cells using cell surface molecule expression profiles. Using real-time RT-PCR and mRNA isolated from the purified cell populations, we found that activated microglial cells and peripheral macrophages expressed similar levels of CB_2 receptor mRNA, which was 10-fold higher than resting microglial cells and the encephalitogenic T cells (Fig. 2). The increased level of the CB_2 receptor in the resting versus the activated microglial cells was statistically significant ($p < 0.01$). Attempts to detect levels of CB_2 receptor protein were unsuccessful, as the available monoclonal antibodies do not recognize mouse CB_2 sufficiently.

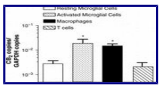

Figure 2.

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CB_2 receptor mRNA is up-regulated in activated microglial cells in the CNS of mice with EAE. Total mononuclear cells were isolated from the CNS of BM chimera mice 10 days following EAE induction, and sorted using cell surface markers into the following populations from which total RNA was isolated: resting microglial cells (open bar), activated microglial cells (dotted bar), macrophages (solid bar) and encephalitogenic T cells (striped bar). CB_2 receptor mRNA was measured using real-time RT-PCR. Data shown are the mean ± SD of three experiments. The asterisk (*) indicates a statistically significant ($p < 0.01$) increase in CB_2 receptor mRNA in activated microglial cells and macrophages compared to resting microglial cells, as determined by the unpaired *t*-test.

Concentrations of spinal cord 2-AG and AA remain constant in the CNS during EAE

We next determined whether the levels of the endogenous ligands for the CB_2 receptor, 2-AG and AEA and their breakdown product AA, were also altered during EAE in the spinal cord. In mice that did not

receive any irradiation, we were not able to detect AEA, while 2-AG was detected at a concentration of 14.4 nm/g of tissue (Table 1). Although this background level dropped to 11.8 nm/g of tissue 10 days after EAE induction, this level was similar to that measured in the irradiation control (10.9 nm/g of tissue) (Table 1). When 2-AG was measured in mice undergoing clinical recovery (day 21) from EAE, the level of 2-AG was identical to the unmanipulated controls (Table 1). We were not able to detect AEA at any time during the EAE clinical disease course (data not shown). We also measured the level of AA in the spinal cord of the same mice and found that both unirradiated mice and mice in EAE recovery had 262 nm/g of tissue AA. Like 2-AG, we observed a modest affect of the irradiation, with AA increasing to 300 nm/g in the mice irradiated for 10 days with or without EAE (Table 1). These data indicate that, in the adoptive transfer model of EAE, the ongoing inflammation and CNS pathology associated with clinical disease does not alter the levels of either 2-AG or AA in the spinal cord.

Table 1. Levels of 2-AG and AA in the CNS of mice do not change during EAE[a]

Group	2-AG (nm/g tissue)[b]	AA (nm/g tissue) [b]	*n*
No irradiation control[c]	14.4 ± 2.4	262 ± 15	3
Irradiation control[d]	10.9 ± 1.4	299 ± 25	3
10 days after EAE induction	11.8 ± 1	300 ± 29	4
21 days after EAE induction	14.8 ± 1.5	262 ± 16	3

- a

  EAE was induced in B10.PL mice as described in the Materials and methods.

- b

  Spinal cords were harvested from individual mice and each spinal cord was analyzed for the level of 2-AG and AA, shown as nm/mg of tissue. The data shown are the mean

- ±

  SEM of the indicated number of mice and is one representative experiment of three.

- c

  [c] B10.PL mice age- and sex-matched to the experimental mice that were not irradiated.

- d

  [d] Spinal cords were harvested from B10.PL mice 10 days after irradiation in mice not induced with EAE, but irradiated on the same day as those in which EAE was induced.

IFN-γ and GM-CSF up-regulate CB_2 receptor mRNA in microglial cells *in vitro*

EAE is characterized by an increased level of the proinflammatory cytokines IFN-γ (Ponomarev *et al.* 2004) and GM-CSF (Ponomarev *et al.* unpublished data) in the CNS at EAE onset. Since both cytokines have been implicated in microglial cell activation, we examined whether these cytokines contribute to CB_2 receptor up-regulation in microglial cells. We examined this using primary microglial cells cultured from the CNS of adult B10.PL mice (Ponomarev *et al.* 2005b). As shown in Fig. 3(a), microglial cells cultured in M-CSF (medium alone) exhibit a characteristic bipolar morphology and constitutively express the CB_2 receptor as measured by real-time PCR (Fig. 3d). The addition of IFN-γ to the microglial cultures resulted in a fivefold increase in CB_2 receptor message, while GM-CSF resulted in a twofold increase (Fig. 3d). The addition of IFN-γ to microglial cell cultures in M-CSF did not alter the biopolar cell morphology (data not shown). However, the addition of GM-CSF resulted in a change to an ameboid morphology (Fig. 3b). The addition of both IFN-γ and GM-CSF had a significant synergistic effect, causing an eight to 10-fold up-regulation of CB_2 receptor mRNA (Fig. 3d; $p < 0.001$) and resulting in a morphology similar to GM-CSF alone (data not shown). As an activation control, LPS was added to the microglial cell cultures and the level of CB_2 receptor was statistically significantly reduced ($p < 0.005$) approximately10-fold, as previously reported for fully activated microglia (Carlisle *et al.* 2002) (Fig. 3d). LPS treatment resulted in a dramatic morphological change in the microglial cells, indicative of activation (Fig. 3c). The LPS treatment did not alter cell survival as determined by trypan blue exclusion, which contained 5 ± 3% dead cells, a level that was identical to control cultures (data not shown).

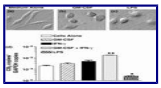

Figure 3.

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CB_2 receptor mRNA is up-regulated in cultured microglial cells in the presence of both IFN-γ and GM-CSF. Microglial cells isolated from the CNS of B10.PL mice were cultured for at least 4 weeks in the presence of M-CSF (10 ng/mL) (a and d, open bar) prior to stimulation with IFN-γ (150 U/mL) (d, dotted bar), GM-CSF (50 ng/mL) (b and d, diagonal stripped bar), both together (d, closed bar) or LPS (100 ng/mL) (c and d, horizontal stripped bar). (d) After 24 h, total RNA was isolated and the level of CB_2 receptor mRNA was determined by real-time RT-PCR. Data shown are the mean ± SD of three separate microglial cell cultures. A double asterisk (**) indicates a statistically significant ($p < 0.001$) increase in CB_2 receptor mRNA in cultured microglial cells and a single asterisk (*) indicates a statistically significant ($p < 0.005$) decrease in CB_2 receptor mRNA compared with cells alone, as determined by the unpaired *t*-test. All photomicrographs are 400×.

Discussion

The eCB and their receptor, CB_2, are thought to be involved in immune regulation. However, there is little known about their role in CNS inflammation during MS and EAE. The goal of this study was to determine whether the level of the eCB and the CB_2 receptor are altered in the CNS during ongoing autoimmune disease. We showed that while the CB_2 receptor mRNA was highly up-regulated in the spinal cord at EAE onset and peak of disease, the content of 2-AG remained constant. By examining the cell types expressing the CB_2 receptor in the CNS during EAE we were able to show that the majority of the expression was due to activated microglial cells and peripheral macrophages. The elevated expression of CB_2 receptor mRNA by activated versus resting microglial cells is the first direct evidence that CB_2 is up-regulated as a consequence of microglial cell activation during CNS autoimmunity. We next determined that the proinflammatory cytokines, IFN-γ and GM-CSF, synergized in up-regulating CB_2 receptor mRNA in cultured microglial cells. These cumulative data show that proinflammatory cytokines present in the CNS during early EAE could potentially regulate the expression of the CB_2 receptor on microglial cells, whose expression level is likely to be important in the regulation of inflammation in the CNS during autoimmunity.

Because CB_2 receptor expression is confined to cells of the immune system and not neurons or other glia, little is known about its function within the CNS. The only hematopoietic cell present in the resting CNS is the microglial cell and although the CNS has been reported to lack expression of the CB_2 receptor, we have shown in this study that its expression is detectable in the spinal cord and isolated CNS microglial cells by real-time RT-PCR (Fig. 2). This previous inability to detect the CB_2 receptor in the brain is likely due to sensitivity of the assays. We also could not detect the CB_2 receptor in spinal cord when using conventional RT-PCR and detecting the PCR product using an agarose gel and ethidium bromide for visualization (data not shown). Because real-time PCR is light-based it is more sensitive than conventional PCR, northern blotting or *in situ* hybridization used in previous studies (Munro *et al*. 1993; Galiègue *et al*. 1995). In addition, we examined the spinal cord, which could have higher levels of the CB_2 receptor than the brain (Munro *et al*. 1993; Galiègue *et al*. 1995). Previously, we and others have shown that cultured rat microglial cells constitutively express the CB_2 receptor (Carlisle *et al*. 2002; Carrier *et al*. 2004). In addition, it was shown that microglial cells cultured from newborn mice also express transcripts for the CB_2 receptor (Walter *et al*. 2003). Since both our rat and cultured newborn mouse microglial cells exhibit an activated phenotype (Becher and Antel 1996; Rademacher *et al*. 2004), we extended our study to examine the CB_2 receptor on resting microglial cells. We found that *ex vivo* isolated microglial cells, and microglial cells cultured from adult mice that maintain a resting phenotype (Ponomarev *et al*. 2005b), also express mRNA for the CB_2 receptor (Figs 2 and 3). These results are consistent with a report showing the detection of CB_2

receptors in perivascular microglial cells of the human cerebellum using western blotting and immunohistochemistry (Núñez *et al*. 2004). Although perivascular and parenchymal microglia are both from the monocytic lineage, those in the perivascular location are generally considered to be macrophages (Guillemin and Brew 2004), which have been shown to express the CB_2 receptor (Munro *et al*. 1993; Galiègue *et al*. 1995; Lee *et al*. 2001). Thus, these cumulative data suggest that microglial cells, whether in the resting state in the CNS or cultured *in vitro*, express the CB_2 receptor.

In addition to the healthy CNS, the CB_2 receptor has been observed both abundantly and selectively in neuritic plaque-associated astrocytes and microglia in Alzheimer's disease (Benito *et al*. 2003), which is a chronic degenerative disorder of the human brain. In a chronic pain model associated with peripheral nerve injury, the expression of the CB_2 receptor was induced in a highly restricted and specific manner in the lumbar spinal cord (Zhang *et al*. 2003), a finding consistent with our detection of the CB_2 receptor in the spinal cord (Fig. 1b). This study also noted that the detection of mRNA for the CB_2 receptor coincided with microglial cell activation. However, they did not specifically confirm the relationship (Zhang *et al*. 2003). Although B cell activation has been shown to be associated with increased CB_2 expression (Lee *et al*. 2001), a relationship between CB_2 receptor expression and microglial cell activation state *in situ* has not been specifically examined. We examined this question, and found that microglial cells exhibiting a typical activation phenotype expressed 10-fold greater CB_2 receptor message than microglial cells with a resting phenotype (Ponomarev *et al*. 2005a) from the CNS of mice with ongoing EAE (Fig. 2). While the function of resting microglial cells is not specifically known, upon activation they have been shown to change morphology and become efficient antigen presenting cells, and they are thought to actively participate in inflammatory processes in the CNS (Aloisi 2001; Carson 2002; Nelson *et al*. 2002). We have shown that during CNS autoimmune inflammation microglial cells proliferate and localize to sites of inflammation, indicating their migratory potential (Ponomarev *et al*. 2005a). In support of a role for the eCB and their ligands in microglial proliferation is our study showing that 2-AG enhanced microglial cell proliferation through a CB_2 receptor-dependent mechanism (Carrier *et al*. 2004). In addition, two separate studies showed that microglial cell migration is increased with CB_2 receptor ligands (Franklin and Stella 2003; Walter *et al*. 2003). When synthetic cannabinoids were administered to mice with encephalomyelitis induced by Theiler's virus infection, signs of microglial cell activation were reduced in the CNS compared with controls. However, the number of infiltrating CD4 cells were also reduced (Arévalo-Martín *et al*. 2003). Thus, it is not clear whether the synthetic cannabinoids were acting at the level of the T cell or microglial cell or both. These cumulative studies provide evidence that the eCB system plays an important role in the regulation of CNS inflammation.

In this study, we found no alteration in eCBs in the spinal cord that was specific to EAE clinical disease (Table 1). This is in agreement with others, who found no change in 2-AG or AEA in spinal cords from Theiler's murine encephalomyelitis virus-infected mice (Mestre *et al*. 2005). While the lesions of EAE are not necessarily localized to one specific region of the spinal cord, it is possible that small local

increases in 2-AG were diluted in these studies, and specifically examining different lumbar, thoracic or cervical sections of EAE spinal cord may yield different results. Here, 2-AG levels found in the spinal cord are in agreement with previously published reports (Baker *et al*. 2001), although we were unable to detect AEA in our samples. AEA in the spinal cord has previously been detected in picomolar quantities, relative to the nanomolar amounts of 2-AG present (Baker *et al*. 2001; Witting *et al*. 2004; Mestre *et al*. 2005), and therefore levels may be too low for detection using our methods of tissue preparation and LC/MS. To date, not all the cellular sources of 2-AG have been identified in the CNS during EAE and in addition to neurons, cells of the immune system, including microglial cells and macrophages and dendritic cells that migrate into the CNS, are likely sources (Di Marzo *et al*. 1999; Matias *et al*. 2002; Mestre *et al*. 2005). Both macrophages and dendritic cells have been shown to increase production of 2-AG upon activation with LPS (Di Marzo *et al*. 1999; Matias *et al*. 2002) while decreasing CB_2 receptor levels (Carlisle *et al*. 2002). Thus, activation of immune cells from the myeloid lineage alters expression of both the receptors and ligands of the CB system, allowing multiple layers of regulation.

Since 2-AG is present at relatively high concentrations in the normal CNS (Sugiura *et al*. 2002), a finding we confirmed in our EAE model (Table 1), and the level of the CB_1 receptor also did not change during EAE, it is likely that any role for the eCB system in EAE is mediated through the CB_2 receptor and 2-AG. Experimental evidence exists supporting a role for 2-AG in the control of immune cell response (Parolaro *et al*. 2002; Klein *et al*. 2003). Particularly in rodent models of MS, both THC and synthetic CB receptor agonists suppressed the clinical signs of disease (Lyman *et al*. 1989; Wirguin *et al*. 1994; Baker *et al*. 2001; Arévalo-Martín *et al*. 2003; Croxford and Miller 2003). However, these studies could not distinguish between a role for the CB_1 or CB_2 receptor, since all the ligands used bind to both receptors. A specific role for the CB_1 receptor in EAE has been implicated using a CB_1 receptor-deficient mouse that exhibited chronic clinical disease associated with increased signs of neurodegeneration (Pryce *et al*. 2003). Although in EAE there is evidence for a suppressive role for the eCB system in neuroinflammation, the studies in MS patients are not as clear. Indeed, recently Killestein *et al.* reviewed all the clinical trials to date examining the effects of cannabinoids on MS and concluded that “the results have been equivocal” (Killestein *et al*. 2004).

In this study, we show evidence that the CB_2 receptor is up-regulated during EAE on activated microglial cells. Since both IFN-γ (Ponomarev 2004) and GM-CSF (Ponomarev *et al.* unpublished data) are expressed in the CNS during microglial cell activation (Ponomarev *et al*. 2005a), we investigated whether either cytokine contributed to CB_2 receptor up-regulation. We found that neither cytokine alone substantially altered CB_2 receptor mRNA levels in cultured microglial cells but when added together, they synergized (Fig. 3). These data are consistent with a role for IFN-γ and GM-CSF in microglial cell activation and a study that showed no alteration in CB_2 receptor message with IFN-γ alone in microglial cells (Carlisle *et al*. 2002). Of interest is the observation that cultured microglial cells express about 10-fold less mRNA for the CB_2 receptor as compared with microglial cells isolated

directly from the CNS (Fig. 3d vs. Fig. 2). This difference could be due to the absence of other CNS resident cells such as astrocytes and neurons in the *in vitro* cultures, suggesting that CB_2 receptor expression by microglial cells is influenced by the CNS microenvironment.

During EAE and MS, it is likely that the inflammatory environment, which includes the production of the inflammatory cytokines IFN-γ and GM-CSF, facilitates microglial cell activation and the up-regulation of the CB_2 receptor. The increased expression of the CB_2 receptor on microglial cells as well as its expression by other immune cells in the CNS during EAE (Fig. 2) suggests that the eCB signaling pathway is important in regulating CNS inflammation. This likely occurs by several mechanisms, one that is stimulatory for microglial cells promoting their proliferation and migration, and perhaps suppressive for the T cells that are required for disease. Either mechanism makes the eCB system an interesting target for therapeutics in the treatment of MS and other neurodegenerative diseases.

Acknowledgements

The authors would like to thank Shelley Morris for help with the animals, Marina Novikova for technical assistance, and Hope Albertz and Corbett Reinbold for help with FACS. This work was supported in part by NIH grants R01 NS046662 (BND) and DA09155 (CJH) and the Blood Center Research Foundation (BND).

Ancillary

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Suppression of experimetal autoimmune encephalomyelitis by cannabinoids

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https://doi.org/10.1016/0162-3109(94)90056-6Get rights and content

Abstract

The effect of Δ^8-THC on experimental autoimmune encephalomyelitis (EAE) was examined. Δ^8-THC is an analogue of Δ^9-THC, the psychoactive component of marihuana. It is more stable and less psychotropic than Δ^9-THC and like the latter it binds to the brain cannabinoid receptor. Two strains of rats were incolulated for EAE, and Δ^8-THC (40 mg/kg) was administered for up to 21 days. Δ^8-THC significantly reduced the incidence and severity of neurological deficit in both rat strains. The beneficial influence of Δ^8-THC only occured on oral administration and not with parenteral injection. Serum corticosterone levels were twofold elevated in rats with EAE chronically treate with Δ^8-THC. These results suggest that suppression of EAE cannabinoids may be related to their effect on corticosterone secretion.

Keywords

Cannabinoids

Δ-Tetrahydrocannabinol
HU-211
Experimental autoimmune ecephalomyelitis
Corticosterone

Abbreviations

CNS
central nervous system
CS
corticosterone
EAE
experimental autoimmune encephalomyelitis
MS
multiple sclerosis
MBP
myelin basic protein
THC
tetrahydrocannabinol

https://academic.oup.com/brain/article/126/10/2191/314489/Cannabinoids-inhibit-neurodegeneration-in-models

Cannabinoids inhibit neurodegeneration in models of multiple sclerosis

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Brain (2003) 126 (10): 2191-2202.
DOI:
https://doi.org/10.1093/brain/awg224
Published:
01 October 2003
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Abstract

Multiple sclerosis is increasingly being recognized as a neurodegenerative disease that is triggered by inflammatory attack of the CNS. As yet there is no satisfactory treatment. Using experimental allergic encephalo myelitis (EAE), an animal model of multiple sclerosis, we demonstrate that the cannabinoid system is neuroprotective during EAE. Mice deficient in the cannabinoid receptor CB_1 tolerate

inflammatory and excito toxic insults poorly and develop substantial neurodegeneration following immune attack in EAE. In addition, exogenous CB_1 agonists can provide significant neuroprotection from the consequences of inflammatory CNS disease in an experimental allergic uveitis model. Therefore, in addition to symptom management, cannabis may also slow the neurodegenerative processes that ultimately lead to chronic disability in multiple sclerosis and probably other diseases.

Keywords: cannabinoids; excitotoxicity; experimental allergic encephalomyelitis; multiple sclerosis; neuroprotection

Abbreviations: 2-AG = 2-arachidonoyl glycerol; CB = cannabinoid receptor; CREAE = chronic relapsing experimental allergic encephalomyelitis; EAE = experimental allergic encephalomyelitis; EAU = experimental allergic uveitis; ELISA = enzyme-linked immunosorbent assay; i.p. = intraperitoneal; IRBP = interphotoreceptor retinoid binding protein; NMDA = *N*-methyl-d-aspartate; Δ^9-THC = tetrahydrocannabinol

Topic:

- cannabinoid
- multiple sclerosis
- nerve degeneration
- neurodegenerative disorders
- uveitis
- cannabis
- mice
- palliative care
- agonists
- disability
- neuroprotection
- inflammatory disease of the central nervous system

Introduction

Multiple sclerosis is a chronic disease of the CNS, where autoimmunity is thought to drive the development of inflammatory lesions that induce the primary demyelination, which results in the inhibition of normal neurotransmission (Compston and Coles, 2002). However, the observation that disability often continues to worsen despite immunotherapy, which reduces blood–brain barrier dysfunction and relapse rate (Coles *et al*., 1999; SPECTRIMS Study Group, 2001; Wiendl and Hohlfeld, 2002) underscores that neurodegenerative changes are of major importance in disease progression (Barnes *et al*., 1991; Ferguson *et al*., 1997; Trapp *et al*., 1998; Coles *et al*., 1999). This correlates with gross atrophy of the CNS, axonal loss and the accumulation of permanent disability (Bjartmar *et al*., 2000; Compston and Coles, 2002). Axonal pathology is an early feature of multiple sclerosis lesions and is initially associated with inflammation (De Stefano *et al*., 2001; Filippi *et al*., 2003); likewise, axonal damage is a feature in experimental allergic encephalomyelitis (EAE), an autoimmune model of multiple sclerosis (Baker *et al*., 1990; Wujek *et al*., 2002). During multiple

sclerosis and EAE, destruction of myelin results in the redistribution and aberrant expression of axonal ion channels, and demyelinated axons are particularly sensitive to the damaging effects of free-radicals and glutamate excitotoxicity, which may additionally contribute to chronic neurodegeneration in CNS autoimmune disease (Foster *et al*., 1980; Black *et al*., 2000; Pitt *et al*., 2000; Smith *et al*., 2000, 2001; Werner *et al*., 2001; Lo *et al*., 2002; Kapoor *et al*., 2003). Therapeutic strategies in multiple sclerosis have concentrated on immunomodulation (Wiendl and Hohlfield, 2002). There is an urgent need for agents that can inhibit progressive multiple sclerosis.

Cannabis contains many compounds but it has been found that the major psychoactive ingredient is Δ^9-tetrahydrocannabinol (Δ^9-THC) (Mechoulam and Gaoni, 1967). Δ^9-THC mediates the majority of its activities through stimulation of cannabinoid receptors (CB), notably CB_1, which are expressed throughout the CNS (Matsuda *et al*., 1990; Howlett *et al*., 2002). Following the discovery of the receptors, fatty acid endogenous ligands, such as anandamide and 2-arachidonoyl glycerol (2-AG), and a degradation system including a re-uptake mechanism and hydrolytic enzymes have been identified (Devane *et al*., 1992; Deutsch and Chin, 1993; Mechoulam *et al*., 1995; Dinh *et al*., 2002). The cannabinoid system functions to regulate synaptic neurotransmission (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001) and tonically controls clinical signs such as spasticity and tremor that develop in chronic EAE (Baker *et al*., 2000, 2001). This provides objective evidence to support the claims of multiple sclerosis patients that cannabis may have a benefit in symptom management (Consroe *et al*., 1997), a claim further supported by some recent clinical trials of medical cannabis extracts (Killestein *et al*., 2002; Robson *et al*., 2002; Vaney *et al*., 2002). There is *in vitro* evidence that cannabinoids can also regulate glutamate release, oxidant free radicals and calcium influxes (Twitchell *et al*., 1997; Hampson *et al*., 1998; Kreitzer and Regehr, 2001; Howlett *et al*., 2002), which, in excess, can cause neuronal death in neuroinflammatory disease (Pitt *et al*., 2000; Smith *et al*., 2000; Kapoor *et al*., 2003). The lack of specificity of all available cannabinoid reagents (Howlett *et al*., 2002) and the potential presence of additional CB-like receptors (Di Marzo *et al*., 2000; Breivogel *et al*., 2001; Monory *et al*., 2002) means that gene-deleted transgenic mice (Ledent *et al*., 1999; Zimmer *et al*., 1999) provide powerful tools to definitively investigate the potential role of the cannabinoid system in neuroprotection.

Material and methods

Animals

Biozzi ABH and CB1 gene (*Cnr1*)-deficient mice were from stock bred at the Institute of Neurology. They were fed RM-1(E) diet and water *ad libitum*. Congenic (N5) ABH.*Cnr1* –/– –/+ and +/+ were generated from CD1.*Cnr1*–/– knockout mice (Ledent *et al*., 1999) and screened as described previously (Brooks *et al*., 2002). B10.RIII mice were purchased from Harlan Olac, Oxford, UK. All experiments were ethically performed according to the UK Animals (Scientific Procedures) Act (1984), under the control of the UK Government, Home Office.

Chemicals

The cannabinoid receptor agonists *R*(+)-WIN55,212 and CP55,940 were purchased from Tocris (Bristol, UK). *N*-methyl-d-aspartate (NMDA) and the NMDA receptor antagonist MK-801 were obtained from Sigma (Poole, UK). The CB_1 selective antagonist rimonabant (SR141617A; Rinaldi-Carmona *et al*., 1994) and Δ^9-THC were from the National Institute for Drugs and Abuse (NIDA) drug supply program. These were dissolved in ethanol : cremophor : PBS (1 : 1 : 18) and 0.1–0.2 ml was injected intraperitoneally (i.p.) daily.

Induction of chronic relapsing EAE (CREAE)

Mice were injected subcutaneously in the flank on day 0 and 7 with 1 mg mouse spinal cord homogenate in complete Freund's adjuvant [60 μg *Mycobacterium tuberculosis* H37Ra, *Mycobacterium butyricum* (4 : 1) per injection] on day 0 and 7 (Baker *et al*., 1990). Clinical disease was assessed daily and scored: 0 = normal, 1 = limp tail, 2 = impaired righting reflex, 3 = paresis of hindlimbs, 4 = complete paralysis of hindlimbs and 5 = moribund/death (O'Neill *et al*., 1992). The activity of animals was monitored over 5 min in a 27 × 27 cm open-field activity chamber (Brooks *et al*., 2002). Tissues were either snap-frozen or formaldehyde-fixed for immunohistology (Baker *et al*., 1990; Ahmed *et al*., 2002), western blotting for caspase activity (Ahmed *et al*., 2002) and enzyme-linked immunosorbent assay (ELISA) detection of CNS proteins.

Neurofilament ELISA

Whole spinal cords were homogenized on ice by trituration and sonication in 500 μl of barbitone buffer [11 mM barbital, 63 mM sodium barbital, 1.2 mM EDTA (Sigma)] containing a protease inhibitor cocktail and 4 mM EGTA. Lipids were extracted from the sample by adding di-isopropyl-ether (Sigma) at 1 : 5000 and centrifuging for 5 min at 20 000 *g*. The supernatant was frozen and stored in aliquots at –70°C, and the total protein was measured using the standard Lowry method. Ninety-six-well microtitre plates (Maxisorp; Nunc, Rochester, NY, USA) were coated overnight at 4°C with the SMI35 coat monoclonal antibody (SMI35; Sternberger Monoclonals Inc., Lutherville, MD, USA) against neurofilament heavy chain diluted in 0.05 M sodium carbonate (pH 9.6). This was followed by a wash step with barbitone buffer containing 5 mM EDTA, 1% bovine serum albumin and 0.05% Tween-20 (Sigma). Non-specific protein binding was blocked by incubation with 1% bovine serum albumin in barbitone buffer for 1 h at room temperature, followed by a wash with wash buffer as above. Spinal cord homogenates were serially diluted to 1 : 10 000 in barbitone buffer containing 5 mM EDTA, and incubated at room temperature for 2 h. After washing, a rabbit polyclonal anti-neurofilament H antibody (N-4142; Sigma), diluted 1 : 1000, was incubated at room temperature for 1 h. Following another wash, horseradish peroxidase-conjugated anti-rabbit immunoglobulin diluted 1 : 1000 was incubated for 1 h at room temperature. The tetramethylbenzidine chromogenic reagent (R & D Systems Europe, Minneapolis, MN, USA) was used, signal development stopped using 1 M phosphoric acid, and the plate read at 450 nm, with a reference reading at 620 nm. The antigen concentration for each sample was calculated from an internal standard curve ranging from 0 to 250 ng/ml (high-performance

liquid chromatography-purified bovine neurofilament H; Affiniti Bioreagents, Golden, Colorado, USA). All samples were analysed in duplicate.

Induction of experimental allergic uveitis (EAU)

B10.RIII mice were injected subcutaneously with 25 μg interphotoreceptor retinoid binding protein ($IRBP1^{51–181}$) peptide in complete Freund's adjuvant (as used in EAE experiments) on day 0 and 7, disease was assessed histologically, by haematoxylin and eosin-stained 5 μm paraffin wax sections, for the level of infiltration (score 0–6) and structural damage (score 0–5), as described previously (Hankey *et al*., 2001).

Glutamate excitotoxcity

NMDA-induced Ca^{2+} influx

Cerebellar neurons obtained from 6-day-old wild-type ABH.*Cnr1*+/+ and CB_1 knockout mice were cultured for 9 days in poly-d-lysine coverslips as described previously (Evans and Pocock, 1999). At 36 h and 6 days, 10 μM cytosine arabinoside was added to inhibit non-neuronal proliferation (Evans and Pocock, 1999). Cells were loaded with 5 μM of the fluorescent Ca^{2+} indicator dye fura-2 acetoxymethyl ester (Calbiochem, Nottingham, UK), prior to ionotropic glutamate receptor stimulation with 100 μM NMDA and subsequent inhibition with the NMDA receptor antagonist, 10 μM MK-801. This concentration of MK-801 is required to give maximal block of imaged NMDA-induced Ca^{2+} influxes in cerebellar neurons (Pocock and Nicholls, 1998). The CB_1 receptor agonist CP55,940 (0.01–5 μM) was added 5 min before imaging commenced and was present throughout the experiment. A 12-bit digital camera acquired images and the output visualized with a Life Science Resources Merlin Imaging system, version 1.8630 (Perkin Elmer Life Science, Cambridge, UK). Data were analysed by calculating the 340/380 nm fluorescence ratios with time.

Kainic acid induced lesion

Mice were deeply anaesthetized with halothane and stereotactically injected unilaterally (bregma 2.5 mm, medial-lateral 1.7 mm and dorsoventral 1.6 mm) with 1.5 nmol of kainic acid in 30 μl of 0.9% saline over 30 s; the injection needle was retained for 1 min to prevent reflux of fluid (Chen and Strickland, 1997).

Statistical analysis

Non-parametric data were assessed using the Mann–Whitney *U*-test with Minitab software (Coventry, UK), parametric data were assessed using *t*-tests with Sigmastat software. The group score represents the maximal clinical grade developed by all animals within the group.

Results

Development of chronic paresis in EAE is associated with accumulation of axonal loss

Following actively, spinal cord homogenate-induced CREAE, ABH mice develop a relapsing–remitting disease progression of distinct paralytic disease episodes followed by remission with an increasing residual deficit (Baker *et al*., 1990, 2000). Although histological axonal damage occurs in the initial acute phase of CREAE in ABH mice, this becomes much more evident, particularly in the spinal cord, following the development of relapsing disease (Baker *et al*., 1990; Ahmed *et al*., 2002). Whilst EAE has classically been assessed using a subjectively scored scale of paralysis (Table 1; Fig. 1A) (Baker *et al*., 1990; Smith *et al*., 2000; Lo *et al*., 2002), accumulating residual deficit could be quantitatively demonstrated through assessment of mobility of remission animals in an open-field activity chamber (Fig. 1B). Whilst ABH mice remitted to exhibit more immobility after one episode of paralysis ($P < 0.001$; clinical score 0.5), movement activity was further reduced ($P < 0.001$) after three to four episodes (clinical grade 2.5–3), where animals had chronically developed residual hind limb paresis upon recovery from relapsing paralytic episodes (Fig. 1B). This immobility was associated with accumulating axonal loss, which could be quantitatively assessed using a neurofilament ELISA (Fig. 2A), and demonstrated histologically (Fig. 2B and C).

The cannabinoid system regulates EAE-induced neurodegeneration

Wild-type (ABH) and congenic wild-type homozygous (ABH.*Cnr1*+/+), heterozygotes (ABH.*Cnr1*+/–) and CB_1-deficient (ABH.*Cnr1*–/–) mice developed EAE with comparable incidence, severity and onset (Table 1). There was, however, an increased frequency (>5%) of EAE-induced mortality in CB_1-deficient mice and also in heterozygotes, which have reduced CB_1 expression (Selley *et al*., 2001). However, most strikingly CB_1-deficient mice exhibited significantly ($P < 0.05$) more immobility and residual paresis (Fig. 1) and axonal pathology (Fig. 2A and D–F) than wild-type mice following recovery after the first paralytic episode. These animals relapse and accumulate more deficits that rapidly reach an acceptable severity limit, including the development of permanent hindlimb paralysis. Consistent with the enhanced neurodegeneration after a single attack, spasticity (Baker *et al*., 2000) developed early in ABH.*Cnr1*–/– mice, which in wild-type ABH mice usually only occurs after three to four attacks (Baker *et al*., 2000). Injection of wild-type mice with the CB_1 antagonist (twice daily with 5 mg/kg rimonabant i.p. from onset; $n = 8$) induced greater mortality than usually occurs (<5%). However, using clinical signs of remission, the level of neurodegeneration was not as consistent as that found in CB_1-deficient animals (data not shown). Although multiple pathways contribute to axonal damage in EAE, the final effector mechanism in neuronal death is probably toxic ion influxes (Ca^{2+}) and caspase-3-mediated apoptosis (Ahmed *et al*., 2002), and consistent with this, ABH.*Cnr1*–/– mice exhibited significantly ($P < 0.001$) elevated levels of active caspase-3 during acute-phase EAE compared with wild-type ABH.*Cnr1*–/– mice, although levels of caspase-1 activity were comparable

(Fig. 2F). Caspase-3 could be detected immunocytochemically in dying axons and these axons demonstrated many transections, which is a feature of multiple sclerosis (Trapp *et al*., 1998) (Fig. 2D and E). Therefore, neurodegeneration is clearly elevated in CB_1-deficient mice following inflammatory insults, suggesting that CB_1 agonism should have neuroprotective potential in CB_1-wild-type animals, in addition to controlling neurological symptoms such as tremor and spasticity (Baker *et al*., 2000).

Exogenous CB_1 agonism is neuroprotective in inflammatory CNS disease

In CREAE in ABH mice, the neurological deficit accumulates slowly over a number of months and multi-focal lesions can occur anywhere along the neuroaxis (Baker *et al*., 1990), complicating assessment and treatment of neurodegeneration, especially as limited quantities of Δ^9-THC were available for study. In contrast, neurodegeneration is restricted to a focal site and develops rapidly in EAU. Following sensitization of B10.RIII mice with $IRBP^{153–180}$ peptide, the neuroretina is almost completely destroyed within 14–16 days (Hankey *et al*., 2001) (Fig. 3A–E), again associated with caspase-3-induced neuronal pathology (not shown). CB_1 receptor agonism with either *R*(+)-WIN-55,212-2 (Figs 3C and 4A), at doses that demonstrated no immunosuppressive effect in EAE (vehicle: $n = 9/9$, clinical score 3.3 ± 0.4, day of onset 17.6 ± 1.2; compared with 5 mg/kg i.p. *R*(+)-WIN 55,212-2 from day 10–22, $n = 7/8$, clinical score 3.4 ± 0.5, day of onset 17.3 ± 1.8), and Δ^9-THC significantly inhibited photoreceptor damage, without any apparent inhibition of inflammatory infiltrate (Figs 3E and 4B). Therefore, CB_1 agonism can mediate neuroprotection during inflammatory insults.

Cannabinoids regulate/inhibit glutamate excitotoxicity

Although immunosuppression, shown by a reduction in the degree of infiltrate, was not evident following treatment with *R*(+)-WIN-55, 212–2 or Δ^9-THC (Fig. 3) shortly before expression of disease, Δ^9-THC has been reported to have immunosuppressive effects (Lyman *et al*., 1989; Wirguin *et al*., 1994), which could influence neurodegenerative potential. As glutamate excitotoxicity has been implicated in neuronal damage in this and other EAE models (Achiron *et al*., 2000; Pitt *et al*., 2000; Smith *et al*., 2000), glutamate-induced excitotoxicity was examined *in vitro* and following CNS injection of kainic acid *in vivo* to examine neuro protective potential in the absence of a compounding immunomodulation. Following *in vitro* stimulation of NMDA receptors there was a marked (cerebellar) neuronal Ca^{2+} influx, which was more pronounced in CB_1-deficient mice than in controls, suggesting that the cannabinoid system exhibits tonic control of this response (Fig. 5A). In addition, the NMDA receptor antagonist (MK-801) was slower at reducing Ca^{2+} to basal levels in CB_1-deficient mice compared with ABH.*Cnr1*+/+ wild type, suggesting Ca^{2+} dysregulation in the absence of CB_1 receptors had occurred. Exogenous CB_1 agonism by CP55,940 inhibited this NMDA-induced cytosolic Ca^{2+} influx in wild-type animals, maximally at 1 μM using these culture conditions, but was relatively ineffective in CB_1-knockout mice, suggesting that post-synaptic control of NMDA-receptor activation

is lost in CB_1-deficient mice (Fig. 5A). The injection of kainic acid (>0.15 nM) into CB_1-deficient mice induced seizures and caused mortality, usually within 10 min post-injection, which did not occur in wild-type ABH and ABH congenic wild-type (Fig. 5B) mice ($P < 0.01$), despite using 50-fold higher doses of kainic acid. This elevated mortality was also evident in CD1.*Cnr*1–/– mice (Fig. 5B), and therefore CB_1 receptors are also clearly regulating ionotropic glutamate receptor activity (Fig. 5), which has been implicated in neural exocitoxicity.

Discussion

Neurological disability in multiple sclerosis correlates with spinal cord axonal loss (∼50–70% in paralysed multiple sclerosis patients) and reduced *N*-acetyl aspartate (NAA) levels in chronic multiple sclerosis patients (Bjartmar *et al*., 2000). This study provides the first and definitive evidence that the cannabinoid system controls the development of neurodegeneration, which occurs as a result of inflammatory insult of the CNS. This provides a novel avenue for neuroprotection in multiple sclerosis and other neurodegenerative diseases.

In diseases such as EAE and multiple sclerosis, it is unlikely that there is a single route to neurodegenerative events, and these may change during the disease course. The clinical outcome will be determined by the rate that these accumulate and how the genetic background of the individual enables them to adapt to the insult. Whilst axonal loss occurs very early in the course of multiple sclerosis (Filippi *et al*., 2003), it can remain clinically silent for some time, and irreversible neurological disability appears to develop when a threshold (15–30% in mice) of axonal loss is reached and compensatory CNS resources are exhausted (Confavreux *et al*., 2000; Wujek *et al*., 2002). In this model, marginal, statistically non-significant axonal loss occurred after the initial neurological attack, assessed here using a novel, relatively rapid neurofilament ELISA and by changes in NAA levels (Preece *et al*., 1994). Once chronic paresis was evident there was significant axonal loss (∼40%) as shown here by ELISA and also by magnetic resonance spectroscopy of spinal cord NAA levels (R. A. Page, H. G. Parkes, D. Baker, G. Giovannoni and C. A. Davie, unpublished observations). However, most interestingly, CB_1-deficient mice accumulated significant axonal loss (∼36%) even after a single acute episode, indicating that the presence of CB_1 was mediating a degree of neuroprotection during autoimmune attack. Surprisingly, analysis of spinal cord axonal content indicated that apparently normal, CB_1-deficient ABH mice have fewer spinal nerves than wild-type animals ($P < 0.001$), which may be reflective of CB_1 involvement in neural plasticity during development (Kim and Thayer, 2001), or there could be inherent neurodegeneration in these animals. This requires further study.

In EAE and, at least initially, in multiple sclerosis axonal damage occurs at least concordantly with inflammation (Ferguson *et al*., 1997; Trapp *et al*., 1998), which produces many potentially damaging elements such as cytokines and oxidative stress (Koprowski *et al*., 1993; Werner *et al*., 2001; Lock *et al*., 2002). Ionotropic glutamate receptor systems can also signal damaging mechanisms, at the blood–brain barrier and within the neural microenvironment, in EAE and multiple sclerosis (Bolton and Paul,

1997; Achiron *et al*., 2000; Pitt *et al*., 2000; Smith *et al*., 2000; Kalkers *et al*., 2002). As shown here, cannabinoids can tonically regulate NMDA glutamate receptor activity *in vitro* and support the *in vivo* observation that CB_1 regulates NMDA-induced and ischaemic excitotoxicity (Nagayama *et al*., 1999; Parmentier-Batteur *et al*., 2002). We also show definitively that CB_1 receptor activity regulates kainate glutamate receptor activity *in vivo*. Cannabinoids also have anti-oxidant properties that could further limit damaging events during inflammation (Hampson *et al*., 1998; Howlett *et al*., 2002). In addition, cellular changes such as neural and oligodendrocyte death and gliosis will change the CNS microenvironment, for example through redistribution of ion channels on demyelinated nerves (Foster *et al*., 1980; Black *et al*., 2000), loss of trophic support and the formation of compensatory neural pathways, which may contribute to excitotoxic stress and induce further degeneration. This could amplify as the disease progresses, possibly largely independent of inflammation (Compston and Coles, 2002), and may have similarities to nerve destruction in other neurodegenerative conditions, such as Huntington's chorea, Alzheimer's disease and amyotrophic lateral sclerosis, where nerve loss accumulates slowly. The cannabinoid system acts as a regulator of many different neurotransmitters and ion (K^+ and particularly Ca^{2+}) channels (Henry and Chavkin, 1995; Twitchell *et al*., 1997; Howlett *et al*., 2002) and appears to be particularly important when CNS homeostasis is in imbalance, as occurs in disease (Baker *et al*., 2000). Therefore, CB_1 can act at many levels within the death cascade, which will ultimately lead to toxic ion influxes, cell metabolic failure and activation of death effector molecules, such as caspase-3 (Ahmed *et al*., 2002). This would be consistent with the rapid neurodegeneration that accumulates in CB_1-deficient mice. This also implicates a role for endocannabinoids in neuroprotection. The nature of the endogenous neuroprotective cannabinoid has yet to be definitively resolved and may involve more than one CB_1-mediated pathway, possibly dependent on the neural circuit involved. Whilst in head trauma it has been suggested that 2-AG may mediate neuroprotection (Panikashvili *et al*., 2001), in a similar study anandamide, not 2-AG, was shown to be active (Hansen *et al*., 2001). However, as both anandamide and 2-AG are elevated in chronic EAE lesions (Baker *et al*., 2001) both may participate in endogenous neuroprotective mechanisms. This will be elucidated once suitable agents to dissect these pathways become available.

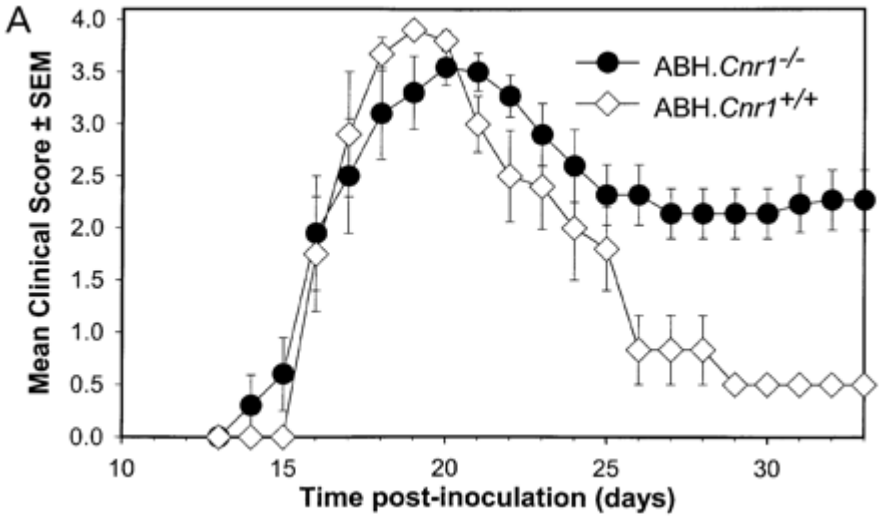
Previous studies in non-demyelinating EAE models have demonstrated that high-dose Δ^9-THC, often administered during the induction process, has clinical disease ameliorating effects, due to prevention of infiltrate reaching the CNS (Lyman *et al*., 1989; Wirguin *et al*., 1994). Furthermore, Δ^9-THC had no effect on the clinical course, which in Lewis rats is usually naturally self-limiting, when treatment was initiated at disease onset (Lyman *et al*., 1989). Acute phase paralysis in most rodent EAE models is rapidly reversed and can occur largely independent of any demyelination and axonal loss, and more probably reflects conduction block (Wujek *et al*., 2002). Therefore, inhibition of acute phase paralysis (Lyman *et al*., 1989) may reflect inhibition of the immune process that leads to damage in addition to neural effects. Indeed, CB agonists have recently been reported to have immunomodulatory effects in a viral model of multiple sclerosis (Arevalo-Martin *et al*., 2003; Croxford and Miller, 2003). Without the use of tissue-specific CB_1 conditional knockouts (Marsicano *et al*., 2002), it is probably not possible to

completely exclude some influence of cannabinoid inhibition of the immune response in the neurodegenerative process. However, in the context of multiple sclerosis, both would be of benefit for inhibition of disease.

The results of this study are important because they suggest that in addition to symptom management, cannabinoids offer the potential to slow the progression of a disease that as yet has no satisfactory treatment. Therefore, if trials are extended to monitor the long-term effects of cannabis use on symptom management, they should be designed to monitor neuronal loss and progression. If CB_1 agonism can be applied whilst limiting the unwanted psychoactive potential, such as through enhancement of endocannabinoid levels (Baker *et al*., 2001), this may provide a new therapeutic route in multiple sclerosis and could be combined with therapies that target the immunological elements of disease. In neurodegenerative diseases including multiple sclerosis, signs appear once significant damage has already accumulated, slowing the degenerative process early following diagnosis may help improve quality of life for many more years.

Acknowledgements

The Multiple Sclerosis Society of Great Britain and Northern Ireland, the Brain Research Trust, Aims2Cure and the National Institute for Drug Abuse (NIDA) chemical supply program supported this work. Support from the Wellcome Trust and the Alzheimers Trust is also gratefully acknowledged. The authors have declared that they have no conflicting financial interests.



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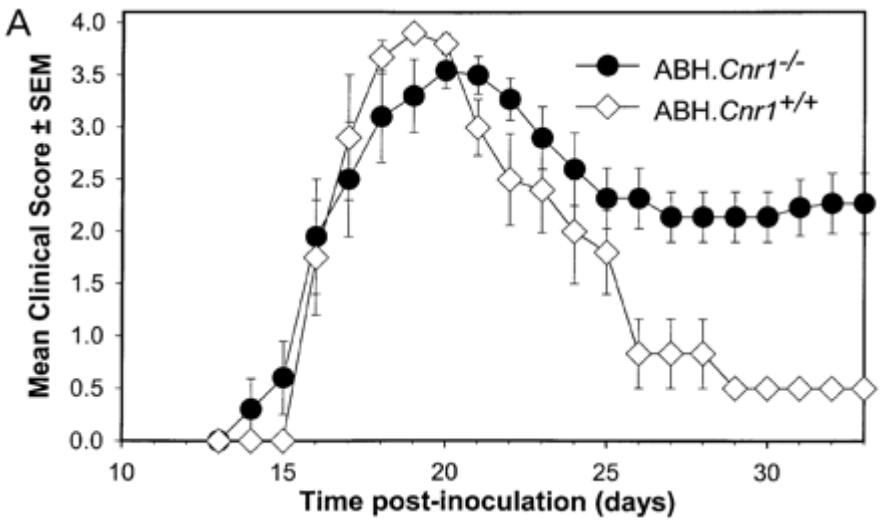

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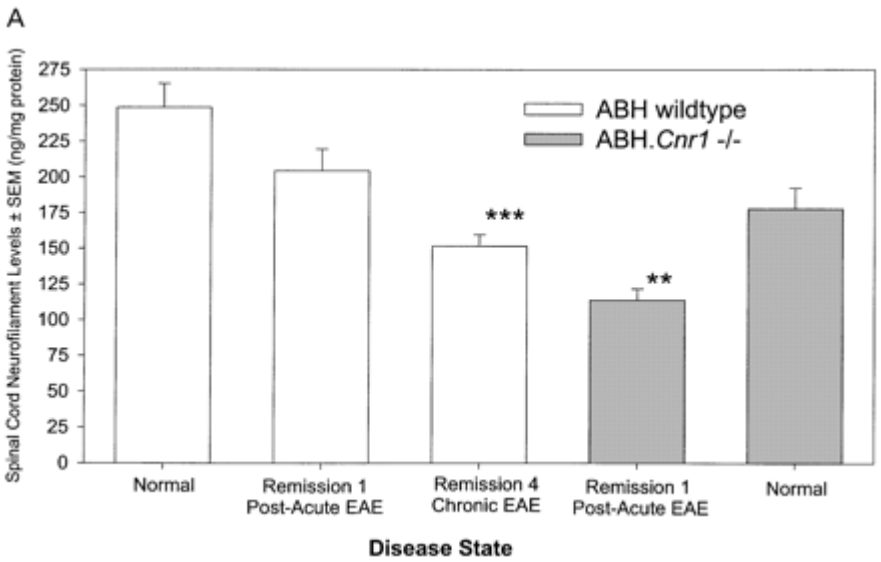
Fig. 1 Cannabinoids limit accumulation of disability in EAE. CREAE was actively induced in wild-type ABH or CB_1 gene (*Cnr1*)-deficient, congenic ABH mice with mouse spinal cord homogenate in complete Freund's adjuvant on day 0 and 7. (**A**) The mean ± SEM daily clinical scores (post-induction) demonstrate that CB_1 knockout mice (filled circles) show poor recovery from paralysis compared with wild-type mice (open diamonds). (**B**) Movement activity of normal and animals with EAE in remission after one or four paralytic disease episodes were measured in an activity chamber over 5 min. The results represent the individual data points (solid circles) and box plot (25–75% percentile) with 5–95% percentiles range of activity wild-type (open box) and CB_1 knockout (shaded box) mice.



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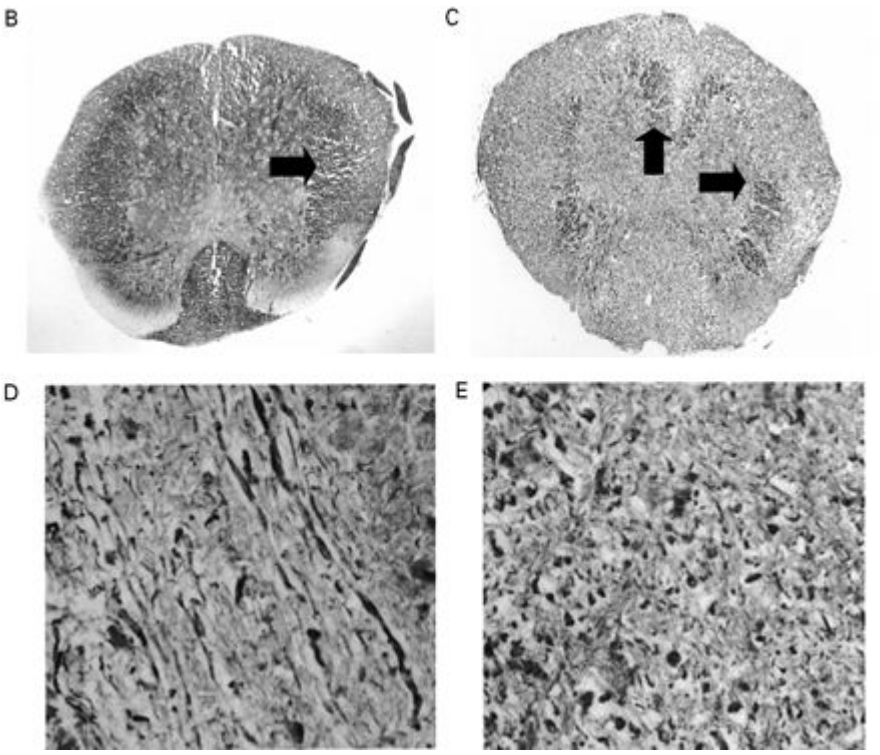

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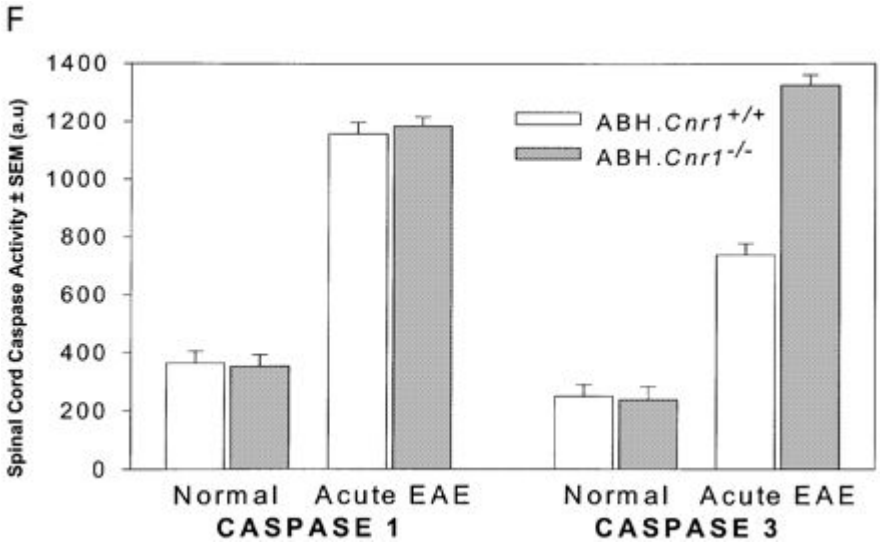

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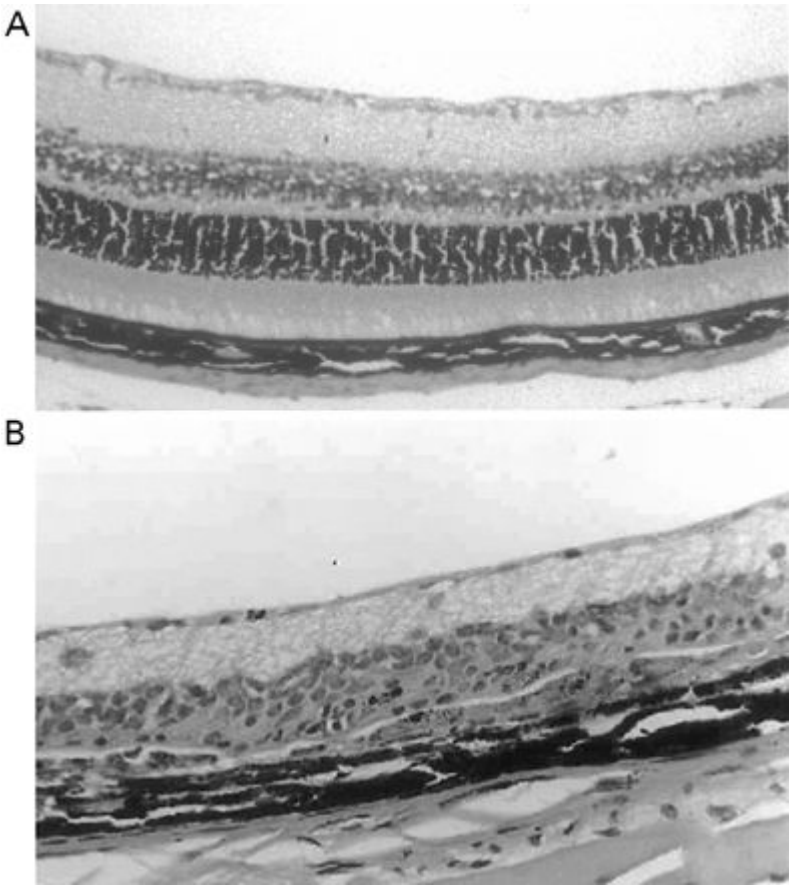
Fig. 2 Cannabinoids mediate neuroprotection in experimental allergic encephalomyelitis. CREAE was actively induced in wild-type ABH or CB_1 gene (*Cnr1*)-deficient, congenic ABH mice with mouse spinal cord homogenate in complete Freund's adjuvant on day 0 and 7, and disease progression in wild-type mice is associated with axonal damage and loss. (**A**) Spinal cord neurofilament levels from tissue homogenates from wild-type (open boxes) and CB_1 knockout (shaded boxes) mice were measured by ELISA from normal and animals with EAE in remission after one or four paralytic disease episodes during EAE. The results represent the mean ± SEM neurofilament levels (n = 6–8 per group). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with respective normal controls. (**B**–**E**) Axonal damage was reflected

histologically. Bielshowsky silver stain of paraffin 5 μm wax sections of lumbar spinal cord in (**B**) normal and (**C**) chronic EAE after four attacks, demonstrating few surviving axons (arrows) and note the loss of axons in the dorsal horn. Neurofilament-specific immunocytochemistry of the spinal cord from a CB_1 knockout mouse (**D**) before and (**E**) after a single paralytic episode of EAE. Note the many transactions of the white matter axons. (**F**) Caspase-1 and -3 levels were assessed using western analysis and caspase-3 levels are significantly ($P < 0.001$) elevated in CB_1 knockout mice (shaded boxes) during acute EAE compared with wild type (open boxes) (Student's *t*-test; $n = 6$ per group).



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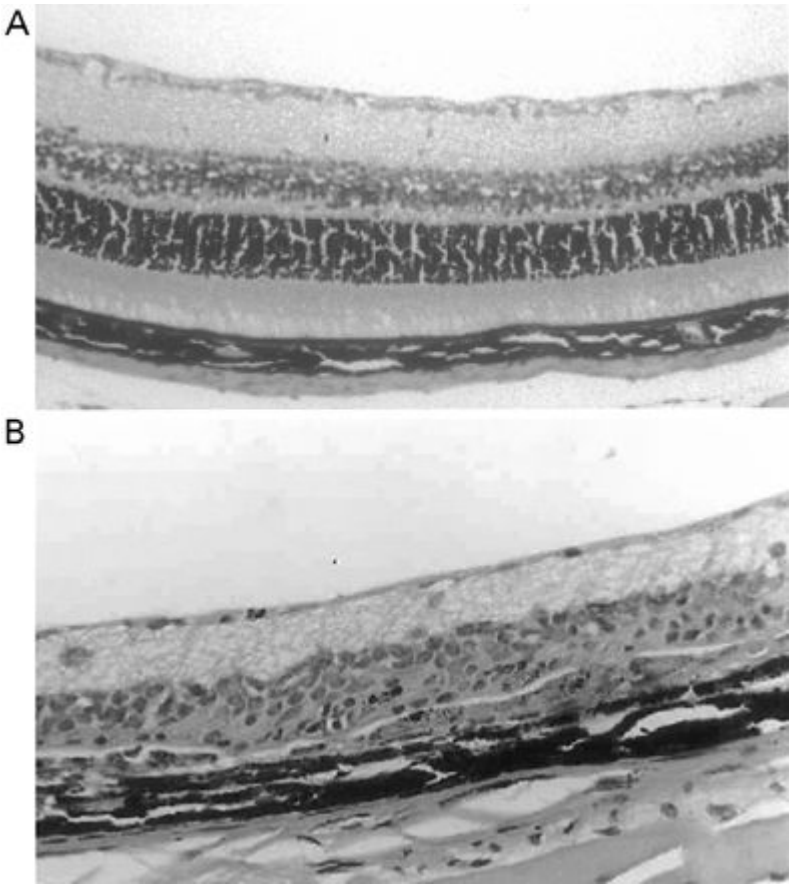

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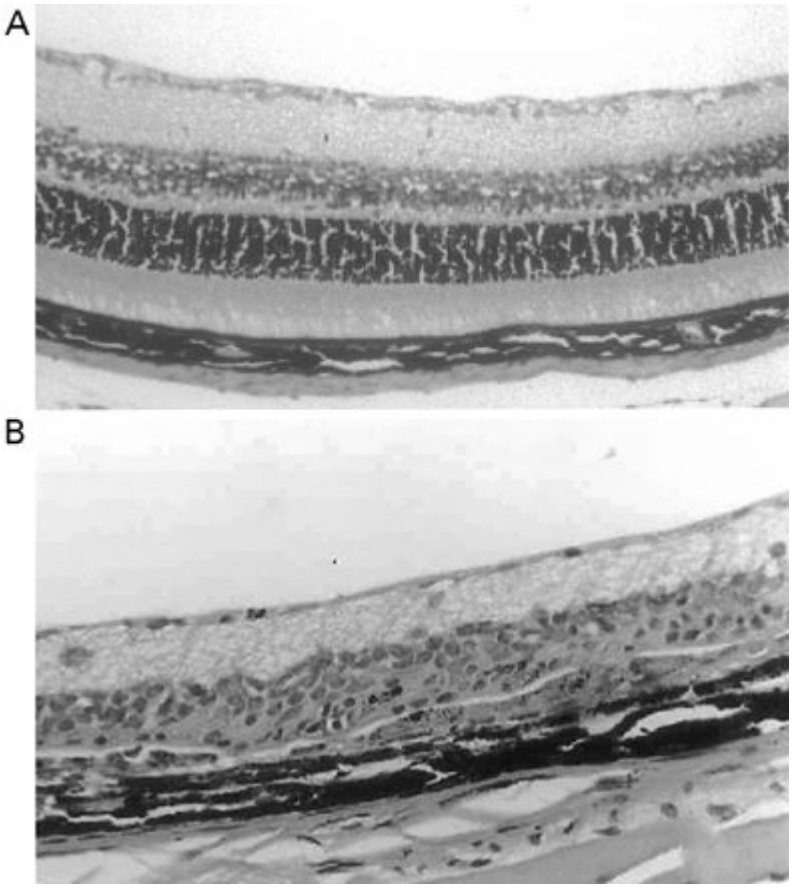

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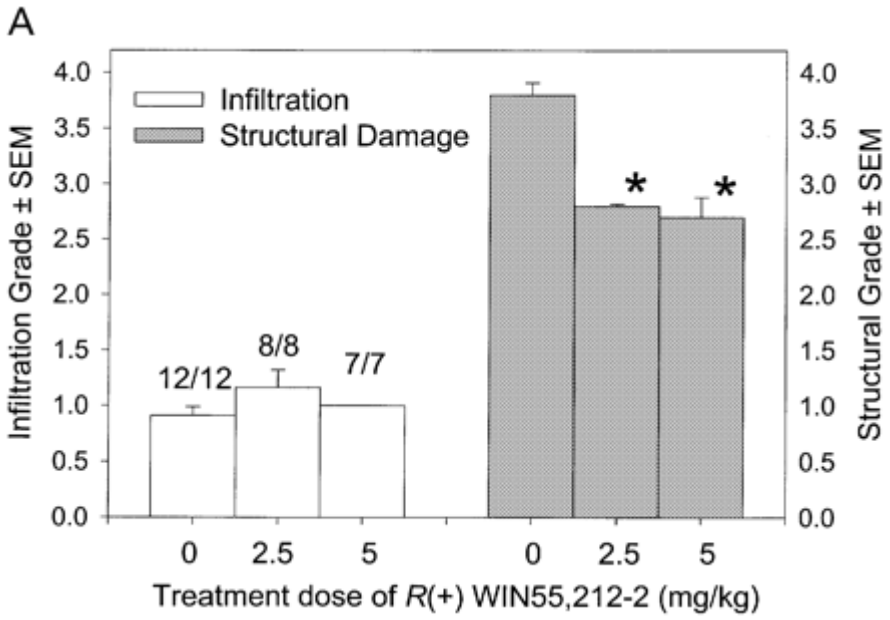
Fig. 3 Cannabinoids limit neurodegeneration of the retina in EAU. (**A**) Paraffin-wax section of normal retina in a B10.RIII mouse. Mice were injected with $IRBP^{153–180}$ peptide in Freund's adjuvant and vehicle (**B**, **D**) or cannabinoid (**C**, **E**) was injected daily from day 8 onwards. Retinas were assessed histologically on either (**B**, **C**) day 15 or (**D**, **E**) day 21 post-inoculation following treatment with either (**C**) 5 mg/kg i.p. *R*(+)-WIN55,212 or (**E**) 20 mg/kg i.p. Δ^9-THC.



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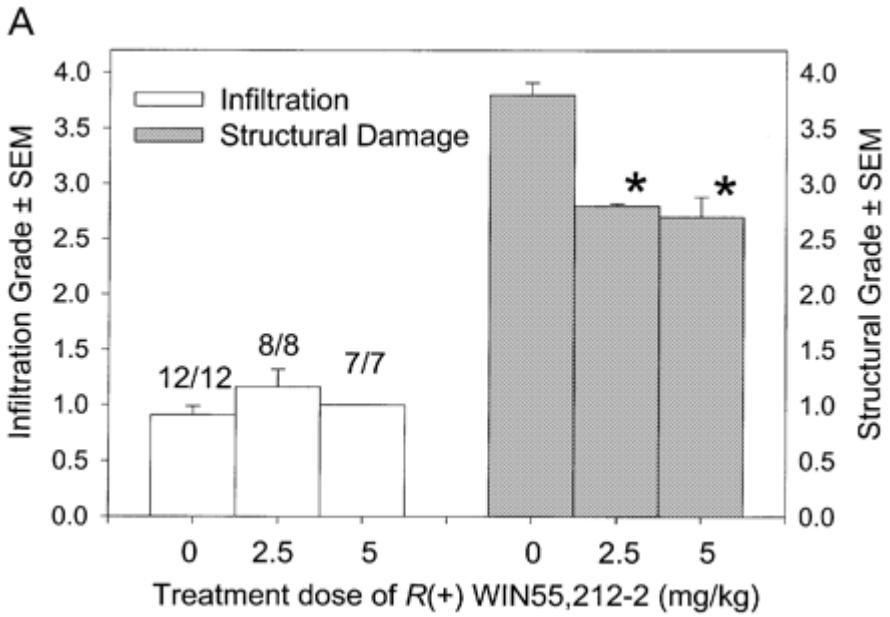

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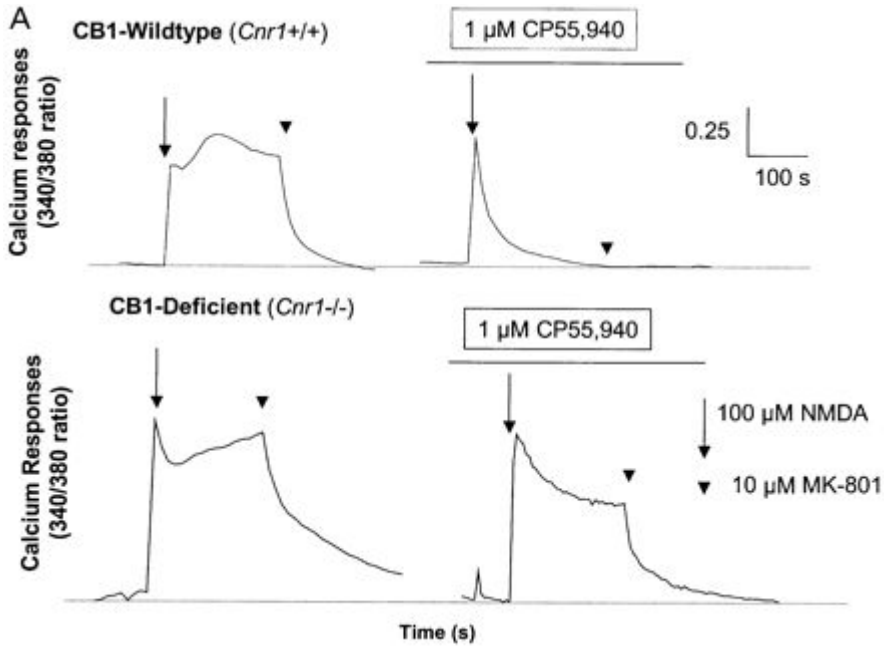
Fig. 4 Cannabinoids mediate neuroprotection in EAU. Following active induction of EAU in B10.RIII mice with $IRBP^{153–180}$ peptide in complete Freund's adjuvant, eyes were processed for routine wax histology and the level of infiltration (scale 0–5) and structural damage (scale 0–6) assessed. Animals received daily injections of (**A**) 5 mg/kg i.p. *R*(+)-WIN55,212 or (**B**) 20 mg/kg i.p. Δ^9-THC, dissolved in ethanol : cremophor : PBS (1 : 1 : 18), from day 8 onwards. The data represent the results on days 15 and 21 post-inoculation, respectively. The number of animals with EAU per group is indicated. **P* < 0.05, ***P* < 0.01 compared with respective control group.



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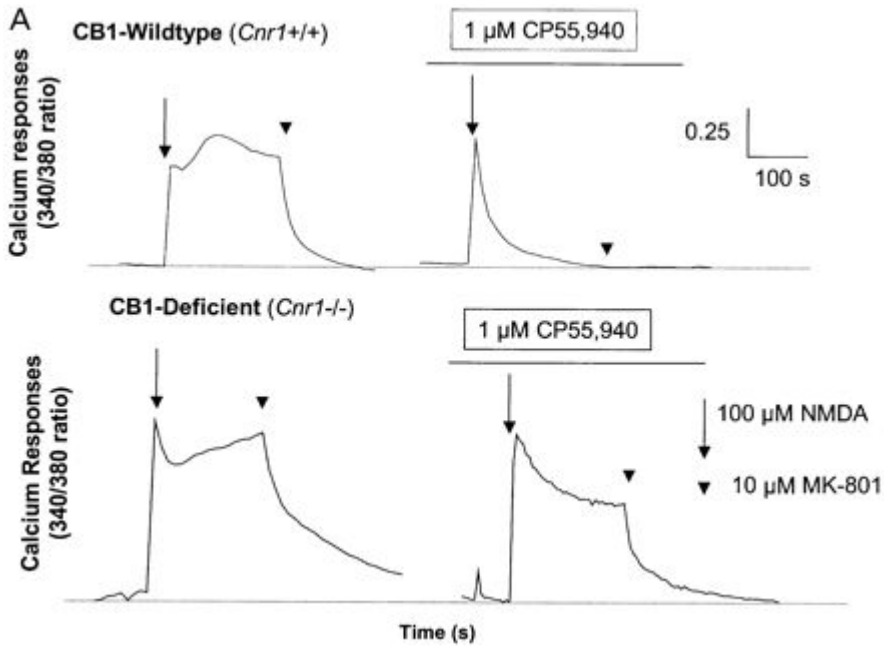

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Fig. 5 Cannabinoids inhibit glutamate-induced excitotoxicity. (**A**) Lack of CB_1 receptors leads to loss of regulation of NMDA-induced calcium influx. Cerebellar neurons from wild-type and CB_1 knockout mice were loaded with the fluorescence Ca^{2+} indicator dye fura-2 prior to ionotropic glutamate receptor stimulation. Each trace is the mean somatic response of 40 individual neurons measured by single-cell fluorescence imaging. The NMDA receptor agonist, NMDA (100 μM), was added at the arrow and the NMDA receptor antagonist, MK-801, was added at the arrowhead. Where indicated, the CB_1 receptor agonist CP55,940 (1 μM) was added 5 min before imaging commenced and was present throughout the experiment. (**B**) Kainate-induced excitotoxicity *in vivo*. Halothane anaesthetized

animals (n = 5 per group) were intracerebrally injected with 15–0.15 nmol of kainic acid. Rapid (1–10 min) mortality (open blocks) developed in ABH.*Cnr1*–/– mice compared with the survival (0 out of 5 mortality; shaded blocks) that occurred in wild type (ABH.*Cnr1*+/+). Seizures consistent with glutamate excitotoxicity were evident. CB_1 knockout animals died within 1–3 min of injection, whereas heterozygotes typically showed adverse effects 5–10 min after injection. Doses lower than the maximal survival dose for each strain were not tested.

Table 1

CB_1-deficient mice are susceptible to the development of EAE

Strain	No. EAE/total	Clinical score (± SEM)	Day of onset (± SD)
ABH (wild type)	15/15	4.0 ± 0.1	14.7 ± 1.0
ABH.*Cnr1*+/+ (wild type homozygous)	6/6	4.0 ± 0.0	16.3 ± 1.8
ABH.*Cnr1*+/– (heterozygous)	9/9	4.2 ± 0.2	15.4 ± 1.0
ABH.*Cnr1*–/– (knockout)	15/15	4.1 ± 0.1	16.3 ± 1.8

The results represent the number of animals within a group that developed clinical EAE, the mean maximal clinical score ± SEM that developed during acute phase EAE (up to day 24 post-inoculation) and the mean day of onset ± SD of clinical EAE (score >0.5) The disease incidence, onset and severity of congenic mice was comparable (P > 0.05) to that found in wild-type ABH mice, as assessed using the Mann–Whitney U-test.

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Anti-inflammatory property of the cannabinoid receptor-2-selective agonist JWH-133 in a rodent model of autoimmune uveoretinitis

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Abstract

Previous studies have shown that cannabinoids have anti-inflammatory and immune-modulating effects, but the precise mechanisms of action remain to be elucidated. In this study, we investigated the effect of JWH 133, a selective agonist for cannabinoid receptor 2, the main receptor expressed on immune cells, in a model of autoimmune disease, experimental autoimmune uveoretinitis (EAU). JWH 133 suppressed EAU in a dose-dependent manner (0.015–15 mg/kg), and the suppressive effect could be achieved in the disease-induction stage and the effector stage. Leukocytes from mice, which had been treated with JWH 133, had diminished responses to retinal peptide and mitogen Con A stimulation in vitro. In vivo JWH 133 treatment also abrogated leukocyte cytokine/chemokine production. Further in vitro studies indicated that JWH 133 down-regulated the TLR4 via Myd88 signal transduction, which may be responsible for its moderate, suppressive effect on antigen presentation. In vivo JWH 133 treatment (1 mg/kg) also suppressed leukocyte trafficking (rolling and infiltration) in inflamed retina as a result of an effect on reducing adhesion molecules CD162 (P-selectin glycoprotein ligand 1) and CD11a (LFA-1) expression on T cells. In conclusion, the cannabinoid agonist JWH 133 has a high

in vivo, anti-inflammatory property and may exert its effect via inhibiting the activation and function of autoreactive T cells and preventing leukocyte trafficking into the inflamed tissue.

http://annualreviews.org/doi/abs/10.1146/annurev.immunol.021908.132653

Recent Advances in the Genetics of Autoimmune Disease

Annual Review of Immunology

Vol. 27:363-391 (Volume publication date 23 April 2009)
First published online as a Review in Advance on December 15, 2008
https://doi.org/10.1146/annurev.immunol.021908.132653

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Abstract

Extraordinary technical advances in the field of human genetics over the past few years have catalyzed an explosion of new information about the genetics of human autoimmunity. In particular, the ability to scan the entire genome for common polymorphisms that associate with disease has led to the identification of numerous new risk genes involved in autoimmune phenotypes. Several themes are emerging. Autoimmune disorders have a complex genetic basis; multiple genes contribute to disease risk, each with generally modest effects independently. In addition, it is now clear that common genes underlie multiple autoimmune disorders. There is also heterogeneity among subphenotypes within a disease and across major racial groups. The current crop of genetic associations are only the start of a complete catalog of genetic factors for autoimmunity, and it remains unclear to what extent common variation versus multiple rare variants contribute to disease susceptibility. The current review focuses on recent discoveries within functionally related groups of genes that provide clues to novel pathways of pathogenesis for human autoimmunity.

https://link.springer.com/article/10.1007/s12026-011-8210-5

Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease

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First Online:

: 10.1007/s12026-011-8210-5

Cite this article as:
Basu, S. & Dittel, B.N. Immunol Res (2011) 51: 26. doi:10.1007/s12026-011-8210-5

Abstract

It has become clear that the endocannabinoid system is a potent regulator of immune responses, with the cannabinoid receptor 2 (CB2) as the key component due to its high expression by all immune subtypes. CB2 has been shown to regulate immunity by a number of mechanisms including development, migration, proliferation, and effector functions. In addition, CB2 has been shown to modulate the function of all immune cell types examined to date. CB2 is a G_i-protein-coupled receptor and thus exhibits a complex pharmacology allowing both stimulatory and inhibitory signaling that depends on receptor expression levels, ligand concentration, and cell lineage specificities. Here, we discuss both in vitro and in vivo experimental evidence that CB2 is a potent regulator of immune responses making it a prime target for the treatment of inflammatory diseases.

Keywords

Autoimmunity Cannabinoid receptor 2 Endocannabinoid GPCR Immune system Immune suppression

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Dronabinol as a treatment for anorexia associated with weight loss in patients with AIDS

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Abstract

The effects of dronabinol on appetite and weight were evaluated in 139 patients with AIDS-related anorexia and ≥2.3 kg weight loss in a multi-institutional study. Patients were randomized to receive 2.5 mg dronabinol twice daily or placebo. Patients rated appetite, mood, and nausea by using a 100-mm visual analogue scale 3 days weekly. Efficacy was evaluatee in 88 patients. Dronabinol was associated with increased appetite above baseline (38% vs 8% for placebo, P = 0. 015), improvement in mood (10% vs -2%, P = 0. 06), and decreased nausea (20% vs 7%; P = 0. 05). Weight was stable in dronabinol patients, while placebo recipients had a mean loss of 0.4 kg (P = 0.14). Of the dronabinol patients, 22% gained ≥2 kg, compared with 10.5% of placebo recipients (P = 0.11). Side effects were mostly mild to moderate in severity (euphoria, dizziness, thinking abnormalities); there was no difference in discontinued therapy between dronabinol (8.3%) and placebo (4.5%) recipients. Dronabinol was found to be safe and effective for anorexia associated with weight loss in patients with AIDS.

Key Words

Dronabinol
cachexia
urine cannabinoid-creatinine ratio

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Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R(+)WIN55,212

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First published April 15, 2003 - More info

Abstract

Theiler murine encephalomyelitis virus–induced demyelinating disease (TMEV-IDD) is a mouse model of chronic-progressive multiple sclerosis (MS) characterized by Th1-mediated CNS demyelination and spastic hindlimb paralysis. Existing MS therapies reduce relapse rates in 30% of relapsing-remitting MS patients, but are ineffective in chronic-progressive disease, and their effects on disability progression are unclear. Experimental studies demonstrate cannabinoids are useful for symptomatic treatment of spasticity and tremor in chronic-relapsing experimental autoimmune encephalomyelitis. Cannabinoids, however, have reported immunosuppressive properties. We show that the cannabinoid receptor agonist, R(+)WIN55,212, ameliorates progression of clinical disease symptoms in mice with preexisting TMEV-IDD. Amelioration of clinical disease is associated with downregulation of both virus and myelin epitope-specific Th1 effector functions (delayed-type hypersensitivity and IFN-γ production) and the inhibition of CNS mRNA expression coding for the proinflammatory cytokines, TNF-α, IL1-β, and IL-6. Clinical trials investigating the therapeutic potential of cannabinoids for the symptomatic treatment of MS are ongoing, and this study demonstrates that they may also have potent immunoregulatory properties.

Introduction

Multiple sclerosis (MS) is the most common human primary demyelinating disease of the CNS and is thought to be an autoimmune disease mediated by myelin-specific $CD4^+$ Th1 cells (1). Although current disease-modifying therapeutic agents, IFN-β and copaxone, reduce relapses in a portion of relapsing-remitting MS patients, they are not curative, and their effectiveness in reducing disability progression in both relapsing-remitting and primary-progressive MS patients is unclear (2). Therefore, there is a need for novel therapeutic agents in the treatment of MS. The endocannabinoid network is a naturally occurring system, wherein endogenous ligands, such as anandamide (AEA), signal through two known cannabinoid receptors, CB1 and CB2 (3, 4). Both of the cannabinoid receptors are present on immune cells, including B lymphocytes, T lymphocytes, macrophages, and natural killer cells, suggesting they may play a role in regulation of immune functions (5). Numerous in vitro studies have described the immunomodulatory properties of cannabinoids, particularly Δ^9-tetrahydrocannabinol (Δ^9-THC), the major psychoactive constituent of marijuana (6). Clinical trials to determine the symptomatic therapeutic potential of Δ^9-THC on spasticity, bladder dysfunction, and mobility in MS patients are underway (7). Cannabinoid receptor agonists have also been shown to alleviate both tremor and spasticity, associated with neurological dysfunction in experimental allergic

encephalomyelitis (EAE), an animal model of MS (8, 9). No studies to date, however, have determined the therapeutic immunosuppressive properties of cannabinoids in viral autoimmune disease models.

MS is thought to have a infectious etiology based on epidemiological evidence that viral infections may play a role in the initiation and/or exacerbation of clinical disease (10, 11). Theiler murine encephalomyelitis virus (TMEV) is a single-stranded RNA virus belonging to the *Picornaviridae* family (12). TMEV-induced demyelinating disease (TMEV-IDD) is a natural chronic-progressive CNS demyelinating disease of susceptible strains of mice, with similarities to primary-progressive MS (13, 14). Although TMEV-IDD is initiated by viral capsid protein-specific $CD4^+$ T cells targeting virus persisting in CNS microglia, autoreactive myelin-specific $CD4^+$ Th1 responses arise by epitope spreading and contribute to chronic pathology (15, 16). Myelin destruction is mediated largely by the production of the proinflammatory cytokines, IFN-γ and TNF-α, secreted from autoreactive T cells and macrophages. Blockade of these inflammatory mediators with TNF-receptor fusion proteins or immunomodulation using anti-inflammatory agents such as IL-10 are effective therapies in EAE (17–19).

We investigated the immunosuppressive potential of R(+)WIN55,212, a cannabinoid receptor agonist, as a treatment for the clinical autoimmune disease associated with TMEV infection. The results show that R(+)WIN55,212 is an effective therapeutic agent for ongoing TMEV-IDD and that R(+)WIN55,212 treatment inhibits the differentiation of Th1 effector cells. The data suggest that the potent immunosuppressive properties of R(+)WIN55,212 may have significant therapeutic potential in ameliorating disability progression in MS patients currently undergoing clinical trials with cannabinoids for the treatment of spasticity and tremor.

Methods

Infection of SJL mice with TMEV. Five- to six-week-old female SJL mice were purchased from Harlan Laboratories (Bethesda, Maryland, USA) and housed in the Northwestern University Center for Comparative Medicine (Chicago, Illinois, USA). Mice were infected by intracerebral injection of 3×10^7 PFU of wild-type TMEV (BeAn 8386 strain) and scored at daily intervals on a clinical scale of 0–5: 0, no signs of disease; 1, mild gait abnormalities; 2, severe gait abnormalities; 3, paralysis in one limb; 4, more than one paralyzed limb; 5, moribund.

Peptides. Proteolipid protein (PLP) peptide $PLP_{139\text{-}151}$ (HSLGKWLGHPDKF) and the TMEV peptide $VP2_{70\text{-}86}$ (WTTSQEAFSHIRIPLPH) were purchased from Peptides International Inc. (Louisville, Kentucky, USA). The amino acid composition was verified by mass spectrometry, and purity was assessed by HPLC.

Administration of WIN55,212. R(+)WIN55,212 and S(–)WIN55,212 (Sigma-Aldrich, St. Louis, Missouri, USA) were dissolved in 10% Tween-80 (Sigma-Aldrich) and PBS to a final concentration of 4 mg/ml or 1 mg/ml. The mixture was then vortexed and passed through a 2-gauge needle. Similar preparations without active compounds were used as vehicle controls. Mice were treated with suspensions (0.1 ml) injected intraperitoneally. Groups of TMEV-infected mice (n = 5–10) were treated with either a high (20 mg/kg) or low (5 mg/kg) dose of WIN55,212 at varying stages of disease.

Delayed-type hypersensitivity response. Delayed-type hypersensitivity (DTH) responses were elicited by injecting mice subcutaneously with 5 μg of the challenge peptides, $PLP_{139-151}$ or $VP2_{70-86}$, into alternate ears following measurement of ear thickness using a Mitutoyo model 7326 engineer's micrometer (Schlesinger's Tools, Brooklyn, New York, USA). Twenty-four hours after peptide challenge, the ears were remeasured, and differences in ear swelling over pre-challenge thickness were expressed in units of 10^{-4} inches plus or minus SEM.

T cell proliferation and cytokine analysis. Spleens were removed from infected mice ($n = 2$) at various times following infection. T cell proliferation and cytokine analysis were performed as described previously (20). Proliferation was determined from triplicate wells for each peptide concentration and then expressed as Δ counts per minute. For IFN-γ and IL-4 cytokine analysis, a duplicate set of proliferation wells were used to collect supernatants at 48 and 72 h, and cytokine concentrations were determined by ELISA.

Virus plaque assays. Brains were removed from infected mice either on day 7 following day 0–5 R(+)WIN55,212 treatment or on day 33 after infection following day 26–31 R(+)WIN55,212 treatment. Organs were weighed, homogenized, and diluted in serum-free DMEM media. Virus plaque assays were performed as described previously (20). Plaques were counted on each plate and multiplied by the dilution and corrected for the quantity of tissue used to give a value in plaque forming units per milligram of tissue.

RT-PCR. Following the induction of TMEV-IDD, mice ($n = 2$ per time point) were anesthetized and perfused with 50 ml of PBS. Spinal cords were extruded by flushing the vertebral canal with PBS. Isolation of mRNA from tissue was performed as described previously (21). First-strand cDNA was generated from 2 μg total RNA using Advantage-RT Kit (CLONTECH Laboratories Inc., Palo Alto, California, USA) using 20 pmol oligo(dT) primer, per the manufacturer's provided protocol, in a total volume of 20 μl. Cytokine PCR and densitometry was performed as described previously (21). The sum intensity and band area were determined for each competitor/wild-type amplicon pair, and a ratio of wild-type/competitor intensity was determined for all cytokines and adjusted to each sample's housekeeping gene, HPRT. The relative levels of mRNA present were measured as the percentage of intensity of the HPRT control for that sample.

FACS analysis of splenic leucocytes. Splenocyte populations were stained using anti-CD4, anti-CD8, or anti–B220-APC-conjugated Ab, anti–F4/80-FITC-conjugated Ab, or anti–CD4-PerCP-conjugated Ab and anti–CD25-APC-conjugated Ab (PharMingen, San Diego, California, USA). Cells were labeled, washed, resuspended in PBS containing 1% FCS and analyzed by flow cytometry on a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View, California, USA). The total number of cells of each population was analyzed individually from three mice per group and then plotted as the mean total number of cells plus or minus SEM.

Statistics. Clinical severity results were presented as the mean group clinical score and the statistical difference calculated by the Mann-Whitney nonparametric ranking test. Analysis of DTH responses, virus titers, proliferation, IFN-γ ELISA, and FACS analysis was performed using the two-tailed Student *t* test.

Results

Cannabinoid therapy delays the onset and reduces clinical severity of TMEV-IDD. The intraperitoneal administration of R(+)WIN55,212 (20 mg/kg) at the time of TMEV infection (day

0–5) significantly delayed the onset of clinical disease and ameliorated both the incidence and severity of clinical disease at equivalent time points up to day 85, compared with either mice treated with the inactive enantiomer S(–)WIN55,212 or untreated TMEV-infected mice ($P < 0.05$) (Figure 1a). Treatment with R(+)WIN55,212 between postinfection day 26–31, i.e., at the onset of TMEV-induced clinical disease, also ameliorated the clinical severity of TMEV-IDD ($P < 0.05$) (Figure 1b). In addition, R(+)WIN55,212 treatment during established disease (postinfection days 50–55) stabilized clinical severity at grade 1 for over 20 days compared with the continued increase of clinical severity in control mice (Figure 1c). Importantly, there was no significant difference in severity, incidence, or onset of TMEV-induced disease between untreated TMEV-infected mice and those treated with the S(–)WIN55,212 enantiomer (Figure 1) or vehicle-treated mice (data not shown). Treatment of TMEV-infected mice with a lower dose of R(+)WIN55,212 (5 or 1 mg/kg) at the time of TMEV infection (postinfection day 0–5) or at the onset of clinical disease (postinfection day 26–31) was unsuccessful at reducing either the incidence or severity of disease compared with control mice (data not shown), indicating the dose dependency of the protection. In addition, mice treated on alternative days (between postinfection day 0–8 or 26–34) also did not show any significant reduction in disease severity (data not shown).

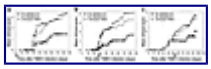

Figure 1

Cannabinoid receptor agonism ameliorates the clinical severity of established TMEV-induced demyelinating disease. Treatment with R(+)WIN55,212 (filled circles) inhibits TMEV-induced clinical disease when administered, at the time of infection (**a**), at the onset of clinical disease (**b**), or during established disease (**c**). Rx, duration of administration of WIN55,212 to mice. *Significant inhibition ($P < 0.05$) of clinical disease score in R(+)WIN55,212-treated mice compared with S(–)WIN55,212 (open circles) or untreated TMEV mice (filled triangles). Data are representative of three separate experiments.

R(+)WIN55,212 treatment increases the viral load in brain tissue at early, but not late time points. To determine whether R(+)WIN55,212 treatment increased the susceptibility of SJL/J mice to TMEV infection, TMEV viral titers were measured in the brains of mice 2 days after the last treatment dose at each of the time points studied (postinfection day 0–5 and 26–31). The viral load was found to be significantly increased in mice treated with R(+)WIN55,212 at the early time point ($P < 0.05$) (Figure 2a), however viral titers following R(+)WIN55,212 treatment initiated at the onset of clinical disease were similar to controls (Figure 2b).

Figure 2

Cannabinoid treatment increases the susceptibility of mice to TMEV infection and is not cytotoxic to splenocytes. R(+)WIN55,212 treatment of mice at the time of infection (**a**) (black bars) showed a significant increase in CNS virus titers compared with either S(–)WIN55,212 (gray bars) or untreated (white bars) TMEV-infected mice. Virus titers were not different from controls in mice treated with R(+)WIN55,212 at the onset of disease (**b**). *Significant increase in viral load in R(+)WIN55,212-treated mice compared with either S(–)WIN55,212 or untreated TMEV mice ($P < 0.05$). No PFUs were observed in naive mice. R(+)WIN55,212 treatment has no cytotoxic effect, as measured by FACS analysis of splenic $CD4^+$, $CD8^+$, $B220^+$, and $CD4^+CD25^+$ lymphocytes, and $F4/80^+$ macrophage populations compared with S(–)WIN55,212

or naive mice (**c**). Results are expressed as the mean total number of cells (n = 3 per group) ± SEM.

In vivo administration of R(+)WIN55,212 is not toxic to lymphocytes. To determine if the disease ameliorating effect of R(+)WIN55,212 was due to a cytotoxic effect on lymphocytes, we examined the effects of the treatment on splenic lymphocyte subpopulations by FACS analysis (Figure 2c). Groups of naive mice (n = 3) mice were treated with either 20 mg/kg of R(+)WIN55,212 or S(–)WIN55,212 intraperitoneally for 5 days. Twenty-four hours after the last treatment, the percentages and numbers of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and $F4/80^+$ macrophages were determined by FACS analysis. There was no significant changes in the total number of cells per spleen or the number of cells within each subpopulation (Figure 2c).

R(+)WIN55,212 treatment inhibits the proliferative capacity of naive, but not activated T cells. Mice treated with R(+)WIN55,212 between postinfection day 0–5 demonstrated a significantly reduced T cell proliferative response following rechallenge with the immunodominant TMEV epitope ($VP2_{70-86}$) compared with controls ($P < 0.05$) (Figure 3a). There was no significant effect on $VP2_{70-86}$-specific proliferative responses, however, in mice with established clinical disease (postinfection day 32 or 56) treated with R(+)WIN55,212 (Figure 3, b and c). Following TMEV infection, peripheral autoreactive $PLP_{139-151}$–specific responses can be detected beginning at postinfection day 40–50. Although early R(+)WIN55,212 treatment (postinfection day 0–5) inhibited $VP2_{70-86}$ proliferative responses, there was no effect on the proliferative responses to $PLP_{139-151}$ when measured at postinfection day 40 (Figure 3d). In fact, R(+)WIN55,212 treatment at all three time points was unsuccessful at inhibiting the proliferative capacity of activated $PLP_{139-151}$–specific T lymphocytes compared with controls (Figure 3, d–f).

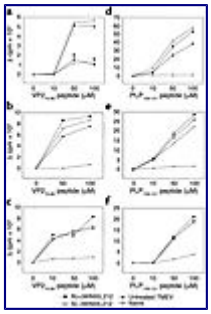

Figure 3

Effects of cannabinoid receptor agonism on T cell proliferation depends upon the cellular activation state. Early R(+)WIN55,212 treatment (filled circles) (postinfection day 0–5) significantly inhibited the proliferation of T lymphocytes compared with S(–)WIN55,212 (open circles) and untreated TMEV-infected mice (filled triangles) upon rechallenge with the TMEV $VP2_{70-86}$ peptide (postinfection day 8) (**a**), but not $PLP_{139-151}$ (**d**). When measured at postinfection day 40, R(+)WIN55,212 treatment did not inhibit T lymphocyte proliferation to either $VP2_{70-86}$ or $PLP_{139-151}$ peptides during established disease (postinfection day 26–31 or 50–55) (**b**, **c**, **e**, and **f**). *$P < 0.05$, compared with either S(–)WIN55,212 or untreated mice.

DTH responses are transiently inhibited by R(+)WIN55,212 treatment. DTH measures the in vivo release of Th1-type cytokines such as IFN-γ, and other mediators of tissue injury and inflammation, from recruited inflammatory Th1 cells. Following treatment with R(+)WIN55,212 or appropriate controls, mice were rechallenged with either the immunodominant TMEV $VP2_{70-86}$ epitope or the myelin $PLP_{139-151}$ epitope (Figure 4). DTH recall responses were measured at postinfection day 38 for the day 0–5 treatment groups and 3 days after R(+)WIN55,212 treatment at the other time points. The DTH responses to $VP2_{70-86}$ and $PLP_{139-151}$ were significantly

reduced compared with controls ($P < 0.05$) (Figure 4, a–c) at all time points. In most cases, the R(+)WIN55,212-treated mice exhibited a total lack of DTH responses. In addition, S(–)WIN55,212-treated and untreated TMEV-infected mice exhibited similar DTH responses at all time points suggesting no inhibitory effect of either the S(–) enantiomer of WIN55,212 or the vehicle (Figure 4, a–c).

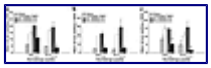

Figure 4

Cannabinoid treatment inhibits both virus and myelin autoimmune inflammatory DTH responses. Treatment of mice with R(+)WIN55,212 (black bars) at the time of TMEV infection (**a**), at the onset of clinical disease (**b**), or during established disease (**c**), significantly reduces DTH responses to rechallenge with either the immunodominant TMEV peptide $VP2_{70-86}$ or the autoreactive myelin $PLP_{139-151}$ peptide. *Significant reduced DTH responses compared with either S(–)WIN55,212 (dark gray bars) or untreated TMEV-infected (white bars) mice ($P < 0.05$). Naive mice (light gray bars) show a low background response to either peptide.

R(+)WIN55,212 treatment inhibits Th1-type cytokine secretion from both virus-specific and autoreactive $PLP_{139-151}$–specific T cells. The ability of R(+)WIN55,212-treated mice to mount Th1-type cytokine responses was measured in vitro by IFN-γ ELISA. Splenocyte cultures from TMEV-infected mice were rechallenged with either $VP2_{70-86}$ or $PLP_{139-151}$. R(+)WIN55,212 treatment of mice either at the time of infection (postinfection day 0–5) or at the onset of clinical disease (postinfection day 26–31), significantly reduced the capacity of T lymphocytes to secrete IFN-γ in response to either $PLP_{139-151}$ or $VP2_{70-86}$ ($P < 0.05$) (Figure 5, a and b). R(+)WIN55,212 treatment during established disease, however, did not significantly inhibit IFN-γ secretion of peripheral T cells in response to either $PLP_{139-151}$ or $VP2_{70-86}$ (Figure 5c). In all experiments S(–)WIN55,212 had little effect upon IFN-γ secretion compared with untreated TMEV-infected mice (Figure 5, a–c).

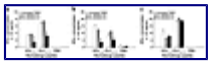

Figure 5

Cannabinoid receptor agonism inhibits Th1-mediated IFN-γ cell secretion. Treatment with R(+)WIN55,212 at the time of infection (**a**) or at the onset of clinical disease (**b**) significantly inhibited IFN-γ secretion from T lymphocytes rechallenged with either $VP2_{70-86}$ or $PLP_{139-151}$ compared with either S(–)WIN55,212 (gray bars) or untreated TMEV-infected mice (white bars). R(+)WIN55,212 treatment during established disease (**c**) had no significant effect on IFN-γ secretion. *Cytokine response was significantly inhibited compared with either S(–)WIN55,212 or untreated TMEV-infected mice. $P < 0.05$.

R(+)WIN55,212 treatment results in reduction of mRNA expression for proinflammatory and antiviral mediators in the spinal cord. Following R(+)WIN55,212 treatment at the onset of infection (postinfection day 0–5), spinal cords were removed on postinfection day 7 and analyzed by RT-PCR for the presence of cytokine mRNA. R(+)WIN55,212 treatment led to a significant reduction in the spinal cord expression of mRNA for the innate antiviral mediators, IFN-α and IFN-β, compared with either control mice, as measured by semiquantitative RT-PCR (Figure 6a). R(+)WIN55,212 treatment at the onset of clinical disease (postinfection day 26–31) resulted in a significant reduction in spinal cord mRNA expression of the proinflammatory mediators, IL-1β,

IL-6, TNF-α, IFN-γ (Figure 6b), and IL-4 (Figure 6c) compared with controls. Interestingly, R(+)WIN55,212 treatment had no effect on mRNA levels in the CNS coding for the IL-12 p40 subunit (Figure 6b) or for IL-10 (Figure 6c).

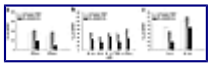

Figure 6

Cannabinoid treatment inhibits mRNA coding for both antiviral and Th1- and Th2-type inflammatory mediators in the CNS. R(+)WIN55,212 treatment at the time of infection (day 0–5) inhibited spinal cord mRNA coding for the innate antiviral mediators, IFN-α, and IFN-β (**a**). R(+)WIN55,212 treatment at the onset of disease inhibited spinal cord mRNA coding for the proinflammatory Th1 mediators, IL-1β, IL-6, TNF-α, and IFN-γ, but not the IL-12 p40 subunit (**b**). Treatment at disease onset also inhibited mRNA coding for the Th2 cytokine IL-4, but not IL-10 (**c**). The mRNA levels are presented as the percentage of intensity of the HPRT control for that sample.

Discussion

Based on the numerous reports of the in vitro immunomodulatory effects of cannabinoids, we investigated the immunotherapeutic effects of R(+)WIN55,212 in TMEV-IDD, a chronic-progressive autoimmune model of MS. SJL mice were treated with R(+)WIN55,212 at three different time points: (a) concomitant with TMEV infection to study effects on virus infection/persistence of the CNS and the associated innate (type 1 IFN) and adaptive ($CD4^+$ T cell) antiviral immune responses; (b) at the onset of clinical disease to determine the immunosuppressive effect upon antiviral and autoreactive anti-myelin T cell responses; (c) during established disease, which has clinical relevance for the treatment of MS. We found that intraperitoneal treatment with R(+)WIN55,212 at all times significantly inhibited progression of clinical disease. The short course of R(+)WIN55,212 treatment (6 consecutive days) appeared to be effective for at least 30 days after treatment. After 30 days, treated mice began to show signs of increasing clinical severity, although still significantly less severe than controls. Interestingly, administration of R(+)WIN55,212 every other day (6 total injections) had little effect on the disease course of TMEV-IDD (data not shown), suggesting R(+)WIN55,212 has a short active half-life in vivo. Importantly, R(+)WIN55,212 is not toxic to lymphocytes as the total number of $CD4^+$, $CD8^+$, $CD4^+CD25^+$, and $B220^+$ lymphocytes, and $F4/80^+$ macrophages in the spleen, were unchanged following treatment. In addition, there was no indication of toxicity in treated versus control groups as measured by a lactose dehydrogenase cytotoxicity assay (data not shown). In contrast to existing treatments for MS, side-effects of R(+)WIN55,212 treatment were mild, consisting of psychoactive effects, primarily ataxia, between 1 and 30 minutes after administration of 20 mg/kg. Similar adverse events were reported in a double-blind study on the safety and efficacy of oral Δ^9-THC for the symptomatic treatment of MS (22).

The therapeutic effect of R(+)WIN55,212 was associated with a reduced capacity of $CD4^+$ T cells to differentiate to Th1 effector cells. The Th1 cytokines IFN-γ and TNF-α promote the activation of the immune system. R(+)WIN55,212 treatment significantly reduced the ability of mice to mount DTH responses to either $VP2_{70-86}$ or $PLP_{139-151}$ following treatment at all three time points. In addition, IFN-γ secretion from $CD4^+$ T lymphocytes from R(+)WIN55,212-treated mice was reduced compared with untreated or S(–)WIN55,212-treated mice. R(+)WIN55,212 treatment at the onset of clinical disease (postinfection day 26–31) also significantly inhibited levels of spinal cord mRNA coding for a number of inflammatory

mediators (IL-1β, IL-6, TNF-α, and IFN-γ) important in the induction and progression of TMEV-IDD, EAE, and presumably MS (18, 23–26). Previous studies from our group have demonstrated that mRNA coding for the proinflammatory cytokines IFN-γ and TNF-α, are expressed at equivalent levels during the onset of disease (postinfection day 33) and late stage disease (postinfection day 103) (21).

R(+)WIN55,212 mediates its effects through CB1 and CB2 receptors. Therefore, some downregulatory effects of R(+)WIN55,212 on CNS cytokine mRNA transcription levels may be directed toward resident CNS cells such as astrocytes, microglial, and endothelial cells. We are currently investigating the effects of R(+)WIN55,212 on cytokine secretion of TMEV-infected glial cells. In addition, recent studies have shown that human dendritic cells express both CB1 and CB2 receptors and fatty acid amide hydrolase, which hydrolyzes endocannabinoids (27). In addition, Δ^9-THC can inhibit in vitro T cell proliferation and Th1 polarization in dendritic/T cell cocultures (28). This indirectly supports the findings in this study, whereby cannabinoids can downregulate the immune response by inhibiting Th1 polarization and therefore effector function during TMEV infection and autoimmune disease. We are currently investigating potential direct immunomodulatory effects of cannabinoids on dendritic cells during TMEV-IDD. Although the endogenous cannabinoid, AEA, can inhibit TMEV-induced TNF-α secretion from astrocytes, IL-6 is upregulated (29, 30). Differences in IL-6 production may be due to the effects of AEA versus R(+)WIN55,212 or the differences between in vitro versus in vivo experiments. Surprisingly, R(+)WIN55,212 had no effect on the levels of mRNA coding for the IL-12 p40 subunit, which is involved in Th1 induction. Although IL-1β, IL-6, IL-12 p40, and TNF-α are all regulated by NF-κB/Rel transcription factors, the IL-12 p40 subunit may require c-Rel, a transcription factor not required for transcription of the other cytokines (31). This suggests that R(+)WIN55,212 may affect different transcription factors, which in turn modulate the expression of cytokines produced. Cannabinoid treatment may also induce the expression of Th2 cytokines, such as IL-4 and IL-10 (32), which have therapeutic effects in EAE (17, 19). Treatment with Δ^9-THC for BALB/c mice infected with *Legionella pneumophila* suppressed IFN-γ secretion and induced IL-4 expression (32). In this study we found no evidence of Th2 cytokine induction, either IL-4 or IL-10, by ELISA or ELISPOT from splenocyte cultures from R(+)WIN55,212-treated mice at all treatment times (data not shown). This may reflect the reduced capacity of SJL mice to produce IL-4 (33). In contrast, mRNA analysis of the spinal cord at the onset of clinical disease showed a decrease in IL-4, but not IL-10, mRNA. Since IL-4 is predominantly derived from Th2 cells, the effects of R(+)WIN55,212 are apparently directed predominantly toward the regulation of Th1 responses. IL-10 is expressed by a number of cell types apart from T lymphocytes and is expressed constitutively by resident CNS cells (19, 34), which may account for its relatively unchanged level in the CNS. Further elucidation of the mechanisms of cannabinoid modulation of both Th1 and Th2 cytokine expression is required to resolve these questions. Both AEA and Δ^9-THC, and potentially R(+)WIN55,212, can also modulate the immune system by regulating the hypothalamo-pituitary axis via CB1 receptors expressed in the hippocampus (35).

The mechanisms underlying the antiproliferative effects of cannabinoids on T cell responses both in vitro and in vivo are still unclear, but appear to be dependent upon timing, dose, and the agonist used (36, 37). Here we report that R(+)WIN55,212 treatment at the time of infection can inhibit the proliferation of $VP2_{70-86}$–specific T lymphocytes. R(+)WIN55,212-treatment at the onset of clinical disease or during established disease, however, had little effect on the proliferative capacity of either $VP2_{70-86}$– or $PLP_{139-151}$–specific T cells. This may reflect differences in cannabinoid efficacy, dependent upon the activation state of the target T cell. The

mRNA coding for CB receptors on T cells has been shown to be either increased or decreased depending on the activating stimuli, and this may account for some of the differences seen between the early and late treatments (38). Therefore, it appears that activated T cells may be more refractory to the inhibitory effect of R(+)WIN55,212 than naive T cells. This may also explain the lack of inhibitory effect of R(+)WIN55,212 on IFN-γ secretion during the late stage of TMEV-IDD. To determine whether R(+)WIN55,212 could also inhibit clinical disease by blockade of Th1 homing to the CNS, we characterized CNS inflammatory infiltrates. Despite the inhibition of Th1 differentiation, immunohistochemical analysis of the CNS following R(+)WIN55,212 treatment suggested that $CD4^+$, $CD8^+$, F4/80, and $B220^+$ cells were present in numbers equivalent to that of untreated or S(–)WIN55,212-treated mice, despite no evidence of increasing severity of clinical disease (Figure 1) and decreased levels of mRNA encoding proinflammatory cytokines in the CNS (Figure 6). At the time of disease onset or during late-stage disease, the CNS will already contain inflammatory infiltrate because disease is ongoing before treatment begins. Therefore, R(+)WIN55,212 may be inhibiting (a) the further polarization of virus and/or myelin epitope-specific Th1 cells in the peripheral lymphoid organs and/or (b) the Th1 cytokine responses in the target organ, i.e., the CNS, either directly at the T cell level or by affecting infiltrating or resident CNS APCs, or both. This observation indicates that the primary effect of the therapy in ongoing disease is at the effector stages of the inflammatory cascade. Collectively, R(+)WIN55,212 influences clinical disease by inhibiting peripheral Th1 differentiation, but not by preventing proliferative or homing capabilities of activated Th1 cells.

Viral infection is thought to play a role in both the initiation and exacerbation/relapse of MS (10, 11). Our results show that R(+)WIN55,212 inhibits the immune response to the immunodominant TMEV peptide, $VP2_{70-86}$; therefore, we determined the effect of R(+)WIN55,212 on the susceptibility of mice to TMEV infection. Following early R(+)WIN55,212 treatment (day 0), there was a marked increase in CNS TMEV titers that may be explained by the downregulation of antiviral Th1 responses (Figures 4 and 5). In addition, CNS levels of spinal cord mRNA coding for the antiviral molecules, IFN-α and IFN-β, were significantly decreased in R(+)WIN55,212-treated mice (Figure 6a). This is similar to the suppression of IFN-α/β seen in Δ^9-THC–treated mice infected with herpes simplex virus (39). Interestingly, despite the increased CNS viral load in R(+)WIN55,212-treated mice, there was decreased clinical disease, confirming our previous data showing the immune response to TMEV initiates CNS damage, not the cytotoxic capacity of the virus itself (15, 16). Infectious CNS TMEV titers reach a maximum 10–12 days after infection and then diminish to a persistent low level by day 30 after infection (40), which may explain the lack of difference in viral titers between the R(+)WIN55,212 and controls in mice following treatment at the onset of clinical disease (postinfection day 26–31). Therefore, viral titers at later time points are unaffected by the R(+)WIN55,212-induced inhibition of antiviral immunity.

Previous studies have investigated the immunosuppressive potential of cannabinoids in animal disease models. Dexanabinol (HU-211), Δ^8- and Δ^9-THC have been shown to be effective at inhibiting EAE (41–43). Although histological examination showed reduced CNS inflammation, no immunological parameters or mechanisms of action were elucidated. Cannabinoids have also been shown to inhibit tremor and spasticity symptoms in EAE (8, 9). In addition, oral administration of cannabidiol, a nonpsychoactive constituent of cannabis, inhibited murine collagen–induced arthritis by inhibiting T cell proliferation and Th1 (IFN-γ and TNF-α) cytokine secretion (44).

Further studies are underway to fully determine the mechanisms of therapeutic action of R(+)WIN55,212 in the TMEV model. In addition to the inhibitory effects on the immune system, the therapeutic effect of R(+)WIN55,212 at the late time point (postinfection day 50–55) may also involve other protective mechanisms. During late-stage EAE, caspase-3 is upregulated and is thought to play an important role in the apoptosis of axons, which leads to the severe hindlimb paralysis seen in that model (45). Caspase-3 is also implicated in the apoptosis of oligodendrocytes (46). It has been shown recently that cannabinoids, including R(+)WIN55,212, can inhibit caspase-3 activity and may protect mice from severe clinical disability (45). In addition to the anti-inflammatory and neuroprotective properties of cannabinoids, they have also recently been shown to promote oligodendrocyte survival (47) and inhibit glutamate release (48) in the CNS, which has been implicated in neurotoxicity (49). Therefore, although the T cell component of the TMEV-induced disease may be refractory to the effects of R(+)WIN55,212 at the late time point, cannabinoid therapy may well affect other ongoing disease processes induced during the chronic stages of TMEV-IDD. We are currently undertaking studies in the TMEV model to determine whether caspase-3 plays a similar role in pathology as was shown in the EAE model.

The use of specific CB receptor antagonists may help elucidate whether the therapeutic effect seen is CB1 and/or CB2 receptor mediated. The current CB receptor antagonists, however, are thought to be “inverse agonists” and capable of delivering a “biological signal” (50, 51); thus, it may be difficult to interpret results seen with these reagents. Furthermore, it is currently unknown what effect TMEV-IDD has on the expression of the cannabinoid receptors on the numerous cell types that express them. In EAE, mRNA coding for CB1 receptors is reduced in certain areas of the brain during disease, although the efficiency of WIN55,212 binding to CB1 is increased (52). Therefore, the effects of exogenous cannabinoids administered during late disease will likely induce different effects because signaling may be occurring only in specific areas of the brain. Furthermore, the expression of CB receptors in the spinal cord during either EAE or TMEV disease is unknown. The spinal cord is the major target for demyelination, and most lesions are located in the white matter. Therefore, it is possible that cannabinoid signaling will be different between the early, onset, and late phases of disease. Finally, studies have determined that endocannabinoid release is upregulated in the brain, and more significantly in the spinal cord, during chronic spastic EAE (8). Therefore, this may also play a role in inducing differential exogenous cannabinoid signaling in the TMEV-IDD model. The presence of additional CB-like receptors may further confuse the issue (53). Therefore, the use of CB-receptor knockout mice and CB receptor–specific ligands should provide important clues as to further mechanisms and the cell types involved.

neuropathic pain (http://www.gwpharm.com/). Correlation of serum levels of R(+)WIN55,212 between mice and humans is difficult. R(+)WIN55,212 has not been tested in humans, and correlation to existing data for the levels of Δ^9-THC in humans is confounded by the fact that drug concentrations in biological fluids are affected by the size of the dose, route of administration, the long-term pattern of drug use, and the individuals' metabolism and excretion rate. In addition, correlation is difficult due to the different structural properties of Δ^9-THC and R(+)WIN55,212, and the affinity and therefore the efficacy of Δ^9-THC and R(+)WIN55,212 for either CB1 or CB2 receptors. Furthermore, other compounds in cannabis may synergize with Δ^9-THC in vivo, or may actually be metabolized to Δ^9-THC (55). Therefore, the quantity of THC present in cannabis extracts used for human clinical trials may not give an accurate indication of the actual bioactive quantity of Δ^9-THC. Finally, the metabolic rate of the mouse is higher than that of humans. Therefore, a direct comparison of serum levels again may not give an accurate correlation. One way to indirectly compare Δ^9-THC levels in humans to R(+)WIN55,212 in mice would be to determine the quantity of compound needed to induce psychoactive effects in the subjects. In this study we observed psychoactive effects with 20 mg/kg R(+)WIN55,212, but not with 5 or 1 mg/kg. In correlation, reported side effects of cannabinoid treatment in humans (60 mg/kg/day orally of nabilone, synthetic Δ^9-THC) include dizziness and euphoria, both psychoactive effects, therefore indirectly implying that 20 mg/kg R(+)WIN55,212 in mice is within the range used for human clinical trials (56).

In summary, treatment with the cannabinoid R(+)WIN55,212 effectively ameliorates progression of established TMEV-IDD, in part by inhibiting the Th1 differentiation as measured by suppressed antigen-specific DTH, IFN-γ secretion, and by inhibiting the production of the proinflammatory cytokines TNF-α, IFN-γ, IL-1β, and IL-6, in the CNS, which are necessary for the induction and progression of clinical disease (summarized in Table 1). In addition, R(+)WIN55,212 may inhibit clinical disease at multiple levels depending upon the stage of disease. During late-stage disease, R(+)WIN55,212 has no inhibitory effect upon T cell proliferation but may limit effector damage of axons in the CNS. This study provides preclinical evidence suggesting that cannabinoids may be promising therapeutic agents for treating autoimmune disorders such as MS by exerting potent immunoregulatory effects, in addition to providing symptomatic relief of spasticity, neuropathic pain, and bladder dysfunction.

Table 1

Differential inhibition of immune response parameters following R(+)WIN55,212 treatment of TMEV-IDD is dependent on the stage of disease

Acknowledgments

This work was supported in part by United States Public Health Service grant NS-23349. J.L. Croxford is a fellow of the National Multiple Sclerosis Society (post-doctoral research fellowship award FG-1456-A-1).

Footnotes

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: multiple sclerosis (MS); anandamide (AEA); Δ^9-tetrahydrocannabinol (Δ^9-THC); experimental allergic encephalomyelitis (EAE); Theiler murine

encephalomyelitis virus (TMEV); TMEV-induced demyelinating disease (TMEV-IDD); proteolipid protein (PLP); delayed-type hypersensitivity (DTH).

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Cannabinoids for Autoimmune Disease

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This unjustly demonized plant is about to change the scope of medicine and disease management as we know it.

Historical Maligning of Marijuana

Although legalizing recreational **marijuana** is a polarizing political issue, the ceremonial and medicinal use of this botanical agent dates back thousands of years (1). Paleobotanical, anthropological, and ethnographic records date the first human interactions with marijuana to 11,000 years ago, in the Holocene era, when human groups in the Eurasian continent used the seeds and stalks as sources of food and fiber, and the resin-laden female flowers within spiritual contexts (2, 3, 4). According to Wei and colleagues (2017), the medicinal actions, as well as the heightened euphoria, modified time perception, intensified sensation, and sense of tranquility produced by consuming the flowers or inhaling their smoke was “intimately woven into religious ritual” (p. 1). Marijuana was likewise used to cement social bonding during weddings, funerals, supernatural rites, and festivals, from cultures ranging to the Scythians inhabiting the Eurasian steppe to the Hindus of the Himalayan mountains (5).

Nonetheless, the plant has been embroiled in a miasma of mythology and its reputation tarnished by its affiliation with counterculture, a segment of the population to which policymakers have harbored historical animus. According to Schafer (1972),

> "Many see the drug as fostering a counterculture which conflicts with basic moral precepts as well as with the operating functions of our society….Marihuana becomes more than a drug; it becomes a symbol of the rejection of cherished values" (p. 9).

Despite its extensive medical use in the United States into the twentieth century, marijuana became entangled with negative cultural connotations and hyperbolic rhetoric after Nixon passed the Controlled Substances Act, which exiled the plant to Schedule One status alongside heroin, mescaline, psilocybin, and LSD, with the implication that marijuana was devoid of medicinal properties, fraught with high abuse potential, and would be inaccessible for clinical research trials (7, 8). The Shafer Commission, appointed to perform a non-partisan, independent appraisal of marijuana, made a formal recommendation that its Schedule One status be repealed, but this was rejected by President Nixon, and future appeals in the matter by independent agencies, advocacy groups, and political leaders have likewise been denied (9).

Prior to the mid-twentieth century, marijuana use was, "…mainly confined to underprivileged socioeconomic groups in our cities and to certain insulated social groups, such as jazz musicians and artists," such that marijuana became conflated with cohorts marginalized by society (6, p.7). However, in the mid-1960s, marijuana use began to encompass mainstream sectors, and became emblematic of a broader social movement, a symbolic rite of protest, challenge to authority, and political demonstration for those involved in anti-war and civil rights efforts (6). Marijuana was adopted as both an "agent of group solidarity" and an instrument of political activism, and became equated with intergenerational and cultural divide as well as "disaffection with traditional society," "political radicalism," and "defiance of the established order" (6, p.10).

Due to its demonization by the Nixon administration during their infamous war on drugs, marijuana was relegated to the realm of street crime, and became ensnared with legal penalties, affixed with **false stereotypes, and had its side effects sensationalized**. False propaganda surrounding its use lingered after the federal prohibition of marijuana in 1937, such that marijuana instilled fear in the public (6). The erroneous narrative surrounding its side effects included aggressive behavior, juvenile delinquency, crime, addiction, insanity, and lethality (6, p. 9). In fact, the independent research panel appointed by Nixon described, "Although based much more on fantasy than on proven fact, the marihuana 'evils' took root in the public mind, and now continue to color the public reaction to the marijuana phenomenon" (6, p. 9).

The Schafer Report, submitted to Nixon to repeal its Class One status, chronicles how the association between marijuana and the undermining of the prevailing social hierarchy led to the plant's fall from grace, as "Such mass deviance was a problem and the scope of the problem was augmented by frequent publicity" (6, p. 8). Thus, when interpreted against the backdrop of institutional defiance, sociopolitical turbulence, campus unrest, communal living, and abdication of traditional moral values, the historical origins of the vitriol against marijuana use by more conservative segments of the population can be contextualized.

The Endocannabinoid System Rekindles Scientific Interest

The early 1990s, however, heralded the discovery of the mammalian endocannabinoid system, comprised of two heterotrimeric G-protein coupled cannabinoid receptors, CB1 and CB2, along with their endogenous lipid-derived ligands, the most important of which were arachidonylethanolamide (anandamide) and 2-arachidonyl glycerol (2-AG) (10). This lipid-signaling regulatory system was based on endocannabinoids derived from arachidonic acid metabolites, which are generated on demand in response to escalating levels of intracellular calcium from phospholipids residing in the lipid bilayer of cell membranes (11).

The revolutionary insight that humans have an endocannabinoid system, which controls executive function, mood, social behavior, and pain, is conserved from invertebrates to high mammals, and can be activated by cannabinoids in marijuana, renewed interest in its medicinal applications (5, 52). Advances in this field of study engendered a platform for the examination of the therapeutic effects of cannabinoids (11).

Marijuana primarily refers to the species *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*, whereas Cannabis species lacking psychoactive cannabinoids are classified as **hemp**, which has been used historically for industrial production of ropes and textiles (7, 6, 8). Approximately one hundred pharmacologically active cannabinoids have been isolated; however, the bulk of scientific research has revolved around two, Δ9-tetrahydrocannabinol (THC), to which the 'high' is attributed, and cannabidiol (CBD), which does not produce this psychoactive effect and may, in fact, counteract it (12).

It was discovered that CB1 cannabinoid receptors are heterogeneously expressed in the central and peripheral nervous systems, and are particularly enriched in areas of the brain such as the cerebellum, cerebral cortex, caudate-putamen, hippocampus, substantia nigra pars reticulata, entopeduncular nucleus, and globus pallidus (10). CB1 receptors are particularly concentrated in regions of the brain that modulate nociception, motor function, and higher executive functions such as memory and cognition (13).

On the other hand, CB2 cannabinoid receptors are primarily localized in non-neuronal tissues, especially immune cells, where they mediate immunosuppressive effects and inhibit pain transmission (13, 10). However, both CB1 and CB2 receptors have been found on immune cells, supporting the notion that cannabinoids contribute to immunoregulation (11). Whereas CBD lacks significant agonist activity at CB1 receptors, which explains its non-psychotropic effects, Δ9-tetrahydrocannabinol (THC), the psychoactive component of marijuana, exhibits comparable activity at both the CB1 and CB2 receptors (10).

Although the endocannabinoid system regulates the autonomic nervous system, microcirculation, and immunity in the periphery, "Studies to date indicate that the main pharmacological function of the endocannabinoid system is in neuromodulation: controlling motor functions, cognition, emotional responses, homeostasis and motivation" (11, p. 2).

Anti-Inflammatory Effects of Cannabinoids

Cannabinoids have been demonstrated to have immunomodulatory effects, and are now being re-conceived as novel anti-inflammatory drugs (11). Although cannabinoids are pleiotropic, functioning through multiple mechanisms, recent studies have elucidated that cannabinoids induce apoptosis, or programmed cell death, in immune cell populations, a process involving molecular and morphological changes required for physiological equilibrium and immunosuppression (14).

In vitro studies have elucidated that THC provokes apoptosis in macrophages and T cells by activating caspases and the apoptosis regulator Bcl-2 (15). Likewise, ex vivo studies have shown that THC is capable of inducing apoptosis in T cell, B cell, and macrophage lineages (51). Importantly, THC has been shown to precipitate apoptosis in dendritic cells, the foremost professional antigen presenting cells that modulate maturation of naive T lymphocytes into effector T cells such as Th1, Th2, or Th17 cells, which can elicit the immune imbalances implicated in autoimmunity (11). CBD also leads to apoptosis of CD4+ and CD8+ T cells by promoting reactive oxygen species (ROS) production and up-regulating activity of caspases, which are integral to the execution phases of apoptosis (16).

On the other hand, “Cannabinoids can protect from apoptosis in nontransformed cells of the CNS, which can play a protective role in autoimmune conditions such as multiple sclerosis” (11, p. 3). Specifically, cannabinoids enhance signaling through the PI3K/AKT pathway, which regulates the cell cycle and promotes brain growth and differentiation (11, 2009). Cannabinoids also inhibit apoptosis of oligodendrocytes, a subset of neuroglial cells injured in multiple sclerosis, which are responsible for producing the myelin sheath, or the fatty insulation of nerves that allows for conduction of electrochemical signals (17, 11).

In addition, although results are highly variable depending on cell population and cannabinoid concentration, cannabinoids have been demonstrated to reduce synthesis of inflammatory intercellular messenger molecules known as cytokines and chemokines, which contribute to the pathogenesis of autoimmune disease. THC, for instance, has been observed to suppress the expression of pro-inflammatory interleukins (ILs) IL-1α, IL-1β, and IL-6, as well as tumor necrosis factor alpha (TNF-α) in cultured rat microglial cells exposed to lipolysaccharide (LPS), the outer cell wall component of Gram-negative bacteria that is implicated in autoimmune and cardiometabolic disorders (18). In similar fashion, synthetic cannabinoids WIN55,212-2 and HU210 reduce synthesis of pro-inflammatory TNF-α and IL-12 while increasing levels of anti-inflammatory IL-10 in a rodent model of LPS challenge (19).

In other studies, CBD blocks the LPS-induced increase in TNF-α in mouse models (20). Moreover, synthetic cannabinoids such as AjA, CP55,940 and WIN55,212-2 have been shown to reduce secretion of IL-6, an inflammatory cytokine that contributes to tissue injury (11). Thus, researchers suggest that these cannabinoid compounds “may have a value for treatment of joint inflammation in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and osteoarthritis” (11, p. 4).

Importantly, some studies have shown that cannabinoids up-regulate activity of FOXP3+ regulatory T cells (Tregs), a subset of immune cells depleted in autoimmunity which promote peripheral immune tolerance, and restore equilibrium between the Th1, Th2, and Th17 arms of the immune system (11).

For instance, in response to low-level stimulation of T cells, CBD up-regulates IL-2 production, which is essential for induction of Treg activity along with TGF-β1 (21). Dhital and colleagues (2017) illustrated that CBD increases populations of Tregs, which results in immunosuppression via robust inhibition of T cell proliferation, effectively protracting the production of lymphocyte populations underlying autoimmune etiology (21).

Lastly, CBD has been shown to inhibit nuclear factor kappa beta (NFκB), a pathway that can be over-active in autoimmune disease (22). NFκB is a master transcription factor that controls hundreds of genes for innate immunity and leads to the expression of downstream pro-inflammatory products (22).

Therapeutic Application to Autoimmune Disease

Cannabinoids are effective in combating many of the hallmark symptoms that accompany autoimmune disease, such as mood disturbance. For example, a double-blind human study revealed that **CBD** modulates neural activity in the limbic and paralimbic systems to significantly reduce subjective anxiety (23). Likewise, CBD may improve motivation and reduce clinical depression and anhedonia (53). Case series and a meta-analysis of eighteen studies have highlighted a role for cannabis in improvement of chronic pain and sleep as well (24, 25). In addition, cannabinoids may improve an array of organ- and tissue-specific autoimmune disorders.

Rheumatoid Arthritis

Potent anti-arthritic properties of cannabinoids have been demonstrated such that their use may be indicated for **rheumatoid arthritis (RA)**, an autoimmune disorder where immune complexes are deposited in the joints, leading to progressive joint pain, stiffness, and deformity. In particular, CBD has been shown to prevent disease progression and protect joints against severe damage in rodent collagen-induced arthritis (20). Ex vivo studies of animal models of rheumatoid arthritis have demonstrated attenuation of immunoproliferative responses in draining lymph nodes with CBD, as well as a reduction in interferon gamma (IFN-γ) production, and diminished release of TNF by knee synovial cells (20). Mitogen-stimulated and antigen-specific clonal expansion of lymphocyte populations was also suppressed by CBD in vivo (20).

In addition, synthetic cannabinoids such as AjA have been shown to reduce secretion of IL-6, and to protect against osteoclastogenesis, or the creation of cells associated with bone resorption (26, 27). AjA also suppresses production of an inflammatory cytokine, IL-1β, in peripheral blood monocytes (PBMs) and synovial fluid monocytes (SFMs) extracted from patients with RA (28).

Type 1 Diabetes

Another autoimmune condition where cannabinoids hold therapeutic promise is type 1 diabetes mellitus (TIDM), or insulin-dependent diabetes, where autoimmune processes result in loss of insulin-producing pancreatic β cells. In rodent models of T1D induced by streptozotocin, it was found that THC mitigated the severity of the immune response, transiently prevented hyperglycemia and loss of

pancreatic insulin, and decreased expression of pro-inflammatory elements including CD3+ T cells, IL-12, IFN-γ and TNF-α (50).

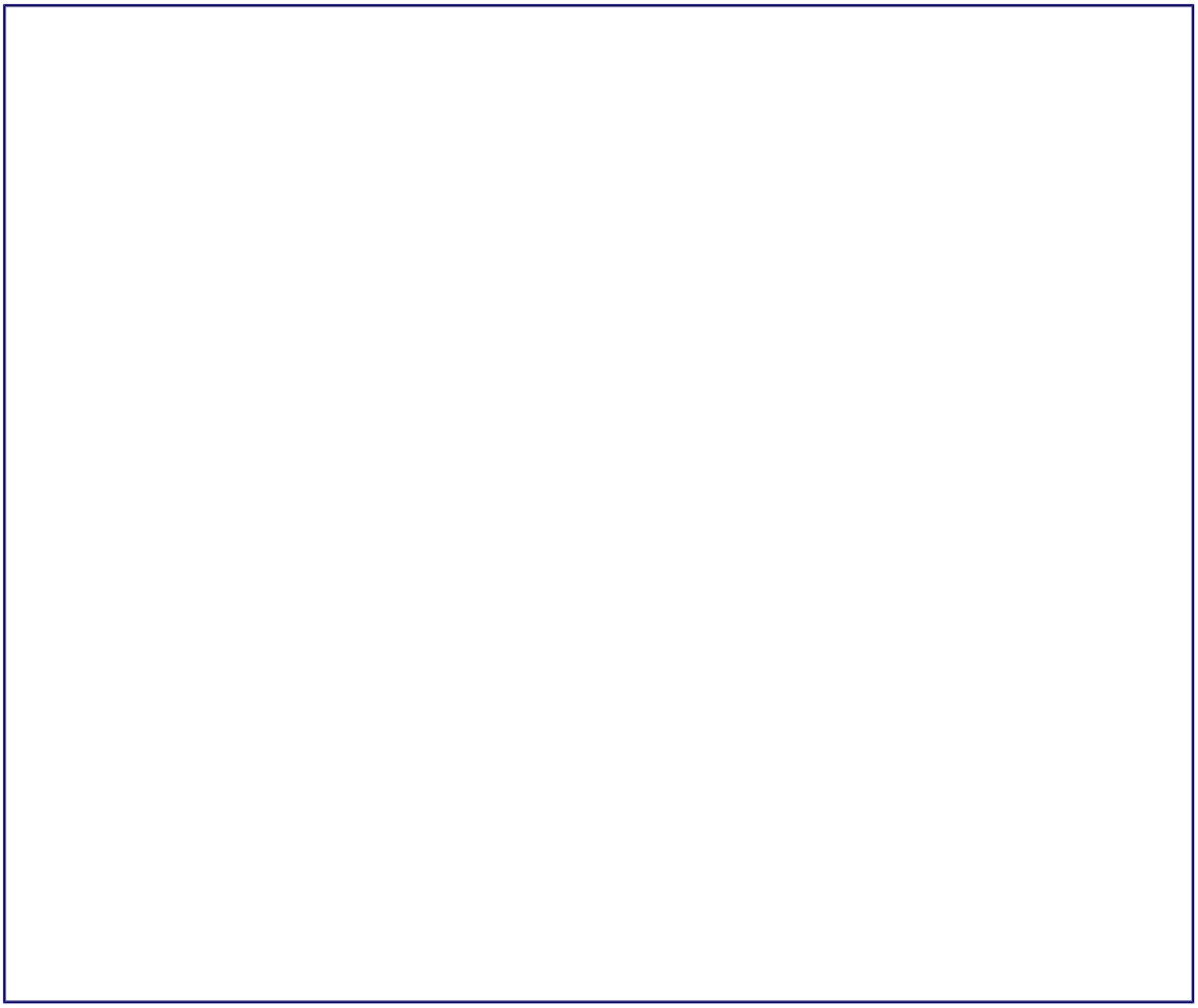
Inflammatory Bowel Disease

Further, data support that cannabinoids confer protection in two forms of **inflammatory bowel disease (IBD), Crohn's and ulcerative colitis**. Crohn's disease is an autoimmune disorder that generally involves the ileum and colon and can be accompanied by intestinal fistulas, granulomas, and strictures, while ulcerative colitis is an autoimmune disease characterized by inflammation in the colonic mucosa and submucosa (29, 30). According to Nagarkatti et al. (2009), "Cannabinoids have been shown to regulate the tissue response to excessive inflammation in the colon, mediated by both dampening smooth-muscular irritation caused by inflammation and suppressing proinflammatory cytokines, thus controlling the cellular pathways leading to inflammatory responses" (p. 7).

CB1 receptors, for instance, are expressed in the colon and ileum and are increased upon inflammation as a protective mechanism (31). Genetic ablation of CB1 receptors enhances sensitivity to inflammatory stimuli in mice (11). In oral dextran sodium sulfate (DSS)-induced rodent models of colitis, pharmacological antagonists, which block stimulation of CB1 receptors, exacerbated colitis, whereas pharmacological activation of cannabinoid receptors with the agonist HU210 ameliorated colitis (11). Likewise, oil of mustard (OM)-induced colitis and sequelae including inflammatory and histological damage, diarrhea, and colonic weight gain were reduced by CB1- and CB2- selective agonists, ACEA and JWH-133, respectively (32). In addition, mice with genetic deletion of fatty acid amide hydrolase (FAAH), the enzyme that degrades anandamide, have significant protection against 2,4-dinitrobenzene sulfonic acid (DNBS)-induced Crohn's due to longer half-life of this endogenous cannabinoid, likely leading to increased cannabinoid receptor stimulation (33).

Ocular Manifestations of Autoimmune Disease

In an in vivo model of experimental autoimmune uveoretinitis (EAU), which is an animal model of human autoimmune ocular disorders, JWH-133, a CB2-selective agonist, elicited an immunosuppressive effect (34, 35). This cannabinoid agonist appeared to work through down-regulation of antigen presentation and T cell proliferation and favorable alterations in cytokine profiles (35, 36). For instance, JWH-133 improved levels of IL-10, which prevents EAU development, and decreased levels of IL-12p40, a cytokine pivotal to EAU pathogenesis (36).



Psoriasis

Cannabinoids are likewise useful in arresting two primary steps in the pathogenesis of psoriasis, an autoimmune skin disease characterized by plaques and lesions. A synthetic cannabinoid JWH-133 is able to suppress angiogenic growth factors and inflammatory cytokines including hypoxia inducible factor-1α (HIF-1α), angiopoitin-2, vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), basic fibroblast growth factor (bFGF), cellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), IL-8,IL-17, and IL-2, all of which are etiological factors in psoriasis development (37).

Multiple Sclerosis

Finally, cannabinoids exert neuroprotective and **immunosuppressive effects in multiple sclerosis (MS)**, a neurological autoimmune disorder wherein nerve fibers and axons are progressively demyelinated (11). At least eight clinical studies have illuminated the benefits of cannabis, THC, and the cannabinoid receptor agonist Nabilone for improving pain, spasticity, tremor, ataxia, and bladder

control in MS (38, 39). A survey of 112 MS patients similarly showed that using cannabis improved these symptoms, as well as depression, in 90% of individuals (11).

At a molecular level, activation of the CB1 receptor suppresses myelin-specific T cells which infiltrate the brain and spinal cord and are responsible for myelin sheath destruction (40). CB2 cannabinoid agonists such as WIN55,212-2 inhibit experimental autoimmune encephalomyelitis (EAE), an animal model of MS, by mediating apoptosis of the encephalitogenic cells that induce brain inflammation (41). The same cannabinoid agonist works through the nuclear receptor nuclear receptor PPAR-γ to reduce endothelial and vascular cell adhesion molecules expression, which effectively inhibits migration of pathogenic CD4+ T cells into the central nervous system (CNS) (42).

Cannabinoids such as AEA also reduce antigen presentation by microglial cells, the macrophages of the CNS, which drive myelin sheath elimination via production of glutamate, nitric oxide, and inflammatory interleukins (11). AEA further functions via CB2 receptors to suppress production of pro-inflammatory cytokines such as IL-12 and IL-23, which promote Th1 proliferation and maintain destructive Th17 cell populations, respectively (43). The vital immunomodulatory role played by the endocannabinoid system in preventing or mitigating MS is underscored by a study where CB2 knockout mice had exacerbated EAE and robust infiltration of myeloid progenitor cells into neuroinflamed tissue (44). Aberrant trafficking of myeloid progenitors, which can be prevented by cannabinoids, replenishes microglia populations, the cell subset which contributes to myelin sheath damage (44). Lastly, because astrocytes harbor CB1 and CB2 receptors, cannabinoids contribute to immunosuppression of these brain cells, which are responsible for augmenting the inflammatory response in MS via nitric oxide, chemokine, and cytokine production (11).

The Future of Medical Marijuana

Grassroots lobbying efforts, activism, and ballot initiatives have mobilized to repeal draconian laws, reclassify marijuana, and decriminalize its use, resulting in unprecedented medical and recreational access to marijuana in approximately half of the states in the United States (8).

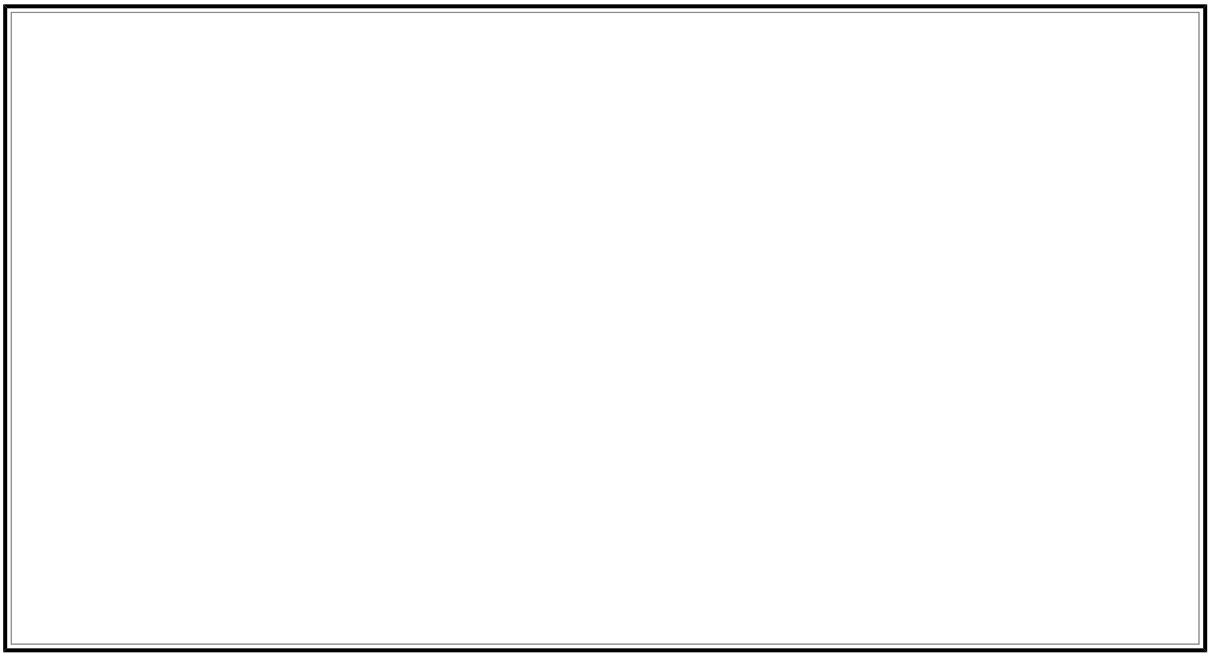
However, in many states, markets are saturated with products unregulated with regard to potency and quality, lacking standardization of their active constituents. Due to cross-breeding, hybridization, and variable cultivation methods, marijuana tends to contain high levels of THC relative to CBD, and unpredictable levels of other cannabinoids (8).

There are several methods of delivery, each of which vary in their onset of action, time to peak effects, and duration of effect (8). Smoking, which “releases the cannabinoid substances into a volatile mixture that is introduced into the lungs via smoke and absorbed though the alveolar membrane and into the pulmonary circulation,” enables compounds to readily translocate across the blood-brain barrier and exert systemic central and peripheral effects (8, p. 241). Although it produces rapid effects and simplifies titration of dose, smoking exposes the user to tar, microbial contaminants, heavy metals, pesticides, and other toxicants, as well as increases the risk of myocardial infarction secondary to coronary artery spasm in susceptible individuals (8). Further, long-term sequelae of marijuana smoking

likely include chronic cough, wheezing, increased sputum production, respiratory tract inflammation, and obstruction of air flow (45, 46).

With vaporization, on the other hand, marijuana leaves are heated just below combustion point, liberating the cannabinoids into vapor and eliciting fast acting effects without the concomitant production of airway-damaging smoke (47). In contrast, edibles, which incorporate marijuana into food products such as baked goods, teas, oils, and lozenges, are convenient to administer but are accompanied by a delay between when the product is ingested and when peak drug effects occur, due to first-pass metabolism in the liver before absorption into the nervous system and systemic circulation (12, 48). Tinctures and sprays of plant extract negate this problem due to their absorption through the oral mucosal membranes, which bypasses the liver, generating relatively rapid peak-effects (8).

For those who live in states where medical marijuana is prohibited, or for individuals wanting to avoid psychotropic effects of marijuana, isolated hemp-derived CBD extracts may be a viable option. As summarized by Kogan and Mechoulam (2007), “The therapeutic value of cannabinoids is too high to be put aside…In view of the very low toxicity and the generally benign side effects of this group of compounds, neglecting or denying their clinical potential is unacceptable” (49, p. 413).



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